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BRIEF REPORT

Improving Molecular Epidemiological Surveillance of Strongyloidiasis Upon Differentiation of *Strongyloides fuelleborni fuelleborni* From *Strongyloides stercoralis*

Lucas J. Cunningham,[1] William D. Nevin,[1,2,3] Jaco J. Verweij,[4] Dora Buonfrate,[5] Salvatore Scarso,[5] Virak Khieu,[6] Angus M. O'Ferrall,[1] Sarah Rollason,[1,7] and J. Russell Stothard[1]

[1]Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Liverpool, Merseyside, United Kingdom; [2]Department of Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, Merseyside, United Kingdom; [3]Academic Department of Military Medicine, Royal Centre for Defence Medicine, Birmingham, United Kingdom; [4]Microvida, Laboratory of Medical Microbiology and Immunology, Elisabeth-Tweesteden Hospital, Tilburg, The Netherlands; [5]Department of Infectious, Tropical Diseases and Microbiology, Istituto di Ricovero e Cura a Carattere Scientifico Sacro Cuore Don Calabria Hospital, Verona, Italy; [6]Ministry of Health, National Centre for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia; and [7]School of Biosciences, Cardiff University, Cardiff, United Kingdom

Molecular epidemiological surveillance for zoonotic strongyloidiasis is confounded by a genus-specific TaqMan probe assay that conflates *Strongyloides fuelleborni fuelleborni* with *Strongyloides stercoralis*. To improve surveillance, we developed and validated a novel duplex species-specific TaqMan probe assay, screening a representative collection of available clinical samples. Our assay was highly discriminatory, evidencing no cross-reactivity, and had a lowest DNA detection limit of 1 pg/μL. However, as the genus-specific DNA assay has greater detection ability, we propose a 2-step protocol where samples are first screened with this assay then, if positive, screened with our species-specific assay, discriminating (co)infections between each threadworm species.

Keywords. zoonosis; threadworms; diagnostics; real-time PCR; TaqMan assay.

Strongyloidiasis is one of the 21 currently listed neglected tropical diseases (NTDs), being caused by infection with nematode threadworms, and is grouped within the soil-transmitted helminthiases (STHs) [1]. Despite occurring worldwide, research interests and control actions on this disease have lagged behind other STHs, earning it an ignominious label of a "neglected" NTD [1, 2]. Major hurdles and impediments include problematic diagnostics, poorly reported distributions, insufficient anthelmintics, and concurrent lack of specific funding [1, 3]. These deficits in research and control make it challenging to accurately target at-risk communities and control this disease globally [3, 4].

Infection with *Strongyloides stercoralis* is the primary cause, although other species such as *Strongyloides fuelleborni fuelleborni*, a common parasite of Old World nonhuman primates (NHPs), are incriminated. This latter species has a sister subspecies, *S fuelleborni kellyi*, found exclusively on Papua New Guinea [2]. While transmission of these threadworms takes place predominantly in the tropics, autochthonous strongyloidiasis can occur in more temperate regions [5]. Indeed, the movement of people across these boundaries can lead to infected migrants presenting to screening clinics within nonendemic countries [6]. Similarly, the movements of infected NHPs, such as baboons, can establish surprising transmission cycles, for example, within a United Kingdom (UK) safari park [7].

Diagnostic methods based upon coproscopy of direct fecal smears or Kato-Katz preparations are unsuitable for strongyloidiasis, owing to the scanty presence of *Strongyloides* larvae (*S stercoralis*) or eggs (*S fuelleborni* ssp) within feces [8]. To increase diagnostic sensitivity, fecal concentration methods are preferred that either isolate motile larvae from larger fecal volumes, with Baermann methods, or raise the total number of worms by incubation in charcoal culture [9]. Although serological diagnostics such as enzyme-linked immunosorbent assay [10] and indirect immunofluorescence antibody tests [11] exist, they are unable to discriminate infecting species [8].

