Comparison of Whole Genome Sequencing versus Standard Molecular Diagnostics for Species Identification in the *Leishmania Viannia* Subgenus

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Abstract. The prognosis and treatment of New World tegumentary leishmaniasis is dependent on the infecting species, yet such species identification in the *Leishmania Viannia* subgenus poses a diagnostic challenge. Currently, speciation relies on standard molecular techniques such as restriction fragment length polymorphism (RFLP) analysis, and Sanger sequencing (SS). Whole-genome sequencing (WGS) is a robust and increasingly cost-efficient tool that may improve *Leishmania* species identification. We evaluated WGS versus standard RFLP-SS for species identification in three reference and five clinical strains of *Leishmania Viannia* spp. Internal transcribed spacer1 (*its1*), cysteine proteinase b (*cpb*), and heat shock protein 70 (*hsp70*) polymerase chain reaction-restriction fragment length polymorphism (RFLP) was performed, followed by SS of the *its2*, *cpb*, *hsp70*, and mannose phosphate isomerase (*mpi*) loci. After de novo assembly, sequences were mapped, and homology compared with both reference strains and reference genomes on National Center for Biotechnology Information. All American Type Culture Collection strains were confirmed to be single-species of *L. V. braziliensis*, *L. V. guyanensis*, or *L. V. panamensis* by WGS. Conversely, RFLP-SS was able to definitively identify one of three isolates to the species level. Clinical samples were identified as either single-species ($N = 3$), mixed ($N = 1$), or hybrid ($N = 1$) infections by WGS, while standard molecular diagnosis required multi-target composite analysis for identification due to loci-dependent results by RFLP-SS. We have corroborated the utility of WGS as a diagnostic tool to speciate members of the *L. Viannia* subgenus and to discriminate between mixed and hybrid infections. WGS is a potentially useful complement to multistaged RFLP-SS for species identification in *Leishmania* infections.

INTRODUCTION

The prognosis and treatment of New World tegumentary leishmaniasis is largely dependent on the infecting species.^{1–3} American tegumentary leishmaniasis (ATL) is a subcategorization of cutaneous leishmaniasis (CL) and mucocutaneous (MCL) or mucosal leishmaniasis (ML) of New World origin identified in Central and South America.⁴ ATL exists on a clinical and immunological spectrum, with the different clinical manifestations, treatment responsiveness, and prognoses often attributed to not only host factors but the many infecting species of the *Viannia* subgenus, including *Leishmania Viannia braziliensis*, *L. V. guyanensis*, *L. V. panamensis*, *L. V. peruviانا*, *L. V. lindenbergi*, *L. V. lainsoni*, and *L. V. shawi*.⁴ In Latin America, for example, it is well recognized that *L. V. braziliensis* portends the greatest risk of downstream MCL/ML following CL, while localized CL (LCL) due to *L. V. panamensis* is much more likely to self-cure than some of the other well-represented species, such as *L. V. braziliensis* and *L. V. guyanensis*.^{1–4} The presence of mixed or hybrid infections within members of the *Viannia* subgenus further complicates an already diagnostically challenging situation not only due to the inability to easily discriminate such infections from one another using standard molecular diagnostics^{5–9} but also because of data implicating such hybrids and mixed infections in more complicated disease.¹⁰

Currently, parasitological methods of diagnosis including culture and Giemsa-stained microscopy remain the gold standard in ATL^{11,12}; however, these techniques are unable to differentiate among members of the *Viannia* subgenus, which can affect patient treatment, long-term risk stratification, and prognosis. Microscopic examination of Giemsa-stained biopsy smears, aspirates, scrapings, or lesion impressions is further limited by poor sensitivity.¹¹ Although culture of tissue specimens is highly specific, poor diagnostic sensitivity, prolonged turnaround time, and required technical expertise limits its widespread utility of culture-based diagnosis in ATL.⁵ Although positive cultures provide a rich source of DNA for species identification, such species-level discrimination requires further downstream testing, amounting to a multi-staged diagnostic approach. Thus, as with all areas of microbiology, rapid, highly sensitive, and specific molecular methods, including polymerase chain reaction (PCR)-based techniques that are ideally performed on the primary specimen, are quickly supplanting parasitological methods for diagnosis and species identification in ATL. PCR-based methods improve diagnostic sensitivity in specimens with low parasite load and can be used on a wide array of specimens including noninvasively collected filter paper lesion impressions^{6,7,13} and cytology brushes,⁸ targeting 18S rRNA and kinetoplast DNA for confirmation. PCR-based methods including restriction fragment length polymorphism (RFLP) and Sanger sequencing (SS) have been evaluated for species identification, with targets including internal transcribed spacer1 (*its1*), internal transcribed spacer 2 (*its2*), heat shock protein 70 (*hsp70*), cysteine proteinase b (*cpb*), and mannose phosphate isomerase (*mpi*) all contributing to the molecular diagnostic approach, even in mixed and hybrid infections in

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patients with ATL.^{7,14–19} *its1*, glycoprotein 63 (*gp63*), *cpb*, and *hsp70* PCR-RFLP, and *its2* and *mpi* SS have all been shown to identify different members of the *Viannia* subgenus, but each target has limitations that necessitate the use of multiple amplified and sequenced loci to assign a final species identity.^{7,18,20–25} Despite such multitargeted speciation, there is a rising issue of species misidentification with such a complex approach, as documented in a comparative study of 16 laboratories across Europe and Asia.^{26,27} Thus, there is a dire need for improved approaches to species identification in *Leishmania Viannia* infections not just for accurate treatment allocation and prognosis but also to inform the molecular epidemiology of leishmaniasis in Latin America.

