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Genotypes and virulence-related activities of *Candida albicans* derived from oral cavity of patients in Hokkaido

Running title

***C. albicans* genotypes, virulence**

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Abstract

Objective: This study aimed to elucidate the difference in virulence of *Candida albicans* derived from oral candidiasis and non-oral candidiasis patients, and its genotype differences in Hokkaido to obtain a clue of a platform to develop new approaches for diagnosis and treatment. **Design:** *C. albicans* strains were collected from patients who visited the Hokkaido University Hospital Dental Center. Each strain was examined 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:** Certain virulence-related protease activities and cytotoxicity were higher in strains derived from patients with oral candidiasis compared with strains derived from patients without oral candidiasis. This is the first report on genotypes and the virulence-related activities, such as some protease secretion, IL-8 induction and cytotoxicity of *C. albicans* in Hokkaido. **Conclusions:** The virulence-related activities of the fungal strain may influence the pathogenesis of oral candidiasis, such as production of secreted aspartyl protease and cytotoxicity. In addition, *C. albicans* genotype C may be important for pathogenicity in Hokkaido, because the ratio of genotype C was increased in strains derived from oral candidiasis patients.

Keywords: *Candida albicans*, oral candidiasis, pathogenesis, genotype

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 35–80% of the population harbor *Candida* in the oral cavity in the world. The most common oral species is *Candida albicans*, which is estimated to be present in 80% of oral fungal isolates (Lewis & Williams, 2017; Talapko et al., 2021). Various factors can disrupt oral homeostasis, changing to abnormal flora (dysbiosis) and leading to microbial overgrowth and the expression of virulence factors (Lewis & Williams, 2017; Zdanavičienė et al., 2017). Infections are typically opportunistic and influenced by host factors such as xerostomia, poor denture cleaning, and topical steroid preparations (Oouchi et al., 2015). In addition, infection can become established when systemic defense mechanisms are abnormal, such as long-term administration of antimicrobials, steroids, anti-cancer drugs, or immunosuppressive drugs, and in immunocompromised patients due to AIDS, diabetes mellitus, or aging (Mane et al., 2012; Soysa et al., 2006; Suryana et al., 2020).

Three forms of oral candidiasis are known to exist: pseudomembranous candidiasis is characterized by a layer of detachable white moss; erythematous candidiasis is smooth and erythematous; and hypertrophic candidiasis has a thickened and hardened mucosal epithelial layer in a speckled pattern because of chronicity (Jayampath

Seneviratne et al., 2015). 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 drugs; however, there are many cases in which this is ineffective. Together with the nature and prevalence of *C. albicans*, the existence of antifungal-resistant candidiasis suggests that there may be key differences between strains. Although there are many reports on the characteristics of *C. albicans* derived from systemic or oral candidiasis, there have been few reports comparing the virulence-related activities of *Candida* in patients with oral candidiasis to those in healthy individuals. In addition, although it has been reported that there are regional differences in genotypes and pathogenicity of *C. albicans* (McCullough et al., 1999), their differences remain unknown in Hokkaido, the northernmost prefecture in Japan. Therefore, this study aimed to elucidate the differences in genotypes and virulence-related activities between *C. albicans* strains from patients with oral and non-oral candidiasis in Hokkaido.

2. Materials and methods

2.1 Subjects

Patients included in the study visited the Hokkaido University Hospital Dental Center 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 45–87 years old (mean 66.5), while the patients with non-oral candidiasis (nOC) were between 47–87 years old (mean 70.7).

2.2 Specimen collection

Specimens were collected by scraping the surface of the tongue ten times with a dental mirror. Specimens were then applied to CHROMagar™ plates (Kanto Chemical, Tokyo, Japan), a selective agar plate that allows the identification of *Candida* by colony coloration and morphology. After sample application, the plate was incubated at 36 °C for 48 h, and colonies that were speculated as *C. albicans* were collected. Selected *C. albicans* strains were incubated in Sabouraud broth (Oxoid-Thermo Fisher Scientific, Waltham, MA, USA) for 24 h, and then their genomic DNA was extracted, determined as *C. albicans* by PCR as described in section 2.5. The identified *C. albicans* were dispensed into 1.5 mL tubes and kept frozen at -80 °C until use. A total of 64 specimens were obtained: 42 OC and 22 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 (Fig. 1): samples resulted in ten or more colonies on CHROMagar™ plates and showed an improvement after treatment with antifungal drugs (Kimori et al., 2009; Ohga et al., 2016; Price et al., 1982; Yamazaki et al., 2011). In detail, the specimens were obtained from all the patients who visited the hospital complaining of abnormalities in the oral mucosa. The specimens were tested for the existence of *Candida* before any diagnosis by dentists. The way of this test is that the dentists rub the dorsum of the tongue with a dental mirror ten times and inoculate the resulting sample onto CHROMagar™ plates by the dental mirror. This method has been used routinely in our hospital, and actually, the samples from patients who have been found to be oral candidiasis mostly present more than ten *Candida* colonies on CHROMagar™ plates. Therefore, as described above, ten is the reference number for the guideline for candidiasis at our hospital (Yamazaki et al., 2011). Then, the patients whose specimens formed more than ten colonies and also who has typical mucosal symptoms, such as white coated tongue or smooth tongue, were speculated as OC and were prescribed antifungal drugs. In addition, any of the cases that the dentists suspected to be candidiasis were

prescribed antifungal drugs regardless of the number of colonies on CHROMagar™ plates. They were patients whose mucosal symptoms were similar to those of typical candidiasis.

