A study on the actual conditions associated with the presence of *Acinetobacter* sp. in a hospital waiting room

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Abstract. In recent years, nosocomial infection, including that by multiple-drug-resistant *Acinetobacter* (MDRA), has become an increasing concern in Japan. Typically, *Acinetobacter* inhabits soil and river water and is usually harmless to humans. However, bacteria of this genus can become pathogens in inpatients with weakened immune systems, resulting in opportunistic infection. Currently, hospital infection by MDRA is spreading not only in Japan, but also worldwide. This is a very serious problem, and little is known about effective countermeasures. The present study sought to examine the microbiome status in a hospital visitor waiting room as a first step in establishing countermeasures for use against *Acinetobacter*. Specifically, we performed a so-called metagenomic analysis to directly analyze DNA collected from the environment, without an intervening cultivation step. Indoor airborne bacteria and surface bacteria were sampled and analyzed. The results show that in a visitor waiting room, the top-ten most-abundant genera included (in decreasing order) *Acinetobacter, Streptococcus, Prevotella, Pseudomonas, Bifidobacterium, Fusobacterium, Neisseia, Porphyromonas, Leptotrichia,* and *Haemophilus.* Thus, *Acinetobacter* constituted the most-detected genus and was present in all collected samples. Furthermore, we assessed the bactericidal effect of ultraviolet C (UVC) against *Acinetobacter* sp. 7206, a hospital isolate. A UVC dose of 4.0 sec·mW/cm^2 was required to kill the 99.9% *Acinetobacter* sp. 7206 on solid culture medium.

1. Introduction

In recent years, nosocomial infection, including that by multiple-drug-resistant *Acinetobacter* (MDRA), has become an increasing concern in Japan. Notably, MDRA and similar bacteria were detected in 15 patients in the Kagoshima University Hospital ICU (intensive care unit) in the summer of 2018. Four of these 15 inpatients died during their hospitalizations, and three of these four mortalities were attributed to MDRA. The hospital administration subsequently revealed that MDRA was recovered from three of sixteen mattresses in this ICU.

Commonly, members of the genus *Acinetobacter* inhabit soil and river water and are usually harmless to humans. However, these bacteria can become pathogens in inpatients with weakened immune systems, resulting in opportunistic infection. However, antibiotics that are typically used for the medical treatment of *Acinetobacter* infection have reduced efficacy against drug-resistant *Acinetobacter* in Japan. Currently, nosocomial infection caused by MDRA is spreading not only in Japan but also worldwide. This is very serious problem, and little is known about effective countermeasures.

The present study sought to examine the microbiome status in a hospital visitor waiting room as a first step in establishing countermeasures against *Acinetobacter*. Specifically, we performed a so-called metagenomic analysis to directly analyze DNA collected from the environment without an intervening cultivation step. Furthermore, we conducted an experiment on the bactericidal effect of ultraviolet C (UVC) as a treatment against *Acinetobacter* sp. 7206, a hospital isolate.

2. Materials and methods

2.1 The outline of the hospital

The investigation site was located at a 778bed hospital that has a total floor area of 56,635 m^2. The assessments described in this report were performed in the visitor waiting room (74m^2 floor area) of neurological diseases. An air-handling unit was installed in the waiting room.

2.2 Field measurement

Field measurement was carried out from January 28 to 31, 2018. The measured parameters included temperature, relative humidity (RH), carbon dioxide (CO$_2$) concentration, and airborne and surface microbial populations. Five measurement locations were selected: the waiting room on the second floor, return and supply air in the air-handling unit, and outdoors. The following

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methods were used to measure the parameters. Air temperature, RH, and CO_2 concentrations were recorded once per min using a data logger (TR-76Ui) during measurement period.

Airborne microbes were collected using an air pump (Air Check XR5000) equipped with a filter (PTFE 0.3 Filter). The sampling volume was 180 L (3 L/min × 60 min).

After measurement of the temperature, RH, and CO_2 level, surface microbes were collected. Surface microbes were recovered by wiping using a swab kit. Adhering microbes were collected by swabbing (separately) a chair and a doorknob in the waiting room, as well as the main filter and pre-filter in the air conditioner. For each item, an area totaling 100 cm^2 was swabbed, including 10 cm ×10 cm surfaces of the chair, the main filter, and the pre-filter, and an 8 cm × 12.5 cm surface of the doorknob before and after UVC operation.

