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Characterization of *Trichosporonoides madida* and Evaluation of Virulence in Laboratory Animals

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Abstract: One of the most important prerequisites of the industrial microorganism is that it should not be virulent to humans or economically important animals or plants. In this investigation, the microbiological characterization of *T. madida* N-5-3 strain was performed. And then, the virulence of the test strain in mouse model was examined systematically. The microbiological characteristics of the test strain were found to be fully consistent with those of typical *T. madida*. The i.p. lethal dose$(LD)_{50}$ of the test strain was greater than $1x10^8$, because there was no dead animal with the challenge doses upto the level of $1x10^8$. When $1x10^8$ yeast cells were challenged to the laboratory mice, *T. madida* N-5-3 strain was completely cleared from the liver and spleen in 4 days after challenge. And no pathological changes in the histological examination of the internal organs from challenged mice was observed. Above results can provide the predictability of the safety of *T. madida* N-5-3 strain for the industrial use in the view point of the public health aspect.

Key Words: *T. madida*, Virulence, Characterization

INTRODUCTION

A various types of yeasts and yeast-like fungi can be isolated from natural sources of environments. The habitats of fungi are quite diverse. Most fungi, however, have terrestrial habitats, in soil or on dead plant matter. A large number of fungi are parasites of plants. In contrast, a few fungi are parasitic on animals[1,3], including humans, although in general fungi are less significant as animal pathogens than are bacteria and viruses.

Most kinds of the yeasts are not related with the human diseases. On the contrary, yeasts are the most important and the most extensively used microorganisms in industry. They are cultured for the cells themselves, for the components, and for the end products that they produce during the fermentation. Recently, with the advancement of scientific knowledge on microbiology and fermentation, the importance of yeasts in industrial fields has been increased with using yeasts for the production of various commercial products of economic value such as alcoholic beverages, pharmaceuticals, organic acids, amino acids, and enzymes from simple carbon and/or nitrogen sources[5].

The classification of yeasts is still subject to change with each new development in classification techniques. A number of indicators can be used to identify yeasts: colony morphology, microscopic morphology produced on

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cornmeal agar, carbon assimilation and fermentation profiles, the presence of certain enzymes such as urease and phenoloxidase, and ascospore formation. Recently, molecular biological techniques of DNA hybridization and the determination of G+C contents in DNA have been applied to the taxonomic classification for the related strains of yeasts[2].

Trichosporonoides spp. are the new xerophilic yeast-like fungi. They are known to be able to grow at water activities at least as low as 0.75[4]. They had been isolated from sweetened orange/mango drink in Australia and from jam[6]. Yeast strains of *Trichosporonoides* spp. are one of the genera of imperfect fungi with no known sexual reproduction phase.

In order to use newly identified strains of yeasts like *Trichosporonoides* spp. in the fields of industry, we should perform thorough and systematic investigations on the properties and the pathogenicity of the organism. An important requisite characteristics of the industrial microorganism is that it should grow rapidly and produce the desired products in a relatively short period time. However, the most important requisite of an industrial microorganism is that it should not be harmful to humans or economically important animals or plants. Because of the large population size in the industrial fermenter, and the virtual impossibility of avoiding contamination of the environmental outside the fermenter, a pathogen would present potentially disastrous problems.

The studies described below were aimed at determining if the isolated strain of yeast display the typical characteristics of *Trichosporonoides madida* and, if so, the isolate is avirulent in laboratory animal model that can predict the safety for the industrial use.

MATERIALS AND METHODS

Microbiological characterization of yeast strain

Microbiological characterization of test strain of *T. madida* N-5-3 was performed with the standard procedures for the identification of yeasts. Test strain was inoculated on GYU agar (20% glucose, 0.5% yeast extract, 0.1% urea, 2% agar) and incubated at 34℃. Cultured yeast strain was examined for the cellular morphology under microscope. At the same time, the production of pseudohyphae was determined, too. Biochemical properties of the yeast strain was performed with the tests of carbon assimilation and fermentation, urease, and nitrate utilization under the protocols of routine yeast identification.

Determination of colony forming units of *T. madida* N-5-3 strain

Test strain of *T. madida* N-5-3 was inoculated into GYU broth, and was cultured at 34℃ for 5 days with vigorous shaking (200 rpm). After the cultivation of yeast strain, yeast cells were harvested by centrifugation. The harvested cells were washed 3 times with sterilized saline, and cells were 10-fold diluted serially with sterilized saline solution. These serially diluted suspensions of test yeast strain were determined the absorbance at 600 nm with spectrophotometer. And then, the series of the 10-fold diluted suspensions of yeast strain were seeded on to triplicate of GYU agar plates. After 5 days of incubation period, colony forming units was determined in the GYU agar plates.

Determination of lethal dose$(LD)_{50}$ of *T. madida* N-5-3 strain

Five-day-grown test strain of yeast cells was harvested, washed and adjusted to $1x10^8$/ml. This solution of yeast suspension was 10-fold diluted serially upto $1x10^1$/ml with sterilized saline solution. Each of the serially diluted yeast suspension was injected to 10 specific-pathogen-free ICR mice, intraperitoneally. The yeast injected mice were placed in a plastic cages according to injected doses and observed for signs of illness to establish specificity of the deaths 2 times daily for 7 days.