Since the advent and introduction of real-time polymerase chain reaction (rtPCR) assays with TaqMan sequence-specific detection probes, there is only one clinically validated rtPCR assay for detection of strongyloidiasis [12]. Originally developed in 2009, it was then designed to be species-specific for *S stercoralis*, but due to the paucity of non-*S stercoralis* reference sequences available at the time, this assay was later appreciated to be less species-specific (ie, more genus-specific) than originally planned. This shortcoming was due to the highly conserved PCR primers and TaqMan probe annealing sites (Supplementary Figure 1). Though this genus-specific assay is routinely used today, having a detection sensitivity comparable to the most sensitive parasitological methods [12], it conflates

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Correspondence: J. Russell Stothard, PhD, Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, Merseyside L3 5QA, UK (russell.stothard@lstmed.ac.uk).

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S stercoralis with *S f fuelleborni*, thus confounding molecular surveillance of zoonotic strongyloidiasis.

To address this diagnostic shortcoming, we developed and validated a species-specific duplex rtPCR with species-specific TaqMan probes on clinical samples representative of typical screening needs.

METHODS

From bioinformatic analysis, the mitochondrial cytochrome c oxidase subunit I (*COX-1*) gene was selected owing to its interspecies variability (Supplementary Figure 2), with the following nontarget reference species used to create alignments to select for species-specific annealing sites for both PCR primers and TaqMan probes: NC028623 (*Strongyloides ratti*), NC028229 (*Strongyloides venezuelensis*), LC459356 (*Strongyloides vituli*), LC536597 (*Strongyloides planiceps*), and LC459360 (*Strongyloides papillosus*). Multiple *COX-1* reference sequences were used for *S stercoralis* (n = 24) and *S f fuelleborni* (n = 11) to represent suitable intraspecies variation within the targeted region (Supplementary Figure 2 and Table 1). The predicted annealing temperatures for the proposed PCR primers and TaqMan probe sites were estimated using the OligoCalc online oligonucleotide properties calculator [13], and the probes were designed to have an estimated annealing temperature of at least 5°C greater than that of the PCR primers.

Selected PCR primers underwent optimization in the laboratory to determine an ideal annealing temperature (58°C–60°C), primer/probe concentration (200–400 nM/100–300 nM), efficiency, and limit of detection (LOD). The latter used the following DNA dilution series: 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 1 pg/µL, 0.1 pg/µL, and 0.01 pg/µL of an in-house titration of reference genomic DNA extracted from charcoal-cultured larvae of each species. From cross-tabulation mixtures of DNA templates, cross-reactivity between *S stercoralis* and *S f fuelleborni* assays was assessed.

The following PCR primers and TaqMan probes were selected for optimization and later validation on a blinded set of diagnostic test samples: *S stercoralis* and *S f fuelleborni* universal forward primer 5′-GTACTAGTTGAACTRTTTATCCTCC-3′, *S stercoralis*–specific reverse primer 5′-CATAGTAATAGCACCAGCT AAAACAGGT-3′, *S stercoralis*–specific TaqMan probe 5′-[FAM]CAGGTCAYCCTGGTTCTAGTGTTG[BHQ1]-3′, *S f fuelleborni*–specific reverse primer 5′-CCYAAAATAGAAC TWACACCAGC-3′, and *S f fuelleborni*–specific TaqMan probe 5′-[HEX]TGGGTCATCCTGGGAGAAGTGTTG[BHQ1]-3′. PCR primer and TaqMan probe orientations within *COX-1* are shown in the schematic in Figure 1. All rtPCR reactions were carried out in a 12 µL total volume, comprising 6 µL of appPROBENo ROX Mix (2×) (Appleton Woods Ltd, Birmingham, UK), 3 µL of template with the rest of the volume comprising the specific optimization PCR primer/TaqMan probe concentrations and adjusted with nuclease-free water. The rtPCR platform used in this study was the Magnetic Induction Cycler from Bio Molecular Systems (London, UK) using the following cycling conditions: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 58°C–60°C for 30 seconds. In addition to a stool negative extraction control, our PCR protocols included both PCR negative (no target DNA) and positive (target genomic DNA) controls in each rtPCR amplification.

