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Clinical Performance of Real-Time Polymerase Chain Reaction for *Strongyloides stercoralis* Compared with Serology in a Nonendemic Setting

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Abstract. *Strongyloides stercoralis* is a nematode endemic to subtropical and tropical regions that may cause asymptomatic carriage, peripheral eosinophilia, cutaneous, gastrointestinal, and pulmonary disease, or hyperinfection syndrome. Conventional diagnostic methods for strongyloidiasis include feces microscopy and culture, with low sensitivity in chronic infection due to the low helminth burden, and serology, which may be prone to false-negative results with immunocompromise and false-positive results with other infections and immunological disorders. We evaluated a laboratory-developed real-time polymerase chain reaction (RT-PCR), detecting the 18S SSU ribosomal RNA gene, compared with conventional diagnostic methods, using serology via ELISA as the gold-standard. The population studied included tertiary hospital inpatients and outpatients residing in a nonendemic area. Seven hundred fifty unfixed stool specimens submitted sequentially between 2014 and 2018 were tested for *S. stercoralis* via microscopy and RT-PCR. Agar plate culture (APC), Harada-Mori culture (HMC), and ELISA were performed in conjunction with 141, 135, and 177 of the specimens, respectively. RT-PCR yielded 13 positive and 730 negative results, with inhibition in seven specimens. ELISA yielded 53 positive, 18 equivocal, and 106 negative results. Results for direct diagnostic methods obtained after treatment with ivermectin were excluded from the performance analysis. Compared with ELISA, RT-PCR, microscopy, APC, and HMC exhibited sensitivities of 38%, 6%, 3%, and 0%, respectively, and specificities of 100%. Given the low sensitivities commensurate with testing a population with remote infection and thus low parasite burden, we recommend a combination of serological and molecular diagnostic testing to achieve the best balance of sensitivity and specificity.

INTRODUCTION

Strongyloides stercoralis is a nematode endemic to tropical and subtropical regions, including northern Australia, that infects humans. Although two other species of the *Strongyloides* genus, *S. fuelleborni* and *S. kellyi*,[1] can also infect humans as well as nonhuman primates, *S. stercoralis* is the more significant pathogen, infecting an estimated 30 to 100 million people worldwide.[2] In Australia, the seroprevalence of strongyloidiasis is as high as 25% among indigenous people living in remote communities[3] and 11% to 36% among migrants from endemic areas.[4–7] Infection occurs when filariform larvae penetrate intact skin. The filariform larvae migrate hematogenously to the lungs then ascend via the tracheobronchial tree to the pharynx where they are swallowed. In the small intestine, filariform larvae develop into adult worms, which mate and lay eggs. The eggs hatch to produce rhabditiform larvae, which either develop into filariform larvae that penetrate the intestine or perianal skin, resulting in autoinfection, or undergo excretion in feces into the environment where they develop into adult worms.[8] Clinical syndromes of strongyloidiasis range from asymptomatic carriage with or without peripheral eosinophilia to cutaneous (e.g., larva currens), gastrointestinal, and pulmonary manifestations, and hyperinfection syndrome, which arises from autoinfection and dissemination of large numbers of filariform larvae to organs resulting in bacteremia, organ dysfunction, or shock and typically affects patients with impaired cell-mediated immunity (e.g., glucocorticoids, hematopoietic stem cell transplantation, HTLV-1 infection).[9] Conventional parasitological diagnostic methods for strongyloidiasis include feces wet mount microscopy or culture, with low sensitivity in chronic infection due to the low helminth burden, and serology, which may be prone to false-negative results with immunocompromize and false-positive results with other infections and immunological disorders.[10,11]

We evaluated the clinical performance of a laboratory-developed real-time polymerase chain reaction (RT-PCR), detecting the 18S SSU ribosomal RNA subunit gene, compared with conventional diagnostic methods, using serology via ELISA for crude antigen as the gold standard. Microscopic identification of *Strongyloides* larvae has been considered the gold standard technique by which to evaluate molecular tests for this pathogen; however, it is possible that RT-PCR is more sensitive, perhaps more so in chronic infestation, which would be more common in nonendemic areas characterized by populations including large numbers of migrants from endemic areas, such as the area the study was conducted in.

