Polish Journal of Microbiology
2017, Vol. 66, No 2, 259–263

SHORT COMMUNICATION

Bactericidal Activity of Octenidine to Various Genospecies of *Borrelia burgdorferi*, Sensu Lato Spirochetes *in Vitro* and *in Vivo*

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Submitted 11 August 2016, revised 10 December 2016, accepted 27 January 2017

Abstract

The aim of our studies was to invent a reliable method for detection of the bactericidal activity of disinfectants against *Borrelia burgdorferi* in suspension (*in vitro*) and in cell line cultures (*in vivo*). In the suspension method, 0.01% octenidine at 20°C and 35°C was bactericidal to *Borrelia afzeli*; *Borrelia garini*, *B. burgdorferi* sensu stricto after 5 minutes treatment. Increase of the temperature to 35°C speed up the bactericidal effect to 1 minute. The bactericidal action of octenidine towards *B. burgdorferi* spirochetes growing in fibroblasts was less effective and needed a longer time to kill them than in the suspension.

Key words: *Borrelia burgdorferi*, octenidine hydrochloride, bactericidal activity

Lyme borreliosis is a zoonosis which can be transferred to humans exclusively by a vector – infected *Ixodes* spp. ticks. Bites by infected ticks introduce *Borrelia burgdorferi* sensu lato spirochetes into the skin (Stanek *et al*., 2012).

B. burgdorferi spirochetes are very fastidious bacteria and do not grow on any known solid and standard liquid bacteriological media. They can be cultivated only in liquid BSK medium supplemented with rabbit serum. They grow on this medium very slowly and results can be obtained after three months of incubation (Stanek *et al*., 2012). Consequently, classical methods for the evaluation of bactericidal activity of chemical disinfectants and antiseptics are not suitable. A method for testing the bactericidal activity of disinfectants against *B. burgdorferi* and other spirochetes has not yet been established. Previously, *in vivo* methods for susceptibility testing of *B. burgdorferi* towards antibiotics in mice, dogs and other mammals were proposed (Embers *et al*., 2012; Hodzic *et al*., 2008; Strabinger *et al*., 1997). However, the obtained results are not clear-cut and rewarding (Embers *et al*., 2012). Recently, the first *in vitro* method for testing the bactericidal activity of some leaf extracts against *B. burgdorferi* has been presented (Theophilus *et al*., 2015).

Reports have also described the *in vivo* and *in vitro* interaction of *B. burgdorferi*, sensu lato spirochetes with different host cells. It has been found that pathogenic strains penetrate mammalian cells (fibroblasts) and can multiply in them (Chmielewski and Tylewska-Wierzbanowska, 2010; Tylewska-Wierzbanowska and Chmielewski, 1997). According to these data, the bactericidal activity of octenidine hydrochloride against spirochetes should be also tested in culture of *B. burgdorferi* in HEL-299 cell line (fibroblasts).

The aim of our studies was to invent a reliable method for detection of bactericidal activity of disinfectants against *B. burgdorferi* spirochetes in suspension, in *in vitro* conditions as well as *in vivo*, in cell line cultures. Moreover, we have examined whether octenidine hydrochloride meets these conditions as the study was conducted in reference to a development of a new antiseptic pharmaceutical product (Octynix®).

Strains *B. burgdorferi* sensu stricto B31 (ATCC 35210), and *B. garinii* 20047 (ATCC 51383), *B. afzelii* VS461 (ATCC 51567) were cultured in BSK-H Medium Complete, supplemented with 6% of rabbit serum (SIGMA-ALDRICH Chemie GmbH, USA). In all tests, suspensions of 1×10^7 bacterial cells/ml (counted in Thoma cell counting chamber) were used. HEL-299 (ATCC-CCL-137) cell line derived from human fibroblasts was cultured in 2 ml Eagle's Minimum Essential Medium (EMEM) with Earle's BSS, 1 mM sodium pyruvate and 2 mM L-glutamine (ATCC 30–2003) (ATCC, Manassas, Canada) supplemented with 5% of fetal calf serum (ATCC), in shell-vials (Bibby Sterilin, Great Britain).

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Octenidine hydrochloride dissolved in methanol was added to tubes containing 990 µl *B. burgdorferi* sensu stricto spirochetes culture in BSK-H Medium Complete to obtain 0.1%, 0.01%, 0.005% and 0.0025% final concentrations in culture medium. The mixtures were incubated obligatorily at 20 ± 1°C for 5 min ± 60 sec., and additionally at 20 ± 1°C and 35°C for 1 min ± 30 sec.; 10 min ± 60 sec; 15 min ± 60 sec., and at 35°C for 5 min ± 60 sec.