2.3 Sterilization by UVC

The experiment testing sterilization of a purified isolate of *Acinetobacter* by UVC was performed under the following conditions.

The test bacterium was *Acinetobacter* sp. 7206, a hospital isolate, plated on solid culture medium at 1600 colony-forming units (cfu) per plate. The UVC intensity at the surface of the culture medium was 0.1 mW/cm^2; irradiation times were 0, 20, 25, 30, 35, 40, or 50 sec.

2.4 DNA extraction, amplification, sequencing

(1) DNA extraction

Each sample was placed in a sterilization bag together with double-distilled water. After processing the samples using a Stomacher 80 Biomaster, the extract was transferred to a test tube and centrifuged (KUBOTA3700). The centrifuged extract was divided into bacterial and viral components, but only bacterial DNA was purified and analyzed in the present study. Bacterial DNA was purified using the NucleoSpin Tissue Kit (740952.50).

(2) DNA amplification, Sequencing, Data analysis

For details of DNA extraction, amplification and sequencing by next generation sequencer, please refer to the previous report.(Yanagi, ex al 2018)

3. Results

3.1 Environment of the waiting room

Air temperature and RH in the waiting room were 26 ± 1ºC and 41 ± 3%, respectively. These RH did not satisfy the Healthcare Engineering Association of Japan standards (temperature: 26 to 27 ºC, RH: 50 to 60%). CO_2 concentration was 697±35 ppm, a value that complied with relevant standards, such as the Japanese 'Law for Environmental Health in Buildings'.

3.2 Detected bacteria

Table 1 shows the number of detected bacteria by phylum, class, order, family, and genus for all prokaryotes with a relative abundance exceeding 1.0% of the total. Compared to outdoor air, a larger number of phylums, classes, orders, families, and genuses were detected in the indoor environment. Notably, multiple phylums, classes, orders, families, and genuses were detected from the upstream and downstream of a coil. Similarly, multiple phylums, classes, orders, families, and genuses were detected from the tested surfaces.

Fig. 1 shows reads of genera that ranked in the top 10 for abundance (excepting reads from outdoor air). The metagenomes included bacteria of the genera *Acinetobacter, Streptococcus, Prevotella, Pseudomonas, Bifidobacterium, Fusobacterium, Neisseria, Porphyromonas, Leptotrichia*, and *Haemophilus.* Notably, the most frequently detected genus was *Acinetobacter*, which was detected in all samples. *Acinetobacter* inhabits soil and river water and usually is harmless to humans. *Acinetobacter* previously has been detected only rarely in indoor environments such as offices. These observations have been used to suggest that the genus *Acinetobacter* exists primarily in hospital settings.

To date, 17 species (including 15 genomic species) have been classified as members of the genus *Acinetobacter* based on metagenomic analyses. Among these species, three (*Acinetobacter baumannii*, *Acinetobacter* genomic species 13TU, and *Acinetobacter* genomic species 3) are considered MDRA.

Table 2 shows detection of species and number of reads in our analysis. Names marked in red represent known strict pathogens; those marked in orange represent known opportunistic pathogens. Our metagenomic analysis did not detect *A. baumannii, Acinetobacter* genomic species 13TU, or *Acinetobacter* genomic species 3. However, only 578 (3.3%) of the reads that were assigned to the genus *Acinetobacter* could be identified at the species level. Among the bacteria of known species, the following opportunistic and strict pathogens were detected.

Table 1. Number of detected bacteria by phylum, class, order, family, and genus

		Phylum	Class	Order	Family	Genus
Air	OA	12	32	32	44	69
	RA	11	33	29	43	80
	Upstream of Coil fin	12	20	31	41	31
	Downstream of Coil fin	10	17	28	40	33
Surface	Chair	11	16	24	32	42
	Doorknob	9	17	25	34	24
	Main filter	6	14	21	31	25
	Prefilter	8	13	21	23	15
	Coil fin	9	15	22	32	23

