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Spiromastigoides asexualis: Phylogenetic Analysis and Evaluation as a Cause of False-Positive *Blastomyces* DNA Probe Test Results

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ABSTRACT This is the first case of *Spiromastigoides asexualis* human infection, and it notably gave a false-positive *Blastomyces* DNA probe laboratory result. We further investigated other *Spiromastigoides* isolates as a cause of false-positive testing results, their phylogenetic relationship, and their susceptibility profiles to clinically available antifungal agents. Other *S. asexualis* isolates also resulted in positive *Blastomyces* DNA probe results, while *Spiromastigoides* species other than *S. asexualis* did not.

KEYWORDS DNA probe, phylogenetic, blastomycosis, mold, fungus, *Blastomyces*, false-positive, fungal, novel, phylogenetics, *Spiromastigoides*

Laboratory identification of fungal species has been traditionally based on colony morphology and characteristic microscopic features, including sporulation patterns. However, similar colonies and sporulation patterns may be observed in disparate pathogens, reducing the specificity of phenotypic methods alone. Confirmatory tests, including exoantigen testing or DNA probes are thus frequently used to ensure an accurate diagnosis. We present a case of false-positive *Blastomyces* DNA probe testing in a patient with *Spiromastigoides asexualis* and present an investigation of *Spiromastigoides* isolates as a cause of false-positive testing results, their phylogenetic relationship, and their susceptibility profiles to antifungal agents.

CASE REPORT

A 68-year-old woman with severe persistent asthma, chronic rhinosinusitis, and bronchiectasis presented with chronic gradual worsening of dyspnea of a 3-year duration. Her notable medications included fluticasone nasal spray, ipratropium-albuterol nebulizer, budesonide-formoterol inhaler, and aclidinium bromide inhaler. She also had been taking 10 mg prednisone daily for the previous 2 years. Her dyspnea had worsened despite ongoing care with her pulmonary physician with adjustments to her inhaler regimen. Prior to evaluation, she additionally noted 2 months of intermittent subjective fever and night sweats.

Of note, she had never traveled outside California. She had been living in a heavily forested area and venturing outdoors frequently. Due to persistent and ongoing symptoms she underwent a bronchoalveolar lavage (BAL) revealing thick purulent secretions in the right middle lobe, right lower lobe, left upper lobe, and left lower lobe. The bronchoalveolar lavage had 5,501 red blood cells/mm^3 and 123 white blood cells/mm^3, with 58% neutrophils and 30% eosinophils. Her BAL cultures grew a mold that phenotypically resembled a *Blastomyces* species on gross examination, so the patient was referred to the infectious diseases clinic.

On presentation, she was afebrile with stable vital signs. Her pulmonary exam

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revealed inspiratory rales and sparse expiratory wheezing. Her most recent laboratory tests a month prior were notable for a white blood cell count of 11,600 cells/mm^3 with an eosinophil count of 800 cells/mm^3. A chest computed tomography scan 1 month previously showed patchy areas of ground glass opacities in the upper lobes bilaterally. Although the patient lived near the identified region of *Blastomyces helicus* (1), diagnostic uncertainty regarding the mold species identification existed, and the specimen was sent for definitive identification and drug susceptibility testing (Fungus Testing Laboratory, San Antonio, TX). We also obtained a *Blastomyces* urine antigen via enzyme immunoassay (ARUP Laboratories, Salt Lake City, UT), which resulted as weakly positive but ultimately indeterminate. The patient was empirically started on 200 mg itraconazole twice daily for possible blastomycosis.

The initial workup of the fungal isolate found a positive chemoluminescent DNA probe for *Blastomyces* (AccuProbe, Hologic, Sunnyvale, CA, USA). However, DNA sequencing identified the organism as *Spiromastigoides asexualis* (UTHSCSA DI20-27). The patient was asymptomatic after 92 days of itraconazole treatment and the medication was discontinued.

Due to concern for *Spiromastigoides* sp. as a previously unidentified cause of false-positive *Blastomyces* DNA probe results, we reviewed all available *Spiromastigoides* isolates from the University of Texas Fungus Testing Laboratory, performed *Blastomyces* DNA probe testing, and queried susceptibility results of these isolates (Table 1).

