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*Case Series*

# Cutaneous and Pulmonary Mucormycosis in Rag1- and Il2rg-deficient Rats

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**Immunodeficient rats are valuable in transplantation studies, but are vulnerable to infection from opportunistic organisms such as fungi. Immunodeficient Rag1- and Il2rg-deficient (RRG) rats housed at our institution presented with dark, proliferative, keratinized dermal growths. Histologic and PCR results indicated that the predominant organism associated with these lesions was fungus from the family *Mucoraceae*, mostly of the genus *Rhizopus*. The *Mucoraceae* family of fungi are environmental saprophytes and are often found in rodent bedding. These fungi can cause invasive opportunistic infections in immunosuppressed humans and animals. We discuss husbandry practices for immunosuppressed rodents with a focus on controlling fungal contaminants.**

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Immunodeficient rodent models are valuable to transplantation studies and may accept immunogenic donor cells or tissues without rejection.[18] Immunodeficient rats are often preferred to mice, as their larger size supports more precise surgical procedures and larger blood and tissue harvests.[18] However, the same immunodeficiency which makes them useful transplantation recipients also makes them susceptible to infection from opportunistic organisms such as fungi. Immunodeficient Rag1- and Il2rg-deficient (RRG) rats housed at our institution presented with dark, proliferative, keratinized dermal growths, consistent with opportunistic and percutaneous invasion of fungi from the family *Mucoraceae* at sites of minor trauma.

RRG rats are a cross between a Rag1-deficient and an Il2rg-deficient line, both on a SD/Crl background.[18] The Rag1-deficient rats have reduced numbers of B and T cells but normal NK cells.[17] Il2rg-deficient rats have severe reductions in their populations of B, T, and NK cells.[18] Therefore, RRG rats have almost no B, T, or NK cells, nor are they able to produce immunoglobulins.[18] RRG rats are able to accept human tumors, human hepatocytes, and skin transplants without rejection.[18]

The *Mucoraceae* are a family of fungi of the order Mucorales, characterized by non- or rarely septate hyphae. These fungi are environmental saprophytes that can cause invasive opportunistic infections in humans and animals.[3,23,24] Pathogenic genera of Mucorales include *Absidia*, *Apophysomyces*, *Mucor*, *Rhizomucor*, *Lichtheimia*, and *Rhizopus*.[2,15] Human patients can contract opportunistic infection with these fungi during immunosuppression, such as from hematologic malignancy, organ transplantation, or diabetes mellitus.[2,6,9,24] *Mucoraceae* can also infect immunocompetent individuals when directly inoculated, as a result of trauma such as needlesticks, stings, bites, or burns.[6,15,24] In humans, mucormycosis (infection with fungi of the order Mucorales) is most commonly rhino-cerebral in presentation, with by pulmonary and cutaneous sequelae.[3,7,24] Cutaneous mucormycosis typically presents with a necrotic eschar.[6,24] Biopsy and culture are recommended, followed by surgical debridement, antifungal therapy such as amphotericin B and azole drugs, and resolution of any predisposing comorbidities.[2,3,6,7,15,24]

Mucormycosis has rarely been reported in veterinary species. *Mucoraceae* have been implicated in infectious keratitis in horses[29] and in pulmonary,[5] cerebral, colonic and splenic lesions.[25] In cats, Mucorales infections have been identified in cases of duodenal perforation,[8] jejunal perforation secondary to intestinal lymphoma,[19] subcutaneous nasal granulomatous disease,[28] and systemic disease.[20]

Spontaneous mucormycosis has not been reported in rodents, although mice[10,12,26,27] and rats[26] have been experimentally infected with this organism. Other types of fungal disease have been reported in both immunocompetent and immunodeficient rats.[4,21] *Pneumocystis carinii* can cause interstitial pneumonia.[21] *Aspergillus fumigatus* or *Aspergillus niger*[4] have been associated with rhinitis[22,23] and tracheobronchitis.[11] *Blastomyces dermatitidis* has been identified as the cause of bronchopneumonia with multifocal pyogranulomas.[4] Dermatophytosis may also occur in rats and is most commonly caused by *Trichophyton mentagrophytes*.[4] Rarely, *Encephalitozoon cuniculi* can infect mice and rats, causing nonsuppurative focal lesions in the brain, kidneys, and liver.[4] In immature rats, phycomycotic meningoencephalitis has also been reported in animals infected with this pathogen.[4]

Here, we describe the presentation and diagnosis of dermal and visceral mucormycosis in an immunocompromised rat strain at our institution. We conclude that the risk of disease from environmental saprophytes to immunocompromised laboratory rodents is significant. Maintenance of these rodents with autoclaved food and bedding was sufficient to prevent recurrence of fungal disease.