Growth curves of *T. madida* N-5-3 strain in laboratory animals

Specific-pathogen-free ICR mice were challenged experimentally with $1x10^8$ cells of *T. madida* N-5-3 strain intraperitoneally. These mice were sacrificed at day 1, 2, 4, 7, 14, and 28 after injection of yeasts. Internal organs of heart, lung, liver, spleen and kidney were removed aseptically from sacrificed mice on each of the test days. Removed organs were weighed and homogenized with tissue homogenizer. The homogenized organs were serially 10-fold diluted. Diluted solutions were inoculated to GYU agar plate and cultured for 5 days. Colony forming units of yeasts were determined after incubation period according to the organ, respectively.

Histopathological examination

A part of removed organs was used for the impression smear. The slide was stained with Wright's or Giemsa stain. After the impression smear, the removed organs were fixed with 10% neutral buffered formalin solution. The fixed organs were dehydrated, embedded with paraffin under the routine histological techniques. Thin sections from paraffin embedded organ specimens were made by microtome, and then these thin sections were stained by hematoxyln-esoin staining techniques. Histopathological changes of the organs of yeast injected mice were observed under microscope.

RESULTS AND DISCUSSION

Microbiological properties of *T. madida* N-5-3 strain

The microbiological biochemical reactions of *T. madida* N-5-3 strain done by conventional methods for the identification of yeast strains are given in Table 1. Macroscopic colonial morphology of test strain on GYU agar were off-white or cream to tan colored with darker center. Matured colonies become finely wrinkled with a lacy appearance. The production of pseudohyphae and blastospore were identified in the microscopic examination of morphology. The microbiological characteristics of test strain were in fully consistent with typical biochemical reactions, macroscopic and microscopic morphology for *T. madida*.

Table 1. Microbiological properties of *T. madida* N-5-3 strain

Microbiological Test	N-5-3 strain
Growth at 37℃	+
Nitrate reduction	+
Urease	+
Production of	
pseudohyphae	+
germ tube	−
capsule	−
Fermentation of	
glucose	+
sucrose	+
lactose	−
galactose	−
maltose	+
Assimilation of	
glucose	+
sucrose	+
lactose	−
galactose	−
maltose	+
raffinose	+
trehalose	+
xylose	+
cellobiose	+
erythritol	+
inositol	−

Enumeration of colony forming units of *T. madida* N-5-3 strain

To determine the colony forming units of *T. madida* suspension, the relationship between absorbance of yeast suspensions at 600 nm and their colony forming units were investigated. The absorbance of diluted suspension of $2x10^8$/ml of test yeast suspension are given Table 2. Upon these relationships, the regression equation between absorbance at 600 nm(X) and colony forming units of *T. madida*(Y) can be

Table 2. Absorbances of diluted suspension of 2x 10^8/ml of T. madida strain

Dilution factor	Absorbance
$1X10^{-1}$	0.346
$1X10^{-2}$	0.044
$1X10^{-3}$	0.006

defined as follows. And R^2 of this equation is 0.9996, showing high significance.

Colony forming units of *T. madida* /ml (Y) = [6 x absorbance (X) - 0.04] x 10^7

Lethal dose$(LD)_{50}$ of *T. madida* N-5-3 strain in laboratory mice

To estimate the virulence of *T. madida* N-5-3 strain, serial dilutions from $1x10^1$/m to $1x10^8$/ ml of test yeast cells were injected to the laboratory mice intraperitoneally. During the observation period of 7 days, none of the yeast injected mice were found to be ill or dead. With these results, the i.p. lethal dose$(LD)_{50}$ of *T. madida* N-5-3 strain was found to be greater than $1x10^8$. This means *T. madida* N-5-3 strain is totally avirulent to the laboratory animal model upto the level of $1x10^8$ cells, and we are able to predict the possible safety for the industrial use in the view point of the public health.

Growth curves of *T. madida* N-5-3 strain in laboratory animals

The virulence of *T. madida* N-5-3 strain was tested by measuring the viable yeast cells in the internal organs of laboratory mice challenged with $1x10^8$ cells of *T. madida* N-5-3 strain intraperitoneally. The colony forming units of viable yeast cells were determined upto 28 days after injection.

When $1x10^8$ yeasts cells were challenged to the laboratory mice, colony forming units of viable *T. madida* N-5-3 strain were dramatically reduced to $1x10^{4.3}$, and $1x10^{3.1}$ in the liver on 1 day and 2 days after challenge, respectively. And colony forming units of viable yeast cells in spleen were $1x10^{3.18}$, and $1x10^{2.87}$ on 1 day and 2 days after challenge, as shown in Fig. 1. However, none of the viable yeast cells was found in other internal organs of heart, lung and kidney. And 4 days after challenge, all of the tested internal organs of heart, lung, liver, spleen and kidney were found to be free of injected *T. madida* N-5-3 strain until the test period of 28 days.

