Evaluation of Newly Developed Oxygen Meters with Multi-Channels and Disposable Oxygen Electrode Sensors for Antimicrobial Susceptibility Testing

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The objective of this study was to investigate the applicability of new oxygen meters with multi-channels and disposable oxygen electrode sensors (DOX-96) on the antimicrobial susceptibility testing of clinical bacterial isolates. The oxygen amount in the wells of 96-well plates was converted into current through electrodes. Bacterial inoculation decreased the oxygen amount in the wells because viable bacteria consume the oxygen. On the other hand, a failure of bacteria to consume oxygen was observed in the presence of potent antimicrobial agents, representing a serious arrest of bacterial metabolism usually leading to stasis or death. Based on these results, the minimum inhibitory concentration was determined by DOX-96 (MICdox). The MICdox showed good agreement with MIC measured by the standard broth microdilution method (98.2%). DOX-96 was also useful for turbid samples such as Mueller-Hinton broth containing 0.1% lipid emulsion. The MICdox in turbid samples showed good agreement with those in clear samples (100.0%). These results indicate that the newly developed DOX-96 is very useful in antimicrobial susceptibility testing even in turbid clinical samples such as colloidal products and turbid biological components.

Key words oxygen electrode; antimicrobial susceptibility testing; oxygen meter; minimum inhibitory concentration; lipid emulsion

The rapid and accurate diagnosis of bacterial infection is of vital importance so that appropriate antimicrobial therapy can be initiated. The broth microdilution method for determining the susceptibility of bacteria to a particular antimicrobial agent is commonly used to obtain reference values.[1)] In this standard procedure for determining the minimum inhibitory concentration (MIC), microplates are incubated overnight for about 16 to 20 h and then read for turbidity.[1)] The disk diffusion method is also used for determining susceptibility.[2,3)] The preceding two methods are the reference standards by which all other procedures are assessed.[4—6)]

There have already been many reports about the application of several types of electrode to bacterial susceptibility testing.[4,7—10)] Amano *et al.* have developed new type of oxygen meters with multi-channels and inexpensive disposable oxygen electrode sensors (DOX-96; DAIKIN ENVIRONMENTAL LABORATORY, LTD., Tsukuba, Japan).[11)] The electrode for DOX-96 is a 96-well plate with three electrodes embedded in each well. The oxygen amount in the well is converted into current through electrodes and the current is drawn on a graph with a laptop computer. The assay procedure is quite similar to that for optical measurements with the conventional 96-well microplate.

In the present study, we investigated the applicability of DOX-96 on the antimicrobial susceptibility testing of clinical bacterial isolates. To address this point, we compared the results obtained by DOX-96 with those by the standard broth microdilution method. In addition, the broth microdilution method cannot be applied to turbid samples because of its optical set-up and the disk diffusion method is qualitative and still insufficient for automated convenience. Therefore, we also focused on measuring oxygen consumption by bacteria using oxygen electrodes as an accurate and convenient method of antimicrobial susceptibility testing for turbid samples.

MATERIALS AND METHODS

Bacterial Isolates *Escherichia coli* ATCC25922 and 15 clinical isolates were used. The clinical isolates were collected in Nagasaki University School of Medicine Hospital, an 829-bed hospital in Nagasaki, Japan. The 15 strains: *Staphylococcus aureus* (2 isolates), *Staphylococcus epidermidis* (1 isolate), *Staphylococcus capitis* (1 isolate), *Enterococcus faecalis* (1 isolate), *Enterococcus avium* (1 isolate), *Enterococcus faecium* (1 isolate), *Escherichia coli* (1 isolate), *Klebsiella pneumoniae* (1 isolate), *Enterobacter cloacae* (1 isolate), *Serratia marcescens* (1 isolate), *Proteus mirabilis* (1 isolate), *Pseudomonas aeruginosa* (1 isolate), *Acinetobacter baumannii* (1 isolate), and *Stenotrophomonas maltophilia* (1 isolate).