As a result of treatment, the patients whose symptoms improved by the treatments were diagnosed with OC patients, and the patients whose symptoms did not improve by antifungal drugs were determined to be nOC patients in this study. The improvement was judged by the effect of antifungal drugs, that is, the effects of ten to 14 days-treatments with antifungal drugs were confirmed whether there were improvements in oral mucosa visually, there were decreases in subjective symptoms, and there were distinct decreases in the number of colonies on CHROMagar™ plates. In addition, although the patients that the dentists suspected to be candidiasis took antifungal drugs regardless of the number of colonies on CHROMagar™ plates, such as less than nine, there were no patients whose symptoms were improved by the antifungal drugs, suggesting there were no OC patients whose specimens formed less than nine colonies in the first test. The problem of antifungal drug-resistant strains arises when determining candidiasis based on the therapeutic effects of antifungal drugs. In fact, when the antifungal drug resistance of *C. albicans* strains was examined, it was found that one OC patient-derived strain was resistant. Although the strain was resistant to some antifungal

drugs, it was susceptible to amphotericin B, which is normally prescribed for the treatment of oral candidiasis. Thus, the existence of the antifungal drug-resistant strain did not affect the diagnosis in this study (data not shown).

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 (Kimori et al., 2009; Shekhany & Jalal, 2022; Tamura et al., 2001). The genomic DNA from *C. albicans* was extracted using GenCheck® DNA Extraction Reagent (Fasmac, Atsugi, Japan) according to the following protocol. *C. albicans* samples were mixed with 100 μ L of GenCheck in a 1.5 mL tube using a vortex mixer. The reaction mixture was then incubated at 95 °C for 10 min in a heat block. The tubes were placed on ice for 1 min, centrifuged at $15,000 \times g$ for 1 min at room temperature, and supernatant collected. Genotyping was performed by polymerase chain reaction (PCR). The primers

used were CA-INT-L (5'-ATAAAGGGAAGTCGGGCAAATAGATCCGTAA-3') and CA-INT-R (5'-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3'). It was possible for these primers not only for genotyping of *C. albicans* but also for discrimination of *C. albicans* and *C. dubliniensis* as described (Tamura et al., 2001). The PCR cycling conditions were: denaturation for 3 min at 94 °C, 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. All reaction products were characterized by electrophoresis on 1% agarose gel in 1 × Tris-borate-EDTA buffer at 100 V for 30 min and stained in a solution of 0.5 µg/mL ethidium bromide (Tamura et al., 2001).

2.6 Measurement of ESE activities

C. albicans secretes various ESEs, including secreted aspartyl protease (SAP), phospholipase (PL), esterase, and hemolysin. The activity of each ESE was determined by changes to the agar surface surrounding the colonies (Fig. S1A), according to protocols from Ikarashi *et.al* (Ikarashi et al. 2020), and Amarasinghe *et al.* (Amarasinghe et al., 2021). The diameter of each colony (a) was measured, as well as the diameter of both the precipitation zone and the colony (b). The Pz value was then calculated as $a \div b$, with smaller Pz values indicating higher enzyme activity. The experiments were performed in

triplicate, and the average Pz values reported (Ikarashi et al., 2020) (Fig. S1B).

2.6.1 SAP activity

SAP activity was measured according to 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 sterilized 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 the Pz values were determined.

2.6.2 PL activity

PL activity was measured according to 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

produce a final concentration of 4% agar. The agar plates were incubated with 5 μ L drops of the bacterial solution for 7 days at 35 °C in an aerobic environment, and the Pz values were determined.

2.6.3 Esterase activity

Esterase activity was measured according to 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 autoclaved at 121 °C for 20 min. The solution was cooled to approximately 50 °C before addition of 0.5 mL Tween-80. The agar plates were incubated with 5 μ L drops of the bacterial solution for 7 days at 35 °C in an aerobic environment, and the Pz values were determined.

2.6.4 Measurement of hemolysin activity

Hemolysin activity was measured according to Manns *et al.* (Manns et al., 1994) and Luo *et al.* (Luo et al., 2001). Modified Sabouraud agar was prepared by adding 7% sheep blood and 3% glucose. The agar plates were incubated with 5 μ L drops of the bacterial solution for 48 h at 37 °C in a 5% CO₂ environment and the Pz values were determined.

2.7 Cell strains

Human buccal mucosa squamous cell carcinoma cells (HO-1-N-1, JCRB) were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Merck Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin (Merck Sigma-Aldrich). Cells were diluted to 2×10^5 cells in 96-well plates and incubated for 24 h. The media was then discarded and new media containing *C. albicans* at a concentration of 2×10^5 cells/well was added. The cells were cultured for a further 20 h before the supernatant was 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 (BioLegend, San Diego, CA, USA) was used according to the manufacturer's instructions. The supernatant was collected as described above and 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

C. albicans induced HO-1-N-1 cell death was assessed using the CytoTox96® Non-Radioactive Cytotoxicity Assay (promega, USA), which measures LDH released from cells. The supernatant was collected as previously described 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 were expressed as the mean $\pm$ SD. Mann–Whitney U test was used to assess the statistical significance, with $p < 0.05$ considered to be statistically significant.

3. Results

3.1 Confirmation and 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 a single strain derived from an nOC-patient; however, it was excluded from the study because it has already been reported that *C. dubliniensis* is determined as genotype D by this PCR analysis (Tamura et al., 2001). Genotype E was not detected in any samples. OC-derived strains consisted of 57% genotype A, 17% genotype B, and 26% genotype C.

nOC-derived strains consisted of 62% genotype A, 19% genotype B, and 19% genotype C. Genotypes A and B were present at higher ratios 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). Therefore, there was no *C. dubliniensis* in our samples, and genotype C was the only strain in which the ratio was increased in OC patients.

3.2 Measurement of ESE activities of *C. albicans*

OC-derived strains showed significant differences in the activities of both SAP and hemolysin compared to those of nOC-derived strains. However, no significant differences were found in the activities of PL and esterase (Fig. 3A). When compared by genotype, genotype A showed significant differences in the activities of SAP and hemolysin and esterase in OC-derived strains compared to those of nOC-derived strains (Fig. 3B). In contrast, genotype B did not show any significant differences in all ESE activities (Fig. 3C). A significant difference was only observed in the activity of hemolysin in genotype C (Fig. 3D).

