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Association between enteric protozoan parasites and gastrointestinal illness among HIV- and tuberculosis-infected individuals in the Chowke district, southern Mozambique

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ABSTRACT

Human immune deficiency virus (HIV) and tuberculosis (TB) infections remain major public health issues globally, particularly in sub-Saharan Africa. Impairment of both cell-mediated and humoral immunity by HIV and/or TB infections may limit the host's defences against other pathogens, including the diarrheagenic protozoan *Cryptosporidium* spp., *Giardia intestinalis*, and *Entamoeba histolytica*. During September–December 2015 a cross-sectional study was conducted to assess the prevalence and molecular diversity of these enteric parasites among HIV- and/or TB-infected patients at a medical reference centre in Chowke district, southern Mozambique. A total of 99 stool specimens were initially screened by direct microscopy and further confirmed and characterised by molecular methods. DNA sequence analyses of the genes encoding the small subunit ribosomal RNA and the 60-kDa glycoprotein were used for the typing and sub-typing of *Cryptosporidium* isolates, respectively. *G. intestinalis*-positive isolates by real-time PCR were subsequently typed at the glutamate dehydrogenase locus. Differential diagnosis of *E. histolytica*/*dispar* was achieved by real-time PCR. *G. intestinalis* (8.1%) was the enteric protozoan more frequently detected, followed by *Cryptosporidium* spp. (7.1%), and *Entamoeba histolytica*/*dispar* (6.1%). Two HIV-infected (but not TB-infected) patients harbour *G. intestinalis* and *Cryptosporidium* spp. co-infections. Two (29%) *G. intestinalis* isolates were successfully characterised, revealing the presence of known AII and novel BIV genotypes. Four (57%) *Cryptosporidium* isolates were unmistakeable assigned to *C. hominis*, identifying two (IbA10G2 and IdA22) sub-types. *Cryptosporidium* infections were not associated to diarrhoea in HIV-positive patients, probably because improved immune function in the affected individuals due to antiretroviral therapy. *G. intestinalis* was considered a non-opportunistic pathogen, whereas the presence of *E. histolytica* could not be confirmed by molecular methods. Based on their common presence in the studied clinical population, we recommend the effective diagnosis and treatment of these enteropathogens for improving the management of HIV and TB patients.

1. Introduction

Mozambique is globally ranked as a high-burden country for human immune deficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis (TB) by *Mycobacterium tuberculosis*. Both infections represent a serious public health concern and have been identified among the leading causes of disease and death in this African country (World Health Organization,

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2014). Under this highly endemic scenario co-infections are common, with up to 42% of TB patients being HIV positive (García-Basteiro et al., 2016). Moreover, because of their impaired immunity and lower CD4 T cell counts, HIV and/or TB patients are particularly susceptible to opportunistic infections by diarrheagenic protozoan pathogens including *Cryptosporidium* spp. and, to a lesser extent, *Giardia intestinalis* (syn. *G. duodenalis*, *G. lamblia*), and *Entamoeba histolytica*, particularly in malnourished subjects (Li et al., 2015). There is little surprise then that up to 90% of individuals living with HIV/AIDS experience diarrhoea in developing countries (Framm and Soave, 1997).

As other enteric pathogens, *Cryptosporidium* spp., *G. intestinalis*, and *E. histolytica* are transmitted via the faecal-oral route through ingestion of contaminated water or food or by direct person-to-person contact, particularly in settings with poor sanitation and limited access to safe drinking water (Nhampossa et al., 2015). The genus *Cryptosporidium* encompasses at least 30 valid species, of which *C. hominis* and *C. parvum* are responsible of the vast majority of human infections (Ryan et al., 2014). The only *Giardia* species that is pathogenic to humans, *G. intestinalis*, is currently regarded as a multi-species complex divided into eight (A to H) distinct genetic variants (assemblages), of which only assemblages A and B are infective to immunocompetent human subjects (Ryan and Cacciò, 2013). Finally, *E. histolytica* is the etiological agent of amoebiasis, a condition normally asymptomatic that in a number of patients may cause diarrhoea, amoebic dysentery, amoebic colitis or abscesses in the liver, lungs or brain. In these particular cases the outcome of the disease is often severe and even fatal (Ximénez et al., 2009).

