Journal of Oral Science, Vol. 55, No. 2, 145-155, 2013

Original

Prevalence of drug-resistant opportunistic microorganisms in oral cavity after treatment for oral cancer

Kaoru Yamashita[1)], Masaru Ohara[2,3)], Taro Kojima[4)], Rumi Nishimura[1)], Tetsuji Ogawa[2)], Takamune Hino[3)], Mitsugi Okada[4)], Shigeaki Toratani[5)], Nobuyuki Kamata[6)], Motoyuki Sugai[7)], and Masaru Sugiyama[1)]

[1)]Department of Public Oral Health, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
[2)]Department of Advanced General Dentistry, Hiroshima University Hospital, Hiroshima, Japan
[3)]Dental Clinic, Hiroshima University Hospital, Higashihiroshima, Japan
[4)]Special Care Dentistry, Hiroshima University Hospital, Hiroshima, Japan
[5)]Department of Oral and Maxillofacial Surgery, Hiroshima University Hospital, Hiroshima, Japan
[6)]Department of Oral and Maxillofacial Surgery, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
[7)]Department of Bacteriology, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

(Received December 25, 2012; Accepted April 23, 2013)

Abstract: Drug-resistant opportunistic infections may cause health problems in immunocompromised hosts. Representative microorganisms in opportunistic infections of the oral cavity are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. We investigated the prevalence of drug-resistant opportunistic microorganisms in elderly adults receiving follow-up examinations after primary treatment of oral cancer. Oral microorganisms were collected from patients satisfactorily treated for oral cancer (defined as good outcomes to date) and a group of healthy adults (controls). After identification of microorganisms, the prevalence of drug-resistant microorganisms was studied. Pulsed-field gel electrophoresis (PFGE) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing were also performed for methicillin-resistant *S aureus* (MRSA). Statistical analysis revealed no significant differences in the prevalences of the three microorganisms between the groups. Surprisingly, 69.2% of *S aureus* isolates showed oxacillin resistance, suggesting that MRSA colonization is increasing among older Japanese. These MRSA isolates possessed SCC*mec* types II and IV but no representative toxin genes. Our results indicate that a basic infection control strategy, including standard precautions against MRSA, is important for elderly adults, particularly after treatment for oral cancer. (J Oral Sci 55, 145-155, 2013)

Keywords: drug-resistant microorganism; opportunistic infection; oral cancer.

Correspondence to Dr. Masaru Ohara, Department of Advanced General Dentistry, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
Fax: +81-82-424-3909 E-mail: mohara@hiroshima-u.ac.jp

Introduction

Opportunistic infections are associated with individuals in poor health (referred to as an immunocompromised host or an opportunistic situation) and are caused by several different microorganisms. Among those that originate in the oral cavity, representative microorganisms include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* (1,2). Most of these organisms have become drug-resistant, which has resulted in difficulties in curing the related infectious diseases. In the 1980s and 90s, methicillin-resistant *S aureus* (MRSA) was preva-

lent among opportunistic and nosocomial infections and caused pneumonia, sepsis, colitis, and urinary tract infection, among other illnesses (3). Methicillin resistance results from production of a unique low-affinity penicillin binding protein (PBP 2a) encoded by the chromosomal gene *mecA*, which is acquired by integration of a gene cassette, termed staphylococcal cassette chromosome *mec* (SCC*mec*), using recombinase (*ccr*) carrying genes on their cassettes. An increasing number of recent reports have identified community-acquired or -associated MRSA (C-MRSA) as a factor in various diseases, such as pneumonia, in young patients and others without classic healthcare-associated risk factors (4).

P aeruginosa is another representative bacterium that causes opportunistic infections (5). It is found in soil, water, and skin flora and can survive in poor environments including hypoxic atmosphere. According to the guidelines of the Japanese Infectious Disease Surveillance Center (IDSC), multidrug-resistant *P aeruginosa* (MDRP) is defined as isolates with the following minimum inhibitory concentrations (MICs) of antibiotics: > 16 μg/mL for imipenem, > 32 μg/mL for amikacin, and > 4 μg/mL for ciprofloxacin. The prevalence of MDRP is increasing, and nosocomial infections have become a serious clinical problem in Japan (6).

The *Candida* species elicit the yeast infectious disease candidiasis, which causes superficial infections such as oral thrush and vaginitis as well as systemic and potentially life-threatening diseases, known as candidemia (7). *C albicans* is a yeast that normally inhabits the human mouth and skin, where it generally uneventfully coexists with a variety of other microorganisms. An infection occurs when the balance of bacteria in the body is disrupted, especially in immunocompromised situations, allowing drug-resistant *Candida* species to proliferate and overwhelm other healthy microorganisms (8).

Immunocompromised situations are frequently seen in older individuals, infants, people infected with HIV, and individuals with cancer; oral cancer can reduce immunity in the maxillofacial region (1,2). The principal treatments for oral cancer are surgical excision, radiotherapy, and chemotherapy, given alone or in combination (9). Despite successful treatment of cancer, local and systemic immunity decreases in affected patients, while surgical excision induces oral dysfunction, due to loss of normal anatomy. For example, swallowing dysfunction after partial tongue excision readily elicits aspiration pneumonia, and radiation and chemotherapy reduce myelocyte function, which may result in an immunocompromised host.

In the present study, we investigated drug-resistant opportunistic microorganisms after primary treatment of oral cancer in elderly adults. A number of reports of opportunistic infections in cancer patients have been published (1,2,5,7). However, the oral conditions of such patients after oral cancer therapy (so-called "follow up-patients") are not well known. We focused on drug-resistant opportunistic microorganisms in the mouth of patients who had received primary treatment for oral cancer. After hospital discharge, attending physicians check not only for cancer recurrence but also for wound healing, local infection, and oral mucositis. Clinicians are also aware that their patients may become carriers of drug-resistant microorganisms and may spread them to others.

We isolated *Staphylococcus*, *Pseudomonas*, and *Candida* species from the oral cavities of elderly adults after oral cancer treatment, attempted to detect MRSA, MDRP, and drug-resistant *Candida* species, and compared prevalences between these follow-up patients and a group of healthy adults. In addition, we discuss SCC*mec* typing and presence of toxin genes in MRSA organisms.

