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Note

Reexamination of the Bactericidal Activity of Chlorhexidine Digluconate against *Pseudomonas aeruginosa*

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In our study, we attempted to confirm a paradoxical effect of chlorhexidine digluconate (CHG). Two test procedures, the phenol coefficient (PC) method based on AOAC and its modified method were used. The cell concentration of test strains, *Pseudomonas aeruginosa* NCTC 7244, *P. aeruginosa* IFO 3445 and some clinically isolated strains were prepared at 10^4-10^9 cells/ml. According to the PC method based on AOAC, 1/800-1/1200 was the greatest dilution rate at which 5% (w/v) CHG was able to kill 10^9 cells/ml of *P. aeruginosa* by contact in 10 min, and 1/10000-1/12000 was the greatest dilution rate in case of 10^4 cells/ml. Increasing concentrations of CHG to kill *P. aeruginosa* were required as the viable counts of the organism increased. By the modified PC method, when undiluted 5% (w/v) CHG was added to the 10^7 cells/ml bacterial suspension, the organisms were killed in 4 or 5 min, and those in the 10^4 cells/ml suspension were killed within 30 s. Therefore, we could not confirm the paradoxical effect of slime production by *P. aeruginosa* as a phenomenon due to the bactericidal activity of CHG.

Key words : Chlorhexidine/Bactericidal activity/*Pseudomonas aeruginosa*.

Disinfectants play an important role in the control of infection in the hospital. Chlorhexidine digluconate (CHG) is a widely used disinfectant which is used to disinfect not only the skin but also medical treatment apparatuses and the hospital environment as well. CHG is able to kill many kinds of bacteria other than *Mycobacterium tuberculosis* and those in the form of bacterial spores. However, it is known that a certain species of *Pseudomonas* has resistance to CHG (Namba et al., 1985). In addition, there are some reports of a peculiar and specific phenomenon in the bactericidal activity of CHG against *P. aeruginosa* (Muto et al.,1984; Tsuji et al., 1986). To kill *P. aeruginosa* with CHG, it would seem that larger bacterial concentrations would require stronger solutions of CHG, or longer contact times in the case of the same CHG concentrations, for CHG to show its bactericidal effectiveness. We should reexamine the usage of CHG if the specific phenomenon involving *P. aeruginosa* is always observed. Then, we examined the bactericidal activity of CHG against *P. aeruginosa* by using two kinds of bactericidal test methods, which are called the customary PC method (AOAC, 1984) and the modified PC method which had been used when the specific phenomenon was first observed (Goto and Tsuji, 1980).

For the experiment 13 strains were used. Of these, there were two standard strains (NCTC 7244 and IFO 3445) and 11 strains isolated from clinical materials at the medical laboratory of Kitasato University Hospital, and all strains had the ability to produce slime substance. 5% (w/v) Hibitane solution(Lot no. ESO 58) was used as the CHG preparation.

The bactericidal effect of CHG against *P. aeruginosa* was examined at 20℃ by using *P. aeruginosa* NCTC 7244 which had an adjusted viable count. The viable count of the bacterial suspension for the modified PC method was adjusted to 10^4, 10^5, 10^6 and 10^7 cells/ml. The bactericidal effects of diluted CHG

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against these different viable counts were compared at the time when no growth of the organism was seen. At each cell concentration (10^4, 10^5, 10^6 and 10^7 cells/ml), *P. aeruginosa* NCTC 7244 could not be killed in 15 min by the disinfectant diluted to 5 times. The diluted CHG became the usual usage concentration (1:50) after being mixed with a bacterial suspension. This result was the same in the solution in which 5%(w/v) CHG solution was diluted to two times. The disinfectant diluted to 1/15 could kill *P. aeruginosa* at 10^4 cells/ml in 5 min after being mixed with a bacterial suspension, though it was not able to kill the bacteria at 10^5 cells/ml or greater even in 15 min. Bactericidal effects were seen by 5% (w/v) CHG solution in 4 or 5 min against the 10^7 cells/ml in one min against the 10^6 cells/ml suspension, in half of a minute to one min against the 10^5 cells/ml suspension, and within 30 sec against the 10^4 cells/ml cell concentration (Table 1). *P. aeruginosa* with low viable counts was killed by CHG in a short contact time compared with the time needed to kill high viable counts. That is, CHG needed a long contact time to kill *P. aeruginosa* with a high viable count.

TABLE 1. Relationship between the cell concentration of the test strain *Pseudomonas aeruginosa* (NCTC 7244) and the time in min for the diluted 5% (w/v) chlorhexidine digluconate to show bactericidal effects as found by the modified PC method.

