Development and evaluation of a molecular diagnostic method to rapidly detect *Histoplasma capsulatum var. farciminosum*, the causative agent of epizootic lymphangitis, in equine clinical samples.

Running title: Molecular detection of *Histoplasma* in equine clinical samples

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Abstract

Histoplasma capsulatum var. farciminosum (HCF), the causative agent of epizootic lymphangitis (EZL), is endemic in parts of Africa. Diagnosis based on clinical signs and microscopy lacks specificity, and is a barrier to further understanding this neglected disease. Here, a nested PCR method targeting the ITS region of the rRNA operon was validated for application to equine clinical samples. Twenty-nine horses with signs of EZL, from different climatic regions of Ethiopia, were clinically examined. Blood samples and aspirates of pus from cutaneous nodules were taken, along with blood from a further 20 horses with no cutaneous EZL lesions. HCF was confirmed in DNA extracts of pus and blood samples from 25 and 17 horses, respectively, of the 29 suspected EZL cases. Positive PCR results were also obtained from heat-inactivated pus (24 horses) and blood (23 horses) spotted onto Whatman FTA cards. Two positives were obtained among blood samples from 20 horses that did not exhibit clinical signs of EZL. These are the first reports of the direct detection of HCF in equine blood, and at high frequency amongst horses exhibiting cutaneous lesions. The nested PCR outperformed conventional microscopic diagnosis, as characteristic yeast cells could only be observed in 14 pus samples. HCF DNA was confirmed by sequencing the cloned PCR products, and while alignment of the ITS amplicons showed very little sequence variation, there was preliminary SNP-based evidence for the existence of two subgroups of HCF. This molecular diagnostic method now permits investigation of the epidemiology of EZL.

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Introduction

Epizootic lymphangitis (EZL), caused by the dimorphic fungus *Histoplasma capsulatum var. farciminosum* (HCF), is traditionally a disease of equids; the related species *H.capsulatum var. capsulatum* causes histoplasmosis in humans and is an important opportunistic pathogen worldwide [1]. The clinical presentation in horses varies, with four forms described [2]: asymptomatic; ocular; cutaneous; respiratory. Mixed forms can occur [3]. The cutaneous form is characterized by multi-focal pyogranulomatous sub-cutaneous nodules that progress along the lymphatic system, with coalescence of nodules producing a 'corded' appearance. If lesions are located on the limbs, progression of the disease can result in severe lameness. The respiratory form is classically characterised by pyogranulomatous lesions within the nasal mucosa and lung parenchyma, with potential for multi-systemic pathology [2-7].

Although eradicated from Europe, EZL is currently prevalent in Ethiopia where, dependent upon region, between 0% and 39% of equids may be infected [8, 9, and 10]. Ethiopia has Africa's largest equine population, where approximately 2 million horses have a crucial role in the economy of both urban and rural communities [11-13]. In two separate participatory studies in different areas of Ethiopia [14, 15], horse owners consistently volunteered EZL as a high priority disease. EZL contributes to extensive morbidity and subsequent mortality due to abandonment of chronically infected animals, and can have a devastating impact on the income of poor families [16, 17].

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Within disease endemic regions, access to treatment is a significant challenge. SPANA (Society for the Protection of Animals Abroad) currently provides free veterinary care within their clinics, however topical treatment with tincture of iodine and oral dosing with potassium iodides, is labour intensive, expensive and of limited efficacy in moderate to severe cases of EZL [18]. It is imperative that animals are diagnosed early in the course of the disease to improve treatment outcomes, conserve resources and reduce the burden of infection within the population. Currently, due to limited available diagnostic technology, veterinarians in Ethiopia diagnose the disease based on clinical appearance and microscopic examination for yeast cells within pus. This has the potential for misdiagnosis as the clinical appearance can mimic other diseases (e.g. ulcerative lymphangitis, sporotrichosis and the cutaneous form of glanders [2, 9, 19, 20]). Culture of HCF from clinical lesions would be definitive, but is challenging and rarely attempted. Therefore, reliable and robust approaches to diagnosis are required to support clinical decision making and enable epidemiological studies to provide the rationale for the development of disease prevention strategies. EZL has recently been highlighted as a priority neglected disease of working equids [21].