Materials and Methods

Setting and patients

This study was carried out from June through December 2010 at Hiroshima University Hospital, Department of Oral and Maxillofacial Surgery and Oral Medicine, after receiving approval from the Ethical Committee of Hiroshima University (Clinical Research No. EKI224). In total, 186 elderly patients (age range, 57-98 years, were identified. After excluding people suffering from systemic diseases that affected infection status (i.e., diabetes, autoimmune diseases, and hepatitis), the patients (n = 83) were divided into two groups. The cancer treatment group (n = 46; 20 men, 26 women; average age 67.4 ± 10.3 years) included patients who had undergone treatment of oral cancer at Hiroshima University Hospital and had good outcomes to date (0 months to 11 years after primary treatment). Therapy for oral cancer consisted of radiotherapy, chemotherapy, and surgery, alone or in combination. Tongue cancer was the most prevalent, followed by gingival cancer of the lower jaw. The second group was the control group (n = 37; 11 men, 26 women; average age, 71.3 ± 9.9 years), who had received treatment for cavities or poorly fitting dentures at the same hospital and had no history of any cancer treatment. Activities of daily living (ADL) did not significantly differ between groups.

Collection of microorganisms and culture conditions

Microorganism samples were collected by scrubbing the

surgical area (in the cancer treatment group), tongue, gingiva, and palate 5 times using a dry sterile cotton swab. Each cotton swab was then immediately inserted into a 1.5-mL sterile tube containing 800 µL of saline for 10 s. After removing the swab, the remaining saline solution was used as the microbial sample. Additionally, 1-mL saliva samples were collected from each subject (saliva sample). In xerostomic participants, the mouth was rinsed with 5 mL of sterile saline for 10 s, and the recovered saline was used as the rinse sample. Each saline sample was centrifuged at 15,000 rpm (20,600 x g) using the angle rotor of a Kubota RA-2724 (Kubota, Tokyo, Japan) with a Kubota 3500 microtube centrifuge at 22°C for 5 min. The resulting precipitate (ppt) was resuspended with 90 µL of saline, after which three 30-µL samples were spread onto three separate agar plates. The microorganisms were cultured on nalidixic acid/cetrimide agar (Eiken, Tokyo, Japan) for the first screening of *Pseudomonas* species, while Mannit-Salt agar (Eiken) was used for the first screening of *Staphylococcus* species and Candida-GS agar (Eiken) was used for the first screening of *Candida* species. The nalidixic acid/cetrimide agar and Mannit-Salt agar plates were incubated at 37°C under aerobic conditions for 2 days, while Candida-GS agar plates were incubated at 30°C for 3 days.

To screen for MRSA, colonies grown on the Mannit-Salt agar plates were collected and spread onto MRSA II selective agar (Eiken) plates, then cultured at 37°C for 2 days. When liquid culture was necessary, trypticase soy broth (Becton Dickinson, Tokyo, Japan) was used for *Staphylococcus* and *Pseudomonas* species, and Sabouraud liquid broth (Becton Dickinson) was used for *Candida* species.

Identification of microorganisms

The shapes of the colonies on the plates were checked, and those with different shapes were collected and gram stained for morphological observation under a light microscope. Genomic DNA from each colony was obtained using a Wizard genomic extraction kit (Promega, Madison, WI, USA). For *Staphylococcus* species, cells were treated with 1 mg/mL of lysostaphin in Tris-EDTA buffer (TE; 10 mM Tris-Cl, 1 mM EDTA, pH 8.0) at 37°C for 1 h before using the kit. Final identification was done by polymerase chain reaction (PCR) amplification with bacterial universal primers for 16S rDNA (for bacteria) or 26S rDNA (for fungus), followed by DNA sequencing (see Supplementary Table).

PCR amplification was carried out using Taq DNA polymerase (Toyobo, Tokyo, Japan) for 25 cycles, with denaturing at 94°C for 20 s, annealing at 50°C for 30 s, and polymerization at 72°C for 1 min. The PCR products were electrophoresed on 1% agarose gels with 0.5 µg/mL ethidium bromide and visualized using an Atto UV illuminator (Atto, Tokyo, Japan). DNA fragments of interest were recovered from each gel using a Geneclean kit (MP Biomedicals, Tokyo, Japan). DNA sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) with a Big Dye Cycle Sequencing reaction kit (AB Applied Biosystems). Identification of experimentally determined nucleotide sequences using sequence databases was performed using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast).

Detection of toxins and related genes in *S aureus*

Toxin genes were analyzed in genome DNA isolated from MRSA organisms by PCR. The detected genes were *PVL* (Panton-Valentine leukocidin), *lukE*, *lukD* (leukocidin), *eta*, *etb*, *etd* (exfoliative toxin), *edinA*, *edinB*, *edinC* (epidermal cell differentiation inhibitor), and *se* (staphylococcal enterotoxin) (see Supplementary Table). PCR amplification was performed using the conditions described above. Enterotoxin genes were detected by multiplex PCR, as previously described (10).

Antimicrobial susceptibility testing

MICs were determined using the microdilution broth method provided by the Clinical and Laboratory Standards Institute, using a MicroScanWalkAway-96 system (11). The antibiotics tested were oxacillin (MPIPC; sensitivity [S] < 0.25 µg/mL, resistance [R] > 2), arbekacin (ABK; S < 4, R > 8), vancomycin (VCM; S < 2, R > 8), teicoplanin (TEIC; S < 8, R > 16), and linezolid (LZD; S < 2, R > 4) for MRSA. For *Candida* species, we tested fluconazole (FCZ; S < 8, susceptible-dose dependent [S-DD] = 16-32, R > 64), 5-fluorocytosine (5-FC; S < 4, intermediate = 8-16, R > 32), itraconazole (ITZ; S < 0.125, S-DD = 0.25-0.5, R > 1), miconazole (MCZ; S < 0.5, R > 1), amphotericin B (AMPH-B; S < 1, R > 2), voriconazole (VRCZ; S < 1, R > 4), and micafungin (MCFG; S < 1, R > 2). For *Pseudomonas* species, imipenem/cilastatin (IMP/CS; S < 16, R > 16), amikacin (AMK; S < 32, R > 32), and ciprofloxacin (CPFX; S < 4, R > 4) were tested.

Statistical analysis

Differences between groups were assessed using Pearson's $\chi 2$ test, followed by Yate's correction.

