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Evaluation of Pathogenesis and Immune Response to *Coccidioides posadasii* Infection in U.S. Feral Swine (*Sus scrofa*)

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Abstract: The dimorphic, soil-dwelling fungus *Coccidioides* is the causative agent of coccidioidomycosis and is a reemerging pathogen of human health concern. Despite increasing incidence and isolations of the organism outside of its theoretical environmental range, much of *Coccidioides* epidemiology, including complete host range, remains unknown. Feral swine (*Sus scrofa*) are an invasive species distributed throughout the United States that may play a role in fungal ecology due to their behavioral associations with soil. To address this, we evaluated seven feral swine for susceptibility to *Coccidioides*. After intranasal inoculation with $10^{6.2}$ arthroconidia of *C. posadasii*, no pigs displayed signs of clinical disease, nor did any exhibit antibodies by AGID testing. Histopathologic evaluation did not reveal the presence granulomatous lesions, and fungal spherules or endospores were not observed in any of the tissue sections examined, although individuals had significant comorbidities, most notably *Metastrongylus* nematodes in the lungs. Despite the absence of lesions and organisms histologically, *C. posadasii* was isolated by culture from the lung and mediastinal lymph node of two pigs, indicating active infection. These results suggest that feral swine are mildly susceptible to acute *Coccidioides* infection and may aid in fungal dispersal due to the presence of spherules in tissues post-mortem.

Keywords: *Coccidioides*; coccidioidomycosis; Valley Fever; feral swine; *Sus scrofa*; experimental inoculation; fungal ecology

1. Introduction

Coccidioides immitis and *posadasii* are largely indistinguishable pathogenic fungi and are the causative agents of coccidioidomycosis, or Valley Fever, a potentially deadly disease in humans [1]. *Coccidioides spp*. primarily rest in soil profiles, with fungal spores or arthroconidia infecting hosts chiefly through the respiratory route. This mode of transmission is thought to be driven primarily by soil disruption and aerosolization of infectious arthroconidia, with large-scale fungal dissemination

mediated by extreme weather events like dust storms [2]. Based on the seminal work performed by Edwards and Palmer in the 1950s [3,4], it's believed that in the United States, *Coccidioides* is endemic to the Central Valley of California, southern Arizona, and western Texas, and that desert rodents and armadillos likely play a role in fungal ecology by providing organic materials within their burrows, as well as being incidental hosts themselves [5,-8].

Reports and isolations of *Coccidioides* have been made outside of the defined endemic region within the last decade, suggesting that the organism is expanding geographically, yet despite this, an animal reservoir has not been definitively identified [3,9,10]. Ongoing study of *Coccidioides* distribution and ecology has proven difficult, as distribution within soil profiles is not homogenous even in large regions considered disease "hot spots" based on human incidence, and coccidioidomycosis is not a reportable disease in all states [9,11,12]. Furthermore, although attempts have been made to better define environmental suitability, resulting evidence has been inconclusive, and reliable isolation of *Coccidioides* from the environment remains a challenge [9,12-14].

Besides humans, *Coccidioides* appears able to infect most vertebrates with varying levels of disease [15]. Experimental infection studies in wild rodents including Merriam's kangaroo rat (*Dipodomys merriami*) and the desert pocket mouse (*Chaetodipus penicillatus*) have revealed they may be infected but rarely die from coccidioidomycosis, and serological studies have detected antibodies in the serum of kangaroo rats, desert woodrats (*Neotoma lepida*) and deer mice (*Peromyscus maniculatus*), suggesting many desert rodents are potential reservoirs or incidental hosts [3,6]. It has thus been broadly hypothesized that burrowing mammals play a role in fungal ecology due to their association and close interactions with soils that may be contaminated with fungal hyphae [5,6,9,13]. Similarly, infection in domestic dogs is thought to be more prevalent than in humans because of their behavioral tendency to disrupt soil [16,17].

Observational studies in several livestock species suggest that clinical infection is rare in horses, cattle, sheep, and swine. *Coccidioides*-associated lesions, when observed, are incidentally detected during slaughter or meat processing, and likely do not reflect true incidence [17-20]. Limited reports for domestic swine indicate that they are susceptible to *Coccidioides,* but rarely develop clinical signs or succumb to disseminated disease, and past serological surveys have suggested that at least 12% of domestic swine in some endemic regions can be positive for *Coccidioides* antibodies [17,18]. Like canids, pigs have behavioral tendencies to disrupt soil, such as rooting for food or wallowing for thermoregulation [21]. Despite this, susceptibility and transmission dynamics in pigs have yet to be fully elucidated.

Feral swine (*Sus scrofa*) are a proliferative invasive species with widespread distribution across the United States and are known for their disruption of soil through rooting and wallowing [22,23]. Taxonomically identical to domestic swine, feral swine have yet to be considered for their potential role in *Coccidioides* ecology and epidemiology, despite population overlap with regions exhibiting high and low incidence of Valley Fever [24,25]. We thus evaluated a group of feral swine for susceptibility to *C. posadasii* and documented clinical signs, serological response, and fungal presence in tissues after intranasal exposure.

