博 士 論 文

Comparison of the virulence of *Candida albicans* strains oral candidiasis and non-oral candidiasis

口腔カンジダ症由来株と非口腔カンジダ症由来株の *Candida albicans* の病原性の比較

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Comparison of the virulence of *Candida albicans* strains on oral candidiasis and nonoral candidiasis

· Running title: Comparison of *C. albicans* pathogenicity

· Keywords: *Candida albicans*, fungi, oral candidiasis, pathogenesis

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[2] Department of Oral Molecular Microbiology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan **Abstract**

Objective

Oral candidiasis is an opportunistic infection caused by the fungus *Candida*. The underlying cause of candidiasis is therefore more likely to be host factors than the presence of the fungus. However, the pathogenicity of the fungus may also be important. We therefore hypothesised that the pathogenicity of *Candida albicans* may be different between strains, and aimed to compare the differences of virulence.

Materials and Mehotds
Candida albicans from patients who visited the Hokkaido University Hospital Dental Centre and were tested for *Candida* to i) identify the *Candida albicans* genotype by PCR,
ii) measure the strain's extracellular secretory enzyme activity, iii) determine the strain's ability to induce the production of interleukin-8, and iv) determine the strain's ability to induce cell death.

Results

We found that certain virulence-related activities were higher in strains derived from patients with oral candidiasis compared with strains derived from patients without oral candidiasis.

Conclusion

These results suggest that differences in the virulence of the fungal strain may influence the pathogenesis of oral candidiasis.

1. Introduction

Candidiasis is the most common fungal infection of the oral cavity, and is caused by *Candida* spp. Previously, it was thought that between 35% and 80% of the population harbour *Candida* in the oral cavity. The most common oral fungus is *Candida albicans*,

which is estimated to be present in 80% of oral fungal isolates (Talapko *et al*, 2021), (Lewis and Williams, 2017). Various factors can disrupt oral homeostasis, resulting in a change from normal to abnormal flora (dysbiosis). These changes can lead to microbial overgrowth and the expression of virulence factors (Lewis and Williams, 2017), (Zdanavičienė *et al*, 2017). Infections are typically opportunistic, and are often influenced by host factors (Oouchi *et al*, 2015) such as xerostomia, poor denture cleaning, and topical steroid preparations. In addition, the infection may be established because of abnormal systemic defence mechanisms, such as long-term administration of antimicrobials, steroids, anti-cancer drugs, or immunosuppressive drugs, as well as immunocompromise due to AIDS, diabetes mellitus, or aging (Suryana *et al*, 2020), (Mane *et al*, 2012), (Soysa *et al*, 2006). Three forms of oral candidiasis are known to exist: pseudomembranous candidiasis, which is characterised by a layer of detachable white moss; erythematous candidiasis, which is smooth and erythematous; and hypertrophic candidiasis (Jayampath Seneviratne *et al*, 2015), in which the mucosal epithelial layer becomes thickened and hardened in a speckled pattern because of chronicity. Clinical symptoms often include pain (mainly on the tongue), xerostomia, dysgeusia (such as tasting bitterness), and oral discomfort (such as a sticky sensation). Candidiasis is typically treated with antifungal agents to eradicate the fungus; however, there are many cases in which this is ineffective.

Together with the endemic nature and prevalence of *C. albicans,* these cases suggest that there may be key differences between strains. To investigate this, we examined the differences in pathogenicity of *C. albicans* strains derived from patients with and without oral candidiasis.

2. Materials and methods

2.1 Subjects

The subjects chosen were patients who visited the Hokkaido University Hospital Dental Centre with complaints of oral pain, oral discomfort, abnormal taste, or oral mucosal disease from 2020–2021. All subjects were provided with a detailed oral and written explanation of the study, and gave their written consent. The study was approved by the Human Ethics Committee of Hokkaido University Hospital (approval number: 020-0049). The patients with oral candidiasis (OC) were between 45 and 87 years old, while the patients with non-oral candidiasis (nOC) were between 47 and 87 years old.

2.2 Specimen collection

Specimens were collected by scraping the surface of the tongue approximately ten times with a dental mirror, and were then applied to CHROMagar™ medium (Kanto Chemical, Tokyo, Japan), a selective medium that allows the identification of *Candida*

by the coloration and morphology of the colonies. After sample application, the medium was incubated at 36 °C for 48 hours, and colonies that were identified as *C. albicans* were then collected. Selected *C. albicans* strains were incubated in Sabouraud liquid medium (Oxoid-Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours, dispensed into 1.5 mL tubes, and kept frozen at -80 °C until use. A total of 64 specimens were obtained: 42 from OC patients and 22 from nOC patients. Of the OC-derived strains, 19 patients had pseudomembranous candidiasis, 22 had erythematous candidiasis, and one had black hairy tongue.

2.3 Diagnostic Methods

OC patients were defined as having symptoms consistent with candidiasis (according to the department's diagnostic criteria), with samples resulting in ten or more colonies in CHROMagar™ medium, and showing an improvement after treatment with antifungal drugs. Patients with samples that resulted in nine or fewer colonies in CHROMagar™ medium, and who showed no improvement in symptoms following treatment with antifungal drugs, were designated as nOC. In this study, there were no patients with nine or fewer colonies that showed a symptomatic improvement following antifungal drug treatment.