Genotyping of staphylococcal cassette chromosome *mec*

Genotypes of staphylococcal cassette chromosome *mec* (SCC*mec*) were defined by two elements: the *mec* region (*mec* class A, B, C) and *ccr* region (*ccr* types 1-4) (12). SCC*mec* was classified by PCR amplification, using DNA extracted from MRSA (Supplementary Table), under the conditions described above. PCR products were electrophoresed on 1% agarose gels with 0.5 μg/mL ethidium bromide and visualized using an Atto UV illuminator.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed using a previously described method (13). Agarose gels embedded with *S aureus* were treated with 1 mg/mL lysostaphin in TE at room temperature for 30 min, followed by incubation with 1% sarkosyl in TE at 37°C for 1 h. Sarkosyl-treated samples were incubated in 1 mg/mL protease K in TE at 50°C overnight. After in-gel digestion of chromosomal DNA with the restriction enzyme *Sma*I (New England BioLabs Inc., Ipswich, MA, USA) at 37°C for 18 h, the resulting fragments were separated using a CHEF Mapper PFGE system (Bio-Rad, Tokyo, Japan). Each gel was stained with 0.5 μg/mL ethidium bromide at room temperature for 1 h. After destaining in water for 30 min, the DNA fragment pattern was visualized using an Atto Printgraph UV illuminator. Images were captured using an Atto Image Saver AE-6905 and analyzed with the Dice coefficient and unweighted pair group methods using GelCompar II software (Applied Maths BVBA; Sint-Martens-Latem, Belgium).

Results

Detection of opportunistic microorganisms in the oral cavity

In samples from the 83 subjects, 57 *Candida* species, 71 *Staphylococcus* species, and 7 *Pseudomonas* species were isolated (Fig. 1a). In some cases, a single participant had more than two species. For example, *S aureus* was detected in saliva and *S lugdunensis* in a tongue swab from the same individual. Thirty-nine participants had *Candida* species, 28 had *Staphylococcus* species, and seven had *Pseudomonas* species (Fig. 1b).

In a comparison between groups of isolated microorganisms, *Candida*, *Staphylococcus*, and *Pseudomonas* species were detected in 39 (68.4%), 31 (43.7%), and 4 (57.1%) participants, respectively, in the cancer treatment group, and in 18 (31.6%), 40 (56.3%), and 3 (42.9%) participants, respectively, in the control group (Fig. 1a). Statistical analysis revealed no significant difference between groups in the number of isolated microorganisms or the number of participants with these isolates.

Identification of the isolated organisms by 16S or 26S rDNA sequencing indicated that *C albicans* (33.3% of *Candida* species) and *C glabrata* (31.5%) were dominant among the 57 isolates of *Candida* species (Fig. 1c). *C guilliermondii* was isolated from subjects in the control group but not from those in the cancer treatment group. *C krusei* was found in one participant in the cancer treatment group, and *C africana* was detected in one subject in each group. The numbers of *C albicans* and *C glabrata* organisms isolated in the cancer treatment group were greater than those isolated in the control group, although the difference was not significant.

Among *Staphylococcus* species, *S aureus* (18.3% of *Staphylococcus* species) and *S epidermidis* (19.7%) were dominant, followed by *S haemolyticus* (15.5%) and *S lugdunensis* (12.7%) (Fig. 1d). Several minor strains, e.g. *S sciuri*, *S auricularis*, and *S warner*, were only detected in the control group. In a comparison of each *Staphylococcus* strain between groups, the number of *S aureus* organisms isolated in the cancer treatment group was greater than that in the control group, although the difference was not significant. All *Pseudomonas* organisms isolated in this study were *P aeruginosa*.

The presence of two or more microorganisms in patients was also analyzed. No subject had organisms from all three species, although 18 (21.4%) participants had organisms from two species: 11 in the cancer treatment group and seven in the control group. Among these patients, 14 (16.7%; 9 in the cancer treatment, 5 in the control group) had a combination of *Candida* and *Staphylococcus* species, one from each group had a combination of *Candida* and *Pseudomonas* species, and one from each group had a combination of *Staphylococcus* and *Pseudomonas* species.

Prevalence of antibiotic- and antifungal-resistant opportunistic microorganisms

Because there was no difference between groups in the prevalence of the three opportunistic microorganisms, we next investigated the prevalence of antibiotic-resistant microorganisms in the oral cavity. Determination of MICs for *Candida* species revealed 17 strains with resistance to at least one antifungal agent (Fig. 1c, Table 1). Among drug-resistant *Candida* species, 14 (82.4%) were *C glabrata* isolates (11 in the cancer treatment group, 3 in the control group) resistant to itraconazole. *C krusei*, isolated from subject #60-1, was resistant to three antifungals (5-FC, FCZ, MCZ; Table 1), which is an unsurprising finding because *C krusei* has been

