

Flow Cytometry Analysis of the Activity of Disinfecting Agents Tested with *Staphylococcus aureus* and *Escherichia coli*

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Abstract

The efficacy of five disinfecting agents was tested by a new microbiological method, *i.e.* the flow cytometry. The method is based on kinetic measurements of the effects of a disinfecting agent on the percent of live/dead cells detected with propidium iodide. *E. coli* ATCC52922 strain and *S. aureus* ATCC 29213 strain were used in the experiment. From the measurements the killing time 50 (KT50) was estimated as the period of time needed to kill 50% of microorganisms in the disinfected volume. KT50 for ethanol 70% was 11s and it was the most efficient disinfecting agent among all examined. Other commercial preparations were compared with ethanol 70% and were traced throughout the period of 5 min. The results were obtained rapidly, frequently in less than 10 min. In conclusion, the effectiveness of a particular antibacterial disinfectant preparation may be estimated quantitatively within a few minutes by the flow cytometry. The method proved to be very useful for a fast comparison of the effectiveness of various disinfectant preparation against pathological microorganisms.

Key words: Flow cytometry, disinfecting agents, *Escherichia coli*, *Staphylococcus aureus*, propidium iodide

Introduction

The definition of a widely recognized disinfection characterizes the process as a destruction of the pathogenic microorganisms found on the surfaces of medical instruments and parts of hospital equipment. Despite the enormous progress in the management of infectious diseases, the problem of nosocomial infection spread has to be controlled steadily. The washing and disinfection of the hands and skin, medical instruments, parts of the equipment, working surfaces and floors with a view to eradicating pathogenic microorganisms are the essential elements of sanitary hygiene in the hospital and the physician's office. The disinfectants are normally not active over the whole spectrum of microorganisms and may be inactivated by the presence of organic substances (*e.g.* blood). The disinfectants may be highly toxic and pose a health risk to the medical personnel or the patient (Spicher, 1998).

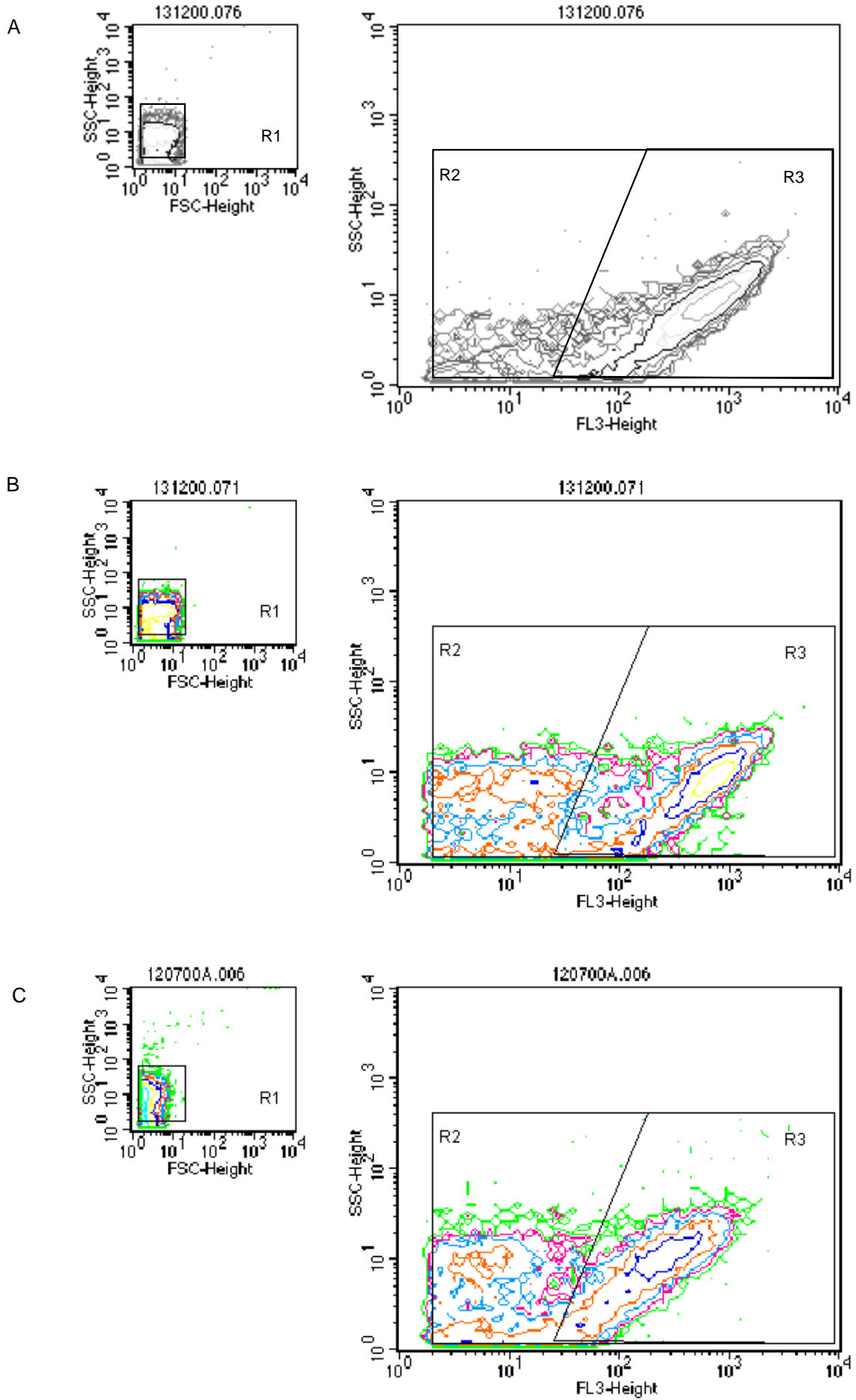
An ideal disinfectant should: -have the ability to eliminate completely all pathogenic microorganisms, -be efficient in a short time, -be safe for humans and animals, -not adversely affect the environment, -remain active in the presence of organic substances (*e.g.* blood, pus), -do not have an unpleasant odour, -have as wide range of applications as possible (Ozcan *et al.*, 2003; Sander *et al.*, 2002).