The optimized duplex assay was then validated against a blinded collection of available historical extracted fecal DNA samples that were previously confirmed to be positive for *Strongyloides* using the rtPCR genus-specific TaqMan probe assay [12]. A total of 96 clinical samples, provided by the authors, likely representative of 24 countries, were again rescreened with the genus-specific assay, then our novel duplex species-specific rtPCR assay (Figure 1). In Italy, amplifications were performed in a Bio-Rad CFX96 real-time PCR detection system with CFX Maestro software (Bio-Rad Laboratories) and in the UK amplifications were performed as described above. All clinical samples from which genomic DNA was extracted from feces were gifted for diagnostic research, after patient names were anonymized, and formed a historical archive.

RESULTS

The results of the optimization experiments found an optimum annealing temperature of 58°C, with this temperature having the lowest average cycle threshold (Ct) value at 24.4 and 25.2 for *S stercoralis* and *S f fuelleborni*, respectively. The optimum concentration of primers and probes was found to be 400 nM for the universal forward primer, 400 nM for the *S stercoralis* species-specific reverse primer, 300 nM for the *S f fuelleborni* species-specific reverse primers, and 300 nM for both the *S stercoralis* and *S f fuelleborni* species-specific probes. The LOD for both *S stercoralis* and *S f fuelleborni* in a duplex reaction was 1 pg/µL; no cross-reactivity was observed between the *S stercoralis* and *S f fuelleborni* templates and their respective species-specific assays. Efficiency of the species-specific duplex assay was calculated as 87.4% and 93.6% for *S stercoralis* and *S f fuelleborni*, respectively, using a 1:5 dilution series from 1 ng/µL–8 pg/µL of target templates.

Of the 96 clinical samples examined, using the novel species-specific duplex assay, 55 (57.3%) were identified as positive for either *S stercoralis* or *S f fuelleborni* or both (Table 1). Among these, 36 samples (37.5%) were positive alone for *S stercoralis*, 17 (17.7%) were positive alone for *S f fuelleborni*, and 2 (2.1%) showed mixed positivity for both *S stercoralis* and *S f fuelleborni*. The average Ct difference between the *Strongyloides* generic rtPCR assay and the species-specific rtPCR assay was −3.2, with a range of −3.5 to −5.6 cycles. Samples positive in both the generic and species-specific assays

Table 1. Results of the 96 Clinical Samples Screened Using the Duplex Species-Specific Assay

Country rtPCR Undertaken (n = Generic Positive)	No. of Samples Assigned to Each Threadworm Species			
	S stercoralis	*S f fuelleborni*	*S stercoralis* and *S f fuelleborni*	None
UK (n = 45)	11 (11.5)	13 (13.5)	0 (0.0)	21 (21.9)
Italy (n = 51)	25 (26)	4 (4.2)	2 (2.1)	20 (20.8)
Total (n = 96)	36 (37.5)	17 (17.7)	2 (2.1)	41 (42.7)

Data are presented as No. (%) unless otherwise indicated. All samples were previously identified as positive using the *Strongyloides* generic rtPCR assay although having a range of different cycle threshold values.

Abbreviations: rtPCR, real-time polymerase chain reaction; *S stercoralis*, *Strongyloides stercoralis*; *S f fuelleborni*, *Strongyloides fuelleborni fuelleborni*; UK, United Kingdom.

had an average Ct value of 28.4 for the *Strongyloides* genus-specific rtPCR, whereas samples that were positive with the generic assay but negative with the species-specific assay had a higher average generic real-time PCR Ct value of ≥31.1. The distribution of each encountered threadworm species for the 24 countries is displayed in Figure 1.

DISCUSSION

Our novel duplex rtPCR assay successfully differentiated *S f fuelleborni* from *S stercoralis* within a variety of typical test samples, representative of routine migrant screening needs, augmenting the current diagnostic armamentarium [8]. Importantly, the assay demonstrated high specificity, with no cross-reactivity observed between the 2 target threadworm species. However, its absolute detection sensitivity was lower compared to the widely used genus-specific real-time PCR assay, likely due to differences in primer-probe binding efficiency and target DNA abundance between the nuclear 18S and mitochondrial *COX-1* genes. The average Ct value difference of 3.2 cycles between the genus-specific and species-specific assays suggests a sensitivity gap of approximately 10-fold, which likely contributed to an inability to detect *S stercoralis* or *S f fuelleborni* DNA within 42 of 96 (43.8%) of the test samples. As other research and clinical laboratories adopt our novel duplex species-specific TaqMan probe assay, further optimization, such as inclusion of additional reagents (eg, bovine serum albumin), may improve its sensitivity but this should be mindful of future cross-laboratory standardization if accredited for routine clinical use. The accredited genus-specific assay has greater diagnostic sensitivity but lacks species-specific specificity, and is still a powerful frontline diagnostic tool to first detect strongyloidiasis.