3.3 Measurement of IL-8 production

When compared without genotype discrimination, no significant differences were

observed between OC- and nOC-derived strains in the IL-8 production-inducing levels of HO-1-N-1 cells. No significant differences were observed in genotype A and C between OC- and nOC-derived strains. However, genotype B showed a significant difference (Fig. 4A).

3.4 Measurement of cytotoxicity of *C. albicans*

When HO-1-N-1 cells were co-cultured with *C. albicans*, the LDH levels in the supernatant were significantly higher in the co-cultures with OC-derived strains compared with nOC-derived strains, suggesting that OC-derived strains possessed significantly higher cytotoxicity than nOC-derived strains (Fig. 4B). Interestingly, OC-derived strains possessed significantly higher cytotoxicity than nOC-derived strains in the cases of genotype A and C, whereas nOC-derived strains possessed significantly higher cytotoxicity than OC-derived strains genotype B (Fig. 4B).

3.5 Comparison of strains derived from patients with pseudomembranous and erythematous candidiasis

Of the 42 OC specimens, 19 were from pseudomembranous patients, 22 were from erythematous patients, and one was from black hairy tongue patient (Fig. 5A). Strains of

genotype A were present in 11 pseudomembranous, 12 erythematous, and the single black hairy tongue cases. Strains of genotype B were present in one pseudomembranous and six erythematous cases. Strains of genotype C were present in four pseudomembranous and seven erythematous cases. These data show that genotypes B and C had a high erythematous rate compared with strains of genotype A (Fig. 5B).

When ESE activity was examined, there were no significant differences in activities of all ESEs between pseudomembranous and erythematous candidiasis derived strains (Fig. 6A).

We observed no significant difference in IL-8 production activity between strains derived from erythematous and pseudomembranous candidiasis; whereas, a significant difference was observed in LDH levels in the culture supernatant between these strains, suggesting that cytotoxicity but not IL-8 inducing-activity of *C. albicans* is an important factor for erythematous candidiasis (Fig. 6B).

3.6 Comparison of genotypes A, B, and C

Since both genotypes B and C had a high erythematous rate compared with genotype A, we next investigated differences between these genotypes. Genotype A showed high PL activity, but no significant differences were observed in the activities of all ESEs (Fig. 7A).

No differences were observed in IL-8 production. However, there was a significant difference in LDH levels in the culture supernatants between strains of genotype A compared with genotypes B and C (Fig. 7B). Genotypes B and C had higher erythematous rates (Fig. 5B) than genotype A, but the activities of ESEs (Fig. 6A, 7A) did not show the same trend, nor did the IL-8 production-inducing activities (Fig. 6B, 7B). In contrast, LDH levels (Fig. 6B, 7B) showed the same trend, suggesting that the cytotoxicity of *C. albicans* genotypes B and C is an important pathogenic factor in erythematous candidiasis.

4. Discussion

In this study, we compared strains of *C. albicans* that were derived from different sources and examined a number of different factors. These factors were examined because, although many studies have been conducted on the association between oral candidiasis development and prevalence of other diseases, few reports have compared the virulence-related activities of *C. albicans* derived from patients with oral candidiasis with those from non-oral candidiasis. Generally, higher levels of the activities of SAP, hemolysin, and cytotoxicity were detected from OC-derived strains than nOC-derived strains of *C. albicans* (Fig. 3A and Fig. 4B), and genotype B showed peculiar characteristics in IL-8 production-inducing activity and cytotoxicity compared with genotypes A and C (Fig. 4A

and B).

In this study, it was found that various properties differed between OC- and nOC-derived *C. albicans*, suggesting that there may be differences in pathogenicity. A number of ESEs that represent *Candida* growth factors were investigated, 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, inhibit phagocytosis by polymorphonuclear neutrophils, and induce the inflammatory response. SAP degrades proteins, including human albumin and keratin, and is involved in the destruction of host tissues. Moreover, SAPs cause increased vascular permeability by activating one or more kallikrein-kinin system steps to release bradykinin, leading to inflammatory reactions and clinical symptoms (Sanitá et al., 2014). The main mechanism of action of PL is to target and digest the phospholipids in the cell membrane, leading to lysis. Since PLs are concentrated mostly in the tips of the hyphae, host cell injury would be expected to facilitate the penetration of the infecting fungi (Sanitá et al., 2014). Esterase hydrolyses fatty acids and is involved in cellular destruction and adhesion to host cells. Hemolysin is another virulence factor produced in *Candida* species which can lyse blood cells and support and promote infection in blood tissue. By producing a hemolytic factor, *C. albicans* can acquire erythrocyte-derived iron for growth (Nouraei et al., 2020;

Sardi et al., 2012). In this study, esterase was the only factor which had no significant differences between OC- and nOC-derived strains of *C. albicans*, suggesting that esterase is not a significant factor for the development of oral candidiasis in Hokkaido. Significant differences in the activity of PL were only observed between genotype A and B with genotype C. On the basis of these results, 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 characterized by inflammation and infiltration of neutrophils and inflammatory cells to the mucosa, with oral epithelial cells being 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 activates the neutrophils and monocytes to kill fungi. Studies using SCC4 and SCC15 cells, 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 shown that IL-8 induction is increased in a fungus-dependent 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 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 & Fidel Jr., 2002). In the present study, we observed that the OC-derived strains induce significantly higher levels of IL-8 production than nOC-derived strains in genotype B (Fig. 4B), although no significant difference was observed in other genotypes (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 h after *Candida* infection (Villar and Zhao, 2010). Furthermore, it has 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 cell, as well as the change from yeast to hyphae, is critical for invasion (Hasebe et al., 2018; Villar & Zhao, 2010). Since the amount of LDH released from lysed cells reflects the degree of oral epithelial cell destruction, LDH levels should be related to *C. albicans* virulence. We observed significantly higher levels of LDH secretion by oral epithelial cells after

incubation with *C. albicans* genotypes A and C but not with genotype B from OC-derived strains compared with nOC-derived strains, indicating that infection with *C. albicans* genotypes A and C induces epithelial cell damage and cell death (Fig. 4B). Therefore, cytotoxicity may be an important factor in the development of candidiasis by *C. albicans* strains of genotypes A and C. It is interesting why genotype B shows peculiar activities, such as the IL-8 production-inducing activity and cytotoxicity, compared with genotypes A and C. Further study is needed to elucidate these differences.