2.4 Comparison of *C. albicans* characteristics

A comparative study of *C. albicans* was conducted to examine genotypes, extracellular secretory enzyme (ESE) activities, interleukin (IL)-8 production-inducing activity, and cytotoxicity.

2.5 *C. albicans* genotype identification

Previous studies have shown that *C. albicans* can be classified into five genotypes: A, B, C, D, and E (Tamura *et al*, 2001) (Shekhany and Jalal, 2022).

The genomic DNA from *C. albicans* was first extracted using GenCheck® DNA Extraction Reagent (Fasmac, Atsugi, Japan) according to the following protocol. *C. albicans* samples were mixed with 100 µL of GenCheck in a 1.5 mL tube using a vortex mixer. The mixture was then heated at 95 °C for 10 minutes in a heat block. The tubes were placed on ice for 1 minute and then centrifuged (15,000 *g*, 1 minute, room temperature) to collect the supernatant. The genotyping for *C. albicans* was performed by PCR. The primers used were CA-INT-L (5′-ATAAAGGGAAGTCGGGCAAAATAGATCCGTAA-3′) and CA-INT-R (5′CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3′). The PCR conditions were as

follows: denaturation by incubation for 3 min at 94 °C prior to 30 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2.5 min, followed by a final incubation at 72 °C for 10 min in a thermal cycler. All reaction products were characterised by electrophoresis on 1% agarose gels in 1 × Tris-borate-EDTA buffer at 100 V for 30 min and were then stained in a solution of 0.5 μg/mL ethidium bromide (Tamura *et al*, 2001).

2.6 Measurement of ESE activities

C. albicans secretes ESEs, including secreted aspartyl protease (SAP), phospholipase (PL), esterase, and haemolysin. The activity of each ESE was determined according to protocols from Ikarashi *et al.* (2020) and Amarasinghe *et al*. (2021). The activity of the ESE was determined by changes to the agar surface surrounding the colonies (Fig. 1A). The diameter of each colony was measured (A), as well as the diameter of both the precipitation zone and the colony (B). The Pz value was then calculated as A ÷ B, with smaller Pz values indicating higher enzyme activity. The experiments were performed in triplicate, and the average Pz values are given (Ikarashi et al, 2020) (Fig. 1B). All reagents were obtained from commercial sources and were of analytical or reagent grade.

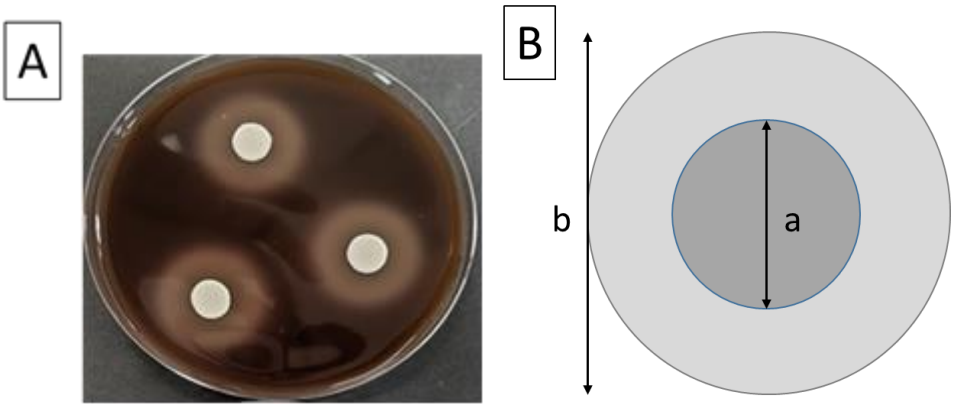

Figure 1. Measurement of ESE activities. (A) A typical picture of the agar plate for the measurement of ESE activity. Large white colonies of C. albicans and destained or precipitated zones around them were observed because of the activity of ESEs. (B) The diameter of the colonies (denoted as A) and the diameter of the destained or precipitated zones (denoted as B) were measured. The Pz values were calculated as A ÷ B.

2.6.1 SAP activity

SAP activity was measured according to the protocol given in Aoki *et al.* (Aoki *et al*, 1990). Solution A consisted of 60 mL sterile purified water, 0.04 g magnesium sulphate 7-hydrate, 0.5 g dipotassium phosphate, 1.0 g sodium chloride, 0.2 g yeast extract, 4.0 g glucose, and 0.5 g bovine serum albumin (Merck Sigma-Aldrich, St. Louis, MO, USA). The pH was adjusted to 3.5 with 1 M HCl, and the solution was sterilised by filtration. Solution B consisted of 3.0 g bacterial agar (Eiken Chemical, Tokyo, Japan) dissolved

in 140 mL purified water and autoclaved at 121 °C for 20 min. After cooling, solution B was mixed with solution A to prepare agar plates. The agar plates were incubated with 5 μL drops of the bacterial solution for 7 days at 35 °C in an aerobic environment, and then the Pz values were determined.