Shell-vials with HEL-299 cell line confluent monolayer in 2 ml EMEM, supplemented with 5% of fetal calf serum (ATCC), were incubated at 35°C with 5% CO_2 atmosphere for 2 days. Next, EMEM medium was removed and replaced with 1.9 ml of BSK-H medium. After that, cell line cultures were inoculated with 100 µl of spirochetes cultured in BSK-H. Infected cell lines were incubated for 24 hrs. After that time, 20 µl of consecutive dilutions of octenidine hydrochloride in methanol to obtain 0.01%, 0.005%, 0.0025% final concentrations, were added for 5, 10, 15 minutes. Next, the cell lines were washed with fresh BSK-H medium to remove octenidine hydrochloride. Washed cell lines were incubated in 5% CO_2 at 35°C for 72 hours. Every 24 hours, the number or healthy and dead bacterial cells in the medium, outside the cell line, were counted.

Immediately, when incubations were terminated, slides were prepared and evaluated under dark field microscope (10 × 25). The evaluation of bactericidal activity of the tested substance was based on the observation of motility and the loss of motility of the examined spirochetes. One hundred cells were counted and the numbers of mobile (live) and motionless (dead) spirochetes were estimated (in triplicate). The influence of octenidine hydrochloride diluents (methanol, 2-phenoxyethanol) and octenidine hydrochloride neutralizer on the viability of spirochetes and HEL-299 cells was tested to exclude their side effects.

Decimal logarithm reduction for each product concentration and each experimental condition was calculated and recorded according to the formula: $lgR = lgN_o - lgN_a$ (lgR – decimal logarithm reduction; N_o – number

Table I
Bactericidal activity of octenidine to *B. burgdorferi*, sensu lato strains at 20°C

Genospecies of *B. burgdorferi*, sensu lato	Octenidine concentration*	Reduction** after treatment with octenidine for the following time				
		1 min	5 min	10 min	15 min	60 min
B. afzelii	0.1	0	0	0	0	0
		>7	(>7)	(>7)	(>7)	(>7)
	0.01	84	0	0	0	0
		(0.08)	(>7)	(>7)	(>7)	(>7)
	0.005	83	4	0	0	0
		(0.08)	(1.4)	(>7)	(>7)	(>7)
	0.0025	89	71	5	5	0
		(0.05)	(0.15)	(1.3)	(1.3)	(>7)
B. garinii	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	5	0	0	0	0
		(1.3)	(>7)	(>7)	(>7)	(>7)
	0.005	30	0	0	0	0
		(0.52)	(>7)	(>7)	(>7)	(>7)
	0.0025	79	72	68	56	6
		(0.1)	(0.16)	(0.17)	(0.25)	(1.22)
B. burgdorferi, sensu stricto	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	5	0	0	0	0
		(1.3)	(>7)	(>7)	(>7)	(>7)
	0.005	83	59	52	52	0
		(0.08)	(1.69)	(0.28)	(0.28)	(>7)
	0.0025	90	75	70	61	6
		(0.05)	(0.12)	(0.15)	(0.41)	(1.22)

* minimum bactericidal concentration (%) of octenidine in bacterial suspension in BSK-H medium
** expressed as percentage % of viable bacterial cells and in decimal logarithm (lgR)

Table II
Bactericidal activity of octenidine to *B. burgdorferi*, sensu lato strains at 35°C

Genospecies of *B. burgdorferi*, sensu lato	Concentration (%) of octenidine	Reduction of viable bacteria after treatment with octenidine for the following time**				
		1 min	5 min	10 min	15 min	60 min
B. afzelii	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	16	0	0	0	0
		(0.8)	(>7)	(>7)	(>7)	(>7)
	0.005	70	0	0	0	0
		(0.15)	(>7)	(>7)	(>7)	(>7)
	0.0025	86	7	0	0	0
		(0.06)	(1.15)	(>7)	(>7)	(>7)
B. garinii	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.005	12	0	0	0	0
		(0.92)	(>7)	(>7)	(>7)	(>7)
	0.0025	87	49	29	20	0
		(0.06)	(0.31)	(0.54)	(0.7)	(>7)
B. burgdorferi, sensu stricto	0.1	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)
	0.01	5	0	0	0	0
		(1.3)	(>7)	(>7)	(>7)	(>7)
	0.005	81	28	0	0	0
		(0.09)	(0.55)	(>7)	(>7)	(>7)
	0.0025	94	80	63	56	8
		(0.03)	(0.1)	(0.2)	(0.25)	(1.1)

