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Diagnosis of *Taenia solium* infections based on “mail order” RNA-sequencing of single tapeworm egg isolates from stool samples

Henrik Sadlowski[1], Veronika Schmidt[2,3], Jonathan Hiss[1], Christian G. Schneider[1], Gideon Zulu[4], Alex Hachangu[4], Chummy S. Sikasunge[4], Kabemba E. Mwape[4], Andrea S. Winkler[2,3], Markus Schuelke[1,5]

[1]Charité-Universitätsmedizin Berlin, corporate member of the Freie Universität Berlin and Humboldt-Universität zu Berlin, NeuroCure Cluster of Excellence, Berlin, Germany;

[2]Department of Neurology, Centre for Global Health, Klinikum rechts der Isar, Technical University M unich (TUM), Munich, Germany;

[3]Centre for Global Health, Institute of Health and Society, University of Oslo, Oslo, Norway;

[4]School of Veterinary Medicine, University of Zambia, Lusaka, Zambia;

[5]Charité-Universitätsmedizin Berlin, corporate member of the Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Neuropediatrics, Berlin, Germany

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Henrik Sadlowski

Here we present a detailed protocol for the identification of *Taenia solium* based on the few *Taenia* spp. eggs found in diagnostic stool samples. Our approach is based on "mail order" RNA sequencing of single eggs and can be performed in laboratories equipped with basic tools such as a microscope, a Bunsen burner, and access to an international post office for shipping samples to a next-generation sequencing facility.

This protocol describes sample collection and transport, isolation of individual *Taenia* spp. eggs, reliable disruption of individual *Taenia* eggs, and important considerations for shipping samples to a next-generation sequencing facility. We provide images and videos to help prepare the tools needed for the protocol. Additional information on our rationale for designing the critical steps can help implement the protocol in new environments.

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protocol

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Tapeworm, species identification, taeniasis, stool microscopy, Taenia asiatica, Taenia solium, Taenia saginata, Neglected tropical diseases, SMART-seq 2, Single cell RNA sequencing, parasitology

protocol ,

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MATERIALS

Triton X-100

Sigma Catalog #93426

RNAse inhibitor

Murine **NEB Catalog #M0314S**

dCTP

(100mM) **Promega Catalog #U1225**

dATP

(100mM) **Promega Catalog #U1205**

dGTP

(100mM) **Promega Catalog #U1215**

dTTP

(100mM) **Promega Catalog #U1235**

Borosilicate Glass Pipet **VWR**

International Catalog #14673-010

Any ordinary Borosilicate Pasteur pipet can be used.

Carry gloves, goggles, and protective clothing during the whole procedure. Be aware that you are handling potentially infectious materials.

Routine Diagnostic Workflow

1 Place 1 gram of a fresh native stool sample into a 15 mL Falcon tube and resuspend it in 10 mL PBS.

Carry gloves, goggles, and protective clothing during the whole procedure. Be aware that you are handling potentially infectious materials.

! Many different procedures exist to prepare samples for diagnostic stool microscopy. Sample preparations deviating from steps 1-5 of this procedure are likely to be compatible with our overall RNA-sequencing workflow of single tapeworm egg isolates if the stool sample remains native and is not exposed to organic solvents or high ion / salt concentrations.

2 Centrifuge **2000 x g, Room temperature, 00:20:00**

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3 Remove the supernatant and all floating particles. Resuspend 1 volume of sediment in 2 volumes of PBS, mix well, and place a drop of the suspension on a microscopic specimen slide. Cover the sample with a cover slip. In some cases more PBS has to be added in order to reduce the density of the stool matrix.

4 Screen the sample on a microscope with 20x phase contrast lens for characteristic *Taenia* spp. or other helminth eggs.

5 If tapeworm eggs are detected, remove the coverslip and move the matrix with the PBS into an Eppendorf tube using a braod gauged pipette (e.g. cut the tip of a normal 200 µL laboratory pipette to increase the opening and to prevent blockage).

Clinical sample stability, transportation and storage

6 Store both, the Eppendorf tube with the matrix from the microscope slide and the stool sample at 4°C

! We have found that cool storage for up to 8 days (4°C in the refrigerator or in cold packs) guarantees high-quality sequencing results. Even longer storage is possible, as eggs remain infectious for a very long time in the wild. However, for sequencing purposes, we have tested a maximum of 8 days.

7 Transport samples on cool packs (-20°C) in a styrofoam box to a laboratory that is connected to an international postal or courier service which accepts shipments on dry ice.

! We found that a transport of up to 3 days in a tropical climate with temperatures between 20 and 35°C had no negative effects on the samples. During this transport the cold packs were not changed.

! Samples should be kept cold, however **without freezing** them. A freeze-thaw cycle with subsequent storage at 4°C resulted in degradation of the RNA.