2.6.2 PL activity

PL activity was measured according to the protocol given in Price *et al.* (Price *et al*, 1982) . The medium was prepared by mixing 45 g malt agar medium (Nissui Pharmaceutical, Tokyo, Japan) in a solution of 1 M sodium chloride, 0.005 M calcium chloride, and egg yolk solution, to a produce a final concentration of 4% agar. The agar plates were incubated with 5 μL drops of the bacterial solution for 7 days at 35 °C in an aerobic environment, and then the Pz values were determined.

2.6.3 Esterase activity

Esterase activity was measured using the method given in Ziccardi *et al*.(Ziccardi *et al*, 2015). A solution consisting of 100 mL distilled water, 1.0 g hypolipeptone (Nihon Pharmaceutical, Osaka, Japan), 0.5 g sodium chloride, 0.01 g calcium chloride, and 1.5 g bacterial agar was generated and then autoclaved at 121 °C for 20 minutes. The solution was cooled to approximately 50 °C, then 0.5 mL of Tween-80 was added. The

agar plates were incubated with 5 µL drops of the bacterial solution for 7 days at 35 °C in an aerobic environment, and then the Pz values were determined.

2.6.4. Measurement of haemolysin activity

Haemolysin activity was measured according to the protocols given in Manns *et al.* and Luo *et al*. (Manns *et al*, 1994) (Luo *et al*, 2001). Modified Sabouraud agar medium was prepared by adding sheep blood and glucose at 7% and 3% respectively. The agar plates were incubated with 5 µL drops of the bacterial solution and then incubated for 48 hours at 37 °C in a 5% CO_2 environment. The Pz values were then determined.

2.7 Cell strains

Human buccal mucosa squamous cell carcinoma cells (HO-1-N-1, JCRB) were used in this study. The medium was Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Merck Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum and penicillin-streptomycin (Merck Sigma-Aldrich). Cells were diluted to 2×10^5 cells in 96-well plates and incubated for 24 hours. The media was then discarded and new media containing *C. albicans* was used to dilute the cells to 2×10^5. The cells were then cultured for a further 20 hours. The supernatant was then collected and used to measure IL-8 production and lactate dehydrogenase (LDH) release.

2.8 Measurement of IL-8 production

The human IL-8 ELISA kit was purchased from BioLegend (San Diego, CA, USA) and used according to the manufacturer's instructions. The supernatant was collected as described above and then the absorbance at 450 nm was measured using a microplate reader (Dongari-Bagtzoglou and Kashleva, 2003).

2.9 Cytotoxicity of *C. albicans* to HO-1-N-1 cells

Cell death of HO-1-N-1 cells induced by *C. albicans* was assessed using the CytoTox96® Non-Radioactive Cytotoxicity Assay, which measures LDH released from cells. The supernatant was collected as above and the absorbance at 490 nm was measured according to the manufacturer's instructions (Villar *et al*, 2004).

2.10 Statistical Analysis

Each experiment was performed in triplicate, and the results are expressed as the mean ± SD. A Student's t-test was used to assess the statistical significance of the difference. Values with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 Genotypic identification of *C. albicans*

For both OC- and nOC-derived strains, genotype A was detected with the highest prevalence, followed by genotype C and then genotype B. Genotype D was detected in one case from a strain derived from an nOC-patient, but was excluded from the study because it did not result in colonies on the agar medium used to measure enzymatic activity. Genotype E was not detected. The OC-derived strains consisted of 57% genotype A, 17% genotype B, and 26% genotype C, while the nOC-derived strains consisted of 62% genotype A, 19% genotype B, and 19% genotype C. Genotypes A and B were present at a higher ratio in nOC-derived strains compared with OC-derived strains, whereas genotype C was present at a higher ratio in OC-derived strains (Fig. 2A, B).

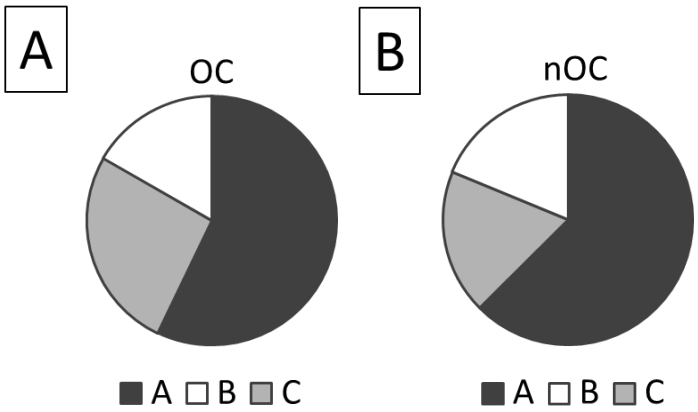

Figure 2. The ratio of C. albicans genotypes. (A) The ratio of genotypes of OC-derived C. albicans. (B) The ratio of genotypes of nOC-derived C. albicans.