Antimicrobial Agents The following antimicrobial agents were tested: vancomycin (VCM; Shionogi Pharmaceutical Co., Osaka, Japan), cefazolin (CEZ; Fujisawa Pharmaceutical Co., Osaka, Japan), ampicillin (ABPC; Meiji Seika Co., Tokyo, Japan), piperacillin (PIPC; Toyama Chemical Co., Tokyo, Japan), cefoperazone-sulbactam (SBT/CPZ; Pfizer Pharmaceuticals Inc., Tokyo, Japan), piperacillin-tazobactam (TAZ/PIPC; Toyama Chemical Co.), aztreonam (AZT; Eisai Co., Tokyo, Japan), ceftazidime (CAZ; Tanabe Pharmaceutical Co., Osaka, Japan), cefpirome (CPR; Chugai Pharmaceutical Co., Tokyo, Japan), imipenem (IPM; Banyu Pharmaceutical Co., Tokyo, Japan), gentamicin (GM; Schering-Plough Co., Osaka, Japan), minocycline (MINO; Wyeth-Lederle Co., Tokyo, Japan), ciprofloxacin (CPFX; Bayer Yakuhin Co., Tokyo, Japan), and levofloxacin (LVFX; Dai-

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ichi Pharmaceutical Co., Tokyo, Japan).

DOX-96 System Figure 1 shows the structure of the electrode for DOX-96. The oxygen amount in the sample is converted into current by the following the reaction, $4H^+ + O_2 + 4e^- = 2H_2O$, and the current is drawn on a graph with a laptop computer.[11] As viable bacteria consume the oxygen, the oxygen amount in the sample decreases. The electrode for DOX-96 is a 96-well plate with three electrodes embedded in each well.

MIC Determination by Microdilution Method MIC was determined by a microdilution method with cation-adjusted Mueller–Hinton broth (BBL Microbiology Systems, Cockeysville, MD, U.S.A.) according to the recommendations of the National Committee for Clinical Laboratory Standards.[12]

MIC Determination by DOX-96 System MIC was determined by DOX-96 (MICdox). Antimicrobial agents were diluted two-fold in Mueller–Hinton broth, and dispensed into the wells (100 μl/well) of an electrode plate. Fresh Mueller–Hinton broth (90 μl) was added to the wells. Antimicrobial agent concentrations ranged from 0.5 to 16 μg/ml. All the wells except the negative control wells were inoculated with 10 μl of each bacterium in Mueller–Hinton broth to yield a final inoculum size of 1×10^5 colony-forming units (CFU)/ml. The negative control wells received 10 μl of Mueller–Hinton broth only. The positive control wells received Mueller–Hinton broth instead of antimicrobial agent. The electrode plate was set on DOX-96 and was incubated for 999 min (16.65 h) at 35 °C. The measurement of the current in each well when compared with their respective positive and negative controls was taken as oxygen consumption. If bacteria exist in the sample, remarkable oxygen consumption was observed within 999 min (16.65 h) by proliferation of bacteria. The remarkable oxygen consumption was suppressed by bactericidal activity of antimicrobial agent. The lowest concentration of antimicrobial agent at which the remarkable oxygen consumption was suppressed during 999 min (16.65 h) was determined as MICdox.

Measurement in Turbid Solution The turbid solution was prepared by the addition of a lipid emulsion (Intralipid®, Otsuka Pharmaceutical Co., Tokyo, Japan) to each well at 0.1%. Oxygen consumption was measured by the method described above.

RESULTS

Measurement of Oxygen Consumption Using DOX-96 Figure 2A shows the current profiles of samples with or without *Escherichia coli* ATCC25922 determined by DOX-96. The profile in the negative control without *Escherichia coli* ATCC25922 showed a two-phase elimination in oxygen consumption. The electrodes embedded in the 96-well plate were stabilized and reached equilibrium within 60 min at the first phase. An addition of *Escherichia coli* ATCC25922 into the samples decreased the oxygen amount in the well because viable bacteria consumed the oxygen. The current rapidly declined at the second phase. The differential profiles of current curves are shown in Fig. 2B. The peaks in Fig. 2B indicate the time points of remarkable oxygen consumption. An increase in the inoculum size of *Escherichia coli* ATCC25922 shortened the time to reach the peak.

