

## THE LABORATORY DIAGNOSIS OF STRONGYLOIDES STERCORALIS

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# The laboratory diagnosis of *Strongyloides stercoralis*



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**It is estimated that over 30 million people worldwide are infected by the nematode, *Strongyloides stercoralis*<sup>1</sup>. It is endemic in sub-tropical and tropical parts of Australia, with high rates of infection documented in some indigenous communities<sup>2</sup>. Due to the potential for chronic autoinfection, that may persist for decades, migration leads to the presence of the infection in non-endemic areas<sup>1</sup>. Transmission to humans is generally through the penetration of larvae through the skin, following contact with faecally contaminated soil<sup>1</sup>. Disease severity ranges from asymptomatic chronic carriage to an overwhelming illness, where large numbers spread throughout the body, usually triggered by immunosuppression<sup>1</sup>.**

Clinicians are advised to consider strongyloidiasis in patients prior to immunosuppression, or with indicative symptoms, if there is a history of probable exposure in an endemic area, regardless of the elapsed time since exposure<sup>3,4</sup>. Eosinophilia is not an accurate marker of strongyloidiasis, with a retrospective study finding that only a quarter of patients with *Strongyloides* infection had a raised eosinophil count<sup>5</sup>. The detection of strongyloidiasis is optimised by appropriate test ordering, clinical notes, specimen transportation, and processing by the receiving laboratory.

\*These two authors contributed equally.

The gold standard for the diagnosis of strongyloidiasis is the morphological identification of larvae in stool, tissue biopsies, and other clinical specimens such as bronchoalveolar lavage. However, in chronic infections, detection can be limited by low larval output in stool, leading to false negative results<sup>6</sup>. Consequently, in validation studies for serological and nucleic acid tests there is a tendency to define heavier infections as ‘true positives’. This affects serological cut-offs, measurements of sensitivity and specificity, and positive and negative predictive values<sup>7</sup>. Recognition of these limitations is important for the interpretation of negative diagnostic test results, where clinical suspicion remains. Here, we will give an overview of currently available conventional and molecular tests for the diagnosis of strongyloidiasis.

## Stool microscopy and culture methods

Specimen transport and storage have a major impact on the efficacy of culture techniques in the laboratory diagnosis of *S. stercoralis* from faecal samples. Fresh, unrefrigerated samples should be delivered to the laboratory for culture as soon after collection as possible, as the viability of larvae decreases incrementally with storage at 4°C over a 72-h period<sup>8</sup>. Rhabditiform and filariform larvae will be found along with free-living adults of *S. stercoralis* in

older cultures (Figure 1). Larval stages must be differentiated from those of hookworms, which may also be recovered.

Microscopic methodologies such as examination of Kato-Katz preparations, FLOTAC, and formalin/ethyl acetate concentrates have a low yield compared to culture<sup>9,10</sup>. A modified formalin/ethyl acetate method proposed by Anamnart *et al.* improved rates of detection<sup>9</sup>. Overall, however, microscopic techniques alone are insensitive and not sufficient for the exclusion of strongyloidiasis. In one of these studies, though 30 of 254 participants were diagnosed with strongyloidiasis by either agar plate culture (APC; Figure 2) or Baermann culture techniques, no infections were identified by microscopy using the Kato-Katz technique<sup>11</sup>.

APC is possibly the easiest culture to perform in the context of high volume diagnostic testing. Results are available within two days, although extended incubation up to four days increases yield<sup>8,11,12</sup>. Two studies comparing 48 h APC with Baermann culture found an improved recovery of *S. stercoralis* larvae in APC<sup>11,12</sup>. Recovery rates improve markedly with multiple stool cultures<sup>6,11,13</sup>.