MATERIALS AND METHODS

DNA extraction and sequencing. Harvested mycelia from 7 of 10 isolates were sent for DNA extraction and sequencing as previously described (2). Two isolates, namely, UTHSCSA DI13-1 and UTHSCSA DI13-2, were previously sequenced and identified as *Spiromastigoides asexualis* (3). Isolate UTHSCSA DI20-20 was not included because the extant culture was no longer available. The internal transcribed spacer ribosomal DNA (rDNA) region (ITS) and the D1/D2 domains of the large subunit rDNA (LSU) genes were amplified and sequenced to compare with sequences of the same loci in previous studies (4). PCR and sequencing were carried out using primer pairs BMBC-R and NL4R for ITS and LSU (5, 6). The generated sequences were used to perform BLASTn searches in GenBank (7). BLASTn search results were considered significant with an E value of 0.0, at 90% to 100% identity, and from 90% query coverage.

Phylogenetic analysis. BLASTn searches of the obtained sequences presumptively identified the isolates as species belonging to the genus *Spiromastigoides*. Thirty-three ITS and LSU sequences (including the outgroup, *Aleuria aurantia* AFTOLID65) were aligned using MUSCLE (8) as implemented in Sequencher v. 5.4.6 Build 46289 (Gene Codes Corporation, Ann Arbor MI, USA) and manually checked in PAUP v. 4.0a Build 167 (PAUP) (9). Two data sets, namely, the ITS and combined ITS and LSU, were analyzed together with reference and type strains of the genus *Spiromastigoides* genus and representative species in the family *Ajellomycetaceae* (4, 10, 11). The alignments were analyzed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). The maximum likelihood tree was constructed in IQ-TREE (12) with 1,000 resamplings of standard nonparametric bootstrapping (BT) implemented in IQ-TREE as UFBoot (13). TPM2u+I+G for both ITS and combined ITS and LSU loci was the best fit substitution model for ML, as determined by the ModelFinder implemented in IQ-TREE using the corrected Akaike information criterion (AIC) (14). Bayesian posterior probabilities (PPs) were calculated using MrBayes 3.2.5 (15). The substitution model for Bayesian analysis was determined as described above. However, TPM2u+I+G is not implemented in MrBayes 3.2.5 and was replaced by GTR+I+G as an alternative model (16). The analyses for each data set ran for 2×10^7 generations each, with 2 parallel runs with 4 chains; every 1,000th tree was sampled until convergence was reached when the standard deviation of split frequency was <0.01. The first 25% of trees were discarded as burn-in, and the remaining trees were combined into a single tree following the 50% majority rule consensus. Thresholds of $\geq$0.90 PP and $\geq$75% BT on nodes were considered significantly supported.

Susceptibility testing was performed by broth dilution in accordance with the CLSI M38Ed3 methodology (17). DNA probe testing of *Spiromastigoides* isolates was performed using the manufacturer's instructions (18).

Data availability. The sequences determined in this study were deposited in GenBank under accession numbers KJ880031, KJ880032, LN867603, LN867607, MT957837 to MT957839, MT957886 to MT957890, and MT957894 to MT957898 (see Table 1).

RESULTS

BLASTn searches of ITS and LSU sequences showed matches with *Spiromastigoides* species, ranging from 90% to 99% identities. Based on BLAST results, phylogenetic analyses were done to determine the relationships of these isolates with *Spiromastigoides* species.

TABLE 1 Characteristics of *Spiromastigoides* isolates and susceptibility results

