Resistogram typing of oral *Candida albicans* isolates from normal subjects in three successive trials

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Summary

Sixty-six oral strains of *Candida albicans*, which had been consecutively isolated from 22 normal, young females in three isolation trials at intervals of one to three weeks, were biotyped by their susceptibility to boric acid, cetrimide, silver nitrate, sodium periodate and sodium selenite. The 66 isolates were grouped into 13 resistogram types. An identical biotype strain was found three times and twice in seven and six each of the 22 subjects in the three isolation trials, respectively. In the remaining nine subjects, different strains were found at the three trials. These results suggested that certain strains tended to persist in the oral cavity of the normal subjects although changes in the biotype of oral *C. albicans* strains occurred to a certain extent.

Key words *Candida albicans*, Normal subjects, Oral isolates, Resistogram

Resistograma de cepas orales de *Candida albicans* aisladas en personas sanas en tres veces consecutivas

Resumen

Sesenta y seis cepas orales de *Candida albicans* fueron aisladas tres veces en 22 mujeres sanas en un periodo de una a tres semanas. Las cepas aisladas fueron biotipficsadas según sus sensibilidad al ácido bórico, cetrimida, nitrato de plata, periodato de sodio y selenito de sodio. Según los resultados obtenidos las cepas fueron agrupadas en 13 diferentes resistogramas. El mismo biotipo fue observado tres veces en siete casos y dos veces en seis casos de las 22 mujeres examinadas por tres veces. Las nueve mujeres restantes mostraron diferentes biotipos de *C. albicans*. Los resultados obtenidos muestran que algunas cepas de *C. albicans* persisten en la cavidad oral de personas sanas a pesar de un posible cambio de biotipo oral.

Palabras clave *Candida albicans*, Personas sanas, Cepas orales, Resistograma

The opportunistic fungal pathogen, *Candida albicans*, is the most frequent cause of superficial and deep-seated candidiasis [1,2]. The risk of infection by this opportunistic yeast has increased in compromised patients, in particular, in HIV-infected and AIDS patients during recent years [3-8].

The biotyping of invasive *C. albicans* strains is essential for tracing the source and spread of candidal infections. In addition, a relationship between the biotypes and a possible role in inducing oral cancer has been speculated. The catalytic potential to form the esophageal carcinogen, N-nitrosobenzylmethylamine, from its precursors [9] has been demonstrated in *C. albicans* strains of certain biotypes [10,11]. Therefore, it is important to know the biotype of invasive strains not only in respect to epidemiology and but also to the pathogenesis of candidiasis.

C. albicans is known to be harbored in the oral cavity of a high percentage of normal subjects [1]. Biotyping studies of oral *C. albicans* strains isolated from normal subjects and patients have been reported [12-14]. Moniaci *et al.* [15] and Bruatto *et al.* [16] reported changes in the biotypes of *C. albicans* strains isolated from HIV-infected patients with recurrent oral candidiasis. We previously attempted to isolate oral yeasts from 109 normal, young female students in three successive trials [17]. *C. albicans* was recovered from 26-45% of their mouths. Among the subjects tested, 23, 10 and 4 individuals were respectively positive for *C. albicans* three times, twice and once in the three isolation trials. Thus, it was considered important to know whether the biotypes of these oral isolates changed during the period of our experiments. In the present study, the oral strains isolated in the previous study [17] were differentiated by a resistogram typing method.

MATERIALS AND METHODS

Strains. Sixty-six oral strains of *C. albicans* were examined in the present study. They had been isolated

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from 14 healthy young female students (18 to 20-year-old; average 18.5 years) from a dental hygienist school (DH group) and 8 nurses (18 to 31-year-old; average 20.1years) from a training school (NT group) in three isolation trials at intervals of one to three weeks by using a swabbing method, as reported previously [17]. Briefly, surfaces of the tongue, teeth, buccal mucosa and gingiva were swabbed with a sterile cotton swab (Nippon Menbo, Japan) for 2 min and the specimens obtained were seeded on Sabouraud dextrose agar (SDA) (Nissui, Japan). The agar plates were incubated at 30ºC for 3-4 days. Single colonies of yeasts were transferred onto fresh SDA. The isolates were identified by standard morphological (formation of germ tubes and chlamydospores) and physiological tests using the MicroScan Rapid Yeast Identification Panel (Baxter Diagnostics, USA). The isolates were maintained on SDA slants.