3.2 Measurement of ESE activities of *C. albicans*

OC-derived strains showed higher activities for the enzymes SAP, esterase, and haemolysin compared with nOC-derived strains, and significant differences were observed for SAP and haemolysin (Fig. 3A–D).

When compared by genotype, genotype A showed higher SAP, esterase, and haemolysin activities in the OC-derived strains compared with the nOC-derived strains, with significant differences in SAP and haemolysin in particular (Fig. 4A–D). In comparison, OC-derived genotype B strains tended to have higher SAP, PL, and haemolysin activities; however, the differences were not significant (Fig. 5A–D). Within genotype C, the esterase and haemolysin activities of OC-derived strains tended to be higher; however, only haemolysin showed a significant difference (Fig. 6A–D).

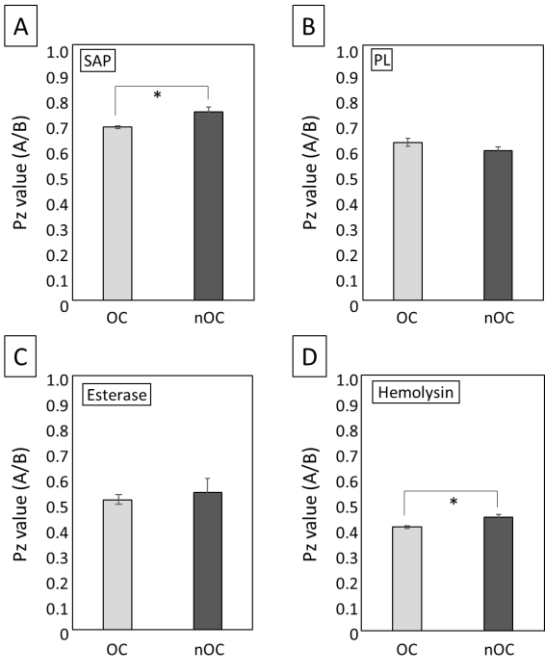
A
SAP
Pz value (A/B)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
OC
nOC
B
PL
C
Esterase
D
Hemolysin

Fig.3

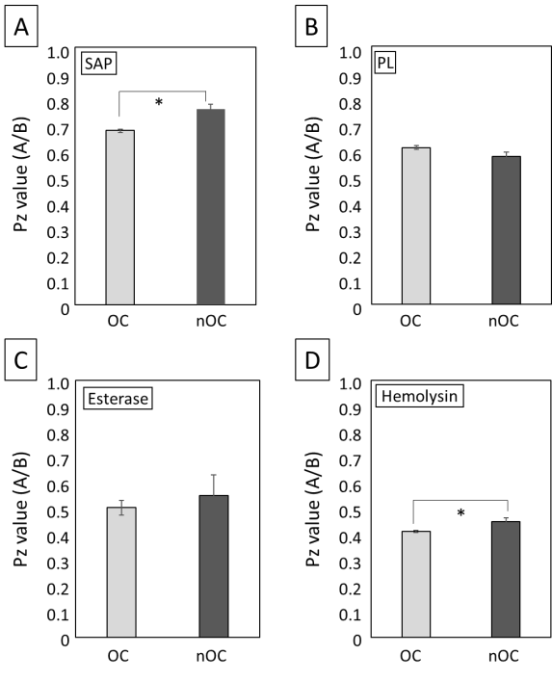
A
SAP
Pz value (A/B)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
OC
nOC
B
PL
C
Esterase
D
Hemolysin

