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Application of real-time PCR for the detection of *Strongyloides* spp. in clinical samples in a reference center in Spain

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ABSTRACT

Strongyloidiasis is one of the major intestinal helminthic infections in humans with a worldwide distribution, affecting especially tropical and subtropical regions. This disease can occur without any symptoms or as a potentially fatal hyperinfection or disseminated infection. Definitive diagnosis of *Strongyloides stercoralis* infection relies mainly on demonstration of larvae in stool, but at present there is no gold standard for this diagnosis. Our main objective was to evaluate a real-time PCR targeting the 18S rRNA gene of *Strongyloides* spp. and to compare it with routine parasitological methods. DNA from *Strongyloides venezuelensis* was used to optimize PCR protocols obtaining an analytical sensitivity of 0.1 pg of parasite DNA per sample. Sensitivity and specificity of real-time PCR on fecal samples from 231 patients screened for suspected strongyloidiasis attending two hospitals in Madrid were 93.8% and 86.5%, respectively. No significant differences were found when comparing Ct-values of positive PCR between parasitological positive and negative samples. This study showed that real-time PCR is an effective tool for diagnosing strongyloidiasis and could be applied in association with parasitological methods in epidemiological studies in endemic areas. It would be also important to assess its performance in immunocompromised populations who are at risk of fatal disease.

1. Introduction

Strongyloidiasis is a human helminthic intestinal infection mainly caused by *Strongyloides stercoralis* and, to a lesser extent, by *Strongyloides fuelleborni*. *S. stercoralis* has a cosmopolitan distribution, whereas *S. fuelleborni* occurs in Africa and *S. fuelleborni* kellyi in Papua island. Human infections by both species are associated to poor sanitary conditions and a warm and humid climate, but accurate information on the geographical distribution and the global burden of strongyloidiasis is frequently lacking (Siddiqui and Berk, 2001). Strongyloidiasis is one of the neglected tropical diseases listed by the World Health Organization (WHO, 2014) with an estimated global prevalence ranging from 30 to 100 million cases (Bethony et al., 2006). In Spain, most of the reported human infections are imported cases (González et al., 2010), although an endemic area persists in the Autonomous region of Valencia on the Eastern Mediterranean coast (Román et al., 2001). In this area, a human strongyloidiasis prevalence rate of 12.4% has been found in agricultural workers (Román et al., 2003). Additional Spanish national cases have also been documented in renal transplant recipients (Beltrán et al., 2009; Rodríguez et al., 2009).

Chronic human strongyloidiasis is usually asymptomatic in healthy individuals, where the infection is associated with low intestinal worm burdens and intermittent larval excretion (Olsen et al., 2009). Also, the parasite has the ability to replicate (autoinfection), resulting in sustained parasitation, facilitating parasite dispersal and increasing the risk of infection among the community (Gill et al., 2004). In addition, in patients with defective cell mediated immunity, increased parasite burdens (hyperinfection syndrome) may lead to the dissemination of the infection to adjacent organs including lung, liver and central nervous system. These cases usually present the most severe clinical manifestations with associated mortality rates ranging from 15% to 87% (Marcos et al., 2008).

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Detection of the infection in asymptomatic patients is crucial to prevent hyperinfection syndrome. To date, there is no gold standard for diagnosing *S. stercoralis* human infections. The presence of the parasite should be suspected by clinical signs and symptoms (e.g. eosinophilia, hyperglobulinemia E, allergic reactions) compatible with the infection, although many of them are non-pathognomonic of the disease. The definitive diagnosis relies on the demonstration of the parasite's larval stages in stool samples (Siddiqui and Berk, 2001). Nevertheless, direct diagnostic procedures examining stool samples, including Baermann method, agar plate stool culture or Harada-Mori technique, are often hampered with variable or low sensitivities. On the other hand, most serological tests offer good sensitivities in individuals with chronic infections, but are less effective in hyperinfections and in returning travelers from endemic areas (Sudarshi et al., 2003). In addition, *Strongyloides* spp. antibody tests are known to cross-react with other helminthic infections. A combination of some of the above mentioned methods may yield highest sensitivity, especially if consecutive stool samples are examined. However, these procedures are rather cumbersome and time consuming (Siddiqui and Berk, 2001), so new diagnostic approaches are highly needed.

DNA-based methods for the detection of a variety of intestinal parasites have been developed in recent years, showing excellent sensitivity and specificity values. The introduction of real-time PCR has significantly contributed to the improvement of the diagnostic performance of microscopy and other conventional parasitological techniques, especially in those cases where parasitic material is excreted at low concentrations or in a fluctuating manner (Tanriverdi et al., 2002). Specific detection of *Strongyloides* spp. DNA in human stool samples by real-time PCR has been recently proposed as a promising alternative diagnostic approach (Verweij et al., 2009; Kramme et al., 2011; Sultana et al., 2013), but this methodology is not commercially available and at the moment is only performed in research centers.