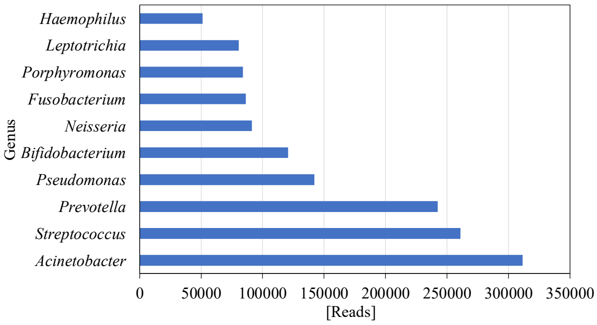

Fig. 1. Most abundant (>1% of sequences) genera

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Acinetobacter lwoffii: This species has been detected primarily in humans, where the organism has been identified as an opportunistic pathogen in meningitis cases, especially in post-operative meningitis. *A. lwoffii* was detected all samples in the present study.

Acinetobacter segnis is a pathogen associated with infectious diseases of the intraoral and digestive systems, and has been recovered from cases of periodontitis, cholecystitis, appendicitis, and pancreatic abscess, and as a causative bacterium in primary sepsis. In the present study, *A. segnis* was detected primarily from the main air filter and in both front and rear filter spaces.

Bacteroides uniformis typically coexists with humans but serves as an opportunistic pathogen within the peritoneal cavity. In the present study, *B. uniformis* was detected in large amounts from RA on a single day (January 30, 2018).

Bacteroides fragilis is one of the predominant bacteria constituting the human gastrointestinal microbiome and is present from the human oral cavity through the intestine. This organism has low pathogenicity in general but does cause disease when the body's resistance is decreased. In the present study, *B. fragilis* was detected in the RA and on the doorknob.

Capnocytophaga ochracea is present in human oral and non-oral cavities and is related to periodontitis in both children and adults. This organism rarely causes serious infections, but has been recovered from cases of endocarditis, endometriosis, osteomyelitis, abscess, peritonitis, and keratitis. In the present study, *C. ochracea* was detected in large amounts from the chair on a single day (January 30, 2018).

Haemophilus influenzae is a bacterium that is normally present in the upper respiratory tract of humans and is a causative agent of various infectious diseases associated with the respiratory system. In the present study, *H. influenzae* was detected primarily from the main air filter and in both front and rear filter spaces.

Neisseria subflava is an opportunistic pathogen that is commonly observed in the human oral and respiratory tract. In the present study, *N. subflava* was detected on the majority of surveyed surfaces.

Prevotella nigrescens is an opportunistic pathogen that is commonly detected in the human oral and respiratory tract. In the present study, this organism was detected in large amounts from the rear of the filter space on a single day (January 30, 2018).

Rothia mucilaginosa is present in the human oral cavity but in rare cases serves as a causative organism for infectious diseases such as bacteremia, meningitis, and pneumonia. In the present study, this species was detected in large amounts on all surveyed surfaces.

Streptococcus anginosus has been detected from various human sites and can cause infections and abscesses.

Three UV lamps were set forward of a coil fin on the air-handling unit. A clear effect with UVC irradiation was not detected at the level of the number of metagenomic reads. This analysis is considered an example of the potential use situation for sterilization in the waiting room, given that the level of bacterial contamination in the RA would be changing at every moment.

