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## Original Research

# Experimental Infection of Mice with *Veronaea botryosa* as a Model for Human Phaeohyphomycosis

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*Veronaea botryosa* is a ubiquitous, dematiaceous mold capable of causing cutaneous and subcutaneous lesions in humans. In the last decade, *V. botryosa* has been associated with emergent systemic fungal infections in aquatic animals, including cultured sturgeon (*Acipenser* spp.), captive amphibians, and wild reptiles. Recently, repetitive extragenic palindromic PCR (rep-PCR) fingerprinting has demonstrated intraspecific variability among *V. botryosa* isolates from different clinically affected hosts and geographic regions. However, little is known regarding the pathogenic potential of the different genetic clades, and no mammalian model currently exists to investigate *V. botryosa* phaeohyphomycosis. In this study, we inoculated immunocompetent heterozygotic (*nu/+*) and immunodeficient homozygotic (*nu/nu*) Hsd:ATHymic Nude-Fox1<sup>tm</sup> mice subcutaneously or through orogastric gavage with 1 of 3 representative *V. botryosa* strains that had been recovered from white sturgeon (*Acipenser transmontanus*), green sea turtle (*Chelonia mydas*), and human hosts and typed by using rep-PCR analysis. Daily mortality and morbidity were recorded, and dissemination of the fungus was investigated through culture of splenic samples and histologic analysis of the injection site, regional lymph nodes, salivary gland, spleen, liver, mesenteric lymph node, and gastrointestinal tract. No differences in survival, fungal burden, or dissemination were observed between fungal strains, routes of inoculation, or host immune status. Fungal infection was observed after subcutaneous inoculation only, was localized to the inoculation site, and was identified in both *nu/nu* and *nu/+* mice. Fungal strain variability was not associated with virulence in a murine model of infection, and this novel mouse model of *V. botryosa* phaeohyphomycosis recapitulates the human clinical condition.

**Abbreviation:** rep-PCR, repetitive extragenic palindromic PCR fingerprinting

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*Veronaea botryosa* is a saprobic, dematiaceous fungus found globally in water and soil samples.<sup>2,12,17,18</sup> *V. botryosa*-associated disease has been sporadically reported as localized cutaneous infections in humans for decades,<sup>21</sup> but only recently has it been identified as a systemic pathogen of poikilotherms, including those farmed for human consumption.<sup>6,10,18,19</sup> The manifestation and severity of disease differ substantially by host species, and currently no mammalian model exists for investigating the pathogenesis, transmission, and treatment of *V. botryosa*.

Since 1990, *V. botryosa* has been identified as the etiologic agent of at least 12 human cases of phaeohyphomycosis in China, Libya, the Philippines, France, Taiwan, Japan, the United States, and Mexico.<sup>1,2,4,5,11,12,15,17,20-22</sup> There appears to be no age or sex predilection, and although immunocompromise can exacerbate infection, it does not appear to be necessary.<sup>1,2,7,21</sup> The overwhelming similarity among human cases is the presence of lesions in the dermis, submucosa, or subcutis of the head or extremities. Lesions often consist of verrucous, dermal or

submucosal nodules or plaques with variable black pigmentation, pain, and pruritus.<sup>2,21</sup> The route of dematiaceous fungal infection, typically due to *Exophiala* spp. or *Bipolaris* spp.,<sup>2,7</sup> is hypothesized to be through inoculation of open wounds or ingestion of the organism.<sup>2,9</sup>

In veterinary medicine, cultured sturgeon (*Acipenser* spp.) appear particularly susceptible to *V. botryosa* infection. Aquaculture of these fish for meat, caviar, and other products, is of great commercial value for many countries, making losses due to *V. botryosa* of significant economic impact.<sup>18</sup> Unlike in humans, fish develop a systemic mycosis that manifests as emaciation, skin and ocular lesions, and coelomic fluid accumulation, which is colloquially known in sturgeon as ‘fluid belly.’ In addition, there are no FDA-approved treatments for fungal disease in aquaculture species destined for human consumption.<sup>18,19</sup> Like these fish, some amphibians and aquatic reptiles have shown susceptibility to systemic infections with *V. botryosa*.<sup>6,10</sup>

