Identification of a Novel Rhizopus-specific Antigen by Screening with a Signal Sequence Trap and Evaluation as a Possible Diagnostic Marker of Mucormycosis

Kanako Sato, Ken-Ichi Oinuma, Mamiko Niki, Satoshi Yamagoe, Yoshitsugu Miyazaki, Kazuhisa Asai, Koichi Yamada, Kazuto Hirata, Yukihiro Kaneko, Hiroshi Kakeya

Description	【概要】 研究グループは、生命予後が極めて悪いことで知られている難診断深在性真菌症であるムーコル症の早期診断法の開発に世界で初めて成功しました。 ムーコル症は、ムーコル目に属する真菌感染症の総称で、発生頻度はそれほど高くありませんが、発症すると急性に進行し大多数が死に至るため、早期に診断し治療することが最重要とされています。しかし、特徴的な臨床症状に乏しく、実用化された血清診断法がないため、確定診断には真菌の培養や病理組織検査が必要であるというのが現状でした。そこで研究グループは、ムーコル症を引き起こす代表的な真菌である Rhizopus oryzae（リゾプス・オリゼ）の菌体蛋白に注目し、「シグナルシークエンス・トラップ法」を用いて、血液検査で抗原（菌体の一部）を検出する方法を開発しました。 本検査方法は世界で初めて開発された「ムーコル症の血清診断法」です。また、本研究に使用した「シグナルシークエンス・トラップ法」が真菌研究においても新しい抗原を探すことに有用であることを明らかにしました。 '世界初！難診断深在性真菌症（ムーコル症）の早期診断法の開発に成功'. 大阪市立大学. https://www.osaka-cu.ac.jp/ja/news/2016/170215-1. (参照 2017-02-15)
Note	本研究は特許出願済です。

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Running title: **A *Rhizopus*-specific Marker of Mucormycosis**

Kanako Sato,[1,2] Ken-Ichi Oinuma,[1,3] Mamiko Niki,[1,3] Satoshi Yamagoe,[5] Yoshitsugu Miyazaki,[5] Kazuhisa Asai,[2] Koichi Yamada,[3,4] Kazuto Hirata,[2] Yukihiro Kaneko,[1,3] and Hiroshi Kakeya[3,4]

[1]Department of Bacteriology, [2]Department of Respiratory Medicine, [3]Research Center for Infectious Disease Sciences, and [4]Department of Infection Control Science, Osaka City University Graduate School of Medicine, Osaka, Japan; [5]Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases, Tokyo, Japan

Correspondence to:

Hiroshi Kakeya, MD, PhD

Department of Infection Control Science, Osaka City University, Graduate School of Medicine

1-4-3, Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

Tel.: +81 6-6645-3784; Fax: +81 6-6646-6056;

E-mail: kakeya@med.osaka-cu.ac.jp

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Abstract

Mucormycosis is the second most common mold infection, often indistinguishable from other invasive mold infections such as aspergillosis. Although an appropriate antifungal therapy is effective at an early stage of the infection, there is no reliable diagnostic method for decision-making. Thus, it is necessary to develop an efficient method that can detect mucormycosis rapidly and accurately. We searched for secreted or membrane-bound proteins of *Rhizopus oryzae*, which is the most common pathogen of mucormycosis, using the method of a signal sequence trap by retrovirus-mediated expression (SST-REX). Among the identified proteins, a *Rhizopus*-specific antigen was selected as a candidate, and efficacy of this specific antigen was evaluated using *R. oryzae*-infected mice. Of 302 clones obtained from the SST-REX library, a hypothetical protein (23 kDa, named "protein RSA") was selected as a candidate because of its highest prevalence of clones. Protein RSA was detected at significantly higher concentrations in serum and in lung homogenates of the infected mice as compared to those of uninfected mice. Our study indicates that protein RSA may be a promising biomarker of *R. oryzae* infection. SST-REX may be useful for comprehensive screening of prospective eukaryotic biomarkers of intractable mold infections.

Keywords. Mucormycosis; *Rhizopus oryzae*; Signal sequence trap; Biomarker.

