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Persistence of Schistosoma japonicum DNA in a kidney-liver transplant recipient

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Abstract:	Emerging issues for schistosomiasis control include concern about drug resistance and evolution of new animal parasite hybrids. We report a patient with undiagnosed schistosomiasis for more than 35 years. A digital droplet PCR assay that targets the schistosome NADH dehydrogenase 1 mitochondrial gene amplified parasite DNA extracted from colon biopsies containing uncalcified eggs, persistent despite multiple praziquantel treatments over seven years. Analysis of parasite DNA revealed that it was a Philippines strain of S. japonicum. Future molecular studies using DNA from patients such as this will provide insight into why some persons do not respond well to praziquantel.
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Cover Letter

MEDICAL COLLEGE OF WISCONSIN

July 24, 2018

To: The Editor, *The Journal of Infectious Diseases*

Dear Editor:

With this cover letter we submit for review a manuscript formatted as a **Brief Report** and is entitled "*Persistence of Schistosoma japonicum DNA in a kidney-liver transplant recipient.*" We report our findings after eight years caring for a unique patient with schistosomiasis who went undiagnosed and untreated for more than thirty five years. We feel that our investigation fits well with *The Journal of Infectious Diseases* for many reasons. In particular, we note with great interest two papers dealing with schistosomiasis published in the August 1 2018 issue of *The Journal of Infectious Diseases:* Colley and Loker discuss in their Editorial Commentary "*New Tools for Old Questions: How Strictly Human are "Human Schistosomes – And Does It Matter?"* Catalano et al. reported on "*Rodents as Natural Hosts of Zoonotic Schistosoma Species and Hybrids: An Epidemiological and Evolutionary Perspective from West Africa.*" Our investigations address many of the same important issues raised in these papers, but also provide a concrete example of how advances in parasite genomic studies in Asia can be used to speciate an unknown parasite and identify the country where a person became infected. In addition, we believe that future molecular studies of unique patients such as this will provide answers to important unanswered questions such as why some persons do not respond well to treatment with praziquantel, the drug of choice to treat schistosomiasis in man and animals throughout the world. At present, there is no consensus amongst the schistosomiasis research community on human or parasite markers of praziquantel resistance.

Thank you in advance for consideration of our manuscript by *The Journal of Infectious Diseases*.

As per instructions for authors:

This manuscript has not been submitted or accepted elsewhere.
All authors fulfill the criteria for authorship.
No writing assistance was provided in the preparation of this manuscript.

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Persistence of *Schistosoma japonicum* DNA in a kidney-liver transplant recipient

Running title: Persistence of *S. japonicum* DNA

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Emerging issues for schistosomiasis control include concern about drug resistance and evolution of new animal parasite hybrids. We report a patient with undiagnosed schistosomiasis for more than 35 years. A digital droplet PCR assay that targets the schistosome NADH dehydrogenase 1 mitochondrial gene amplified parasite DNA extracted from colon biopsies containing uncalcified eggs, persistent despite multiple praziquantel treatments over seven years. Analysis of parasite DNA revealed that it was a Philippines strain of *S. japonicum*. Future molecular studies using DNA from patients such as this will provide insight into why some persons do not respond well to praziquantel.

Keywords: *Schistosoma japonicum*, diagnosis, treatment, immunopathology, transplantation

Notes

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Schistosomiasis is an important neglected tropical parasitic disease that affects more than 250 million persons worldwide and induces organ specific immune mediated pathology as well as growth stunting and impaired cognition [**1**]. Emerging issues in the control of schistosomiasis include concern about development of drug resistance after more than forty years of using the same medication, praziquantel, to treat humans and animals. In Africa human schistosomes have hybridized with close non-human animal species, with the potential to confound control strategies [**2**]. Rodents have been identified as natural hosts of both zoonotic schistosome species and hybrid schistosomes in West Africa [**3**]. Similar concerns exist in Asia where carabao (water buffalo), dogs and rats harbor the human parasite, *Schistosoma japoncium* and non-human species such as *S. bovis*. The existence of and impact of hybrid schistosome species has not been as well studied in Asia.