Two basic kinds of disinfection are distinguished: the chemical disinfection and heat disinfection. In the chemical disinfection, the active chemical agents serve as disinfecting factors. By combining various active substances the gaps in the preparations of antimicrobial spectrum of activity can be avoided.

The heat disinfection is a simple, quick and safe method to use and control. The inactivation of microorganisms is achieved through exposing them to the high temperature and water steam. This method can be employed for the devices and materials resistant to heat and for disinfecting bed linen and medical personnel's white coats. The remaining disinfection procedures are carried out using bactericidal chemical substances.

The traditional methods of bacteriology require the isolation of the bacteria prior to identification and other testing procedures. In most cases the bacterial culture results are available within 48 to 72 h

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(Alvarez-Barrientos *et al.*, 2000; Woźniak-Kosek *et al.*, 2003). The flow cytometry (FCM) makes it possible to detect single or multiple bacteria in samples in an easy and reliable way. The bacteria can be identified (live/dead) on the basis of their light scatter cytometric parameters and by the binding of certain fluorochromes. (Woźniak-Kosek *et al.*, 2003). The purpose of the experiment was a comparison of different disinfecting agents activity on the viability of *Escherichia coli* and *Staphylococcus aureus* as model organisms by the flow cytometry. The damaged cells were identified by the fluorescence of propidium iodide (PI) after its binding to the bacterial DNA.