Introduction

Invasive mold infections are frequently life-threatening in immunosuppressed hosts, such as patients with a hematological cancer, a solid organ transplant, uncontrolled diabetes, or penetrating trauma.[1] Invasive mucormycosis is the second most common mold infection after invasive aspergillosis (IA),[2] and several studies showed increasing incidence of mucormycosis among the above-mentioned immunocompromised patients.[3,4] Timely surgical treatment of the site of mucormycosis, especially of the rhino-orbito-cerebral and soft tissue, is recommended to reduce the mass of the infecting mold and to prevent expansion of mucormycosis to adjacent structures.[5] Nonetheless, this surgical intervention is not recommended for most patients with hematological cancer because of severe thrombocytopenia. In addition, therapeutic options against mucormycosis are limited because voriconazole and caspofungin, which are antifungal drugs that are safer than amphotericin B,[6] are ineffective. Although posaconazole may be active against Mucorales, posaconazole monotherapy is not recommended as a first-line treatment of mucormycosis[7] and is commercially unavailable in Japan. Some reports suggest that a high dose of liposomal amphotericin B (L-AMB) is effective as a first-line treatment of mucormycosis but poses a high risk of drug-related renal toxicity.[8] In addition, mucormycosis is often indistinguishable

from other invasive mold infections such as aspergillosis because the clinical and radiographic findings of pulmonary mucormycosis are nonspecific. Currently, the diagnosis of invasive mold infections is usually based on clinical-sample culture and detection of fungal hyphae during histopathological examination. In many cases, it is difficult to perform invasive examinations for the diagnosis, for example, transbronchial and surgical lung biopsies, because of the poor general condition of such patients. The *Aspergillus* antigen (galactomannan) test is available for IA. Some studies have shown the potential value of quantitative real-time polymerase chain reaction (qPCR) testing for circulating Mucorales DNA (cmDNA) in human serum and plasma samples for early diagnosis,[9,10] but there are no standardized serum assays available for the detection of Mucorales-specific antigens. As a result of delayed diagnosis and intervention, prognosis of mucormycosis remains poor as compared to aspergillosis, and therefore it is important to accurately distinguish mucormycosis from IA and to commence an appropriate treatment as soon as possible.

Our main aim was to develop a rapid diagnostic test for mucormycosis. Here, we identified an antigen specific for *Rhizopus oryzae*, which is the most common pathogen and responsible for up to 70% of all cases of mucormycosis.[11,12] This antigen may serve as a candidate biomarker and was identified by the method of a signal sequence trap by

retrovirus-mediated expression (SST-REX).[13] Diagnostic evaluation in mice revealed this marker's efficacy.

Materials and Methods

The Microbe

R. oryzae TIMM 1327 was originally provided by Teikyo University Institute of Medical Mycology. These isolates were grown on potato-dextrose-agar (PDA) plates for 2 weeks at 30°C. On the day of infection, conidia were aseptically dislodged from PDA plates and resuspended in sterile saline supplemented with 0.05% of Tween 80 and passed through 70-μm nylon cell strainers (BD Falcon, Heidelberg, Germany) for removal of any hyphal fragments. The conidia were counted with a hemocytometer to prepare the final inoculum.

Cloning of the Rhizopus oryzae-specific Antigen by the Method of a Signal Sequence Trap by Retrovirus-mediated Expression

An *R. oryzae* cDNA library was screened by SST-REX, which detects signal sequences coded in cDNA fragments on the basis of their ability to redirect a constitutively active mutant cytokine receptor to the cell surface, resulting in interleukin 3 (IL-3)-independent growth of Ba/F3 cells.[13] Briefly, cDNA was synthesized from total RNA of *R. oryzae* cells that were cultivated in the YPD medium (0.5% yeast extract, 1% peptone, 2% glucose) overnight. The synthesized cDNA was separated on the basis of

size, and fractions >600 bp were inserted into *BstX*I sites of the pMX-SST vector by means of *BstX*I adapters (Invitrogen, Carlsbad, CA, USA). Ba/F3 cells were infected with high-titer retroviruses expressing the *R. oryzae* cDNA library, and the integrated cDNA fragments were isolated from factor-independent Ba/F3 clones by genomic PCR. All cDNA fragments were sequenced and analyzed. After that, the *R. oryzae* cDNA library in the pME18S vector was screened by means of the ^{32}P-labeled cDNA fragment of a clone to isolate the entire coding region.

The most frequent gene among the genes identified by the above method was selected as a source of the candidate protein, and this candidate protein was named "protein RSA" and used in the subsequent experiments. We developed an enzyme-linked immunosorbent assay (ELISA) based on this protein. The signal sequence was predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

Animals

Six-week-old female ICR mice (>20 g) were purchased from SLC (Hamamatsu, Japan) and were allowed to acclimate for a week. They were given irradiated feed and sterile water. All experimental procedures were conducted in accordance with the Japanese

Physiological Society and National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The study protocol was approved by the Committee on Animal Care and Use at Osaka City University. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation at Osaka City University.