Non-PCR- or -sequencing-based detection assays, such as multilocus enzyme electrophoresis (MLEE) and MALDI-TOF mass spectrometry, are alternate methods that have emerged over the past decade, but they are limited to regions with substantial laboratory infrastructure, technical expertise, large volumes of cultured material, and funding for platform maintenance and consumables.^{10,28,29} Whole-genome sequencing (WGS), on the other hand, is a robust and increasingly cost-efficient molecular tool that may improve *Leishmania* spp. identification, particularly in scenarios where single target sequences are conserved across closely related species, necessitating sequencing of multiple targets.

In this study, we evaluated WGS as a potential complement to the standard molecular assays, PCR-RFLP and SS, of *cpb*, *hsp70*, *its1*, *its2*, and *mpi* loci in a cohort of three reference and five clinical strains of the *Viannia* subgenus to provide confirmatory species identification. Further, we assessed whether WGS could differentiate potential mixed and hybrid infections within the *Viannia* subgenus.

METHODS

Clinical specimens and reference strains. Five primary clinical specimens submitted to the Public Health Ontario Laboratory for *Leishmania* spp. diagnostic testing that were positive for *Viannia* spp. and culture positive were included in this analysis. The following WHO reference strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and used as reference strains: *L. V. braziliensis* ATCC 50135 (MHOM/BR/75/M2903), *L. V. guyanensis* ATCC 50126TM (MHOM/BR/75/M4147), and *L. V. panamensis* ATCC 50158TM (MHOM/PA/71/LS94). ATCC strains and clinical specimens were denoted as Day 0 and Passage 0 upon receipt in our laboratory (S1 Figure).

***Leishmania* cultures.** Clinical and ATCC cultures of *Leishmania* spp. promastigotes were cultured in Tobie's diphasic culture medium consisting of a blood agar (0.24% Laboratory-Lemco Powder, Oxoid; 0.5% Bacto Peptone, Difco; 1.5% Bacto Agar, Difco; 0.8% sodium chloride; and 340 mL of whole horse blood in a total of 1 L) with a liquid overlay (0.8% sodium chloride; 0.02% potassium chloride; 0.02% calcium chloride; 0.03% monopotassium phosphate; 0.25% dextrose; and 100 units/mL of penicillin and 100 µg/mL of streptomycin (ThermoFisher Scientific, Waltham, MA) in T25 culture flasks (Nunc, ThermoFisher Scientific) and incubated at 22 to 26°C under standard atmospheric conditions. Cultures were propagated until they reached at least 50 million promastigotes ("Baseline") to allow extraction of sufficient DNA (5 µg) for WGS. For clinical samples, this could take 40

to 82 days from primary specimen. Promastigotes were collected at two time points 6 weeks apart for analysis (Supplemental Figure 1). An interval period of 6 weeks was chosen to allow sufficient time for promastigotes to achieve the required number of cells for the next DNA extraction (Supplemental Figure 1). All samples were analyzed at two time points except for clinical isolate #5, which was analyzed at only one time point.

Leishmania cultures were centrifuged at 800g for 5 min, following which most of the supernatant was aspirated out, leaving ≈1 mL residual, which was stored at –80°C until DNA extraction.

DNA extraction. DNA was extracted from cultured samples using the DNA mini kit (Qiagen, Germantown, MD) with slight modification from the manufacturer's protocol as follows: cells were lysed at 56°C for 1 h; mixing with pipette rather than vortexing after the lysis step to avoid shearing of DNA; DNA was eluted with 60 µL of nuclease free water and quantified by Qubit 2.0 fluorometer with broad range dsDNA kit (ThermoFisher Scientific) and 260/280 ratio by Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific).

PCR and RFLP assays. *its1* PCR was performed on DNA extracted from cultured isolates using 370 nM of primers LITSR and L5.8S^{20,21} and 3 µL of DNA in a final volume of 27 µL with proofreading polymerase Accuprime Pfx Supermix (ThermoFisher Scientific). Cycling conditions were 95°C for 5 min followed by 38 cycles of 95°C for 15 s, 52°C for 30 s, and 68°C for 1 min followed by a final extension of 68°C for 5 min in Veriti Thermal Cycler (Applied Biosystems, Waltham, MA). After confirmation of amplification indicated by a ≈350 bp band on 1% agarose gel, 10 to 15 µL of PCR product was digested in 1× FastDigest Green Buffer and 1 µL of HaeIII FastDigest enzyme (Thermo Scientific) in a final volume of 30 µL for 1 h to overnight at 37°C. Digested PCR products were resolved in 2% agarose gel in 1× Tris-borate-EDTA (TBE) and run at 80 V for 2 h in the presence of ethidium bromide. *its2* PCR was performed with Accuprime Pfx Supermix and 370 nM each of primers LGITSF2 and R2.^{18,30} Cycling conditions for *its2* were 95°C for 5 min followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, extension at 68°C for 30 s and a final extension step of 68°C for 5 min.