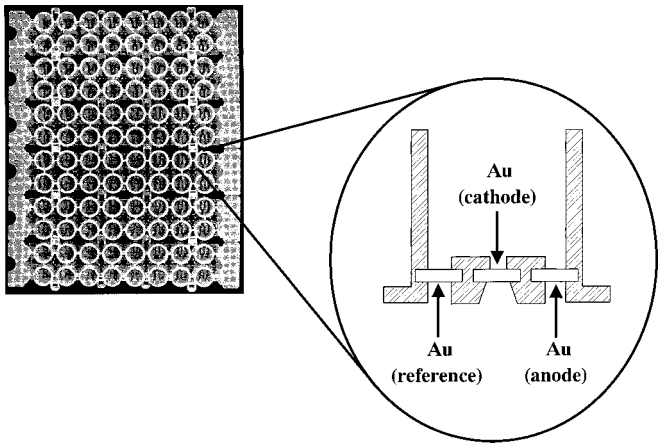

Fig. 1. Structure of the Electrode for DOX-96

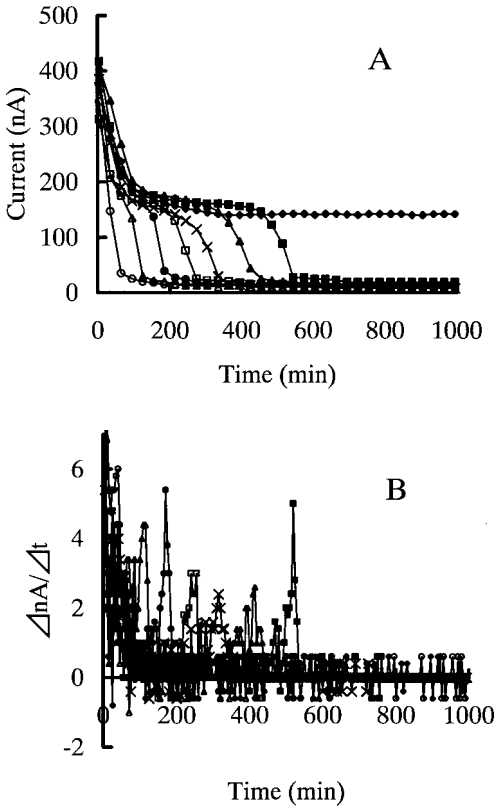

Fig. 2. Current Profiles of Oxygen Consumption of *Escherichia coli* ATCC25922 Determined by DOX-96 (A) and Differential Profiles of Current Curves (B)

Inoculum size was 1×10 CFU/ml (■), 1×10^2 CFU/ml (▲), 1×10^3 CFU/ml (×), 1×10^4 CFU/ml (□), 1×10^5 CFU/ml (●), 1×10^6 CFU/ml (△), 1×10^7 CFU/ml (○) and none (negative control, ◆).

Figure 3 shows the relationship between the time when remarkable oxygen consumption occurred and the logarithmic value of the inoculum size of *Escherichia coli* ATCC25922. The time when remarkable oxygen consumption occurred highly correlated with the logarithmic value of the inoculum size ($R^2=0.996$) in the range of 1×10 to 1×10^7 CFU/ml. The average of slope is 31.5 ± 1.4 (average±S.D., Cv=4.6%).