Immunosuppression

The mice were injected with cortisone acetate (250 mg/kg, *Wako* Pure Chemical Industries, Osaka, Japan) subcutaneously on days −2 and 0 and with cyclophosphamide (100 mg/kg, *Sigma-Aldrich*, *Tokyo*, Japan) intraperitoneally on days −2, 0, and 2 (Figure 1). To prevent bacterial infection, tetracycline hydrochloride (Wako Pure Chemical Industries, Osaka, Japan) was administered with drinking water at a concentration of 0.8 mg/mL starting on day −2 of the experiment.[14]

Infection

The mice were subdivided into 2 groups: (i) the control group: 5 mice in 1 cage, (ii) the *R. oryzae*-infected group: 27 mice in 5 cages (including 10 mice in 2 cages for survival monitoring).

The control group was challenged intratracheally on day 0 with 50 μL of sterile

saline containing 0.05% of Tween 80, and the *R. oryzae*-infected group was challenged intratracheally on day 0 with 10^7 conidia of *R. oryzae* (TIMM 1327) in 50 μL of sterile saline containing 0.05% of Tween 80 (Figure 1). All the mice were challenged only after sedation with isoflurane.

The Fungal Burden in the Infected Lungs and a Serological Antigen Detection Test

For fungal-burden and serological examinations, the mice were euthanized on day 4. Blood samples were extracted from the murine heart, and the lungs were surgically removed under anesthesia. The lungs were homogenized in 1 mL of sterile normal saline after weighing, and the homogenates were quantitatively cultured on PDA plates. The resulting colonies were counted after 24-h incubation at room temperature. Homogenized lung samples were diluted 10- to 1 000 000-fold with sterile normal saline, and 100 μL of each dilution was spread on PDA plates. Thus, the detection limit was 100 colony-forming units (CFU)/mL. These values were expressed as $\log_{10}$ CFU. The homogenates and blood samples were centrifuged at 1700 × *g* for 10 min. Serum and the supernatants of lung homogenates were used for an assay of the *R. oryzae*-specific antigen.

ELISA

Two custom-made polyclonal antibodies against protein RSA (which was predicted as the most abundantly secreted protein by the SST-REX method) were supplied by Medical & Biological Laboratories Co., Ltd. (formerly known as ACTGen), Nagoya, Japan. These antibodies, one for capture and the other for detection, were designed to recognize different epitopes of the antigen for a sandwich ELISA. Microtiter plates (Nunc MaxiSorp®, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 µL of the capture antibody in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 3 µg/mL with incubation at room temperature for 2 h. The unbound antibody was removed by washing the wells 3 times with phosphate-buffered saline (PBS) containing 0.05% of Tween 20 (PBS-T). The remaining protein-binding sites in the wells were blocked with blocking buffer (PBS containing 4% of bovine serum albumin (BSA) and 0.2 M sucrose) at 4°C overnight. After 3 washes, 100 µL of serum samples diluted 5-fold or of supernatants from the lung homogenates diluted 5-fold in incubation buffer (PBS containing 0.1% of BSA) were added to the wells in triplicate and were incubated for 1 h at room temperature.

To construct a standard curve, serial dilutions of the recombinant antigen (protein RSA, 1.25 to 20 ng/mL) were prepared in incubation buffer containing 20% of mouse

serum (Sigma-Aldrich, Tokyo, Japan). After we washed the wells, 100 μL of the biotin-conjugated detection antibody (3 μg/mL) in incubation buffer was added to each well and incubated for 1 h at room temperature. The microplates were washed, and a streptavidin/peroxidase conjugate (Pierce, Rockford, IL) diluted 1:10 000 in incubation buffer was added (100 μL/well), followed by incubation for 30 min at room temperature. The microplates were then washed, and 100 μL of the SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA) was added to each well. The microplates were incubated for 10 min at room temperature. The reaction was stopped by addition of HCl, and absorbance was measured at 450 nm using a Multiskan-JX (Thermo Fisher Scientific, Yokohama, Japan).

Statistical Analysis

All calculations were performed in the JMP software, version 10.0.0 for Windows (SAS Institute Inc., Cary, NC, USA). The statistical significance level was set to 5%. Survival curves for the mice after infection were plotted using the Kaplan-Meier analysis. To compare 2 groups, we performed Wilcoxon's rank-sum test, and to assess a correlation between protein RSA levels and lung weight, Spearman's rank correlation coefficient was calculated.

Results

Candidate Proteins Obtained by Means of a Signal Sequence Trap by Retrovirus-mediated Expression

We identified 302 genes encoding transmembrane and secreted proteins in the SST-REX library and selected a hypothetical protein (23 kDa) that had the highest prevalence: 163 clones (Supplementary Table 1). This protein was named protein RSA; it consists of 226 amino acid residues, and the first 18 residues were predicted to be a signal sequence (Table 1). A BLASTP search indicated that this protein is unique to *R. oryzae*, i.e., is absent in the common pathogenic fungi such as *Aspergillus* spp. and *Candida* spp.