Cpb and *hsp70* PCR was performed using 370 nM *cpb* fwd and rev and *hsp70* fwd and rev primers, respectively.^{7,25,31} PCR conditions were the same as *its2* except the extension step for each cycle was 90s instead of 30s. PCR product (10–15 µL) was digested in 1× FastDigest Green Buffer and 1 µL of TaqI FastDigest enzyme for *cpb* and HaeIII FastDigest enzyme for *hsp70* (Thermo Scientific) in a final volume of 30 µL for 1 h to overnight at 65°C (TaqI) and 37°C (HaeIII), respectively, and 10 to 15 µL of *hsp70* PCR product was digested in 0.5 µL of BclI and 1× CutSmart buffer, in a final volume of 30 µL at 37°C overnight (New England Biolabs, Ipswich, MA). Digested PCR products were resolved in 2.5% agarose gel in 1× TBE and run at 80V for 1.5 h with ethidium bromide. Two PCRs targeting *mpi* gene were performed using Accuprime Pfx Supermix, 370 nM of each primer and 3 µL of DNA in a final volume of 27 µL. PCR specific to *L. V. peruviana* was amplified with pan-*Viannia* fwd and *L. V. peruviana* specific rev primers; PCR specific to the other *Viannia* species was amplified with 370 nM of pan-*Viannia* fwd and rev primers.⁷

Cycling conditions were 95°C for 5 min followed by 40 cycles of 95°C for 15 s, and 68°C for 45 s, with a final extension of 68°C for 5 min in Veriti Thermal Cycler (Applied Biosystems). Ten microliters of PCR product were run in 1% agarose gel in 1× TAE with ethidium bromide at 100 V for 30 min for confirmation.

SS and National Center for Biotechnology Information Basic Local Alignment Search Tool. SS was performed for *its2*, *cpb*, *hsp70*, and *mpi* regions using 1 µL of PCR product (diluted in nuclease free water depending on band intensity) with 1.5× sequencing buffer, 0.25 µL BigDye Cycle Sequencing v3.1, and 1,000 nM primer in a total volume of 10 µL (ThermoFisher Scientific) for both forward and reverse primers. Cycling conditions were followed as per manufacturer's protocol and subsequently purified with BigDye Xterminator Purification Kit (ThermoFisher Scientific) before analysis using ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled using Vector NTI Advance 11 software (ThermoFisher Scientific) and aligned using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for species identification. As *cpb* and *hsp70* give longer assembled sequences of greater than 1,000 bp, for consistency with the analysis of these two regions, all sequences of these two amplicons were trimmed at the same position before BLAST. For *cpb*, sequences were trimmed from nucleotide position (nt) 172 to 1173 of the gene with reference to NCBI GenBank accession# XM_001562090.1, GQ180933.1, EU289030.1,^{32–34} and for *hsp70*, from nt 35 to 1351 of the gene with reference to NCBI accession# FN395040.1, FN395052.1, FN395055.1^{35–37} before BLAST analysis. In case SS-BLAST of the *its2* amplicon was not determined, single nucleotide polymorphism analysis was performed for speciation.¹⁸

WGS and bioinformatics analysis. WGS and bioinformatics analysis were conducted as a fee-for-service by the Center for Applied Genomics (The Hospital for Sick Children, Toronto, Canada). WGS was conducted by first preparing a DNA library by Illumina Low input DNA library prep kit, then sequenced in Illumina HiSeq 2500 with V4 chemistry paired end sequencing of 2 × 126 bases. Cutadapt was used to trim low quality bases (using quality cutoff: 20) and to trim sequencing adapters. Quality and adapter trimming were performed at both (5' and 3') ends. Trimmed reads with length less than 36 bp were filtered out. Trimmed reads for all samples were aligned to human genome (b37 decoy) using GSNAP aligner. Reads mapping to human genome were filtered out, and remaining reads were retained to be processed by next step. Reads were de novo assembled using SPAdes and ABySS and assemblies subsequently merged using Quickmerge. Unique reads from all samples were mapped to the same ATCC strains we genome sequenced and to NCBI reference genomes: *L. braziliensis* MHOM/BR/75/M2904 (ASM284v2), *L. panamensis* MHOM/PA/94/PSC-1 (ASM75516v1), and *L. peruviana* SAMEA2767111 (PAB-4377_V1).^{38–40} Percentage number of reads mapped without any mismatch to the aligning genomes were determined.

Arbitration of species identification, composite reference standard, and statistical analysis. A composite reference analysis of *its2*, *cpb*, *hsp70*, and *mpi* RFLP-SS BLAST was conducted whereby final species identification was assigned if at least two assays supported that final

species identification. The composite was used as the reference standard against which WGS results were compared.