MATERIALS AND METHODS

Specimens and study design. Seven hundred fifty sequential unfixed stool specimens were submitted to the microbiology laboratory at Concord Repatriation General Hospital (CRGH), a tertiary referral hospital in Sydney, Australia, for microscopy and RT-PCR testing to detect *S. stercoralis* between 2014 and 2018. The vast majority of specimens were obtained from inpatients and outpatients from CRGH, with a small number of specimens from other hospitals and private laboratories in metropolitan Sydney. Medical records and pathology request forms associated with these patients were reviewed to determine whether patients had been previously treated with ivermectin or other agents for *Strongyloides* infection and for peripheral blood eosinophilia or comorbidities. The direct parasitological

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methods of wet mount microscopy, laboratory-developed RT-PCR, agar plate culture, and Harada-Mori culture were performed on these specimens by scientists trained and experienced in parasitology. One hundred seventy-seven patients had a serology result available, and excluding those previously treated for *Strongyloides*, direct methods were compared with serology as the best available gold standard to calculate sensitivity and specificity; equivocal ELISA results were considered negative for this purpose. Patient factors associated with equivocal ELISA results were analyzed.

Wet mount microscopy. Unfixed stool specimen was homogenized in phosphate-buffered saline then centrifuged, and the pellet was then examined via light microscopy.

Agar plate culture. One hundred forty-one unfixed stool specimens were inoculated onto 2% agar, 0.3% meat extract, 0.5% peptone, and 0.5% sodium chloride plates incubated for 10 days at 35°C and examined for furrows with or without bacterial growth via stereomicroscopy at days 2, 4, 5, and 7. At day 10, the plate was flooded with sodium acetate, acetic acid, and formalin (SAF) fixative, which was then collected and centrifuged and the pellet examined for larvae via stereomicroscopy.

Harada-Mori culture. One hundred thirty-five unfixed stool specimens were inoculated onto filter paper above sterile water such that the filter paper contacted the water and incubated for 10 days at 30°C. The water was sampled and centrifuged, and the pellet was examined for larvae via stereomicroscopy at days 4 and 10.

Real-time PCR. *Nucleic acid extraction.* Feces specimens were sampled with a cotton-tipped swab and mixed in 1 mL of sterile water, and 500 μL of well-mixed sample was placed into BeadBug tubes, containing 0.5 g of 0.1-mm glass beads. These were homogenized for three minutes at 400× speed on the BeadBug high-energy benchtop homogenizer for cell disruption and lysis (PathTech, Melbourne, Australia). Tubes were heated for 10 minutes at 96°C in a heating block, then centrifuged for 3 minutes at 13,000 × *g*. DNA was extracted from 200 μL of supernatant, with 5 μL of Equine Herpes virus (EHV) solution added as an internal and extraction control, (CSIRO Australian Animal Health Laboratory, Geelong, Australia), using the EZ1 Tissue Kit (Qiagen, Germany), to a final volume of 50 μL.