The Survival Rate of Mice Infected Intratracheally with Conidia of *Rhizopus oryzae*

For survival monitoring, 10 mice in 2 cages were infected with *R. oryzae* according to the same protocol as in the *R. oryzae*-infected group. They started to die on day 4, and only 1 mouse had survived until day 14, when we finished the monitoring. Median survival time was 5 days, and 10% of the mice survived (Figure 2). No mice died in the control group.

Survival and Growth of *Rhizopus oryzae* in the Lungs of Mice

Seventeen mice in 3 cages were challenged intratracheally with conidia of *R. oryzae* for evaluation, and 16 mice had survived until euthanasia (day 4). We assessed survival and growth of *R. oryzae* by measuring lung weight and by calculating the CFUs present in the homogenized lungs (Table 2). Thirteen mice of the *R. oryzae*-infected group yielded *Rhizopus* colonies in the cultures of homogenized lungs, and 2 mice of the *R. oryzae*-infected group yielded other bacterial colonies in the cultures of homogenized lungs: a suspected coinfection. Mice without detectable hyphae were excluded from further analyses. Control mice and 3 mice challenged with conidia of *R. oryzae* had undetectable CFUs. Mean $\log_{10}$ CFU in homogenized lungs in the *R. oryzae*-infected group was 5.2 (range, 2.51–5.49), and *R. oryzae*-infected mice showed significantly increased lung weight (Table 2).

Evaluation of the *Rhizopus oryzae*-specific Antigen (Protein RSA) Concentration in Serum

Antigen titers in serum obtained from control mice were 57.7 ± 12.3 ng/mL (mean ± SD; range 50.2–61.3 ng/mL). Antigen titers in serum obtained from *R. oryzae*-infected

mice were 161.1 ± 117.7 ng/mL (mean ± SD; range 53.5–413.6 ng/mL), and these titers were significantly higher than those of control mice ($P < 0.05$, Figure 3A). Detection limit was approximately 50 ng/mL, and thus antigen titer elevation in control mice was presumed to be nonspecific. The serum antigen concentration had a tendency to correlate positively with lung weight ($P = 0.085$, r = 0.50, Figure 4A).

Evaluation of the Protein RSA Concentration in Lung Tissue

Antigen titers in homogenized lungs obtained from control mice were 66.1 ± 4.9 ng/mL (mean ± SD; range 62.5–76.9 ng/mL). Antigen titers in homogenized lungs of *R. oryzae*-infected mice were 307.0 ± 64.4 ng/mL (mean ± SD; range 95.1–426.7 ng/mL). These titers were significantly higher than those of control mice ($P < 0.05$, Figure 3B). Protein RSA levels in lung tissues were found to have a significant correlation with lung weight ($P < 0.0001$, r = 0.89; Figure 4B).

We reproduced the finding of a higher protein RSA concentration in the serum and lung tissue of *R. oryzae*-infected mice using the same protocol (Supplementary Table 2).

Discussion

Since a signal sequence trap was first reported as a cloning strategy for secreted proteins and type I membrane proteins, this method has been applied to discovery of biomarkers such as cancer markers.[15] Most studies have been conducted on mammalian cells including human ones, and only several studies examined proteins from other eukaryotic sources. Although the structure of signal sequences and the functioning of the secretory machinery are well conserved from prokaryotes to eukaryotes,[16] the above method has never been applied to fungal pathogens of humans.

Because there is not an established method for rapid diagnosis of invasive fungal infections and because it is especially difficult to distinguish aspergillosis and mucormycosis, we tried to use this method to find a novel mucormycosis biomarker. Mucors from clinical samples are generally difficult to cultivate, with the culturability rate being only 2%.[17] Diagnosis is mostly based on histopathological analysis (85.7%) and microscopy (12.3%), and therefore most cases are not accurately diagnosed.[17] Bronchoalveolar lavage (BAL), a lung biopsy, and blood and urine samples are the major clinical samples available in pulmonary invasive mycoses. BAL and a lung biopsy usually require bronchoscopy; therefore, blood and urine samples are more practical. Recently, qPCR assays for cmDNA in serum were developed for rapid

detection of Mucorales,[9,10] but a serum antigen test for Mucorales has never been reported. Our results suggest that detection of protein RSA in serum may be a promising diagnostic test for *R. oryzae* mucormycosis, as effective as qPCR assays. In addition, we showed that the antigen concentration is also elevated in infected murine lungs, suggesting that the antigen in the lung biopsy samples can be useful as a biomarker. Although lung antigen levels and lung weight correlated well, serum antigen concentration and lung weight did not. Because lung weight can reflect severity of pulmonary damage, so can lung antigen concentrations. On the other hand, the lack of a correlation between the serum antigen concentration and lung weight may mean that this secreted protein can be detected irrespective of the severity of lung damage because Mucorales tend to invade blood vessels, frequently resulting in thrombosis and subsequent tissue necrosis.[18,19]