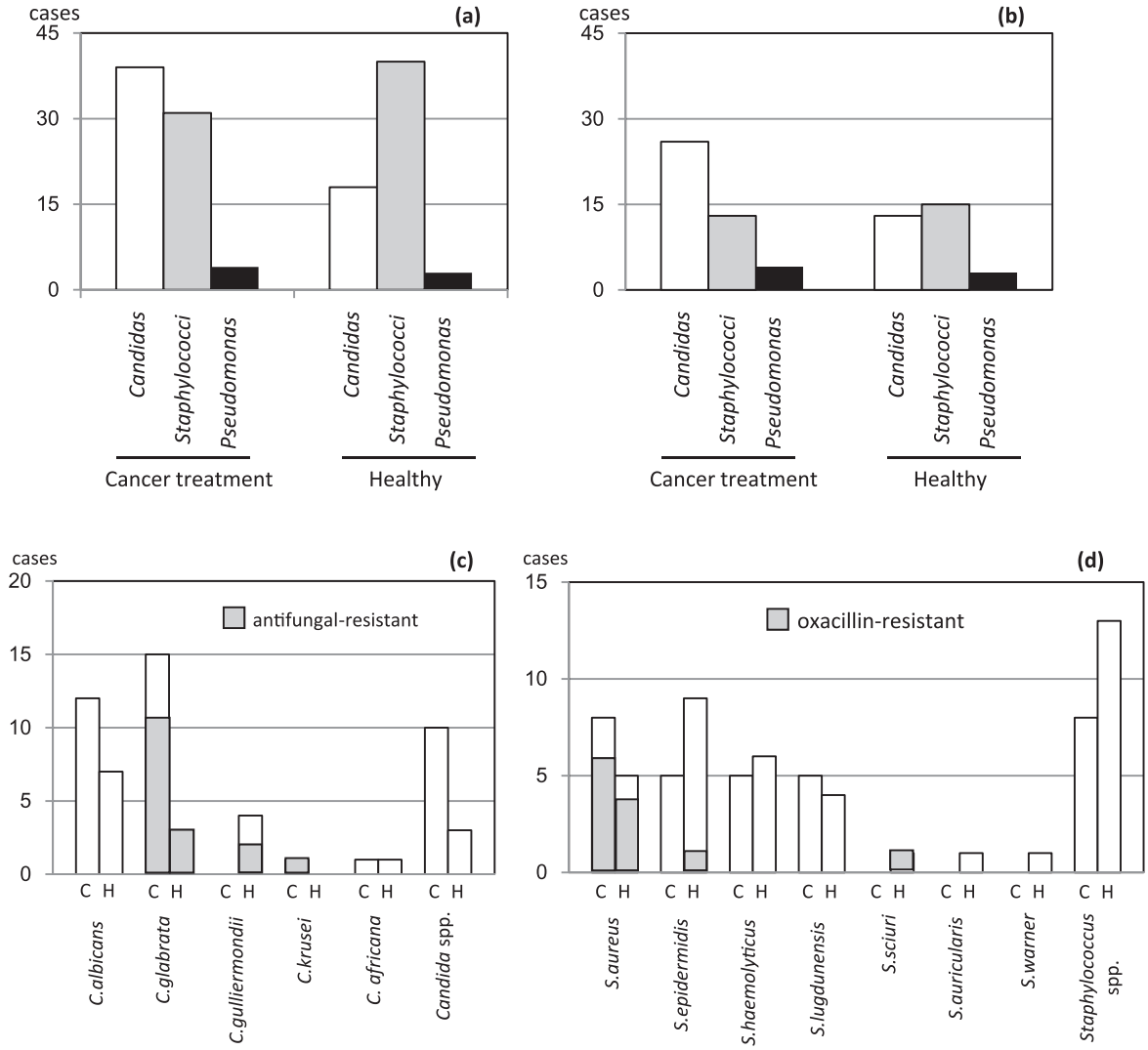

Fig. 1 Prevalence of drug-resistant opportunistic microorganisms in cancer treatment and control groups.
Candida species (*Candidas*), *Staphylococcus* species (*Staphylococci*), and *Pseudomonas* species (*Pseudomonas*) were isolated from both groups. The cancer treatment group (Cancer treatment) included 46 patients who had received oral cancer treatment and had good outcomes. The control group (Healthy) included 37 patients who had no history of any cancer and were being treated for cavities or poorly fitting dentures. Panel (a) shows the numbers of microorganisms isolated from the subjects. Panel (b) shows the number of subjects who harbored at least one opportunistic microorganism.
The numbers of *Candida* subspecies isolated are shown in panel (c). Grey bars indicate the numbers of strains that showed resistance to at least one antifungal agent. Minimum inhibitory concentrations (MICs) were measured in *Candida* species. Of the 18 *C glabrata* isolates (15 in the cancer treatment group, 3 in the control group), 11 in the cancer treatment (C) and 3 in the control (H) group were drug-resistant. Panel (d) shows the numbers of *Staphylococci* subspecies and oxacillin-resistant strains isolated. Six of 8 (75%) *S aureus* organisms in the cancer treatment group and 3 of 5 (60%) in the control group were MRSA. In total, 9 (69.2%) of 13 *S aureus* isolates were MRSA.

reported to be an intrinsically drug-resistant fungus in which antifungals bind the target molecule cytochrome P-450 with very low affinity (14). The drug resistance of *C glabrata* is likely partly due to the association with increased expression of genes encoding *CDR* drug efflux pumps (15,16). However, all the present strains showed susceptibility to MCFG and VRCZ (Table 1). Interestingly, one major fungus in the oral cavity, *C albicans*, did not show resistance to the antifungals used in this study. Furthermore, only four men carried drug-resistant strains; the other 13 were isolated from women.

Among the *Staphylococcus* species isolates, *S aureus* was detected in 13 participants. Among these 13 isolates, 9 (69.2%) were MRSA, namely 6 (75%) MRSA isolates among eitht participants with *S aureus* in the cancer treatment group and three MRSA isolates among five participants with *S aureus* in the control group (Fig. 1d, 2). Among *S epidermidis* isolates from the control group, 1

Table 1 Antifungal susceptibility of drug-resistant *Candida* species

No	Age, y	Sex	Sampling site	Cancer treatment	Identification	AMPH	5FC	FCZ	MCZ	ITZ	MCFG	VRCZ
60-1	62	M	saliva	surgery&RT	*C.krusei*	S	R	R	R	R	S	S
88-46	79	F	surgical scar	surgery&chemo&RT	*C.glabrata*	S	S	S-DD	I	R	S	S
43-3	70	F	saliva	surgery&chemo&RT	*C.glabrata*	S	S	S-DD	I	R	S	S
22-67	69	F	saliva	surgery&chemo&RT	*C.glabrata*	S	S	S-DD	I	R	S	S
53-8	79	F	saliva	surgery&chemo	*C.glabrata*	S	S	S-DD	I	R	S	S
12-3	45	M	saliva	surgery&chemo	*C.glabrata*	S	S	S-DD	I	R	S	S
11-8	81	F	surgical scar	surgery&chemo	*C.glabrata*	S	S	S-DD	I	R	S	S
94-9	81	F	saliva	surgery	*C.glabrata*	S	S	S-DD	I	R	S	S
47-13	87	M	saliva	surgery	*C.glabrata*	S	S	S-DD	I	R	S	S
36-12	62	F	saliva	surgery	*C.glabrata*	S	S	S-DD	I	R	S	S
25-18	91	F	saliva	surgery	*C.glabrata*	S	S	S-DD	I	R	S	S
9-7	74	F	saliva	surgery&RT	*C.glabrata*	S	S	S	I	R	S	S
55-9	66	F	saliva	healthy	*C.glabrata*	S	S	S-DD	I	R	S	S
66-11	75	F	saliva	healthy	*C.glabrata*	S	S	S-DD	I	R	S	S
79-5	80	F	saliva	healthy	*C.glabrata*	S	S	S	S	R	S	S
65-3	77	F	saliva	healthy	*C.guilliermondii*	S	S	S-DD	R	R	S	S
80-43	74	M	saliva	healthy	*C.guilliermondii*	S	S	S	R	R	S	S

AMPH: amphotericin B, 5FC: 5-fluorocytosine, FCZ: fluconazole, MCZ: miconazole, ITZ: itraconazole, MCFG: micafungin, VRCZ: voriconazole, S: sensitive, S-DD: susceptible-dose dependent, I: intermediate, R: resistant, chemo: chemotherapy, RT: radiotherapy