Fig.4

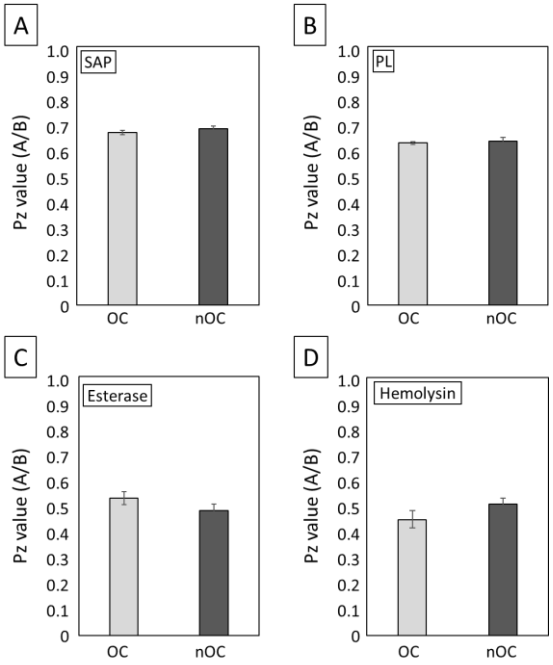

Fig.5

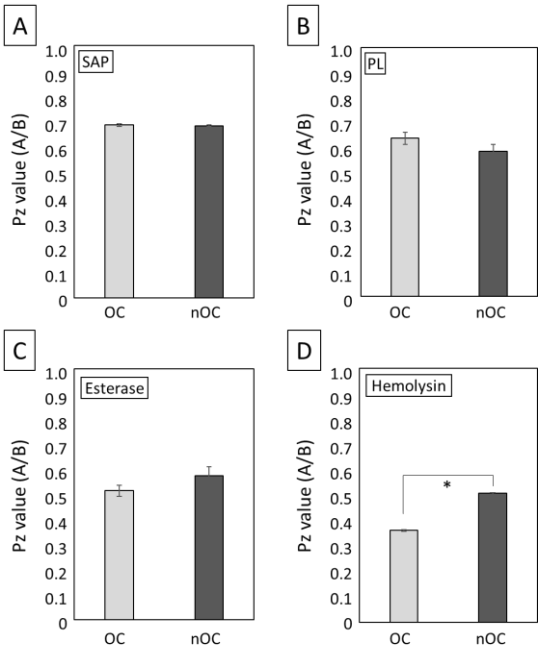

Fig.6

Figure 3. The ESE activities of OC-derived and nOC-derived strains of C. albicans. The activities of SAP (A), PL (B), esterase (C), and haemolysin (D) are shown in the graph

as Pz values. The smaller the value, the higher the activity. *Indicates a statistically significant difference ($p < 0.05$).

Figure 4. The ESE activities of OC-derived and nOC-derived C. albicans strains of genotype A.

The activities of SAP (A), PL (B), esterase (C), and haemolysin (D) are shown in the graph as Pz values. The smaller the value, the higher the activity. *Indicates a statistically significant difference ($p < 0.05$).

Figure 5. The ESE activities of OC-derived and nOC-derived C. albicans strains of genotype B.

The activities of SAP (A), PL (B), esterase (C), and haemolysin (D) are shown in the graph as Pz values. The smaller the value, the higher the activity. *Indicates a statistically significant difference ($p < 0.05$).

Figure 6. The ESE activities of OC-derived and nOC-derived C. albicans strains of genotype C.

The activities of SAP (A), PL (B), esterase (C), and haemolysin (D) are shown in the graph as Pz values. The smaller the value, the higher the activity. *Indicates a statistically significant difference ($p < 0.05$).

3.3 Measurement of IL-8 production

The OC-derived strains showed slightly higher levels of IL-8 production when exposed to HO-1-N-1 cells compared with nOC-derived strains; however, these differences were not significant (Fig. 7A). Furthermore, all genotypes of *C. albicans* showed slightly higher levels of IL-8 production in the OC-derived strains compared with the equivalent nOC-derived strains; however, only genotype B showed a significant difference (Fig. 7B–D).

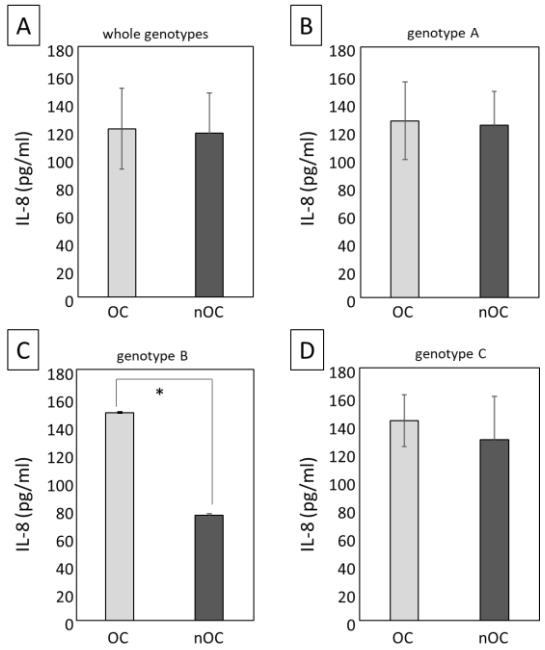

Fig.7

Figure 7. The induction of IL-8 production by C. albicans in HO-1-N-1 cells.

IL-8 production-inducing activity of OC-derived and nOC-derived strains of C. albicans (A), and then according to genotype (genotype A: B, genotype B: C, and genotype C: D). *Indicates a statistically significant difference ($p < 0.05$).

3.4 Measurement of cytotoxicity of *C. albicans*

When HO-1-N-1 cells were co-cultured with *C. albicans*, the levels of LDH in the cell culture supernatants were significantly higher in the co-cultures with OC-derived strains compared with nOC-derived strains (Fig. 8A). Moreover, in contrast to IL-8 productioninducing activity, significantly higher amounts of LDH were detected in co-cultures containing OC-derived strains of genotypes A and C than genotype B. (Fig. 8B–D).