In the present study we evaluated the diagnostic performance (sensitivity, specificity, and predictive positive and negative values) of an optimized real-time PCR protocol based on that described by Verweij et al. (2009). The results obtained by real-time PCR were compared with those obtained by routine parasitological methods for the diagnosis of strongyloidiasis.

2. Materials and methods

2.1. Human stool samples and epidemiological questionnaires

Stool samples were submitted from outpatients (including Spanish nationals, immigrants, tourists and aid workers) attending Severo Ochoa and Gregorio Marañón Hospitals in Madrid (Spain) between June 2010 and June 2012, showing significant levels of IgE, eosinophilia or other symptoms suggestive of strongyloidiasis. Eosinophilia was defined as an eosinophil count of more than 800 per mm^3. Patients who received any parasitic treatment in the previous 3 months were excluded. A total of 231 patient samples were included in the study: 135 from the Severo Ochoa Hospital and 96 from the Gregorio Marañón Hospital. A control group was composed of 25 healthy Spanish individuals with no history of travel to endemic area. Stool samples were collected according to routine procedures and sent to the Instituto de Salud Carlos III (ISCIII) for further analyses.

A standardized epidemiological questionnaire covering demographic (age, gender, country of birth, history of past exposure in an endemic area) and clinical (Ig E level in serum, eosinophil count) information was also obtained from each participant included in the study.

All the patients with confirmed strongyloidiasis were treated with ivermectin according to the protocols of each hospital.

This study received the approval of the Committee of Research Ethics and Animal Welfare from the Instituto de Salud Carlos III (PI No.: CEI PI06_2012-v2).

2.2. Microscopy and parasitological methods

Microscopic examination for the presence of rhabditiform larvae was performed using formalin-ethyl acetate concentration technique from each sample, according to the standard routine procedures at the diagnostic laboratory of the hospitals. The coproculture (agar culture) was carried out as previously described in the Proceedings in Clinical Microbiology edited by the Spanish Society of Infectious Diseases (Cañavate et al., 2009). Briefly, 1 g of each stool sample was placed on a nutrient agar plate, sealed with adhesive tape to prevent desiccation and incubated at 28 °C up to 10 days. On average, positive cases usually appeared between days three and five. Cultures were evaluated daily for visible tracks created by larvae carrying bacteria over the agar. Morphological identification was conducted by competent microbiologists at the hospital facilities. For this, if traces were seen on the agar plate, the plate was flushed with 10% formalin and a smear loop was passed over the surface to recover the helminths present. Then, the liquid was transferred to a tube, centrifuged and further examined for morphological identification. In the Harada Mori technique (HM), 0.5 to 1 g of each stool sample was used. Filter paper containing fresh fecal material was placed in a test tube with water to allow soaking of the filter paper by capillarity at 30 °C for 15 days. Then, larvae that crawled out of the fecal suspension and migrated into the warm water were collected by centrifugation and visualized by microscopy.

2.3. DNA isolation

Prior to DNA extraction, 1 g of each stool sample was suspended in 8 ml of saline solution and concentrated using Bioparapred-Midi columns (Leti Diagnostics, Barcelona, Spain). Then, the supernatants were discarded and 200 mg of the pellets were used for total DNA extraction using the QiaAmp DNA stool Mini kit (Qiagen, Hilen, Germany) following the manufacturer's instructions.

Purified genomic DNA from *Strongyloides venezuelensis* L3 (kindly provided by IBSAL-CIETUS, Salamanca, Spain) was prepared as described elsewhere (Simpson et al., 1982) and used as positive control. The concentration of all DNA samples obtained was measured by duplicate using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, NY, USA) to obtain an average concentration. DNA samples were further stored at −20 °C until use.

2.4. Real-time PCR amplification

2.4.1. Optimization of real-time PCR

Real-time PCR was standardized using the pair of primers designed from *S. stercoralis* 18S rRNA gene sequence (AF279916.2) by Verweij et al. (2009) using as template DNA purified from *S. venezuelensis* L3, DNA from stool samples of patients with strongyloidiasis confirmed by agar culture, genomic DNA extracted from other different parasite species (*Cryptosporidium parvum*, *Trichinella spiralis*, *Anisakis simplex* and *Taenia saginata*) and DNA from clinical samples of patients with different infections (*Giardia duodenalis*, *Entamoeba histolytica*, *E. dispar*, *Trichuris trichura*, *Schistosoma mansoni*, *Ascaris lumbricoides*, *Wuchereria bancrofti*, *Loa loa* and hookworm).

Amplification and detection of *Strongyloides* spp. DNA was performed in a final volume of 25 μl using 1X of Quantimix Easy Master Mix (Biotools B&M Laboratories, Madrid, Spain) which