2. Materials and Methods

2.1. Animals

Seven wild-caught juvenile feral swine of mixed-sex were evaluated for susceptibility to *C. posadasii*. Feral swine sounders were trapped by the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (USDA) personnel in Burnet County, Texas using baited corral traps from April 25-27, 2021. Juvenile swine were preferentially selected from sounders to minimize the chance of previous fungal exposure and were defined as less than 2 months of age, where age was estimated based on the absence of incisors and permanent canines on the lower jaw [26]. Upon capture, seven individuals were selected based on these criteria and transported overnight to Fort Collins, Colorado. Animals were then group-housed in an Animal Biosafety Level 3 (ABSL-3) facility at Colorado State University, in one room (12 ft. x 18 ft) with natural lighting and

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controlled climate. Once in the ABSL-3, swine had access to food and water ad libitum throughout the study period.

Animals were allowed to acclimate for six days before being given a thorough physical examination, where an initial blood sample was taken, and individuals screened externally for ectoparasites, sexed, and implanted with thermally sensitive microchips (Lifechips, Destron-Fearing) for identification and temperature measurement. Due to significant numbers of ectoparasites observed on arrival, a thin application of deltamethrin insecticide (Delta Dust, Bayer) was also applied to the coat of each pig during the initial exam. All procedures and use of animals were performed with the approval of Colorado State University's Institutional Animal Care and Use Committee (Protocol #1360).

2.2. Fungal Challenge

Prior to inoculation, baseline body temperatures were recorded for all individuals (day 0). Wild-type *C. posadasii* strain Silveira spores diluted in phosphate-buffered saline (PBS) were then administered into the nares of six individuals using a pipette (500ul per nare) under manual restraint. One individual was not inoculated and left as a contact control. Immediately following animal challenge, back titration was performed on 1 X GYE medium (1% glucose, 0.5% yeast extract, and 1.5% agar) and confirmed that animals received $10^{6.2}$ arthroconidia of *C. posadasii*.

2.3. Clinical Observations

All animals were observed daily and included an assessment of temperament and the presence or absence of clinical signs of disease, including but not limited to ocular and nasal discharge, ptyalism, coughing/sneezing, dyspnea, diarrhea, lethargy, anorexia, and moribund status. Body temperature was recorded starting the day prior to challenge and continued to seven days post-inoculation (DPI); after day seven temperature was measured again at 14, 28 and 42 DPI. In addition to monitoring for overt clinical signs, we also monitored behavior by observing animals behind double-paned glass, assessed food intake as well as response to provided enrichment, including playing with toys and consuming treats.

2.4. Sampling

We evaluated feral swine for acute pathologic changes, fungal dissemination in tissues, and serological immune response after *Coccidioides* exposure. Blood samples (3-5 ml into serum separator tubes) were collected from all individuals on 14-, 28-, and 42-DPI. At each timepoint two animals were also euthanized and tissues harvested for fungal culture and formalin fixation. The exception to this process was 42 DPI, where three individuals were euthanized; this included the last two inoculated pigs as well as the uninoculated contact control. Blood was collected from the anterior vena cava vein under manual restraint, and animals that were euthanized were sedated with 1-2 mg/kg xylazine and 15-20 mg/kg ketamine before being removed from the room and euthanized by captive bolt. During necropsy, approximately 100 mg samples of lung, liver, spleen, and mediastinal lymph node tissue were collected in 1 ml of PBS with two stainless steel balls for fungal culture. Approximately 1-5 g of the same tissues, in addition to kidney, pancreas, small intestine, and heart were collected in 10% neutral-buffered formalin for histopathology.

2.5. Serology

Serum was extracted from all blood samples within two hours of collection and passed through a 0.22 μm filter before being taken out of the ABSL-3 and stored at -80°C. Sera were shipped to the Veterinary Medical Diagnostic Laboratory in Missouri, where they were evaluated for the presence of *Coccidioides* antibodies by porcine agar gel immunodiffusion assay (AGID).

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2.6. Fungal Culture

All manipulations of viable cultures and tissue specimens were performed within the ABSL-3. Tissues collected for culture were homogenized using a Qiagen Tissuelyser at 25 cps for 5 minutes and 100µl of homogenate was plated onto 1 X GYE medium supplemented with selective fungicide and antibiotics (1% glucose, 0.5% yeast extract, 1.5% agar, and 50µg/ml each of cycloheximide, chloramphenicol, and streptomycin). Plates were allowed to incubate for a maximum of 1 week at 37°C before any suspicious fungal growths were harvested into 1 ml of PBS and stored at -80°C pending DNA extraction and PCR.