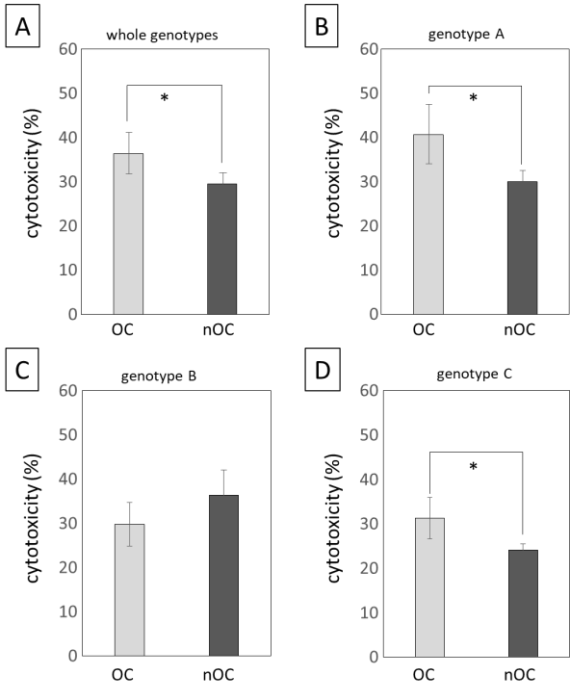

Fig.8

Figure 8. The cell death inducing-activity of C. albicans against HO-1-N-1 cells. Cytotoxicity of OC-derived and nOC-derived strains of C. albicans (A), and then according to genotype A (genotype A: B, genotype B: C, and genotype C: D). *Indicates a statistically significant difference ($p < 0.05$).

3.5 Comparison of strains derived from pseudomembranous candidiasis and erythematous candidiasis.

Of the 42 OC specimens, 16 were pseudomembranous, 25 were erythematous, and one had black hairy tongue (Fig. 9A). Strains of genotype A were present in 11 cases of

pseudomembranous, 12 cases of erythematous, and the single case of black hairy tongue. Genotype B was present in one case of pseudomembranous and six cases of erythematous, and genotype C was present in four cases of pseudomembranous and seven cases of erythematous. These data show that genotypes B and C had a high erythematous rate compared with strains of genotype A (Fig. 10A–C). When we examined ESE activity, we observed that SAP, PL, and esterase were more active in the strains derived from pseudomembranous candidiasis, while haemolysin was more active in the strains derived from erythematous candidiasis (Fig. 11A–D). We observed no differences in IL-8 production activity between strains derived from erythematous and pseudomembranous candidiasis (Fig. 12A); whereas, we observed significant differences in levels of LDH in the culture supernatants between these strains (Fig. 12B).

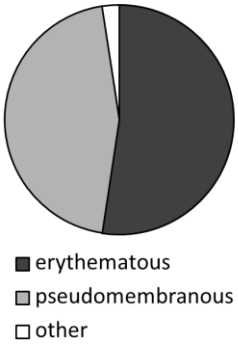

Fig.9

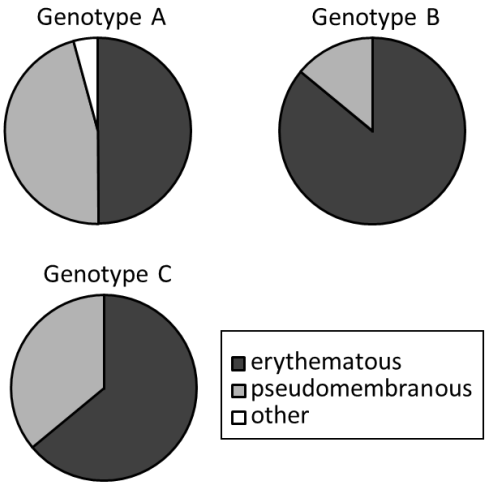

Fig.10

Figure 9. The ratio of symptoms of oral candidiasis classified by erythematous, pseudomembranous, and others.

Figure 10. The ratio of symptoms of oral candidiasis classified by erythematous, pseudomembranous, and others, for genotypes A, B, and C.