Here we report a patient who acquired schistosomiasis in Asia at least 35 years before diagnosis. She was unaware of where or when she became infected. Persistence of non- calcified eggs in colon biopsies despite multiple praziquantel treatments over seven years during which time she underwent a combined kidney-liver transplant, raised the suspicion that she may have been infected with an animal schistosome, a new hybrid schistosome or a strain of *S. japonicum* that was praziquantel resistant. Schistosome genome data collected for analysis of parasite evolution in Asia proved useful to speciate the parasite and determine its country of origin [**4**]. Presently there are no consensus genetic markers of praziquantel resistance in humans or parasites; however, we propose that future molecular studies of well characterized patients such as this will provide insight into why some persons with schistosomiasis do not respond well to appropriate drug treatment.

METHODS

Human subjects Research

Written informed consent was obtained to identify the parasite species and its country of origin. Institutional review board approval was obtained for research to extract parasite DNA from formalin fixed explanted liver and ethanol fixed colon biopsies (Project number PRO00028065).

Specimen collections

A portion of explanted formalin fixed liver from 2016 and unused ethanol fixed colon biopsy samples from 2017 were utilized for molecular studies. Study samples were shipped to the Berghofer Queensland Institute of Biomedical Research (QIBR Berghofer), Molecular Parasitology Laboratory.

DNA extraction, digital droplet and conventional polymerase chain reactions (PCR).

The Qiagen DNA Micro Kit was used per manufacturer's guidelines for extraction of genomic DNA from laser-microdissected tissues. Total DNA quantity and quality was assessed using a NanoDrop 2000 (Thermofisher Scientific). DNA was utilized in a digital droplet PCR (ddPCR) assay with primers which amplify the NADH dehydrogenase I (NAD1) mitochondrial gene as described previously [**5,6**]. Reaction mixtures of 25 µl were prepared containing 12 µl of EvaGreen (Bio-Rad), 1 µl each of forward and reverse primer (final concentration 200 nM), 9 µl of water, and 2 µl of template DNA. Reaction mixtures were added to the ddPCR cartridge along with 70 µl oil and the cartridge placed in the droplet generator and droplets generated. Droplets were pipetted into a 96 well twin tec plate (Eppendorf) and placed in a thermocycler (C1000, Bio-Rad) with the following cycling condition; 2 minutes initialization at 95°C, 10 minutes denaturation at 95°C, followed by 40 cycles of 15 sec denaturation at 50°C, 20 sec annealing at 60°C, and 20 sec extension at 72°C, followed by a final dissociation phase at 60-95°C. After amplification the plate was placed in the droplet reader (QX200, Bio-Rad). Non-template controls using water in place

of DNA, and positive controls using DNA from *S. japonicum* eggs isolated from the liver of an infected mouse were used for all PCR assays. Conventional PCR was performed on ddPCR positive samples using primers SJ1 and SJ2 which amplify a portion of the mitochondrial NAD1 gene. Conserved single nucleotide polymorphisms in this region of Chinese *S. japonicum* allow design of PCR primer sets (SJ1, SJ2) that differentiate Chinese and Philippines strains of *S. japonicum* as previously described [6]. Amplified products were sequenced at Berghofer QIMR using the in house Big Dye terminator sequencing service. Sequences were viewed in Finch TV 1.4.0 (Geospiza) and analyzed in Sequencher 5.4.6 (Gene Codes). DNA sequence of the patient's parasite DNA was analyzed using BLASTn (nucleotide-nucleotide BLAST, National Center for Biotechnology Information) and compared to known sequences of *S. japonicum* from various locations in China and the Philippines, *S. mekongi*, *S. bovis* and *Fasciola giganta*.

RESULTS

Case Report

The patient is a fifty-seven year-old female who presented in 2010 for evaluation of progressive liver failure, abdominal pain, diarrhea and colitis. Her past history was significant for living in China, Korea and the Cebu region of the Philippines; however she had not travelled back to those countries since 1975. She was unaware of when or where she acquired schistosomiasis. Multiple stool samples for ova and parasites were negative and serology for schistosomiasis performed twice by a regional reference laboratory was negative. Serological testing by the Centers for Disease Control and Prevention identified antibody for *S. japonicum* or *S. mekongi* by immunoblot. Esophagogastroduodenoscopy showed two columns of grade 1-2 esophageal varices at the gastroesophageal junction. Colonoscopy revealed that the entire length of the colon was tubular, pale, opaque, with decreased vascular pattern and mild friability especially in the rectosigmoid. Computerized axial tomography imaging revealed