## Case Series

In June 2018, 2 naïve 6-mo-old male intact rats developed dark, proliferative, keratinized dermal growths. Rat no. 1 had

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a 1 cm growth on the soft tissue of the left mandible, and porphyrin secretion was evident in his nares (Figure 1 A). Rat no. 2 had a 1 cm lesion behind his right pinna, and a flat, crateriform dermal growth covered with hair on his ventral abdomen (Figure 1 B). The rats were grooming the lesions and appeared uncomfortable when the lesions were palpated.

One week later, 2 female 6-mo-old rats from the same cohort were found with similar growths. Rat no. 3 had a lesion on the right muzzle, ventrolateral to the nose (Figure 1 D), and rat no. 4 had a moist, ulcerative lesion on her dorsal neck behind the right pinna (Figure 1 B, inset). Three unaffected female rats housed in the same cage (rats no. 5 to 7) were moved to a quarantine facility.

In July 2018, 2 of the 3 female cage mates of rats no. 3 and 4 (no. 5 and 6) developed growths. Rat no. 5 had a moist, ulcerative 1.5 cm diameter mass at the base of her tail (Figure 1 E), and rat no. 6 had dry, dermal lesion over her right scapula (Figure 1 C inset). Their cage mate, rat no. 7, had no detectable lesions.

In August 2018, an 8-mo-old male rat (rat no. 8) presented with a dark, crusty dermal growth caudal to his right pinna, and a 0.5 cm diameter red-brown crusty dermal lesion near the left commissure of the mouth. He had porphyrin in both eyes. This rat was euthanized by the laboratory and was not submitted for necropsy.

In October 2018, an adult breeding male (rat no. 9) developed 2 dark dermal plaques on his right side, measuring 1 and 1.5 cm in diameter. The nursing female and pups in the cage did not have lesions.

Rats no. 1 to 7 and 9 were euthanized and submitted for diagnostic necropsy within 24 h of when the lesions were first reported to veterinary staff.

## Materials and Methods

**Animals.** The founders of the RRG colony at our institution consisted of 2 female and 2 male RRG rats that were received in quarantine at our institution from Janvier Labs on July 20, 2017. RRG rats are the property of the TRIP platform, Nantes, France[18] and were maintained under SPF conditions at Janvier Labs. Rats were group housed in static microisolation cages (Allentown, Allentown PA) with corncob bedding (1/8 in., catalog number 7092, Harlan, South Easton, MA). Based on recent vendor reports, all rats were free of rat coronavirus, hantaviruses, mouse adenoviruses, rat parvoviruses, pneumonia virus of mice, reovirus type 3, Sendai virus, Theiler-like virus, *Bordetella bronchiseptica*, cilia-associated respiratory bacillus, *Clostridium piliforme*, *Cornebacterium bovis*, *Corynebacterium kutscheri, Encephalitozoon cuniculi, Helicobacter* species, *Klebsiella oxytoca, Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Actinobacillus* species, *Haemophilus* species, *Mannheimia hemolytica, Pasteurella multocida, Pasteurella pneumotropica, Pasteurella trehalosi, Pneumocystis* species, *Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella* species, *Staphylococcus aureus*, *Streptobacillus moniliformis*, Beta *Streptococcus* species, *Streptococcus pneumoniae*, *Entamoeba* species, flagellates, coccidia, cestodes, nematodes, and ectoparasites.

