Published in final edited form as:

Mycopathologia. 2020 February ; 185(1): 145–159. doi:10.1007/s11046-019-00391-2.

Investigating the Role of Animal Burrows on the Ecology and Distribution of *Coccidioides* spp. in Arizona Soils.

Daniel R. Kollath¹, Marcus M. Teixeira^{1,2}, Aubrey Funke³, Karis J. Miller¹, Bridget M. Barker^{1,@}

¹Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, USA

²School of Medicine, University of Brasília, Brasília-DF Brazil

³Imaging and Histology Core Facility, Northern Arizona University, Flagstaff, AZ, USA

Abstract

The lack of knowledge regarding the ecology of *Coccidioides* spp. makes both modeling the potential for disease outbreaks and predicting the distribution of the organism in the environment challenging. No single ecological parameter explains the biogeography of the pathogen. Previous investigations suggest an association with desert mammals, but these results should be confirmed with modern molecular techniques. Therefore, we used molecular tools to analyze soils associated with animal activity (i.e. burrows) to better define the ecology and biogeography of *Coccidioides* spp. in Arizona. Soils were collected from locations predicted to have favorable habitat outside of the established endemic regions to better understand the ecological niche of the organism in this state. Our central hypothesis is that soils taken from within animal burrows will have a higher abundance of *Coccidioides* spp. when compared to soils not directly associated with animal burrows. Our results show that there is a positive relationship with *Coccidioides* spp. and animal burrows. The organism was detected in two locations in northern Arizona at sites not known previously to harbor the fungus. Moreover, this fungus is able to grow on keratinized tissues (i.e. horse hair). These results provide additional evidence that there is a relationship between *Coccidioides* spp. and desert animals, which sheds new light on *Coccidioides*' ecological niche. These results also provide evidence that the geographic range of the organism may be larger than previously thought, and the concept of endemicity should be reevaluated for *Coccidioides*.

Introduction

The disease coccidioidomycosis, which is commonly known as valley fever (VF), is caused by two species of dimorphic fungal pathogens: *Coccidioides immitis* and *C. posadasii* [1, 2].

@ **Corresponding author:** Bridget M. Barker, bridget.barker@nau.edu. Pathogen and Microbiome Institute, Northern Arizona University, Applied Research & Development Building, 1298 S Knoles Drive, Flagstaff, Arizona 86011-4073, Phone 928-523-6074, Fax 928-523-4015.

Compliance with ethical standards

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Publisher's Disclaimer: This Author Accepted Manuscript is a PDF file of an unedited peer-reviewed manuscript that has been accepted for publication but has not been copyedited or corrected. The official version of record that is published in the journal is kept up to date and so may therefore differ from this version.

The disease outcome in human patients is highly variable with 60% of cases being asymptomatic after the establishment of infection and the other 40% of cases having symptoms ranging from mild pneumonia to severe disseminating disease involving the spleen, liver brain, bone, and other host tissues [2-4]. However, the infection primarily involves the lungs and is defined by the morphological switch from an inhaled arthroconidia into a large pathogenic endospore-forming structure called a spherule. Complications in diagnosis, treatment failure and unusual presentations may result in severe disease progression and even death. The causes for variability in disease outcome are not well understood, but are influenced by genetic factors of the host, environmental conditions, and variation in virulent fungal genotypes [4, 5].

Both species are soil-inhabiting fungi that are endemic to arid and semi-arid regions throughout North and South America; including California, Arizona, New Mexico, Texas, Mexico, and portions of Central and South America [6]. Genetically distinct species appear to have discrete geographic distributions. Genetically distinct populations of *C. immitis* inhabit Central and Southern California, Northern Mexico as well as a newly identified novel genotype in eastern Washington State. Within *C. posadasii*, genetically isolated populations are found in Arizona, parts of Mexico, New Mexico, Texas, Central and South America [2, 6-8]. Both species are found in soils that are slightly alkaline, but previous studies revealed that the sporadic distribution of *Coccidioides* spp. cannot be explained by soil characteristics alone [4, 9-12]. This observation suggests that additional factors play a role in the patchy distribution of the fungus. To date conditions required for growth and maturation in the environment are unknown, but enriched keratin sources, temperature, and precipitation all play a role in the fungal development [13-15].

Coccidioides spp. belong to the fungal phylum Ascomycota in the order Onygenales. Most Ascomycete fungi are saprotrophic (extracellular digestion of decaying organic material) in the environment and many have a direct or indirect connection with plants, but *Coccidioides* spp. and other Onygenales are associated with animals [13, 16]. During the mycelial phase of the life cycle, *Coccidioides* spp. are thought to be saprotrophic soil dwelling fungi with sporadic and irregular dispersal driven by abiotic soil factors (e.g. pH and electrical conductivity) and/or by biotic associations with mammals (e.g. rodents, bats and armadillos) [13, 17-20]. Lacy and Swatek investigated *C. immitis* around California archeological sites and revealed that sandy-textured soils made up of 98% of positive samples and 96.7% of positive soils were alkaline [21, 22]. Fisher *et al.* analyzed multiple abiotic factors and suggested that soils with low water content are more favorable for filamentous fungi because hyphae may reach water pockets that are unavailable to bacteria and yeast forming fungi. Thus, bacteria and yeast are predicted to be potential competitors for resources in wetter conditions where they outcompete filamentous fungi [20]. It is important to note that the relatively few studies that have examined the role of abiotic factors had small sample sizes and relied on culture-based approaches for fungal identification and, thus, do not provide strong evidentiary support for soil characteristics that can be used to predict the growth pattern and distribution of *Coccidioides* spp. Studies have also shown a lack of support for a correlation between a high prevalence of the pathogen and an association with certain flora [9, 10, 16, 22, 23].