Effect of Antimicrobial Agents The effects of antimicrobial agents on the oxygen consumption by bacteria were

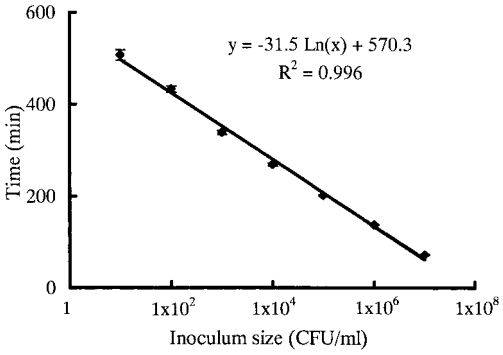

Fig. 3. Relationship between the Time When Remarkable Oxygen Consumption Occurred and the Logarithmic Value of Inoculum Size of *Escherichia coli* ATCC25922

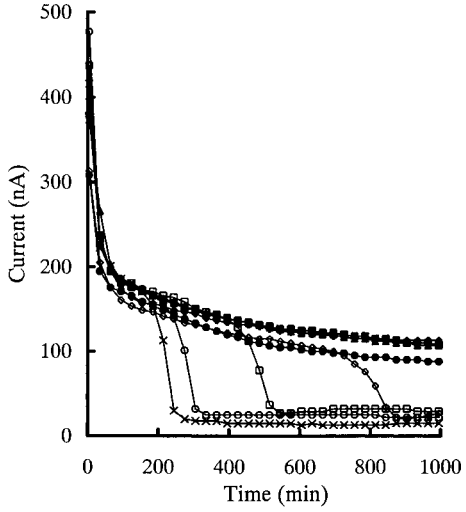

Fig. 4. Effect of MINO on Oxygen Consumption of *Serratia marcescens*

Concentrations of MINO were 16 μg/ml (◆), 8 μg/ml (▲), 4 μg/ml (●), 2 μg/ml (◇), 1 μg/ml (□), 0.5 μg/ml (○), 16 μg/ml without *Serratia marcescens* (negative control, ■) and none (positive control, ×).

determined with DOX-96. The effect of MINO on the oxygen consumption of *Serratia marcescens* as current profiles is shown in Fig. 4 as a typical example. An increase in the concentration of MINO lengthened the time to reach remarkable oxygen consumption. Remarkable oxygen consumption was not observed at the concentrations of MINO over 4 μg/ml during 999 min (16.65 h), as well as in the negative control without bacteria. Therefore, 4 μg/ml was considered to be MICdox of MINO for the *Serratia marcescens* isolate.

The MICdox and MIC of 11 antimicrobial agents for 15 clinical bacterial isolates were determined by oxygen consumption using DOX-96 and by the standard broth microdilution method, respectively, and those results are shown in Table 1. Complete agreement of MICdox with MIC was 80.0% (132 data/165 data) and agreement within a two-fold difference was 18.2% (30 data/165 data). The overall agreement of MICdox with MIC was 98.2%.

Effect of Turbid Sample Figure 5 shows the effect of CPR on the oxygen consumption by *Pseudomonas aeruginosa* as current profiles in Mueller–Hinton broth with or without 0.1% lipid emulsion. Addition of 0.1% lipid emulsion caused the Mueller–Hinton broth to become turbid but did not affect the current profiles of *Pseudomonas aeruginosa* in the presence of CPR.

The MICdox of 14 antimicrobial agents for 5 clinical isolates were determined in Mueller–Hinton broth with or without 0.1% lipid emulsion by oxygen consumption using DOX-96. The results in the turbid sample are compared with those in the clear sample in Table 2. Complete agreement of MICdox in the turbid sample with those in the clear sample was 87.1% (61 data/70 data) and agreement within a two-fold difference was 12.9% (9 data/70 data). The overall agreement of MICdox in the turbid sample with that in the clear sample was 100.0%.