The rats were prophylactically treated for fur mites (one-part ivermectin 1% solution diluted in 9 parts water and sprayed topically, Noromectin, Norbrook, Overland, KS) and pinworms (fed a diet containing 150 ppm fenbendazole for 5 wk, Envigo Teklad TD.01432, Indianapolis, IN). Serology of contact sentinels after a 3-wk exposure to quarantined rats was negative for rat coronavirus, pneumonia virus of mice, Sendai virus, rat parvovirus, and *Mycoplasma pulmonis*. Examination of cecal and colonic contents of sentinel rats confirmed the absence of pinworms and pelage examination was negative for fur mites. The rats were released from quarantine and transferred to colony housing on August 31, 2017. In colony housing, the 4 founding rats and their subsequent generations of offspring were housed in individually ventilated caging (Allentown, Allentown PA) with unautoclaved corncob bedding (1/8 in., catalog number 7092, Harlan, South Easton, MA) on a 12:12-h light:dark cycle. They received ad libitum unautoclaved rodent chow (diet 2018, EnvigoTeklad). In both quarantine and colony housing facilities, hyperchlorinated water (4 to 6 ppm) was offered ad libitum through the racks' integrated auto-water system. Room temperature and relative humidity were maintained between 22.2 ± 1.1 °C (72 ± 2 °F) and 50% ± 10%, respectively. Rats were maintained in accordance with the Guide for the Care and Use of Laboratory Animals,[13] and on protocols approved by the Yale University IACUC. Yale University is an AAALAC-accredited institution.

**Anatomic pathology.** Gross pathologic examinations were performed on rats #1 to 7 and 9. Systemic histopathology was performed on rats no. 1, 2 and 9; tail, subcutis, and muscle for rat no. 5; and skin and subcutis only for rats no. 3, 4, and 6. After fixation in 10% neutral buffered formalin, all tissues underwent routine paraffin processing, followed by sectioning at 5 µm and staining with Hematoxylin and Eosin (H and E), Periodic Acid Schiff, or Gomori Methenamine Silver (Yale Mouse Research Pathology Core in the Department of Comparative Medicine, Yale School of Medicine; mrp.yale.edu).

**Bacterial and fungal culture.** Bacterial culture was performed on tissues from rats no. 1 and 2. Swabs from lesions were cultured for aerobic bacteria by streaking on blood agar and McConkey agar plates, followed by incubation at 35 °C. Bacteria were identified using Gram staining and standard biochemical techniques.[14,29] Fungal culture was performed on tissues from rats no. 5 to 7, bedding from rat no. 9's cage, unautoclaved bedding, clean autoclaved bedding with no animal exposure, and autoclaved bedding from lesion-free RRG rat cages. Samples were cultured by plating onto potato dextrose agar (Remel Microbiology Products, Lenexa, KS) at 25 °C for 7 to 14 d. The fungal growth was then suspended in filamentous fungi (FF) inoculating fluid and cultivated in 96 well FF-microplate under the same conditions, and read in a microSTATION (Biolog, Hayward, CA). Cultured fungi were also identified by PCR and genetic sequencing.

**Polymerase chain reaction.** Fungal PCR was performed on lesions from all rats. Fungal PCR was also performed on corncob bedding from cages containing a RRG rat with lesions (rat no. 9), RRG rats without lesions, and immunocompetent rats from the same housing room, as well as clean autoclaved and unautoclaved corncob bedding. Masses, skin, liver, lung and tongue were homogenized in minimal essential media (10% w/v) and DNA was extracted from homogenates, hair or bedding using the DNeasy kit (Qiagen, Valencia, CA). Nested fungal PCR panels containing generic fungal primers (FD1/2) and primers specific for Eurotionmycetes/Dothideomycetes (ASPER1/2), Saccharomyceteles (CAND1/2), Mucorales (MUCOR1/2) and Sordariomycetes (FUSAR1/2) were performed as previously described.[29] Negative and positive control samples were included in each assay. PCR products were sequenced by the WK Keck facility at Yale University and sequence homologies were assessed using NCBI Blastn.