To understand the ecology of *Coccidioides*, it is necessary to have a well-validated method to detect the presence of both species in the soil. However, detection in soil via classical microbiological methods is a difficult task as those are shown to be insensitive (thousands of soils with no/few cultured strains) and mouse inoculation is expensive and time consuming with variable results [10, 11, 17, 21, 23, 24]. Greene et al. attempted to isolate the fungus from 535 soil samples from the endemic region of California and only three were culture positive [11]. One difficulty in culturing *Coccidioides* spp. from soil is overgrowth by fast-growing fungi on antibiotic media plates [11]. Molecular technologies (PCR, DNA sequencing, and real-time qPCR) and more reliable and easy to use soil DNA extraction methods improve detection of the organism in the environment. However, even with sensitive molecular techniques, detection of the organism is still difficult. Previous studies have a detection rate from 0% to 8.42% [11, 24-26]. Some molecular methods require additional amplification and sequencing of the internal transcribed spacer (ITS) regions for identification [17, 24, 27, 28]. A newly developed real-time qPCR method that targets a repetitive sequence of a unique *Coccidioides*-specific transposon was highly sensitive in soil [29, 30]. The main benefit of using this approach is the ability to screen a large number of soil samples in a relatively short amount of time with high specificity to *Coccidioides* spp., which improves ability to perform ecological modeling. Using this methodology, it is possible to better understand the ecology of these organisms and make more informed recommendations about possible high exposure areas or activities.

Based on previous research, we hypothesize that burrowing desert mammals play an important role in the distribution and maintenance of *Coccidioides*. To test this hypothesis, we collected soils from animal burrows and outside of animal burrows from 5 different locations throughout Arizona and determined the presence of *Coccidioides* spp. in these soils samples using real-time qPCR assays. Our goal was to determine if animal burrows may be the preferred microhabitat for *Coccidioides* spp. by determining if the fungi were present at higher frequency in soils from burrows compared to nearby soil outside of burrows in Arizona. Moreover, we investigate the ability of this fungus to degrade keratin under “*in vitro*” conditions, and we defined the animal species that are common at the positive endemic sites.

Materials and Methods

Soil Collection

Soil samples were collected during the spring and summer months, pre- and post-monsoon season at five different locations throughout Arizona to encompass different elevations, life zones, and climatic conditions (Table 1). The site names and locations are as follows: Tucson site is located within Tucson, Arizona city limits, Tom Mix site is located between Florence and Tucson, Arizona, POW site is located in Florence, Arizona city limits, Flagstaff site is located just south of the Flagstaff, Arizona city limits, and Lake Mead is located southeast of Lake Mead National Recreation Area. Soils were collected using a garden trowel and put directly into sterile 50 milliliter collection containers; trowels were sterilized with 10% bleach between soil samples. A 20 × 20-meter plot was established by randomly picking a location at each site and every animal burrow within that transect was

sampled. Five soils samples were taken from inside animal burrows and one sample was taken from outside of the animal burrow to compare the prevalence of *Coccidioides spp.* inside burrows to outer sites. Due to the difficulty in detecting the pathogen and to increase the chance of observing a pattern more soils were taken from inside the burrow. Soils collected from inside animal burrows were taken from approximately 100-1000 centimeters depth (depending on size of burrow) carefully to avoid collecting soil from outside of the burrow. This depth was determined by depth of burrow, soil itself was taken at 10cm depths. Soils taken from outside of the animal burrow were collected approximately 1 meter away from the entrance to the burrow at a depth of approximately 10 centimeters. 456 total soil samples (n=456) were collected from 90 different animal burrows at five different locations in Arizona. A total of 385 soils were collected from within animal burrows and 77 were taken outside of burrows on the soil surface. Soils were stored at room temperature in sealed containers and processed within one to three months after collection.

Soil DNA Extraction

DNA from soil samples was extracted using the DNeasy PowerSoil Pro kit (QIAGEN, Valencia, CA). Due to potential infectious propagules in the soil, DNA extractions were performed in a biological safety level two laboratory inside a biological safety cabinet. The manufacturer's protocol was followed except for a 10-minute heat step at 65° C that was added before the homogenization step [24]. Two DNA extractions per soil sample were performed, and one microliter was used to estimate purity and concentration with FungiQuant real-time qPCR [31]. Although NanoDrop was used as well, estimated concentration measurements were unreliable. Remaining DNA was stored at -20°C until *Coccidioides*-specific molecular analysis was performed.

Molecular Detection of *Coccidioides spp.* by qPCR

Two real-time qPCR-based assays were used to detect *Coccidioides* DNA extracted from the collected soil samples. These TaqMan-based assays are highly sensitive, and target a repetitive region of DNA that is only known to occur in the two species within the *Coccidioides* genus [29]. The CocciDX assay targets a 106-bp sequence present in multiple copies within a transposable element in the genome of both *Coccidioides* species and is specific to this genus [30, 32]. In addition, the CocciENV assay was used to refine detection by adding additional target alleles based on newly genomes sequenced isolates of *Coccidioides* [29]. Both assays were performed on the Applied Biosystems QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Each reaction was performed in 20 µl-volume containing a mixture of 2X TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), 1x of either CocciENV or CocciDX at 100µM concentration of Cocci Assay oligo/probe mix and two µl of DNA template. PCR cycling conditions were performed as followed: Two minutes of activation step at 50° C, 10 minutes at 95° C for denaturation step, followed by 45 cycles of 15 seconds at 95°C and one minute at 60° C. Each sample was performed in triplicate and the average Cycle threshold (Ct) value was taken if amplification occurred. Control samples included purified DNA from *Coccidioides posadasii* strain Silveira as a positive control and molecular grade sterile water was used as negative no-template DNA control. Soils were considered positive if the Ct value was < 40 and negative for a Ct value >40. A burrow was considered negative when zero out of the five

soils samples taken from it did not show detectable real-time qPCR results. A burrow was considered “low positive” when one out of five soils was positive, “moderate positive” two or three out of five were positive, and “high positive” when greater than three out of five soil samples were positive.