Characteristic	Data for isolate: UTHSCSA DI20-20	UTHSCSA DI13-2	UTHSCSA DI13-1	UTHSCSA DI20-23	UTHSCSA DI20-27*	UTHSCSA DI20-21	UTHSCSA DI20-22	UTHSCSA DI20-24	UTHSCSA DI20-25	UTHSCSA DI20-26
Species	*Spiromastigoides* sp.	*S. asexualis*	*S. asexualis*	*S. asexualis*	*S. asexualis*	*S. minimus*	*S. minimus*	*S. minimus*	*S. minimus*	*S. minimus*
Source	Human finger nail	Human BAL[a]	Canine disc biopsy	Human sputum	Human BAL	Human bronchial wash	Human bronchial wash	Human skin scraping	Human bronchial wash	Human BAL
State of origin	Unknown	TX	AZ	AZ	CA	PA	WI	LA	TX	MD
Blastomyces exoantigen probe	Negative	Positive	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
ITS GenBank accession no.	NA[b]	LN867607	KJ880032	MT957837	MT957838	MT957886	MT957887	MT957888	MT957889	MT957839
D1/D2 GenBank accession no.	NA	LN867603	KJ880031	MT957837	MT957890	MT957895	MT957896	MT957897	MT957898	MT957894
MIC (μg/ml)										
Amphotericin B	0.06	ND[c]	0.06	0.5	0.5	4	2	8	2	ND
Micafungin	>8	ND	>8	>8	ND	>8	>8	>8	>8	ND
Fluconazole	>64	ND	64	>64	32	64	64	32	>64	ND
Itraconazole	4	ND	0.25	0.125	$\leq$0.03	0.125	0.125	0.125	4	ND
Posaconazole	1	ND	0.06	0.125	0.06	0.06	0.06	0.06	1	ND
Voriconazole	16	ND	2	1	0.25	1	1	1	16	ND
Isavuconazole	16	ND	16	4	0.25	2	2	2	16	ND

[a]BAL, bronchoalveolar lavage.
[b]NA, not available.
[c]ND, not determined.

A single most parsimonious tree was obtained for each data set. ITS alignment was 807 characters with 264 (32.7%) parsimony informative characters, and the tree scores were as follows 0.617 (consistency index [CI]), 0.780 (retention index [RI]), and 0.383 (homoplasy index [HI]). The combined ITS and LSU alignment was 1,385 characters with 368 (26.5%) parsimony informative characters with tree scores of 0.627 (CI), 0.804 (RI), and 0.373 (HI). The topologies of the single parsimonious trees of the two data sets generated with MP and ML trees were congruent with the BI trees (not shown). In the best ML scoring tree for both data sets (Fig. 1; see Fig. S1 in the supplemental material), UTHSCSA DI20-27 and UTHSCSA DI20-23 grouped with *Spiromastigoides asexualis* UTHSCSA DI13-1^T with strong nodal support (1.00 PP/100% BT [ITS]; 1.00 PP/100% BT [combined ITS and LSU]). Isolates UTHSCSA DI20-21, UTHSCSA DI20-22, UTHSCSA DI20-24, and UTHSCSA DI20-25 grouped together in one clade. This finding was strongly supported at 1.00 PP/100% BT (ITS) and 1.00 PP/99% BT (combined ITS and LSU) and was closest to *S. minimus* CBS 138268 with strong support at PP 1.00 for both data sets but weak BT support at 94 and 81% for ITS and combined ITS and LSU, respectively. UTHSCSA DI20-26 sits on its own branch and is closest to *S. minimus* with PP at 1.00 but also weak BT support at 94% and 89% for ITS and combined ITS and LSU. *Spiromastigoides* species are shown to be phylogenetically well delineated and resolved in well-supported clades except for two species, namely, *Spiromastigoides kosraensis* and *Spiromastigoides alatospora*, which are shown in a clade with 1.00 PP/99% BT support (Fig. 1). ITS alone and combined ITS and LSU are not able to separate these two species; however, the use of combined multiple loci, i.e., ITS, LSU, ACT, and TUB, may be able to distinguish these two as separate species (4). As shown in the phylogenetic trees, there are two possible putative new species close to *Spiromastigoides minimus*, i.e., *Spiromastigoides* sp. 1 UTHSCSA DI 20–26, and *Spiromastigoides* sp. 2 (UTHSCSA DI 20–21, UTHSCSA DI 20–22, UTHSCSA DI 20–24, and UTHSCSA DI 20–25). However, analysis of additional gene loci is needed for a definitive decision on their taxonomies. The ITS locus alone would be adequate to delineate 80% of the *Spiromastigoides* species in the present taxonomy. This is promising from the practical point of view of the clinical mycological laboratory, without resorting to sequencing extra loci, such as beta-tubulin actin and partial LSU.

Blastomyces DNA probe testing of all available *Spiromastigoides* isolates ($n = 10$) revealed positive results only with *S. asexualis* isolates ($n = 4$), while other *Spiromastigoides* species ($n = 6$) were negative. Susceptibility results revealed (Table 1) antibiotics with the following MIC ranges: amphotericin B, 0.06 to 8 μg/ml; micafungin, >8 μg/ml; fluconazole, 32 to >64 μg/ml; itraconazole, <0.03 to 4 μg/ml; posaconazole, 0.06 to 1 μg/ml; voriconazole, 1 to 16 μg/ml; and isavuconazole, 2 to 16 μg/ml.