massive splenomegaly, extensive periportal and intrahepatic venous collaterals consistent with portal hypertension. A liver biopsy in 2010 revealed hepatic fibrosis, multifocal bridging fibrosis and numerous round to oval partially calcified and non-calcified ova. Non-calcified ova also were present in the colonic mucosa, submucosa, the lamina propria and muscularis with preserved crypt architecture, intact epithelium, and no evidence of intraepithelial lymphocytosis. There were no egg granulomas, cryptitis, crypt abscess, dysplasia or malignancy. Anti-cytokine antibody panel was negative, as was testing for MSMD (Mendelian susceptibility to Mycobacterial Disease). Quantitative immunoglobulins were normal and HIV/HTLV serology was nonreactive. The patient exhibited normal activation of Interferon gamma stimulated STAT1 in monocytes. Flow cytometry confirmed lymphopenia with severely decreased absolute B cells, CD_4 T cell counts and CD_8 T cell counts, and excessive T cell activation.

Between 2010 and her kidney-liver transplant in 2016, the patient received six separate courses of high dose praziquantel (60/mg/kg). The explanted liver demonstrated extensive fibrosis and partially calcified and non-calcified eggs (Figure 1). After transplantation the patient experienced several bouts of severe colitis and repeat colonoscopy biopsies in 2017, forty two years after leaving schistosomiasis endemic countries, demonstrated persistence of non-calcified schistosome ova**.**

DNA sequence analysis.

No human or parasite DNA was recovered from formalin fixed explanted liver. Total genomic DNA was extracted successfully from ethanol fixed colon biopsy tissue. ddPCR successfully amplified parasite DNA from colon biopsy samples utilizing NAD1 primers and amplicons were sequenced. A phylogenetic tree of DNA sequences was constructed using the Maximum Likelihood Method and demonstrated that the unknown parasite was *S. japonicum* and not *S. bovis* or *S. mekongi* (Figure 2.) No amplification of

DNA occurred with SJ1 primers (Chinese *S. japonicum*) however, two of three samples produced amplicons of the expected size (242 bp) using SJ2 primers (Philippines *S. japonicum*).

DISCUSSION

Our investigations were successful in solving most of the unknown features of this patient with longstanding schistosomiasis. Schistosomiasis has been eradicated from Japan and the coastal plains of China but remains elsewhere where *S. japonicum and S. mekongi* are the major human pathogens. In 1975, more than twenty-four provinces throughout the Philippines reported endemic *S. japonicum.* Cebu is adjacent to the island of Leyte that has remained a hyperendemic focus for decades. In spite of more than 30 years of mass anti-helminthic treatments with praziquantel in the Philippines, *S. japonicum* remains endemic for a variety of reasons, including logistic issues with drug delivery to rural areas/islands, reinfection and the existence of animal reservoirs of *S. japonicum,* predominantly cattle and carabao [**7**].

Examination of stool for ova using a fecal concentration method historically has been the primary screening method for suspected schistosome infections [**1**]. Egg deposition however can be intermittent or light depending on the intensity of infection and immune status of the subject, and thus various antigen, DNA and serological assays using purified adult worm antigens or recombinant proteins have undergone evaluation in cases where suspicion is high but standard diagnostics are negative [**8**]. The ddPCR method used in this report to identify a Philippines strain of *S. japonicum* also has proven useful to detect schistosome egg DNA in stool samples [**6**]. The two negative serological tests for schistosomiasis in this patient is a reminder of the difficulties inherent with serodiagnosis of *S. japonicum.* Most schistosome antibody detection tests in the United States done by reference laboratories use commercial kits that are not universally standardized. At the Centers for Disease Control a FAST-ELISA method to detect schistosome antibody is 99% specific for *S. mansoni* and 90%

specific for *S. hematobium*, however detection of specific antibody against *S. japonicum/S. mekongi* requires the use of an immunoblot and different adult antigen preparations [8].