Repetitive extragenic palindromic PCR fingerprinting (rep-PCR) analysis has revealed genetic variability between white sturgeon (*Acipenser transmontanus*), green sea turtle (*Chelonia mydas*), and human isolates of *V. botryosa*,<sup>18</sup> however, little is known regarding the pathogenic potential of the various genetic clades. Given the emerging nature of *V. botryosa* in humans and animals, its ubiquitous presence in the environment, and its

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potential for zoonotic transmission through cultured meat and caviar, the current study aimed to demonstrate the effectiveness of a mouse model for the characterization of mammalian *V. botryosa* infection and to investigate *V. botryosa* strain-associated virulence. We hypothesized that mice would adequately simulate *V. botryosa* phaeohyphomycosis as it manifests in humans, that immunodeficiency would be associated with systemic infection, and that *V. botryosa* strain variability would be associated with pathogenicity.

## Materials and Methods

**Fungal isolation.** Frozen stocks of *V. botryosa* recently isolated from a cultured sturgeon, human, and sea turtle were kept at  $-80^{\circ}\text{C}$ .<sup>18</sup> Isolates were revived on potato flake agar (Biologic Media Services, University of California, Davis, CA) and incubated at  $25^{\circ}\text{C}$  in aerobic conditions for 15 d. On the day of challenge, the agar plates were flooded with PBS. Spores were purified from recovered PBS by vacuum filtration through a Miracloth (EMD Millipore, Billerica, MA) at pore sizes of 22 to  $25\ \mu\text{m}$ . Once spores were isolated, counts were confirmed by hemocytometer under light microscopy and adjusted by using PBS. The final concentration used for inoculation was  $5.73 \times 10^6$  spores per mouse. Viability was confirmed through culturing on potato flake agar and counting colonies after 5 to 7 d.

**Mouse infection.** Animal protocols were approved by the UC Davis Institutional Animal Care and Use Committee. Male and female, immunocompetent heterozygotic (*nu/+*) and immunodeficient homozygotic (*nu/nu*) Hsd:ATHymic Nude-Fox1<sup>tm</sup> mice (age, 6 wk) were obtained from an intramural breeding colony. Sentinel animals exposed to bedding from research animals tested negative for mouse hepatitis virus, mouse parvovirus, minute virus of mice, *Mycoplasma pulmonis*, Theiler murine encephalomyelitis virus, ectromelia virus, epizootic diarrhea of infant mice virus, murine adenovirus types 1 and 2, lymphocytic choriomeningitis virus, and reovirus 3 throughout the study. Mouse strains were selected to parallel previous murine phaeohyphomycosis studies.<sup>8,14</sup> Inoculation was performed by either subcutaneous injection over the right shoulder or through orogastric gavage. Mock-infected mice (PBS) were included for both genotypes. All control ( $n = 4$  per strain) and experimental groups ( $n = 3$  per group) were housed separately in polycarbonate microisolation caging in a HEPA-filtered room with controlled temperature ( $22$  to  $24^{\circ}\text{C}$ ) and humidity (30% to 70%) on a 12:12-h light:dark cycle. Health checks were conducted on all mice at least once daily. Mice were observed through day 30 before being euthanized by carbon dioxide inhalation. All animals underwent a complete necropsy. Splenic samples (approximately 20 mg) were collected immediately after euthanasia, placed on potato flake agar plates, and incubated at  $25^{\circ}\text{C}$  under aerobic conditions for 15 d.