DISCUSSION

Our patient is the first reported case of *Spiromastigoides asexualis* infection in a human, and our results revealed that *Spiromastigoides asexualis* can cause a false-positive result in the *Blastomyces* species DNA probe and questionable results with urine antigen testing.

Kuehn and Orr discovered the soilborne *Spiromastix* genus in 1962, which was isolated from a wheat field soil in Australia, and the first species was named *Spiromastix warcupii* (19). In 2013, the genus *Spiromastix* was renamed *Spiromastigoides*, as the former name had been used to name an alga (20). Since its discovery, additional species have been identified, as follows: *Spiromastix alatospora*, *Spiromastix albida*, *Spiromastix curvata*, *Spiromastix frutex*, *Spiromastix kosraensis*, *Spiromastix saturnispora*, *Spiromastix pyramidalis*, *Spiromastix sphaerospora*, and *Spiromastix sugiyamae* (19, 21–25). Pathogenicity had been presumed low due to the lack of reported infections in either humans or animals until 2014 when Rizzo et al. described discospondylitis in a German shepherd dog by *S. asexualis*, which was the first time this species was identified based on DNA sequencing (3). This species is similar to *S. warcupii*, as it produces groups of curved appendages, but in contrast, it has neither ascomata nor ascospores and produces an anamorph akin to that of *Chrysosporium* members (3). Since 2014, the