Classically, members of the genus *Histoplasma* have been classified into three separate varieties, *capsulatum* (HCC)*, duboisii* and *farciminosum* (HCF) defined by host species and pathogenesis [22]. However, *Histoplasma spp.* have more recently been grouped into eight clades based upon MLST of isolates combined with geo-spatial distribution of their sources [23]. HCF has long been considered an equine specific pathogen, but the application of molecular biology techniques has identified a broader host and geographic range for HCF with clinical cases reported in dogs [24], badgers [25] and even humans [26]. The phylogeny of *Histoplasma*

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spp. has been examined using a range of different gene loci [23, 24, 26-31]. As has been frequently reported for fungi, sequence variation in the ITS region of the ribosomal RNA operon provides sufficient resolving power to discriminate between closely related species and variants, and can contribute to the design of specific PCR-based detection protocols [26, 28, 32, 33]. For HCF, a nested PCR protocol for specific identification of cultures has been designed [32] and is adapted here for use with clinical material. Isolates and/or sequences of both equine and human origin from Africa are under-represented in studies on the phylogeny of histoplasmas [26, 27, 30, 34-36], which is at odds with their prevalence in these regions [37-40]. Phylogenetic analysis of a handful of historic cultures from equine clinical cases has been described [26, 28], but no studies have reported the application of PCR to diagnose HCF directly in clinical specimens.

This study has two primary objectives: 1) to establish and validate the use of DNA extraction and PCR amplification protocols to rapidly identify HCF directly from equine clinical specimens, and stored clinical samples and 2) to generate ITS region sequences that may provide an insight into strain diversity.

Methods

The methods for extracting DNA from equine pus were optimised prior to field sampling as follows. DNA extraction was tested on pus samples collected from horses in the UK (incisional site infection and sinusitis samples collected at surgery) and pus samples spiked with a 1/10 (v/v) cell culture suspension of *Saccharomyces cerevisiae* as a proxy for HCF in order to demonstrate that fungal DNA could be recovered from pus. DNA preparations were obtained using the Qiagen blood and tissue kit according to manufacturers' instructions but using a starting volume of 50µl of pus and extending the incubation at 56^0C to 2-3 hours to ensure adequate lysis of the sample. The DNA yield was assessed by Nanodrop followed by running the genomic DNA extract on a 2% agarose gel stained with Midori green.

Case selection and sampling

Field sampling of clinical cases presented to the SPANA mobile veterinary clinical team was undertaken in Ethiopia between February and April 2014. Cases were selected from each of 7 SPANA clinic sites on the basis of clinical signs suggestive of infection with HCF provided that they presented palpably fluctuant and unruptured nodules. Sampling regions varied by altitude, topography and climate (Table S1). Verbal informed consent was sought from all participating owners, and the study was approved by the University of Liverpool Research Ethics Committee and the Addis Ababa University, College of Veterinary Medicine and Agriculture's ethics board prior to commencement. The horses of all owners attending SPANA clinics received free treatment regardless of their decision to volunteer for the study.

A 10ml jugular blood sample was taken from each case into EDTA and plain Vacutainer tubes. The area surrounding two unruptured cutaneous nodules was shaved, aseptically prepared, and the contents aspirated with a ½ inch 16 gauge needle. Aspirates were immediately transferred into separate sterile Eppendorf tubes and stored in a cool box prior to transfer to a refrigerator, and were processed within 24 h. Horses with respiratory signs were sampled using a nasal swab inserted into the rostral 10cm of the nasal mucosa. The swabs were placed directly into a universal bottle containing 20ml sterile saline. Where necessary, horses were sedated to facilitate sampling and allow subsequent treatment.

Blood samples were collected from a further 20 horses from 5 highland regions where little or no EZL had previously been reported (4 horses from each region, Table S1). These horses did not have any cutaneous lesions suggestive of EZL and were randomly selected for comparison from a larger cohort of 350 horses being sampled for the presence of respiratory pathogens. Alongside the clinical sampling and treatment, a short questionnaire was delivered to owners to gather information on the clinical presentation, history of infection, and any previous treatment. The signalment, case presentation and clinical examination findings (performed by a veterinary surgeon/animal health professional) were recorded and lesion location recorded on equine silhouettes (Fig. 1). Suspected EZL cases were categorized as mild, moderate or severe based on a previously developed grading system [18] (Table S2).

Sample processing in Ethiopia

Slide preparations of pus, blood and nasal swab impression smears were stained with Giemsa using a 1:20 working solution [41]. The pus samples were examined for the presence of yeast cells suggestive of *Histoplasma* infection [42] at x1000 magnification under oil immersion.

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Differential blood cell counts were calculated [43], the haematocrit (PCV) measured with capillary tubes, and the total protein measured with a hand-held refractometer [44].

Genomic DNA preparations from pus and blood were made using a Qiagen DNeasy Blood and Tissue kit in the clinical laboratory at SPANA, Debre Zeit, Ethiopia. Blood and 50µl pus samples were processed according to the manufacturer's protocol. For the lysis step, the pus samples were heated in a water bath at 56^{o}C for 2-3 hours, until the lysate appeared clear. The eluted DNA preparations were dried by evaporation from the Eppendorf tubes at 40^{o}C. Eighty DNA extractions from blood and pus samples were transported to the University of Liverpool under UK (DEFRA) approved licensing.