2.7. DNA Extraction

DNA extraction was achieved by placing approximately 1cm^2 of mycelia in a 2 ml screw-cap tube containing 0.5 mm diameter acid-washed glass beads (Fisher Scientific, #501461310) and 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8.0], 100 mM NaCl, 0.5% sodium dodecyl sulfate, and 50 mM dithiothreitol) and subjected to mechanical disruption by vortexing on a Qiagen Tissuelyser at 25 cps for 5 minutes. Samples were then incubated at 65°C for a minimum of 30 minutes before being centrifuged at 10,000 rpm for 2 minutes. Nucleic acids were extracted from the supernatant with buffered phenol:chloroform:isoamyl alcohol pH 8.0 (25:24:1) and again with cholorform:isoamyl (24:1) and precipitated from the aqueous layer with 0.1 volume of 0.2 mM NaCl and 0.1 volume of ethanol. The resulting suspension was then centrifuged at 14,000 rpm for 10 minutes, washed again with ethanol, re-suspended in 300 µL of TE buffer (10 mM Tris-HCl [pH 7.6], 1mM EDTA [pH 8]), with 100ug/mL RNAse A (Thermo Fisher, #EN0531), and incubated at 37°C for 30 minutes to degrade extraneous RNA. DNA of interest was then precipitated out with the addition of 300µl of 5M LiCl followed by a 10-minute incubation on ice, before being centrifuged for 14,000 rpm for 10 minutes. Supernatants were then transferred to 1.2 ml of ethanol and incubated on ice for an additional 10 minutes and centrifuged one final time at 14,000 rpm for 10 minutes. The resulting pellet was then washed with 70% ethanol and allowed to air dry before being suspended in 100µl of TE buffer. Final suspensions were stored at -20°C until analysis by PCR.

2.8. PCR

All suspicious fungal growths isolated from tissues collected at necropsy were confirmed by conventional PCR, using primers targeting the ITS region of *Coccidioides* described previously [27]. Singleplex PCR was performed using Invitrogen PCR Supermix (Cat. #10572014), according to the manufacturer's instructions. Briefly, 50µl reactions consisting of 0.2µM of the ITS primer set and 1µl template DNA was used. The PCR program consisted of initial denaturation at 94°C for 2 minutes, followed by 35 cycles each of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, extension at 72°C for 1 minute, and one cycle of final extension at 72°C for 7 minutes. Amplification products as well as 1-kilobase ladder (Invitrogen, #10787018) were then loaded onto a 1% agarose stained with ethidium bromide and electrophoresed for 1 hour at 100 V. Band presence at 223 bp was considered positive for *C. posadasii* when viewed under UV light.

2.9. Histopathology

Tissues collected for histopathology were fixed in 10% neutral-buffered formalin for 15 days before being transferred to 70% ethanol. Tissues were then processed for paraffin embedding and sectioned for staining with hematoxylin and eosin. All slides were interpreted by a veterinary pathologist blinded to animal treatments and history.

3. Results

None of the feral swine exhibited outward clinical signs of disease and maintained stable body temperatures throughout the study period. None of the animals displayed abnormal behavior compared to the acclimation period. Furthermore, AGID testing of sera collected prior to inoculation, as well as on 14, 28, and 42 DPI revealed that none of the pigs were positive for *Coccidioides* antibodies

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prior to inoculation, nor did any seroconvert after experiencing either a primary or (regarding the contact control) secondary exposure event.

Animal demographics, day of termination, and the results of culture and subsequent colony PCR, as well as a summary of histologic findings are depicted in Table 1. During necropsy, four pigs (#3916, 7639, 7652, and 7670) displayed grossly noticeable congestion in the lungs, with three of four animals exhibiting regions of congestion diffusely across all lung lobes, and one (#7670) with congestion localized only to the lower respiratory tract in both caudal lobes. No additional gross lesions were observed in any other tissues. Culture of tissue homogenates resulted in four suspect colonies being harvested from the right middle lung lobe of one pig (#7639) and two colonies each from the mediastinal lymph node of two additional pigs (#7670 and 7648). Analysis by PCR however revealed that only those colonies harvested from pigs 7648 and 7639 were *Coccidioides*. Histopathology did not reveal the presence of fungal spherules or endospores in any tissues, yet inflammatory processes were observed in most tissue types for all animals (Table 1). Most commonly, interstitial pneumonia, lympho-histiocytic inflammation, lympholysis, and alveolar edema were observed in the lungs. Notably, *Metastrongylus* nematodes were also detected in the lung sections of three individuals (#3916, 7648, and 7670). Due to inadequate sample collection at necropsy, lung tissue was only cultured and not examined by histopathology for pig 7632 (Table 1).