To optimize the application of this species-specific assay in more routine services, we recommend a 2-step diagnostic protocol: (1) initial screening with the genus-specific assay, which has greater diagnostic sensitivity, and if found positive, (2) followed by confirmatory testing with our duplex species-specific assay. For those test samples that fail to be identified with the species-specific assay, recourse to DNA sequencing, for example with *Strongyloides*-specific barcoding primers for the nuclear ribosomal 18S hypervariable region IV or other regions of the mitochondrial cytochrome oxidase subunit 1 gene, among others, would be advised [8]. Despite its limitation LOD, our duplex assay's species specificity provides a new molecular surveillance tool to better reveal zoonotic strongyloidiasis. This was clearly evidenced upon the detection of *S f fuelleborni* within just under a fifth of the clinical samples examined, which would have been otherwise overlooked. These migrants had a putative travel history of visiting Brunei, Malawi, Kenya, Senegal, Bangladesh, and Guinea-Bissau (Figure 1).

Two of the test samples appeared to be mixed-species coinfections, each originating from Bangladesh and Guinea-Bissau. By contrast, other samples from Brunei, Malawi, Kenya, and Senegal were positive alone for *S f fuelleborni*. From this first application on available samples from migrants, the presence and distribution of zoonotic strongyloidiasis, as caused by *S f fuelleborni*, is now better understood against a background of the more common and widespread *S stercoralis* [3]. This observation underscores the practical need for better understanding species-level differentiation across the world, travel-related (co)infections, and generation of new surveillance data to help implementation research that could lead to better tailored public health actions and control interventions [3, 6].

Although both *S stercoralis* and *S f fuelleborni* are treated with ivermectin [14], their distinct epidemiological patterns require separate approaches for public health prevention and control, particularly given their different zoonotic hosts. Dogs are putative reservoirs of *S stercoralis* [8], for example, while NHPs are well-known reservoirs for *S f fuelleborni* [7]. Additionally, the life cycle of these 2 threadworm species within the human host likely differs in the role that autoinfection plays and the potential for disseminated strongyloidiasis, particularly for *S stercoralis* and not for *S f fuelleborni* [8]. From discussions among authors arising from a wider diagnostic appraisal, we surmise that serological assays appear more unreliable in *S f fuelleborni* infection, needing closer scrutiny, to ensure that appropriate anthelminthic treatment is allocated and its antiparasitic impact better monitored. Furthermore, the precise species relationships predicting severe clinical outcomes, such as severe strongyloidiasis triggered by an expanded prescription of dexamethasone for coronavirus disease 2019 [15], needs a new appraisal. Due to the diverse origins and patient anonymity of our clinical samples examined, it was