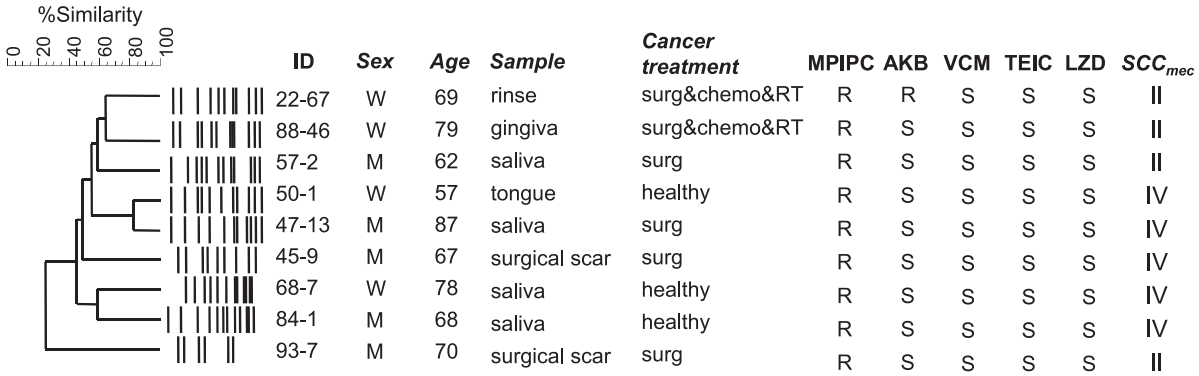

Fig. 2 Phylogenic tree, antibiotic susceptibility, and genotyping of staphylococcal cassette chromosome *mec* (SCC*mec*).
Pulsed-field gel electrophoresis (PFGE) of the *Sma*I-digested MRSA genome revealed a phylogenic tree of 9 MRSA organisms. Information on subjects, including MIC, is shown. Abbreviations – MPIPC: oxacillin, ABK: arbekacin, VCM: vancomycin, TEIC: teicoplanin, LZD: linezolid, S: sensitive, I: intermediate, R: resistant, surg: surgery; RT: radiotherapy.

of 9 was oxacillin-resistant (so-called coagulase-negative methicillin-resistant *Staphylococcus* [MRCNS]), while one MRCNS *S sciuri* strain was isolated from the control group (Fig. 1d). Susceptibility tests were performed for antibiotics frequently used against MRSA in hospitals (Fig. 2). Isolated MRSA and MRCNS strains were all sensitive to vancomycin, teicoplanin, and linezolid, which are the agents of last resort against MRSA infections. One MRSA strain (subject #22-67) was resistant to arbekacin.

Antibiotic susceptibility of *P aeruginosa* was examined using the criteria of the Japanese Infectious Disease Surveillance Center (IDSC). However, no multidrug-resistant *P aeruginosa* (MDRP) organisms were found.

As noted above, 14 of our 83 subjects had both *Candida* and *Staphylococcus* species. Of those, four had both MRSA and drug-resistant *Candida* species, three of whom were in the cancer treatment group.

History of antibiotic and antifungal treatment

There was variation in the doses and durations of antibiotic treatment during oral cancer treatment. Flomoxef, a second-generation oxacephem antibiotic, was the most frequently used antibiotic in this study, followed by

Table 2 Prevalence of opportunistic microorganisms in cancer patients who had undergone surgery, chemotherapy, and radiation therapy

No.	Age, y	Sex	Cancer site	Surgery/chemotherapy/radiotherapy	Candidas	Staphylococci (R or S)	Pseudomonas (R or S)
4-1	72	F	tongue	partial resection + 5FU + external RT 70 Gy	*C.albicans*(S)		
8-5	67	M	tongue	partial resection, RND, skin graft + TS1, 5FU, DCT, CDDP + external RT 60 Gy	*C.albicans*(S)		
15-1	67	F	tongue	partial resection, titanium plate + 5FU + internal RT 60 Gy	*C.albicans*(S)		
30-2	66	F	oral floor	partial resection + CDDP, 5FU + external RT 66 Gy	*C.albicans*(S)		*P.aeruginosa* (S)
31-3	74	F	lower gingiva	block resection, PND, titanium plate + 5FU + external RT 60 Gy			
36-12	62	F	upper gingiva	partial resection, RND, skin graft + 5FU + external RT 49 Gy	*C.glabrata*(R)	*S.aureus*(S)	
43-3	70	F	upper gingiva	partial resection + 5FU + external RT 60 Gy	*C.glabrata*(R)		
60-1	60	M	palate	partial resection, RND + 5FU + external RT 60 Gy	*C.krusei*(R)	*S.epidermidis*(S)	
88-46	79	F	lower gingiva	partial resection + 5FU + internal RT 60 Gy	*C.glabrata*(R)	*S.aureus*(R), *S.lugdunensis*(S), *S.haemolyticus*(S)	
89-3	58	M	tongue	partial resection + CDDP + internal RT 30 Gy, external RT 32 Gy		*S.epidermidis*(S)	

PND, partial neck dissection; RND: radical neck dissection; 5FU: fluorouracil; CDDP: cisplatin; DCT: docetaxel; TS1: tegafur; RT, radiotherapy; R, drug-resistant; S, drug-sensitive

cefazolin, a first-generation cephalosporin antibiotic. In all but two cases, first- and second-generation cephalosporin antibiotics were given. Meropenem, which belongs to the carbapenem subgroup, was given to one patient (#22-67, Fig. 2), who carried drug-resistant *C glabrata* and ABK-resistant MRSA, as described above. In the other case (#15-1, Table 2), clindamycin hydrochloride, a lincosamide antibiotic, was given simultaneously with flomoxef. No drug-resistant organism was detected in this patient. The duration of administration was 3-7 days. There was no association between prevalence of drug-resistant organisms and duration of antibiotic administration.

Prevalence of drug-resistant opportunistic microorganisms among follow up-patients treated for severe oral cancer

Ten patients in the cancer treatment group had required all three therapeutic modalities (surgery, chemotherapy, and radiotherapy) for treatment of their primary cancer (Table 2). Partial or block resection of primary lesions had been performed. Fluorouracil (5FU), cisplatin (CDDP), docetaxel (DCT), and tegafur (TS1), either singly or combined, were given as chemotherapy. In some cases, intra-arterial injection was selected, to kill cancer cells more effectively. Radiotherapy included external beam therapy (teletherapy) and internal therapy (brachytherapy) with implanted unsealed radioisotopes. Total dose was 9-70 Gy.