Species identification by WGS was based on percentage mapped reads (homology) to the ATCC strains and NCBI reference genomes. A threshold of 70% homology was used to identify a strain as a unique species (Supplemental Table 1). A unique mono-species identification would be made if reads were ≥ 70% homology for a particular species, with ≥ 10% difference between the next closest species. A mixed infection was determined if reads achieved ≥ 70% homology for the mixed species. Lastly, a hybrid was determined if reads achieved < 70% homology to the hybrid species. Seventy percent homology was used as the cutoff based on the WGS "Percentage mapped reads to the same species" (Supplemental Table 1). This analysis revealed that the lower percent range for mapping to the reference strains with NCBI reference genomes was 71%; thus, 70% was selected as the cutoff to determine if samples mapped to certain species.

The sensitivity/accuracy for species identification of the standard molecular assays and WGS was determined by comparing with the composite reference standard, where WGS "true positives" were those corroborated by at least two standard molecular assays, and WGS "false negatives" were those discordant with at least two standard molecular assays. As there were no "true negatives" in this analysis, specificity and negative predictive value were not calculated. Statistical analyses were performed by MEDCALC Diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS

Standard molecular speciation analysis. The discriminatory level of species identification with standard molecular assays varied for the different loci (Table 1 and Supplemental Table 3). *Its1*-HaeIII RFLP was only able to identify included strains to the *Viannia* subgenus level. The combination of *cpb*-TaqI and both HaeIII- and BclI-based *hsp70* RFLP narrowed all ATCC and clinical strains to one species except clinical specimen #2, which had an overlapping *hsp70*-HaeIII banding pattern suggesting a hybrid or mixed infection (Table 2). Different loci of SS BLAST analysis gave different species identities. SS BLAST of the *cpb* loci for ATCC 50158 *L. V. panamensis* and clinical specimens #3, 4, and 5 determined these isolates to be *L. V. guyanensis*, whereas analysis of *its2* and SS BLAST of *hsp70* loci determined them to be *L. V. panamensis* (Table 2 and Supplemental Table 2). Further sequence analysis of *cpb* locus with ATCC 50126 *L. V. guyanensis*, ATCC 50158 *L. V. panamensis*, *L. V. guyanensis* (NCBI GenBank accession# GQ180933.1), and *L. V. panamensis* (NCBI GenBank accession# EU289030.1) revealed intraspecies single nucleotide polymorphism heterogeneity of ATCC 50158 *L. V. panamensis* likening it more so to *L. V. guyanensis* rather than *L. V. panamensis* at this particular locus (Figure 1). As opposed to all other samples analyzed by *hsp70* -SS BLAST in this study, *hsp70* SS BLAST failed to discriminate clinical specimen #2 to a single species further supporting that this isolate is a hybrid (Table 2 and Supplemental Table 2). Although two *mpi* end-point PCRs were included in this study to differentiate *L. V. peruviana* from the other *Viannia* species, *Mpi* SS BLAST could not narrow down to a single species level for some strains,

TABLE 1
Comparison of standard molecular assays to whole-genome sequencing (WGS) for species identification of strains of *Leishmania Viannia* spp by day of P in culture