Typing. The resistogram typing of the isolates was performed according to the method developed by McCreight and Warnock [12] with modifications. The chemicals (Wako Pure Chem. Indust., Japan) and the concentrations (mg/ml SDA) used for the resistogram typing were: boric acid, 1.15, 1.3, 1.45, 1.6; cetrimide, 0.06, 0.08, 0.1, 0.12; silver nitrate, 0.0075, 0.01, 0.0125, 0.015; sodium periodate, 0.01, 0.02, 0.03, 0.04; and sodium selenite, 0.1, 0.2, 0.3, 0.4. To prepare stock solutions, all of the chemicals, except silver nitrate, were dissolved in sterile distilled water at 20 mg/ml, while the silver nitrate was dissolved at 2 mg/ml. Aliquots of the stock solutions were added to 40-ml amounts of molten SDA at 55ºC to give the above final concentrations These mixtures were poured into 13.5 x 9.5-cm plastic dishes (Eiken, Japan). Cells of each strain grown on SDA at 37ºC for 24 h were suspended in sterile distilled water and the optical density at 540 nm was adjusted to 0.45. Five microliters of the suspensions were inoculated on a SDA plate (control) and chemical-containing SDA plates by using a multichannel pipette (Socorex, Switzerland). After incubation for 40 h at 37ºC, the growth of each strain was measured.

Reading of results. Growth of the tested strains on chemical-containing plates was recorded as 'full confluent growth' or 'non-confluent/no growth'. Among the four plates containing a chemical at different concentrations, the plate that most clearly differentiated the growth of the tested strains was used to read results (see Results). To describe the resistogram, sodium selenite, boric acid, cetrimide, sodium periodate and silver nitrate were respectively lettered A to E, according to McCreight and Warnock [12]. The 'full confluent growth' and 'non-confluent/no growth' were expressed as a capital letter (A to E) and a hyphen (-), respectively [12]. Thus, for example, the resistogram "AB - - E" signifies that the tested strain was resistant to sodium selenite (A), boric acid (B) and silver nitrate (E), but sensitive to cetrimide (-) and sodium periodate (-).

RESULTS AND DISCUSSION

As mentioned above, four plates, which contained different concentrations of each chemical, were used to examine the growth of each isolate. In preliminary trials, we used the four concentrations of each chemical originally reported by McCreight and Warnock [12]. However, results of the trials showed that the concentrations of sodium selenite, sodium periodate and silver nitrate were found to be too high to obtain clear differentiation of the tested strains under our conditions. Thus, the concentration of these chemicals was moved to a lower range. In the present study, the following concentrations (mg/ml SDA) among the four concentrations of each chemical gave clearest differentiation of the tested strains and were used to read results: sodium selenite, 0.3; boric acid, 1.3; cetrimide, 0.06; sodium periodate, 0.03 and silver nitrate, 0.0125. In our experience, the concentration of the chemicals to give clearest discrimination of the tested strains tended to vary slightly in repeated trials. This shift may be due to minor variation in the chemical concentration from run to run [12]. Thus, results obtained in a same experimental run were shown below.

The results of the resistogram typing of the 66 oral *C. albicans* strains are shown in table 1. Among the 22 subjects, who were positive for *C. albicans* in all of the three isolation trials, 7 (student nos. 1, 3, 5, 7, 10, 14 and 18) harbored an identical biotype strain, 6 (student nos. 2, 9, 13, 17, 19 and 22) gave an identical strain twice in the three trials, and 9 yielded different biotype strains in each of the isolation trials.

The resistograms found in the 66 oral isolates from the DH and NT groups are listed in table 2. The 66

Table 1. Resistograms of 66 oral strains of *Candida albicans* obtained from 22 normal subjects in three successive isolation trials.

Group	Student number	Isolation trials*		
		1st	2nd	3rd
DH	1	-B-DE	-B-DE	-B-DE
	2	--CDE	-BCDE	-BCDE
	3	-BCDE	-BCDE	-BCDE
	4	-BC-E	-BCDE	-B--E
	5	-B-DE	-B-DE	-B-DE
	6	---DE	-BCDE	-B-DE
	7	---DE	---DE	---DE
	8	-B-D-	-B--E	-B---
	9	AB-DE	AB--E	AB--E
	10	-BC-E	-BC-E	-BC-E
	11	----E	-BCDE	-B-DE
	12	--C-E	-BCDE	---DE
	13	--CDE	--CDE	---DE
	14	---DE	---DE	---DE
NT	15	--CDE	----E	---DE
	16	-BCDE	-BCD-	---DE
	17	--CDE	---DE-	-CDE
	18	--CDE	--CDE	--CDE
	19	--CDE	-BCDE	--CDE
	20	--CDE	--C-E	----E
	21	-BCDE	-B-DE	---DE
	22	--CDE	---DE	---DE