Table 1. Results of fungal culture, colony PCR, and summary of histologic findings for feral swine inoculated with *C. posadasii.*

Animal ID	Sex	Treatment[1]	Necropsy Day (DPI)	Fungal Culture (Number of Colonies/100mg of Tissue Type)[2]	Colony PCR (+/-)[3]	Histologic Findings
7652	F	Infected	14	--	NA	Interstitial and perivascular lympho-histiocytic inflammation in lungs. Perivascular lympho-histiocytic inflammation in heart and liver. Lympholysis in spleen.
7670	F	Infected	14	2/Mediastinal Lymph Node	−	*Metastrongylus* nematodes in lungs. Alveolar edema, interstitial pneumonia, bronchitis, and BALT hyperplasia with lympholysis in lungs. Mild hyperplastic arteriosclerosis and scattered individual hepatocellular necrosis in heart.
7620	M	Infected	28	--	NA	Interstitial and perivascular lympho-histiocytic inflammation in lungs, liver, and heart. Lympholysis in spleen and lymph node. Granulomatous lymphoid hyperplasia in lymph node.
7632	M	Infected	28	--	NA	Lungs not examined. Lympholysis and resorbed neutrophils, and mild edema in lymph node. Lympholysis in spleen. Interstitial edema in heart.
3916	F	Uninfected	42	--	NA	*Metastrongylus* nematodes in lungs. Interstitial pneumonia, lympho-histiocytic inflammation, alveolar and interstitial hemorrhage and edema in lungs. Lympho-histiocytic inflammation in

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						kidney and spleen. Myocardial interstitial edema in heart.
7648	F	Infected	42	2/Mediastinal Lymph Node	+	*Metastrongylus* nematodes in lungs. Interstitial pneumonia and bronchitis and alveolar edema in lungs. Lympho-histiocytic inflammation in lung and liver. Lympholysis in spleen and kidney.
7639	M	Infected	42	4/Right Middle Lung	+	Interstitial pneumonia, alveolar edema, and lympho-histiocytic inflammation in lungs. Lympholysis in spleen. Interstitial edema in heart.

[1]"Uninfected" denotes contact-control. [2] Fungal culture results are expressed as number of colonies harvested per 100 mg of tissue type indicated; "--" denotes no colonies observed in any tissues examined. [3] A positive (+) colony PCR indicates confirmation of colonies as *C. posadasii* by conventional PCR; NA: Not Applicable.

4. Discussion

After intranasal exposure with $10^{6.2}$ arthroconidia of *C. posadasii,* feral swine, as suggested for their domestic relatives, appear resistant to developing and succumbing to acute coccidioidomycosis [17,18]. Despite lack of overt clinical signs and the absence of significant lesions at necropsy, *C. posadasii* was cultured from tissues of two pigs, indicating that active infection was achieved. That infection was only detected in two exposed animals, however, was somewhat surprising given the substantial dose of the inoculum. This low infection rate may be due to the route of inoculation used; by nature of instilling PBS-suspended spores into the nasal cavity, some inoculum could have passed into the oropharynx and been swallowed rather than inhaled into the respiratory tract. Alternatively, it is possible that infection was simply missed by sampling insufficiency, as only a small amount of tissue was cultured, and the organism is not always easy to detect nor evenly distributed in organs [5,28]. The route of inoculation likely mimics a natural exposure event from feral swine rooting within contaminated soil and inadvertently inhaling infectious materials [29]. We additionally examined the likelihood of animal-to-animal transmission by leaving one individual uninoculated, but group-housed with exposed animals, as swine are very gregarious, often making nose-to-nose contact with littermates or other members of their sounder [21]. Despite regular nose-to-nose contact between our animals throughout the study period, we did not detect any signs of *Coccidioides* infection in the contact control, suggesting animal-to animal transmission after intranasal exposure is unlikely in feral swine. This is unsurprising given that coccidioidomycosis is not considered contagious, and transmission events between vertebrates are extremely rare [18,30,31].

Interestingly, none of the pigs developed antibodies to *Coccidioides* as assayed by AGID following inoculation. Lack of seroconversion in animals without associated active infection is not altogether surprising, however no marked change in the serology of the two culture positive pigs through 42 DPI is somewhat puzzling. These results, however, may be explained by the challenges associated with *Coccidioides* serology. While AGID is highly specific and remains the gold standard serological test for both humans and animals [17,32], its relatively low sensitivity can be problematic when testing the serum from individuals with self-limiting infections or those who are immunocompromised [33-35]. Indeed, as seen with many canine coccidioidomycosis cases, dogs with regular or chronic clinical symptoms can be serologically negative by AGID [33,36]. Given that all study pigs displayed general inflammation across organ systems, seroconversion as well as subsequent serology may have been confounded to some degree by the occurrence of coinfection in some or all of the pigs, particularly those coinfected with detectable parasites [37-39]. Alternatively, the lack of seroconversion as well as fungal bodies in tissues examined histologically may be due in part to the relatively short duration of our study. Primary pulmonary infections in humans are typically symptomatic between one and four weeks after exposure, while disseminated disease can occur months or years later [18]. While the timeline for pathogenesis in pigs may not be the same,

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follow-up studies will be required to further assess the susceptibility and transmission potential of feral swine experiencing chronic infection with *Coccidioides,* or results of infection past the 42-day period examined here.