Specimen	its1 HaellI	cpb TaqI	hsp70 HaellI	hsp70 BclI	its2 SNP	its2 Sequence BLAST	cpb Sequence BLAST	hsp70 Sequence BLAST	mpi Sequence BLAST	WGS
ATCC 50135, Wk 0, W 45, P 7	L. V. subgenus	L. V. braziliensis	L. V. braziliensis L. V. peruviana	L. V. braziliensis L. V. peruviana	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis
ATCC 50135, Wk 6, day 87 P 12	L. V. subgenus	L. V. braziliensis	L. V. braziliensis L. V. peruviana	L. V. braziliensis L. V. peruviana	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis
ATCC 50126, Wk 0, BD 86	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. braziliensis L. V. peruviana	L. V. guyanensis	L. V. guyanensis	L. V. guyanensis	L. V. guyanensis	L. V. braziliensis	L. V. guyanensis
ATCC 50126, Wk 6, day 128, P 19	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. braziliensis L. V. peruviana	L. V. guyanensis	L. V. guyanensis	L. V. guyanensis	L. V. guyanensis	L. V. braziliensis	L. V. guyanensis
ATCC 50158, Wk 0, BD 45, P 7	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. guyanensis	L. V. panamensis	L. V. braziliensis	L. V. panamensis
ATCC 50158, Wk 6, day 87, P 12	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. guyanensis	L. V. panamensis	L. V. braziliensis	L. V. panamensis
Clinical 1, Wk 0, baseline, day 67, P 5	L. V. subgenus	L. V. braziliensis	L. V. braziliensis L. V. peruviana	L. V. braziliensis L. V. peruviana	L. V. braziliensis	Not Determined	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis L. V. guyanensis L. V. panamensis	L. V. braziliensis L. V. peruviana mixed infection
Clinical 1, Wk 6 day 109, P 9	L. V. subgenus	L. V. braziliensis	L. V. braziliensis L. V. peruviana	L. V. braziliensis L. V. peruviana	L. V. braziliensis	Not Determined	L. V. braziliensis	L. V. braziliensis	L. V. braziliensis L. V. peruviana	L. V. braziliensis L. V. peruviana mixed infection
Clinical 2, Wk 0 day R2, P 9	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. braziliensis L. V. peruviana L. V. panamensis L. V. guyanensis hybrid or mixed infection	L. V. braziliensis L. V. peruviana L. V. panamensis	L. V. guyanensis/ L. V. panamensis	Not Determined	L. V. guyanensis	L. V. braziliensis L. V. guyanensis L. V. panamensis hybrid or mixed infection	L. V. braziliensis	L. V. guyanensis L. V. panamensis hybrid
Clinical 2, Wk 6, day 124, P 14	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. braziliensis L. V. peruviana L. V. panamensis L. V. guyanensis hybrid or mixed infection	L. V. braziliensis L. V. peruviana L. V. panamensis	L. V. guyanensis/ L. V. panamensis	Not Determined	L. V. guyanensis	L. V. guyanensis L. V. panamensis hybrid or mixed infection	L. V. braziliensis L. V. guyanensis L. V. panamensis	L. V. guyanensis L. V. panamensis hybrid
Clinical 3, Wk 0, BD 69, P 3	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. guyanensis	L. V. panamensis	L. V. braziliensis L. V. guyanensis L. V. panamensis	L. V. guyanensis L. V. panamensis hybrid
Clinical 3, Wk 6, day 111, P 7	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. guyanensis	L. V. panamensis	L. V. braziliensis L. V. guyanensis L. V. panamensis	L. V. panamensis
Clinical 4 Wk 0, BD 67 P 5	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. guyanensis/ L. V. panamensis	Not Determined	L. V. guyanensis	L. V. panamensis	L. V. braziliensis	L. V. panamensis
Clinical 4, Wk 6, BD 109, P 8	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. guyanensis/ L. V. panamensis	Not Determined	L. V. guyanensis	L. V. panamensis	L. V. braziliensis	L. V. panamensis
Clinical 5, Wk 0, BD 40, P 2	L. V. subgenus	L. V. guyanensis L. V. panamensis L. V. peruviana	L. V. guyanensis L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. panamensis	L. V. guyanensis	L. V. panamensis	L. V. braziliensis	L. V. panamensis

BD = baseline day; P = passage; SNP = single nucleotide polymorphism; Wk = week.

FIGURE 1. Sequence alignment of *cpb* locus. Alignment of *cpb* locus displayed intraspecies heterogeneity of ATCC50158 *L. V. panamensis* strain. This figure appears in color at www.ajtmh.org.

likely due to its low discriminatory level (Table 2 and Supplemental Table 2). Only ATCC 50135TM *L. V. braziliensis* had a definitive species identification consistent in all RFLP and SS BLAST assays (Table 2 and Supplemental Table 1). Compared with the composite reference, standard molecular assays correctly identified all three ATCC strains and differentiated the clinical isolates into single species within the *Viannia* subgenus with the exception of clinical specimen #2, which was likely a hybrid or mixed infection (Table 2).

WGS speciation analysis. Unique reads from WGS of the specific ATCC strains mapped to themselves had an average of 83.2% (range 79.3–85.3%) homology and mapping to the same species of ATCC strains and NCBI reference genomes had an average of 80.8% (range 71.3–85.3%) homology (Supplemental Table 1). Intra-genome variability of the mapped reads during the 6 weeks of culture had an average of 2.0% difference (range 0.8–3.0%) (Supplemental Table 1).

Mapping of WGS reads of ATCC 50135 *L. V. braziliensis* to itself and to NCBI *L. V. braziliensis* reference genome at baseline was 79.3% and 71.3%, respectively, whereas to NCBI *L. V. peruviana* reference genome was 68.3%, which was less than our threshold of 70% (Table 3). Upon culture to week 6, homology of all mapped reads increased, with ATCC 50135 *L. V. braziliensis* WGS reads mapped to itself increased to 82.2%, NCBI *L. V. braziliensis* reference genome to 75.7% and NCBI *L. V. peruviana* reference genome to 72.4%. Because the homology to *L. V. braziliensis* was approximately 3% to 10% higher than to *L. V. peruviana*, ATCC 50135 *L. V. braziliensis* was determined as the final species by WGS analysis.

ATCC 50126 *L. V. guyanensis* and ATCC 50158 *L. V. panamensis* strains both mapped to their corresponding species with more than 70% homology at both time points (Table 3). Although similar to ATCC 50135 *L. V. braziliensis*, ATCC 50126 *L. V. guyanensis* also mapped to more than 70% to *L. V. panamensis*, it had 10% higher homology to *L. V. guyanensis* (84.3% and 85.1%) (Table 3). On the basis of WGS analysis, ATCC 50126 *L. V. guyanensis* and ATCC 50158 *L. V. panamensis* were determined to be *L. V. guyanensis* and *L. V. panamensis* respectively.