## Serological diagnosis

Several tests for the serological diagnosis of strongyloidiasis have been described, using both crude and recombinant antigens. Two commercial ELISA kits employing somatic antigens are available from BORDIER (*Strongyloides ratti* antigen) and IVD Research (*S. stercoralis* antigen), respectively<sup>14</sup>. Recently, two recombinant antigens (32 kD recombinant antigen, called NIE and *S. stercoralis* immunoreactive antigen, SsIR) have been employed for serological testing in both ELISA and luciferase immunoprecipitation system assay (LIPS) platforms<sup>15</sup>. The reported sensitivity and specificity of various serological platforms ranges from 56-100% and 29-100%, dependent upon the method, antigens, cut-offs, study populations, and reference methods employed<sup>16</sup>. *Strongyloides* serology using a crude larval extract antigen was shown in one study to be less sensitive for the diagnosis of returned travellers (73%) compared to patients who have lived for an extended period in an endemic area (98%)<sup>17</sup>. No definitive study of serological methods has been conducted to date, and much of the available data is subject to flaws in methodology, particularly the use of microscopy only as a reference



Figure 1. Life stages of *Strongyloides stercoralis* in agar plate culture: (a) rhabditiform larva; (b) a free-living adult male with filariform larva adjacent; and (c) a gravid, free-living adult female with filariform larva adjacent.

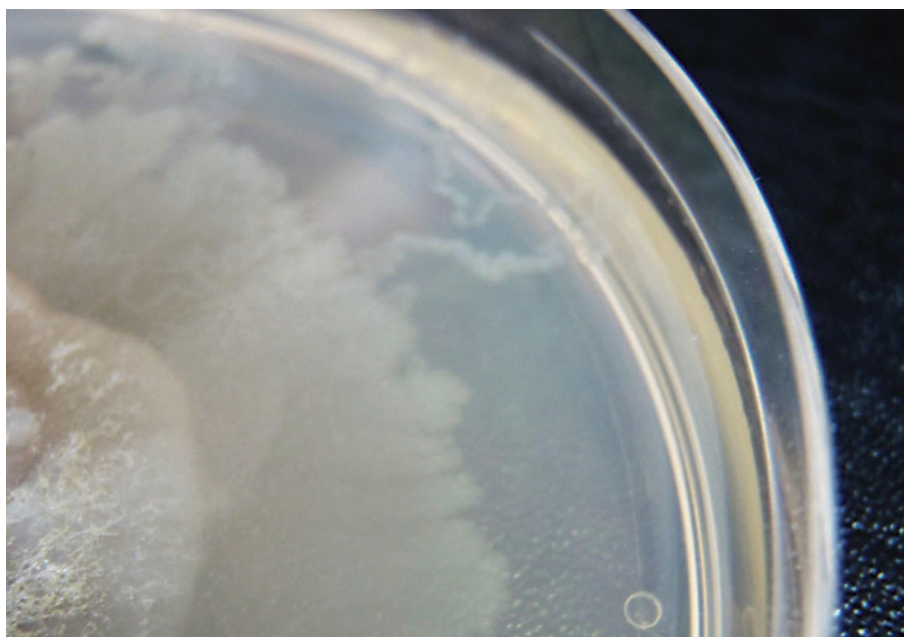


Figure 2. Larval tracks of *Strongyloides stercoralis* on Koga agar plate culture.

standard for positive specimens and varying *S. stercoralis* exposure rates amongst tested serum groups<sup>16</sup>. A 2010 study with a reference standard of a combination of three culture methods and sedimentation concentration found NIE LIPS had a sensitivity of 97.8% (cut-off 37.89 LU) in a study population with high endemicity but from regions without filarial infection<sup>15</sup>. Lower sensitivity resulted when testing the same samples by NIE ELISA (84%), NIE-SsIR LIPS (91.2) and a *S. stercoralis* crude antigen extract ELISA (97%)<sup>18</sup>. All assays tested showed 100% specificity in this study<sup>15</sup>. A more recent study compared an in-house crude *S. stercoralis* filariform larvae immunofluorescent antibody test (IFAT) with the ELISAs from IVD Research, Bordier, and a recombinant antigen NIE ELISA and LIPS<sup>14</sup>. This study used reference samples identified as positive by culture as well as microscopy, and also a composite reference standard of concordant results in at least three of five serological tests<sup>14</sup>. The in-house IFAT was found to be the most sensitive (93.9%) when used in a test subject group with no known previous exposure to *S. stercoralis* and using the composite reference standard, whilst NIE LIPS was found to be the most specific test (100%)<sup>14</sup>. Furthermore, when tested against subjects with potential previous exposure and using the composite reference standard, NIE LIPS was almost 100% specific and 84.6% sensitive (cut off value 1388 LU)<sup>14</sup>. In testing against the same sample group, the Bordier and IVD ELISAs maintained a high specificity (almost 100%), but a lower sensitivity (70% and 79%, respectively) and the NIE ELISA showed the highest specificity (99%), but a low sensitivity (45%) (cut-off 76.5 U/mL)<sup>14</sup>.