We inoculated all study pigs with wild-type *C. posadasii* spores, despite *C. immitis* also known to be present in regions of the United States. It's currently thought that the largest difference between the two species is their relative geographical distribution, with *C. immitis* primarily occurring in Central and Southern California, and *C. posadasii* in the desert regions of Nevada, Arizona, New Mexico, and West Texas [13]. Otherwise, *C. immitis* and *C. posadasii* are morphologically identical, and no clinical differences have been identified between species to date; however, no investigations have assessed phenotypic variation in the context of pathogenicity [13,16]. Additional experiments will therefore be necessary to determine if any differences exist in infectivity and transmission dynamics in suspected reservoir species.

Lastly, all individuals appeared to have considerable comorbidities; notably, half of inoculated individuals possessed nematodes within the bronchioles of the lungs, and all animals exhibited generalized inflammation within most tissues. While nematodes were not detected in the tissue sections of all animals, the systemic inflammation observed in others is more likely indicative of a parasitic infection, rather than disseminated coccidioidomycosis [5,40-43]. The fact that all animals also exhibited similar pathology may be due to pigs used here originating from the same geographic area, and thus experiencing similar environmental exposures, or direct transmission from infected individuals, either while grouped together while in transportation to Fort Collins or while being group-housed for the duration of the study [44,45].

While it is largely accepted that burrowing mammals contribute to *Coccidioides* ecology because of their interactions with soils that may contain fungal hyphae, the role that additional species play in fungal persistence and spread is underexplored. Moreover, increases in both human coccidioidomycosis cases and isolation of *Coccidioides spp*. from soils outside of historically defined endemic regions have implied that the metrics used to determine endemicity and environmental suitability are outdated [9,46]. Feral swine, like desert rodents, are behaviorally drawn to soil and are additionally known for their opportunistic interactions with humans, domestic animals, and native wildlife, which in some instances can have implications for the transmission of pathogens and disease [23,45]. In the case of *Coccidioides*, we postulate that feral swine, like rodents and armadillos, may be reservoirs and disseminators of the fungus on the landscape, particularly if they experience infection and expire with viable spherules within their tissues. This mode of fungal dissemination could prove significant, as others have proposed that animal carcasses can serve as mediums for the growth of *Coccidioides* mycelia in the soil [47-50]. Based on viable *Coccidioides* being cultured from the tissues of two study pigs post-mortem, feral swine carcasses may therefore pose a risk to human health, particularly for wildlife managers removing feral swine for invasive species management and hunters, who may regularly manipulate feral swine carcasses [51,52]. This risk could also extend to other wildlife or domestic animals who may scavenge or inspect infected carcasses and come into direct contact with infectious material [47].

Coccidioidomycosis is a potentially severe disease and its causative agent an understudied pathogen [53]. Knowledge gaps concerning *Coccidioides* environmental requirements, host range, and epidemiology currently make mediation of human infection difficult. Additionally, it is likely that changing climates, increased urbanization, and spread of invasive species may be modifying *Coccidioides* epidemiological patterns and increasing the risk of human disease [5,11]. The expansion of invasive species susceptible to and capable of disseminating *Coccidioides* post-mortem such as feral swine may contribute to fungal ecology and should not be overlooked as potential sources for environmental contamination of *Coccidioides spp.* in the United States, particularly in regions where the pathogen is emerging.

5. Conclusions

After intranasal exposure to $10^{6.2}$ arthroconidia *C. posadasii*, feral swine appear mildly susceptible to infection, although resistant to acute disease, and could potentially serve as a reservoir species for *Coccidioides*. Lack of overt clinical signs as well as gross lesions in internal tissues may allow active *Coccidioides* infection to go unnoticed by wildlife managers or hunters that may be manipulating feral swine carcasses in the field and could facilitate secondary human exposure. Furthermore, death of feral swine with viable spherules or endospores present in tissues may also contribute to fungal dissemination and establishment of *Coccidioides spp*. into novel environments by providing a starting growth medium for fungal establishment into soils. Movement of feral swine from known endemic to non-endemic *Coccidioides* environments could therefore pose a threat to more susceptible vertebrate species, including humans.