Vertebrate DNA Collection and Genotyping

Animal identification was achieved by DNA analysis of fecal and hair samples collected at four of the five previously described study sites. A total of 32 hair and fecal samples were collected during spring and summer months at each location that soil was collected from. Useable DNA was extracted from 24 of the 32 samples. Samples were collected using sterile tweezers or a garden trowel that was sterilized with 70% ethanol in between samples. The fecal and hair material were placed in sterile 50mL collection containers and stored at 4° C until DNA extraction. Samples were processed within 1-2 months of collection. DNA was extracted using the DNeasy Powersoil Pro kit (QIAGEN, Valencia, CA) with the addition of a 10-minute incubation at 65° C. Primers developed by Xie et al. were used to amplify short (211 and 107 bp) highly polymorphic fragments of the 12s rRNA gene of the mitochondrial DNA of mammalian species from degraded samples[33]. The two sets of primers used in a single reaction master-mix L899 (5-GTAAATTTTCGTGCCAGCCACCG-3) and H1066 (5-GTGGGGTATCTAATCCCAGTTTG-3) for the longer fragment (12S-Long); L1497 (5-ACACACACCGCCC GTCACCCTC-3) and H1559 (5 - CCA GTATGCTTACCTTGTTACGAC-3) for the shorter fragment (12S-Short) [33]. The amplification was performed in a volume of 25ul containing 5 ng of template DNA, 200nM concentration of each primer and 12.5 µl of 1x PCR supermix from ThermoFisher. PCR was carried out with an initial denaturation step at 94° C for 5 minutes and followed by 30 cycles of 94°C for 30 seconds, 62° C for 30 seconds, 72° C for 15 seconds and a final extension at 72° C for 10 minutes. 24 PCR products underwent Sanger-sequencing using the aforementioned primer set. Sequencing was performed on the ABI 3130 genetic analyzer (ThermoFisher Scientific). The sequences were aligned and assembled using Sequencher® version 5.4.6 DNA sequence analysis software, (Gene Codes Corporation, Ann Arbor, MI USA). A Basic Local Alignment Search Tool (BLASTn) search was used to identify the species of each sequence by using the NCBI database [7].

Keratin Degradation

Experiments involving live *Coccidioides* spp. isolates were performed at the Biosecurity Level 3 (BSL3) laboratory at the Pathogen and Microbiome Institute, Northern Arizona University following pre-approved protocols. In order to determine if *C. posadasii* can germinate and proliferate on a keratin source we inoculated 0.5 × 0.5 cm mycelial plugs of the strains CPA0001 or CPA00020 into Soil Extract Agar plates [17, 34] containing autoclaved horse hair as keratin source. Strains CPA0001 and CPA00020 are soil isolates that are maintained at the Pathogen and Microbiome Institute in Flagstaff, Arizona, originally isolated from soil using mouse passage [17]. After inoculation, the plates were incubated at 28° C for 8 weeks and the fungus was killed using vapor formaldehyde in a sealed container for 48 hours. The cells were sterility-checked by plating part of the killed mycelial cells onto a fresh 2X-GYE plate for 72hs. The aerial mycelial were mounted on

specimen stubs and coated with an ultra-thin layer of gold. Morphology was analyzed by SEM in an UltraDry EDS and MagnaRay WDS spectrometer (Thermo Scientific).

Statistical Analysis

The data that we obtained were binary, meaning that the response variable was coded as either a 1 or 0, corresponding to the presence or absence of *Coccidioides* spp., respectively. For this reason, the chosen statistical model was a binomial regression model with presence/absence of the fungus in a soil sample being the response variable and burrow/topsoil, sample site, and sub-site all (sample location within sample site) being predictor variables. A model with random effects was chosen because individual soil samples taken from the same burrow can be considered dependent observations and our sampling protocol called for more samples from the burrow than outside the burrow. The random effects in the model addresses these sampling issues. Random effects were also chosen because soil is very heterogeneous and a subsequent experiment will not be able to sample the exact same soil due to destructive sampling. The random effects that were chosen were the sample site (location) and sub-site (burrow). The chosen alpha value was 0.05. The assumptions for binomial regression models are as follows. The outcome should be discrete and dichotomous (e.g. present versus absent), there should be no outliers in the data (z scores below -3.29 or greater than 3.29 should be removed), the predictor variables should not be inter-correlated (correlation coefficients among predictor variables are less than 0.90), and a large sample size is beneficial for this type of model [35]. All of these assumptions for a binomial regression model were met. All statistical analyses were done in R (R Core Team 2013).

Results

qPCR Detection of *Coccidioides* spp. in Arizona Soils

Of a total of 456 soil samples collected, 105 had real-time qPCR amplification for *Coccidioides* spp. (23% positive) using both assays employed. All five sample locations in Arizona had real-time qPCR amplification for *Coccidioides* DNA. The Lake Mead location had 8 out of 32 samples test positive (25%), Flagstaff had 6 out of 81 samples test positive (7.4%), Tucson had 77 out of 187 samples test positive (41.1%), Florence had 1 out of 60 samples test positive (1.6%), and the location located between Florence and Tucson Arizona had 13 out of 96 test positive (13.5%) (Figure 1).

qPCR Detection of *Coccidioides* spp. inside and outside Animal Burrows

Of the 105 soils that had real-time qPCR amplification for *Coccidioides* spp., 95 soils were taken from within animal burrows and 10 were taken from outside of animal burrows. Figure 2A shows the percentage of positive soils from within burrows and outside of burrows. Statistical analysis shows that there is a positive relationship between animal burrows and the presence of *Coccidioides* spp. (p-value=0.0152). The probability of detecting *Coccidioides* spp. in Arizona soils within animal burrows was calculated in the statistical model and is approximately 5.7% with a 95% confidence interval of 2.5% to 12.5% (Figure 2B). The probability of detecting the fungus in Arizona soils outside of animal burrows is approximately 2.01% with a 95% confidence interval of 0.5% to 6.8% (Figure 2B). The statistical model calculated the odds ratio on a log odds ratio scale. The calculated odds ratio of soils within animal burrows to soils outside of animal burrows was 2.93, meaning that it is approximately 3 times more likely to detect *Coccidioides* spp. in soils within animal burrows than soils that are located outside of animal burrows. Ten soils amplified *Coccidioides* spp. DNA outside of animal burrows. Nine of those soils were taken outside of a burrow that detected *Coccidioides* spp. within the burrow and only one of those soils were taken from a site not near any positive animal burrows (Figure 2C).