FTA card preparation

To examine the use of Whatman FTA cards as a convenient method to capture, transport and store DNA, pus and blood samples from case horses were placed onto classic Whatman FTA indicating cards (GE Healthcare). Aliquots (0.5ml) of pus diluted with 0.5ml of sterile saline were inactivated by heat treatment at 95^{o}C for 30 min in a water bath prior to applying 200µl of sample to the card [45]. Two 200µl blood spots and two heat-inactivated pus samples from each horse were placed onto separate sample zones and the loaded FTA cards were air dried overnight before being microwaved at full power (800 Watt) for 30 s, left to stand for one min, and microwaved for a further 30 s. A glass beaker containing >200ml of water was placed alongside cards in the microwave to dissipate heat. Heat inactivated cards were individually placed into sealable plastic bags and despatched to the University of Liverpool where they were stored at 4^{o}C upon arrival.

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Validation of a nested-PCR protocol for detecting HCF in DNA preparations

DNA preparations from HCF reference strain CBS 539.84 and HCC reference strain CBS 137.72 were obtained from CBS-KNAW Fungal Biodiversity Centre, Netherlands and used as positive controls. DNA extracts of *Saccharomyces cerevisiae*, *Escherichia coli* and pus collected at surgery from a horse in the UK were used as negative controls.

Precipitated DNA samples (Qiagen) were rehydrated with DNA and RNA-free water, and analysed by Nanodrop. Aliquots of 50ngμl^{-1} DNA were prepared and stored at -20^{o}C before use. A nested PCR protocol targeting the ITS region coding for rRNA genes was adapted from that published by Jiang *et al.* 2000 [32]. Both sets of primers (P3 / 2R8 and F5 / 2R5) spanned the ITS1-5.8S-ITS2 region [32]. The optimum annealing temperatures for each primer pair were determined with control HCF template DNA. Each reaction mixture contained 50ngμl^{-1} of template DNA and 10pmol concentrations of PCR primers P3 and 2R8 added to Biomix red (Bioline) in a 25µl volume reaction as follows: 12µl Biomix red; 2µl forward and 2µl reverse primer; 8µl H_2O; 1µl DNA template. The first round PCR using P3/2R8 primers (P3 5'-CGGAAGGATCATTACCACGCCG-3', 2R8 5'-CAGCGGGTATCCCTACCTGATC-3') was performed on the following thermocycler programme; 94^{o}C for 10 min (denaturation), then 35 cycles of 94^{o}C for 1min, 49^{o}C for 1min then 72^{o}C for 1min followed by a final extension cycle of 72^{o}C for 10min. The product for the 1st round was expected to be 587bp and this was visualised by electrophoresis on a 2% agarose gel stained with Midori green compared to a 1kb hyperladder (Bioline). A 1 in 10 (v/v) dilution of product from this first reaction was added to fresh master mix including 10pmol concentrations of primers F5 and 2R5 (F5 5'-CTACCCGGCCACCCTTGTCTAC-3', 2R5 5'-CCTACCTGATCCAGTCAACC-3'). The thermocycler

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programme for the second round was the same except the annealing temperature was raised to 55°C for 1 min. The expected product was 514bp and was visualised on a 2% (w/v) agarose gel stained with Midori green (2µl per 100ml agarose) via electrophoresis at 70V for 30 minutes. Excess primers and nucleotides were removed from PCR amplicons using ExoSAP-IT (USB products) and the forward sequence determined (GATC Biotech).

DNA extraction from pus and blood samples on Whatman FTA cards

The FTA cards were prepared for analysis using a hole punch (Knippex 9070220, Germany). The following protocol produced the most satisfactory DNA yield from both blood and pus samples on Whatman FTA cards and was applied throughout. For each pus and blood sample spot, 4 x 5mm hole punches were produced. Between samples, the hole punch was sterilised by immersion in 100% ethanol followed by flaming. The FTA punches were placed into a screw cap tube containing 0.5g acid washed glass beads (425-600µm diameter), 0.5 ml H_2O and 0.5 ml Phenol Chloroform Isoamyl alcohol (25:24:1 pH8). The solution was mechanically disrupted using a Powerlyser homogeniser at 2100 rpm for 5 min and then centrifuged at 8000xg for 3 min. The uppermost aqueous phase was extracted into a sterile tube and 0.5 ml chloroform added. The mixture was centrifuged at 8000xg for 3 min and the aqueous phase was again collected. DNA was precipitated by adding 1 ml of ethanol and 5µl of 200mM sodium acetate and incubating the mixture at -20°C for > 2h. The ethanol precipitate was microcentrifuged at 13,000xg for 10 min and the supernatant removed and discarded. The DNA pellet was air dried then reconstituted in 50µl sterile DNA and RNA free H_2O. The resulting DNA preparation was

analysed using a Nanodrop prior to being tested for the presence of HCF DNA by nested PCR as described above.