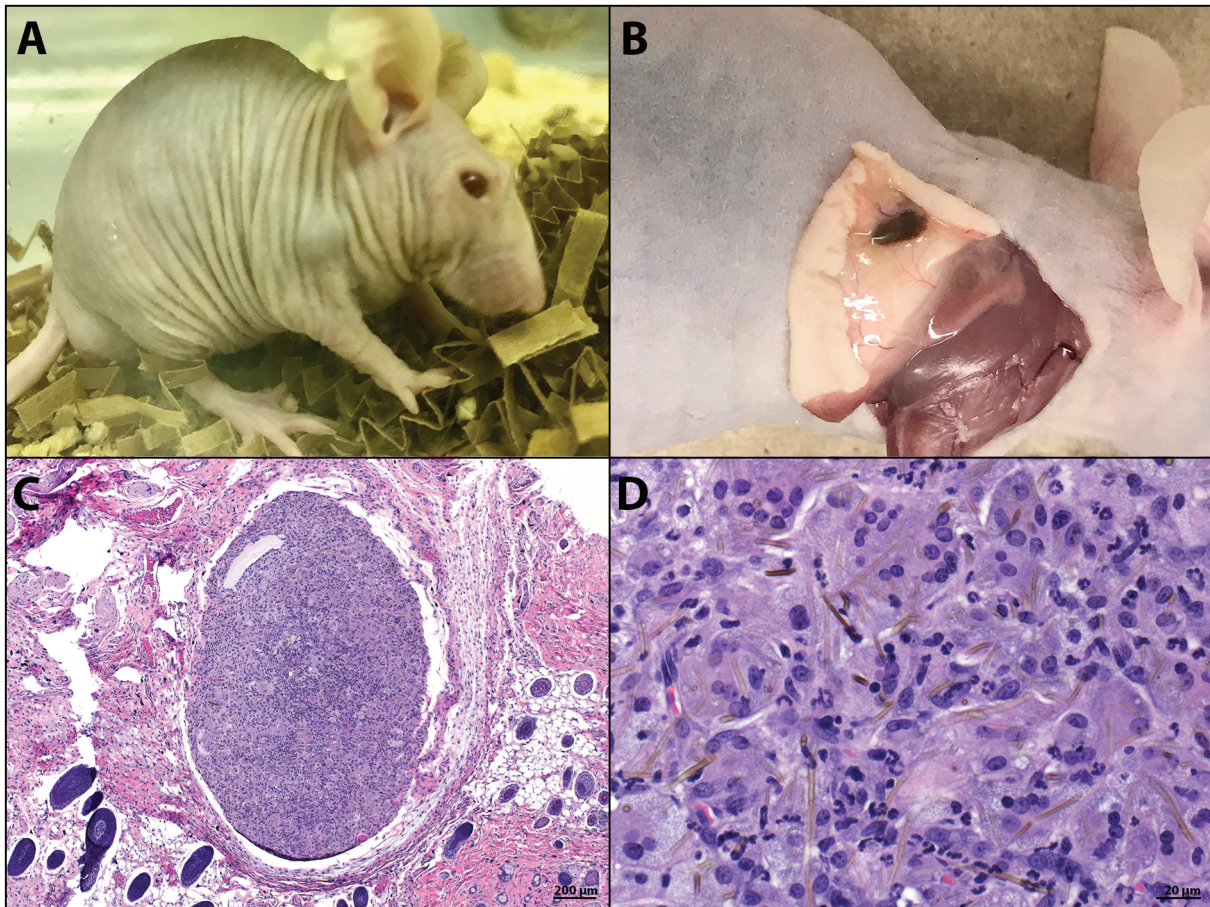
**Histopathology.** Tissues were fixed in 10% neutral buffered formalin. Injection site, regional lymph node, salivary gland, spleen, liver, mesenteric lymph node, and gastrointestinal tract were processed routinely, stained with hematoxylin and eosin for light microscopic examination, and analyzed by a board-certified veterinary pathologist (DMI) who was blinded to treatment group. Binomial scores were assigned for the presence (1) or absence (0) of organisms. A semiquantitative, criterion-based scoring system for pathogen burden in an area encompassing 10 high-power fields (magnification, 400 $\times$ ) was applied: 1, few (10 organisms or fewer); 2, moderate (11 to 30 organisms); 3, marked (31 to 50 organisms); and 4, severe (more than 50 organisms). Severity of inflammation was quantified according to the area of involvement in the section examined.

**Statistical analysis.** Statistical analysis was performed by using Prism version 7.0d (GraphPad Software, San Diego, CA). Binomial scores for the presence of pathogens were compared by using the  $\chi^2$  test. Ordinal data measuring pathogen burden were compared by using the Kruskal–Wallis test. The effects of mouse strain, *V. botryosa* isolate, and their interactions on pathology score (load) and inflammation were analyzed using 2-way (factorial) ANOVA in JMP Pro 14.2, (SAS Institute, Cary, NC). Statistical significance was defined as a *P* value of less than 0.05 for all analyses.