In summary, we detected *Coccidioides* spp. DNA in all sampled areas in Arizona for animal burrows. Five out of five animal burrows sampled were positive for *Coccidioides* DNA from Lake Mead (three low positives and two moderate positives). Three out of fourteen animal burrows were positive from Flagstaff with one high positive and two low positives. Nineteen out of thirty-one animal burrows tested positive from Tucson with three low positives, nine moderate positives, and seven high positives. Eight animal burrows out of 16 were positive from Tom Mix wash with three low positives and five moderate positives. One animal burrow out of 10 was a low positive from POW camp, Florence (Table 2). These data provide strong evidence that there is an association between *Coccidioides* spp. and the animal burrow environment.

qPCR Detection of *Coccidioides* spp. throughout Arizona

We compared the detection of the pathogen at each of the 5 locations that soil was collected from throughout Arizona. Lake Mead had 25% soils that were positive, Flagstaff had 7.4% soils that were positive, Tucson had 41.1% soils that were positive, Florence had 1.6% soils that were positive, and the site located between Florence and Tucson had 13.5% soils that were positive. The calculated probabilities of detecting the pathogen at each location as well as a 95% confidence interval are as follows, Lake Mead 8.46% CI 1.87%-3.09%, Flagstaff 1.22% CI 0.21%-6.88%, Tucson 23.9% CI 11.4%-43.4%, Florence 0.3% CI 0.02%-4.2%, Florence/Tucson 4.7% CI 1.2%-16.2%. The probability of detecting the pathogen is greatest in Tucson compared to other areas sampled within Arizona. At all locations the prevalence of the pathogen is greatest within animal burrows.

Molecular Detection of Mammalian Species at Burrows

To identify animals that may associate with burrows at our study sites and, which may be reservoir hosts, animal species were molecularly identified from hair and fecal samples. We identified seven different species across four of our study sites (no samples collected from

Flagstaff). We identified *Canis latrans* (coyote), *Canis lupus familiaris* (domestic dog), *Lepus californicus* (Black-tailed jackrabbit), *Neotoma albigula* (White-throated woodrat), *Neotoma mexicana* (Mexican woodrat), *Sylvilagus audubonii* (Desert cottontail), and *Xerospermophilus tereticaudus* (Round-tailed ground squirrel) at our sampling locations. The proportion of each species detected at the four sites is shown in Figure 3. Of the seven species identified at our sites, five species were found at burrows that were positive for *Coccidioides* spp. (Table 2). The species' DNA found at each positive burrow are displayed at Figure 3. We cannot determine if each species inhabited a given burrow at the time of soil collection. These data suggest that the fungus may not be specific to one host, but rather may be a generalist pathogen.

***C. posadasii* Growth in Keratin-Enriched Nutrient Source**

In order to explore whether *C. posadasii* has predilection for growth on animal hair, SEM microscopies were performed of the fungus growing on horsehair. We observed that both *C. posadasii* CPA0001 and CPA0020 strains are able to grow on keratin-enriched material (Figure 4). The horse hair filaments are completely dominated by *C. posadasii* mycelial cells and appear to invade the hair follicles (Figure 4B). The period of growth is long enough to produce arthroconidia on standard glucose-enriched 2X-GYE media however, only filamentous mycelia cells were observed suggesting that the growth on abundant keratin-enriched substrate does not induce sporulation in those *C. posadasii* strains after two months of growth (Figure 4).

Discussion

Increased soil disturbance due to land development and natural phenomenon in endemic areas has been hypothesized as the cause of increased morbidities due to VF, but the lack of knowledge about areas of the true prevalence of the organism makes exposure risk management a difficult task [36]. The distribution of the pathogen has been described as irregular and unpredictable, and no one factor has been found that explains the biogeography of the organism [37]. The majority of data on biogeographic distribution of the *Coccidioides* spp. was gathered indirectly from skin testing done on humans and cattle in the 1950s [38]. Clearly, the metrics used to determine the geographical distribution of the organism are outdated. Many studies have tried to ascertain abiotic conditions, such as soil texture, soil salinity, pH, soil and air temperature, and water concentration that aid in the proliferation of the fungus in the environment but the evidence was inconclusive with weak correlations [12, 17, 20-23].

In this study we detected the presence of *Coccidioides* spp. at five different locations throughout Arizona using molecular diagnostics. One caveat to molecular testing is the inability to determine pathogen viability. Direct plating and injecting soil solutions in mice have been utilized in several of the previous studies [17] but with limited success. Thus, while we feel that molecular methods allow for higher throughput and greater sampling across low-endemic areas, future ecological modeling will need to consider this variable. Although the pathogen was detected at higher frequencies within the true endemic area (Southern Arizona), *Coccidioides* spp. is present throughout Arizona, which has public

health implications. The three locations in southern Arizona had the highest prevalence, which agrees with previous studies that first described the endemic region of the pathogen [38]. These data are also in agreement with current case reporting data, with the highest reported incidences of disease in southern Arizona counties (Pima, Pinal, and Maricopa). However, we also detected the pathogen in soils in two locations in northern Arizona, an area of low endemicity, and reported cases in this area are thought to be mostly travel-related. Although more work is still needed, our data suggest that *Coccidioides* spp. have a greater geographic range in Arizona than previously thought and the endemic range needs to be reevaluated. This information can also help to alert medical professionals to test patients with VF symptoms when they have no travel history to the highly endemic regions.

The data from this study also provide evidence that *Coccidioides* spp. is more prevalent within animal burrows, and the realized niche may be the burrow microhabitat. Burrows are considered to be a stable environment with relatively narrow daily and seasonal shifts in temperature and relative humidity, hold moisture, and are shielded from direct UV radiation [39, 40]. For example, in the peak of summer in the Texas desert ambient temperatures ranged from 23-58°C, whereas animal burrow temperatures stayed within the range of 28-32°C [41]. Even when soil moisture is low, burrows tend to maintain a high vapor pressure (6.1-33.9 mm Hg) which keeps the burrow interior relatively moist [42]. The Sonoran desert in Arizona has a surface temperature that fluctuates throughout the year by more than 80°C, but one meter below the surface (common depth of rodent burrows) the annual temperature fluctuation is only 12°C and the maximum temperature at that depth rarely exceeds 31°C, while the surface temperature easily can reach 50°C in the summer [43, 44]. Although the desert conditions are extreme, the microclimate of animal burrows maintains a more strict homeostasis. Burrows also have associated animal activity, so the amount of keratin is likely higher inside animal burrows than outside. However, no information on keratin concentrations was found in the literature. Because we observed a *lack* of conidiation on a keratin rich source (hair), it is difficult to assess how growth at keratin-rich locations would affect conidia burden in the environment with other organisms competing for the same resources.