Formation of egg granulomas is an almost universal characteristic of chronic *S. japonicum* infection in immunocompetent humans and animal models. The absence of calcified eggs or egg granulomas in this patient despite repeated praziquantel treatments over seven years was a major concern for praziquantel failure. On the other hand, it is widely accepted that a major component of praziquantel's effectiveness is dependent on an intact host immune response [**9**]. The absence of markers for a congenital immunodeficiency led us to conclude that the failure of granuloma formation observed some four decades post infection was due to the patients' longstanding acquired immunodeficiency from progressive liver and renal failure. In immune competent animals and humans, calcification of eggs can take two years or more, however there is limited data in immunocompromised hosts with schistosomiasis.

After praziquantel was discovered in the 1970's it became the global veterinary and human drug of choice to treat schistosomiasis. Praziquantel remains the drug of choice to treat all major *Schistosoma* species [**10**]. Recommendations for treatment of *S. japonicum*, S*. mekongi* and/or accidental infection with an animal schistosome, suggest that higher doses or repeated treatments may be necessary. Praziquantel primarily kills adult worms and has little to no effect on miracidia inside viable eggs. Concern about praziquantel resistance of human species is supported by reports of low cure rates in some populations [**11**]. In murine models of *S. japonicum* with praziquantel resistance, infected mice appear to have higher parasite egg production than drug-susceptible isolates, which in turn suggests a greater pathogenicity due to the damaging effects of the host immune response to eggs, and potentially greater transmissibility [**12**]. The primary mechanism(s) of action of action(s) of praziquantel remains unclear. Several mechanisms have been proposed, including disruption of parasite calcium mobilization, inhibition of glutathione S - transferase, and stimulation of human and parasite

serotoninergic G-protein coupled receptors [**13**]. The normal life span of adult schistosomes is less than 10 years, however several cases of human schistosomiasis lasting more than 30 years have been reported. The longevity of schistosomes is a classic example of the successful evolution of parasite mechanisms that promote survival and evasion of the host immune response [**14**].

Solid organ transplantation programs in countries with endemic schistosomiasis report good outcomes in patients with preexisting, treated schistosomiasis infections, and even in the occasional unsuspected infected organ donor [**15**]. In Egypt, a fifteen year follow up of morbidity and mortality of persons receiving liver transplantation for schistosomiasis due to *S. mansoni* indicates that as long as effective praziquantel treatment is completed, there was no significant difference in outcomes between recipients with or without schistosomiasis.

In conclusion we anticipate this patient will continue to do well with no new egg deposition in the transplanted liver. In the future when there are human or parasite genetic markers for praziquantel resistance, further analysis of stored DNA from this patient will help to answer this final question of drug resistance.

Figure 1. Panel A. Gross appearance of explanted liver from patient with longstanding *Schistosoma japonicum* infection. Panel B. Histological findings in liver and subsequent colon biopsies demonstrated many partially calcified and non-calcified ova without granuloma formation. Egg morphology was irregular but consistent with a species of *Schistosoma* (magnification x 400)**.**

Figure 2. Panel A**.** Phylogenetic tree constructed using maximum likelihood method to compare *S. japonicum* strains based on mitochondrial NAD1 sequences. Included in this comparison are sequences from the patient, *S. japonicum* from China (Hunan, Jiangzi) and the Philippines (Sorsogon, Mindoro, Leyte), *S. mekongi, F. giganta* and *S. bovis*. Phylogenetic tree demonstrates that the patient's parasite is most closely related to *S. japonicum*, but does not identify the country of origin. This method does not take into account the single nucleotide polymorphisms used to design PCR primer pairs (SJ1 and SJ2) that distinguish between Chinese and Philippines strains of *S. japonicum*. Accession numbers are listed for each strain at the left of each sequence. Panel B**.** DNA **s**equence alignments representing *Schistosoma japonicum* strains from China (Jianxi, Hunan) and the Philippines (Leyte, Sorsogon, Mindoro) compared to the DNA sequence obtained from our patient. Single nucleotide polymorphisms (highlighted in gray) in specific regions of the NADH dehydrogenase 1 mitochondrial gene allow for the design of PCR primer sets that differentiate Chinese *S. japonicum* from *S. japonicum* found in the Philippines. Accession numbers are listed at the left of each sequence. Panel C**.** Agarose gel electrophoresis of PCR products identified *S. japonicum* DNA in two of three colonoscopy biopsy samples (lanes 3 and 4) using SJ2 primers but no amplicons were generated using SJ1 primers (lanes 5, 6, 7) specific for Chinese *S. japonicum*. Lane 1 contains positive control PCR products using SJ2 primers with Philippines *S. japonicum* and Lane 8 shows positive control PCR products obtained with SJ1 primers and *S. japonicum* DNA from China. DNA molecular weight markers are displayed on the far left.