Cell concn (cells/ml)	Dilution rate (×1)				
	10	15	20	25	50
10^7	4−5	>15	>15	>15	>15
10^6	1	>15	>15	>15	>15
10^5	0.5−1	>15	>15	>15	>15
10^4	0.5	3−5	>15	>15	>15

On one hand, the viable counts of cultured broth for the PC test method based on AOAC were adjusted to 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 cells/ml each. The greatest dilution rate of 5% (w/v) CHG solution which was able to kill 10^9 cells/ml of *P. aeruginosa* NCTC 7244 after contact for 10 min was 1/1200, and 1/2000 in case of 10^8 cells/ml, 1/3000 in case of 10^7 cells/ml, 1/5000 in case of 10^6 cells/ml, 1/8000 in case of 10^5 cells/ml and 1/10000 in case of 10^4 cells/ml (Table 2). The concentration of CHG which could kill *P. aeruginosa* NCTC 7244 when the bactericidal time is fixed increased as the viable count of organism increased.

The bactericidal time of CHG was examined by the modified PC method using 11 strains of *P. aeruginosa* which were isolated from clinical materials. When 5% (w/v) CHG solution was added to bacterial suspension made so that the viable count was adjusted to 10^7 cells/ml, no strain was killed after treatment for 15 min. When this solution was added to the 10^4 cells /ml bacterial suspension, there were 6 strains which were killed with 5 min of treatment. However, 4 strains were not killed even with treatment for 15 min (Table 3).

With the PC test based on AOAC, the greatest dilution rate of 5% (w/v) CHG solution which was able to kill 10^8 cells/ml of test organisms was from 1/400 to 1/1200, and differences in effective dilution rates of as many as 3 times existed for the different strains used. Bactericidal effects against 10^6 cells/ml of organisms were seen with dilutions of 1/4000 to 1/8000, and in case of 10^4 cells/ml they were seen with dilution of 1/8000 to 1/12000 (Table 4). Differences in the resistance of *P. aeruginosa* isolated from clinical materials against CHG did not appear in an obvious manner. However, large viable counts of *P. aeruginosa* were killed by high concentrations of CHG, and small viable counts were killed by low concentrations of CHG.

The phenol coefficient has been generally used to compare the bactericidal activity of the disinfectant. However, the phenol coefficient method has been heavily criticized. For example, Goto and Tsuji (1980) have pointed out four matters of concern regarding the PC method. These four problems were that the species of test strain must be limited, that it is not easy to understand on practical use dilution, that it should be possible to display the dilution rate of the disinfectant accurately, and that for some disinfectants the bactericidal activity decreases remarkably by using the cultured broth as it is. Therefore, some test methods have been designed (Goto and Tsuji, 1980; Jono et al., 1985). One such method is the modified PC method which had been used when the paradoxical bactericidal phenomenon was found, and is the method used in this experiment. The bactericidal activity of CHG was first reportd by Davies (Davies et al., 1954), since then, many research reports concerning the bactericidal activity of CHG have been published. In 1984, it was reported by Muto (1984) that the special bactericidal phenomenon of CHG against *P. aeruginosa* was due to slime production by *P. aeruginosa*. Moreover, in 1986, it was concluded by Tsuji et al. (1986) that the DNA in the slime produced by *P. aeruginosa* at high cell concentrations increased the sensitivity of the organism to CHG. It had already been known that *P. aeruginosa* produces a slime substance in liquid media (Haynes, 1951). However, this specific bactericidal phenomenon has not been confirmed by other researchers. Then, the

TABLE 2. Effect of the cell concentration of *Pseudomonas aeruginosa* (NCTC 7244) on the bactericidal activity of 5% (w/v) chlorhexidine digluconate as found by the PC method based on AOAC at 20℃.

