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Manual for the Screening of Trypanosoma cruzi, HTLV, Strongyloides stercoralis, and Intestinal Parasite Infections in HIV-infected Patients in the Peruvian Amazon V.1

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Disclaimer

The Ethics Committee of Loreto Regional Hospital in Iquitos (Peru) **(EXP: ID-018-CIEI-2013**) approved this study. After being informed about the study, individuals who volunteered to participate provided written consent to be included. We maintained all the results in strict confidentiality, and those who tested positive for intestinal parasites received free treatment and follow-up by their HIV healthcare provider.

Abstract

Over time, our country has faced public health issues. Moreover, the high prevalence rates have become a significant challenge in low-resource areas, with intestinal parasitism being a particularly aggravating factor for the population's health. The presence and spread of intestinal parasitism—especially in young children and immunosuppressed individuals—is made possible by adverse community health factors such as open defecation, poor environmental sanitation, poverty, and low levels of education. Intestinal parasites are protozoa or helminths that can be found in stool, secretions, bodily fluids, and perianal smears in their various developmental stages. These parasites affect the intellectual and nutritional development of affected populations, further impacting their economic wellbeing. For the study of intestinal parasitic infections, there is a need for a manual to ensure timely and accurate diagnosis of these infections. This will enable prompt corrective action, such as treatment with appropriate medications or preventive and hygiene education. The Institute of Health and Biomedical Research of Alicante, through the Selva Amazonica Civil Association Clinic, has prepared this manual as a useful tool for organizing laboratory procedures. The Manual for the Screening of Trypanosoma cruzi, HTLV, Strongyloides stercoralis, and Intestinal Parasite Infections in HIV-Infected Patients in the Peruvian Amazon presents the protocol for the ongoing Tryp-Peru 3.1 prospective study. It includes a compilation of the most commonly used and useful methods for diagnosing intestinal parasitic infections and is intended as a practical, easy-to-use guide for laboratory technicians and professionals.

Guidelines

The Ethics Committee of Loreto Regional Hospital in Iquitos (Peru) (EXP: ID-018-CIEI-2013) approved this study. After being informed about the study, individuals who volunteered to participate provided written consent to be included. We maintained all the results in strict confidentiality, and those who tested positive for intestinal parasites received free treatment and follow-up by their HIV healthcare provider.

Materials

Stool Samples: The sample (4-8 grams of stool) must be as fresh as possible (within 90 minutes if testing for Entamoeba histolytica), and always collected on the same day of stool processing. It should be placed in a wide-mouth, screw-cap container, properly labeled with patient identification. The sample should be collected before administering antiparasitic medication, or 2 to 5 days after such treatment. Stool that has been passed on the ground is not suitable for diagnosis due to potential contamination with biological elements such as larvae resembling human intestinal parasites, nematode larvae, mite or insect eggs, etc. If the patient typically does not have regular bowel movements and passed stool the night before the test, it is recommended to store the sample in a refrigerator or cool, shaded area to preserve parasitic forms. If there will be a delay of several hours or days in transporting the sample to the laboratory, a fixative and/or preservative should be added (e.g., PAF, PVA, 10% formalin, SAF, sodium acetate, etc.). Blood Samples: Collect 5 mL of blood in an EDTA tube, labeled with the patient code. Keep samples in a cooler until patient inclusion is complete for the day. Transport under refrigerated conditions to the Selva Amazonica Civil Association (ACSA) starting at 11:30 AM. Samples are received and registered at the ACSA Laboratory around 12:00 PM. ACSA lab technicians will centrifuge, separate, and store samples into two to three aliquots: One aliquot will be used for serological testing. Another will be preserved for possible further testing. Storage: All aliquots will be kept at -80°C until analysis.

General Information

1 Objectives: To determine the seroprevalence of Trypanosoma cruzi, HTLV, Strongyloides stercoralis, and intestinal parasites in HIV-infected patients in the Peruvian Amazon.