Protein RSA is annotated as a hypothetical protein carrying a conserved signal sequence in the public database for *R. oryzae*, and secretion of this protein into the culture medium was confirmed experimentally (data not shown). BLAST analysis showed that this protein is well conserved among *Rhizopus* spp. but does not exist in *Aspergillus* spp. Because other Mucorales do not have this protein, it may serve as a specific marker of *Rhizopus* infection. We are currently tempted to use the SST-REX

method to find specific antigens of other pathogenic filamentous fungi, including other Mucorales and *Aspergillus* spp. There is an established diagnostic test (involving a galactomannan antigen) for aspergillosis, but the sensitivity is inadequate. If the new assay is developed successfully, more accurate diagnosis using another antigen may lead to more accurate selection of treatment.

To determine whether the method we developed is indeed applicable to clinical practice, further studies are necessary including testing on clinical serum samples, evaluation of murine or human BAL samples, and examining the possible relation between the disease severity and antigen titer. In addition, functional analysis of this protein in the pathogenesis should be conducted because this research may lead to a novel therapeutic strategy.

In conclusion, we identified an *R. oryzae*-specific secreted protein and showed that this protein may be a serum biomarker for early diagnosis of *R. oryzae* mucormycoses. Our results also suggest that SST-REX can help to identify other filamentous-fungus-specific antigens and may be applied to comprehensive screening of prospective eukaryotic biomarkers.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

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Figure Legends

Figure 1. The experimental design of the mouse model of invasive pulmonary mucormycosis. Each mouse was treated with cyclophosphamide and cortisone acetate as indicated. For prevention of bacterial infection, water containing tetracycline hydrochloride (0.8 mg/mL) was provided throughout the experiment. On day 0, 10^7 conidia of *Rhizopus oryzae* (TIMM 1327) resuspended in saline containing 0.05% of Tween 80 were inoculated into the mice in the infection group, while the same amount of sterile vehicle was administered to the control group. The mice were euthanized on day 4 to obtain blood and lung samples for subsequent experiments.

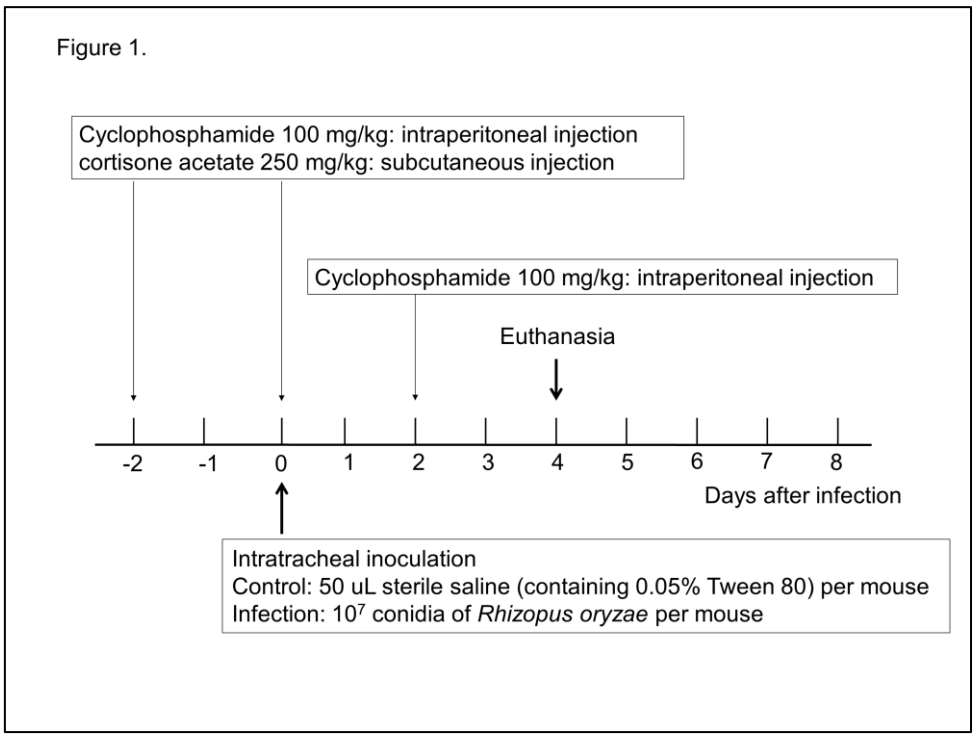

Figure 2. Kaplan-Meier probability of survival after *Rhizopus oryzae* infection. Survival curves for the mice after infection were plotted using Kaplan-Meier analysis. Ten mice in the *R. oryzae*-infected group were used for survival monitoring until day 14. Median survival time was 5 days, and only 10% of the mice survived.