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Data Availability Statement: All relevant data is contained within this article.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Thompson III, G.R. Pulmonary coccidioidomycosis. *Seminars in Respiratory and Critical Care Medicine* **2011,** *32*, 754-763. https://doi.org/10.1055/s-0031-1295723.
2. Zender, C.S. ; Talamantes, J. Climate controls on valley fever incidence in Kern County, California. *Int J Biometeorol* **2006**, *50*, 174-182. https://doi.org/10.1007/s00484-005-0007-6.
3. Barker, B. M., Litvintseva, A.P., Riquelme, M., & Vargas-Gastélum, L. Coccidioides ecology and genomics. *Med Mycol* **2019**, *57*(Supplement_1), S21-S29. https://doi.org/10.1093/mmy/myy051.
4. Edwards, P. Q.; Palmer, C.E. Prevalence of sensitivity to coccidioidin, with special reference to specific and nonspecific reactions to coccidioidin and to histoplasmin. *Dis Chest* **1957**, *31*, 35-60. https://doi.org/10.1378/chest.31.1.35.
5. Nguyen, C.; Barker, B.M.; Hoover, S.; Nix, D.E.; Ampel, N.M.; Frelinger, J.A.; Orbach, M.J.; Galgiani, J. N. Recent advances in our understanding of the environmental, epidemiological, immunological, and clinical dimensions of coccidioidomycosis. *Clin Microbiol Rev* **2013**, *26*, 505-525. https://doi.org/10.1128/cmr.00005-13.
6. Taylor, J.W.; Barker, B.M. The endozoan, small-mammal reservoir hypothesis and the life cycle of Coccidioides species. *Med Mycol* **2019**, *57*(Supplement_1), S16-S20. https://doi.org/10.1093/mmy/myy039.
7. Eulalio, K.D.; de Macedo, R.L.; Salmito Cavalcanti, M.D.A.; Soares Martins, L.M.; Lazéra, M.D.S.; Wanke, B. Coccidioides immitis isolated from armadillos (Dasypus novemcinctus) in the state of Piauí, northeast Brazil. *Mycopathologia* **2001**, *149*, 57-61. https://doi.org/10.1023/A:1007273019647.
8. Kuti, H.O. Role of the armadillo Dasypus novemcinctus in the epidemiology of paracoccidioidomycosis. Mycopathologia **1998**, *144*, 131-133. https://doi.org/10.1023/A:1007034215003.

9. Kollath, D.R.; Teixeira, M.M.; Funke, A.; Miller, K.J.; Barker, B.M. Investigating the role of animal burrows on the ecology and distribution of Coccidioides spp. in Arizona soils. *Mycopathologia* **2020**, *185*, 145-159. https://doi.org/10.1007/s11046-019-00391-2.
10. Marsden-Haug, N.; Goldoft, M.; Ralston, C.; Limaye, A.P.; Chua, J.; Hill, H.; Jecha, L.; Thompson, III.; Chiller, T. Coccidioidomycosis acquired in Washington state. *Clin Infect Dis* **2013**, *56*, 847-850. https://doi.org/10.1093/cid/cis1028.
11. Johnson, S.M.; Carlson, E.L.; Fisher, F.S.; Pappagianis, D. Demonstration of Coccidioides immitis and Coccidioides posadasii DNA in soil samples collected from Dinosaur National Monument, Utah. *Sabouraudia* **2014**, *52*, 610-617. https://doi.org/10.1093/mmy/myu004.
12. Catalán-Dibene, J.; Johnson, S.M.; Eaton, R.; Romero-Olivares, A.L.; Baptista-Rosas, R.C.; Pappagianis, D.; Riquelme, M. Detection of coccidioidal antibodies in serum of a small rodent community in Baja California, Mexico. *Fungal Biol* **2014**, *118*, 330-339. https://doi.org/10.1016/j.funbio.2014.01.006.
13. Kirkland, T.N.; Fierer, J. Coccidioides immitis and posadasii; A review of their biology, genomics, pathogenesis, and host immunity. *Virulence* **2018**, *9*, 1426-1435. https://doi.org/10.1080/21505594.2018.1509667.