*In the DH group, the intervals between the three isolation trials were three and two weeks, while the interval for the NT group was one week [17]. The above results of the DH and NT groups were obtained in a same experimental run.

strains were grouped in 13 resistogram types among the 32 potential biotypes that could be typed by the method used in the present study. The three resistogram types, -BCDE, - - CDE and - - - DE, were the major ones among the 13 biotypes observed. However, different features of the resistogram type were observed between the DH and NT groups. About 40% isolates (17 strains) of the DH group were distributed in the resistogram types - BCDE and - B - DE in which only about 17% (4 strains) of the NT group were included. Inversely, about 42% isolates (10 strains) of the NT group were included in the - - CDE type which was a minor type in the DH group. As mentioned above, the resistogram typing of the isolates from the two group was determined in a same experimental run to minimize experimental variation. We have no reasonable explanation for the differences in the resistogram type between the two groups.

Several methods have been described to differentiate the biotypes of oral *C. albicans* isolates [18]. We used the resistogram typing method reported by

McCreight and Warnock [12] with modifications, because this method is convenient and easy for biotyping a large number of *C. albicans* isolates. McCreight's group differentiated oral isolates of *C. albicans* from normal subjects [12], patients with denture-induced stomatitis [13] and those with oral and laryngeal cancer [14] using the resistogram method. They reported that 198 oral isolates from 22 normal subjects sampled at one month intervals for 12 months were grouped into 16 resistograms [13]. Their results showed that certain strains persisted in individual subjects although there were slight shifts of the resistogram during the 12-month period. Unfortunately, however, changes of the biotypes in each individual in the successive isolation trials were not described in the report. A direct comparison of the resistogram between the previously reported results [12-14] and ours is difficult because of differences in the chemical concentrations used.

In our study, identical biotype strains were isolated three times from 7 (32%) of the 22 subjects in the three isolation trials and twice from 6 (27%) subjects. Similarly, of the 10 subjects, who were positive for *C. albicans* twice in the three isolation trials, 7 individuals gave identical strains (data not shown). These results revealed that a particular strain tends to persist in the oral cavity of normal subjects although changes of the *C. albicans* biotype occur to a certain extent, supporting the results reported previously [13].

A group at the University of Turin, including two authors of the present paper, reported that changes in the biotype of *C. albicans* were significant in recurrent oral candidiasis in HIV-infected patients [15,16]. The changes were discussed in relation to antifungal therapy in patients; replacement of the initial biotype by a new biotype or by an antifungal drug-resistant one. Our results show that shifts in the resistogram biotypes of oral isolates of *C. albicans* are not frequent, but they do occur to a certain extent in normal subjects. Further studies are required to reveal whether the changes are due to the replacement of the initial strains or by variations of their physiological characteristics.

As performed in previous biotyping studies of *C. albicans* isolates [12-14], we picked up one colony of *C. albicans* grown on primary isolation plates of each individual [17]. Thus, it is difficult to exclude completely the possibility that the resistogram change in each individual observed above might be due to the methodological reason. This problem in sampling of *C. albicans* strains is shared in other biotyping methods, as discussed previously by McCreight and Warnock [12]. A number of colonies grown on primary isolation plates must be examined to reveal whether strains more than one biotype are isolated from each individual at one occasion. Thus, our results cannot contradict to assume that an identical resistogram strain might persist in an individual from whom different biotype strains had not been isolated. This sampling problem remains to be solved in further studies.

Table 2. Resistograms of 66 oral strains of *Candida albicans* isolated from 22 normal subjects in the DH and NT groups.

Resistogram	Number of strains		
	DH group	NT group	Total
AB-DE	1	0	1
AB--E	2	0	2
-BCDE	9	3	12
-BCD-	0	1	1
-BC-E	4	0	4
-B-DE	8	1	9
-B-D-	1	0	1
-B--E	2	0	2
-B---	1	0	1
--CDE	3	10	13
--C-E	1	1	2
---DE	9	6	15
----E	1	2	3
Total	42	24	66

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