Experimental

Materials and Method

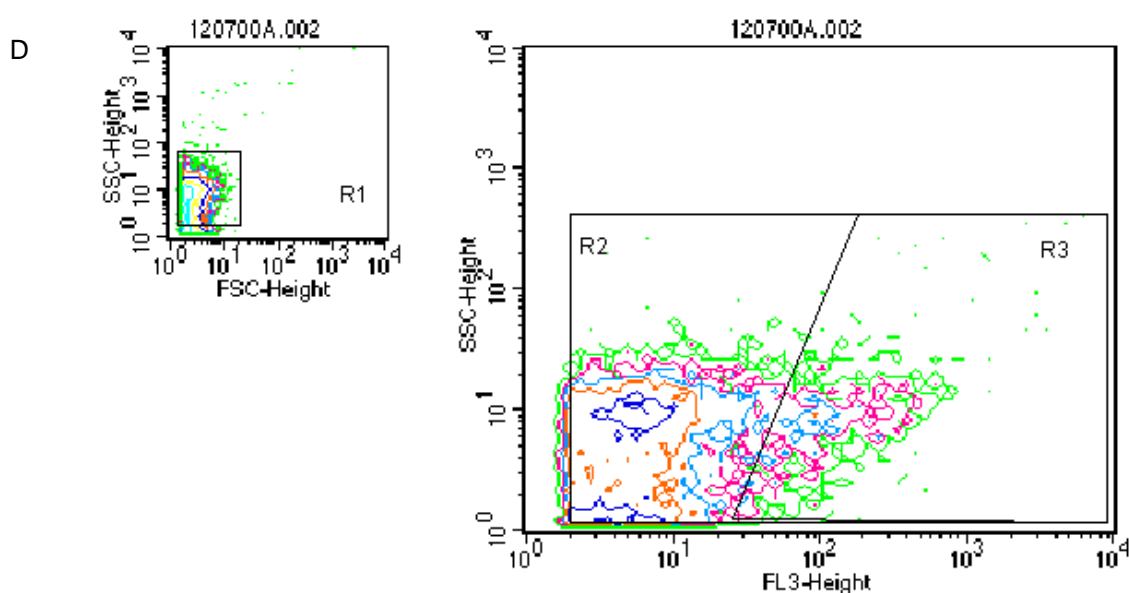
Bacteria. In the present studies *E. coli* ATCC 52922 strain and *S. aureus* ATCC 29213 strain were used. The culture of bacteria in a nutrient broth medium was incubated at 35°C for 24 h. Then the bacteria were transferred on broth 3x and finally transferred on the Agar for the additional 24 h and cultured at 35°C. For the flow cytometry the bacteria were transferred from agar to sterile water. The bacteria suspension density 0.625 was set with the use of the Beckman spectrophotometer at $\lambda = 600$ nm, an equivalent of 10^9 CFU/ml of bacteria concentration (Woźniak-Kosek *et al.*, 2002). For the analysis, the suspension of bacteria was diluted to the 10^5 CFU/ml concentration.

Disinfecting agents. The following disinfecting agents were tested: Substance number 1–70% and 50% ethanol at the final concentration used for surgical disinfection of hands, objects and surfaces; substance number 2–50% isopropanol at the final concentration used for the disinfection of small, difficult of access objects and surfaces; substance number 3 – the commercial preparation containing ethanol, isopropanol, benzyl alcohol, glycerin, demineralized water, used for the surgical disinfection of hands; substance number 4 – the commercial preparation containing ethanol, greasing substance, ethereal oils used for the disinfection of small, difficult of access objects and surfaces; substance number 5 – the commercial preparation containing ethanol, isopropanol, glutaric aldehyde used for the disinfection of small, difficult of access objects and surfaces.

Preparation of samples for cytometry. 0.5 ml of bacteria suspension and 5 μ l of propidium iodide solution (PI) (500 μ g/ml) were added to the cytometric tubes. After 10 min of incubation at room temperature and aquisition of zero time sample, the tested preparation was added. For comparison of the result with the activity of 70% ethanol, 1.5 ml of 99% ethanol was added to one tube with 0.5 ml of the bacteria suspension. The kinetic activity of the tested preparation was traced by the aquisition of samples every 20 s (first 3 samples) followed by samples aquisition every 1 min for the total time of 5 min.

Cytometric analysis. Light scatter parameters (FSC and SSC) and the fluorescence of propidium iodide were analysed using FACSCalibur flow cytometer (Becton Dickinson) equipped with an argon ion laser (488 nm). The FSC and SSC were set on logarithmic amplification. List mode data were collected for 10 000 events with the event rate amounting to 200–600 per second. The instrument cleaning cycle with water, detergent and ethanol was a routine procedure before and after the aquisition of the cells.

For the analysis of viable/dead cells CELLQuest, version 1.2 software system was used. The percent of dead bacteria was read as PI+ events against the control sample of live cells (the first sample in the kinetic measurements). The readings were taken from FL3/SSC graphs after the bacteria gating according to FSC and SSC parameters.