## Results

**Gross and Histopathologic analysis.** The spectrum of presenting cutaneous clinical lesions for rats no. 1 to 6 are illustrated in

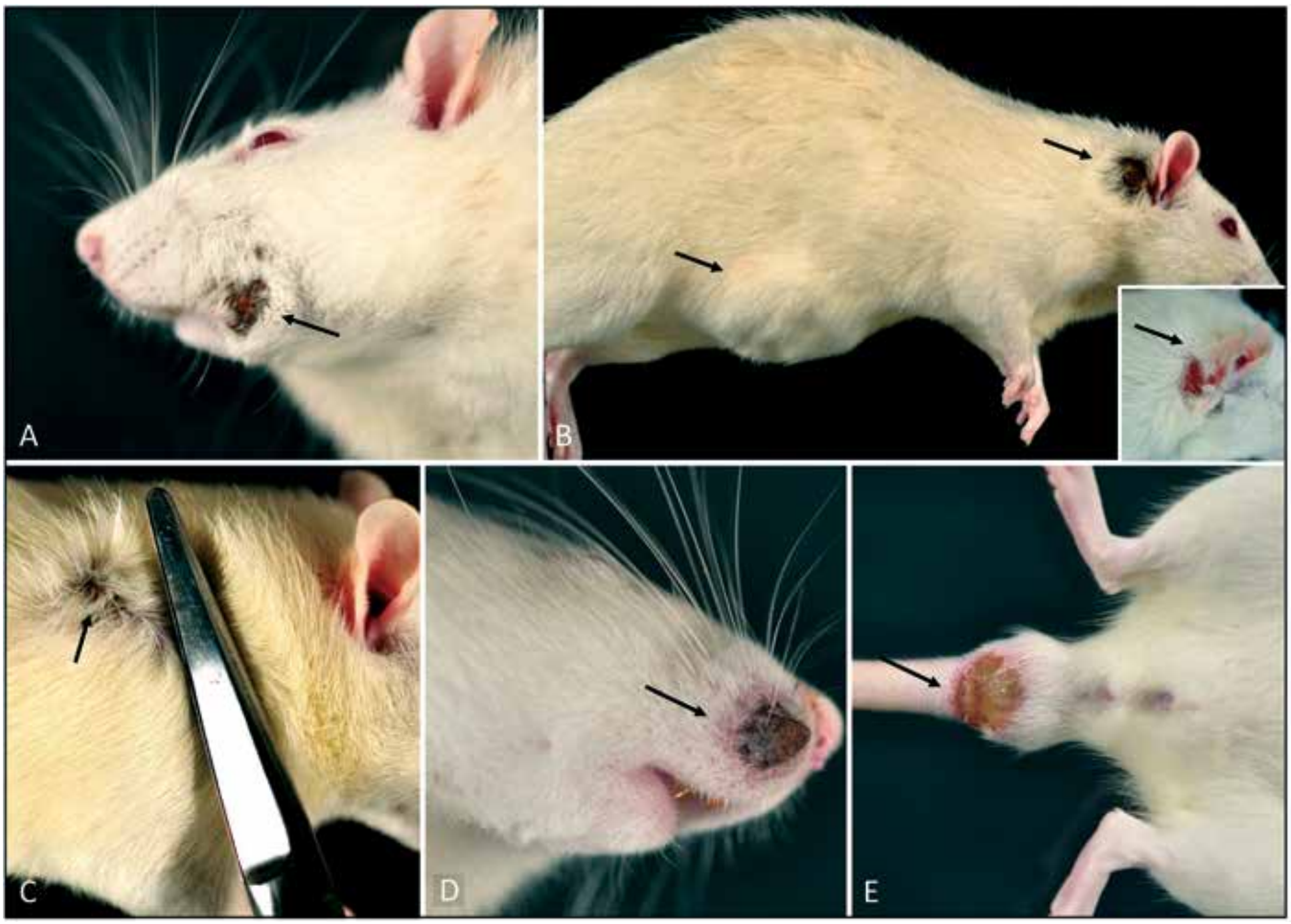


**Figure 1.** Spectrum of clinical presentations. Cutaneous lesions presented most commonly as firm raised ulcerated masses located around the mouth (A, rat no. 1), base of the ears (B, rat no. 2 and inset, rat no. 4) and nose (D, rat no. 3). Less common presentations included firm subcutaneous masses (B, abdominal mass), small flaky lesions (C, rat no. 6) and a proliferative ulcerated lesion around the base of the tail (E, rat no. 5).