1.1 Describe risk factors for acquiring these infections.

1.2 Assess the serological status in relation to HIV disease stage.

1.3 Correlate positive results with stool-based parasitological testing for S. stercoralis.

2 Scope of Application: Applicable to laboratories at the Selva Amazonica Civil Association Clinic and antiparasitic natural product laboratories of the Amazon, as part of the Research Center for Natural Resources of the Peruvian Amazon — CIRNA.

3 Responsibilities: The Institute of Health and Biomedical Research of Alicante and the Selva Amazonica Civil Association Clinic, through its Executive Directorate, are responsible for authorizing the design, development, review, and updating of this manual. Directors are responsible for authorizing, providing necessary resources, and appointing staff to apply the procedures contained in this manual. Staff are responsible for planning, organizing, implementing, training, and overseeing their actions in accordance with the manual's guidelines. Laboratory heads or supervisors must ensure internal quality control, staff competency, proper equipment, materials, and reagents, optimal use of human resources, and the appropriate layout of facilities. Professional, technical/operational, and support personnel are responsible for following the specifications outlined in the manual and performing the indicated procedures. Once laboratory procedures are completed, staff responsible for handling biological samples, as well as the facility director, are responsible for the neutralization and/or proper disposal of waste materials.

Biosafety Measures

4 Responsibilities: Personnel involved in various diagnostic processes for parasitic infections and/or diseases must meet the requirements and implement the measures established in the Biosafety Standards Manual (Technical Standards Series No. 18), published by the National Institute of Health.

5 Scope of Application: Necessary measures must be adhered to and monitored, especially the following:

5.1 Personnel Guidelines: Wear gloves when collecting, receiving, and processing fresh samples. Dispose of fresh material promptly or fix it if it needs to be preserved.

5.2 Types of Agents: Fresh samples and those from HIV-infected patients should be considered high-risk materials.

5.3 Clothing: Always wear a lab coat inside the laboratory and remove it before entering other areas.

5.4 Sample Examination Area: A designated, single area within the laboratory must be used for examining samples.

5.5 Sending Samples to Another Laboratory: The only fresh material permitted for inter-lab transfer is culture material. All other samples must be fixed before transport.

5.6 In Case of Accidents: Laboratories must neutralize and dispose of biological material. Decontamination should occur in each lab, and dirty materials must be either discarded or washed.

Methodology

6 This is a cross-sectional prospective study conducted in collaboration between the Institute of Health and Biomedical Research of Alicante (ISABIAL) — General University Hospital of Alicante (Spain) and the Regional Hospital of Loreto (Iquitos, Peru) over a 12-month period (approximately 2 months of planning, 5 months of fieldwork, and 5 months for data analysis and publication). The study has been approved by the corresponding Research and Ethics Committees.

7 Inclusion Criteria: Patients over 14 years old living with HIV who are seen at the Regional Hospital of Loreto in Iquitos, Peru, either during outpatient follow-up visits or inpatient stays. Sociodemographic, epidemiological, and clinical data will be collected using a semi-structured oral questionnaire.

8 Sample Collection: Venous blood samples will be taken for serological testing of T. cruzi (two different serological tests), HTLV-1, and S. stercoralis. In patients positive for Chagas disease, PCR testing will be added. In patients positive for HTLV-1, proviral RNA testing will be performed. All patients will provide a stool sample, which will be analyzed using: Direct microscopy, Kato-Katz technique, Modified Baermann funnel method, Charcoal culture, Rapid immunochromatographic test for Cryptosporidium, Giardia lamblia, and Entamoeba histolytica/dispar. A small portion will be preserved using the filter paper method to allow for future molecular testing.

9 This research project is part of an international development cooperation effort aligned with the 2030 United Nations Sustainable Development Goals, specifically Goal 3: Health and Wellbeing. It is conducted under the agreement 'University Cooperation for Development 2022 — Miguel Hernandez University of Elche.'

10 Patient Inclusion Process: The study will be conducted at the Regional Hospital of Loreto (Iquitos) in the TARGA clinic, where HIV-positive patients receive outpatient follow-up care.

10.1 Research schedule: 7:30 AM to 11:30 AM (Monday through Wednesday, potentially extended by 1 hour if needed).

10.2 Expected inclusion rate: 7-10 patients per day.

10.3 Personnel involved: Seyer Mego Campos (researcher) and Anderson Pizango Panaifo (phlebotomist).

Steps to Follow

11 Study Information and Informed Consent: Patients will receive a clear explanation of the study and will sign two sections of the informed consent form—one for participation and one for storage and future use of samples. Both the patient and the researcher will sign, and each party will keep a copy.

12 Patient Code Assignment: Each participant will be assigned a code starting with 'TP' followed by a sequential number (e.g., TP-1). This code will be used on the consent form, data sheet, blood and stool collection tubes, and across all sample handling steps.

13 Epidemiological and Clinical Data Collection: Data will first be recorded in a physical notebook and later transferred to an Excel spreadsheet. Weekly data will be sent to the lead investigator in Spain (Dr. Otero), who will upload them to the RedCap international research database.

14 Data Collected Will Include:

14.1 Patient and Inclusion Data: Study code, case number, medical record number, initials, inclusion date, and site.

14.2 Sociodemographic Data: Sex, date of birth, current occupation, education level, place of residence (rural or urban).

14.3 Epidemiological Data: Exposure to triatomine bugs, history of breastfeeding, past blood transfusions, domestic animals, barefoot walking in rural areas, housing material.

14.4 Clinical Data: Substance use, pregnancy, comorbidities, previous infections, current diarrhea or other symptoms.

14.5 HIV Infection Data: Information on mode of transmission and current status (latest CD4 count and viral load).

Sample Collection

15 General Conditions: Stool Samples: The sample (4-8 grams of stool) must be as fresh as possible (within 90 minutes if testing for Entamoeba histolytica), and always collected on the same day of stool processing. It should be placed in a wide-mouth, screw-cap container, properly labeled with patient identification. The sample should be collected before administering antiparasitic medication, or 2 to 5 days after such treatment. Stool that has been passed on the ground is not suitable for diagnosis due to potential contamination with biological elements such as larvae resembling human intestinal parasites, nematode larvae, mite or insect eggs, etc. If the patient typically does not have regular bowel movements and passed stool the night before the test, it is recommended to store the sample in a refrigerator or cool, shaded area to preserve parasitic forms. If there will be a delay of several hours or days in transporting the sample to the laboratory, a fixative and/or preservative should be added (e.g., PAF, PVA, 10% formalin, SAF, sodium acetate, etc.).

16 Blood Samples: Collect 5 mL of blood in an EDTA tube, labeled with the patient code. Keep samples in a cooler until patient inclusion is complete for the day. Transport under refrigerated conditions to the Selva Amazonica Civil Association (ACSA) starting at 11:30 AM. Samples are received and registered at the ACSA Laboratory around 12:00 PM. ACSA lab technicians will centrifuge, separate, and store samples into two to three aliquots: One aliquot will be used for serological testing. Another will be preserved for possible further testing. Storage: All aliquots will be kept at -80°C until analysis.

Laboratory Procedures for Blood Parasitological Diagnosis

17 Once a total of 95-100 frozen samples is collected, they will be transferred to the CIRNA-UNAP Laboratory to perform the following serological tests:

18 CHAGATEST ELISA Recombinant v.3.0 and v.4.0 (Wiener Lab, Rosario, Argentina): These are third-generation enzyme immunoassays for the detection of antibodies against Trypanosoma cruzi. The test uses recombinant antigens (SAPA 1, 2, 13, 30, 36), obtained via recombinant DNA technology from epimastigote and trypomastigote stages of T.

cruzi, and designed to include conserved regions across different strains. If the sample contains specific antibodies, they bind to the immobilized antigens. Unbound material is removed by washing, followed by the addition of anti-human antibodies for detection. The test offers reliable, specific, and highly sensitive results due to the standardized antigen mixture.

19 HTLV I+II ELISA Recombinant v.4.0 (Wiener Lab, Rosario, Argentina): Microplate wells are coated with recombinant antigens from HTLV I and II. If anti-HTLV antibodies are present in the diluted patient sample, they will bind to the antigens. After washing, a conjugate (HTLV antigens linked to peroxidase) is added to form a detectable complex. After incubation with tetramethylbenzidine and hydrogen peroxide, a blue color develops in reactive samples, turning yellow upon addition of sulfuric acid (stop solution).

20 ELISA for Detection of IgG against Strongyloides stercoralis (DRG Instruments GmbH, Marburg, Germany): A qualitative enzyme immunoassay for detecting IgG antibodies against S. stercoralis in human serum or plasma. Microplate wells are coated with a soluble fraction of filariform L3 larval antigens. This test is intended only for use by trained laboratory personnel.

21 Interpretation of Serological Results and Confirmation: Chagas Disease (T. cruzi): Considered seropositive if both ELISA tests are positive. Considered seronegative if both tests are negative. Indeterminate if results are discordant; in this case, the patient will be notified, and a new blood sample will be taken for PCR testing. HTLV-1: Considered positive if the ELISA test is reactive. The patient will be informed and asked to provide a new sample for proviral RNA testing, performed at the Alexander von Humboldt Tropical Medicine Institute, Universidad Peruana Cayetano Heredia, Lima. Strongyloides stercoralis: Considered positive if the ELISA is reactive. The result will be correlated with stool examination findings.

Laboratory Procedures for Stool Parasitological Diagnosis

22 Testing should be performed as soon as possible and in an appropriate area, away from direct sunlight. Diarrheic samples and those containing blood must be examined both macroscopically and microscopically immediately upon arrival at the lab. All samples will undergo the corresponding diagnostic methodology, using a six-step diagnostic approach. The laboratory report should include the full name of the parasite, its evolutionary stage, and its density in the sample.

23 Filter Paper Method: 100 filter papers are available, each capable of preserving samples from 4 patients (4 circles per sheet), with a shelf life of up to 6 months for future molecular studies on geohelminths and protozoa (e.g., multiplex PCR in Spain). Materials: Filter paper for stool samples, Swab and distilled water or PBS, Ziplock bags and silica gel. Procedure: Use unprocessed stool. Amount should be between 180 and 220 mg (if liquid, measure in mL). If weighing is not possible, use about the size of a pea. Wet a swab with water or PBS, coat it with the stool sample, and apply it to the circle. Allow it to

air dry completely at room temperature to prevent mold growth. Label each circle with the patient's code. Store the circles together in a zip bag or cut them individually and place in zip bags, preferably with silica gel. Keep dry, out of sunlight, and in a dark or opaque container for transport.

24 Direct Microscopic Examination: Primarily used for fresh samples, to identify motile evolutionary forms of microscopic parasites: Protozoa: Entamoeba histolytica, Giardia lamblia, Balantidium coli, etc. Helminths: larvae and eggs of S. stercoralis, Ancylostoma duodenale, Necator americanus, Trichostrongylus spp., Paragonimus, Fasciola, etc. Materials: Glass slides and cover slips, Wooden or glass applicator, Optical microscope, Glass marker, Saline solution, Lugol's iodine, brilliant green, neutral red. Procedure: Place a drop of saline on one end of the slide. Add 1-2 mg of stool using an applicator, emulsify it, and cover with a coverslip. On the other end, add a drop of Lugol's solution and repeat the process. With saline: observe natural morphology of trophozoites and cysts. With Lugol: observe internal structures, such as nuclei and vacuoles. Observation: Use microscope at 10x or 40x (avoid 100x oil immersion). Move the slide systematically, e.g., right to left or top to bottom.

25 Baermann Method (Concentration by Migration): Used to detect motile larvae and trophozoites, especially S. stercoralis and Balantidium coli. Relies on their geotropism, thermotropism, and hydrotropism. Materials: Conical cups (200-300 mL), Metal sieve or mesh, Pasteur pipettes, Gauze, Depression slides, Saline solution. Procedure: Place sieve lined with 2-3 layers of gauze inside the cup. Add 4-6 g of fresh stool on top. Pour warm saline (37°C) into the cup. Let stand at room temperature or in an incubator (28-37°C) for 30-50 minutes. Remove the sieve, collect 1 mL of sediment with a pipette. Place on a depression slide and examine under microscope. Observation: Look for motile larvae and trophozoites. Occasionally, adult male forms of E. vermicularis may be seen. For better larval differentiation, use Harada-Mori method.

26 Kato-Katz Quantitative Method (Eggs per Gram — EPG): Quantifies helminth eggs in stool using the Kato technique. Results are reported as eggs per gram of stool (EPG). Materials: Glass slides (2.5 × 7.5 cm), Absorbent paper (towel or newspaper), Applicator, Glycerin-malachite green-soaked cellophane, Plastic mold with 6 mm diameter hole, White mesh or nylon (0.09 mm). Procedure: Transfer 0.5-1g of stool to absorbent paper using applicator. Place mesh over the stool and press to filter it. Place the plastic mold on a slide and fill the hole with filtered stool. Lift mold, leaving a 'cylinder' of stool on the slide. Place glycerin-malachite green cellophane over sample and press gently. Let clear at room temperature for 30-45 minutes. Observation: Observe under microscope for helminth eggs.

27 Charcoal Culture (Dancescu Method): Enhances sensitivity for diagnosing S. stercoralis when combined with previous methods. Materials: Distilled water, Charcoal, Petri dish, Vinyl tape. Procedure: Mix 4g of fresh stool with distilled water. Then mix with equal amount of granulated charcoal. Place mixture in center of Petri dish, seal with vinyl tape.

Incubate in dark room at 30°C. Examine under microscope at 48 hours and 7 days for S. stercoralis larvae.

28 Rapid Immunochromatographic Test (ICT) for Protozoa: CerTest Combo: One-step ICT for simultaneous detection of Cryptosporidium, Giardia, and Entamoeba. Materials: CerTest Crypto+Giardia+Entamoeba combo card, Sample dilution tubes with buffer, Stool collection container, Disposable gloves, Timer. Procedure: Open dilution tube and use stick to collect stool. Close and shake tube to mix sample with buffer. Open CerTest and apply 3 drops to each test window. Read results after 10 minutes.

Data Analysis

29 To analyze the data collected during the parasitological survey, we calculate the following epidemiological indicators for each of the parasitic species found:

29.1 Prevalence (P): The percentage of infected individuals in the population examined.

29.2 Intensity (I): The average number of parasites (or eggs, cysts, or oocysts) found per infected individual.

29.3 Abundance (A): The average number of parasites (or eggs, cysts, or oocysts) per individual examined, regardless of whether they are infected.

29.4 Polyparasitism (%): The percentage of individuals infected with two or more different species of parasites.

29.5 Frequency (%): The percentage of times a parasite species appears in all positive cases (i.e., how common a particular parasite is among those who are infected).

30 These indicators help determine the distribution, severity, and transmission patterns of parasitic infections in the studied population and are crucial for planning effective public health interventions.

31 Sample Size: To calculate a prevalence of approximately 1% for T. cruzi, with a 95% confidence level and a 2% margin of error, in a population of 1,500 patients with HIV infection and a 6% attrition, we must take a sample of 323 individuals. To calculate a prevalence of approximately 2% for HTLV, with a 95% confidence level and a 2% margin of error, in a population of 1,500 patients with HIV infection and a 6% attrition, we must take a sample of 534 individuals. To calculate a prevalence of approximately 15% for Strongyloides, with a 95% confidence level and a 2% margin of error, in a population of 1,500 patients with HIV infection and a 6% attrition, we must take a sample of 717 individuals.

32 Data Analysis: Statistical analysis of the data will be performed using GraphPad Prism 5.0 software and SPSS 22. The presence of positive serology for T. cruzi, HTLV-1, and Strongyloides will be analyzed with epidemiological and clinical variables to determine the associations between serology and these variables. Variables found to be significant in the univariate analysis will be included in a multivariate analysis. The measure of association will be calculated as the odds ratio (OR), 95%.

33 Study Limitations: This study will be conducted at the Loreto Regional Hospital and the Iquitos Support Hospital. Clinicians will be trained to perform screening and collect epidemiological data. However, despite training, the variables may not be captured correctly.

Quality Control

34 Ten percent of negative samples and all positive samples will be kept refrigerated and sent to ACSA every 48-72 hours for a second quality control analysis.