Coccidioides spp., as well as other Onygenales, can degrade animal-derived material and utilize it as a nutrient source [13, 16, 45]. The genome of *Coccidioides* has a reduced gene family of fungal-cellulose binding domains that aid in plant cell wall destruction and give the ability to digest plant material as a nutrient source [13]. The subtilisin N-domain-containing proteins are expanded in the *Coccidioides* genome as well as in close relatives and upregulated during the pathogenic stage of this fungus [13]. This gene family contains the peptidase S8 family domain, which encodes several keratinolytic subtilases (keratinases) expanded in *Coccidioides* when compared to other related taxa [13]. This genomic information suggests that unlike the sister order Eurotiales, which are often associated with plants and plant material, *Coccidioides* and other Onygenales utilize animal-derived substrates, and may have reduced ability to thrive on a plant-based diet.

An animal reservoir has not been identified for *Coccidioides* spp. but there is evidence of an association with desert rodents or armadillos, and both pathogenic and non-pathogenic relatives of *Coccidioides* have been shown to be associated with a variety of different

animals [9, 10, 28, 46]. Coccidioidal antibodies were detected in deer mice in the Baja California region of Mexico, and isolation of *Coccidioides* spp. from wild rodents in Arizona and California [9, 10, 46]. *Paracoccidioides brasiliensis*, a close pathogenic relative of *Coccidioides*, has been isolated from bat (*Artibeus lituratus*) guano, in South America and has been found within internal organs of nine-banded armadillos (*Dasypus novemcinctus*) [47, 48]. *Coccidioides* has also been isolated from bat lungs in Brazil [18]. *Blastomyces* spp., and *Histoplasma* spp. have also been isolated from bat guano and various other mammal species [49-52]. Thus, fungi within the order Onygenales are linked with wildlife, either *in vivo* or *in situ*, and this close relationship may be a clue to pathogenic potential in humans and other animals.

While our data provide evidence that *Coccidioides* spp. preferentially occupy animal burrows at our tested sites, there is not enough evidence to say that the fungus is infecting the animals that occupy the burrow. Our data show that the probability of finding the organism in a burrow is greater than outside a burrow. The burrowing desert animals may be the natural reservoirs that host *Coccidioides* spp., or the burrows may represent a stable microclimate in the environment. The classical definition of a disease reservoir states that the reservoir needs to have the ability to maintain the pathogen in the environment and have a feasible transmission route between the target population (reservoir) and non-target population (human) [53]. We propose an expanded definition of the classical disease reservoir. The *Coccidioides* reservoir system is likely the development of arthroconidia in soil in the burrows. This would maintain the fungus in the environment, and support transmission to a naive host through airborne arthroconidia from burrows (Figure 5).

Taylor and Barker 2019 have proposed a new hypothesis suggesting that *Coccidioides* spp. is actually living as an endozoan inside mammals, forming a granuloma, and are rendered inactive by the mammalian immune system until which time the animal dies and the fungus transforms into vegetative spore-forming hyphae [16]. There are numerous examples of many species of burrowing animals that are dying naturally within their burrows or dens [54, 55] allowing the fungus to transform into growing hyphae and establish outside of the carcass in the soil. This is a similar system to entophytic plant fungi that live inside of plant tissues without causing apparent disease that then decompose the tissue when the plant dies or the leaf falls off the plant [56].

We detected seven different mammal species at our sampling sites across Arizona. DNA from these animals was found in the vicinity of *Coccidioides* positive burrows and could possibly be the species that are harboring the fungus as an endozoan. *Coccidioides* spp. is not restricted to infecting a single species or even closely related group, but rather is a generalist and can take advantage of a wide variety of species. Many closely related human pathogens from the Onygenales order, such as *Blastomyces*, *Emmonsia*, *Paracoccidioides*, and *Histoplasma*, also have been isolated from a wide variety of animals (wild mice, bats, birds, armadillos, etc.) and have the ability to cause devastating mycoses in humans [47-52, 57]. A generalist pathogen can infect a wide variety of host species with varying severities and the host range of the pathogen is determined by the physiological, behavioral and ecological characteristics of the host that determines the ability of a pathogen to infect the host and complete its lifecycle [58-60]. The data show that a variety of animal species are

associated with burrows where *Coccidioides posadasii* is prevalent in Arizona. These species are excellent candidates for possible *Coccidioides* reservoirs and targets for further analysis.

Finally, the currently defined endemic region for this pathogen needs to be reexamined. There is a population of *Coccidioides* in Washington State that was recently discovered, an area well outside of the historic endemic region [32]. This study detected the pathogen in new locations in Northern Arizona suggesting that *Coccidioides* spp. may have a much broader geographic distribution than previously thought. More studies need to examine new areas that the fungus may be located at low concentrations such as New Mexico, Utah, Texas, and areas of Mexico, and South America. The methods from this study (directed sampling in and around animal burrows) may aid in detecting the pathogen in proposed potential habitat and help better understand the distribution of this fungus.

Conclusion

The ecology of *Coccidioides* spp. is vastly understudied. With disease numbers increasing every year the public health implications of information about the ecology of the organism are becoming progressively more important. Existing data do not accurately predict disease outbreaks and inform the public about specific areas and activities that can put them at high risk for being infected. Furthermore, there are few predictions of how the changing climate will affect the geographical distribution of the organism and the potential naive hosts that are at risk for becoming infected. Ecological studies form the necessary foundational support to establish better predictive models. This ecological modeling may then expand the true endemic region, and sharing this new information with clinicians so that they consider testing for VF could reduce delayed diagnoses and misdiagnoses and improve public health interventions.

Acknowledgments

Thanks to Austin Blackmon and Kaitlyn Parra for technical support. Thanks to Dr. David Wagner and Dr. Matthew Bowker for advisement and consultation throughout this study. This work was supported by an Arizona Biomedical Research Centre grant (ABRC 16-162415) to BMB.

