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Original papers

Critical diagnosis of complicated strongyloidosis with nested-PCR and High Resolution Melting analysis (HRM)

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ABSTRACT. Diagnosis of strongyloidosis is sometimes problematic and requires novel techniques. Here, critical diagnosis of a complicated case of strongyloidosis using molecular methods is reported. A young woman referred to the Diagnostic Laboratory of Strongyloidiasis in School of Public Health, Tehran University of Medical Sciences. She had taken albendazole before referring to the laboratory. She had cerebral edema, behavior disorders, hypereosinophilia and titer of IgE >2000 IU/mL. The patient had history of intestinal and skin disorders and steroid therapy. For detection of *Strongyloides stercoralis* infection, parasitological techniques and novel methods of nested-PCR and HRM analysis were applied on stool samples upon admission and during the following up. On the samples provided upon first admission, parasitology showed negative results, while both molecular methods revealed infection with *S. stercoralis*. After specific treatment, during the following up, the patient general health was much improved and the results of all parasitological and molecular tests were negative for strongyloidosis. Application of novel sensitive diagnostic methods for detection of *S. stercoralis* is necessary, especially once parasitological techniques have lack of sensitivity.

Keywords: complicated strongyloidosis, diagnosis, HRM, nested-PCR

Introduction

Strongyloidosis is one of the soil-transmitted helminthic infections caused by *Strongyloides stercoralis* [1]. This parasite is most common in tropical and subtropical areas; however, migration and globalization have changed this distribution [2]. It has been estimated that about 30–100 millions of people are infected worldwide with this nematode [3]. Most infected immunocompetent individuals only present eosinophilia, but strongyloidosis manifestations have various clinical presentations, ranging in severity from asymptomatic to hyper infection and disseminated infections [2]. Dissemination is the results of migration of the infective larvae away from lung and gastrointestinal tract into other organ systems that can lead to the death of individuals [4]. Immunosuppressive conditions, especially corticosteroids therapies are the most important predisposing risk factors for such devastating strongyloidosis [2]. Early diagnosis and proper treatment before initiation of suppressive therapy in people with history of residency or travel to endemic areas is necessary to prevent such complicated consequences [4,5].

For diagnosis of strongyloidosis, various parasitological techniques have been used on stool samples; however, detection rate of conventional methods is low and repeated examinations of stool over a number of consecutive days are essential [3]. There are different serological antibody detection tests, some of which are commercially available [6], but in immunosuppressed individuals, they may yield false negative results [7]. Recently, molecular methods have been proved highly sensitive and specific for detection of *S. stercoralis* in fecal samples [8]; among all, in a study, nested-PCR was reported to be 100% sensitive and 91.6% specific in

diagnosis of strongyloidosis [9]. Real-time PCR with a high resolution melting (HRM) analysis has also been used for detection or differentiation of parasites such as *Echinococcus* species [10] and *Schistosoma mansoni* from *Schistosoma haematobium* [11] with indicator of amplicon production and physical behavior of DNA melting at specific temperature in real time.

Here, critical diagnosis of a complicated case of strongyloidosis using nested-PCR and HRM analysis is reported. The patient had brain involvement and being under steroid therapy; she had history of frequent travelling to *S. stercoralis* endemic areas. Parasitology failed to detect the infection, due to the use of insufficient anti-helminthic therapy; however, diagnosis was fulfilled using these novel methods.

Materials and Methods

Case report. In June 2018, a 30-year-old woman referred to the Diagnostic Laboratory of Strongyloidiasis in School of Public Health, Tehran University of Medical Sciences, suspected to have nervous *larva migrans* based on the result of her brain biopsy pathology. She had history of frequent travelling to Mazandaran Province in northern Iran, which is endemic for strongyloidosis [12].