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Figure 1

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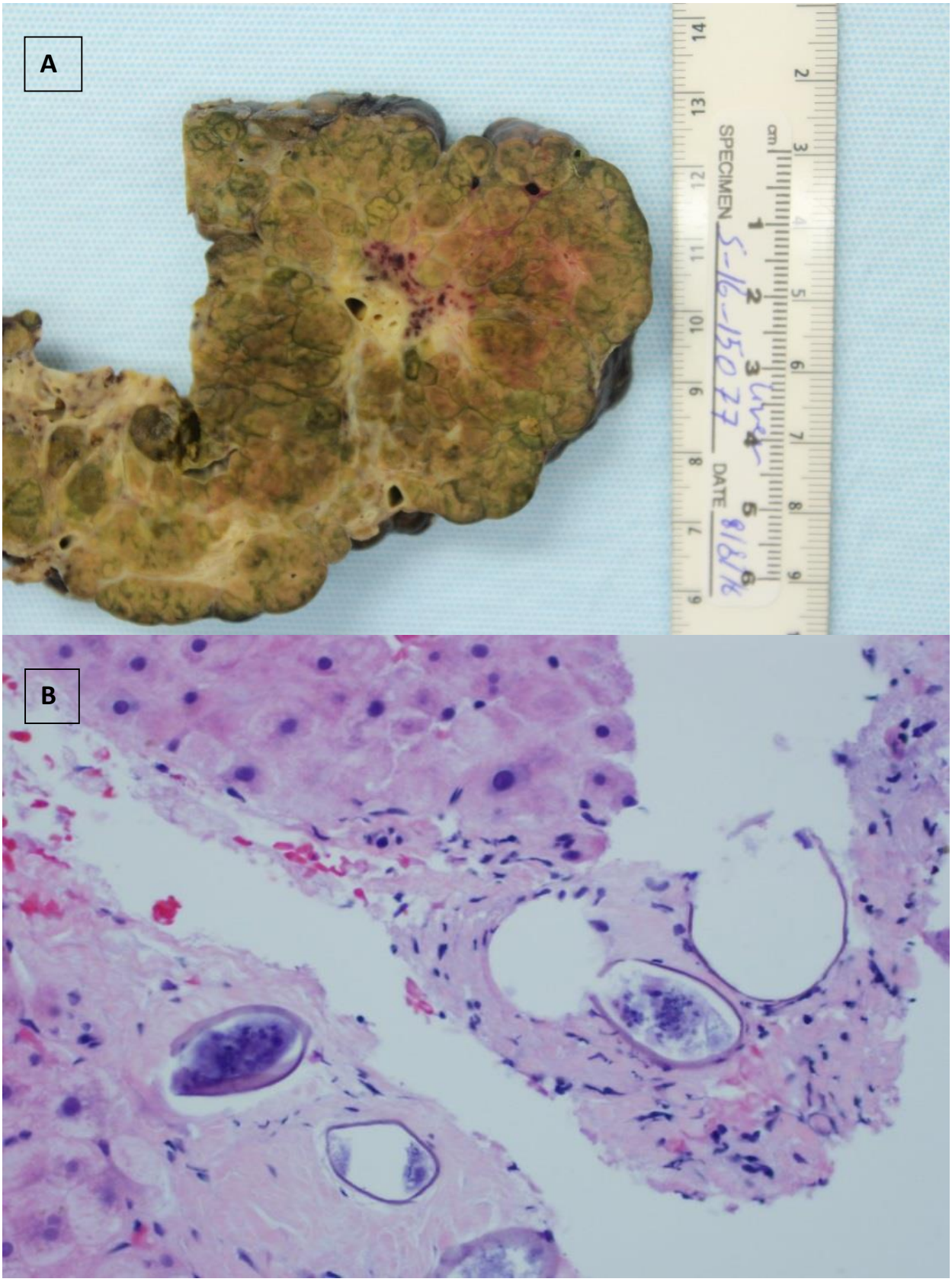