## Results

**Sturgeon-derived *V. botryosa* infection in mice.** Two of the 3 subcutaneously inoculated immunodeficient mice gradually developed an injection site swelling (maximal diameter, 5 mm) that was indurated, spherical, and clinically palpable for approximately 20 d after inoculation before receding (Figure 1 A). At necropsy, reflection of the skin over the injection site in one mouse (Figure 1 B) revealed a single, subcutaneous, black nodule remnant containing multiple moniliform, pigmented, fungal hyphae (Figure 1 C and D). No significant gross lesions were noted at necropsy in the other 2 immunodeficient mice that were subcutaneously inoculated with sturgeon-derived *V. botryosa*. Two of the 3 subcutaneously inoculated immunocompetent mice gradually developed indurated injection site swellings that did not appear until 25 d after inoculation and grew to 4 mm in diameter. At necropsy, subcutaneous abscesses were noted at the sites of inoculation and were found to contain granulomatous inflammation. Histopathologic evaluation of the subcutaneous inoculation site in the immunodeficient mouse with the black nodule remnant revealed an established infection, with a heavy fungal burden and mild inflammation, whereas the infections in the remaining 2 immunocompetent mice demonstrated a relatively lighter fungal burden and greater inflammation (Figures 2 and 3). Although qualitative differences were noted, neither pathogen burden nor inflammation differed significantly between the subcutaneously challenged immunocompetent and immunodeficient mice. None of the aseptically obtained spleen samples yielded *V. botryosa* on culture, regardless of immune status or infection route. Orogastrically inoculated mice did not develop any clinical sign of disease, did not establish infection and did not exhibit any histopathologic changes in any of the analyzed tissues.

**Sea turtle-derived *V. botryosa* infection in mice.** None of the immunodeficient mice subcutaneously inoculated with sea turtle-derived *V. botryosa* showed any clinical signs, developed infection, or developed any histopathologic lesions (Figures 2 and 3). One of the 3 subcutaneously inoculated immunocompetent mice gradually developed an indurated injection site swelling that became apparent at 27 d after inoculation; this swelling corresponded to a spherical, subcutaneous abscess approximately 2 mm in diameter at necropsy and an extensive, granulomatous inflammatory response. Histopathologic assessment of the subcutaneous injection site in the immunocompetent mice revealed a moderate pathogen burden in 1 of the 3 mice (Figure 2) and mild to severe inflammation in all 3 mice (Figure 3). Neither pathogen burden nor inflammation differed significantly between the immunocompetent and immunodeficient mice subcutaneously inoculated with turtle-derived *V. botryosa*. None of the aseptically obtained spleen samples yielded *V. botryosa* on culture, regardless of immune status or infection route. Orogastrically inoculated mice did not develop any clinical signs of the disease, become infected, or exhibit any histopathologic changes in any of the analyzed tissues.



**Figure 1.** Focal phaeohyphomycosis in an immunodeficient mouse inoculated subcutaneously with sturgeon-derived *V. botryosa*. (A) Injection site swelling on day 5 after inoculation. (B) A pigmented granuloma at the injection site, as seen at necropsy after reflection of the skin, (C) was confirmed to be chronic granulomatous cellulitis. Hematoxylin and eosin stain; magnification, 40 $\times$ . (D) Microscopically, the inflammatory reaction was composed of multinucleated giant cells, macrophages, and few neutrophils admixed with and engulfing numerous pigmented fungal hyphae. Hematoxylin and eosin stain; magnification, 400 $\times$  magnification.