According to the patient medical records, she had been under alternative courses of corticosteroid therapy for several years due to cosmetic operations, the last one of which had been performed about five months earlier. The month later, the patient presented mild intestinal complains in the form of alternative diarrhea and constipation, nausea, vomiting and indigestion as well as presence of skin rash, without taking medication or visiting any physician for these disorders. However, about two month thereafter she presented sudden progressive behavior disorders followed by incontinence of urine and stool. Since then, she had been hospitalized to do many imaging and laboratory tests including MRI and CT-Scan of brain, serological assays for various infectious and non-infectious diseases. MRI had shown multiple shape enhancing lesions in both centum semi vale and white matter subcortical portion of both frontal lobe and right parietal lobe with visualization of surrounding edema. CT-Scan had shown diffuse hypodense area in the white matter of right posterior parietal and occipital lobe suggestive of edema. At that time, the patient had been given corticosteroids for cerebral edema. Chest X-ray of axillary lymph nodes had revealed bilateral reactive response. CT-Scan of thorax with and without IV contrast had normal shape. There were no pulmonary symptoms, and density of lungs, pulmonary hila, and cardio mediastinal complex were normal, without any mass lesion, abscess or pulmonary infiltrations. CT-Scan of abdomen and pelvis indicated normal shape and density for liver, biliary channels, gall bladder, spleen, pancreas and both kidneys, without any abdominal or pelvic solid masses.

Culture and PCR of CSF for *Mycobacterium tuberculosis* and Herpes virus were negative. Serologic tests included of *Toxoplasma* (IgG and IgM) (performing twice in a week), rheumatoid factor, wright, V.D.R.L, CRP, FANA, Anti-DNA and HIV were all-negative. However, peripheral blood eosinophil showed a gradual increase from 1% to 10.8%, and ultimately to 14.5% during a week, but white blood cell counts at all these occasions were at normal range (4.8–6.6× 1000/mm^3). Subsequently, a brain biopsy sample was prepared and pathological findings indicated infection and allergic granulomatous type reactions suggestive of *larva migrans*. Based on these findings, the patient took albendazole for 10 days followed by interruption of steroid therapy. Serological tests for *Toxocara* IgG and *Fasciola* Ab revealed negative results, whereas the titer of IgE was >2000 IU/Ml. Then, she referred to our laboratory in order to find the etiological agent for her nervous *larva migrans*.

On her arrival, she was still suffering from behavior disorders and in continence of urine and stool, in addition to worsening of her intestinal and skin disorders, followed by onset of physical movement impairment in consecutive days. ELISA test on the sera for *S. stercoralis* total Ab, by a private diagnostic laboratory, showed negative titer of 5.5 NTU (< 9: negative). In our laboratory, the patient's stool samples were tested for detection of infection with *S. stercoralis* by both conventional parasitological techniques and novel methods of nested-PCR and HRM that these later molecular methods yielded positive results. Soon after, in the hospital that she was confined in, the patient received a combination of ivermectine and albendazole therapy, in addition to the administration of corticosteroids one week after initiations of anti-helminthic due to the cerebral edema. Then, a week later the patient's general health was much improved and she was discharged

from the hospital with continuous chemotherapy for one more week at home. About one month later during the following up, MRI showed no enhancing lesion and hydrocephalus or midline shift, and peripheral blood eosinophil was 2.1%. On this occasion, the results of our laboratory tests using all above-mentioned parasitological and molecular methods on stool samples were negative for *S. stercoralis*; and the patient physical and behavior disorders as well as intestinal and skin manifestation were resolved.

Samples collection. At first admission, triple fresh stool samples of consecutive days were provided by the patient accompany. After recovery, during the following up, double fresh stool samples were delivered to the laboratory.

Parasitological methods. All the stool samples were examined using direct smear preparation, formalin-ether concentration as well as nutrient agar plate culture. The culture method was performed as described previously [13]; i.e. about 3 g of every fecal sample was placed in the center of nutrient agar plates, incubated at 28–30°C for 48–72 h followed by examination of the plates using stereomicroscope.

DNA extraction. About 3 g of each stool sample was treated by distilled water and passed through the filter membrane into the collection tube. Later genomic DNA of the fecal samples were extracted using DNA Extraction kit YTA (Yekta Tajhiz Azma Co. Lot: DNAST 2016. 223002), according to the manufacturer's protocol, and stored at -20°C until the performance of molecular methods.

Nested-PCR. Nested-PCR protocol was performed for amplification of a partial mitochondrial cytochrome c oxidase subunit 1 (*Cox*1) gene of *S. stercoralis*. In the primary PCR round, external primers namely TJ5207 F (5′ -TTTG ATTGTTACCTGCTTCTATTTT -3′) and TJ5208 R (5′-TTTTACACCAGTAGGAACAGCAA -3′) [14] were used for amplification of a 650-bp target. For the second amplification round the primers COX F (5′- TGGTTTGGGTACTAGTTG -3′) and COX R (5′- GATGAGCTCAAACTACACA -3′) were used to amplify a 509-bp internal fragment [9].