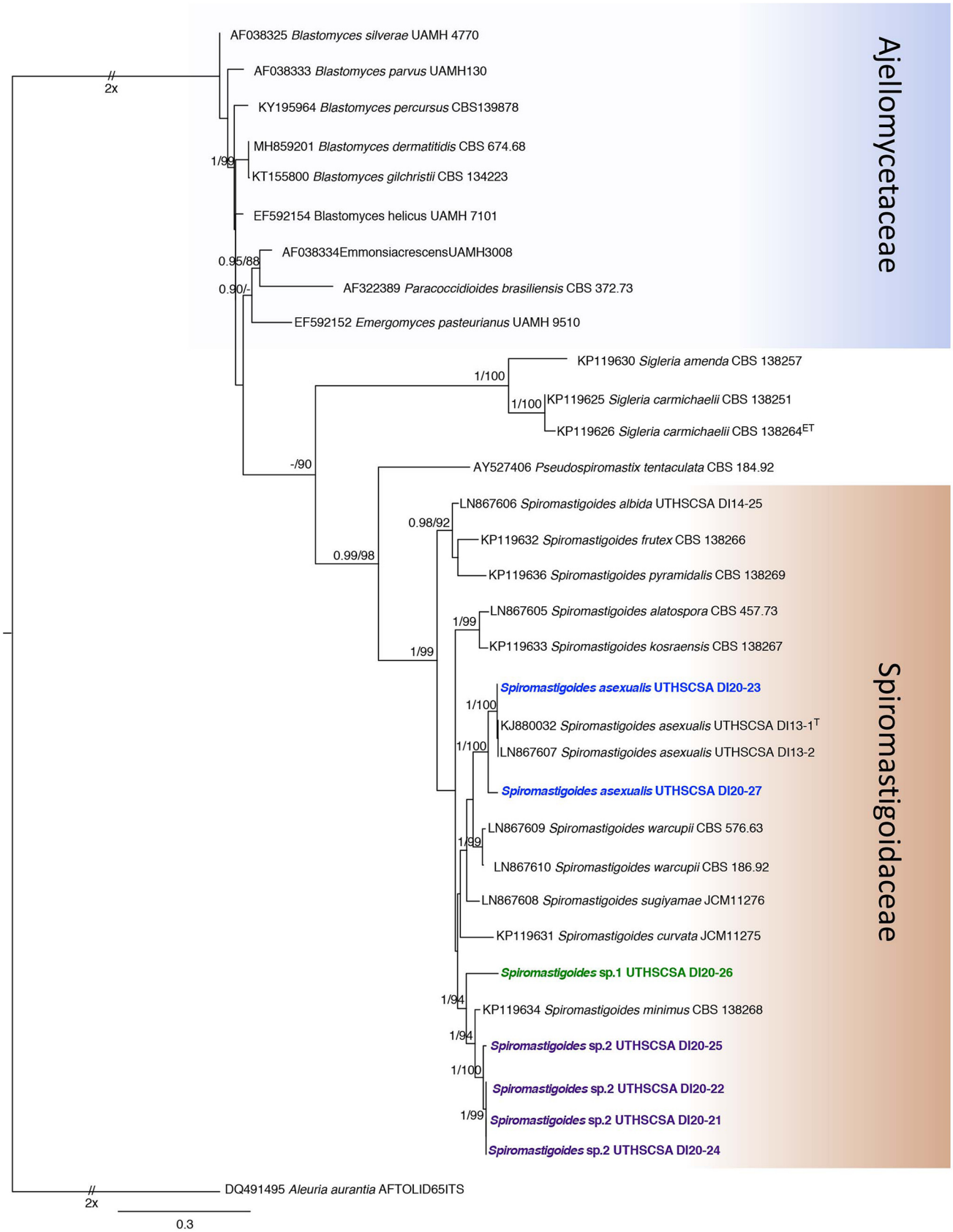

FIG 1 Phylogenetic relationships of *Spiromastigoides* strains using maximum likelihood analysis of ITS sequences. Bayesian posterior probabilities (≥0.90 PP, left) and maximum likelihood bootstrap support (≥75% BT, right) are shown on the nodes. The tree is rooted with *Aleuria aurantia* AFTOLID65.

advent of DNA sequencing has led to a proliferation of species identification under this genus (4, 10).

Our case illustrates that *S. asexualis* can cause infection in the appropriate host, as pathogenicity and virulence depend on the interaction with the host immune system (26). Our patient was immunosuppressed with prolonged systemic and inhaled corticosteroids, of which both had been known to predispose patients to invasive fungal infections (27–29). We suspect our patient was inoculated with the organism from her frequent exposure to soil, and her suppressed immunity allowed for the organism to cause disease. We deemed the presence of *S. asexualis* as a true infection in her lungs, as opposed to colonization or contamination, based on evidence of thick purulent secretions visualized on bronchoscopy with compatible radiographic findings, without growth of other organisms, and clinical improvement on itraconazole. However, as infection was not proven by histopathologic evidence, we can provide evidence of only possible infection overall.

Notably, *S. asexualis* caused a questionable result from the *Blastomyces* urine antigen assay and a positive result from the *Blastomyces* species DNA probe. We were initially skeptical of these *Blastomyces* testing results given the lack of epidemiologic exposure, and although *B. helicus* has been identified within the Rocky Mountain region, it has not been previously observed within the Lake Tahoe area. The *Blastomyces* urine antigen assay, although sensitive, is nonspecific, with one study showing cross-reactivity in 96.3% of patients with histoplasmosis, 100% of patients with paracoccidioidomycosis, and 70% of patients with talaromycosis (30). The specificity of the DNA probe for *Blastomyces* species is also imperfect; for example, *Paracoccidioides brasiliensis*, *Emergomyces canadensis*, and *Gymnascella hyalinospora* can result in false-positive test results (31–33). Ultimately, testing of other *S. asexualis* isolates similarly caused false-positive DNA probe testing, while other *Spiromastigoides* species did not cause such false positivity. Susceptibility results showed considerable variability between isolates, although MICs for itraconazole and posaconazole were generally favorable.

As the first case of a *S. asexualis* infection in a human, our case demonstrates even a typically nonpathogenic environmental organism, such as *S. asexualis*, can become an opportunistic infectious agent in a sufficiently immunosuppressed host. A thorough evaluation and ascertainment of high-fidelity microbiological cultures, such as a bronchoscopy with bronchoalveolar lavage, remain of utmost importance for diagnosing such infections. As *S. asexualis* is a mold that grossly resembles *Blastomyces* species and causes false positivity of the DNA probe assay and questionable results on urine antigen testing, our identification of it underscores the importance of understanding epidemiological risk factors for endemic infections and having a low threshold for utilizing advanced molecular genetics to investigate and identify the true causative organism. The clinical availability of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry and isolate sequencing will undoubtedly identify additional novel pathogens over the next decade.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare no conflict of interest.

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