Clinical sample #1 showed similar percentages of > 70% homology to both *L. V. braziliensis* and *L. V. peruviana* at both time points (Table 3). The intra-genome culture variability was within range when compared with the ATCC strains suggesting that the homologies between the two time points were consistent. Furthermore, the difference in homology of mapped reads between *L. V. braziliensis* and *L. V. peruviana* was less than 1% suggesting that this strain could be a mixed infection of both of these species.

Clinical sample #2 may represent a signature profile of hybrid species of *L. V. guyanensis* and *L. V. panamensis* as the percentages mapped to both were less than 70% homology, respectively, and there was only 4% homology difference between the two species (Table 3).

Clinical sample #3 resembled a hybrid species of *L. V. guyanensis* and *L. V. panamensis* at baseline (week 0); however, homology to *L. V. panamensis* was ~65%, whereas to *L. V. guyanensis*, the homology was 55%, which was 10% less (Table 3). Further analysis at week 6 confirmed it to be a single *L. V. panamensis* species with more than 80% homology (Table 3). The large difference in homology between baseline and week 6 suggests potential selection of strains in culture.

Clinical sample #4 mapped to be single *L. V. panamensis* species with over 80% homology at both time points, whereas clinical sample #5 mapped to *L. V. panamensis* at one time point and had at least 10% higher homology than to *L. V. braziliensis*, *L. V. guyanensis*, or *L. V. peruviana* (Table 3).

Performance characteristics of standard molecular assays and WGS. *Its1*-RFLP, *hsp70*-RFLP, and *hsp70*-SS all had 100% (95% confidence interval [CI] 63.1–100%) accuracy (Table 2). *Cpb* and *mpi* SS failed to provide an accurate species identification with only 37.5% (95% CI 8.5–75.5%) and 50% (95% CI 15.7–84.3%) sensitivity, respectively. WGS had an accuracy of 87.5% (95% CI 47.4–99.7%). Standard molecular analysis of clinical specimen #1 revealed it to be a mono *L. V. braziliensis* species, however, WGS suggested that it was a mixed infection (Table 3). This specimen lowered the accuracy of WGS, however, given the robust analysis of the whole genome in WGS compared with only a small fraction of DNA in the standard molecular assays, WGS provided a higher confidence with the true identity of this specimen being a *L. V. braziliensis* and *L. V. peruviana* mixed infection.

DISCUSSION

Causative species identification in New World tegumentary leishmaniasis is critical to optimal patient treatment allocation, prognostication, and risk stratification.³ However, the strains correlated to the most complex, recalcitrant, and relapsing infections in Latin America are those that are genetically similar members of the *Leishmania Viannia* subgenus, the discrimination of which has proven challenging for even major diagnostic reference laboratories.^{14,20,27} Thus, more sophisticated and innovative diagnostic platforms, and all the efficiencies that such interventions entail, for the true allocation of species in such tegumentary leishmaniasis infections are needed. We have herein demonstrated the ability of WGS to aid in the species identification and particularly differentiation of mixed and hybrid leishmaniasis infections from mono-infection in the *Viannia* subgenus using ATCC and NCBI reference strains. WGS's performance is at least comparable and possibly superior to that of current standard molecular diagnostics, with a simpler laboratory workflow algorithm. Instead of sequentially performing PCR, RFLP, and sequencing analyses on multiple conventional molecular targets, which can take up to 2 weeks to conclude, WGS offers a single analytic intervention, and the potential for high-throughput once an efficient

TABLE 3
Percentage of Unique Reads Mapped to American Type Culture Collection (ATCC) strains and National Center for Biotechnology Information (NCBI) reference genomes

	ATCC 50135, Wk 0, BD 45, P 7	ATCC 50135, Wk 6, BD 87, P 12	ATCC 50126, Wk 0, BD 86, P 14	ATCC 50126, Wk 6, Day 128, P 19	ATCC 50158, Wk 0, BD 45, P 7	ATCC 50158, Wk 6, Day 87, P 12	C-1, Wk 0, BD 67, P 5	C-1, Wk 6, Day 109, P 9	C-2, Wk 0, BD 82, P 9	C-2, Wk 6, Day 124, P 14	C-3, Wk 0, BD 69, P 3	C-3, Wk 6, Day 111, P 7	C-4, Wk 0, BD 67, P 5	C-4, Wk 6, Day 109, P 8	C-5, Wk 0, BD 40, P 2
	Unique mapped reads with no mismatch (%)														
ATCC 50135 <i>L. V. braziliensis</i>	79.3	82.2	54.9	55.0	51.1	53.4	73.2	71.5	57.3	61.1	42.6	55.6	53.3	57.7	56.8
ATCC 50126 <i>L. V. guyanensis</i>	52.1	54.2	84.3	85.1	64.9	67.8	55.2	53.8	61.6	65.3	55.0	69.4	67.7	71.4	69.9
ATCC 50158 <i>L. V. panamensis</i>	52.4	54.5	69.8	70.4	83.0	85.3	55.4	54.0	63.9	67.4	65.3	80.3	80.6	83.1	80.3
NCBI <i>L. V. braziliensis</i>	71.3	75.7	56.0	56.3	51.7	54.4	72.8	70.7	57.9	61.9	42.3	56.9	54.4	59.0	57.6
MHOM/BR/75/M2904															
NCBI <i>L. V. panamensis</i>	53.6	56.1	70.7	71.6	78.8	82.7	57.0	55.3	65.7	69.3	65.2	82.1	81.6	84.7	81.9
MHOM/PA/94/PSC-1															
NCBI <i>L. V. peruviana</i>	68.3	72.4	55.1	55.4	50.9	53.4	73.1	70.9	57.4	61.3	41.7	55.9	53.5	58.0	56.6
SAMEA2767111															