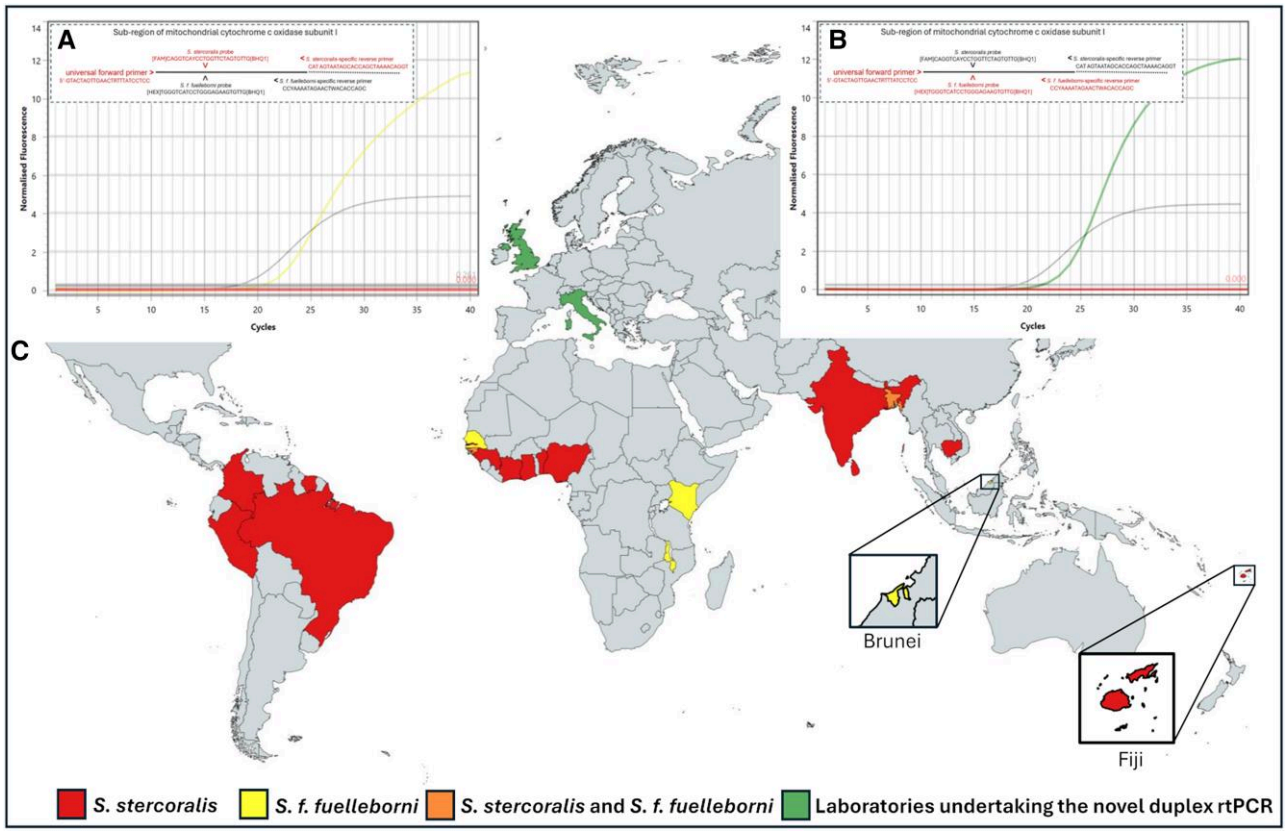

Figure 1. Map showing countries of origin for the clinical samples used in this study and the schematic orientation of the duplex primers and TaqMan probes for each species-specific assay, *Strongyloides fuelleborni fuelleborni* (*A*) and *Strongyloides stercoralis* (*B*). Our geographical map is only a snapshot of the potential endemicity of the 2 species from the samples inspected here. Associated amplification plots are also shown, depicting the difference in relative detection sensitivity against the genus-specific real-time polymerase chain reaction (rtPCR) assay. *C*, Geographical display of countries in which rtPCR was undertaken. The Italian laboratory identified *S stercoralis* (red) from 11 countries (Benin, Brazil, Colombia, Cote d'Ivoire, The Gambia, Ghana, Guinea, India, Italy, Nigeria, and Peru) and *S f fuelleborni* (yellow) from 2 countries (Kenya and Senegal), alongside *S stercoralis* and *S f fuelleborni* coinfections (orange) in a further 2 countries (Bangladesh and Guinea-Bissau). The UK laboratory identified *S stercoralis* from 4 countries (Suriname, Sri Lanka, Fiji, and Cambodia) and *S f fuelleborni* from 2 countries (Brunei and Malawi). Additionally, 2 genus-specific positive samples, originating from the clinical collection in the Netherlands, had no country of origin records. Map generated using www.mapchart.net.

beyond the scope of our study to correlate morbidity indicators against our species-specific identifications.

In summary, our novel duplex rtPCR assay is robust and able to differentiate *S f fuelleborni* and *S stercoralis*, improving surveillance of strongyloidiasis and future clinical appraisals of zoonotic disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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