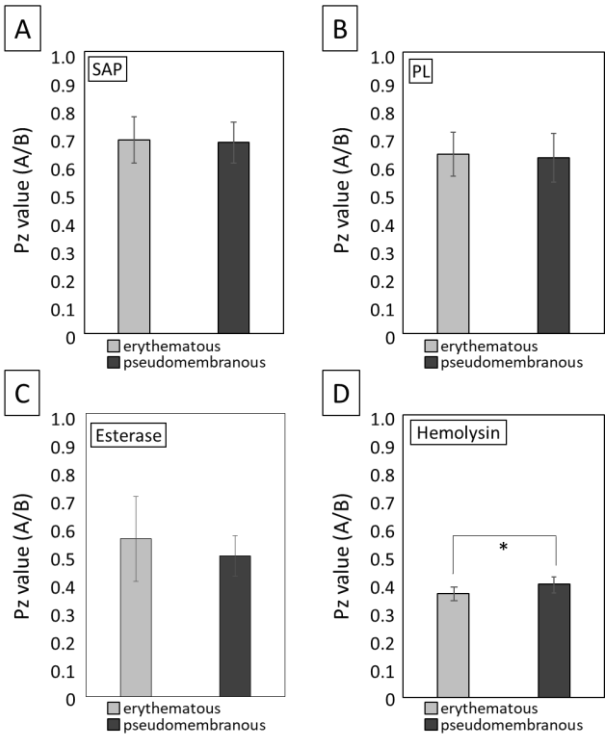

Fig.11

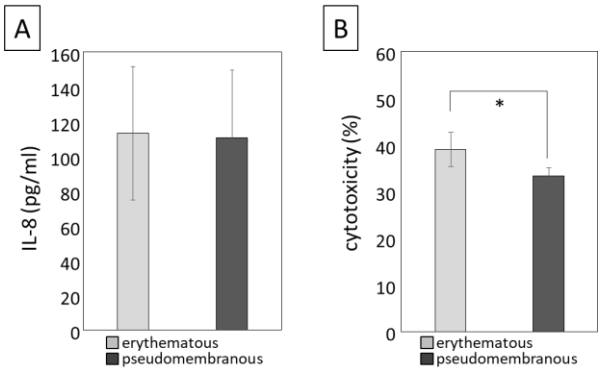

Fig.12

Figure 11. The ESE activities of erythematous candidiasis-derived and pseudomembranous candidiasis-derived C. albicans.

The activities of SAP (A), PL (B), esterase (C), and haemolysin (D) are shown as Pz values. The smaller the value, the higher the activity. *Indicates a statistically significant difference ($p < 0.05$).

Figure 12. The IL-8 production-inducing activity and the cell death inducing-activity of C. albicans against HO-1-N-1 cells.

The IL-8 production-inducing activity (A) and the cytotoxicity (B) of erythematous candidiasis-derived and pseudomembranous candidiasis-derived C. albicans. *Indicates a statistically significant difference ($p < 0.05$).

3.6 Comparison of genotype A with B and C

Since both genotypes B and C have a high erythematous rate compared with genotype A, we next looked for differences between these genotypes. Genotype A showed high PL activity, but we observed no significant differences in the activities of SAP, esterase, or haemolysin (Fig. 13A–D). We also found no differences in IL-8 production (Fig. 14A); however, there were significant differences in LDH levels in the culture supernatants between strains of genotype A compared with genotypes B and C (Fig.

14B).

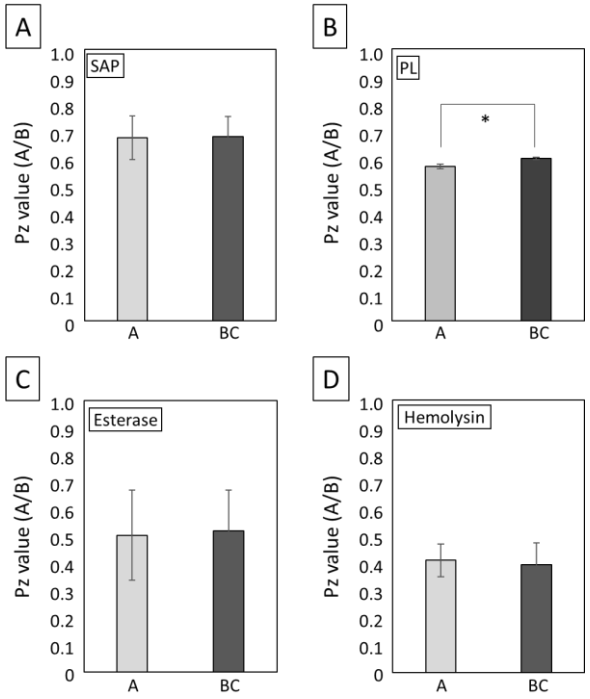

Fig.13

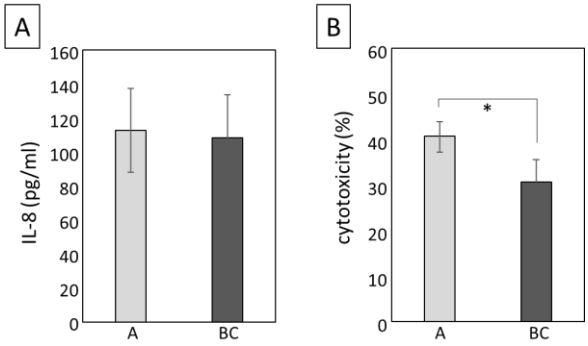

Fig.14

Figure 13. The ESE activities of C. albicans for genotypes A, B, and C.

The activities of SAP (A), PL (B), esterase (C), and haemolysin (D) are shown as Pz values. The smaller the value, the higher the activity. *Indicates a statistically significant difference ($p < 0.05$).

Figure 14. The IL-8 production-inducing activity and the cell death inducing-activity of C. albicans genotypes A and B compared with C in HO-1-N-1 cells.

Comparing the IL-8 production-inducing activity (A) and the cytotoxicity (B) of C. albicans genotypes A and B with genotype C. *Indicates a statistically significant difference ($p < 0.05$).

4. Discussion

In this study, we compared strains of *C. albicans* that were derived from different sources, and examined a number of different factors. Together, these data suggest that there may be differences in pathogenicity between strains of *C. albicans* derived from OC and nOC patients. On the basis of this study, we will be able to analyse the characteristics of pathogenic *C. albicans* strains in more detail.