3.3 Principal coordinate analysis (PCoA)

Table 2. Detected species

	2018/1/28					2018/1/29												
UVLamp						before	after	before	after	before	after	before	after					
Sampling point	MF	PF	CF	Ch	Dk	FIL f	FIL f	FIL r	FIL r	RA	RA	OA	OA	MF	PF	CF	Ch	Dk
Acinetobacter lwoffii	97	0	18	406	51	77	8	113	26	80	141	86	8	55	1	62	197	56
Acinetobacter rhizosphaerae	1	0	0	2	0	1	0	0	0	7	0	7	0	0	0	2	1	4
Aggregatibacter segnis	9	0	6	0	0	2	0	0	0	4	0	0	0	128	0	0	0	29
Akkermansia muciniphila	6	0	0	2	3	0	1	2	1	1	0	0	0	0	0	2	4	0
Anoxybacillus kestanbolensis	1	0	1	60	50	0	0	0	0	233	98	0	10	25	1	26	1	1
Bacillus humi	0	0	0	0	0	0	0	0	0	0	0	0	76	0	0	106	0	3
Bacteroides uniformis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteroides fragilis	0	0	0	0	0	0	0	0	0	859	13	0	0	72	0	0	0	199
Bifidobacterium adolescentis	29	0	236	359	64	18	2	32	33	5	123	359	854	131	0	32	44	29
Brachybacterium conglomeratum	1	0	0	0	3	0	0	0	0	0	0	1	2	1	0	2835	0	0
Capnocytophaga ochracea	0	0	0	0	0	0	0	5	0	0	0	0	1	0	0	0	0	0
Corynebacterium durum	1	0	941	1	0	0	0	2	0	0	0	0	0	0	0	0	16	98
Deinococcus geothermalis	380	0	0	0	0	0	0	0	0	0	0	1681	0	0	0	0	0	0
Eggerthella lenta	0	0	0	0	0	0	0	0	0	0	0	0	0	699	0	41	0	0
Enterococcus cecorum	0	0	1	0	0	0	1	0	235	0	2	0	0	0	0	0	35	1
Faecalibacterium prausnitzii	4	0	0	0	3	0	0	0	0	0	46	0	0	0	0	0	1	49
Geodermatophilus obscurus	0	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus parainfluenzae	19	0	2	91	8	13	0	0	0	8	163	1	1	163	1	13	8	55
Haemophilus influenzae	83	0	1	0	23	11	0	0	0	51	1	0	0	0	0	0	0	73
Kocuria rhizophila	2	3	20	195	2	74	0	16	0	0	45	2	643	0	0	0	34	250
Kocuria palustris	0	0	0	79	37	1	0	0	6	0	2	1	157	0	0	0	10	0
Lactobacillus iners	164	0	1	0	2	992	0	0	0	0	1	0	0	298	0	1	1	0
Lysobacter brunescens	0	0	0	0	0	0	1078	0	0	0	0	0	17	0	0	1	0	1
Methylobacterium organophilum	0	0	92	0	0	0	0	0	0	0	86	0	1	0	0	0	0	0
Methylotenera mobilis	0	0	19	0	0	0	0	0	0	0	0	0	0	1	3841	1	0	0
Neisseria subflava	9	0	8	23	51	2	63	314	39	26	53	2	42	51	0	26	35	16
Parabacteroides distasonis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Paracoccus aminovorans	1	0	21	0	10	0	1	1411	0	0	0	0	0	0	0	142	0	0
Porphyromonas endodontalis	0	0	0	0	0	0	0	0	116	0	24	1	0	37	0	0	0	0
Prevotella melaninogenica	1943	8	4431	2270	1234	10	17072	10120	12804	5246	4843	15530	7354	4306	19	4540	10258	4414
Prevotella nanceiensis	2838	0	1250	159	751	2914	1	252	1	2	808	36	357	87	0	2775	7420	2008
Prevotella nigrescens	1	0	0	0	0	0	0	1388	0	0	30	0	0	0	0	0	0	0
Prevotella copri	364	0	7	52	89	0	6	0	2	252	1	0	0	3	0	4	14	3
Prevotella intermedia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Propionibacterium acnes	419	7	192	23	47	8	21	24	325	5	425	39	84	49	0	261	197	420
Pseudomonas viridiflava	0	0	1	0	0	0	532	2	1	0	0	0	0	0	0	0	0	0
Psychrobacter sanguinis	0	0	8	0	1	0	1	0	0	0	0	0	0	149	0	0	0	32
Roseomonas mucosa	10	2	1171	2	0	1358	1	1	1	1	566	650	0	155	0	0	7	152
Rothia mucilaginosa	2504	1	286	355	182	3	1549	51	236	1259	581	6350	2678	1065	0	721	317	327
Rothia aeria	1	0	24	105	0	1	178	449	1	1863	30	290	1	60	0	3	0	25
Rothia dentocariosa	2	0	76	182	1	0	1	0	0	30	72	11	2	21	0	112	15	69
Selenomonas noxia	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	32	54
Staphylococcus epidermidis	0	0	0	0	14	0	1	0	0	0	1	0	408	0	0	0	0	0
Streptococcus anginosus	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	21
Veillonella parvula	804	0	992	543	541	36	90	878	96	389	380	349	2	18	1	1168	1291	266
Veillonella dispar	4	0	7	2	2	0	0	4	0	0	2	2	0	0	0	6	1	2