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 drugs 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 whether mixed infection of oral epithelial cells is highly pathogenic and interferes with treatment (Hato et al., 2022).

Given that the ability of *C. albicans* to induce of oral epithelial cell inflammatory cytokine production depends on its ability to form hyphae, the relationship between the

proportion of yeast and hyphae types, speed of hyphal formation, and pathogenicity of *C. albicans* requires investigation. Future studies in these areas may help 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 analyzing erythematous and pseudomembranous candidiasis-derived *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 the present study, we have elucidated some virulence-related properties of *C. albicans* between *C. albicans* from patients with or without oral candidiasis in Hokkaido. However, as described below, there are still many pathogenicity-associated characteristics of *C. albicans* left to be revealed, such as hyphal formation, biofilm formation, cell surface hydrophobicity, antifungal drug resistance, and cell-wall stress. The hyphal formation is necessary for *C. albicans* to induce IL-1 β production in the macrophages (Hasebe et al., 2018), and to invade host tissue (Mayer et al., 2013). In addition, hyphal formation is critical for the early stage of biofilm formation. Although there are many factors related to the biofilm formation by *C. albicans*, one of the important factors is its cell surface hydrophobicity, which influences adhesion to host cells. (Ramage et al., 2009;

Samaranayake et al., 2003). Once *C. albicans* biofilm is formed, it becomes difficult to remove completely due to its ability to resist conventional antifungal drugs and host immune systems (Blankenship & Mitchell, 2006). In addition to these biofilm's abilities, difficulty in the treatment of *C. albicans* infection is increasing due to the increase of antifungal drug-resistant strains. Not only antifungal drugs but many substances and situations damage the cell walls of *C. albicans*. It has been reported that some mutation in *C. albicans* is sufficient to render its hypersensitivity to cell wall stress, and impair septation, hyphal development, and pathogenesis (Norris et al., 2021). Therefore, studies on *C. albicans* cell wall stress may also contribute to elucidating the pathogenicity of *C. albicans*. Taken together, we must reveal these many pathogenicity-associated characteristics in *C. albicans* to develop new approaches for the diagnosis and treatment of candidiasis.

We think we could elucidate some of the differences in the virulence-related properties between *C. albicans* from patients with oral candidiasis and patients with non-oral candidiasis in Hokkaido by this study. Our next project is in progress which elucidates the rate of biofilm formation and distribution of antifungal drug-resistant strains using the same strains in this study. We will also try elucidating other pathogenicity-associated characteristics, such as cell-wall stress and cell surface

hydrophobicity of *C. albicans*. We hope to increase the study size to better elucidate the pathogenicity of refractory and recurrent oral candidiasis, and contribute to developing efficient methods for diagnosis and treatment by analyzing the characteristics of pathogenic *C. albicans* strains in more detail.

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Conflicts of interest

There are no conflicts of interest related to this study.

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Figure Legends

Figure 1. The flow chart on determining candidiasis in this study.

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

Figure 3. The ESE activities of *C. albicans* (SAP, PL, esterase, and hemolysin). The ESE activities of (A) OC- and nOC-derived strains of *C. albicans*, (B) OC- and nOC-derived *C. albicans* strains of genotype A, (C) OC- and nOC-derived *C. albicans* strains of genotype B, (D) OC- and nOC-derived *C. albicans* strains of genotype C . Smaller values represent higher activity. * indicates a statistically significant difference ($p < 0.05$).

Figure 4.

(A) The induction of IL-8 production by *C. albicans* in HO-1-N-1 cells. IL-8 production-inducing activity of OC- and nOC-derived strains of *C. albicans* for all and individual genotypes. * indicates a statistically significant difference ($p < 0.05$). (B) Cell death inducing-activity of *C. albicans* against HO-1-N-1 cells. Cytotoxicity of OC- and nOC-derived strains of *C. albicans* for all and individual genotypes. * indicates a statistically

significant difference ($p < 0.05$).

Figure 5.

(A) The ratio of symptoms of oral candidiasis classified as erythematous, pseudomembranous, and others forms. The ratio of symptoms of oral candidiasis classified as erythematous, pseudomembranous, and others forms, separated into genotypes A, B, and C.