References

1. Canteros CE, et al., [Genetic characterization of the fungus involved in the first case of coccidioidomycosis described by Alejandro Posadas in 1892]. *Medicina (B Aires)*, 2009 69(2): p. 215–20. [PubMed: 19435693]
2. Fisher MC, et al., Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. *Mycologia*, 2002 94(1): p. 73–84. [PubMed: 21156479]
3. Lee CY, et al., *Coccidioides* Endospores and Spherules Draw Strong Chemotactic, Adhesive, and Phagocytic Responses by Individual Human Neutrophils. *PLoS One*, 2015 10(6): p. e0129522. [PubMed: 26070210]
4. Smith CE, Beard RR, and et al., Varieties of coccidioidal infection in relation to the epidemiology and control of the diseases. *Am J Public Health Nations Health*, 1946 36(12): p. 1394–1402. [PubMed: 20278046]
5. Thompson GR 3rd, et al., Current Concepts and Future Directions in the Pharmacology and Treatment of Coccidioidomycosis. *Med Mycol*, 2019 57(Supplement_1): p. S76–S84. [PubMed: 30690601]

6. Teixeira MM and Barker BM, Use of Population Genetics to Assess the Ecology, Evolution, and Population Structure of *Coccidioides*. *Emerg Infect Dis*, 2016 22(6): p. 1022–30. [PubMed: 27191589]
7. Altschul SF, et al., Basic local alignment search tool. *Journal of molecular biology*, 1990 215(3): p. 403–410. [PubMed: 2231712]
8. Engelthaler DM, et al., Local Population Structure and Patterns of Western Hemisphere Dispersal for *Coccidioides* spp., the Fungal Cause of Valley Fever. *MBio*, 2016 7(2): p. e00550–16. [PubMed: 27118594]
9. Emmons C and Ashburn L, The isolation of *Haplosporangium parvum* n. sp. and *Coccidioides immitis* from wild rodents. Their relationship to coccidioidomycosis. *Public Health Rep*, 1942 57(46): p. 1715–1727. [PubMed: 19315895]
10. Emmons CW, Coccidioidomycosis in wild rodents. A method of determining the extent of endemic areas. *Public Health Reports (1896-1970)*, 1943: p. 1–5. [PubMed: 19315902]
11. Greene DR, et al., Soil isolation and molecular identification of *Coccidioides immitis*. *Mycologia*, 2000 92(3): p. 406–410.
12. Kolivras KN and Comrie AC, Modeling valley fever (coccidioidomycosis) incidence on the basis of climate conditions. *Int J Biometeorol*, 2003 47(2): p. 87–101. [PubMed: 12647095]
13. Sharpton TJ, et al., Comparative genomic analyses of the human fungal pathogens *Coccidioides* and their relatives. *Genome Res*, 2009 19(10): p. 1722–31. [PubMed: 19717792]
14. Tamerius JD and Comrie AC, Coccidioidomycosis incidence in Arizona predicted by seasonal precipitation. *PLoS One*, 2011 6(6): p. e21009. [PubMed: 21701590]
15. Untereiner WA, et al., The Ajellomycetaceae, a new family of vertebrate-associated Onygenales. *Mycologia*, 2004 96(4): p. 812–21. [PubMed: 21148901]
16. Taylor JW and Barker BM, The endozoan, small-mammal reservoir hypothesis and the life cycle of *Coccidioides* species. *Med Mycol*, 2019 57(Supplement_1): p. S16–S20. [PubMed: 30690603]
17. Barker BM, et al., Detection and phylogenetic analysis of *Coccidioides posadasii* in Arizona soil samples. *fungus ecology*, 2012 5(2): p. 163–176.
18. Cordeiro Rde A, et al., *Coccidioides posadasii* infection in bats, Brazil. *Emerg Infect Dis*, 2012 18(4): p. 668–70. [PubMed: 22469192]
19. Eulalio KD, et al., *Coccidioides immitis* isolated from armadillos (*Dasypus novemcinctus*) in the state of Piauí, northeast Brazil. *Mycopathologia*, 2001 149(2): p. 57. [PubMed: 11270394]
20. Fisher FS, et al., *Coccidioides* niches and habitat parameters in the southwestern United States: a matter of scale. *Ann N Y Acad Sci*, 2007 1111: p. 47–72. [PubMed: 17344527]
21. Elconin AF, Egeberg RO, and Egeberg MC, Significance of Soil Salinity on the Ecology of *Coccidioides Immitis*. *J Bacteriol*, 1964 87: p. 500–3. [PubMed: 14127564]
22. Lacy GH and Swatek FE, Soil ecology of *Coccidioides immitis* at Amerindian middens in California. *Appl Microbiol*, 1974 27(2): p. 379–88. [PubMed: 4856715]
23. Swatek FE and Omieczynski DT, Isolation and identification of *coccidioides immitis* from natural sources. *Mycopathol Mycol Appl*, 1970 41(1): p. 155–66. [PubMed: 5520711]
24. Lauer A, et al., Detection of *Coccidioides immitis* in Kern County, California, by multiplex PCR. *Mycologia*, 2012 104(1): p. 62–9. [PubMed: 21933931]
25. Chow NA, et al., Molecular detection of airborne *Coccidioides* in Tucson, Arizona. *Med Mycol*, 2016 54(6): p. 584–92. [PubMed: 27143633]
26. Johnson SM, et al., Demonstration of *Coccidioides immitis* and *Coccidioides posadasii* DNA in soil samples collected from Dinosaur National Monument, Utah. *Med Mycol*, 2014 52(6): p. 610–7. [PubMed: 24847036]
27. Alvarado P, et al., Detection of *Coccidioides posadasii* from xerophytic environments in Venezuela reveals risk of naturally acquired coccidioidomycosis infections. *Emerg Microbes Infect*, 2018 7(1): p. 46. [PubMed: 29593263]
28. Baptista-Rosas RC, et al., Molecular detection of *Coccidioides* spp. from environmental samples in Baja California: linking Valley Fever to soil and climate conditions. *fungus ecology*, 2012 5(2): p. 177–190.