Cloning PCR products

A sub-sample of 9 *Histoplasma* positive amplification products of the second round of the nested PCR were cloned in E.coli [46]. DNA bands of the expected size from the second round of the nested PCR were excised and extracted using Bioline PCR and Gel Kit (Bioline). 2 µL of each of the DNA preparations were mixed with 5 µl ligation buffer (60mM Tris-HCl [pH 7.8], 20mM $MgCl_2$, 20mM DTT, 2mM ATP, 10% PEG), 1 µl pGEM-T Easy plasmid vector containing an ampicillin resistant gene (Promega), 1 µl T4 DNA Ligase preparation and made up to 10 µl with deionised water. This reaction was then left overnight at 4°C and 4 µl of the ligation reaction mixture was then added into 100 µl aliquots of thawed Top10 *Escherichia coli* competent cells and placed on ice for 20 min. The cells were subsequently heat shocked at 42°C for 45-50 s, returned to ice for a further 2 min and 950 µl of LB then added to each aliquot before incubation at 37°C with shaking for 1.5 h. 100 µl of cell suspension were then pipetted onto LB agar plates containing 100 $\mu g ml^{-1}$ Ampicillin, 100 µl of IPTG (100 mM), and 20 µl of X-Gal (50 mg/ml) for blue/white screening. White colonies (containing inserts) were then picked and each inoculated into 10 ml LB plus 100 $\mu g ml^{-1}$ Ampicillin, and incubated for 16-24 h at 37°C. Five colonies from each of the 9 samples were randomly selected, and the plasmids then purified from the *E. coli* cultures (Plasmid Mini Kit – Isolation of High-Copy plasmid DNA, Bioline) and the presence of the target DNA in the plasmid confirmed by amplification with the primer pair F5/2R5 used above. Each plasmid DNA preparation was then sequenced (GATC Biotech AG). All

sequence data were analysed using BLAST search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cloned sequence data were edited to remove any plasmid DNA, leaving fragments of ca. 514 nucleotides in length encoding the 18S rRNA internal transcribed spacer regions. These fragments were then aligned using ClustalW2 software.

Screening for Corynebacterium species by multiplex PCR.

As *Corynebacterium pseudotuberculosis* (causing ulcerative lymphangitis) is one of the differential diagnoses for the cutaneous presentation of EZL [2, 9], all 80 DNA extracts from pus and blood samples were screened by multiplex PCR for the presence of *Corynebacterium spp*. A freeze dried culture of *Corynebacterium pseudotuberculosis* (DSMZ No. 20689) was obtained from the Liebniz institute DSMZ (www.dsmz.de/), cultured in brain heart infusion media with glucose (5mgml^{-1}), and incubated at 37^{o}C with agitation. DNA was extracted [47] and a multiplex PCR was used to amplify the 16S rRNA gene (816 bp), *rpoB* (encoding the RNA polymerase β-subunit) (446 bp) and *pld* (encoding an exotoxin) (203 bp) present in the genome of *C. pseudotuberculosis* using the six oligonucleotide primers described in Pacheco *et al.* [48]. The amplification products were visualised on a 1% (w/v) agarose gel stained with Midori green.

Statistical analyses

Descriptive statistics of the clinical examination data were produced and comparisons made between case and control horses using SPSS software, with the cut-off for statistical significance set at $p<0.05$. The different diagnostic methods (nested PCR, microscopy and pattern recognition of clinical signs) were compared using diagnostic test comparison tables [49]. Univariable logistic regression analysis was used to compare clinical signs.

Results

In total, 29 case horses with suspected EZL and 20 horses, selected as comparisons from highland regions (>2,300 msl) in Ethiopia where EZL has not previously been reported, comprised the study. This included 28 geldings, 18 stallions, 1 mare and 2 unrecorded. Supplementary Table S3, summarises the questionnaire and clinical examination findings for all case and control horses. Among the 29 presumptive EZL cases, 11 (38%) were classified as early, 12 (41%) as moderate and 6 (21%) as severely affected cases (Table S2 and Fig. 1).