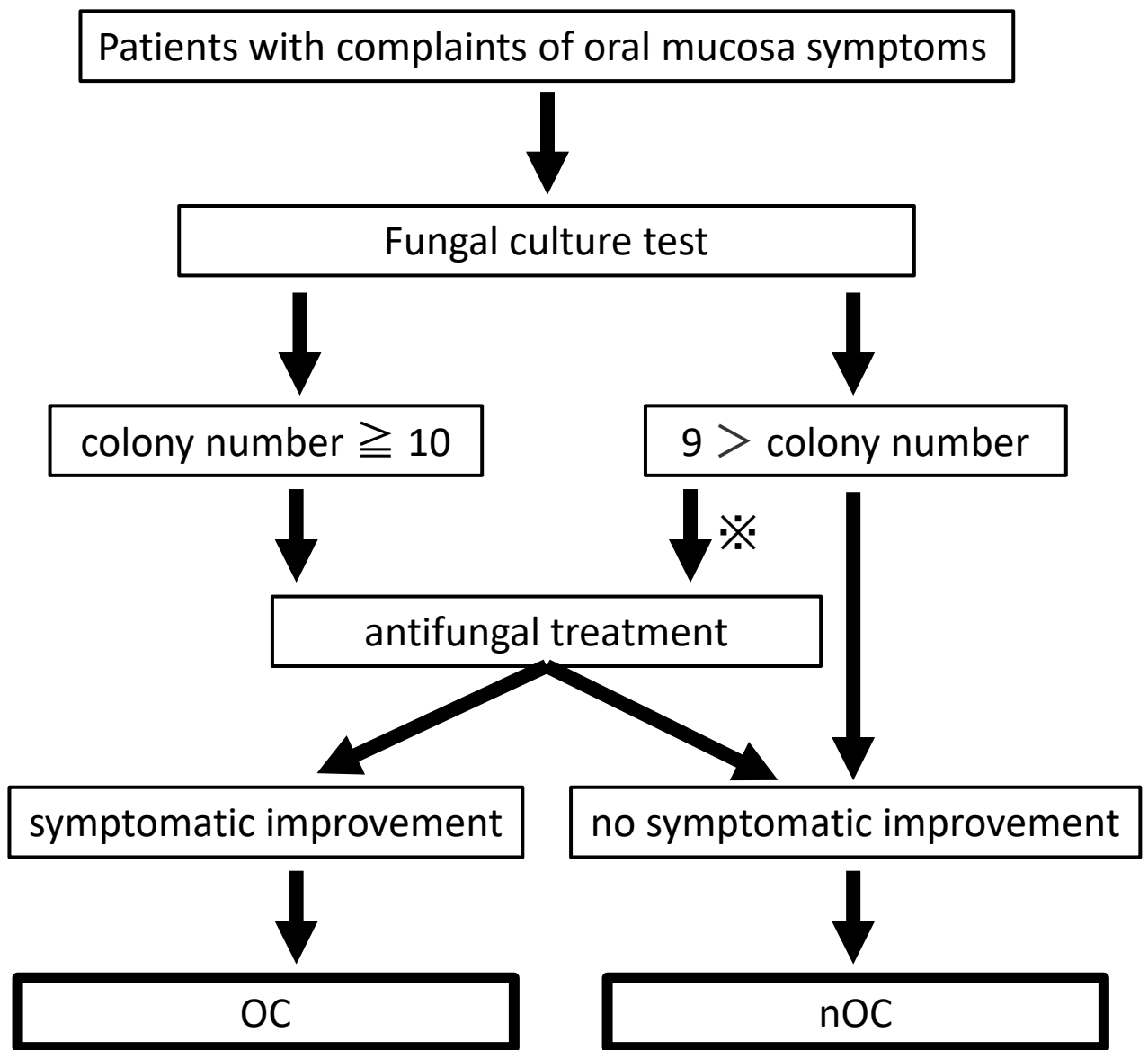
Figure 6.

(A) The ESE activities of erythematous and pseudomembranous candidiasis-derived *C. albicans*. Pz values are shown for the activities of SAP, PL, esterase, and hemolysin. Smaller values represent higher activity. (B) 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 and cytotoxicity of erythematous and pseudomembranous candidiasis-derived *C. albicans*. * indicates a statistically significant difference ($p < 0.05$).

Figure 7.

(A) The ESE activities of *C. albicans* for genotype A compared with genotypes B and C.

Pz values are shown for the activities of SAP, PL, esterase, and hemolysin. Smaller values represent higher activity. (B) The IL-8 production-inducing activity and the cell death inducing-activity of *C. albicans* genotype A compared with genotypes B and C in HO-1-N-1 cells. * indicates a statistically significant difference ($p < 0.05$).



※If clinical symptoms resemble oral candidiasis.

Fig.1

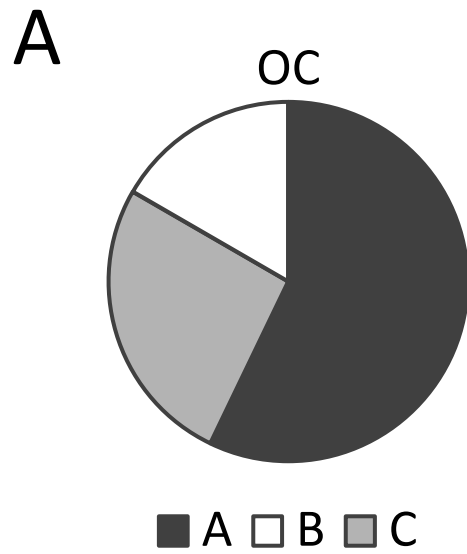
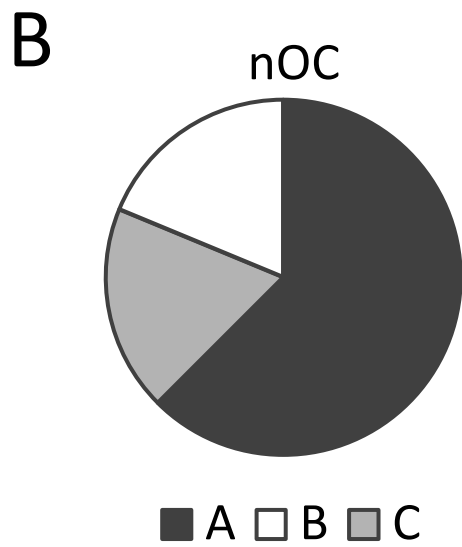