Seroreversion following treatment of many, but not all, patients was noted in a study using a *Strongyloides ratti* antigen ELISA<sup>3</sup>.

However, this effect is not universal and varies between studies<sup>3,17</sup>. Immunosuppression was demonstrated to cause a reduction in serological sensitivity (62% vs previously determined 92%), when testing haematological patients on antineoplastic therapies<sup>18</sup>. NIE LIPS did not cross-react with antigens from other parasites in the study by Bisoffi *et al.*, whereas IFAT and the two commercial ELISAs did yield false positives, particularly from *Mansonella perstans* infection<sup>14</sup>. Such cross-reaction may be decreased by pre-incubation of serum in an extract of *Onchocerca gutturosa*<sup>16</sup>.

## Nucleic acid tests

Nucleic acid tests complement non-molecular methodologies for the diagnosis of *S. stercoralis*, and allow the use of refrigerated, frozen, or preserved specimens<sup>11,19,20</sup>. This simplifies specimen transportation, particularly where collection occurs some distance from the testing laboratory, and there is no risk of laboratory-acquired strongyloidiasis<sup>21</sup>. DNA extraction and amplification can be performed within 1 day, however, laboratories may batch specimens according to demand.

## DNA extraction

It is important that DNA extraction methods for stool specimens are effective at removing the numerous nucleic acid test inhibitors in stool<sup>22</sup>. A comparison of 5 methods of DNA extraction demonstrated that two column-based methods were the most effective for the PCR detection of DNA from *Strongyloides ratti* that had been spiked into human stool. These were the MoBio PowerSoil kit (MoBio Laboratories, Carlsbad, CA, USA) and a method based on modifications of



the QiaAmp Tissue kit (Qiagen, Hilden, Germany) by Verweij *et al.*, which has been successfully automated<sup>23</sup>. The comparison found that bead beating prior to the use of the NucliSens EasyMag (BioMerieux, Marcy l'Etoile, France) was less effective, which indicates the method of sample pretreatment prior to automated extraction will impact upon test sensitivity. Other investigators have used a variety of different extraction methods for *Strongyloides* PCR, including in-house methods, the Qiagen stool kit (unmodified and modified), and the Nucleospin Soil kit (Macherey-Nagel, Duren, Germany)<sup>10,24–30</sup>.

One of the inherent limitations of the molecular diagnosis of *S. stercoralis* is the sampling error that can occur when relatively small amounts are extracted in the context of low larval output<sup>6</sup>. For example, 2g of stool can be used for agar plate culture, whereas 250 mg of specimen is recommended for the MoBio PowerSoil kit<sup>31</sup>. Methods that concentrate larger amounts of stool prior to DNA extraction have the potential to increase test sensitivity, if they remove inhibitors and retain larvae<sup>29</sup>.

## PCR

Current PCR methods most commonly target one of four regions: the 18S rRNA small subunit (SSU); the internal transcribed spacer region 1 (ITS-1); the 28S rRNA gene; or the cyclooxygenase gene (*cox1*)<sup>19,23–30,32–37</sup>. Published sensitivities and specificities for *Strongyloides* PCR vary according to the reference methods and are listed in Table 1. The majority of *Strongyloides* PCR publications have used a real-time method with primers and probe published

by Verweij *et al.*<sup>19</sup>. This has also allowed for the development of multiplexed PCR<sup>10,30,34,38</sup>. Some studies evaluating the diagnostic accuracy of these PCR methods have used both morphological diagnosis and detection of PCR products as their reference standards, and are not reviewed here. Their methodology precludes the calculation of sensitivity and specificity based on gold-standard, according to an FDA Guidance<sup>39</sup>.