Figure 1. Lesions ranged from 4 to 5 cm nodular subcutaneous masses covered by intact skin (Figure 1 B), or most commonly, as 0.5 to 1 cm diameter ulcerated masses that formed horn-like eschars (Figure 1 A, B, and D). In their mildest manifestation, lesions appeared as a superficial crusted or ulcerated lesion, 0.5 cm or smaller (Figure 1 B, and C). Lesions were located in a variety of regions (on the muzzle, behind the ear, over the shoulder, ventral abdomen or tail base) that suggested transmission via grooming or biting.

The typical subgross appearance of the masses (Figure 2 A) was firm, expansile and well-demarcated, with the masses extending into underlying subcutis and muscle. Skin was typically ulcerated. Dark red discoloration of the superficial aspect of the mass overlying a yellowish base extending into deeper tissues was typical. In lesions with visible external ulceration, hyphae were seen histologically in hair follicles and within intraepidermal abscesses and penetrated through epidermis to invade superficial dermis (Figure 2 B). In all cases, a dense mat of fungal hyphae occupied the superficial dermis. Hyphae varied from 5 to 20 microns in width, were rarely septate and had irregularly parallel walls characteristic of the *Mucoraceae*. The dermal tissue reaction was characterized by necrosis, macrophage (including giant cells) and neutrophil dominated infiltrate, and fibroplasia (Figure 2 C). Hyphae were locally invasive and extended into deep dermis, subcutis (Figure 2 D), and muscle. In larger nodular lesions, overlying tissue was necrotic, forming an eschar.

Rats no. 5 and 9 had unique presentations. In the tail skin of rat no. 5 (Figure 1 E), an intensely destructive lesion was characterized by massive infiltration of hyphae (Figure 3 D inset), attendant subcutaneous inflammation and necrosis, and necrosis of vertebrae (Figure 3 D). Circumferential reactive new bone and cartilage formation and ulceration was evident (Figure 3 D). Rat no. 9 presented with gross dermal plaques on his right side, and necropsy revealed the lung almost entirely replaced with necrotic tissue adherent to the pericardium (Figure 3 A). Hyphae extended through the pulmonary tissue into the epicardium (Figure 3 B) and pulmonary vein (Figure 3 C).

Besides those already described, no further lesions were seen in the systemic histopathology for rats no. 1, 2 and 9. Rat no. 7 had normal skin and no histologic lesions were evident.

**Culture and PCR Results.** Bacterial culture yielded *Klebsiella oxytoca* from rat no. 1's left mandibular mass. Rat no. 2's pinna and abdominal masses grew *Staphylococcus xylosus* and *Hafnia alvei*.

Fungal culture detected *Rhizopus microsporus* in rat no. 5's tail mass. No fungal growth was noted in samples from the cutaneous mass from rat no. 6 or the normal skin from rat no. 7.

Fungal PCR detected *Rhizopus oryzae* in masses taken from rats #1 to 6 and 9. *Rhizomucor pusillus* was detected in rat no. 6. *Fusarium* sp. was detected in the masses from rats #3 and 9 and *Cladosporium* sp. was detected in the mass from rat no. 9. *Rhodotorula* sp. were detected in hair from rat no. 9. Rat no. 9 also had detectable *Rhizopus* in the liver and lung.