Fig.2

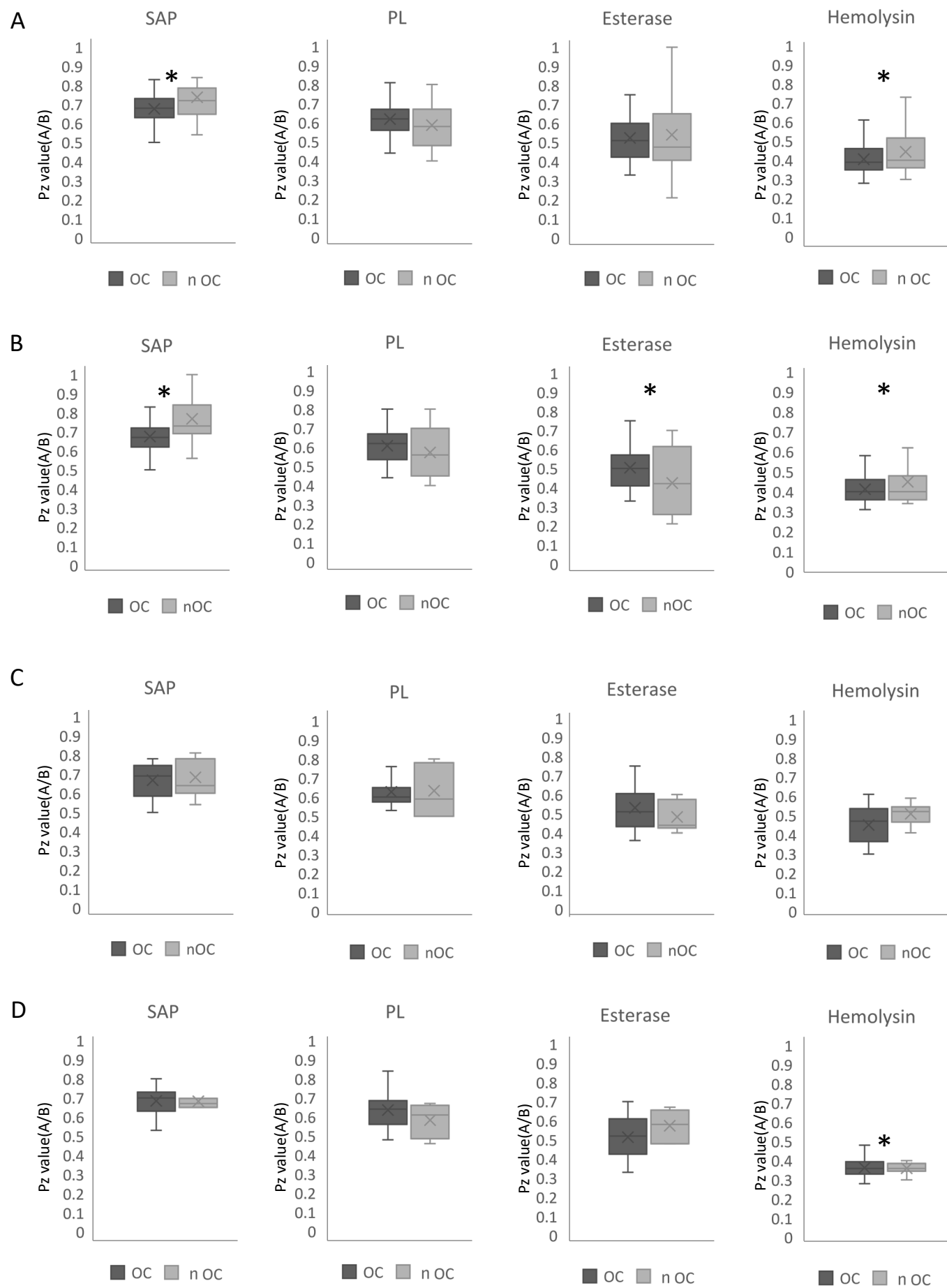


Fig.3

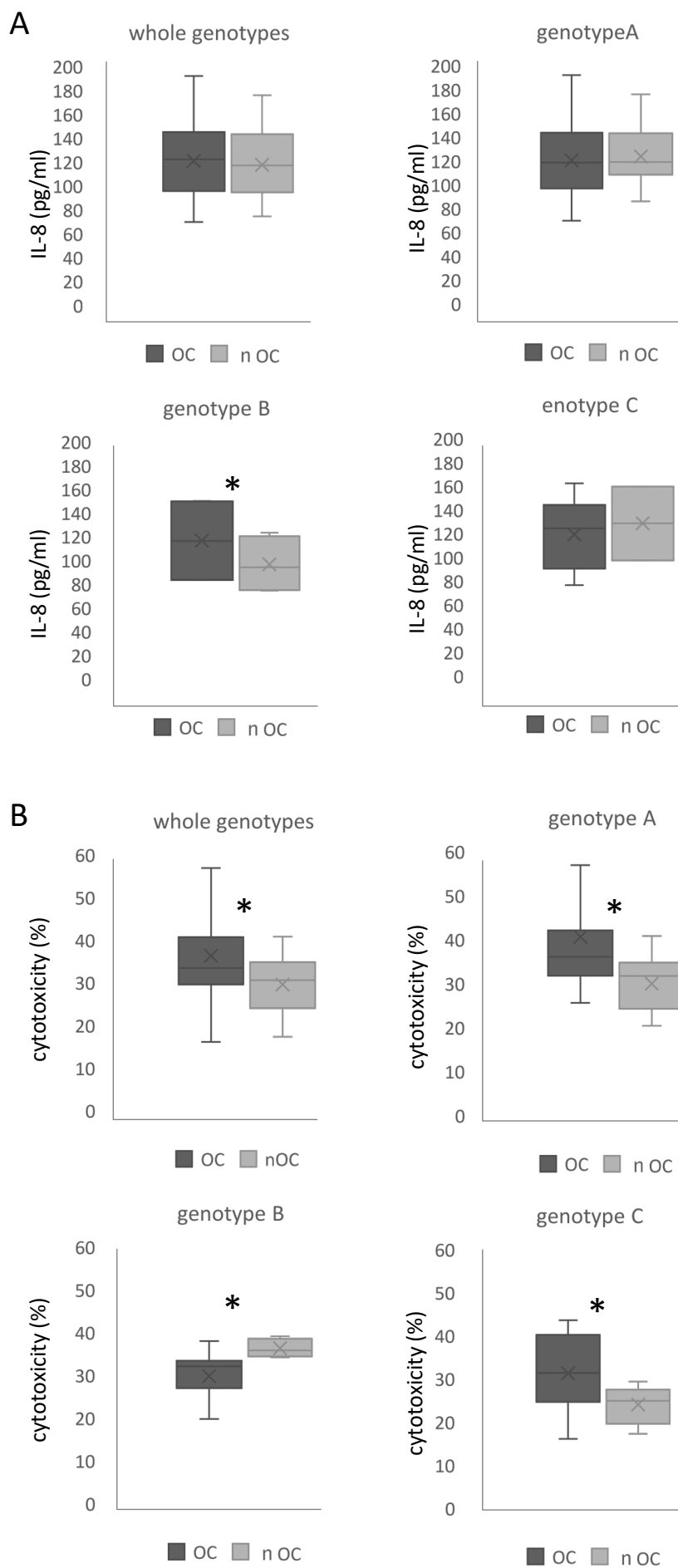
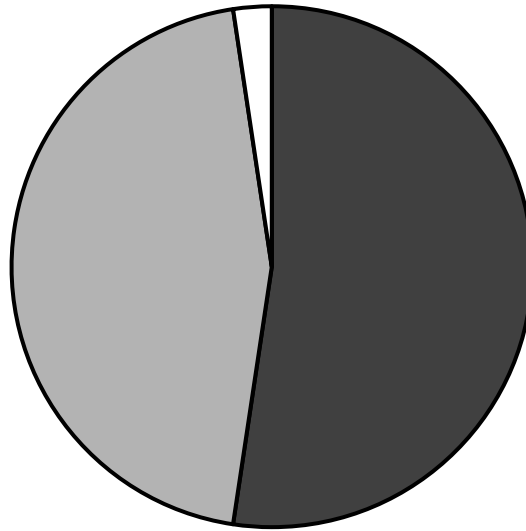


Fig.4

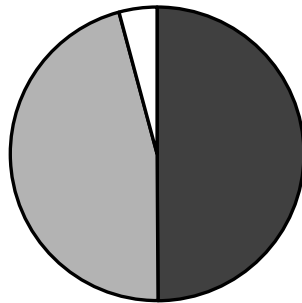
A



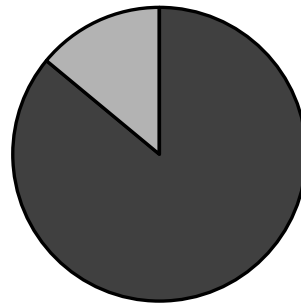
■ erythematous
■ pseudomembranous
□ other