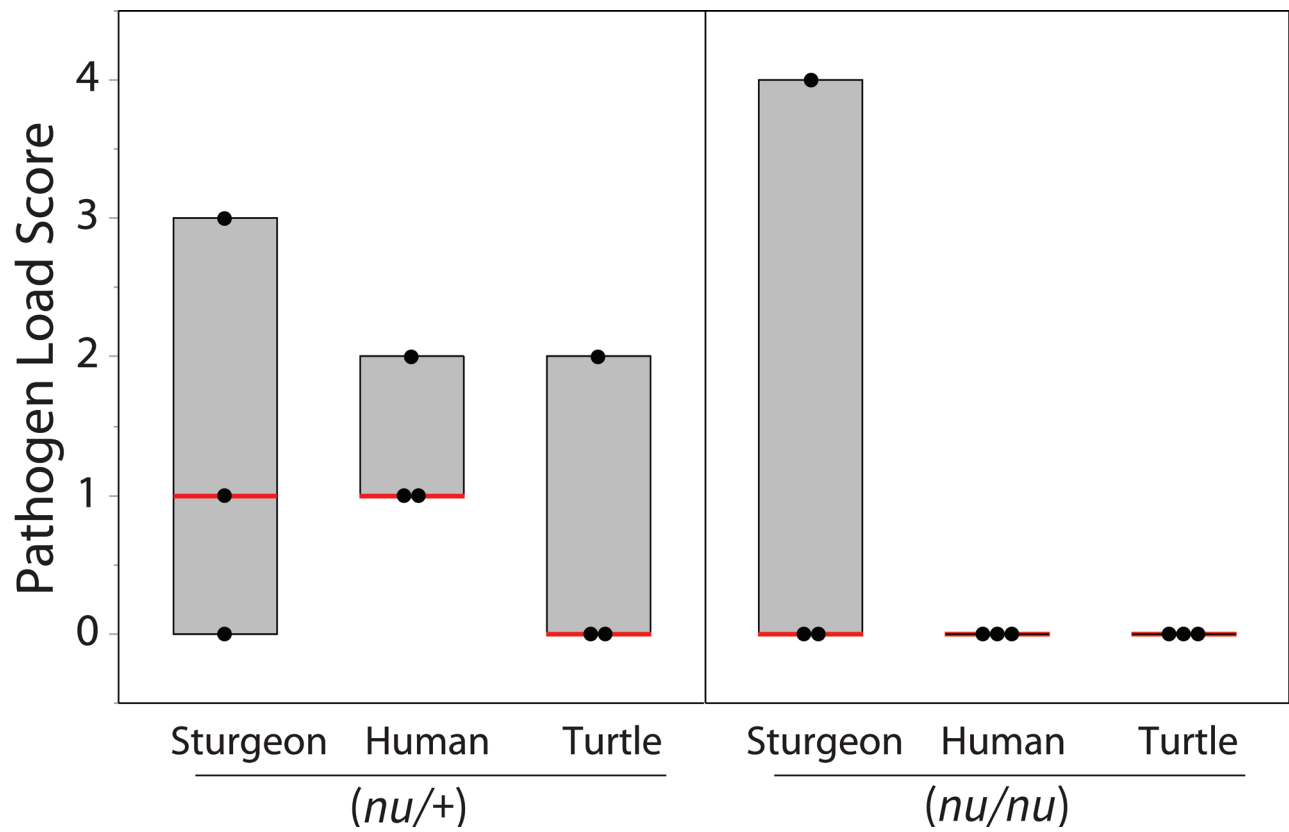
**Human-derived *V. botryosa* infection in mice.** None of the immunodeficient mice subcutaneously inoculated with human-derived *V. botryosa* showed any clinical signs, developed infection, or had histopathologic lesions. All 3 of the subcutaneously inoculated immunocompetent mice gradually developed injection site swellings (maximal diameter, approximately 6 mm) that were spherical, indurated, and clinically palpable beginning on day 20 to 25 after inoculation and continuing until necropsy (Figure 4 A), at which time they were noted grossly as abscesses (Figure 4 B). Granulomatous inflammation was noted at the site of subcutaneous inoculation and contained a few moniliform, pigmented, fungal hyphae (Figure 4 C and D). Assessment of the histopathology at the subcutaneous injection site revealed that the inflammation of all 3 mice ranged from moderate to severe (Figure 3), and all animals had a light to moderate pathogen burden (Figure 2). Neither pathogen burden nor inflammation differed significantly between the subcutaneously inoculated immunocompetent and immunodeficient mice infected with human-derived *V. botryosa*. None of the aseptically obtained spleen samples yielded *V. botryosa* on culture, regardless of immune status or infection route. Orogastrically inoculated mice did not develop any clinical sign of disease, did not become infected, and did not exhibit any histopathologic changes in any of the analyzed tissues. In addition, 2-way ANOVA did not

show a statistically significant effect of isolate on the inflammatory response scores. However, immune status had a significant effect on inflammation score (mean  $\pm$  SEM), which was higher ( $P = 0.04$ ) in immunocompetent (*nu/+*) mice ( $-6.88 \pm 2.67$ ) than in immunodeficient (*nu/nu*) mice ( $-0.14 \pm 0.08$ ).