The current situation of cryptosporidiosis, giardiasis, and amoebiasis in Mozambique remains largely unknown. The epidemiological data available come from few surveys conducted in the provinces of Maputo and Nampula specifically targeting paediatric or student populations (Fonseca et al., 2014; Guidetti et al., 2011; Mandomando et al., 2007; Nhampossa et al., 2015). Reported prevalence rates based on conventional microscopy or immunoassay methods varied from 1 to 20% for *Cryptosporidium* spp., 2 to 50% for *G. intestinalis*, and 1 to 12% for *Entamoeba histolytica*/*dispar*. No molecular epidemiological studies assessing the occurrence and genetic diversity of these protozoan enteropathogens have been carried out in other human populations at risk of infection such as immunocompromised patients. In order to fill this knowledge gap we report here the prevalence and molecular diversity of the most relevant diarrheagenic protozoan species among HIV- and tuberculosis-infected patients seeking medical care at a reference centre in southern Mozambique.

2. Material and methods

2.1. Compliance with ethical standards

This study has been approved by the Research Ethics Committee of the Valencia University (reference number: H1474970118385). Written informed consent was waived as the stool samples used were exclusively intended for routine clinical diagnostic procedures. However, verbal informed consent was obtained from all the participants prior to stool collection and examination.

2.2. Study area

This survey was conducted at the Carmelo Hospital (CH), a medical unit of reference for HIV and TB located in the rural town of Chokwe in Gaza province (Mozambique), about 230 km north of the capital city of Maputo. The CH serves a catchment population of more than 187,000 people only in the Chokwe district, and approximately 1.4 million people in the Gaza province. Agriculture is the main economic activity, involving more than 80% of the active population. Drinking untreated water is usually obtained from underground wells.

2.3. Stool sample collection

A cross-sectional study was conducted during September–December 2015 among all TB- and/or HIV-infected individuals receiving medical care at the CH. A single fresh stool sample was collected per patient. Individual specimens were divided and stored in two different preservatives. An aliquot was placed in a commercial faecal concentrator device (REAL Mini-system, Durviz, Valencia, Spain) containing sodium acetate-acetic acid-formalin (SAF) solution intended for the transport, concentration, and subsequent microscopic examination of enteric parasites. A second aliquot was placed in a vial containing 70% ethanol and was specifically intended for extracting and purifying genomic DNA. A set of sub-samples was shipped to the Pharmacy Faculty (Valencia University, Spain) and the National Centre for Microbiology (Majadahonda, Spain), respectively, for further molecular analyses. Basic demographic (gender, age, origin) and clinical (symptoms, other non-enteric protozoan infections, stool consistency) parameters were also compiled from each patient at the time of sample collection.

2.4. Direct microscopy

Detection of parasite eggs, cysts, oocysts, and trophozoites was performed by direct smear microscopic examination by experienced staff on each concentrated stool sample. Thin smears were also prepared and stained by Ziehl–Neelsen technique for the specific identification of *Cryptosporidium* oocysts and other Coccidia. Examination was conducted at 100–400 magnification.

2.5. DNA extraction and purification

Total DNA was extracted from ~1 g of each faecal sub-sample preserved in 70% ethanol using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples (200 μL) were stored at −20 °C until use.

2.6. Molecular detection and characterization of Giardia intestinalis

Detection of *G. intestinalis* DNA was achieved using a real-time PCR method targeting a 62-bp region of the small subunit ribosomal RNA (*SSU* rRNA) gene of the parasite (Verweij et al., 2003b). Amplification reactions (25 μL) were conducted using a TaqMan® expression assay mix (Applied Biosystems, CA, USA) on a Corbett Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany). *G. intestinalis* isolates that tested positive by real-time PCR were subsequently tested by a semi-nested-PCR protocol (Read et al., 2004) targeting a ~432-bp fragment of the glutamate dehydrogenase (*GDH*) locus. PCR reaction mixtures (25 μL) were carried out using MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany) on a 2720 thermal cycler (Applied Biosystems). Sequences for the primers and probes used in these assays are shown in Supplementary material 1. Laboratory-confirmed positive and negative DNA samples were routinely used as controls and included in each round of PCR. PCR products were visualized on 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda). Positive-PCR products were directly sequenced in both directions using the internal primer set described above. DNA sequencing was conducted by capillary