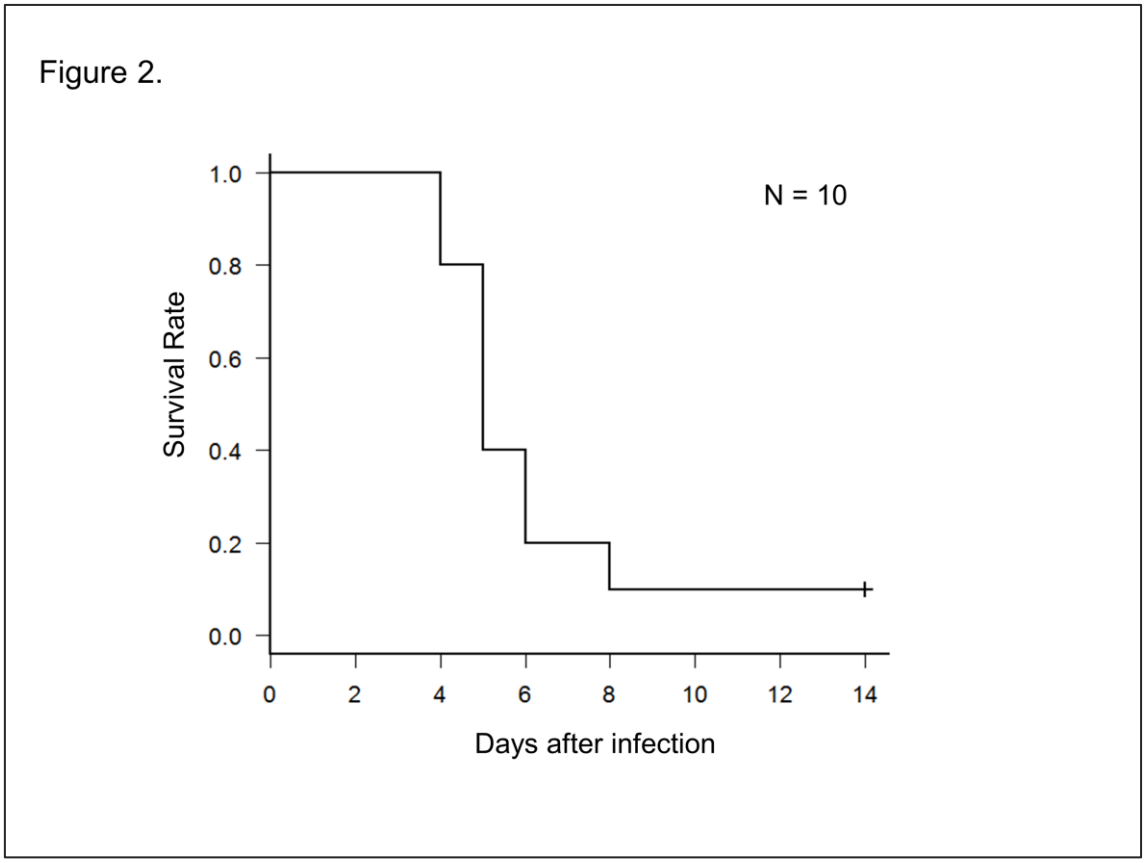

Figure 3. Protein RSA's titer in serum and lungs. Each plot indicates means of triplicate measurements, and the bars denote the means of each plot. Protein RSA concentration was significantly higher in serum (A) and in homogenized lungs (B) of *R. oryzae*-infected mice (n = 13) compared to the control mice (n = 5). *Significantly different ($P < 0.05$) from control mice. The figure shows a representative result from 2 independent experiments.