Table 2. Detected species, continue

	2018/1/30													2018/1/31							
UVLamp	before	after	before	after	before	after	before	after						before	after	before	after	before	after	before	after
Sampling point	FIL f	FIL f	FIL r	FIL r	RA	RA	OA	OA	MF	PF	CF	Ch	Dk	FIL f	FIL f	FIL r	FIL r	RA	RA	OA	OA
Acinetobacter lwoffii	146	175	215	221	19	11	57	54	1	29	101	39	60	106	99	58	28	96	226	34	10
Acinetobacter rhizosphaerae	14	2	34	8	0	0	6	0	0	0	4	1	160	2	1	4	0	6	1	1	1
Aggregatibacter segnis	36	65	9	6	2	9	0	3	0	37	10	0	4	0	144	0	0	0	0	0	0
Akkermansia muciniphila	44	10	71	153	1	0	4	3	0	0	0	1	0	446	4	1007	0	0	2	0	95
Anoxybacillus kestanbolensis	123	114	18	16	0	0	0	57	0	121	20	0	0	1	9	78	0	17	0	0	0
Bacillus humi	0	14	0	50	1	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0
Bacteroides uniformis	1	1	26	0	0	2698	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
Bacteroides fragilis	0	0	6	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
Bifidobacterium adolescentis	21	150	90	148	157	64	70	278	0	58	339	64	59	86	63	42	52	35	16	16	13
Brachybacterium conglomeratum	11	70	45	101	0	0	6	6	5	0	0	72	0	0	0	543	1	0	0	0	0
Capnocytophaga ochracea	30	173	24	0	0	0	101	1	0	91	0	841	3	0	0	0	3	0	0	0	0
Corynebacterium durum	31	122	4	19	1	0	0	1	3	102	146	1	1	0	1	0	0	0	0	0	0
Deinococcus geothermalis	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	205	0	0	0	0	0
Eggerthella lenta	0	134	10	0	0	0	0	0	0	0	0	0	0	0	100	1	0	0	0	0	0
Enterococcus cecorum	0	0	6	0	0	0	0	0	0	2	1	2	0	0	0	0	0	520	0	13	0
Faecalibacterium prausnitzii	119	209	82	71	1	1	5	5	0	0	3	0	2	2	7	4	0	146	0	0	0
Geodermatophilus obscurus	0	0	0	0	0	0	0	0	0	0	0	0	191	0	1	0	0	35	0	0	0
Haemophilus parainfluenzae	7	21	6	78	71	137	7	3	0	12	7	4	9	17	32	66	22	2	100	0	1
Haemophilus influenzae	52	28	37	32	0	0	48	1	0	8	4	0	1	4	12	143	0	2	13	0	9
Kocuria rhizophila	78	232	234	63	0	1	3	2	3	198	1	12	1	171	159	0	7	86	32	86	809
Kocuria palustris	4	151	16	22	1	0	1	0	0	38	0	0	40	27	10	1	3	12	3	3	7
Lactobacillus iners	2	6	108	123	0	52	2	2	4	0	2	0	1	0	2	0	0	0	0	0	0
Lysobacter brunescens	0	0	0	0	0	0	0	0	0	1	0	0	0	83	0	0	57	0	0	1	0
Methylobacterium organophilum	0	0	2	20	0	0	3	0	0	228	0	0	0	0	302	0	0	0	62	0	0
Methylotenera mobilis	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria subflava	41	20	38	21	6	48	1	32	0	40	6	33	100	45	18	33	20	16	7	148	32
Parabacteroides distasonis	0	0	5	0	0	0	0	1	0	0	0	0	1	0	0	370	0	2	0	0	0
Paracoccus aminovorans	50	4	1	11	0	0	0	0	0	49	0	1	0	0	0	105	0	66	0	1	0
Porphyromonas endodontalis	20	121	45	170	0	0	789	0	0	120	0	6	0	0	397	0	0	0	0	0	0
Prevotella melaninogenica	4416	5106	3126	4172	3813	9159	5235	1679	18	7479	5901	4560	6662	7427	4318	7723	3230	2473	884	7096	5421
Prevotella nanceiensis	831	917	743	972	293	3	23	248	2	2326	1032	12	111	453	578	7	101	1053	8941	2701	31
Prevotella nigrescens	14	0	1	23	1	1	0	4	1	0	0	0	0	95	2	0	0	284	0	0	0
Prevotella copri	186	35	189	98	2	0	26	55	2	0	13	13	8	946	21	9	3	80	2	5	1
Prevotella intermedia	0	0	1	0	0	0	1	0	0	0	1	0	0	0	118	0	0	0	0	0	0
Propionibacterium acnes	317	318	174	187	90	17	942	136	17	113	50	127	483	151	101	877	181	138	179	153	86
Pseudomonas viridiflava	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Psychrobacter sanguinis	142	131	79	123	0	2	66	5	0	0	1	1	14	1	39	55	0	0	1	2	165
Roseomonas mucosa	274	540	304	897	1783	1228	3	4	0	860	3	6	119	2	363	602	1	1	0	313	4
Rothia mucilaginosa	741	1185	593	980	126	6	856	966	0	2728	613	3592	1723	2130	1243	950	73	645	769	1040	550
Rothia aeria	144	117	83	82	0	1	51	7	0	484	153	309	343	226	45	66	0	1	1	35	408
Rothia dentocariosa	16	20	36	3	0	1	161	259	0	97	9	31	10	7	177	1	0	0	169	0	17
Selenomonas noxia	0	5	2	1	0	0	2	2	0	0	0	1	36	0	0	1	0	773	0	0	0
Staphylococcus epidermidis	1	0	1	0	15	0	0	0	0	2	0	0	0	0	1	0	0	1	20	0	0
Streptococcus anginosus	0	45	3	0	0	0	878	0	0	0	1	0	0	0	1	0	0	0	0	1	0
Veillonella parvula	828	365	487	472	1079	483	17	1233	0	796	31	1624	916	392	644	15	300	3958	2951	8	299
Veillonella dispar	4	0	6	1	0	1	0	12	0	7	0	16	2	56	1	0	3	212	1854	0	2