The findings regarding the prevalences of opportunistic microorganisms in these 10 patients were unremarkable. Three patients had both *Candida* and *Staphylococcus* species. One patient (#88-46; Fig. 2, Tables 1, 2) carried drug-resistant *C glabrata* and MRSA. To date, this patient has not developed any subjective or objective health problems.

We also examined the prevalence of opportunistic organisms in the 17 follow-up patients who underwent reconstructive surgery (data not shown). Reconstructive surgery on bone defects included iliac bone graft and titanium plate implantation. Soft tissue reconstructions included pectoralis major myocutaneous (PMMC) flap, temporoparietal fascia flap, rectus abdominis myocutaneous flap, and skin graft. Our analysis revealed no significant findings.

Phylogenic analysis of isolated MRSAs

Because drug-resistant *Candida* species seem to be primarily inherited, we next focused on how the *mec*A gene was acquired among methicillin-resistant *Staphylococci* and the possibility of nosocomial transmission of MRSA. To investigate the origin of MRSA and

nosocomial infection, we performed a PFGE of the *Sma*I-digested MRSA genome. The results are shown as a phylogenic tree in Fig. 2. A very similar PFGE pattern (an approximately 80% match) was seen in samples #50-1 and #47-13. These subjects had not been admitted to a hospital during the same period and did not live near each other. The other samples showed no such similarity.

The methicillin-resistant phenotype is transferred by acquiring staphylococcal cassette chromosome *mec* (SCC*mec*) elements, which carry the penicillin-binding protein 2a (*mec*A) and cassette chromosome recombinase (*ccr*) genes. SCC*mec* of MRSA has been classified into more than eight genotypes (17). Nine isolates of MRSA were classified by PCR, which showed that four MRSA isolates were type II and five were type IV (Fig. 2).

One of the most important toxins produced by MRSA is Panton-Valentine leukocidin (PVL), which induces pore formation in the membranes of host defense cells, especially white blood cells, monocytes, and macrophages. PVL was reported to be present in most community-associated methicillin-resistant *S aureus* (C-MRSA) infections (18). In the present study, all MRSA strains isolated from oral cavities were PVL-negative (data not shown). Other toxins, including staphylococcal enterotoxins (*sea-selr* and *tst-1*), were also negative in all tested strains.

Discussion

We performed a microbiological study of the major opportunistic microorganisms in the oral cavities of healthy volunteers and adults treated for oral cancer. In the analysis of *Candida*, *Staphylococcus*, and *Pseudomonas* species, we found no significant differences between groups in the numbers of microorganisms isolated. However, 9 (69.2%) of 13 *S aureus* isolates were MRSA, suggesting that MRSA colonization is increasing among elderly Japanese.

Because we wanted to develop a protocol for assessing risk factors related to oral health after cancer therapy, we chose to study patients who had been treated for oral cancer, had good outcomes, and were receiving periodic examinations at our department. Previous studies investigated mucositis in cancer patients by searching published reports; however, some of the findings were controversial (19,20). One report noted that oral mucositis showed no clear pattern in relation to type of cancer treatment or antimicrobial agents (21). Other studies investigated microbiological changes in oral flora among leukemia patients. Systematic reviews of published reports were inconsistent with regard to qualitative or quantitative changes in oral flora during cancer chemotherapy and showed no clear pattern or association between mucositis and oral flora changes (2,22). In contrast, several studies have reported qualitative changes in oral flora during chemotherapy (23-25). Although such changes in oral flora are not well understood, the incidence of *Candida* species seems to increase after chemotherapy and radiotherapy for cancer (26), a trend also seen in oral flora obtained from immunocompromised hosts (27). In the present study, we found no significant differences in the prevalence of opportunistic microorganisms between the cancer treatment and control groups. It is likely that qualitative changes in oral opportunistic microorganisms do not occur in patients who have satisfactorily recovered after oral cancer treatment. We expected that, as compared with the controls, opportunistic microorganisms would be much more plentiful in the mouths of the cancer patients, but this was not the case, probably because of immunological recovery and improvement in the oral ecosystem after successful cancer therapy. We do not know how long it takes for the oral ecosystems of patients to recover their oral flora. However, once commensal flora return to the mouths of patients, there may be no difference between the cancer treatment group and control group in the detection rate of opportunistic microorganisms.

Because we found no difference in microorganisms or oral mucosal condition between groups, our focus shifted to the prevalence of drug-resistant opportunistic microorganisms, especially MRSA. MRSA prevalence in the oral cavity is a frequent topic of study. According to a study performed in the United Kingdom, 13 (41.9%) of 31 *S aureus* isolates from the oral mucosa and pockets of patients with gingivitis/periodontitis were *mec*A-positive (28). Another study, in the United States, reported that the prevalence of MRSA organisms in the nasal and oral cavities of nursing home residents was 20-35% (29). Furthermore, a Japanese group investigated MRSA colonization in neonatal intensive care units and found that 207 (49.9%) of 415 newborns had MRSA organisms (30). As compared with these results, the MRSA prevalence (69.2%) in the present study was quite high, possibly due in part to the tendency to prescribe long-term, high-dose antibiotic treatment in Japan (31).

Genotyping of SCC*mec* showed that types II and IV were prevalent in our subjects. A previous Japanese study reported that the prevalent SCC*mec* genotype changed from I or IV (frequently detected in the early 1980s) to type II, which carries a large number of drug-resistant genes on the SCC*mec* locus (3). Our genotyping results seem to confirm this pattern. SCC*mec* is also carried by MRCNS organisms, which can be carriers of drug-resis-

tant genes. In the present study, 2 MRCNS organisms, *S epidermidis* and *S sciuri*, were detected during the experiments. In the future, we intend to analyze the molecular characteristics of MRCNS, particularly in isolates from immunocompromised hosts.

Although our analysis of antibiotic use showed no significant relation between prevalence of antibiotic-resistant organisms and antibiotic dose/duration, clinicians should be mindful of antibiotic selection and dose. Continuous monitoring and a basic infection control strategy, including standard precautions, are important for older individuals, especially those receiving follow-up care for oral cancer, as there is always a risk that they may become immunocompromised hosts if cancer recurs or metastasizes. Clinicians must also pay careful attention to systemic illnesses (such as diabetes, hypertension, heart disease) and local conditions (including oral hygiene and smoking status; number of teeth; number of decayed, missing, and filled teeth; saliva volume; and severity of periodontal disease).