* minimum bactericidal concentration (%) in bacterial suspension in BSK-H
** expressed as percentage of viable bacterial cells and in decimal logarithm (lgR)

of cells in control test suspension/ml; N_a – number of survived bacterial cells in the test mixture/ml). At least one concentration of octenidine hydrochloride per test shall demonstrate a 5 lg or more reduction (lgR ≥ 5) in motility (viability) of spirochetes and at least one concentration shall demonstrate a lg reduction of less than 5. Equivalence of lgR ≥ 5: lack of live spirochetes treated with octenidine hydrochloride in fresh medium after 5 days of incubation. The above criteria are in accordance with EN 1276/2009.

Octenidine hydrochloride activity toward *B. burgdorferi* spirochetes was dependent on time of exposure and to some extent on temperature. It was found that at 20°C 0.01% octenidine hydrochloride kills all three tested genospecies of *B. burgdorferi*, sensu lato within 5 minutes (Table I). Increase of the temperature to 35°C speed up the bactericidal effect to 1 minute (Table II). Generally, in the suspension method, octenidine hydrochloride in concentration 0.01% was bactericidal to all three tested genospecies of *B. burgdorferi* sensu lato (*B. afzelii*; *B. garinii*, *B. burgdorferi* sensu stricto) after 5 minutes treatment.

Octenidine hydrochloride in concentration 0.01% was bactericidal after 5 minutes treatment for *B. afzelii* and *B. garinii* spirochetes growing in eukaryotic HEL-299 cell line.

At this time, cells of the *B. burgdorferi* sensu stricto strain treated for 5 min. with 0.02% octenidine hydrochloride, were still weakly motile. Cells of *B. burgdorferi,* sensu stricto strain lost their motility (viability) totally within 24 hours after treatment with 0.02% octenidine hydrochloride for 15 minutes. They were motionless (dead) 48 hours later meaning that under these conditions they are not able recuperate and to grow and multiply. These results indicate that the bactericidal concentration of octenidine hydrochloride against *B. burgdorferi*, sensu lato spirochetes may vary depending on genospecies and strains (Table III).

A method for testing the bactericidal activity of disinfectants against *B. burgdorferi* and other spirochetes

Table III
Bactericidal activity of octenidine to *B. burgdorferi*, sensu lato strains growing in mouse fibroblasts at 35°C

Genospecies of *B. burgdorferi*, sensu lato	Concentration of octenidine (%) in culture medium*	Percentage of viable bacteria treated with octenidine for the following minutes** after 24 hrs			Percentage of viable bacteria treated with octenidine for the following minutes** after 48 hrs			Percentage of viable bacteria treated with octenidine for the following minutes** after 72 hrs		
		5	10	15	5	10	15	5	10	15
B. afzelii	0.0025	4	4	0	10	5	1	6	5	0
		(1.4)	(1.4)	(>7)	(1.0)	(1.3)	(2.0)	(1.33)	(1.3)	(>7)
	0.005	2	0	0	0	0	0	5	0	0
		(1.7)	(>7)	(>7)	(>7)	(>7)	(>7)	(1.3)	(>7)	(>7)
	0.01	0	0	0	0	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)
B. garinii	0.0025	3	0	0	6	1	0	2	5	0
		(1.52)	(>7)	(>7)	(1.22)	(2.0)	(>7)	(1.7)	(1.3)	(>7)
	0.005	0	0	0	1	0	0	1	0	0
		(>7)	(>7)	(>7)	(2.0)	(>7)	(>7)	(2.0)	(>7)	(>7)
	0.01	0	0	0	0	0	0	0	0	0
		(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)	(>7)
B. burgdorferi, sensu stricto	0.005	29	18	6	48	41	68	41	4	4
		(90.54)	(0.74)	(1.22)	(0.32)	(0.39)	(0.17)	(0.39)	(1.4)	(1.4)
	0.01	8	2	1	23	0	0	10	0	0
		(1.1)	(1.7)	(2.0)	(0.64)	(>7)	(>7)	(1.0)	(>7)	(>7)
	0.02	3	2	0	0	0	0	0	0	0
		(1.52)	(1.7)	(>7)	(>7)	(>7)	(>7)	**(>7)**	(>7)	(>7)

* Treated with octenidine spirochetes were culture in HEL-299 cell line for 72 hours
** minimum bactericidal concentration against *B. burgdorferi* in cell cultures expressed as percentage (%) of viable bacterial cells and in decimal logarithm (lgR) – bold numbers

has not yet been established (no publication on this topic has been found in PubMed).