We looked at a number of ESEs that represent *Candida* growth factors and which have been shown to be involved in host tissue destruction and tissue penetration. SAP and PL secreted by *Candida* act to degrade immunoglobulins and proteins of the extracellular matrix, to inhibit phagocytosis by polymorphonuclear neutrophils, and to induce the inflammatory response. SAP degrades proteins, including human albumin and keratin, and is involved in the destruction of host tissues, while PL degrades phospholipids in biological membranes and promotes cell lysis and adhesion, thereby contributing to *Candida* invasion and the destruction of host tissues. Esterase hydrolyses fatty acids and is involved in cellular destruction and adhesion to host cells. Haemolysin is secreted by the fungus after it acquires iron, and promotes mycelial invasion and the development of disseminated candidiasis (Sardi *et al*, 2012). In this study, we showed that strains of *C. albicans* derived from OC patients had significantly higher SAP and haemolysin activity

compared with strains from nOC patients. We further observed higher levels of esterase activity in OC-derived strains compared with nOC-derived strains; however, these differences were not significant. We saw no differences in PL activity. These results suggest that protein degradation and mycelial invasion may be more important in the pathogenesis of oral candidiasis than the degradation of phospholipids and fatty acids in the cell membrane.

Oral candidiasis is characterised by inflammation and infiltration of neutrophils and inflammatory cells to the mucosa. Oral epithelial cells are the primary target of *Candida* adherence and invasion. This invasion results in the initial release of chemotactic molecules to recruit innate immune effector cells (Villar *et al*, 2004). IL-8 is a chemokine that attracts human neutrophils and native lymphocytes to the infected area, and further activates the neutrophils and monocytes to kill target fungi. Studies using SCC4 and SCC15, which are derived from highly differentiated squamous cell carcinomas of the oral epithelium, have reported that direct contact between epithelial cells and fungi is required for the production of IL-8 in response to *C. albicans* infection. These studies have further shown that IL-8 induction is increased in a fungusdependent manner (Dongari-Bagtzoglou and Kashleva, 2003): IL-8 secretion was induced approximately seven to eight-fold more when the effector-to-target (E/T) ratio

(the ratio of *Candida* to cell number) was one (Dongari-Bagtzoglou and Kashleva, 2003). In contrast, a different study showed that oral epithelial cells did not significantly enhance IL-8 secretion, which was proposed to be due to the high ratio of yeast cells to epithelial cells (E/T ratio = 100) (Steele and Fidel, 2002). In the present study, we observed that the OC-derived strains tended to induce higher levels of IL-8 production, although no significant difference was observed (E/T ratio = 1). The discrepancies between these studies may be due to the differences in the mammalian cells used, as different oral epithelial cell lines may have different effects on IL-8 production. The CytoTox96® assay quantitatively measures levels of LDH, a stable cytosolic enzyme released from lysed cells. It has been reported that oral epithelial cells die approximately 18 hours after *Candida* infection (Villar and Zhao, 2010). Furthermore, it has also been shown that the ability of *C. albicans* to kill oral epithelial cells depends on a physical interaction with these cells: direct contact between the fungus and the cell as well as the form change from yeast to hyphae is critical for invasion (Villar and Zhao, 2010) (Hasebe *et al*, 2018).

We hypothesised that the amount LDH released from lysed cells may reflect the degree of oral epithelial cell destruction, and may therefore be related to the virulence of *C. albicans.* We observed significantly higher levels of LDH secretion by oral epithelial

cells after incubation with *C. albicans* from OC-derived strains compared with nOCderived strains, indicating that infection with *C. albicans* induces epithelial cell damage and cell death. Other studies have shown that levels of LDH released by human oral epithelial cells infected with both *C. albicans* and *C. glabrata* species were significantly increased compared with cells infected by *C. albicans* alone. *C. albicans* has been shown to facilitate *C. glabrata* invasion of the oral mucosa. *C. glabrata* strains are also often more resistant to azole antifungal agents than many other *Candida* species, including *C. albicans* (Silva *et al*, 2011). In clinical practice, the detection rate of *C. glabrata* is the second highest after *C. albicans* (Fidel *et al*, 1999). Future studies are required to address the question of mixed infection of oral epithelial cells, as mixed infections of these two species are thought to be highly pathogenic and may interfere with treatment (Hato *et al*, 2022). In addition, given that the ability of *C. albicans* to induce inflammatory cytokine production by oral epithelial cells depends on its ability to form hyphae, we plan to investigate the relationship between the proportion of yeast and hyphae types, as well as the speed of hyphal formation, and the pathogenicity of *C. albicans*. Future studies in these areas may help us identify key differences in the virulence of the different fungi (Villar *et al*, 2004). In addition, more information is required to examine the relationship between erythematous candidiasis and oral pain by

analysing erythematous candidiasis-derived and pseudomembranous candidiasisderived *C. albicans:* the disease status of oral candidiasis in outpatient charts showed that erythematous candidiasis was associated with a higher incidence of oral pain complaints and other symptoms compared with patients with pseudomembranous candidiasis.

In future, we hope to increase the study size to better elucidate the pathogenicity of refractory and recurrent oral candidiasis, and to provide a platform through which to develop new approaches to diagnosis and treatment.

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Conflicts of interest

There are no conflicts of interest related to this study.

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