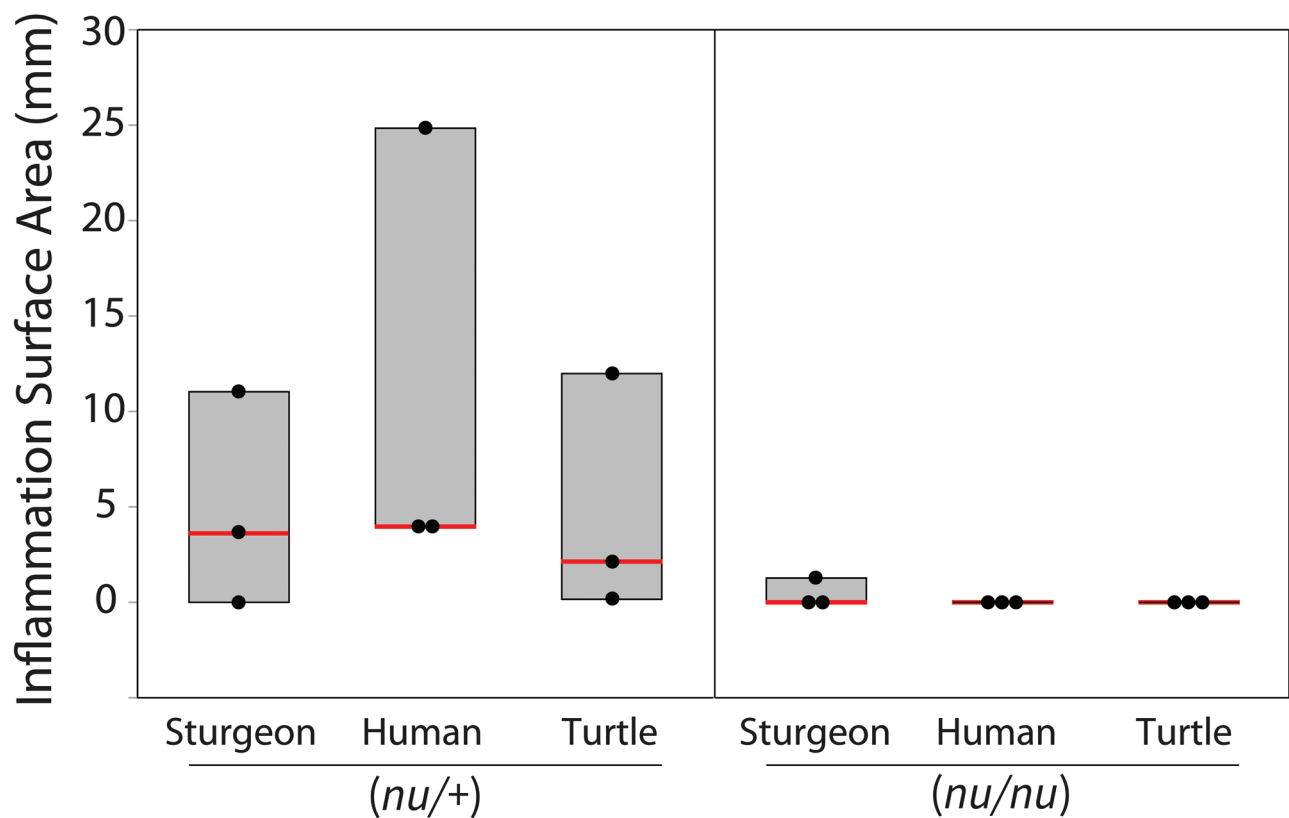
Mock-infected mice inoculated subcutaneously or through gavage did not develop any clinical signs of disease or histopathologic lesions. None of the aseptically obtained spleen samples yielded *V. botryosa* after culture, regardless of the immune status of the mouse or infection route.