In our method, evaluation of the bactericidal activity of tested substance is based on observation of motility and loss of motility of the examined spirochetes. In the proposed bactericidal activity evaluation method of antiseptics and disinfectants against spirochetes, the test conditions such as temperatures of incubation, time of exposure to the tested substance, reduction of number of alive bacterial cells, *etc.* followed the rules included in European Standard EN 1276/2009. These regulations have applied to chemical disinfectants and antiseptics and characterize a quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. The obligatory test conditions have been set at: 20 ± 1°C for 5 min ± 60 sec.

According to PN-EN 1276/2009, the mixtures were incubated obligatorily at 20 ± 1°C for 5 min ± 60 sec. and additionally, at 20 ± 1°C for 1 min ± 30 sec., 10 min ± 60 sec., 15 min ± 60 sec., and at 35°C for 1 min ± 30 sec., 5 min ± 60 sec., 10 min ± 60 sec. and 15 min ± 60 sec.

It has been assumed that observed motile/motionless spirochetes mean live/dead spirochetes. *B. burgdorferi* are highly motile and invasive microorganisms that disseminate widely throughout their arthropod and vertebrate hosts (Groshong and Blevins, 2014; Wolgemuth, 2015). It has been discovered that motility and chemotaxis are critical for multiple stages of infection. There is an absolute requirement for cell motility during any stage of the *B. burgdorferi* life cycle (Motaleb *et al.*, 2015).

Motion ability of spirochetes is a necessary condition to survive in both vector as well as in consecutive reservoirs. Loss of ability to move leads to the death of the bacterial cell (Coburn *et al.*, 2013; Harman *et al.*, 2012).

It has been shown previously that *B. burgdorferi* spirochetes are able to enter and multiply in Vero cell and mouse fibroblasts (HELL-299) (Chmielewski and Tylewska-Wierzbanowska, 2010; Tylewska-Wierzbanowska and Chmielewski, 1997). It has been observed that *B. burgdorferi* can attach to the vascular endothelium and actively migrate between these cells to disseminate into other tissues (Coburn *et al.*, 2013; Harman *et al.*, 2012). Ticks as a vector inject spirochetes directly into the skin of animals and humans. This means that the skin protects the bacteria to some extent against disinfectants applied on its surface. The results of our *in vivo* bactericidal test suggest this possibility. *B. burgdorferi* growing in mouse fibroblasts were

less sensitive to octenididine. The bactericidal action of this substance towards *B. burgdorferi* spirochetes growing in fibroblasts was less effective and needed a longer time to kill them than in the suspension.

In conclusion: in the proposed suspension method, octenidine hydrochloride in concentration 0.01% has shown bactericidal activity to all three tested genospecies of *B. burgdorferi*, sensu lato (*B. afzelii*; *B. garinii*, *B. burgdorferi*, sensu stricto) after 5 minutes treatment.

Octenidine hydrochloride in concentration 0.01% was bactericidal after 5 min treatment for cells of *B. afzelii* and *B. garini* spirochetes growing in eukaryotic cell line whereas *B. burgdorferi*, sensu stricto strain was resistant to octenidine hydrochloride under these conditions. Moreover, cells of the *B. burgdorferi*, sensu stricto strain treated for 5 min with 0.02% octenidine hydrochloride, 24 hours later were still weakly motile. They lost motility (viability) 48 hours later meaning that under these conditions they were not able to grow and multiply. The cells of *B. burgdorferi*, sensu stricto strain totally lost their motility (viability) within 24 hours after 15 minutes treatment with 0.02% octenidine hydrochloride.

These results indicate that the method for detection of bactericidal activity of octenidine against spirochetes is sensitive and has potential usefulness for other disinfectants.

Acknowledgements

The research was supported in part by The National Centre for Research and Development and European Union within European Regional Development Fund and company InnoVitas Polska Sp. z. o.o. (Program INNOMED/I/3/NCBR/2014) and National Institute of Public Health-NIH funds.

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