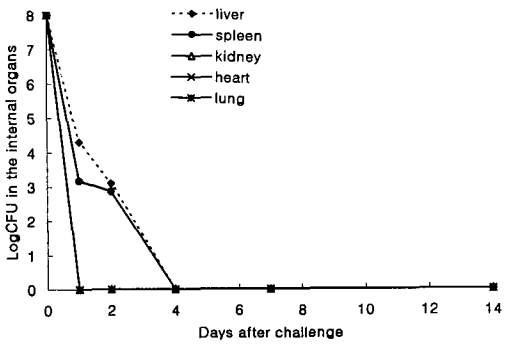

Fig. 1. Growth curves of *T. madida* N-5-3 strain in the internal organs of mice after i.p.-administration of $1x10^8$ yeasts.

These results strongly indicate that *T. madida* N-5-3 strain can be successfully eliminated in laboratory mouse model in less than 4 days and this avirulence of injected yeast strain might provide the predictability of the safety of *T. madida* N-5-3 strain for the industrial use.

Histopathological examination of *T. madida* challenged mice

To examine the *T. madida*-induced histopathological changes of internal organs of laboratory mice, internal organs of lung, heart, liver, spleen and kidney were removed from sacrificed animals according to the time schedule. After fixing and staining, these specimens were examined under microscope. The hyperplasia of germinal centers and slight increase in the cell infiltration around the germinal centers of spleens from challenged mice were confirmed (Data not shown). These results can be attributed to the production of humoral immune

responses to the challenged *T. madida* strain. During the whole test periods of 28 days postchallenge, none of the challenged *T. madida* N-5-3 was found in the histological specimens from internal organs of lung, heart, liver, spleen and kidney even though challenged yeast cells were isolated on microbiological media in the range between $1x10^{4.3}$ and $1x10^{2.87}$ in the liver and spleen of mice after challenge. However, these results are not uncommon because usually more than $1x10^{5}$ cells per gram specimen can be successfully identified in the histological specimens.

REFERENCES

1. Campbell GD, Currier RD, and Busey JF (1981): Survival in untreated cryptococcal meningitis. *Neurology,* **31:** 1154-1157.
2. De Hoogs GS and Gutsho E (1984): Deoxyribonucleic acid base composition and taxonomy of *Moniella* and allied genera. *Antonie Van Leewenhoek,* **50**(2): 135-141.
3. Domer JE and Carrow EW (1989): Candidiasis. pp. 57-92, *In* Coxs RA(Ed.), "Immunology of the Fungal Diseases", CRC Press, Boca Raton.
4. Hocking AD and Pitt JI (1981): *Trichosporonoides nigrescens* sp. nov., a new xerophilic yeast-like fungus. *Antonie Van Leewenhoek,* **47**(5): 411-421.
5. Phaff H (1981): Industrial microorganisms. *Scientific American,* **245**(3): 77-89.
6. Ramirez C (1989): A new species of *Trichosporonoides* isolated from sweetened orange/mango drink in Australia. *Mycopathologia,* **108**(1): 25-30.

=국문초록=

Trichosporonoides madida 균주의 성상 및 실험동물에 대한 병원성

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*Trichosporonoides madida*와 같이 자연계에서 새로 분리된 균종을 산업 분야에서 이용하기 위하여 이들 균종의 제반 성상을 명확히 확인하여야 함은 물론 분리균주의 병원성에 관한 연구가 필수적이다. 본 실험에서는 자연계에서 분리된 *T. madida* 분리균주의 제반 성상을 확인하고 실험동물에 대한 병원성 여부를 체계적으로 조사하여 산업미생물로서의 이용 가능성에 대한 안전성을 검증하였다. 본 실험에 사용한 *T. madida* N-5-3 분리균주의 생화학적, 형태학적인 특성은 전형적인 *T. madida* 균주의 미생물학적인 특징과 모두 일치하였다. *T. madida* N-5-3 균주의 실험용 mouse에서의 복강내 투여 치사량 (LD_{50})은 실험에 사용한 $1x10^1$에서부터 $1x10^8$ 투여 시까지 사망하는 동물이 관찰되지 않아 $1x10^8$ 이상인 것으로 확인되었다. 또 $1x10^8$의 시험균주를 실험용 mouse의 복강에 접종한 후 각 시기별로 심장, 폐, 간, 비장 및 신장에서의 시험균주의 증식여부를 조사한 결과 간과 비장에서만 접종 후 2일까지 $1x10^{4.3}$에서 $1x10^{2.87}$의 시험균주가 분리되었으나 접종 4일째부터는 시험기간 완료일까지 접종 균주가 전혀 관찰되지 않아 실험동물 체내에서 신속히 제거되는 것으로 나타났다. 한편 시험균주 접종에 따른 mouse 내부장기의 조직학적인 변화를 조사한 결과 특징적인 병리학적 소견을 관찰할 수 없었다. 이상의 결과를 종합하여 볼 때 *T. madida* N-5-3 분리균주는 산업분야에서 이용시 공중보건학적인 관점에서 비병원성 균주이며 안전한 균주인 것으로 판단된다.

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