DISCUSSION

A conventional polarographic oxygen electrode is composed of electrodes, an electrolytic solution and an oxygen penetrating membrane. Measurement is carried out in stirred

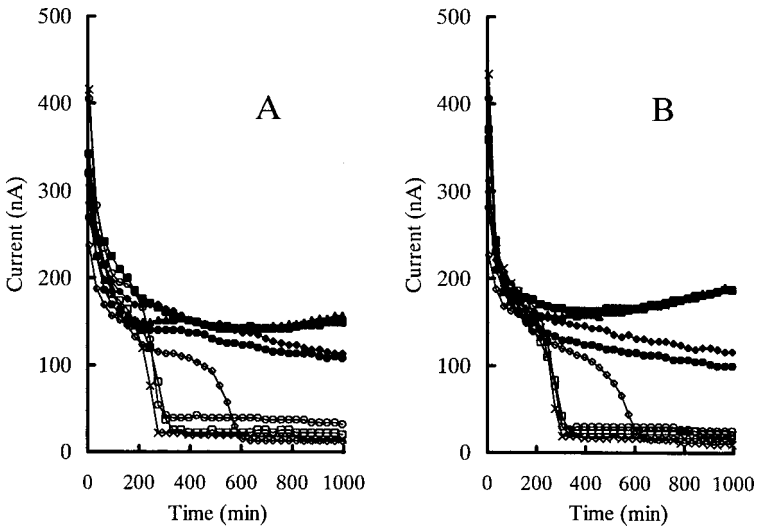

Fig. 5. Effect of CPR on Oxygen Consumption of *Pseudomonas aeruginosa* in a Clear Sample (A) and a Turbid Sample (B)

The clear sample was Mueller–Hinton broth only. The turbid sample was Mueller–Hinton broth including 0.1% lipid emulsion. Concentrations of CPR were 16 μg/ml (◆), 8 μg/ml (▲), 4 μg/ml (●), 2 μg/ml (◇), 1 μg/ml (□), 0.5 μg/ml (○), 16 μg/ml without *Pseudomonas aeruginosa* (negative control, ■) and none (positive control, ×).

Table 1. Comparison of MICdox (Determined by DOX-96) and MIC (Determined by the Broth Microdilution Method) of 11 Antimicrobial Agents for 15 Clinical Bacterial Isolates