14. Fisher, F.S.; Bultman, M.W.; Johnson, S.M.; Pappagianis, D.; Zaborsky, E. Coccidioides niches and habitat parameters in the southwestern United States: a matter of scale. *Ann N Y Acad Sci* **2007**, *1111*, 47-72. https://doi.org/10.1196/annals.1406.031.
15. Lewis, E.R.; Bowers, J.R.; Barker, B.M. Dust devil: the life and times of the fungus that causes valley fever. *PLoS Pathog* **2015**, *11*, e1004762. https://doi.org/10.1371/journal.ppat.1004762.
16. Kollath, D.R.; Miller, K.J.; Barker, B.M. The mysterious desert dwellers: Coccidioides immitis and Coccidioides posadasii, causative fungal agents of coccidioidomycosis. *Virulence* **2019**, *10*, 222-233. https://doi.org/10.1080/21505594.2019.1589363.
17. Shubitz, L.F. Comparative aspects of coccidioidomycosis in animals and humans. *Ann N Y Acad Sci* **2007**, *1111*, 395-403. https://doi.org/10.1196/annals.1406.007.
18. Spickler, A.R. Coccidioidomycosis. Available online: http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php (accessed on 1 Nov 2022).
19. Prchal, C.J.; Crecelius, H.G. Coccidioidomycosis in swine. *J Am Vet Med Assoc* **1966**, *148*, 1168-1169.
20. Maddy, K.T. Coccidioidomycosis in animals. *Vet Med* **1959**, *54*.
21. Graves, H.B. Behavior and ecology of wild and feral swine (Sus scrofa). *J Anim Sci* **1984**, *58*, 482-492. https://doi.org/10.2527/jas1984.582482x.
22. Bevins, S.N.; Pedersen, K.; Lutman, M.W.; Gidlewski, T.; Deliberto, T.J. Consequences associated with the recent range expansion of nonnative feral swine. *Biosci* **2014**, *64*, 291-299. https://doi.org/10.1093/biosci/biu015.
23. APHIS. Feral Swine Damage. Available online: https://www.aphis.usda.gov/aphis/resources/pests-diseases/feral-swine/feral-swine-damage (accessed 19 Jan 2021).
24. APHIS. History of Feral Swine in the Americas. Available online: https://www.aphis.usda.gov/aphis/resources/pests-diseases/feral-swine/sa-fs-history (accessed on 19 Jan 2021).
25. Ashraf, N.; Kubat, R.C.; Poplin, V.; Adenis, A.A.; Denning, D.W.; Wright, L.; McCotter, O.; Schwartz, I.S.; Jackson, B.R.; Chiller, T.; Bahr, N. C. Re-drawing the maps for endemic mycoses. *Mycopathologia* **2020**, *185*, 843-865. https://doi.org/10.1007/s11046-020-00431-2.
26. USDA-WS. Wildlife Services' Comprehensive Feral Swine Disease Surveillance Procedures Manual, Fiscal Year 2020. Procedure Manual for Comprehensive Feral Swine Disease Surveillance.
27. Greene, D.R.; Koenig, G.; Fisher, M.C.; Taylor, J.W. Soil isolation and molecular identification of Coccidioides immitis. *Mycologia* **2000**, *92*, 406-410. https://doi.org/10.1080/00275514.2000.12061175.
28. Malo, J.; Luraschi-Monjagatta, C.; Wolk, D.M.; Thompson, R.; Hage, C.A.; Knox, K.S. Update on the diagnosis of pulmonary coccidioidomycosis. *Ann Am Thorac Soc* **2014**, *11*, 243-253. https://doi.org/10.1513/AnnalsATS.201308-286FR.
29. Studnitz, M.; Jensen, M.B.; Pedersen, L.J. Why do pigs root and in what will they root?: A review on the exploratory behaviour of pigs in relation to environmental enrichment. *Appl Anim Behav Sci* **2007**, *107*, 183-197. https://doi.org/10.1016/j.applanim.2006.11.013.
30. Gaidici, A., Saubolle, M.A. Transmission of coccidioidomycosis to a human via a cat bite. *J Clin Microbiol* **2009**, *47*, 505-506. https://doi.org/10.1128/jcm.01860-08.