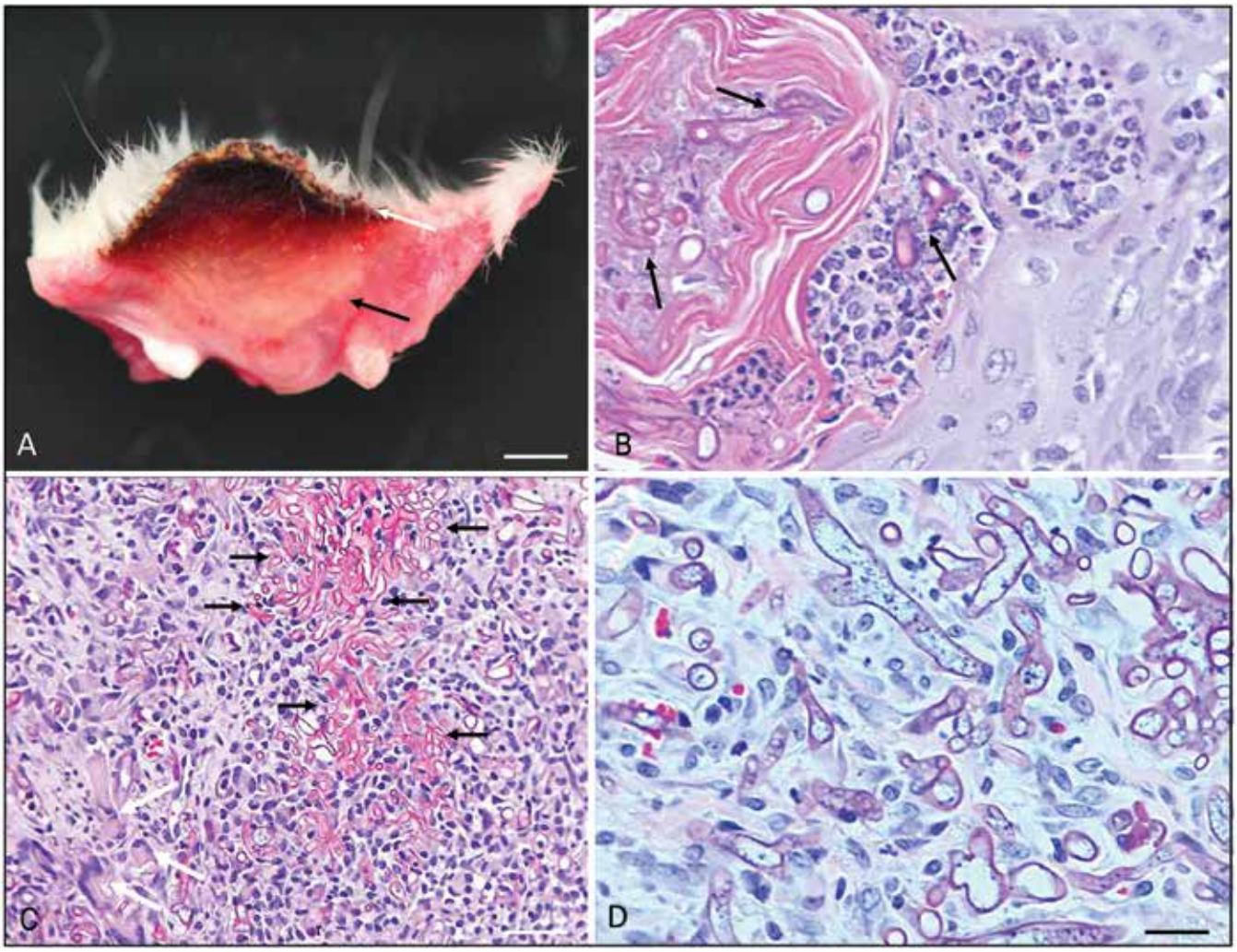


**Figure 2.** Subgross tissue and histopathology of cutaneous disease. (A) Typical subgross appearance is illustrated. A firm, expansile and well demarcated mass extending into underlying subcutis and muscle (black arrow) was evident regardless of whether skin was ulcerated (white arrow) or not. Dark red discoloration of the superficial aspect of the mass was typical. (B) Cutaneous invasion of hyphae through follicular epithelium is evident (arrows) and is accompanied by necrosis and neutrophilic infiltration. (C) Invasive hyphae (black arrows) incite an adjacent mixed inflammatory and giant cell (white arrows) response. (D) Irregular, branching 5-20 µm diameter rarely septate hyphae characteristic of the Mucorales were abundant in subcutis. Hematoxylin and eosin (B), Periodic Acid Schiff (D) Bar =2 mm (A), 50 µm (B), 20 µm (B, D).

*Debaryomyces hansenii* grew in culture from both unautoclaved and autoclaved clean corncob bedding from the facility in which the rats were housed. Used bedding from RRG rats without lesions grew *Debaryomyces*, *Candida*, *Fusarium,* and *Penicillium* sp. Bedding from Rat no. 9's cage was positive by PCR for *Trichocomaceae (Aspergillus, Eurotium or Penicillium sp.*), *Debaryomycetaceae* (*Debaryomyces, Candida or Kurtzmaniella sp.*), *Fusarium sp.* and *Rhizopus orzae* and positive on fungal culture for *Penicillium* sp. Bedding from immunocompetent rats in the same housing room was positive for *Fusarium sp* and *Sarocladium sp.* by PCR, but no other fungal species were detected.