Cell concn (cells/ml)	Time (min)	Dilution rate (×100)																
		4	8	10	12	15	16	20	30	40	50	60	70	80	100	120	140	200
10^9	2.5	−	+	*	+	*	+	+	*	*	*	*	*	*	*	*	*	*
	5.0	−	−	*	+	*	+	+	*	*	*	*	*	*	*	*	*	*
	10.0	−	−	*	−	*	+	+	*	*	*	*	*	*	*	*	*	*
	15.0	−	−	*	−	*	−	+	*	*	*	*	*	*	*	*	*	*
10^8	2.5	*	*	+	*	+	*	+	+	+	*	*	*	*	*	*	*	*
	5.0	*	*	−	*	−	*	+	+	+	*	*	*	*	*	*	*	*
	10.0	*	*	−	*	−	*	−	+	+	*	*	*	*	*	*	*	*
	15.0	*	*	−	*	−	*	−	−	+	*	*	*	*	*	*	*	*
10^7	2.5	*	*	+	*	*	*	+	+	+	+	*	*	*	*	*	*	*
	5.0	*	*	−	*	*	*	−	+	+	+	*	*	*	*	*	*	*
	10.0	*	*	−	*	*	*	−	−	+	+	*	*	*	*	*	*	*
	15.0	*	*	−	*	*	*	−	−	−	+	*	*	*	*	*	*	*
10^6	2.5	*	*	*	*	*	*	*	−	+	+	+	+	*	*	*	*	*
	5.0	*	*	*	*	*	*	*	−	−	+	+	+	*	*	*	*	*
	10.0	*	*	*	*	*	*	*	−	−	−	+	+	*	*	*	*	*
	15.0	*	*	*	*	*	*	*	−	−	−	−	+	*	*	*	*	*
10^5	2.5	*	*	*	*	*	*	*	*	−	*	+	*	+	+	+	*	*
	5.0	*	*	*	*	*	*	*	*	−	*	−	*	+	+	+	*	*
	10.0	*	*	*	*	*	*	*	*	−	*	−	*	−	+	+	*	*
	15.0	*	*	*	*	*	*	*	*	−	*	−	*	−	−	+	*	*
10^4	2.5	*	*	*	*	*	*	*	*	*	*	−	*	+	+	*	+	+
	5.0	*	*	*	*	*	*	*	*	*	*	−	*	−	+	*	+	+
	10.0	*	*	*	*	*	*	*	*	*	*	−	*	−	−	*	+	+
	15.0	*	*	*	*	*	*	*	*	*	*	−	*	−	−	*	−	+

+, Growth; −, no growth. *, Not tested.

TABLE 3. Time needed for bactericidal effects of 5% (w/v) chlorhexidine digluconate to be seen against clinical isolated strains of *Pseudomonas aeruginosa* according to the modified PC method.

Strain no.	Cells per ml		Strain no.	Cells per ml	
	10^7	10^4		10^7	10^4
2589	>15	>15	2879	>15	4
2656	>15	2	2882	>15	≧15
2739	>15	1	2892	>15	15
2740	>15	2	2911	>15	1
2849	>15	1	3012	>15	>15
2853	>15	>15			

reconfirmation was atetempted there by using two test methods: a PC method based on AOAC and the modified PC method by which had been used when the specific bactericidal phenomenon was found. As a result, the differences in sensitivity of *P. aeruginosa* to CHG appeared according to the test strains regardless of which examination method was used, but it was not possible to reconfirm the specific bactericidal phenomenon of CHG against the organism, since CHG needed a long time to kill high concentrations of the organism and a short time to kill low cell concentrations. Because the specific bactericidal phenomenon of CHG had not been reproduced despite using strains isolated not only from standard strains (NCTC 7244, IFO 3445) but also from clinical materials, we were unable to examine the action of DNA in slime produced by *P. aeruginosa*.

TABLE 4. The greatest dilution rate[a] of 5% (w/v) chlorhexidine digluconate to kill 11 clinically isolated strains of *Pseudomonas aeruginosa* as evaluated by the PC test method based on AOAC at 20℃.

Strain no.	Cells per ml			Strain no.	Cells per ml		
	10^8	10^6	10^4		10^8	10^6	10^4
2589	×800	×8000	×12000	2879	×1200	×4000	×12000
2656	×400	×5000	×8000	2882	×800	×6000	×10000
2739	×400	×6000	×12000	2892	×1200	×6000	×12000
2740	×1200	×8000	×12000	2911	×1200	×6000	×10000
2849	ND[b]	×6000	×8000	3012	×800	×4000	×10000
2853	×1200	×6000	×8000				

[a] Growth of the test organism was evident within 5 min of contact, but not after 10 min.

[b] Not done. This strain did not proliferate even at a cell concentration necessary for the usual cultivation.

There was a big difference in the evaluation of the bactericidal activity of CHG against *P. aeruginosa* as examined by the two methods. With the modified PC method, the concentration of CHG able to kill 10^7 cells/ml of *P. aeruginosa* in 5 min was 5000 μg/ml, but it was only 25 μg/ml with the PC method based on AOAC. It was reported by Satuta et al. (1983) that the bactericidal activity of CHG against *P. aeruginosa* was not able to expected. In that report, they adopted the same examination method as the modified PC method. Their method, like ous, also involved adding nine portions of bacterial suspension to one portion of diluted disinfectant. When the volume of bacterial suspension was far larger than the volume of the diluted CHG solution like that in the modified PC method, it seemed that the organism was not sensitized easily by a drug such as CHG.

When the bactericidal activity of the disinfectant is evaluated, the differences in the evaluation caused by examination method used confuse the persons who try to use the disinfectant. Therefore, careful consideration is necessary when the evaluation method of bactericidal activity is newly designed.

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