Figure 2

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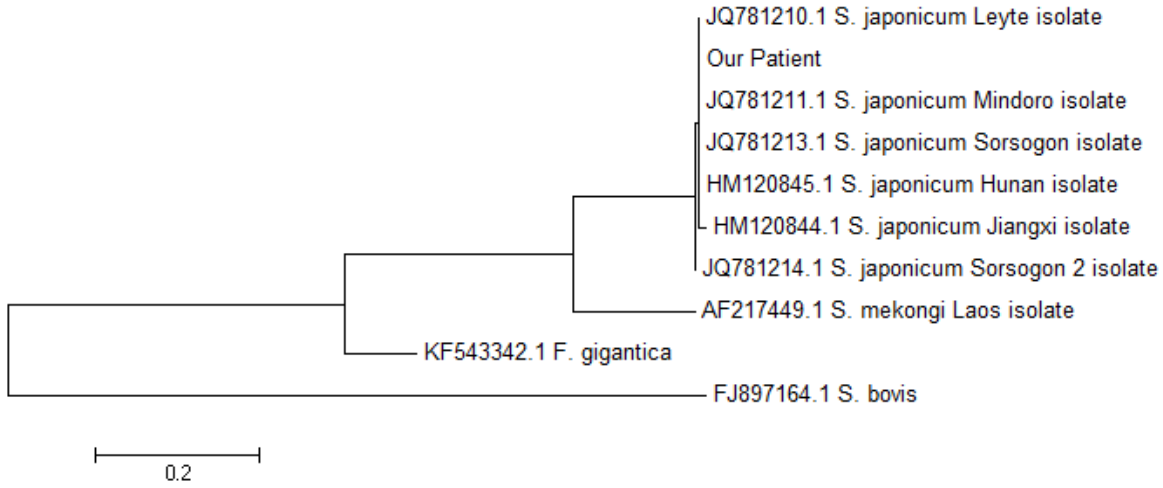

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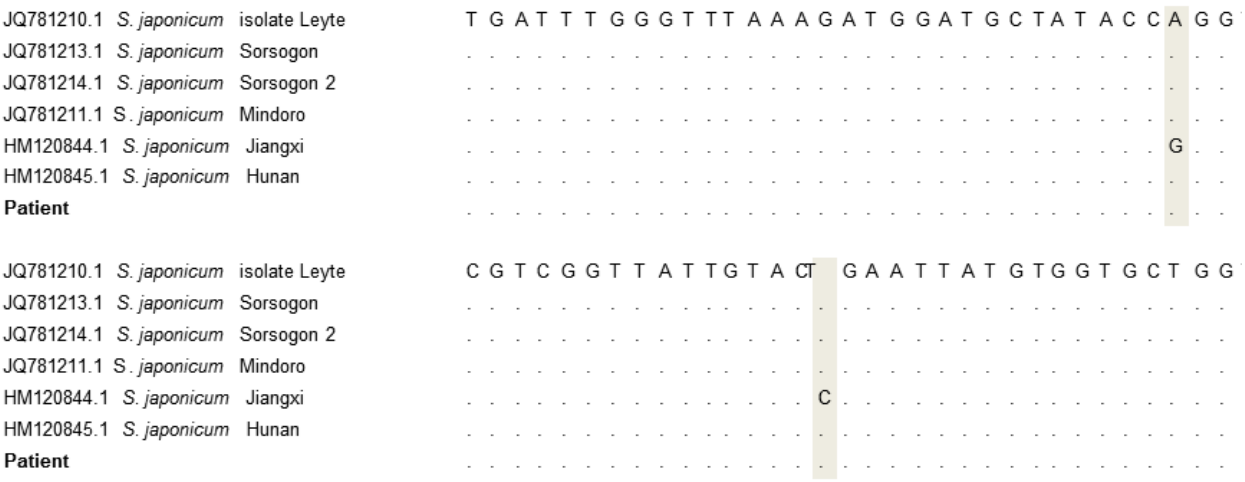

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