Acknowledgments

We thank Professor Tetsuji Okamoto for assistance in collecting bacterial samples, Ryoichi Kuwabara for PFGE analysis, and Hokkaido System Sciences and BML for identification of microorganisms.

References

1. Belazi M, Velegraki A, Koussidou-Eremondi T, Andreadis D, Hini S, Arsenis G et al. (2004) Oral Candida isolates in patients undergoing radiotherapy for head and neck cancer: prevalence, azole susceptibility profiles and response to antifungal treatment. Oral Microbiol Immunol 19, 347-351.
2. Napeñas JJ, Brennan MT, Bahrani-Mougeot FK, Fox PC, Lockhart PB (2007) Relationship between mucositis and changes in oral microflora during cancer chemotherapy. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 103, 48-59.
3. Ma XX, Ito T, Chongtrakool P, Hiramatsu K (2006) Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant Staphylococcus aureus strains isolated in Japanese hospitals from 1979 to 1985. J Clin Microbiol 44, 4515-4527.
4. Hidron AI, Low CE, Honig EG, Blumberg HM (2009) Emergence of community-acquired meticillin-resistant Staphylococcus aureus strain USA300 as a cause of necrotising community-onset pneumonia. Lancet Infect Dis 9, 384-392.
5. Ohara M, Kouda S, Onodera M, Fujiue Y, Sasaki M, Kohara T et al. (2007) Molecular characterization of imipenem-resistant Pseudomonas aeruginosa in Hiroshima, Japan. Microbiol Immunol 51, 271-277.
6. Kouda S, Fujiue Y, Watanabe Y, Ohara M, Kayama S, Kato F et al. (2011) Sporadic isolations of a multi-drug resistant Pseudomonas aeruginosa clone during a 14-month epidemic in a general hospital in Hiroshima. Infection 39, 247-253.
7. Repetto EC, Giacomazzi CG, Castelli F (2012) Hospital-related outbreaks due to rare fungal pathogens: a review of the literature from 1990 to June 2011. Eur J Clin Microbiol Infect Dis 31, 2897-2904.
8. Li L, Redding S, Dongari-Bagtzoglou A (2007) Candida glabrata: an emerging oral opportunistic pathogen. J Dent Res 86, 204-215.
9. Myers EN, Suen JY, Myers JN, Hanna EY (2003) Cancer of the head and neck, 4th ed, Saunders, Philadelphia, 675-684.
10. Mizumachi E, Kato F, Hisatsune J, Tsuruda K, Uehara Y, Seo H et al. (2011) Clonal distribution of enterotoxigenic Staphylococcus aureus on handles of handheld shopping baskets in supermarkets. J Appl Microbiol 110, 562-567.
11. Clinical and Laboratory Standards Institute (2011) Performance standards for antimicrobial susceptibility testing. Twenty-first informational supplement. M100-S21. CLSI, Wayne.
12. Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J et al. (2007) Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. Antimicrob Agents Chemother 51, 264-274.
13. Yamaguchi T, Yokota Y, Terajima J, Hayashi T, Aepfelbacher M, Ohara M et al. (2002) Clonal association of Staphylococcus aureus causing bullous impetigo and the emergence of new methicillin-resistant clonal groups in Kansai district in Japan. J Infect Dis 185, 1511-1516.
14. Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Nagy E, Dobiasova S et al. (2008) Candida krusei, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. J Clin Microbiol 46, 515-521.
15. Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20, 133-163.
16. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J (2012) Candida glabrata, Candida parapsilosis and Candida tropicalis: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol Rev 36, 288-305.
17. Li S, Skov RL, Han X, Larsen AR, Larsen J, Sørum M et al. (2011) Novel types of staphylococcal cassette chromosome mec elements identified in clonal complex 398 methicillin-resistant Staphylococcus aureus strains. Antimicrob Agents Chemother 55, 3046-3050.
18. Nastaly P, Grinholc M, Bielawski KP (2010) Molecular characteristics of community-associated methicillin-resistant Staphylococcus aureus strains for clinical medicine. Arch Microbiol 192, 603-617.
19. Bergmann OJ (1991) Alterations in oral microflora and pathogenesis of acute oral infections during remission-induction therapy in patients with acute myeloid leukaemia. Scan J Infect Dis 23, 355-366.
20. O'Sullivan EA, Duggal MS, Bailey CC, Curzon MEJ, Hart P (1993) Changes in the oral microflora during cytotoxic

chemotherapy in children being treated for acute leukemia. Oral Surg Oral Med Oral Pathol 76, 161-168.
21. Donnelly JP, Bellm LA, Epstein JB, Sonis ST, Symonds RP (2003) Antimicrobial therapy to prevent or treat oral mucositis. Lancet Infect Dis 3, 405-412.
22. Napeñas JJ, Brennan MT, Coleman S, Kent ML, Noll J, Frenette G et al. (2010) Molecular methodology to assess the impact of cancer chemotherapy on the oral bacterial flora: a pilot study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 109, 554-560.
23. Fainstein V, Rodriguez V, Turck M, Hermann G, Rosenbaum B, Bodey GP (1981) Patterns of oropharyngeal and fecal flora in patients with acute leukemia. J Infect Dis 144, 10-18.
24. Minah GE, Rednor JL, Peterson DE, Overholser CD, DePaola LG, Suzuki JB (1986) Oral succession of gram-negative bacilli in myelosuppressed cancer patients. J Clin Microbiol 24, 210-213.
25. Sixou JL, De Medeiros-Batista O, Gandemer V, Bonnaure-Mallet M (1998) The effect of chemotherapy on the supragingival plaque of pediatric cancer patients. Oral Oncol 34, 476-483.
26. Almståhl A, Wikström M, Fagerberg-Mohlin B (2008) Microflora in oral ecosystems in subjects with radiation-induced hyposalivation. Oral Dis 14, 541-549.
27. Dahlén G, Blomquist S, Carlén A (2009) A retrospective study on the microbiology in patients with oral complaints and oral mucosal lesions. Oral Dis 15, 265-272.
28. Cuesta AI, Jewtuchowicz VM, Brusca MI, Mujica MT, Rosa AC (2011) Antibiotic susceptibility of Staphylococcus aureus isolates in oral mucosa and pockets of patients with gingivitis-periodontitis. Acta Odontol Latinoam 24, 35-40.
29. Hall DL (2003) Methicillin-resistant Staphylococcus aureus and infection control for restorative dental treatment in nursing homes. Spec Care Dentist 23, 100-107.
30. Uehara Y, Kikuchi K, Nakamura T, Nakama H, Agematsu K, Kawakami Y et al. (2001) Inhibition of methicillin-resistant Staphylococcus aureus colonization of oral cavities in newborns by viridans group streptococci. Clin Infect Dis 32, 1399-1407.
31. Takeda S, Yasunaka K, Kono K, Arakawa K (2000) Methicillin-resistant Staphylococcus aureus (MRSA) isolated at Fukuoka University Hospital and hospitals and clinics in the Fukuoka city area. Int J Antimicrob Agents 14, 39-43.