Primers, probes, and thermocycling conditions. Four microliters of extracted DNA solution was added to 16 μL of mastermix, comprising 10 μL SensiFAST Probe No-ROX kit (Meridian Bioscience, Sydney, Australia), 1 μL each of *Strongyloides* forward and reverse primer (10 μM) and probe (5 μM), and 1 μL each of EHV forward and reverse primer (10 μM) and probe (5 μM) (5′-3′ EHV F-primer GCGAAGAGACTGTGGTAATTTACG, EHV R-primer TGGCAAGTTTACATTTCCTAATTGG, EHV probe HEX—TCTCGCTTCTATACACCGTAC—BHQ1, Sigma Aldrich, Merck, Darmstadt, Germany). Amplification of a 101 base-pair region of the 18S small subunit ribosomal RNA *Strongyloides* gene (AF279916) was performed using forward primer 5′-GAATTCCAAGTAAACGTAAGTCATTAGC-3′, reverse primer 5′-TGCCTCTGGATATTGCTCAGTTC-3′, and probe 5′-ACACACCGGCCGTCGCTGC-3′ labeled with fluorescein amidite (FAM) dye at the 5′ end and Black Hole Quencher-1 (BHQ1) dye at the 3′ end. Primers were selected based on a study conducted in northern Ghana, which demonstrated excellent sensitivity and specificity.[12] The reaction mix was run on the Rotorgene Q real-time PCR thermocycler (Qiagen, Hilden, Germany) at 95°C for 15 minutes, then 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Results from the RotorGene software were interpreted by trained and experienced scientists, and considered positive if exponential curves were seen. If indeterminate amplification was seen, the sample underwent repeat extraction and RT-PCR and resulted appropriately. If no exponential curve was seen for EHV, the sample was considered inhibited and therefore invalid. An in-house positive control, sourced from a patient with strongyloidiasis confirmed by experienced microscopists at multiple laboratories, positive serology for *Strongyloides*, and a negative control were included in each run. Results were considered valid if positive, and negative controls demonstrated consistent results. Analytical specificity was assessed during the validation of the assay, with no cross reactivity with stool specimens containing *Salmonella*, *Shigella*, *Campylobacter*, *Clostridioides difficile*, *Entamoeba histolytica*, *Giardia*, *D. fragilis*, *Entamoeba nana*, *Entamoeba hartmanii*, *Entamoeba coli*, or *Cryptosporidium*.[13] During validation, the *Strongyloides* PCR product (when tested without EHV) underwent Sanger sequencing (NSW Health Pathology, Department of Molecular Medicine, Royal Prince Alfred Hospital) and BLAST search of the GenBank nucleotide database (National Center for Biotechnology Information, Washington, DC) demonstrated a 98% match with *Strongyloides stercoralis* 18S ribosomal RNA gene (GenBank accession M84229[14]).[13]

ELISA. Serum ELISA was performed in conjunction with 177 of the unfixed stool specimens using *Strongyloides ratti* larvae crude antigen via the method described by Carroll et al.[15] at NSW Health Pathology, Center for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia. The optical density of the tested sera relative to reference positive serum was determined and defined as negative (< 0.8), equivocal (0.8–1.2), or positive (> 1.2).

RESULTS

ELISA yielded 53 positive, 18 equivocal, and 106 negative results. Of the 18 equivocal ELISA results, none were associated with a positive culture, microscopy, or RT-PCR result. Three of the 18 equivocal ELISA results represented a decrease in the IgG titer in patients with a previous positive ELISA result and treated with ivermectin previously. One patient with an equivocal ELISA result was diagnosed with and treated for neurocysticercosis. The remaining equivocal ELISA results were obtained from patients with hematological and/or immunological disorders. Some of these patients were treated empirically with ivermectin, but subsequent ELISA results remained equivocal. APC, HMC, microscopy, and/or RT-PCR results associated with a positive ELISA result were excluded from the analysis if the patient had previously received ivermectin therapy. This was the case for 19 specimens with a negative microscopy and RT-PCR results, 16 specimens with a negative APC result, and 15 specimens with a negative HMC result. No specimens with a positive APC, HMC, microscopy, or RT-PCR result were obtained after treatment with ivermectin.

RT-PCR yielded 13 positive and 730 negative results, whereas assay inhibition occurred in seven specimens.

Microscopy yielded only two positive results and 748 negative results, APC yielded only one positive result and 116 negative results, and HMC yielded 0 positive results and 111 negative results. Compared with positive ELISA results after the exclusion of ivermectin-treated patients, RT-PCR, microscopy, APC, and HMC exhibited sensitivities of 38% (13/34), 6% (2/33), 3% (1/29), and 0% (0/24), respectively, and specificities of 100% (see Table 1). There were no microscopy positive samples that were PCR negative. Peripheral blood eosinophilia was observed in 74% (25/34) of patients with a positive ELISA result.