In the absence of a consistent gold standard in chronic infection, positive nucleic acid test results, where conventional tests are negative, may be due to greater sensitivity or false positive results<sup>6</sup>. No PCR studies have reported false positive results when analytical specificity has been tested using DNA extracted from bacteria, viruses, fungi, protozoa, and other helminths<sup>19,23,24,27,29,30</sup>. Studies have also assessed the specificity of the PCR products by sequence analysis, with all finding 100% sequence homology with the target sequence of *S. stercoralis*.<sup>24,25,27,29</sup> Sitta *et al.* found a number of false positives, using published genus and species-specific primers, based on non-target sized bands on gel electrophoresis<sup>19,25</sup>. The genus-specific primers amplified sequences that generated non-target bands on electrophoresis in specimens that contained *Blastocystis* and other helminths on microscopy, and the species-specific primers amplified sequences that generated non-target bands on electrophoresis in specimens positive for hookworm on microscopy<sup>25</sup>. Similar accounts of cross-reactivity have not yet been reported, so further data will be useful to monitor the specificity of PCR in different populations.

Table 1. Sensitivity and specificity of stool PCR for human strongyloidiasis.

Reference	Target	Sensitivity	Specificity	Reference method
19	18S	61.0%	92.4%	Coproculture; Baermann
32	18S	58.6%/96.6% <sup>A</sup>	ND	McMaster
26	18S	61.0%	92.7%	APC; Baermann
34	18S	100%	100%	Direct microscopy
23	18S	33.0%	99.0%	Harada-Mori
25	18S	84.8%/78.8% <sup>A</sup>	ND	APC
10	18S	11.6% 83.3%	90.6% 96.2%	Baermann FLOTAC
29	18S	93.8%	86.5%	FEAC; APC; Harada-Mori
36	18S	90.0%	85.7%	APC
27	18S/ <i>cox1</i> <sup>B</sup> 18S	100% 84.7%	91.6% 95.8%	FEC; APC

<sup>A</sup>The first value relates to a species-specific primer, the second to a genus-specific primer.

<sup>B</sup>Nested PCR. APC, agar plate culture; FEAC, formalin-ethyl acetate concentration; FEC, formalin-ether concentration.

## LAMP

Loop-mediated isothermal amplification (LAMP) is an additional nucleic acid detection method. LAMP uses a DNA polymerase with strand-displacement activity, so it doesn't require the temperature cycling of PCR, and can be performed with a simple source of constant temperature such as a heating block<sup>40</sup>. LAMP has been successfully applied in resource limited-settings for the detection of pathogens<sup>40</sup>.

The *Strongyloides* LAMP assay uses primers that are genus specific and bind to the 28S rRNA gene<sup>20</sup>. The reaction runs at 60°C for 1 hour. Pre-heating of the reagents and DNA template to 95°C, prior to the addition of enzyme, increases the limit of detection and eliminates the need to pre-heat the template and keep it at 4°C<sup>20</sup>. A novel use of Syto-82 dye (Life Technologies, Carlsbad, CA, USA) enables the detection of positive results in real-time or visually on completion of the reaction<sup>20</sup>. Analytical sensitivity and specificity are comparable to PCR, according to the method of Verweij *et al.*<sup>19,20,23</sup>. When 28 human stool specimens that were microscopy and PCR positive for *S. stercoralis* were tested with the LAMP method, 27 were positive<sup>20</sup>. The negative specimen had a high cycle threshold (38.44) on PCR<sup>20</sup>. Further validation of the LAMP assay with clinical specimens is currently in progress.

## Conclusion

The diagnosis of strongyloidiasis can be made through the morphological identification of larvae, usually in the stool, serological testing, and nucleic acid tests. While each methodology has advantages, there are limitations that need to be taken into account when assessing the significance of negative test results. Often the most important aspect of patient management is to consider the possibility of *S. stercoralis* infection.

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## Biographies

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**Gemma Robertson** is a final year microbiology trainee at Melbourne Pathology. She has an interest in tropical medicine and parasitology, and will be undertaking a PhD to continue her research into soil-transmitted helminthiasis in Aboriginal communities.

**Dr Richard Bradbury** is an Australian Parasitologist with an interest in all fields of parasitology. He was recently appointed as the Team Lead in the Parasite Diagnostics and Biology Laboratory of the Centers for Disease Control and Prevention in Atlanta, USA. He is writing this work in both his personal capacity and in his capacity as an adjunct academic at Central Queensland University.

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