Supplementary Table Primers used in the study

Gene type	Gene name	gene	primer name	sequence 5'-3'	size
rDNA	bacterial 16s rDNA		16S-Top500(Fw):	agagtttgatcctggctcag	
			16S-Top500(Rv):	attaccgcggctgctggc	526bp
	fungal 26s rDNA		NL1(Fw):	gcatatcaataagcggaggaaaag	
			NL4(Rv):	ggtccgtgtttcaagacgg	varies
SCC*mec*	PBP2a	*mec* A	TY356	actgctatccaccctcaaac	
			TY357	ctggtgaagttgtaatctgg	163bp
	mec complex	classA *mec*	mI4	caagtgaattgaaaccgcct	
			mI3	caaaaggactggactggagtccaaa	ca.180bp
		classB *mec*	IS5	aacgccactcataacatatggaa	
			mA6	tataccaaacccgacaac	ca.2kb
		classC *mec*	IS2	tgaggttattcagatatttcgatgt	
			mA2	aacgttgtaaccaccccaaga	ca.3kb
	ccr complex	type 1 *ccr*A	beta2	attgccttgataatagccitct	
			alpha1	aacctatatcatcaatcagtacgt	695bp
		type 2 *ccr*A	alpha2 (+beta2)	taaaggcatcaatgcacaaacact	937kb
		type 3 *ccr*A	alpha3 (+beta2)	agctcaaaagcaagcaatagaa	1791kb
		*ccr*C	gammaF	cgtctattacaagatgttaaggataat	
				cctttatagactggattattcaaaatat	518bp
		*ccr*B4A4	alpha4	gtatcaatgcaccagaactt	
			beta4	ttgcgactctcttggcgttt	1287bp
toxins	exofoliative toxin	*eta*	TY1	ctatttactgtaggagctag	
			TY2	atttatttgatgctctctat	741bp
		etb	TY3	atacacacattacggataat	
			TY4	caaagtgtctccaaaagtat	629bp
		etd	TY122	aactatcatgtatcaagg	
			TY123	cagaatttcccgactcag	376bp
	Panton-Valentine Leukocidin	*PVL*	PVL1	atcattggtaaaatgtctggacatgatcca	
			PVL2	gcatcaastgtattggatagcaaagc	433bp
	epidermal differentiation inhibitor	*edinA*	TY45	taaatgggggaataaactta	
			TY46	cgatacttgtcaaataatct	248bp
		edinB	TY370	ttagtttgatagatacatctt	
			TY371	atttagttttgacacaagcg	422bp
		edinC	TY372	agactcataagcaaatcaagt	
			TY373	gaagaaaaaccttaacactgg	926bp
	Leukocidin	*lukE*	TY834	ggatatgaattaactaaacg	
			TY835	tggaccatttggactttgtacg	368bp
		lukD	TY836	acatcaaatgcctttattggcgc	
			TY837	ttagtttctttagaatccgtacc	263bp
	enterotoxin A	*sea*	SEA-3	cctttggaaacggttaaaacg	
			SEA-4	tctgaaccttcccatcaaaaac	127bp
	enterotoxin B	*seb*	SEB-1	tcgcatcaaactgacaaacg	
			SEB-4	gcaggtactctataagtgcctgc	477bp
	enterotoxin C	*sec*	SEC-3	ctcaagaactagacataaaagctagg	
			SEC-4	tcaaaatcggattaacattatcc	271bp
	enterotoxin D	*sed*	SED-3	ctagtttggtaatatctcctttaaacg	
			SED-4	ttaatgctatatcttatagggtaaacatc	319bp
	enterotoxin E	*see*	SEE-3	cagtacctatagataaagttaaaacaagc	
			SEE-2	taacttaccgtggacccttc	178bp
	enterotoxin G	*seg*	SEG-1	aagtagacatttttggcgttcc	
			SEG-2	agaaccatcaaactcgtatagc	287bp
	enterotoxin H	*sehe*	SEH-1	gtcataatggaggtacaacact	
			SEH-2	gacctttacttatttcgctgtc	213bp
	enterotoxin I	*sei*	SEI-1	ggtgatattggtgtaggtaac	
			SEI-2	atccatattctttgcctttaccag	454bp
	enterotoxin J	*selj*	SEJ-1	atagcatcagaactgttgttccg	
			SEJ-2	ctttctgaattttaccaccaaagg	152bp
	enterotoxin P	*selp*	SEP-3	tgatttattagtagaccttgg	
			SEP-4	ataaccaaccgaatcaccag	396bp
	enterotosin K	*selk*	SEK-1	taggtgtctctaataatgcca	
			SEK-2	tagatattcgttagtagctg	293bp
	enterotoxin M	*selm*	SEM-1	ggataattcgacagtaacag	
			SEM-2	tcctgcattaaatccagaac	379bp
	enterotoxin O	*selo*	SEO-1	tgtgtaagaagtcaagtgtag	
			SEO-2	tctttagaaatcgctgatga	214bp
	toxic shock syndrome toxin 1	tst1	TST-3	aagccctttgttgcttgcg	
			TST-6	atcgaactttggcccatacttt	447bp
	enterotoxin L	*sell*	SEL-1	taacggcgatgtaggtccagg	
			SEL-2	catctatttcttgtgcggtaac	383bp
	enterotoxin N	*seln*	SEN-1	tatgttaatgctgaagtagac	
			SEN-2	atttccaaaatacagtccata	282bp
	enterotoxin Q	*selq*	SEQ-1	aatctctgggtcaatggtaagc	
			SEQ-2	ttgtattcgttttgtaggtattttcg	122bp
	enterotoxin R	*selr*	SER-1	ggataaagcggtaatagcag	
			SER-4	gtattccaaacacatctaac	166bp