The PCR amplification was carried out in a final reaction volume of 30 µl containing 15 µL of PCR mix included of 1.25 U Taq DNA polymerase, 200 µM of dNTPs and 1.5 mM MgCl2 (2x Master Mix RED Ampliqon, Denmark), 10 pmol of each primer and 3 µL of DNA sample for the first PCR round. Subsequently, 1µL of 1/40 diluted of the first round PCR product was subjected to a second PCR round. Negative controls (distilled water) were included for each PCR run. The cycling conditions for first round of PCR reaction were an initial denaturation step at 95°C for 6 minutes followed by 35 cycles of 95°C for 45 seconds (denaturation), 52°C for 60 seconds (annealing), and at 72°C for 90 seconds (extension) with a final extension of 72°C for 6 minutes. PCR conditions for the second round comprised of 95°C for 2 min, followed by 25 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, plus a final extension at 72°C for 6 minutes. Subsequently, 3 µL of each nested-PCR product was electrophoresed on a 1.5% agarose gel and visualized using a UV transilluminator after staining with 0.3 µg/mL safe stain (Fig. 1). DNA sequencing was performed using an ABI 3130xlplatform (Applied Biosystems, Foster City, California, USA).

HRM assay. PCR was performed in 25 µL final reaction volume containing 5 µL HOT FIREPol® EvaGreen® (ROX) qPCR Mix Plus (Solis Bio Byne, Estonia), 14 µL distilled water, 0.5 µL of 5 pmol of each primer, and 5 µL of DNA. The PCR *Cox*1 gene was amplified using specific primers CNF (5′- TTCTAGTGTTGATTTGGCT -3′) and CNR (5′- TTACCACCAAA ACTAGGATC -3′) which produced a 261-bp target [9]. PCR cycling for HRM curve obtainment and amplification detection were performed using a StepOnePlus^{TM} (Applied Bio system, CA, USA), under the conditions as follows: an initial denaturation step at 95°C for 12 min, followed by 45 cycles at 95°C for 15 s (denaturation), 50°C for 20 s (annealing), 72°C for 30 s (extension), and a final extension step at 72°C for 5 min. Fluorescence signals were measured after each amplification cycle. The reaction products were melted by increasing the temperature from 60°C to 95°C, with an increment of 0.3% °C every 15 sec, to obtain melting profiles. All samples were examined in triplet in 96 well plates. The quantitative detection and T_m values measured using the mentioned system. Extracted DNA from filariform larva of *S. stercoralis* (GenBank accession no. MG251326) was used as positive control.

Results

Parasitological methods

All parasitological methods, including direct smear preparation, formalin-ether concentration as

well as nutrient agar plate culture for triple samples of first admission and double samples of second admission during the following up, yielded negative results for infection with *S. stercoralis* and other intestinal helminths.

Nested-PCR

An initial fragment of genomic DNA was amplified using TJ5207and TJ5208 primers, followed by nested-PCR using COX F and COX R primers to amplify an internal fragment of 509 bp (Fig. 1). The PCR products were specific for *S. stercoralis*, and no band was observed in negative control. Sequence results were edited and analyzed by the Chromas software and compared with sequences present in GenBank database. The current sequence was identified as *S. stercoralis* and deposited in GenBank with the accession number KP663661. It had 100% identity with *S. stercoralis* isolates from northern (MK049079 and MG251321) and southern (MK049107 and MG251324) areas of Iran.

To follow up the patient after treatment for strongyloidosis, DNA from two stool samples of consecutive days was extracted, using DNA extraction kit, and nested-PCR was performed. Based on the results, no special band of *S. stercoralis* was observed on agarose-gel electrophoresis.

HRM assay

According to the analysis of HRM-PCR curve profiles of the replications, the melting temperature peaked at 77.8°C ± 0.3 and 77.9°C ± 0.25 for the patient's sample – obtained during the course of strongyloidosis infection – and the standard *S. stercoralis*, respectively (Fig. 2). However, application of HRM analysis, with the same procedure as mentioned above, on two stool samples of the patient during the following up yielded negative results for strongyloidosis.