Figs. 1. Flow cytometry analysis of the viability of bacteria with PI in mixed populations of live and heat killed *S. aureus* or *E. coli* bacteria. The populations were read in a constant FSC/SSC gate. Compare the results in Table 1. FL3 is fluorescence of PI. A. 91.0% of *S. aureus* dead cells, B. 48.4% of *S. aureus* dead cells, C. 52.5% of *E. coli* dead cells, D. 8.5% of *E. coli* dead cells

Results

The flow cytometry method was standardized with the use of mixtures of live and killed (80°C for 15 min) bacteria (Fig. 1). The samples containing 100, 90, 50, 40 and 10 % of “live” cells were analysed. To 1 ml of the bacteria mixture 5 µl of PI was added and the acquisition was started after 10 min of incubation at room temperature. The results of *E. coli* or *S. aureus* live/dead mixed suspensions are presented in the Table I. A temperature of 80°C kills 95% of bacteria in 15 min. A small percentage of dead cells in each “live” bacteria suspension was observed routinely. The results of cytometric reading were with the error of 1.2% for *E.coli* and 3.1% for *S. aureus*.

The crucial problem in examining disinfecting agent is the time after which the disinfected area sterility is achieved. From the kinetics, Killing Time 50 (KT 50) was estimated as the period needed to kill 50% of microorganisms in the disinfected volume (area). The most efficient disinfecting agent among all examined was 70% ethanol. It proved to be effective for both *E. coli* and *S. aureus* (Figs 3, 4). KT50 for ethanol 70% was 11 s and over 98% of bacteria were dead after 5 min (Fig. 2). The result of KT50 for 70% ethanol was compared with KT50 for other disinfecting preparations (Table II). Ethanol 50% was not as effective as ethanol 70%. KT50 for anti *S. aureus* was 2 min. and for anti *E. coli* 5 min. The substance number 3 in its final 50% concentration was efficient *S. aureus*. KT50 was 40 s, but it was not as effective *E. coli* (KT50 240 s).

The effect of various disinfecting agents on *E. coli* viability is presented in Fig. 3. Substance number 5 in its final 50% concentration was as effective with *E. coli* as 70% ethanol. KT50 for both disinfecting agents was about 11 s and after 5 min almost all bacteria were dead. Isopropanol had a similar effect, causing the death 95% of bacteria. However, KT50 was 60 s, which suggests that isopropanol is a considerably slower killing agent. The substance number 5 in its final 50% concentration killed 53% of *S. aureus* bacteria

Table I
Readings of the dead bacteria in the mixtures of live and heat killed *S. aureus* and *E. coli*

% of dead cells in the mixture (A)	Reading of dead cells % of <i>S. aureus</i> (B)	Difference between theoretical and empirical value of dead cells % of <i>S. aureus</i> (B-A)	Reading of dead cells % of <i>E. coli</i> (C)	Difference between theoretical and empirical value of dead cells % of <i>E. coli</i> (C-A)
100	87.7	-12.3	94.9	-5.1
90	91.0*	+1.0	82.3	-7.7
80	74.5	-5.5	75.8	-4.2
60	51.7	-8.3	61.1	+1.1
50	48.4*	-1.6	52.5*	+2.5
40	42.3	+2.3	41.8	+1.8
20	20.2	+0.2	23.8	+3.8
10	9.8	-0.2	8.5*	-1.5

* Reading presented in the Fig.1

Table II
Relative effectiveness of the different disinfectant preparations

Disinfecting agents	Final concentration	<i>S. aureus</i>		<i>E. coli</i>	
		KT50 (seconds)	Relative KT50*	KT50 (seconds)	Relative KT50*
Substance number 1	70% ethanol	11	1.0	11.0	1.0
Substance number 1	50% ethanol	120	10.9	300.0	27.3
Substance number 2	50% isopropanol	60	5.5	60.0	5.5
Substance number 3	50% commercial concentration	40	3.6	240.0	21.8
Substance number 4	50% commercial concentration	80	7.3	144.0	13.1
Substance number 5	50% commercial concentration	11	1.0	11.0	1.0