29. Bowers JR, et al., Direct detection of *Coccidioides* from Arizona soils using CocciENV, a highly sensitive and specific real-time PCR assay. *Med Mycol*, 2019 57(2): p. 246–255. [PubMed: 29534236]
30. Saubolle MA, et al., Multicenter Clinical Validation of a Cartridge-Based Real-Time PCR System for Detection of *Coccidioides* spp. in Lower Respiratory Specimens. *J Clin Microbiol*, 2018 56(2).
31. Liu CM, et al., FungiQuant: a broad-coverage fungal quantitative real-time PCR assay. *BMC microbiology*, 2012 12(1): p. 255. [PubMed: 23136846]
32. Litvintseva AP, et al., Valley Fever: finding new places for an old disease: *Coccidioides immitis* found in Washington State soil associated with recent human Infection. *Clinical Infectious Diseases*, 2014 60(1): p. e1–e3. [PubMed: 25165087]
33. Xie J, et al., Identification of mammalian species using the short and highly variable regions of mitochondrial DNA. *Mitochondrial DNA*, 2015 26(4): p. 550–554. [PubMed: 24438314]
34. Kwon-Chung KJ, Studies on *Emmonsia capsulata*. I. Heterothallism and development of the ascocarp. *Mycologia*, 1973 65(1): p. 109–21. [PubMed: 4686214]
35. Watson JC, Establishing evidence for internal structure using exploratory factor analysis. *Measurement and Evaluation in Counseling and Development*, 2017 50(4): p. 232–238.
36. Colson AJ, et al., Large-Scale Land Development, Fugitive Dust, and Increased *Coccidioidomycosis* Incidence in the Antelope Valley of California, 1999–2014. *Mycopathologia*, 2017 182(5-6): p. 439–458. [PubMed: 28084574]
37. Nguyen C, et al., Recent advances in our understanding of the environmental, epidemiological, immunological, and clinical dimensions of coccidioidomycosis. *Clin Microbiol Rev*, 2013 26(3): p. 505–25. [PubMed: 23824371]
38. Edwards PQ and Palmer CE, Prevalence of sensitivity to coccidioidin, with special reference to specific and nonspecific reactions to coccidioidin and to histoplasmin. *Dis Chest*, 1957 31(1): p. 35–60. [PubMed: 13384171]
39. Bennett N and Jarvis J, The reproductive biology of the Cape mole-rat, *Georchus capensis* (Rodentia, Bathyergidae). *Journal of Zoology*, 1988 214(1): p. 95–106.
40. Mayer W THE PROTECTIVE VALUE OF THE BURROW SYSTEM TO THE HIBERNATING ARCTIC GROUND SQUIRREL, *SPERMOPHILUS-UNDULATUS*. in *Anatomical Record*. 1955 WILEY-LISS DIV JOHN WILEY & SONS INC, 605 THIRD AVE, NEW YORK, NY 10158–0012.
41. Kennerly T, Microenvironmental conditions of pocket gopher burrow. *Texas Journal of Science*, 1964 16(4): p. 395–&.
42. Kay FR and Whitford WG, The burrow environment of the banner-tailed kangaroo rat, *Dipodomys spectabilis*, in southcentral New Mexico. *American Midland Naturalist*, 1978: p. 270–279.
43. Turnage W, Nocturnal Surface-Soil Temperatures, Air Temperatures, and Ground Inversions in Southern Arizona. *Monthly Weather Review*, 1937 65(5): p. 189–190.
44. Vorhies CT, Water requirements of desert animals in the southwest. 1945: College of Agriculture, University of Arizona (Tucson, AZ).
45. Lewis ER, Bowers JR, and Barker BM, Dust devil: the life and times of the fungus that causes valley Fever. *PLoS Pathog*, 2015 11(5): p. e1004762. [PubMed: 25973899]
46. Catalan-Dibene J, et al., Detection of coccidioidal antibodies in serum of a small rodent community in Baja California, Mexico. *Fungal Biol*, 2014 118(3): p. 330–9. [PubMed: 24607357]
47. Restrepo A, et al., Clues to the presence of pathogenic fungi in certain environments. *Med Mycol*, 2000 38 Suppl 1: p. 67–77. [PubMed: 11204166]
48. Vergara ML and Martinez R, Role of the armadillo *Dasypus novemcinctus* in the epidemiology of paracoccidioidomycosis. *Mycopathologia*, 1998 144(3): p. 131–3. [PubMed: 10531678]
49. Baumgardner DJ, et al., Epidemiology of blastomycosis in a region of high endemicity in north central Wisconsin. *Clin Infect Dis*, 1992 15(4): p. 629–35. [PubMed: 1420675]
50. Baumgardner DJ and Paretsky DP, The in vitro isolation of *Blastomyces dermatitidis* from a woodpile in north central Wisconsin, USA. *Med Mycol*, 1999 37(3): p. 163–8. [PubMed: 10421847]

51. Chaturvedi VP, et al., In vitro interactions between *Blastomyces dermatitidis* and other zoopathogenic fungi. *Can J Microbiol*, 1988 34(7): p. 897–900. [PubMed: 3058276]
52. DiSalvo AF, The ecology of *Blastomyces dermatitidis*, in *Blastomycosis*. 1992, Springer p. 43–73.
53. Hallmaier-Wacker LK, Munster VJ, and Knauf S, Disease reservoirs: from conceptual frameworks to applicable criteria: Disease reservoir criteria. *Emerging microbes & infections*, 2017 6(1): p. 1–5.
54. Blanchard R, Blanchard DC, and Flannelly KJ, Social stress, mortality and aggression in colonies and burrowing habitats. *Behavioural processes*, 1985 11(2): p. 209–213. [PubMed: 24895927]
55. Reichman O and Smith SC, Burrows and burrowing behavior by mammals. *Current mammalogy*, 1990 2: p. 197–244.
56. Petrini O, Fungal endophytes of tree leaves, in *Microbial ecology of leaves*. 1991, Springer p. 179–197.
57. Baumgardner DJ, Soil-related bacterial and fungal infections. *J Am Board Fam Med*, 2012 25(5): p. 734–44. [PubMed: 22956709]
58. Andreou D and Gozlan RE, Associated disease risk from the introduced generalist pathogen *Sphaerothecum destruens*: management and policy implications. *Parasitology*, 2016 143(9): p. 1204–1210. [PubMed: 27216376]
59. Holmes JC, Parasite populations and host community structure Host-parasite interfaces. Academic Press, New York, 1979: p. 27–46.
60. Solter LF and Maddox JV, Physiological host specificity of microsporidia as an indicator of ecological host specificity. *Journal of Invertebrate Pathology*, 1998 71(3): p. 207–216. [PubMed: 9538025]

Figure 1:

Map of Arizona indicating the locations of each sample site. The total number of samples collected from each site as well as the number of positive samples is in the map legend. F/T represents the Tom Mix site that is located between Florence and Tucson Arizona, lower Sonoran life zone. Florence represents the POW site that is located in Florence Arizona, lower Sonoran life zone. Lake Mead represents the Lake Mead site that is located adjacent to Lake Mead National Recreational Area on the Nevada/Arizona Border. Flagstaff represents the Flagstaff site that is located just south of Flagstaff Arizona, Ponderosa Pine dominated vegetation. This map was created with Environmental Systems Research Institute (ESRI). (2012). *ArcGIS ArcMap Release 10.1*. Redlands, CA.