Isolate	Method	MIC (μg/ml)											Complete agreement	Two-fold difference	Four-fold difference
		ABPC	PIPC	CEZ	CPR	AZT	IPM	SBT/CPZ	GM	VCM	MINO	LVFX			
Escherichia coli	DOX-96 (D)	1	≦0.5	1	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	2	≦0.5	8/11	3/11	
	Microdilution (M)	2	1	1	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	4	≦0.5			
Klebsiella pneumoniae	D	>16	4	2	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	4	≦0.5	10/11	1/11	
	M	16	4	2	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	4	≦0.5			
Enterobacter cloacae	D	>16	1	>16	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	2	≦0.5	9/11	2/11	
	M	16	2	>16	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	2	≦0.5			
Serratia marcescens	D	>16	1	>16	≦0.5	≦0.5	≦0.5	4	≦0.5	>16	4	≦0.5	8/11	3/11	
	M	16	1	>16	≦0.5	≦0.5	≦0.5	2	≦0.5	>16	2	≦0.5			
Proteus mirabilis	D	1	≦0.5	4	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16	16	4	9/11	2/11	
	M	≦0.5	≦0.5	4	≦0.5	≦0.5	≦0.5	≦0.5	1	>16	16	4			
Pseudomonas aeruginosa	D	>16	4	>16	4	8	1	16	≦0.5	>16	8	≦0.5	10/11	1/11	
	M	>16	4	>16	4	8	1	16	1	>16	8	≦0.5			
Acinetobacter baumannii	D	8	16	>16	2	>16	≦0.5	4	≦0.5	>16	≦0.5	≦0.5	11/11		
	M	8	16	>16	2	>16	≦0.5	4	≦0.5	>16	≦0.5	≦0.5			
Stenotrophomonas maltophilia	D	>16	>16	>16	>16	>16	>16	>16	16	>16	≦0.5	≦0.5	10/11	1/11	
	M	>16	>16	>16	>16	>16	>16	>16	16	>16	≦0.5	1			
Staphylococcus aureus (MSSA)	D	≦0.5	≦0.5	1	≦0.5	>16	≦0.5	8	≦0.5	≦0.5	≦0.5	≦0.5	9/11	2/11	
	M	≦0.5	≦0.5	≦0.5	≦0.5	>16	≦0.5	4	≦0.5	≦0.5	≦0.5	≦0.5			
Staphylococcus aureus (MRSA)	D	4	8	8	8	>16	≦0.5	8	>16	≦0.5	1	>16	7/11	3/11	1/11
	M	8	8	16	8	>16	≦0.5	16	>16	≦0.5	4	>16			
Staphylococcus epidermidis	D	16	>16	>16	16	>16	>16	16	>16	1	≦0.5	4	8/11	3/11	
	M	16	>16	>16	>16	>16	>16	>16	>16	2	≦0.5	4			
Staphylococcus capitis	D	≦0.5	≦0.5	≦0.5	≦0.5	>16	≦0.5	1	≦0.5	≦0.5	≦0.5	≦0.5	10/11	1/11	
	M	≦0.5	≦0.5	≦0.5	≦0.5	>16	≦0.5	2	≦0.5	≦0.5	≦0.5	≦0.5			
Enterococcus faecalis	D	1	2	>16	4	>16	1	>16	>16	≦0.5	16	1	9/11	2/11	
	M	1	2	>16	8	>16	1	>16	>16	1	16	1			
Enterococcus avium	D	1	16	>16	>16	>16	2	>16	≦0.5	≦0.5	≦0.5	≦0.5	5/11	6/11	
	M	2	>16	>16	>16	>16	4	>16	1	1	≦0.5	1			
Enterococcus faecium	D	>16	>16	>16	>16	>16	>16	>16	4	≦0.5	4	4	9/11		2/11
	M	>16	>16	>16	>16	>16	>16	>16	4	≦0.5	16	16			

Table 2. Comparison of MICdox of 14 Antimicrobial Agents for 5 Clinical Bacterial Isolates between a Clear Sample and a Turbid Sample

Isolate		MIC (μg/ml)													Complete agreement	Two-fold difference
		ABPC	PIPC	CEZ	CAZ	CPR	IPM	AZT	TAZ/PIPC	SBT/CPZ	GM	VCM	MINO	CPFX	LVFX	
Escherichia coli	Clear (C)	2	1	1	≦0.5	≦0.5	≦0.5	≦0.5	1	≦0.5	1	>16	2	≦0.5	≦0.5	13/14	1/14
	Turbid (T)	2	1	1	≦0.5	≦0.5	≦0.5	≦0.5	1	≦0.5	1	>16	4	≦0.5	≦0.5		
Serratia marcescens	C	>16	1	>16	≦0.5	≦0.5	≦0.5	≦0.5	1	2	≦0.5	>16	2	≦0.5	≦0.5	12/14	2/14
	T	>16	1	>16	≦0.5	≦0.5	≦0.5	≦0.5	1	1	≦0.5	>16	4	≦0.5	≦0.5		
Pseudomonas aeruginosa	C	>16	4	>16	2	4	1	8	8	8	≦0.5	>16	8	≦0.5	≦0.5	13/14	1/14
	T	>16	4	>16	2	4	2	8	8	8	≦0.5	>16	8	≦0.5	≦0.5		
Staphylococcus aureus (MRSA)	C	8	>16	8	>16	16	1	>16	8	16	>16	≦0.5	4	>16	>16	11/14	3/14
	T	8	>16	16	>16	16	≦0.5	>16	8	16	>16	≦0.5	2	>16	>16		
Enterococcus faecalis	C	1	2	>16	>16	4	1	>16	2	>16	>16	≦0.5	16	1	1	12/14	2/14
	T	1	4	>16	>16	4	1	>16	4	>16	>16	≦0.5	16	1	1		

solution.[13,14] This electrode has several advantages although multiple channel measurement is difficult from a cost-effective point of view because of its complicated structure. Amano *et al.* have developed a bare oxygen electrode sensor.[11] The advantage of its simple structure enables the use of DOX-96 with a low cost of production.