Three of the case horses had signs of lower respiratory disease: 1 categorised as mild EZL with lesions on the lateral neck and generally increased respiratory noise detected on thoracic auscultation; 1 categorised as moderate EZL and observed to be coughing with bilateral mucopurulent nasal discharge and retropharangeal lymph node swelling; 1 categorised as severe EZL with bilateral mucopurulent nasal discharge and ulceration apparent on the nasal mucosa. Nasal swabs were collected from the latter 2 horses and tested positive for HCF by the nested PCR diagnostic method reported here. A further seven of the case horses presented with a range of mild upper respiratory signs.

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273 Five of the case horses showed varying degrees of lameness, and 9 had ocular abnormalities
274 present. No typical ocular signs of EZL lesions were seen among these cases, as has been
275 indicated previously [2, 3]. EZL case horses tended to demonstrate eosinopaenia and
276 monocytosis in comparison to controls (Table S3, $p<0.05$), which is consistent with a chronic
277 pyogranulomatous infection.

278 ***Diagnostic tests***

279 Of the 29 case horses, impression smears were prepared from a total of 27 pus and 28 blood
280 samples (in 2 horses with cutaneous lesions it was not possible to aspirate nodules, and for 1
281 horse only a pus sample was taken). Yeast cells were apparent in 14 (52%) pus smear
282 preparations as determined by light microscopy (Fig. 2). No yeast cells were visible on any of
283 the blood smear preparations.

284 A total of 80 Qiagen DNA extractions were made from blood, pus and nasal swabs from 29 case
285 horses (duplicate extractions were prepared for the majority). The overall DNA yield varied
286 between samples, with a median of 68 ngµl^{-1} and A_{260}/A_{280} ratio of 1.8. The DNA yields from the
287 FTA card samples processed by the phenol chloroform extraction method had a median yield of
288 57 ngµl^{-1}, A_{260}/A_{280} ratio 1.2.

289 Samples were scored positive if the nested PCR protocol produced 514bp amplicons, as
290 visualised by agar gel electrophoresis. In all cases, nested PCR was found to be necessary, and
291 amplification products could not be reliably detected after the initial round of amplification
292 with the P3/2R8 primer pair. Similarly, primary amplification with the F5/2R5 primer pair did
293 not yield products, nor did two rounds of amplification with these primers. Control DNA from *H*

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294 *capsulatum var. capsulatum* and *var. farciminosum* cultures could be amplified with the
295 individual primer pairs when used directly, but this was not nearly as reliable as the nested PCR
296 protocol. The detection limits of the nested PCR were tested using serial dilutions of HCC
297 control DNA template. At a 1/100 dilution, it was still possible to detect 0.5ng HCC DNA using
298 this nested PCR.

299 In total, 25 of the 27 EZL case horses tested positive based on analysis of Qiagen DNA extracts
300 from pus, and 17 of the 27 (63%) case horses from blood samples; examples are presented in
301 Fig. 3. Of the FTA card samples, 24 of the 27 pus samples and 23 of the 28 blood samples were
302 positive for HCF as determined by the nested PCR protocol. This all compares very favourably
303 with the low levels of detection of yeast cells (52%) in pus samples taken from these EZL horses.
304 The forward sequence determination for all nested PCR amplification products confirmed their
305 identity as histoplasmas (≥97% similarity by BLAST analysis; data not shown). Of the FTA card
306 blood samples from the 20 control horses (originating from outside of areas where EZL is
307 endemic, and showing no signs of disease), two positives were obtained by nested PCR (Table
308 S4). Sequencing also confirmed that these amplicons were from *Histoplasma* DNA. While the
309 PCR results obtained from the Qiagen extracts were reproducible for each of the 10 horses
310 repeated, the PCR results were not reproducible for all of the pus and blood spots presented on
311 FTA cards (Table S4); 4 of the 27 pus samples, and 9 of the 28 blood samples, gave positive
312 results that were not completely reproducible. Therefore, for the purposes of data analysis, we
313 identified a horse as positive for histoplasmosis if at least one of the pus samples or at least one
314 of the blood samples were positive for HCF on nested PCR.

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315 In addition to simple sequencing of potentially mixed PCR amplification products to confirm the
316 identity of amplicons as *Histoplasma*, we cloned 15 of these to produce a total of 43 clone
317 sequences of the amplified ITS region for more detailed phylogenetic analysis. Bona fide
318 sequence data were obtained for 38 full length 514 bp amplicons, and these were aligned (Fig.
319 S1).

320 Across the entire 514bp fragment of the ITS region sequenced here, there were nine consistent
321 single nucleotide polymorphisms (representing a 1.8% substitution rate) that divided the 38
322 clone sequences into two coherent subgroups. These are: position 83, TC; 249, TC; 328, GC;
323 342, AG; 366, AC; 439, AG; 3 sequential nucleotide substitutions at positions 449-451, CGT in
324 place of GTC. The alignment of these two subgroups and the comparable sequences of *H.*
325 *capsaulatum var. capsulatum* and *var. farciminosum* are presented in Fig. 4. Representatives of
326 each subgroup did occasionally originate from a single horse sample, and analysis of the
327 contextual metadata did not reveal any patterns in origin of sample or stage of disease.