Discussion

Strongyloidosis is one of the most ignored infections among the neglected tropical diseases [1]. It is more prevalent in tropical and subtropical climates throughout the world [2]. In Iran, northern areas including Mazandaran and Guilan Provinces, located along the south coast of the Caspian Sea, have temperate climate and are the most important endemic areas of strongyloidosis in the country

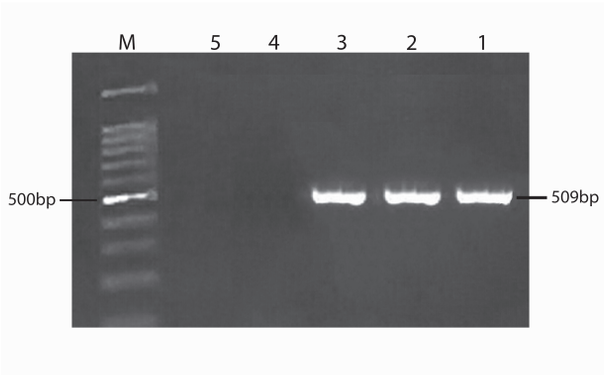

Fig. 1. Agarose-gel electrophoresis of polymerase chain reaction products of *Strongyloides stercoralis* extracted from patient stool samples. Lanes 1, 2, 3: *Strongyloides stercoralis* and lanes 4, 5: negative controls.
M = 100-bp DNA marker ladder.

[12]. The case presented here had frequent travelling to Mazandaran Province. She also had history of interval corticosteroid therapy during previous several years. There are many reports of hyperinfection and disseminated strongyloidosis because of many underlying conditions [4,15,16]. Nevertheless, corticosteroids have a particularly strong and specific association with the development of these manifestations [4], even without the presence of an immunosuppressed condition [17]. Such drugs can directly affect *S. stercoralis* by accelerating the transformation of rhabditiform larvae into invasive, filariform larvae [2]. The larvae disseminate into skin, mesenteric lymph nodes, gallbladder, liver, diaphragm, heart, pancreas, skeletal muscle, kidneys, ovaries and brain [2]. Present case had brain involvement by *S. stercoralis* larvae showing infection and allergic granulomatous type reactions in the brain biopsy, and cerebral edema confined by MRI and CT-Scan. This complication presented sudden progressive

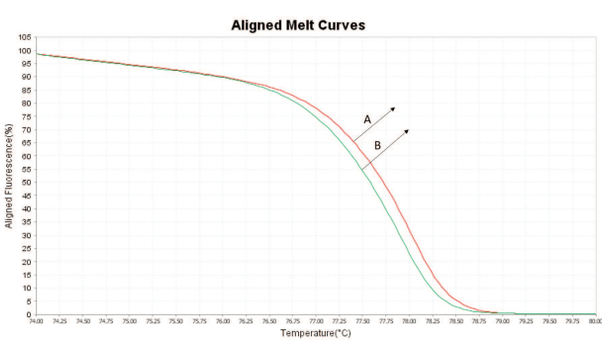

Fig. 2. Representative profiles of the HRM melting curves of *cox*1 amplicons for *Strongyloides stercoralis* from the patient samples (A) and positive control (B) with T_m 77.8°C ± 0.3 and 77.9°C ± 0.25, respectively.

behavior disorders in the patient followed by incontinence of urine and stool and consequently physical movement impairment. It was most likely that the patient acquired the infection following frequent travelling to endemic areas of the country. In spite of her general immunocompetent conditions, strongyloidiasis infection led to complicated disorder due to intermittent immonosupressive therapy for cosmetic surgeries.

In hyperinfection syndrome of strongyloidosis development or exacerbation of gastrointestinal and pulmonary, symptoms are seen [2]. Uncontrolled hyperinfection leads to disseminated strongyloidosis [2,4]. Most disseminated cases are associated with hyperinfection [16,18]. However, extra pulmonary migration of larvae has been reported to cause symptoms without manifestation of hyperinfection syndrome [19], and it does not necessarily imply greater severity of disease [2]. In the presented case, there was no mass lesion of lung, or pulmonary infiltration. In addition, in spite of presence of intestinal disorders, no picture of overwhelming infection was found in the patient, and CT-Scan of abdomen and pelvis indicated normal shape and density for different organs without any abdominal or pelvic solid masses. Therefore, it seems the case is more probably disseminated strongyloidosis involving the brain without presentation of hyperinfection.