B

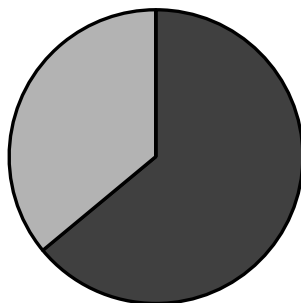
Genotype A



Genotype B



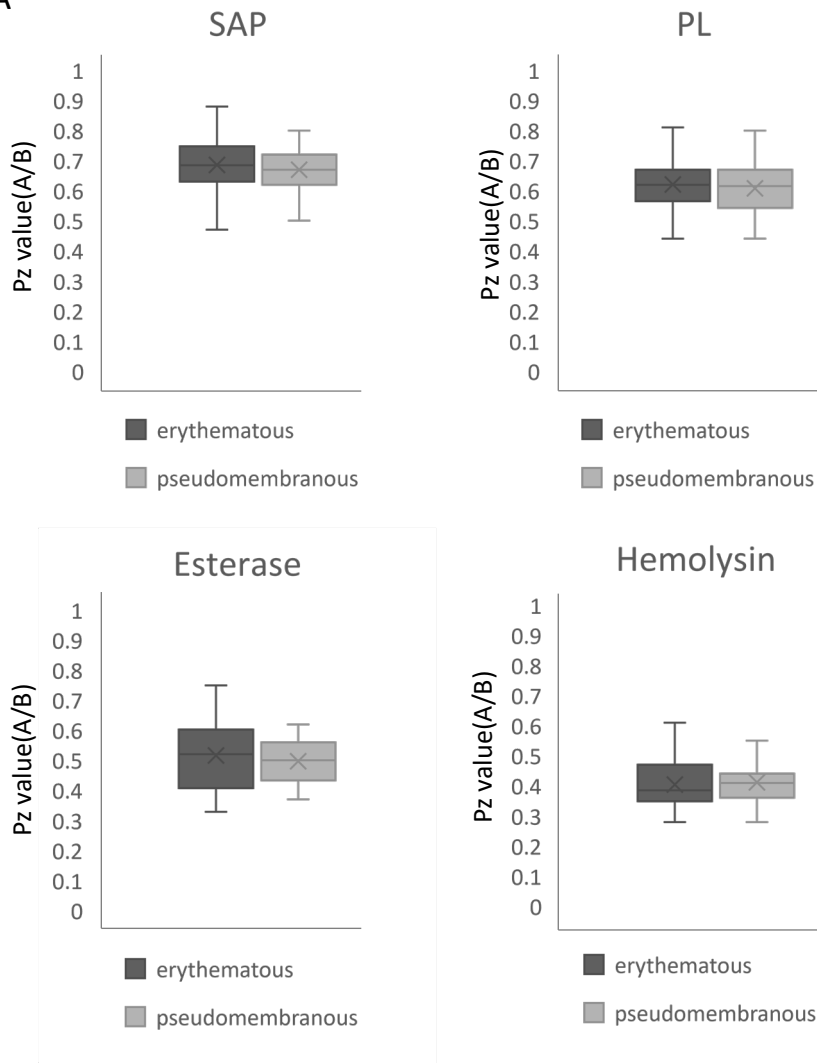
Genotype C



■ erythematous
■ pseudomembranous
□ other

Fig.5

A



B

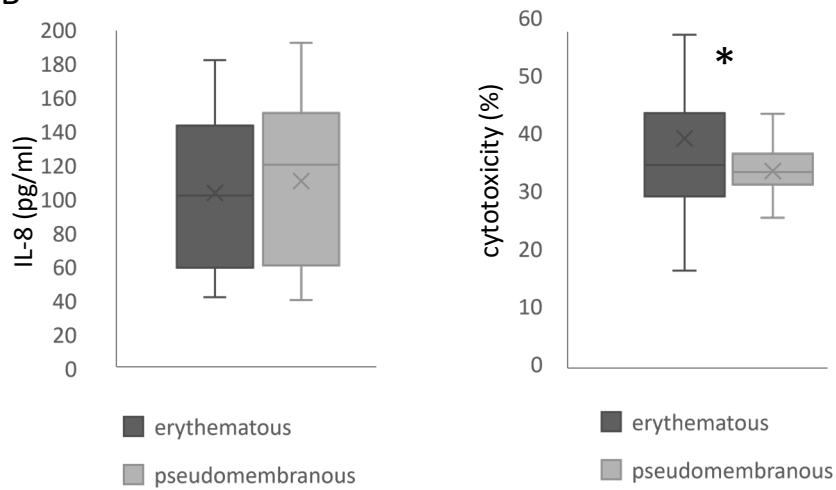
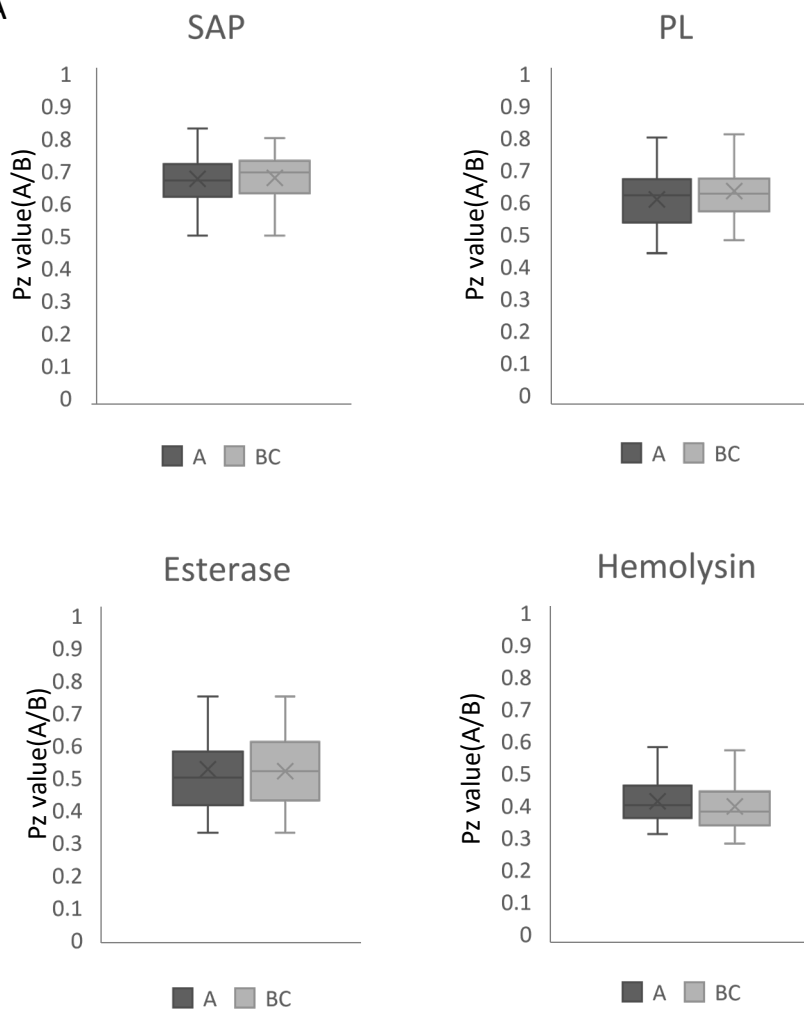


Fig.6

A



B

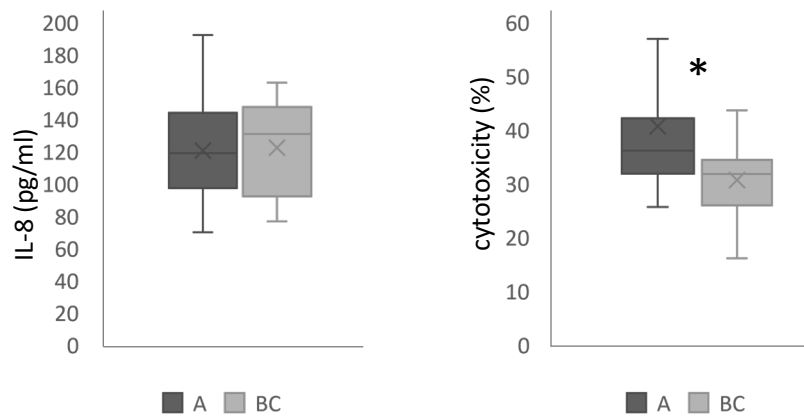


Fig.7