328 Microscopy generally had a lower sensitivity and specificity compared to PCR for both Qiagen
329 and FTA card extracts (Table 1a). In all cases where yeast cells were visible on microscopy, the
330 PCR test was positive on pus samples, except in one instance among the FTA card results. Yeast
331 cells were never observed microscopically in blood samples, however HCF was detected by
332 nested PCR in many of these samples (16 positive among Qiagen extracts and 21 among FTA
333 card samples; Table 1a and S4). Among this case series, if clinical sign were apparent there was
334 >80% probability that the diagnosis would be confirmed by PCR of blood samples (Table 1b).
335 None of the 80 Qiagen DNA extracts from blood and pus samples tested positive for

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Corynebacterium spp. with the multiplex PCR diagnostic test [48] applied here, validated by the inclusion of appropriate positive and negative controls.

Discussion

The nested PCR primer sets developed by Jiang et al. [32] for *Histoplasma capsulatum var capsulatum* cultures were used here to develop a diagnostic test for HCF in clinical samples of horses. Two stage nested amplification was found to be necessary, and this may be due to the biological diversity within the clinical samples that contained a variety of equine DNA including immune cells and degradation factors, and potentially low numbers of yeast cells. This is comparable to other scenarios where nested PCR reactions are required to detect fungal targets in complex clinical samples [50]. Diagnostic reliability of the nested PCR protocol was much superior to conventional microscopy or sole reliance on clinical signs. In all cases, sequencing of the 514bp amplicons demonstrated the presence of *Histoplasma* DNA, and this was further confirmed by sequencing a large sample of clones. PCR methods targeting the ITS region have previously been used to identify HCC [28, 30] and have the additional advantage of enabling strain sequence variation to be explored [32]. In that respect, we also identified a collection of single nucleotide polymorphisms (SNP's) that suggest a delineation of at least two sub-groups of HCF circulating in the working horse population in Ethiopia. At this early stage,

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there is no evidence that the occurrence and distribution of these two sub-groups correlates with clinical signs and severity of disease, geographical location, sample source, or any other identifiable parameter. In fact, both sequence variants were recovered from the same animal on a few occasions. Alignment of HCF and HCC ITS region sequences shows a high degree of conservation, in agreement with the minimal diversity that was reported among *Histoplasma spp.* in the ISHAM ITS barcoding project [33], so it may be more appropriate to target alternative genetic loci in histoplasmas in any search for markers of variation that may have epidemiological utility. Previous molecular taxonomic studies have identified a close link between HCC and HCF [26, 27] and therefore characterising strains from currently endemic regions is appropriate. There are many unanswered questions, eg. can HCF be zoonotic, is there any strain variation that impacts on virulence and clinical presentation, is there host specificity, what are the modes of transmission, are vectors involved and what is the epidemiology of EZL? The biological relationship and functional difference between HCC, a relatively well studied and principally human pathogen, and HCF, primarily a specific pathogen of equids, is unknown. Representative strains of *Histoplasma spp.* (including HCC and HCF) from endemic regions on the African continent are especially lacking, and this is a public health blind spot considering the high burden of infection [37-40].

Another important aspect of this study is the demonstration that the nested PCR assay can be used to detect HCF in inactivated pus and blood samples dried onto Whatman FTA cards. These cards have been used extensively to archive a range of human, animal, plant, bacterial, fungal and viral pathogens, and are a convenient and cost effective method to store DNA and RNA for downstream molecular applications without the need for specialist equipment [45, 51, 52].

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Here, the advantages of Whatman FTA cards for sampling in the field, for inactivating pathogens, and for transporting and storing samples of equine pus and blood for application of molecular diagnostic methods are obvious. They are very suitable for use in countries with limited resources but ideally we would hope to simplify the DNA extraction protocol, especially finding an alternative to phenol chloroform treatment, which was used because it was found to be very effective compared to a number of other methods tested. The stability of DNA in equine pus and blood samples stored on FTA cards also needs to be established; previous studies have identified issues concerning storage and sample longevity [53].

This is the first report of PCR based detection of HCF directly in equine blood. PCR detection of *Histoplasma spp.* directly from human blood samples has been reported previously [54-56] and a fungaemic stage is recognised in human infection [57, 58]. Our data suggest the identification of HCF fungaemia in horses, although further work is required to determine the timing of the development of fungaemia, and investigate the potential for early diagnosis of HCF directly from blood samples. Furthermore, our identification of HCF DNA in two blood samples from horses with no clinical signs of EZL, could be due to subclinical infection and/or asymptomatic carriage, both aspects of EZL about which there is currently no information.