31. Kohn, G.J.; Linné, S.R.; Smith, C.M.; Hoeprich, P.D. Acquisition of coccidioidomycosis at necropsy by inhalation of coccidioidal endospores. *Diagn Microbiol Infect Dis* **1992**, *15*, 527-530. https://doi.org/10.1016/0732-8893(92)90103-Z.
32. Pappagianis, D. Serologic studies in coccidioidomycosis. *Semin Respir Infect* **2001**, *16*, 242-250. https://doi.org/10.1053/srin.2001.29315.
33. Gautam, R.; Srinath, I.; Clavijo, A.; Szonyi, B.; Bani-Yaghoub, M.; Park, S.; Ivanek, R. Identifying areas of high risk of human exposure to coccidioidomycosis in Texas using serology data from dogs. *Zoonoses and Public Health* **2013**, 60, 174-181. https://doi.org/10.1111/j.1863-2378.2012.01526.x.
34. Saubolle, M.A. Laboratory aspects in the diagnosis of coccidioidomycosis. *Ann N Y Acad Sci* **2007**, *1111*, 301-314. https://doi.org/10.1196/annals.1406.049.
35. Saubolle, M.A.; McKellar, P.P.; Sussland, D. Epidemiologic, clinical, and diagnostic aspects of coccidioidomycosis. *J Clin Microbiol* **2007**, *45*, 26-30. https://doi.org/10.1128/jcm.02230-06.
36. Graupmann-Kuzma, A.; Valentine, B.A.; Shubitz, L.F.; Dial, S.M.; Watrous, B.; Tornquist, S.J. Coccidioidomycosis in dogs and cats: a review. *J Am Anim Hosp Assoc* **2008**, *44*, 226-235. https://doi.org/10.5326/0440226.
37. Cox, F.E.G. Concomitant infections, parasites and immune responses. *Parasitol* **2001**, *122*(S1), S23-S38. https://doi.org/10.1017/S003118200001698X.
38. Hassan, A.; Blanchard, N. Microbial (co) infections: Powerful immune influencers. *PLoS Pathogens* **2022**, 18, e1010212. https://doi.org/10.1371/journal.ppat.1010212.
39. Salgame, P. ; Yap, G.S.; Gause, W.C. Effect of helminth-induced immunity on infections with microbial pathogens. *Nat Immunol* **2013**, *14*, 1118-1126. https://doi.org/10.1038/ni.2736.
40. Marruchella, G.; Paoletti, B.; Speranza, R.; Di Guardo, G. Fatal bronchopneumonia in a Metastrongylus elongatus and Porcine circovirus type 2 co-infected pig. *Res Vet Sci* **2012**, *93*, 310-312. https://doi.org/10.1016/j.rvsc.2011.05.016.
41. Soltysiak, Z.; Rokicki, J.; Kantyka, M. Histopathological diagnosis in parasitic diseases. *Ann Parasitol* **2014**, *60*, 127-131.
42. Galgiani, J.N. Coccidioidomycosis: changing perceptions and creating opportunities for its control. *Ann N Y Acad Sci* 2007, 1111, 1-18. https://doi.org/10.1196/annals.1406.041.
43. Huntington Jr, R. Histopathology of Coccidioidomycosis. In Proceedings of Symposium on Coccidioidomycosis, United States Dept. Health Educ. Welfare, Phoenix, Arizona, United States. (1957).
44. Schwartz, B. Controlling Lungworms in Swine (No. 118). US Government Printing Office. 1936.
45. Mayer, J.; Brisbin, I.L. Wild pigs: biology, damage, control techniques and management (No. SRNL-RP-2009-00869). Savannah River Site (SRS), Aiken, SC United States, 2009.
46. Mazi, P.B.; Sahrmann, J.M.; Olsen, M.A.; Coler-Reilly, A.; Rauseo, A.M.; Pullen, M.; Zuniga-Moya, J.C.; Powderly, W.G.; Spec, A. The Geographic Distribution of Dimorphic Mycoses in the United States for the Modern Era. *Clin Infect Dis* **2023**, *76*, 1295-1301. https://doi.org/10.1093/cid/ciac882.
47. del Rocío Reyes-Montes, M.; Pérez-Huitrón, M.A.; Ocaña-Monroy, J.L.; Frías-De-León, M.G.; Martínez-Herrera, E.; Arenas, R.; Duarte-Escalante, E. The habitat of Coccidioides spp. and the role of animals as reservoirs and disseminators in nature. *BMC Infect Dis* **2016**, *16*, 1-8. https://doi.org/10.1186/s12879-016-1902-7.
48. Emmons, C.W. (1942). Isolation of Coccidioides from soil and rodents. *Public Health Rep* **1942**, *1896-1970*, 109-111. https://doi.org/10.2307/4583988.
49. Emmons, C.W.; Ashburn, L.L. The isolation of Haplosporangium parvum n. sp. and Coccidioides immitis from wild rodents. Their relationship to coccidioidomycosis. *Public Health Rep* **1942**, *57*, 1715-1727.
50. Sharpton, T.J.; Stajich, J.E.; Rounsley, S.D.; Gardner, M.J.; Wortman, J.R.; Jordar, V.S.; Maiti, R.; Kodira, C.D.; Neafsey, D.E.; Zeng, Q.; Hung, C.Y.; McMahan, C.; Muszewska, A.; Grynberg, M.; Mandel, M.A.; Kellner, E.M.; Barker, B.M.; Galgiani, J.N.; Orbach, M.J.; Kirkland, T.N.; Cole, G.T.; Henn, M.R.; Birren, B.W.; Taylor, J.W. Comparative genomic analyses of the human fungal pathogens Coccidioides and their relatives. *Genome Res* **2009**, *19*, 1722-1731. https://doi.org/10.1101/gr.087551.108.
51. Peper, S.T.; Hoffarth, A.; Athanasiou, K.; Hawkins, S.L.; Wilson-Fallon, A.N.; Gibson, A.; Webb, C.R.; Gaskamp, J.A.; Webb, S.L.; Haydett, K.M.; Tiffen, H.S.; Presley, S.M. Brucella spp. and Francisella tularensis from an invasive alien species (Sus scrofa) in the southcentral USA. *Ecosphere* **2021**, *12*, e03426. https://doi.org/10.1002/ecs2.3426.

52. Hutton, T.; DeLiberto, T.J.; Owen, S.; Morrison, B. Disease risks associated with increasing feral swine numbers and distribution in the United States. Available online: https://digitalcommons.unl.edu/michbovinetb/59/?a_aid=3598aabf (accessed 6 Feb 2021).
53. Kirkland, T.N. The quest for a vaccine against coccidioidomycosis: a neglected disease of the Americas. *J Fungi* **2016**, *2*, 34. https://doi.org/10.3390/jof2040034.