DISCUSSION

Our results differ from those of a systematic review and meta-analysis of 14 studies in which RT-PCR exhibited a sensitivity of 64% and specificity of 94% compared with parasitological methods and a sensitivity of 57% compared with a combination of parasitological and serological methods.[16] The superior performance of parasitological methods in this meta-analysis may reflect the higher parasite burden in acute infection in endemic areas because only two of the 14 included studies were performed in nonendemic areas. Only one of these two studies compared RT-PCR with parasitological and serological methods. In that retrospective observational study, 223 feces samples were submitted to the Center for Tropical Diseases of Negrar (Verona, Italy), for APC and RT-PCR in conjunction with serological testing via an in-house immunofluorescence antibody test (IFAT). APC, RT-PCR, and IFAT exhibited sensitivities of 45%, 57%, and 96%, respectively.[17] Although our study demonstrated a significantly lower sensitivity for APC, the sensitivity of RT-PCR, at 38%, was comparable.

The lower sensitivity of parasitological methods versus serology in nonendemic areas compared with endemic areas may reflect the lower helminth burden in chronic strongyloidiasis, although it may also be due to the poor specificity of the gold standard serology against which they are compared. In a nonendemic area, the specificity of serological methods is likely to be affected by the low prevalence of the condition. Another possible explanation for the low sensitivity for parasitological methods observed in our study may be because, in the vast majority of cases, patients submitted only one stool specimen. *S. stercoralis* larvae are shed intermittently, and it has been shown that the sensitivity of stool parasitological methods increases with the number specimens examined.[18] Additionally, although ELISA was used as the gold standard diagnostic test, the assay used a crude antigen of *S. ratti* larvae rather than *S. stercoralis* larvae with an unknown specificity, and false-positive results may have erroneously decreased the calculated sensitivity of parasitological methods and RT-PCR. Given the proportion of equivocal results and the high proportion of patients with a hematological or autoimmune diseases within this group, it may be surmised that the specificity of this assay is low. Specificity of a similar assay is 96.1% compared with an indirect immunofluorescence assay (90.1%) when tested on an Iranian population with other parasitic infections.[19]

Assessment of any diagnostic modality for *Strongyloides* is hampered by the lack of a truly sensitive or specific direct or indirect diagnostic method.[20] A recent prospective observational study of 21 patients with strongyloidiasis attending hospitals in Buenos Aires, Argentina, and residing in nonendemic areas also demonstrated results contrary to those of our study. Conventional PCR exhibited a sensitivity of 100% (21/21), whereas agar plate culture and microscopy exhibited a combined sensitivity of 66.7% (14/21). Conventional PCR remained positive throughout the median 730-day follow-up period after treatment with ivermectin, and strongyloidiasis reactivation was detected via parasitological methods on at least one occasion in 66.7% (14/21) of patients and on multiple occasions in 38.1% (8/21) of patients despite repeated treatment with ivermectin.[21] Conversely, none of the specimens tested in our study exhibited positive APC, HMC, microscopy, or RT-PCR after the patient was treated with ivermectin and 19 specimens exhibited negative APC, HMC, microscopy, or RT-PCR despite ELISA remaining positive after treatment with ivermectin. Given these findings, and those described by Requena-Méndez et al.,[20] we recommend employing a combination of molecular and serological methods for diagnosis and screening of strongyloidiasis, including parasitological methods for monitoring for reactivation after treatment.

TABLE 1
Results of direct parasitological methods for detection of *Strongyloides stercoralis* in unfixed stool specimens, compared with serum ELISA using *Strongyloides ratti* crude antigen, excluding results from patients after ivermectin treatment

	RT-PCR		Microscopy		Agar plate culture		Harada-Mori culture	
Serology	+	–	+	–	+	–	+	–
+	13	21	2	31	1	28	0	24
Equivocal	0	18	0	18	0	13	0	13
–	0	104	0	106	0	59	0	59

+ = positive; – = negative; RT-PCR = real-time polymerase chain reaction.

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