Disseminated cases lead to death of infected individuals if not diagnosed early and treated properly [19]. In an analysis of clinical characteristics of *S. stercoralis*, eosinophilia was common both in asymptomatic and symptomatic cases and was the most prevalent reason for suspicious on strongyloidosis [12]. Symptoms of incitement cellular immune system with helminthic infections include a complex interaction of antibody, particularly IgE and peripheral and tissue eosinophils [2]. Present case had hypereosinophila in two occasions of blood tests before specific treatment for strongyloidosis as well as increase of IgE titer. Nevertheless, before suspicion of strongyloidosis, the patient had experienced many other tests of none-infectious and infectious diseases such as *Toxocara*, *Fasciola* and *Toxoplasma*. After obtaining negative results for all those tests, *S. stercoralis* was the last parasite, for which the patient was examined, i.e. about two months after hospitalization and in spite of the presence of intestinal and cutaneous disorders and hypereosinophilia. The prolonged diagnostic sequel of this patient emphasizes that *S. stercoralis* is among the most neglected disease of neglected tropical disease [1], even in such an endemic area.

Various serological tests have been reported for diagnosis of strongyloidosis with variable sensitivity and specificity, depending on antigen preparation and immunoglobulin isotopes [7,19]. The interpretation of serology results may be complicated in immunocompromised patients due to decrease in antibody production [20]. Current case had carried out *S. stercoralis* serology test in a private laboratory using commercial ELISA kit, which yielded negative results. The specificity of the kit and accuracy of performance is not clear to us. Parasitological tests are routine laboratory methods for diagnosis of strongyloidosis [3], but irregular excretion of larvae, needs for multiple fresh stool samples, necessity of the presence of living larvae in culture media [9,13], and difficulty in differentiation from similar nematodes like *Rhabditis* spp. [21] are among limitations of these methods. Additionally, use of anti-helminthic before sampling may hinder the result. As the present case, that due to taking albendazole before stool sample delivery, yielded negative results in the course of disease for all triple samples by either parasitological methods of direct smear, formalin-ether concentration and nutrient agar plate culture. Therefore, utilization of highly sensitive diagnostic tests is crucial to prevent from fatal consequences of devastating strongyloidosis. Critical diagnosis of presented complicated strongyloidosis was ultimately fulfilled in time by utilization of novel molecular methods of nested-PCR and HRM analysis. Fortunately, the patient recovered from the disease and discharged from her admitted hospital following the subsequent combination therapy of ivermectine and albendazole for three weeks.

Recently, molecular methods have been proved highly sensitive and specific for detection of parasitic agents in fecal samples [9,22,23]. In the presented patient, both molecular methods of nested-PCR and HRM were capable of confirmation of strongyloidosis infection and were also useful to follow up the patient after clearance of infection. However, parasitological methods had lack of sensitivity, mostly due to the use of antihelmintics before stool samplings. Failure of such methods to detect *S. stercoralis* may in part be due to the presence of nonviable larvae in those samples [5]. The presence of living larvae in the agar plate culture is necessary [13]. Nested-PCR has been

reported with 100% sensitivity in detection of strongyloidosis in stool samples and with better reliability compared to parasitological methods [9]. HRM provides a rapid, low-cost, and sensitive scanning method to detect variation in DNA using a single step [24]. Its utilization for species differentiation of helminthes like *Echinococcus* spp. [10] and *Schistosoma* spp. [11] have been reported. In present case, *S. stercoralis* in the stool samples was verified by comparison with positive control DNA (MG251326), and the detection was confirmed by product PCR-sequencing.

Application of molecular methods also decreases the number of serial stool samples necessary for diagnosis of *S. stercoralis* with the maximum sensitivity [25]. Confirmatory to this advantage, both molecular methods of nested-PCR and HRM analysis yielded positive results for all triple samples of the present case, during the course of infection, and negative result for the double samples during the following up.

In conclusion, in individuals living in or having history of travelling to endemic areas of strongyloidosis, screening tests needs to be done, especially once they have peripheral eosinophilia or IgE elevated. Application of new diagnostic methods for detection of *S. stercoralis* in reference laboratories is recommended; especially in such similar cases, that due to insufficient anti-helminthic therapy parasitological techniques have lack of sensitivity. Providing stool samples before initiation of anti-helminthic agents improve the chance of recovering larvae by parasitological methods. Awareness of physicians of predisposing factors and related risks is necessary, in addition to population-based studies to assess true prevalence in each endemic area, and screening and proper treatment of infected individuals.

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Conflicts of interest declaration. There are no conflicts of interest.

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