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8 The maximum time investigated for storage under cool conditions (4°C in the refrigerator or in cold packs) was 8 days. For longer storage, samples can be frozen at -80°C.

Setup for egg preparation

9 Before proceeding with this protocol, arrange for shipment of the single cell sequencing reaction kits and/or components listed below on dry ice. We recommend that the following procedure be performed in a laboratory served by international mail or courier services to which the components are to be shipped. The remaining dry ice from these shipments can in turn be used to ship the samples to the sequencing facility.

10 ⚠

Prepare the RNA stabilization buffer by adding the following components to an RNAse free tube:

- 10 µL dNTP mix containing 25 mM dATP, 25 mM dCTP, 25 mM dGTP, and 25 mM dTTP.
- 2.5 µL RNAse Inhibitor (40 U/µL)
- 1 µL 10% Triton X100

Add 0.6 µL of the RNA stabilization buffer to a clean 0.5 mL tube and immediately place it on ice.

! We considered this approach better than using the premixed ready-to-use SMART-seq buffer (BGI). Using the SMART-seq buffer would require breaking the eggs in the SMART-seq buffer itself. Due to the low surface tension of the buffer, this makes handling much more difficult..

11 Prepare two glass pipettes (see cartoon and movie below)

1. Grasp a glass pipette (borosilicate) at both ends and place the middle part over a Bunsen or Butangas burner (**A**) until the glass begins to glow and become viscous (**B**).
2. Pull the two ends slowly apart, causing the glass to taper at the glowing sections (**B**).
3. Continue this movement until only a hair-thin glass thread connects the two ends (**C**).
4. Remove the pipette from the Bunsen burner and, when cooled, breakt it at the tapered point (**D**).

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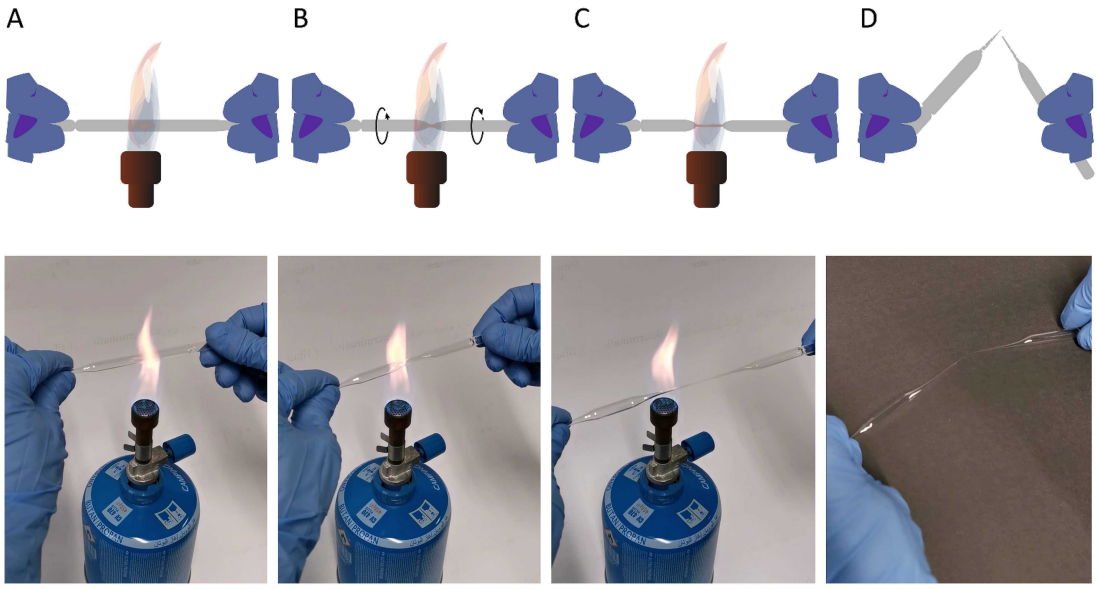

The movie can be watched from here: https://doi.org/10.6084/m9.figshare.16955734

Check if the lumen of the needle is still present (it should not be). An open lumen would be visible by the entry of PBS into the needle by capillary forces when the tip of the needle is dipped into a drop of PBS or buffer. You can securely seal the lumen during the pulling process by simultaneously twisting the two parts of the glass pipette against each other during the final pull of the capillaries (see instructional movie). This may require some manual training.

Preparation of *Taenia solium* eggs for sequencing:

12 Resuspend approximately 50 mg of stool in 250 µL of PBS and apply 40 µL of the sample to a specimen slide. Do not cover the specimen with a coverslip. Focus an individual egg in the center of the field of view.

13 Aspirate 10 µL PBS using a 10 µL pipet tip and a 10 µL Eppendorf pipet. Insert the pipet tip into the stool sample and move it towards the egg in the center of the field of view under constant visual inspection through the ocular lens.