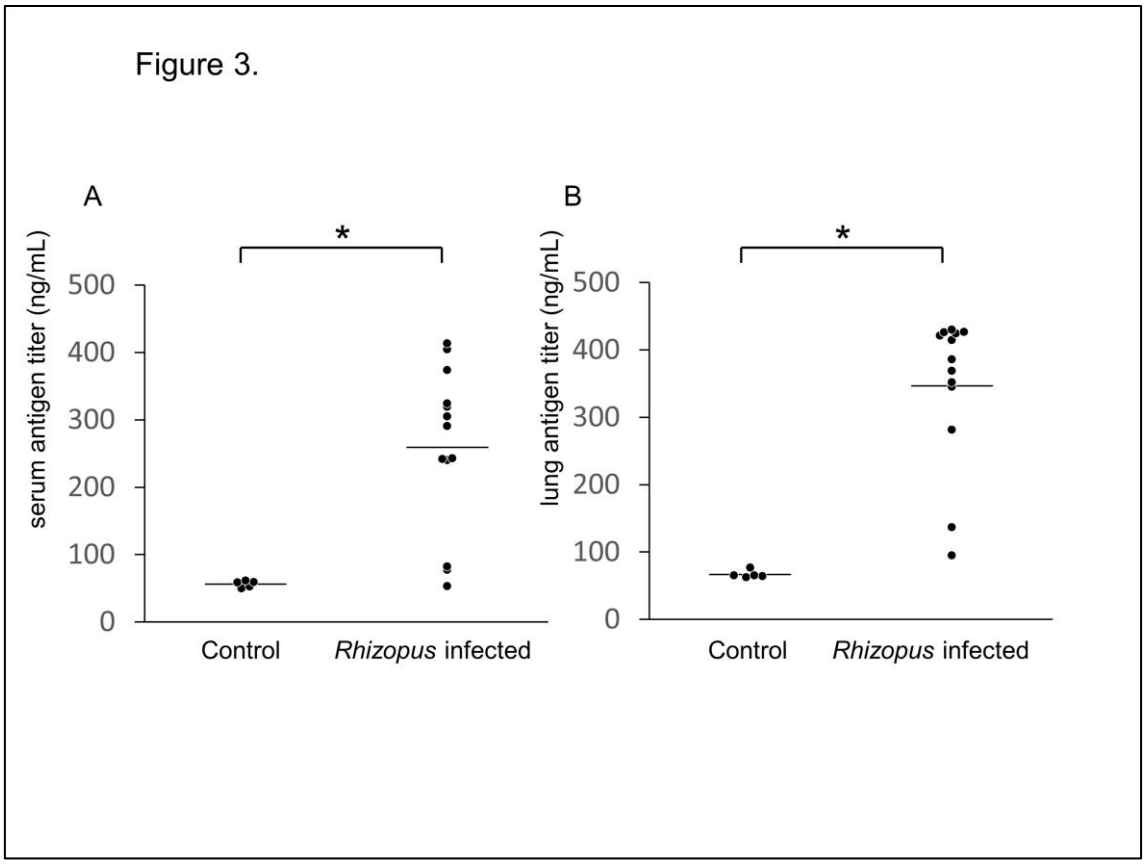

Figure 4. The correlation between protein RSA titer and lung weight. Serum antigen titer did not significantly correlate with lung weight (P = 0.085, r = 0.50) (A) although the lung antigen titer had significant correlations with lung weight (P < 0.0001, r = 0.89) (B).