* as compared with the activity of 70% ethanol

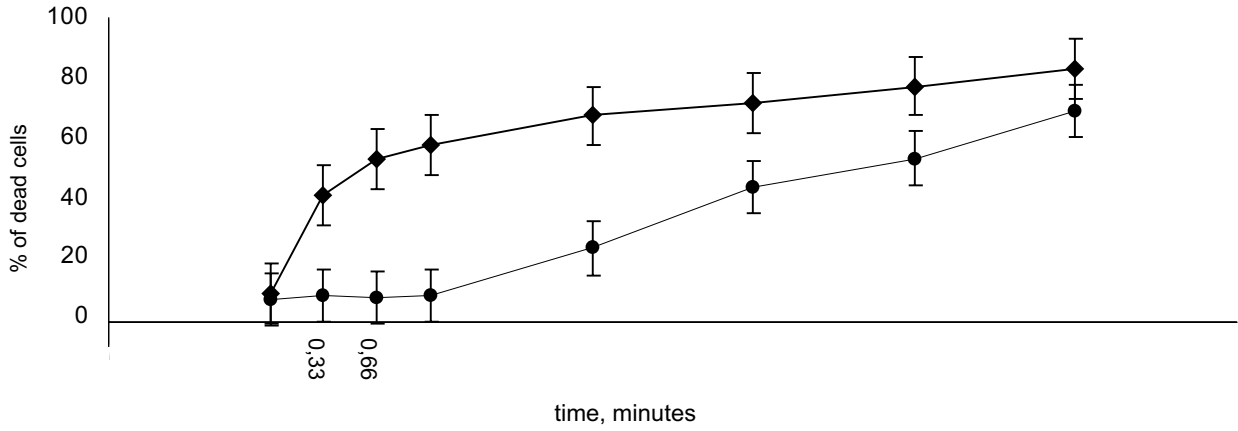


Fig. 2. Effectiveness substance 1 (Ethanol 50%), tested with *S. aureus* or *E. coli*

◆ *S. aureus* ● *E. coli*

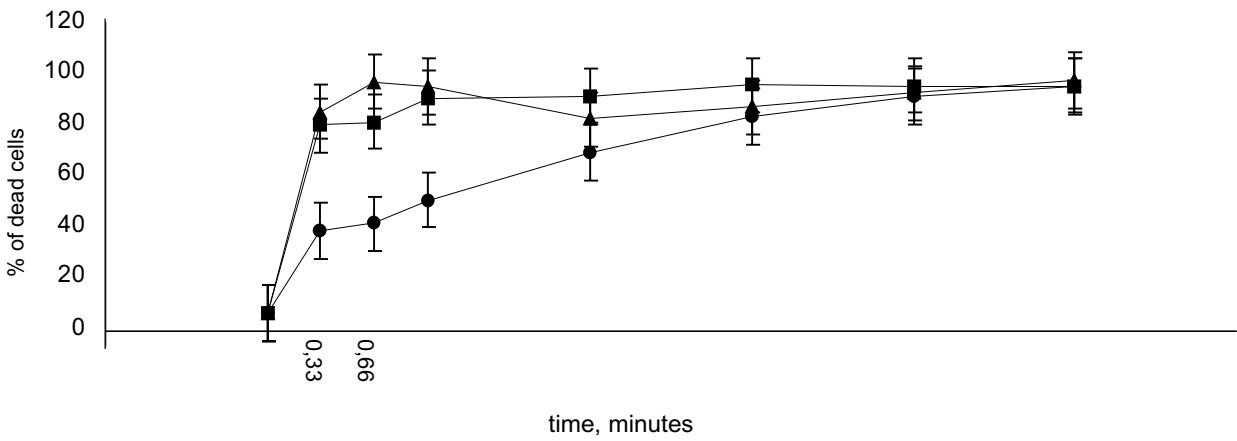


Fig. 3. The kinetic assay of the effectiveness of disinfecting agents tested with *E. coli*.