Bacteria in the well continue to consume the available oxygen by respiration. Therefore, the oxygen consumption level will reflect the continuing growth of the bacteria. The depletion of the available oxygen indicates that an adequate number of bacteria are present and viable. Murray and Murro indicated that an adequate number of bacteria in the logarithmic growth phase must be present to consume 90—100% of the available oxygen in a 15 min post-aeration incubation.[4] Actually, a remarkable oxygen consumption was observed by DOX-96 after the inoculation of bacteria (Fig. 2A). The time of remarkable oxygen consumption, which was determined as differential peaks (Fig. 2B), showed a linear relationship with the logarithmic value of inoculum size in the range of 1×10 to 1×10^7 CFU/ml. (Fig. 3).

On the other hand, a failure of bacteria to consume oxygen was observed in the presence of antimicrobial agents (Fig. 4), representing a serious arrest of bacterial metabolism usually leading to stasis or death. The current profile in the presence of antimicrobial agents over a certain concentration showed a similar profile to the negative control including no bacteria (Fig. 4). There was no difference in the current profile between gram-negative and gram-positive bacteria. And there was no difference in the current profile between bactericide and bacteriostatic agents. A certain concentration was considered to be the MICdox during 999 min (16.65 h). Generally, the MIC of the antimicrobial agents was determined by the broth microdilution method using a bacterial growth period of 16 to 20 h.[1,12]

The MICdox and MIC of 11 antimicrobial agents on 15 clinical bacterial isolates including methicillin-resistant *Staphylococcus aureus* (MRSA) were determined by oxygen consumption using DOX-96 and by the standard method, respectively. Bacterial strains used in the present study were aerobe and facultative anaerobe. In a general way, antimicrobial susceptibility testing for clinical use is also carried out on aerobe and facultative anaerobe under the atmosphere. The data of MICdox showed good agreement with MIC (98.2%, Table 1). These results indicate the clinical usefulness of DOX-96 for antimicrobial susceptibility testing. Further study, however, might be necessary for four-fold different samples to confirm the usefulness of DOX-96.

The development of potentiators of known antimicrobial agents would be an appealing approach if it allowed increased efficacy of antimicrobial agents, a reduction in doses, and a decrease in adverse effects. Liposomes and emulsions, the most studied colloidal drug delivery systems, could be one way of improving the effectiveness of antimicrobial agents.[15—23] The screening of the *in vitro* bactericidal activity of liposomes and emulsions including antimicrobial agents is important to develop useful drug delivery systems.

The current technique using the broth microdilution method,[1] however, could not determine the antimicrobial susceptibility in turbid samples because it is based on the determination of optical absorbance. The procedure described in the present study is based on the respiration of bacteria.

Although the chemiluminescent assay is also based on the respiration of bacteria,[24] there is a possibility that the chemiluminescent assay is influenced by turbidity because of its optical set-up. Therefore, the usefulness of DOX-96 was evaluated for turbid samples such as Mueller-Hinton broth containing 0.1% lipid emulsion. The addition of 0.1% lipid emulsion into the samples did not affect the current profiles of bacteria in the presence of antimicrobial agents (Fig. 5). The data of MICdox in the turbid samples showed good agreement with those in the clear samples (100.0%, Table 2). These results indicate the usefulness of DOX-96 for antimicrobial susceptibility testing even for turbid clinical samples such as colloidal products and turbid biological samples.

Many efforts have focused on the automation of antimicrobial susceptibility testing, and most of the commercially available systems are based on broth culture.[25] We established a new method for antimicrobial susceptibility testing of clinical isolates, including turbid samples, using newly developed oxygen meters, DOX-96.

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