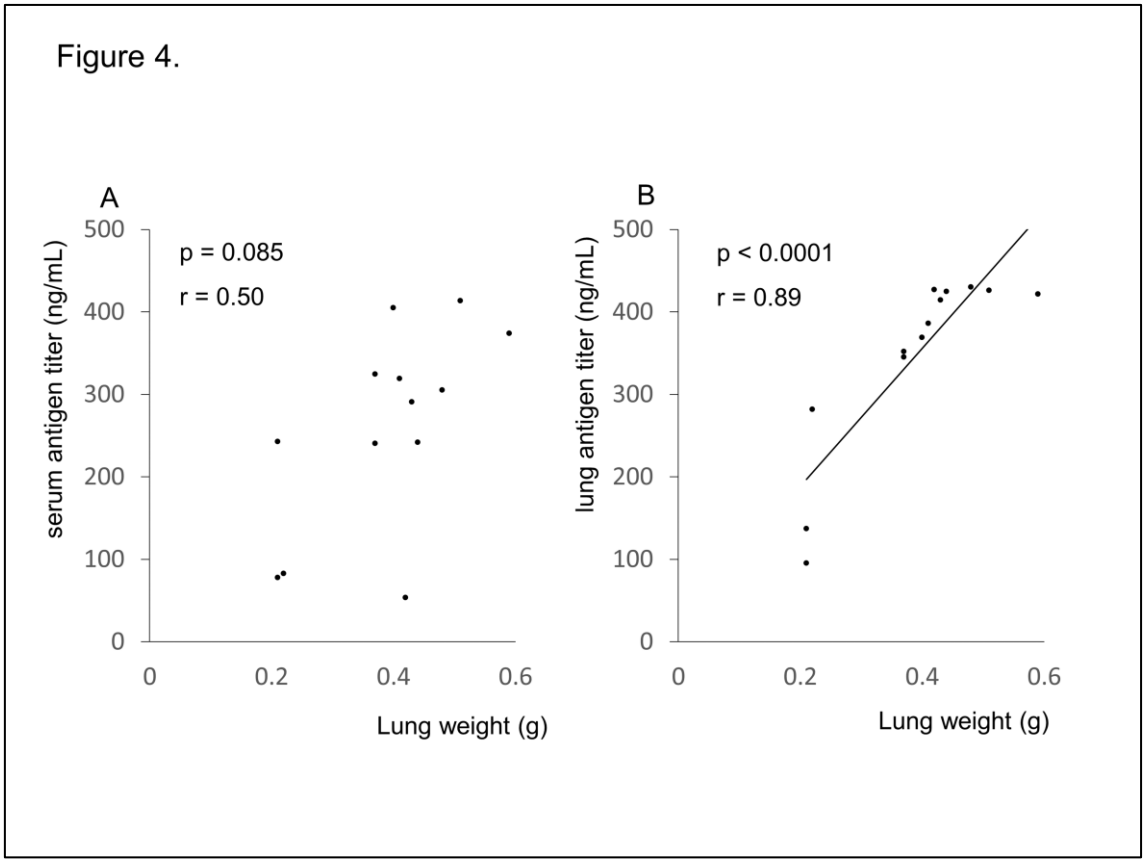

Table 1. Nucleotide and Amino Acid Sequence of Protein RSA (RO3G_17119)

nucleotide sequence (678 nt)

ATGAAGTTATCATTCTCTATCTTGCTCTTAGTTGCTGCTGTTCAAATTCAAGCT
GCCGTCAATCCTAAAGCTGCTGCTGCTTCCAAGTGCATTTCTGGCTCTTCTGG
AAAGGGTAATGGTGATGGTTACAAGGGTTACTGCTGCAAGGACAGCGATGAT
TGCTTTAATACCTGCGTGAAGGGTGTCTGCAATGGATCTTCCAAGCCTGATCC
TACTCCTACTCCTACTCCTACTCCTGGAGGCAAGTGCACTCCTGGATCTAAAG
GTCTTGGTAATGGTGATGGCTACAAGGGATACTGCTGTGATGATAGTGATGAT
TGTATTAACAGCTGTGTAAGCGGTGTCTGCAATGGTCCTACTAACACAGCAAC
CAAGACTACTACTACGAAGACTACTACAGCCACCGCTACTGCTACCCCAGGC
AAGTGTTCCCCTGGATCTCAAGGTCTCAAGAACGGCGATGGCTACAAGGGTG
ATTGTTGTGATACCAGCGATGATTGTGTGAACAGCTGTGTAAACGGTGTCTGC
AATGGCGAAGTTAATCCTAATCCTAATCCTGGTAAATGTATTCCTGGCTACAA
GGACAAAGGCAATGGTAAGGGTCCCTTTAATGCATGCTGTTCAGATAATGAC
GACTGTCAAGAAGCCTGTGTCAGAGGCAGATGTACCAAACCTTAA

amino acid sequence (225 aa)

MKLSFSILLLVAAVQIQAAVNPKAAAASKCISGSSGKGNGDGYKGYCCKDSDDC

FNTCVKGVCNGSSKPDPTPTPTPTPGGKCTPGSKGLGNGDGYKGYCCDDSDDC

INSCVSGVCNGPTNTATKTTTTKTTTATATATPGKCSPGSQGLKNGDGYKGDCC

DTSDDCVNSCVNGVCNGEVNPNPNPGKCIPGYKDKGNGKGPFNACCSDNDDC

QEACVRGRCTKP

The predicted signal sequence is underlined.

Table 2. Lung Weight and Survival and Growth of Hyphae in the Mouse Model of *Rhizopus oryzae* Infection (Mean ± Standard Deviation)[a]

	Control	*Rhizopus* infected	*P* value
n	5	13	
Log_{10} CFU/mL	ND	5.2 ± 0.34	
Lung weight (g)	0.19 ± 0.035	0.30 ± 0.13	0.0121

Abbreviations: CFU, colony-forming units: ND, not detected.

[a]Survival and growth values are expressed as Log_{10} CFU/mL counted on a PDA plate where the supernatant of a homogenized lung was spread. Of the 17 inoculated mice, 4 mice were excluded because the infection failed to get established.