Fig. 3 shows the results obtained from principal coordinate analysis (PCoA). Most of the flora plotted to near positions. However, the flora of 1/28PF (Prefilter), 1/29PF, and 1/30MF (Main filter) plotted as points distinct from the other specimens. These three samples contain flora extracted from the air filter of the air-handling unit, and therefore represent airborne microbes that adhered to the filter surface.

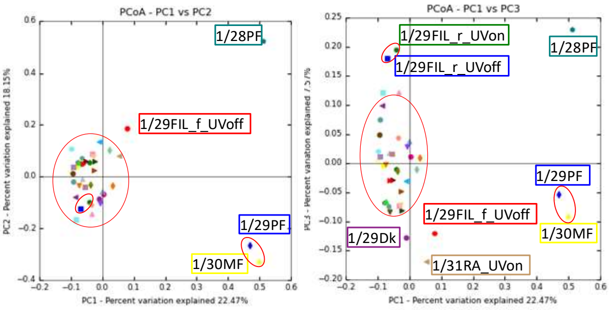

Fig. 3. The results obtained from PCoA.

Table 3. The survival count (cfu) of UVC-irradiated *Acinetobacter* sp. 7206

Elapsed time	Test1	Test2	Test3	Ave
0s	1600	1600	1600	1,600
20s	138	173	286	199
25s	126	99	96	107
30s	66	52	73	64
35s	7	25	40	24
40s	0	0	1	0.3
50s	0	0	0	0

3.3 Bactericidal effect of UVC on Acinetobacter

Table 3 shows the results obtained in an assessment of the bactericidal activity of UVC on *Acinetobacter* sp. 7206 in the laboratory. This table shows acinetobacterial survival as a function of irradiation time. According to Table 6, 99.98% of *Acinetobacter* sp. 7206 strain were killed after 40 sec of exposure, corresponding to a net UVC dose of 4 sec·mw/cm^2.

4. Conclusions

In this study, it was found that many pathogens were detected in a hospital waiting room. Additionally, an in-situ test was performed by installing UV lamps in the air-conditioning equipment of the hospital waiting room, and the effect of UVC irradiation also was assessed in an experiment in which *Acinetobacter* sp. 7206 was directly irradiated in the laboratory. The lab experiment demonstrated that a UVC dose of 4sec·mw/cm^2 achieved sterilization (>3-log decrease in cfu). On the other hand, As the object hospital was in use and the measurements had strong restrictions, a clear effect of UVC was not found in the in-situ test.

References

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