BD = baseline day; C = clinical; P = passage; Wk = week.

workflow and bioinformatic pipeline is established. Our study required 5 µg of DNA for WGS after discussing our objectives with the sequencing and bioinformatics team at The Center for Applied Genomics, a world-renowned next generation sequencing and bioinformatics center. With advancement of WGS technologies and the knowledge we gained from this study, less DNA will be required for further studies and the potential to perform directly on primary clinical specimen. As such, WGS could offer laboratories with more accurate species identification timely for physicians to more appropriately allocate patients to systemic versus local therapy at the time of clinical decision-making.³ Present molecular species identification approaches work well for tegumentary leishmaniasis infections that are acquired in areas where only one or two species are known to circulate, or in situations where lesions are simple and unlikely to lead to downstream mucosal involvement (e.g., Old World cutaneous leishmaniasis from counties such as Afghanistan where *L. tropica* is a common causative species). Under those circumstances, final species identification need not be rendered to deliver appropriate treatment to the patient. Treatment allocation and risk stratification for New World tegumentary leishmaniasis, on the other hand, proves much more challenging, particularly in leishmaniasis from Peru, for example, where multiple species within the *Viannia* subgenus co-circulate and lead to both cutaneous and mucosal disease.^{1,2,6,7,10} In such scenarios, sequences at standard molecular targets, such as *its2*, for example, can differ within species by only a single nucleotide, necessitating a multipronged, very granular, labor intensive, and time-consuming approach to species allocation. WGS has the potential to supplant such approaches and provide clinicians with more rapid, precise, and accurate species identification results, analyzing the whole genome, rather than specific genes, as our molecular analysis has shown, can provide mixed results.

There is enormous genetic diversity within the *Viannia* subgenus in the New World, owing to markedly high levels of clonality and recombination.⁴¹ Traditionally, a number of molecular targets are used to interrogate the identity of strains within the *Viannia* subgenus. *Hsp70* RFLP-SS BLAST assays are viewed as the most accurate standard molecular assays for species identification in the *Viannia* sub-genus. *L. V. braziliensis*, *L. V. guyanensis*, *L. V. panamensis*, *L. V. peruviana*, and *L. V. lainsoni* *hsp70* sequences are readily available in the NCBI database. *Cpb* SS BLAST analysis is another approach to *Viannia* strain interrogation, however, as demonstrated herein, this strategy likely misidentified *L. V. panamensis* ATCC50158 (MHOM/PA/71/LS94) as *L. V. guyanensis*, and similarly did so for clinical specimens 3, 4, and 5, likening them more to *L. V. guyanensis* rather than *L. V. panamensis*. *Mpi* PCR can only discriminate *L. V. peruviana* from other members of the *Viannia* subgenus and perhaps is able to identify hybrid infections. However, this assay must be complemented with SS because the primer set that discriminates *L. V. peruviana* from other *Viannia* strains varies only by a singular nucleotide on the 3' end, leading to assay cross-reactivity.

Use of the *its1*-HaeIII PCR-RFLP assay is limited to differentiation of species in *Leishmania Viannia* subgenus from members of the *Leishmania* subgenus. The *its2* SS assay is limited to detection of *L. V. braziliensis*, *L. V. guyanensis*, and *L. V. panamensis* when using the SS BLAST platform. There is no *its2* sequence available in GenBank for *L. V. peruviana* and

only a partial sequence for *L. V. lainsoni*. Furthermore, *its2* has multiple repeating DNA satellite regions, which can make SS technically challenging, resulting in short reads. The combined *hsp70*-HaeIII and *hsp70*-BclI PCR-RFLP analyses allow us to differentiate out *L. V. guyanensis*, *L. V. lainsoni*, and *L. V. panamensis* from the other *Viannia* species. The addition of yet another restriction enzyme, RsaI, to the *hsp70* PCR-RFLP algorithm can also help to resolve *L. V. peruviana* from *L. V. braziliensis*, in particular, as elegantly described by Montalvo et al. previously,⁴² underscoring the need to balance exhaustive and comprehensive algorithms with the practical operationalization of rapid and efficient species identification. Although our data demonstrated that PCR-RFLP analysis may provide a clearer picture of hybrid or mixed infections compared with SS alone, the interpretation of these assays still requires a collective interpretation, rather than reliance on a single platform approach.