! Maintain a constant, minimal flow of PBS from the pipette by applying slight, continuous pressure to the plunger of the pipette and pushing it down slowly. This flow will wash away the solid components of the stool matrix, while the *Taenia* egg will adhere lightly to the slide and remain in focus. For this procedure, the use of an inverted microscope is

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very helpful.

14 Aspirate the egg with its adhering debris with the pipette in a total volume of around 2 µL.

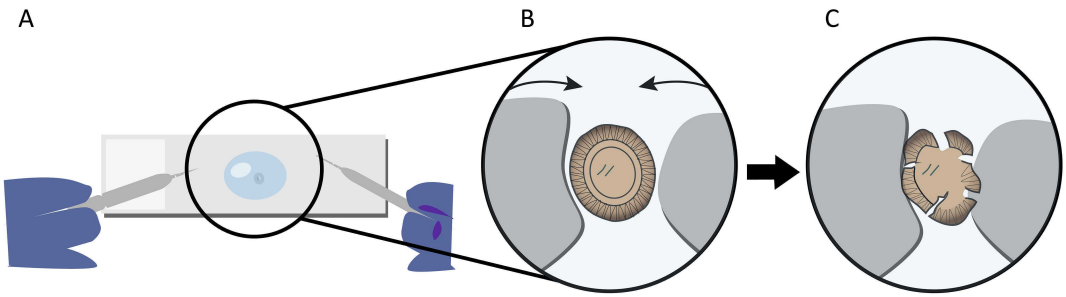
15 Release the aspirated eggs into 40 µL of fresh PBS on the microscopic slide. Exchange the pipet tip for a fresh one and aspirate the eggs again to move them into a fresh drop of PBS. These are the washing steps aiming to remove adherent organic material and bacteria.

Repeat this step until no contamination of solid stool matrix is visible anymore (usually 3 transfers are sufficient).

16 ⚠

Disruption of the eggs and stabilization of the RNA

1. Aspirate two eggs in 4 µL PBS and place them on a fresh specimen slide. Insert the two glass pipettes into the drop of PBS (**A**) at 20x magnification and carefully place them at the left and right side of the egg (**B**). Press the needles carefully against each other to squeeze and ultimately destroy the egg under vision (**C**).
2. Repeat this step with the second egg
3. Aspirate the remaining PBS (usually around 3.5 µL due to evaporation) and add it to the SMART-seq buffer prepared in Step 10.
4. Immediately place the tube on dry ice.
5. Once the eggs are broken, they are not infectious anymore and can be shipped as "noninfectious" biological materials.



From a technological point of view, one intact *Taenia* egg would b e sufficient for the

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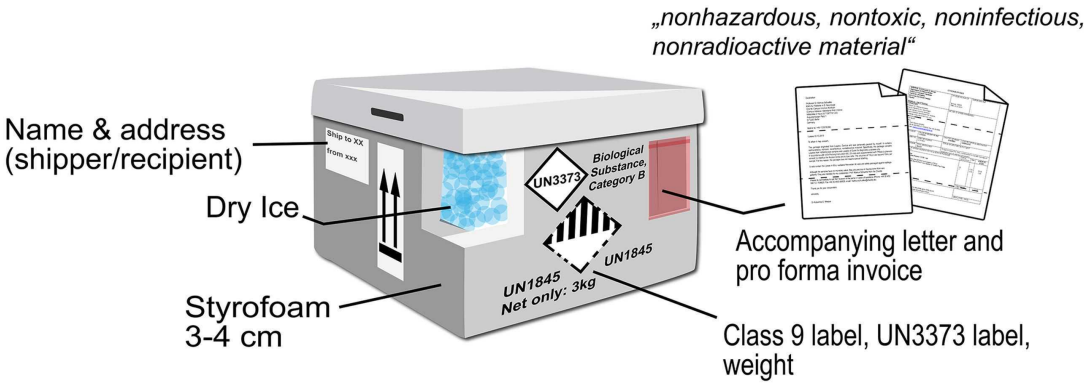
sequencing workflow because the sequencing procedure is highly sensitive. We used two eggs to reduce the risk of an egg appearing microscopically intact but having minor damage resulting in degraded RNA inside the egg. We do not recommend adding more than two eggs to the SMART-seq buffer, as too much input RNA may interfere with the highly sensitive protocol.

Shipment

17 Label a Styrofoam box with the following labels



Description of the labeling and of the documents required for shipping the samples. The tube with the sample should be placed in a plastic bag, well buried between the dry ice inside the Styrofoam box.

18 Add a customs pro forma invoice declaring the value of the samples if they have been purchased or a nominal value of 1 USD if the samples are research samples with no monetary value. Add a letter stating that samples 4 µL are non-infectious lysates not containing any cells or dangerous substances.

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