## Discussion

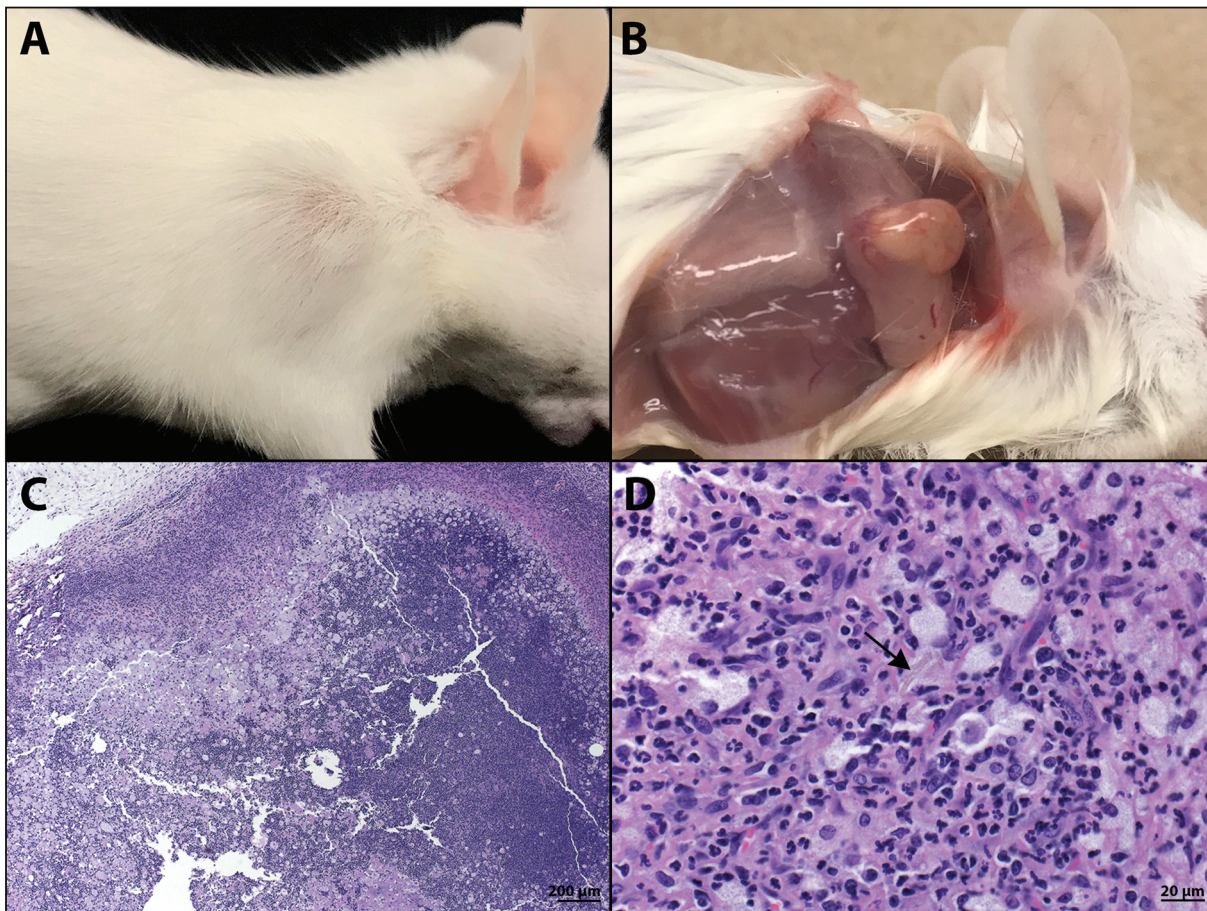
The present study aimed to develop a mammalian model of *V. botryosa* phaeohyphomycosis that could be used to investigate the consequence of inoculation route, host immune status, and virulence of 3 different *V. botryosa* strains. Survival did not differ between fungal strains, routes of inoculation, or host immunocompetence. Fungal infection occurred in subcutaneously inoculated mice only and was localized to the inoculation site; no differences in fungal burden between strains or according to immune status were observed. No evidence of fungal dissemination was observed in any of the treatment groups, and only inflammation surface area was significantly correlated with mouse strain and immunocompetence. Thus, this novel mouse



**Figure 2.** Pathogen burden (score) at the subcutaneous inoculation site of immunocompetent (*nu/+*) and immunodeficient (*nu/nu*) mice at 30 d after inoculation with 1 of 3 *V. botryosa* isolates. Each red line represents the median of the box plot.



**Figure 3.** Inflammatory reaction (affected surface area in millimeters) at the inoculation site of immunocompetent (*nu/+*) and immunodeficient (*nu/nu*) mice at 30 d after receiving a subcutaneous injection of 1 of 3 *V. botryosa* isolates. Each red line represents the median of the box plot.