Figure 2:

Comparing prevalence of *Coccidioides* spp. within animal burrows to outside of animal burrows. A) Percentage of the total positive soils and comparing burrows to non-burrow soils. 105 soils that were positive for *Coccidioides* spp. separated into soils taken from within animal burrows and soils taken outside of animal burrows from the surface of the soil. Greater than 80% of the soils that tested positive for the pathogen came from within an animal burrows. B) Comparing the probability of detecting *Coccidioides* spp. within animal burrows to outside of animal burrows. . The probability of detecting *Coccidioides* spp. in Arizona soils within animal burrows is approximately 5.7% with a 95% confidence interval of 2.5% to 12.5%. The probability of detecting the fungus in Arizona soils outside of animal burrows is approximately 2.01% with a 95% confidence interval of 0.5% to 6.8%. This figure suggests that it is more likely to detect the fungus within animal burrows than outside of burrows. C) 10 soils that were taken from the soil surface outside of animal burrows that were positive for *Coccidioides*. False indicates that the soil was not paired with a positive burrow and true represents that it was paired with a positive burrows. 9 soils were paired with positive burrows and only 1 soil was not paired. This indicates that even though these soils were collected from the soil surface and not inside of animal burrows, the association with animal material still exists and may be a factor on the distribution of the pathogen.

Figure 3:

Number of animal species detected at four out of five sample sites. The number in the figure represents the number of that particular species was detected at each location. A) Lake Mead B) POW located in Florence Arizona C) Tom Mix located in between Florence and Tucson Arizona D) Tucson.

Figure 4:

Electron Micrograph of *Coccidioides posadasii* growing on horse hair. A) Mycelia starting to grow onto the horse hair. B) After 8 weeks of incubation mycelia have proliferated onto the hair. No sign of sporulation. C) Horse Hair D) Mycelia

Figure 5:

This figure represents our proposed sequence of events that may happen in the *Coccidioides* spp. reservoir system. A) The fungus is living within the rodent as an endozoan or on the rodent as a dermatophyte. B) The rodent either dies within the burrow or fungus is shed from the exterior of the rodent and then establishes in the soil of burrow. C) A new rodent is inhabited by the fungus and cycle continues. D) Airborne arthroconidia is dispersed from burrow and inhaled by human or another rodent establishing infection.

Table 1:

Description of the 5 Sample Sites.

n=456					
Site Name and Location	UTM Coordinates NAD3 12n	Elevation	Average Annual Temperature	Average Annual Precipitation	Number of Cases of Coccidioidomycosis Reported in 2017 per county
407-Tucson, Arizona Pima County	Easting:509428 Northing:3577062	865m	21.6°C	30.2cm	Pima-1,022
Tom Mix-Between Tucson and Florence, Arizona Pinal County	Easting:480961.61 Northing:3631473.45	609m	20.8°C	25.5cm	Pinal-518
POW-Florence, Arizona Pinal County	Easting:463757.36 Northing:3658111.77	457m	20.8°C	25.5cm	Pinal-518
Flagstaff, Arizona Coconino County	Easting:441204 Northing:3882722	2,133m	6.5°C	58.7cm	Coconino-32
Lake Mead, Arizona Mojave County	Easting:736014.35 Northing:3982317.18	731m	18.5°C	17.3cm	Mojave-67

Information on the 5 locations in Arizona that soil was collected from. Data was retrieved from National Oceanic and Atmospheric Administration, United States Geological Survey, and Arizona Department of Health Services. Tom Mix site that is located between Florence and Tucson Arizona, lower Sonoran life zone. Florence represents the POW site that is located in Florence Arizona, lower Sonoran life zone. Lake Mead represents the Lake Mead site that is located adjacent to Lake Mead National Recreational Area on the Nevada/Arizona Border. Flagstaff represents the Flagstaff site that is located just south of Flagstaff Arizona, Ponderosa Pine dominated vegetation.

Table 2:

Positive burrows from each site and the degree of positivity.

Location	Burrow	Degree	Species Detected at Positive Burrow
Lake Mead	B1	Low	ND
Lake Mead	B2	Low	ND
Lake Mead	B3	Low	ND
Lake Mead	B9	Moderate	<i>Xerospermophilus tereticaudus</i>
Lake Mead	B10	Moderate	<i>Cornis Lupus familairis</i>
Flagstaff	B1	High	ND
Flagstaff	B6	Low	ND
Flagstaff	B10	Low	ND
Tucson	B1	Moderate	ND
Tucson	B7	Low	ND
Tucson	B9	High	ND
Tucson	B10	High	ND
Tucson	B12	Moderate	ND
Tucson	B14	Moderate	ND
Tucson	B15	Moderate	ND
Tucson	B16	High	<i>Neotoma mexicana</i>
Tucson	B17	Low	ND
Tucson	B19	Low	ND
Tucson	B21	High	<i>Neotoma mexicana</i>
Tucson	B22	Moderate	ND
Tucson	B23	High	ND
Tucson	B24	Moderate	<i>Lepus Californicus</i>
Tucson	B26	High	<i>Syvilagus audubonii</i>
Tucson	B27	High	ND
Tucson	B28	Moderate	ND
Tucson	B30	Moderate	ND
Tucson	B31	Moderate	ND
TomMix	B1	Low	ND
TomMix	B4	Moderate	<i>Xerospermophilus tereticaudus</i>
TomMix	B6	Moderate	ND
TomMix	B7	Moderate	ND
TomMix	B10	Moderate	<i>Xerospermophilus tereticaudus</i>
TomMix	B13	Moderate	ND
TomMix	B14	Low	ND
TomMix	B16	Low	<i>Neotoma mexicana</i>
Florence	B2	Low	ND

The burrows that were positive from each location and the degree of positivity. Degree means how many different soil samples that are positive that were taken from each burrow. Positives were determined by Ct value < 40 . 1 sample that was positive is considered a low positive, 2-3 moderate, and > 3 is considered a high positive burrow. Animal species that were detected at a positive burrow is also included in the last column. This means that hair or fecal samples from these animals were collected at the entrance to a burrow that was positive.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript