Examining human paragonimiasis as a differential diagnosis to tuberculosis in The Gambia

Richard Morter1,2*, Ifedayo Adetifa3,4, Martin Antonio1,5,6, Fatima Touray1, Bouke C. de Jong1,7,8, Charlotte M. Gower9† and Florian Gehre1,8†

Abstract

Objective: Paragonimiasis is a foodborne trematode infection of the lungs caused by Paragonimus spp., presenting clinically with similar symptoms to active tuberculosis (TB). Worldwide, an estimated 20.7 million people are infected with paragonimiasis, but relatively little epidemiological data exists for Africa. Given a recently reported case, we sought to establish whether paragonimiasis should be considered as an important differential diagnosis for human TB in The Gambia, West Africa.

Results: We developed a novel PCR-based diagnostic test for Paragonimus species known to be found in West Africa, which we used to examine archived TB negative sputum samples from a cross-sectional study of volunteers with tuberculosis-like symptoms from communities in the Western coastal region of The Gambia. Based on a “zero patient” design for detection of rare diseases, 300 anonymised AFB smear negative sputum samples, randomly selected from 25 villages, were screened for active paragonimiasis by molecular detection of Paragonimus spp. DNA. No parasite DNA was found in any of the sputa of our patient group. Despite the recent case report, we found no evidence of active paragonimiasis infection masking as TB in the Western region of The Gambia.

Keywords: Paragonimiasis, Paragonimus, West Africa, Foodborne trematodiases, Neglected tropical diseases, Tuberculosis

Introduction

Tuberculosis (TB) is a major global public health concern. The mainstay of TB diagnosis, particularly in developing countries, is sputum smear-microscopy [1]. As a result, verifying differential diagnosis for persons with clinical symptoms and a smear-negative microscopy result is difficult. Besides smear negative, culture positive TB, common alternative diagnoses include non-tuberculous mycobacteria and in some parts of the world, paragonimiasis, a food-borne trematode infection caused by the Paragonimus spp. [2].

Paragonimus is a highly neglected and poorly known infection acquired through ingestion of undercooked crustacea [3, 4], which infects a range of mammals including humans, where symptoms are synonymous with TB infection [5, 6]. Diagnostic confusion between TB and paragonimiasis is common in countries such as India [6] and The Philippines [7], and has resulted in investment in training and integrated diagnostic procedures for all TB suspects in some areas. In West Africa, epidemiological data describing the burden of paragonimiasis is scarce, with studies limited to Nigeria and Cameroon [8, 9]. Though case reports from Liberia, Benin and Cote d’Ivoire have also been documented [10–12]. Whilst the species Paragonimus westermani and P. heterotremus are the most common species worldwide, in Africa only P. africanus and P. uterobilateralis have been found thus far [12, 13].
In The Gambia, West Africa, there is anecdotal evidence of Paragonimus infection and crustaceans are included in the local diet, although there is little documented in the evidence of culinary practices that might increase risk of consuming undercooked crabs. As recently as 2011, there was a confirmed case in a 12 year old Gambian boy returning from Casamance, the Senegalese region immediately south of our study area, who was infected with Paragonimus after reportedly consuming raw crabmeat (Richard Bradbury, personal communication). In addition, four case reports were described in patients originating from Casamance in the 1960s [12, 14]. Therefore, one possible alternative diagnosis for individuals who are smear-negative with TB-like symptoms in The Gambia could be paragonimiasis.

Nested into a cluster-randomised TB enhanced case finding (ECF) study conducted in The Gambia from December 2011 to November 2014, we carried out the first epidemiological study of paragonimiasis amongst smear-negative Gambian TB suspects living in the coastal region bordering Casamance. Using archived sputum samples from the ECF study, our aim was to conduct a rapid appraisal of whether human paragonimiasis was an important differential diagnosis for TB in this region of the Gambia, which might warrant more extensive surveys and investment in training, awareness and diagnostic facilities for this neglected parasite. We further aimed to develop a diagnostic test for paragonimiasis suitable for surveys in West Africa.

Main text
Materials and methods
Study area
For the TB-ECF study, field teams sensitised community members in villages through video presentations in local languages explaining TB symptoms, diagnosis and treatment. All villages were located in the coastal Greater Banjul area of The Gambia and the area bordering Casamance in southern Senegal. Patients with self-reported coughing and production of two sputum samples were enrolled regardless of age and gender. Written informed consent was provided by all participants including written parental consent for minors and assent where appropriate. No additional exclusion criteria applied to those meeting the criteria for inclusion. For the paragonimiasis study, a subset of 300 archived patient sputa negative on two Acid-fast Bacteria (AFB) smears were tested for the presence of Paragonimus DNA, therefore the investigators were not blinded to AFB-smear status. AFB smear microscopy was performed on fresh samples received directly from the field before sample archiving. Sample size calculation for this study was based on the “zero prevalence” model for detection of rare diseases [15]. This model calculates that an absence of cases found in 300 samples would mean it could be stated with 95% confidence that prevalence of an infection is < 1% amongst the study population of smear-negative TB suspects. In order to maximise geographic coverage, twelve anonymised AFB-smear negative sputum samples originating from each of 25 villages in the ECF study were randomly selected for analysis.

Sputum sample processing and DNA extraction
An equivolume of mucolysis solution (0.5% N-acetyl l-cysteine, 2% sodium hydroxide, 1.45% tri-sodium citrate) was added to 1.5 ml of stored sputa, vortexed and incubated at room temperature. After 15 min, each sample was made up to a volume of 50 ml with phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 20 min. The pellet was re-suspended in 200 µl PBS and heat inactivated at 99 °C for 20 min.

DNA was purified from 200 µl of each sample using QIAamp DNA mini kit spin columns (Qiagen Ltd., Hilden, Germany, Ref. 51306) following the standard manufacturer’s protocol for extraction from body fluids. The DNA was eluted in a final volume of 150 µl elution buffer and stored at − 20 °C.

Successful DNA extraction was monitored by real-time amplification of the human ribonuclease P gene (RNaseP) as an internal positive control. Reagents were prepared in 25 µl final volume to the following concentrations: 1 × Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Massachusetts, USA, Ref 1173-025), 0.2 pmol of each primer; RNaseP-F and RNaseP-B, 0.2 pmol probe RNaseP-P, 3.0 mM MgCl₂, 0.1x ROX reference dye (Invitrogen, Ref. 12223-012) and 2.5 µl template DNA. Primer and probe sequences are found in Additional file 1: Table S1. In an ABI7500 real-time PCR system (Applied Biosystems, Massachusetts, USA), samples were amplified with the following cycling parameters: 1 cycle at 50 °C/2 min, 1 cycle at 95 °C/10 min, followed by 45 cycles of 95 °C/15 s and 60 °C/1 min.

Paragonimus spp. PCR assay development
The internal transcribed spacer 2 (ITS-2) region of Paragonimus spp. was chosen as the target region for the PCR assay. Sequences were obtained from GenBank for P. africanaus (accession no. AB298780) and kindly provided by Professor David Blair (James Cook University, Australia) for P. uterobilateralis (unpublished data). The assay used was modified from a PCR-based assay published by Chen et al. (2011). This assay was designed to target the ITS-2 sequence of P. westernmani (primers PW-F and PW-B), for our study of paragonimiasis in The Gambia, however, the reverse primer was redesigned for greater specificity to the endemic species; P. africanaus and P. uterobilateralis.
The same dilution series of positive control DNA was used as template DNA (Fig. 1a). Overall the PCR assay demonstrated sensitivity at levels lower than 0.1 pg/µl when pure positive controls were prepared to the following concentrations: 1000, 100, 10, 5, 1, 0.5, 0.1 pg/µl. 1 µl of DNA in a final reaction volume of 25 µl. PCR conditions included an initial denaturation at 94 °C/5 min, 35 cycles 94 °C/30 s (denaturation), 55 °C/1 min (annealing) and at 72 °C/30 s (extension) and a final extension at 72 °C/5 min. The expected PCR product size was 221 base pairs (bp).

Results
Study population
Descriptive patient characteristics of 300 smear-negative patients from an enhanced case-finding study for TB in the Greater Banjul region of The Gambia whose sputum samples were randomly selected for inclusion in this study are shown in Table 1.

| Age   | N (%) |
|-------|-------|
| < 10  | 9 (3) |
| 10–25 | 57 (19) |
| 26–40 | 75 (25) |
| 41–55 | 89 (29.7) |
| 56–70 | 57 (19) |
| > 71  | 23 (7.7) |

Paragonimus spp. PCR assay validation
The modified assay’s (PW-F/PAU-B) detection limit was estimated using a dilution series of the plasmid positive control DNA. Dilutions of the P. africanus and P. uterobilateralis positive controls were prepared to the following concentrations: 1000, 100, 10, 5, 1, 0.5, 0.1 pg/µl. 1 µl of each was used as template DNA per reaction using primers PW-F/PAU-B. Overall the PCR assay demonstrated sensitivity at levels lower than 0.1 pg/µl when pure positive control alone was used as template DNA (Fig. 1a). The same dilution series of positive control DNA was then used to spike 200 µl decontaminated sputum sediment. Extracted sputum samples, spiked with plasmid positive control displayed sensitivity levels of 5 and 100 pg/µl of DNA for P. africanus and P. uterobilateralis, respectively (Fig. 1b). The sensitivity of the PW-F/PAU-B assay was also shown to be greater for the two West African target species, P. africanus and P. uterobilateralis, compared to P. westermani (Fig. 1c).

Primer specificity was validated by assaying known positive DNA of helminth species closely related to Paragonimus spp. using both PW-F/PW-B and our adapted PW-F/PAU-B primer combinations (Fig. 1d). The Paragonimus spp. samples produced a band of 221 bp as expected. No amplification was detected with either primer pair and S. mansoni, S. haematobium or N.americanus DNA. There was no amplification product using Opisthorchis DNA and the primer combination PW-F/PW-B. Amplifying Opisthorchis DNA using our modified primers PW-F/PAU-B resulted in a substantially larger band (800 bp), clearly distinguishable from Paragonimus bands (221 bp).

PCR analysis of clinical samples
The quality of the DNA extractions was verified in 10% (n = 30) of samples, selected at random. All samples were positive for RNaseP, suggesting DNA was successfully extracted from sputa (data not shown). We selected the PW-F/PAU-B combination for analysis of the clinical samples. All TB smear-negative sputum samples (n = 300) were tested. 10.7% (n = 32/300) samples amplified a band larger than the expected product size, approximately 250 bp. Twenty of these potentially positives were validated by DNA sequencing (Macrogen, Amsterdam, The Netherlands), analysed using Geneious V.7.1 sequence alignment and editing software (Biomatters, Auckland, New Zealand) and it was confirmed none of these amplifications were Paragonimus spp. sequences through BLAST searches against the GenBank (NCBI, MD, USA) database. These results indicate that no Paragonimus infection was detected in the sputa examined in this study. Based on the zero prevalence model, a case detection rate of zero was interpreted (with 95% confidence) as a prevalence less than 1% in our study population.

Discussion
Paragonimiasis is a neglected yet emerging global health problem. It is especially important as differential diagnosis of TB [6, 16] and its potential large cost implications of mis-diagnosis for TB control programs. Although previous reports suggest that the disease is present in the Senegambian region we did not detect any cases of Paragonimus DNA in samples from symptomatic but TB-smear negative patients. The sample size was based
be considered a primary differential diagnosis for adults suspected to have TB in this region of the Gambia. However, it must be remembered that sub-population variation in eating practices may alter the risk in children or in other parts of the Gambia. Further work is needed to identify the source of symptoms in smear negative TB suspects.

Limitations
- Our results are not representative of the whole country although 80% of all TB cases in the country occur in the Greater Banjul area [19].
- We did not study intermediate hosts (crustacea/shellfish) or their mammalian predators and thus our conclusions are limited to the importance of paragonimiasis as a differential diagnosis for TB in this study population, rather than for the generalised presence of paragonimus in other mammals and the potential for occasional human infections.
- This study used archived samples in order to conduct a rapid assessment. Thus we were not able to directly ask participants about individual level eating habits. Blood was not available that would have allowed testing for anti-paragonimus antibodies. Positive control DNA from adult worms was not available.

Additional file

**Additional file 1: Table S1.** Primer and probe sequences used in the study.
ITS-2 region of that was used for the optimisation of the current PCR assay and designed the study. RM, FG and FT carried out molecular assays. RM, CMG and FG analysed the data. IA, MA and BJ conceived and designed the ECF study and contributed reagents and materials. RM, CMG and FG drafted the manuscript. All authors read and approved the final manuscript.

Author details
1 Vaccines and Immunity Theme, Medical Research Council (MRC) Unit The Gambia, Fajara, The Gambia. 2 School of Biological Sciences, The University of Manchester, Manchester, UK. 3 Disease Control and Elimination Theme, Medical Research Council (MRC) Unit The Gambia, Fajara, The Gambia. 4 Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, UK. 5 Microbiology and Infection Unit, Warwick Medical School, University of Warwick, Coventry, UK. 6 Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK. 7 Department of Medicine, New York University, New York, USA. 8 Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium. 9 Department of Infectious Disease Epidemiology, Imperial College London, London, UK.

Acknowledgements
We thank Professor David Blair for kindly providing the DNA sequence of the ITS-2 region of P. vivax that was used for the optimisation of the current PCR assay and Dr Sarah Knowles for statistical advice. We also thank the TB ECF study laboratory and field teams and Abigail Ayorinde for their work in preparing the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data is readily available on request.

Consent to publish
Not applicable.

Ethics approval and consent to participate
The samples used were collected after informed consent within a TB enhanced case finding (ECF) study. Consent was given on behalf of participants aged < 18 by their parents or guardian. Ethical approval for the re-use of samples and written consent to participate in the current study were obtained from the Imperial College Research Ethics Committee (ICREC), Imperial College London, UK. Participants aged ≥ 18 years provided written informed consent to participate in this study.

Funding
CMG was supported by the Royal Society (GB). FG and BD were funded by an INTERRUPTB Grant (311725) of the European Research Council (ERC). IA was supported by a Grant (GBM-T-MRC) of the Global Fund to Fight AIDS, Tuberculosis and Malaria.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 11 September 2017   Accepted: 6 January 2018
Published online: 15 January 2018

Abbreviations
TB: tuberculosis; ECF: enhanced-case-finding; AFB: acid-fast bacteria; DNA: deoxyribonucleic acid; PBS: phosphate buffered saline; PCR: polymerase chain reaction; RNaseP: human ribonuclease P; ITS-2: internal transcribed spacer 2; NTC: no template control; bp: base pairs.

References
1. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, Spector S, Roscigno G, Nikengason G. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. 2011;24(2):514–50.
2. World Health Organisation. Foodborne trematode infections: Paragonimiasis [ONLINE]. Available at: http://www.who.int/foodborne_trematode_infections/paragonimiasis/en/. Accessed 5 Sept 2017.
3. Sachs R, Cumberlidge N. Distribution of metacercariae in fresh-water crabs in relation to Paragonimus infection of children in Liberia, West Africa. Ann Trop Med Parasitol. 1990;84(3):277–80.
4. Shin MH, Kita H, Park H-Y, Seo H-Y. Cysteine protease secreted by Paragonimus westermani attenuates effector functions of human eosinophils stimulated with immunoglobulin G. Infect Immun. 2001;69(3):1599–604.
5. Chang HT, Wang CW, Yu CF, Hsu CF, Fang JC. Paragonimiasis; a clinical study of 200 adult cases. Chin Med J. 1958;77(1):3–9.
6. Singh TS, Mutum SS, Razaque MA. Pulmonary paragonimiasis: clinical features, diagnosis and treatment of 39 cases in Manipur. Trans R Soc Trop Med Hyg. 1986;80(6):967–71.
7. Belizarov V, Guan M, Borja L, Ortega A, Leonarda W. Pulmonary paragonimiasis and tuberculosis in Sorsogon, Philippines. Southeast Asian J Trop Med Public Health. 1997;28(Suppl 1):37–45.
8. Ibanga ES, Eyo VM. Pulmonary paragonimiasis in Oban community in Akamkpa Local Government Area, Cross River State, Nigeria: prevalence and intensity of infection. Trans R Soc Trop Med Hyg. 2001;95(2):159–60.
9. Sam-Abbenyi A. Endemic pulmonary paragonimiasis in Lower Mundani (Fontern district of southwest Cameroon). Results of treatment with praziquantel. Bull Soc Pathol Exot. 1985;78(3):334–41.
10. Aka NA, Allabi ACE, Dreyfuss G, Kinde-Gazard D, Tawo L, Rondelaud D, Boutelle B, Avode G, Aragonou SY, Grinafon M, et al. Epidemiological observations on the first case of human paragonimosis and potential intermediate hosts of Paragonimus sp. in Benin. Bull Soc Pathol Exot. 1999;92(3):191–4.
11. Aka NA, Assoumou A, Adoubryn D, Domoua K, Koudio F, Moyou-Somo R, Nakamura-Uchiyama F, Nawa Y, Rondelaud D, Dreyfuss G. First findings on the seroepidemiology of human paragonimosis at the anti-tuberculosis centre of Divo, Republic of Ivory Coast (West Africa). Parasites &´J De La Societe Francaise De Parasitologie. 2008;15(2):157–61.
12. Aka NDA, Adoubryn K, Rondelaud D, Dreyfuss G. Human paragonimiasis in Africa. Ann Afr Med. 2008;7(4):153–62.
13. Keiser J, Utzinger J. Emerging foodborne trematodiasis. Emerg Infect Dis. 2005;11(10):1507–14.
14. Bowesman C. Surgery and clinical pathology in the tropics. Edinburgh: Livingstone; 1960.
15. Yazici H, Biyikli M, van der Linden S, Schouten HJA. The ‘zero patient’ design to compare the prevalences of rare diseases. Rheumatology. 2001;40(2):121–2.
16. Barennes H, Slesak G, Buisson Y, Odermatt P. Paragonimiasis as an important alternative misdiagnosed disease for suspected acid-fast Bacilli Smear-negatve tuberculosis. Am J Trop Med Hyg. 2014;90(2):384–5.
17. Centers for Disease Control and Prevention. Recommendations of the international task force for disease eradication. MMWR. 1993;42(16):1–38.
18. Traore SG, Odermatt P, Bonfond B, Utzinger J, Aka NA, Adoubryn KD, Assoumou A, Dreyfuss G, Koussemon M. No Paragonimus in high-risk groups in Cote d’Ivoire, but considerable prevalence of helminths and intestinal protozoan infections. Parasites Vectors. 2011;4:10.
19. Touray K, Adetifa IM, Jallow A, Rigby J, Jeffries D, Cheung YB, Donkor S, Adegbola RA, Hill PC. Spatial analysis of tuberculosis in an Urban West African setting: is there evidence of clustering? Tropical Med Int Health. 2010;15(6):664–72.