Ideally, horses in Ethiopia and other countries where EZL has been reported, should not be diagnosed solely on the basis of clinical signs. There are a number of other pathogens causing infections which have similar clinical presentations. Although we were able to exclude ulcerative lymphangitis by demonstrating that all 29 horses were PCR positive for HCF and simultaneously negative for *Corynebacterium* spp., we did not address Sporotrichosis [59] and

the cutaneous (farcy) form of glanders [20] both of which can easily be confused with EZL. Sero-conversion in response to glanders exposure has recently been reported among donkeys within similar regions of Ethiopia [60], which further emphasises the importance of establishing the causative agent of disease in individual horses that present with ulcerative and pyogranulomatous lesions.

Histoplasmosis is not only a problem among horses, but also human populations, where HCC has been recognised as a severely underdiagnosed chronic disease [38, 61-64]. Among a large case-series in people, it was reported that a spectrum of presenting clinical signs could be associated with histoplasmosis and this had implications for delays in clinical recognition, requests for suitable diagnostic tests and the initiation of trial treatment, and ultimately, case outcome [65]. Likewise, Lesion location (Fig.1) and clinical presentation of EZL varied widely among the equine cases presented here, and HCF was also identified in DNA extracts from nasal swab samples from two of the most severe cases with respiratory signs. The well characterised human variant, HCC, is predominantly a respiratory pathogen [37] whereas, the role of HCF as a respiratory pathogen in horses is largely unknown [2, 4, 7, 66].

A recent workshop on infectious disease of working equids called for 'increased research to address technical data gaps, advocacy to secure funding, and improved surveillance at national and international level to allow further understanding of pathogenesis, diagnosis, treatment and prevention of disease such as Epizootic lymphangitis' [21]. Robust, reliable and rapid diagnostic tools are essential for both decision making by veterinarians and to enable large-scale studies that will allow further understanding of the epidemiology, ecology and

transmission of this neglected disease. Ideally, rapid diagnostic techniques could be beneficially utilised within endemic regions and therefore, future work should focus upon examining the practicalities and potential for transfer of technology to regional laboratories, academics and clinicians within Ethiopia, and other regions. Resources to perform PCR are currently available in only some research laboratories within Ethiopia, where diagnostics could be further developed. A WHO report investigating research facilities in national health research systems in sub-Saharan Africa stated that 'major constraints are dominated by financial input for equipment purchase, maintenance and laboratory supplies' [67]. These factors need to be addressed in order to support and action future research development within disease endemic regions.

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Author contributions

C.S., A.M., G.P., and R.C. conceived the study and developed the study design. C.S., T.A., N.A. & A.S. co-ordinated and conducted the data collection and processing of samples in Ethiopia. C.S., P.L. and A.M. developed the laboratory protocols and processed samples in the UK, with further assistance from L.G. for Whatman FTA cards and M.M. for *Corynebacterium* species detection and preparation of the clones of the HCF PCR sequences. C.S. and R.C. conducted the statistical analyses, with bioinformatics analysis of sequence data facilitated by A.M. C.S., A.M. and G.P. prepared the manuscript, and all authors contributed to and approved the final draft.

List of reagents and manufacturers

Qiagen DNeasy blood and tissue kit

Whatman FTA cards, GE Healthcare UK Limited, Buckinghamshire.

Biomix red, Bioline reagents limited, UK.

ExoSAP-IT, USB products, High Wycombe, UK.

Isolate II PCR and Gel Kit, Bioline reagents limited, London UK.

pGEM-T Easy plasmid vector, Promega corporation, Madison, USA.

Isolate II Plasmid Mini Kit, Bioline reagents limited, London UK.

Software

BLAST search engine available at http://blast.ncbi.nlm.nih.gov/Blast.cgi

Webplot digitizer software, Ankit Rohatgi available at http://arohatgi.info/WebPlotDigitizer

R: A language and environment for statistical computing, R foundation for statistical computing, Vienna, Austria.

SPSS Statistics version 21, IBM corporation, USA.

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654 Tables and Figures

655 Table 1a: Comparing microscopic visualisation of yeast cells in pus with nested PCR detection using DNA extracts (from Qiagen and FTA card

656 preparations) of pus and blood from horses suspected of clinical infection with epizootic lymphangitis.