## Discussion

Lesions seen in these 8 RRG rats were consistent with opportunistic and percutaneous invasion of fungi at sites of minor trauma. Histologic and PCR results indicated that the predominant organism associated with the lesions was fungi from the family *Mucoraceae,* mostly of the genus *Rhizopus.* Additional fungi such as *Candida*, *Fusarium*, and *Penicillium* were detected via culture from used unautoclaved bedding from RRG rats, both with and without lesions. *Fusarium sp. and Sarocladium sp.* were also detected via PCR in autoclaved bedding from the cages of immunocompetent rats in the same housing room. After the bedding was autoclaved, *Penicillium* sp. could still be cultured, and *Trichocomaceae (Aspergilllus, Eurotium or Penicillium sp.*), *Debaryomycetaceae* (*Debarymyces candida or Kurtzmaniella sp.*), *Fusarium sp.* and *Rhizopus orzae* were detected by PCR. *Debaryomyces hansenii* was cultured from clean corncob bedding both before and after the bedding was autoclaved.

Environmental saprophytic fungi including the *Mucoraceae* commonly exist on rodent bedding[23] and food,[1,16] especially on the corncob[16,23] bedding used in our facility. For example, we have detected *Rhizopus orzae* by PCR in partially eaten irradiated food in mouse cages. The species that grew in culture were apparently viable, even if the bedding had been autoclaved before culturing. After autoclaving, PCR can continue to detect the DNA in spores even if they are no longer viable and unable to grow in culture.[23] Because *Penicillium* sp. and *Debaryomyces hansenii* were cultured from autoclaved bedding, our results suggest that viable fungal spores remained in corncob bedding even after autoclaving. Our autoclave cycles were designed to eliminate excluded pathogens likely to be present in bedding and food (for example MPV) while keeping cycle times