**Figure 4.** Focal phaeohyphomycosis in an immune competent mouse inoculated subcutaneously with human-derived *V. botryosa*. An injection site swelling, as seen (A) externally and (B) with the skin reflected back, was (C) characterized histopathologically as chronic pygranulomatous cellulitis and myositis. Hematoxylin and eosin stain; magnification, 40 $\times$ . (D) The inflammatory reaction was composed of central necrosuppurative debris with a peripheral rim of foamy macrophages admixed with small numbers of pigmented hyphae (arrow). Hematoxylin and eosin stain; magnification, 400 $\times$ .

model of *V. botryosa* phaeohyphomycosis requires subcutaneous inoculation, does not require the use of immunodeficient mice, and recapitulates the human clinical condition. In addition, genetic variability between *V. botryosa* isolates was not associated with differences in pathogenicity in this murine model of infection.

The relative resistance of the immunodeficient mice to infection by *V. botryosa* was unexpected. Previous studies of phaeohyphomycosis in mice successfully inoculated *Cladosporium trichoides* and *Exophiala dermatitidis*, fungi known to be pathogenic to humans, intravenously<sup>13,14</sup> and found increased susceptibility among immunocompromised mice (*nu/nu*). T-cell-mediated cellular immunity, an immunologic feature absent in *nu/nu* mice, was speculated to play a role in clearing fungal diseases.<sup>13</sup> In the current study, the limitation of small sample size combined with data collection at a single time point in disease progression likely contributed to the apparent relative resistance among *nu/nu* mice. Not surprisingly, inflammation surface area was significantly greater among immunocompetent mice, regardless of *V. botryosa* isolate origin, thus highlighting the susceptibility of immunocompetent mice. Importantly, immunocompetent mice appear sufficient for modeling *V. botryosa* infection and potentially other waterborne pathogens, a feature is beneficial to decreasing research costs and total animal numbers. Future studies exploring innate and adaptive immune

responses to *V. botryosa* may be useful in elucidating the relative resistance to infection seen in the current study.

Affected mice presented similar subcutaneous manifestations as those documented in naturally infected humans.<sup>2,22</sup> Lesions in both immunocompetent and immunodeficient mice developed gradually and became indurated. Mouse lesions were variably noted to be darkly pigmented, consistent with findings in human cases.<sup>12,17</sup> Likewise, granulomatous inflammation and observation of few to many moniliform, pigmented, fungal hyphae within lesions were consistent with human cases of phaeohyphomycosis involving *V. botryosa*. Future studies could use the described mouse model to study the prevention, pathogenesis, and treatment options—such as antifungals and thermo- and cryotherapies<sup>16,22</sup>—for use in human cases.<sup>3</sup>

Other dematiaceous fungi have been speculated to be transmitted by ingestion;<sup>2,9</sup> however, orogastric inoculation with *V. botryosa* failed to produce clinical signs of disease, failed to cause infection, and did not result in any histopathologic lesions, regardless of isolate or host immune status. The high core body temperature of mice (36.5 to 38 °C) compared with poikilotherms likely inhibits the growth and dissemination of this fungus. Previous reports have shown that fish isolates fail to grow at increased temperature (35 °C),<sup>19</sup> and although one group of researchers was able to grow a human-derived isolate of *V. botryosa* at 35 °C, the organism failed to grow at 40 °C.<sup>20</sup> Even

immunodeficient mice inoculated with biologically implausible levels of *V. botryosa* did not experience systemic disease or mortality, as seen in naturally infected poikilotherms. Given these results, ingestion appears to be an ineffective method of transmission in mammals and thus decreases the zoonotic risk of *V. botryosa* transmission through the ingestion of aquaculture products.

In conclusion, this novel mouse model of *V. botryosa* phaeohyphomycosis represents the human clinical condition of cutaneous or subcutaneous infection. *V. botryosa* strain genotype is not associated with pathogenicity in mammalian infection. Transmission of *V. botryosa* through ingestion appears to be unlikely; consequently aquaculture products represent low zoonotic risk. In addition, the methods that we describe herein could easily be applied to other emergent pathogens of aquatic animals and used to study their zoonotic or transmission potential. In addition, this relevant animal model might now be used to study the prevention, pathogenesis, and treatment for human subcutaneous phaeohyphomycosis.

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