	PCR result from Qiagen extracts from pus*		**PCR result from Qiagen extracts from blood***		**PCR result from FTA card preparation of pus***		**PCR result from FTA card preparation of blood spot***	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Yeast cells **Present** in pus on microscopy	14	0	7	7	12	1	11	3
Yeast cells **Absent** in pus on microscopy	11	2	9	2	12	1	10	2
Total	25	2	16	9	24	2	21	5
	Comparison of microscopy with PCR Qiagen pus extracts		**Comparison of microscopy with PCR Qiagen blood extracts**		**Comparison of microscopy with PCR from pus on FTA cards**		**Comparison of microscopy with PCR from blood on FTA cards**	
Sensitivity[1]	0.56		0.44		0.5		0.52	
Specificity[2]	1		0.22		0.5		0.4	
PPV[3]	1		0.5		0.92		0.79	
NPV[4]	0.15		0.18		0.08		0.17	

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Table 1b: Comparing diagnosis based on the presence or absence of clinical signs of EZL with results of nested PCR (using FTA card blood spots from 48 horses including 28 with clinical signs of EZL and 20 with no clinical signs).

	PCR result from blood on FTA cards*	
Presumptive EZL based on clinical signs	Positive	Negative
Yes	23	5
No	2	18
Total	25	23
	Comparing performance of diagnosing based on clinical signs compared with PCR	
Sensitivity[1]	0.92	
Specificity[2]	0.78	
PPV[3]	0.82	
NPV[4]	0.9	

Footnotes for Tables 1a and 1b

The nested PCR tests result is considered as the gold standard here because a known HCF control was used in the test.

*PCR test results based on at least one positive PCR test per horse.

NB no blood was taken from 1 horse with clinical signs suspicious of EZL; one horse did not have a Qiagen DNA extract made from a blood sample.

[1] The sensitivity describes the proportion of microscopy positive samples that are correctly diagnosed compared with the PCR results.

[2] The specificity describes the proportion of microscopy negative samples that are correctly diagnosed compared with the PCR results.

[3] PPV positive predictive value of microscopy compared to PCR, or the probability of a positive result on microscopy given that the test is positive by PCR.

[4] NPV negative predictive value of microscopy compared to PCR, or the probability of a negative result on microscopy given that the PCR result is negative.

678 Figure Legends

679 Figure 1: Cutaneous EZL lesions observed in the infected horses. A) Mild case – lesions evident
680 in only one body area; B) Moderate case - lesions distributed over left forelimb and other body
681 sites, and moderate cording on forelimb; C) Severe case – multiple coalescent nodules over all
682 four limbs and extensive lesions on the face; D) Spatial distribution of cutaneous lesions of EZL
683 across 29 horses with cutaneous lesions suspected to be EZL, plotted onto silhouettes using
684 Webplot digitizer and R software. The plot demonstrates that cutaneous lesions were more
685 densely distributed around the forelimbs, neck, chest and girth regions.

686 Figure 2: Light micrograph of a pus impression smear from an EZL case horse stained with
687 Giemsa and examined for the presence of *Histoplasma* yeast cells. Impression smear of pus
688 aspirated from unruptured subcutaneous nodule viewed at x1000 magnification. The arrows
689 demonstrate clusters of ovoid to lemon shaped yeast cells, 4-5µm diameter with characteristic
690 refractive cell wall. For comparison, an equine neutrophil is approximately 12-15µm in
691 diameter.

692 Figure 3: Gel electrophoresis of nested PCR amplification products obtained from DNA extracts
693 of horse pus and blood samples. DNA preparations were amplified with P3/2R8 primers (first
694 round) then diluted 1 in 10 and subjected to a second round of PCR amplification with F5/2R5
695 PCR primers to generate ITS gene products (514bp) indicative of the presence of *Histoplasma*
696 DNA. All amplification products were subsequently sequenced to confirm >97% identity and
697 closest match to *Histoplasma capsulatus* ITS region DNA. Lanes 1-4 negative controls, DNA
698 extracts from *S.cerevisiae*, *E.coli*, equine pus from UK and DNA and RNA free water; Lane 5, HCC

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control DNA; Lane 6, HCF control DNA; Lanes 7 -25, PCR amplicons of Qiagen DNA extracts of blood and pus from horses with suspected EZL (lanes 7-23 DNA extracts from pus, lanes 24-26 DNA extracts from blood, Lane 9 MW marker).

Figure 4: Sequence alignment of 514 bp cloned fragments of the *Histoplasma* ITS region. The diagram illustrates the 9 consistent SNP's (arrowed) identified here that divide the HCF ITS clones (38 clones) into two sub-groups. Subgroup 1 comprised 23 of the clones, and subgroup 2 the remaining 15. The numbers at the top of the figure are the nucleotide positions in the ITS. The two reference sequences of HCC (*Ajellomyces capsulatus*) and HCF were downloaded from Genbank.