▲ substance 1 (70%) ● substance 2 (50%) ■ substance 5 (50%)

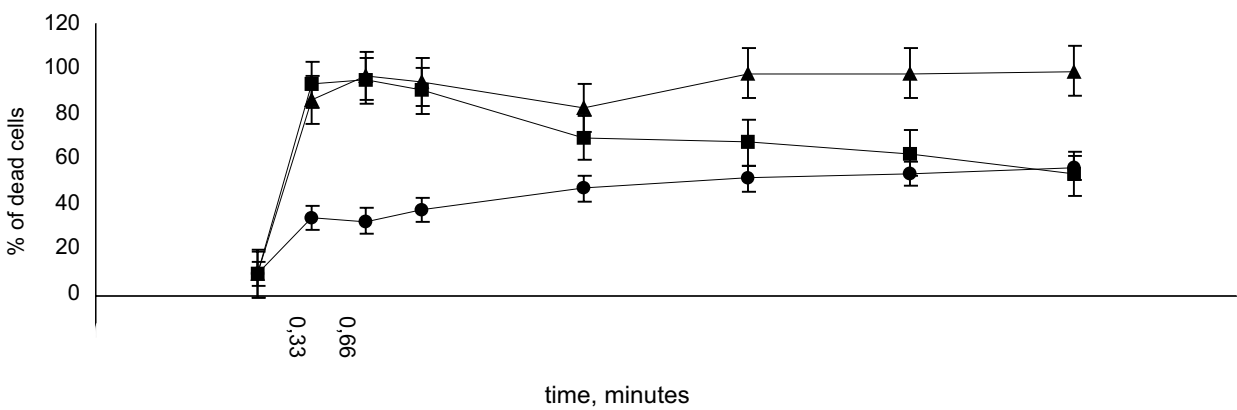


Fig. 4. The kinetic assay of the effectiveness of disinfecting agents tested with *S. aureus*.

▲ substance 1 (70%) ● substance 2 (50%) ■ substance 5 (50%)

after 5 min, while its KT50 is 11 s. This suggests that the disinfecting agent is damaging the cells only partially without killing them, to which a longer time (more than 5 min) would be required.

Isopropanol killed 53% of *S.aureus* cells after 5 min. The initial effect of isopropanol on these cells was slow, but percentage of dead bacteria cells was rising with time. The effect of substances 1, 2, and 5 is

presented in Fig. 4. Substance 4 proved not to be very effective. After 5 min there were 88% of dead *S. aureus* bacteria cells and 73% of dead *E. coli* bacteria cells. KT50 for *S. aureus* was 80 s and for *E. coli* 144 s, respectively.

Discussion

The selection of the appropriate disinfectant is often difficult and requires an extensive specialist knowledge. For the purpose of our study we selected the preparations that are used in the hospital practice and compared their efficiency with the activity of 70% ethanol proved as the most effective disinfectant. The analyzes were carried on the representative gram-positive (G+) and gram-negative (G-) microorganisms (Alcamo, 1998). We demonstrated the differences in the killing efficiency of some disinfectants. The fast observation of the disinfectant preparation effect was possible due to the measurements based on single cell readings by the flow cytometry (Braga *et al.*, 2003; Mortimer *et al.*, 2000; Novo *et al.*, 2002). The bacterial cell damage caused by the thermal treatment or by a chemical agent induces the changes occurring in the cell membrane which results in a diffusion of fluorochrome inside the cell (Jernaes *et al.*, 1994).

The kinetic measurements of the effects of various disinfecting agents within a short period of time is one of the advantages of flow cytometry. The quantitative estimation of Killing Time 50 (KT50) cannot be achieved with standard microbiological methods in a short period of time. We could not find in the bibliography a similar quantitative measure of disinfectant effectiveness which may be estimated within a 10 min. An incorrect selection and/or use of disinfectants, similarly as antibiotics, may be harmful to humans, as well as materials and hospital equipment. In addition, a disinfectant may exhibit an insufficient bactericidal activity against pathogenic microorganisms (Gant *et al.*, 1993; Lindner *et al.*, 2002; Shapiro 2001). With the use of the fast method described here, the activity of the various disinfectant preparations against a particular group of microorganisms may be estimated within a short period of time.

In conclusion, the effectiveness of a particular antibacterial disinfectant preparation may be estimated quantitatively within a few minutes by the flow cytometry. The method effectiveness makes it possible to compare the various disinfecting preparations against microorganisms in a fast and easy way.

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