The necessity for standardization of molecular diagnostics in tegumentary leishmaniasis is made clear by Van der Auwera and colleagues, who compared species identification results among 16 European facilities.²⁷ Similar to our findings, in the absence of a superior analytic method (e.g., WGS), many other studies have demonstrated that *hsp70* SS remains the top choice for species identification methodology.^{17,27,43} However, the reliance solely on *hsp70* can also misallocate closely related *Viannia* species, necessitating clear communication with clinical teams informing them of the chances of error, especially where treatment is species- or complex-dependent, as illuminated previously.²⁷

Of particular interest is the potential use of WGS to help parse mixed and hybrid infections of tegumentary leishmaniasis from mono-infections with closely related species that yield mixed results on the most common standard PCR-RFLP or SS approaches, as illustrated by clinical specimens #1 and #2 in this study. In those specimens, no single or composite standard molecular approach was sufficient to definitively assign mono, hybrid, or mixed infection status, highlighting the need for an expanded repertoire of assays or innovative tools altogether. Clinically, this is an important issue, as we have previously demonstrated that hybrid or mixed infections are overrepresented among those patients with severe forms of American tegumentary leishmaniasis, and further have very high parasite loads in their specimens.¹⁰ Given the lack of sophistication of molecular epidemiology of Latin American strains of *Viannia* to date, we have limited data to support one therapeutic approach over another in such scenarios, but from the data that do exist, we believe that automatic allocation to systemic therapy for such hybrid/mixed infections is warranted.¹⁰

In addition to having diagnostic implications particularly for Latin American strains of tegumentary leishmaniasis, WGS may also help disentangle the challenging issue of treatment failure and drug resistance. Ubeda and colleagues have shown that stochastic rearrangement of repeated sequences and the selection of copy number variants within specific loci is a novel technique by which *Leishmania* is able to react to adaptive environments (such as drug-induced pressures).³⁰ This can all contribute to an increased ability to evolve drug resistance via competent species hybridization, among many other mechanisms. In *L. major*, WGS has already been used to pinpoint novel mutation biomarkers of miltefosine resistance.⁴⁴ Moreover, Teixeira

and colleagues have reported that the whole genomes of symptomatic *L. infantum* in infected patients have a notably higher level of genetic diversity than those of asymptotically presenting individuals.⁴⁵ Using WGS for *L. donovani*, Downing and colleagues have contributed new knowledge to both the population structure and potential mechanisms of drug resistance in strains of *Leishmania*.⁴⁶ The role of sexual reproduction in the establishment of hybrid species of *Leishmania* has become increasingly important in the context of evolution and drug resistance.⁴⁷ Additionally, WGS in *Leishmania donovani* has been shown to have the potential to provide reliable and novel vaccine candidate biomarkers via investigation of virulence attenuation-associated genes.⁴⁸ Ultimately, WGS for species identification within the *Viannia* sub-genus can serve as a powerful tool to study large-scale geographic diversification, evolution, and population-level drug resistance biomarkers.

This study and its associated interpretations are not without limitations. It is well known that the usefulness of SS is dependent on the breadth of the NCBI database, and the accuracy of the correlation of the sequence and species submitted. In our case of the *cpb* loci, we identified *L. V. panamensis* strains with sequence heterogeneity not captured in NCBI GenBank which falsely mis-identified to *L. V. guyanensis*. This phenomenon is not isolated to parasitology: in mycology, as many as 20% of fungal sequences deposited to GenBank can be incorrectly annotated.⁴⁹ We examined eight strains of the *Leishmania Viannia* subgenus due to budget limitations. It is possible that our conclusions may have differed with a study enrolling a larger number of strains or an expanded repertoire of restriction enzymes, such as RsaI, and assays. Use of WGS in this study, required pure culture and a large DNA content. Currently, with the constant advancement of sequencing technology, DNA content requirement has significantly lowered to pg of DNA and potentially performed directly on clinical specimen. High cost of WGS can make it inaccessible to resource-limited settings, not to mention that bioinformatics expertise is required to accurately analyze sequences. Current WGS (including bioinformatics cost) is approximately USD \$500 per sample, whereas the standard molecular diagnostics cost about USD \$30 per sample. With the constantly diminishing cost of WGS over the past decade, it is certainly plausible that the future may bring greater accessibility of the technology and associated expertise.

The ability to discriminate amongst the *Viannia* strain infections in standard *Leishmania* diagnostics is an arduous and often time-consuming process that requires multiple molecular assays to arrive at an often-imprecise result. WGS may be a useful species identification platform that is comparable to our standard composite PCR-RFLP and SS analyses. More data are required to make a definitive conclusion about the role of WGS in *Leishmania* diagnostics. Cost continues to limit access to WGS for *Leishmania* species identification, with a 17-fold higher cost of WGS on a per sample basis compared with PCR-RFLP and SS algorithms. When further optimized, using WGS for species identification may greatly reduce turnaround time as the need for complicated stepwise algorithms with multipronged molecular diagnostics is eliminated.⁵⁰ Having a singular assay, for a singular specimen, through one technological pipeline has its own inherent logistical benefits, unmet by the labor-intensive, intricate, and imprecise stepwise algorithms in current use.⁵⁰

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