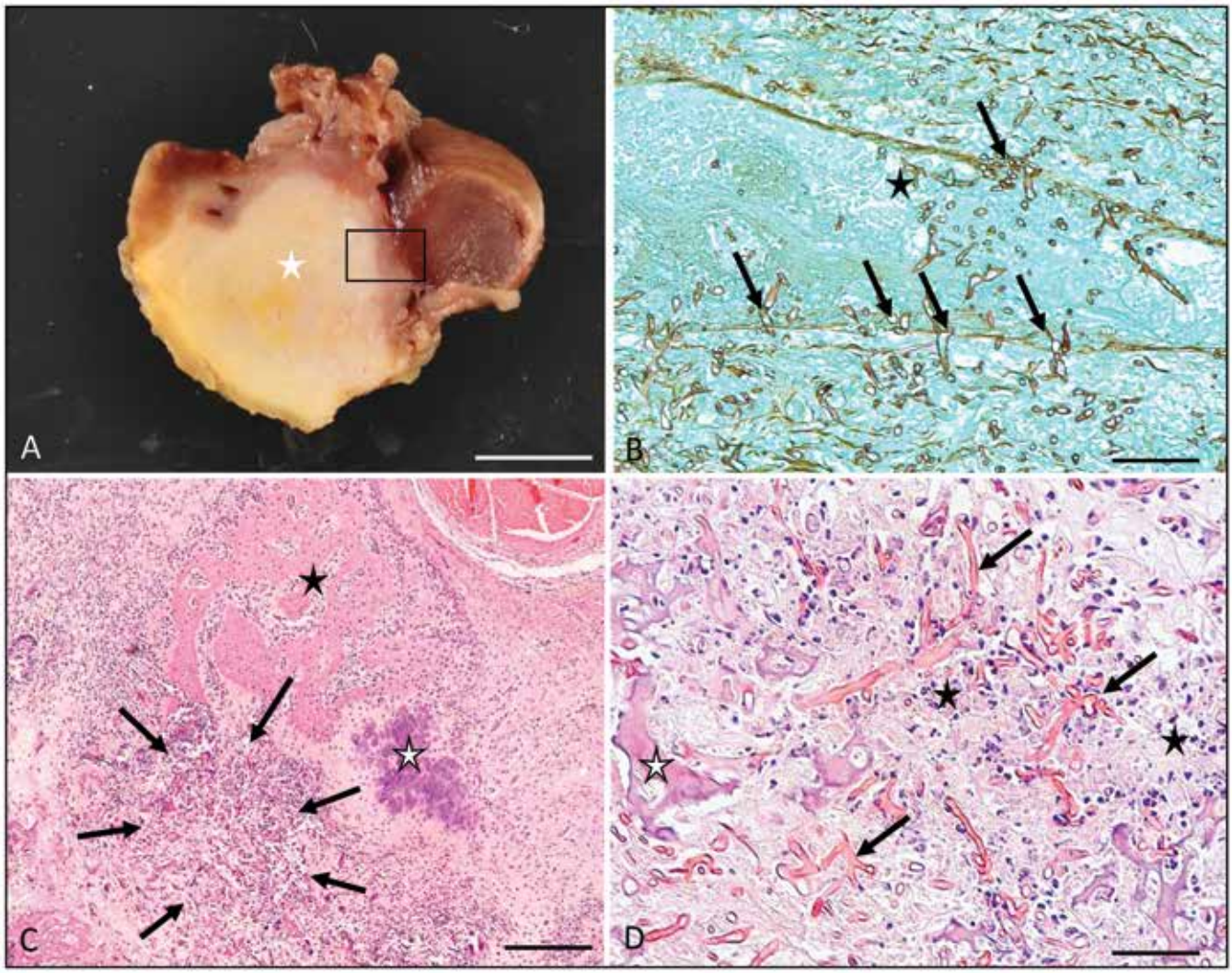


**Figure 3.** Subgross tissue and histopathology of invasive fungal disease. (A) Subgross appearance of lung and heart is shown. Lung is almost entirely replaced with necrotic tissue (star) adherent to the pericardium. (B) A pulmonary vein contains fungal hyphae that invade its wall (arrows) and infiltrate the surrounding parenchyma. (C) Tail lesion. Islands of new bone (black star) and cartilage formation (white star) are evident, adjacent to masses of fungal hyphae (arrows). (D) Tail lesion. Fungal hyphae (arrows) are admixed with necrotic debris (black star) and newly formed bone (white star). Hematoxylin and eosin (C, D), Gomori Methenamine Silver (B) Bar =2 cm (A), 200 µm (C), 100 µm (B), 50 µm (D).

to a minimum to accommodate throughput and save energy. As such, we do not use a full sterilization cycle for autoclaving standard caging prior to use, although a full cycle would be used to process biohazardous waste. Our findings indicate that a full sterilization cycle may be necessary to completely eliminate viable fungal spores from bedding.

All mice and immunodeficient rats at our institution have typically been housed using autoclaved caging, food, and bedding. Our room entry order mandates that immunocompromised animals are handled before immunocompetent animals. Although some institutions house immunodeficient animals in isolators, that practice is not feasible at our institution due to space constraints and the high cost for investigators. However, in general, our standard level of decontamination has been adequate for maintaining the SPF status and health of most rodents, even those with severe immune deficiencies.

In the current case, these rats were initially housed with unautoclaved food and bedding due to an error. The RRG rats were among the few immunocompromised rat strains housed at our institution and the only immunocompromised strain in their home facility. Although husbandry staff are trained to house immunocompromised mice in autoclaved caging, these rats had not been clearly flagged as immunocompromised. To address this error and avoid future problems, all rats in our facility were switched to autoclaved cages, corncob bedding, and food (Envigo 2018S) in August 2018. Currently we are using our standard sterilization cycle which is slightly shorter than a full sterilization cycle. If this cycle at any point proves ineffective in accomplishing our goal of keeping microbial burden sufficiently low to prevent clinical infections, we will consider extending it. No cases of mucormycosis have been detected in the colony from August until October. Most likely, rat no. 9 was infected before husbandry changes, and clinical lesions did not appear for several months. Since rat no. 9 was culled from the colony in October of 2018, we have seen no evidence of mucormycosis or other opportunistic fungal infections in these animals.

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