Short Report

First report of *Pneumocystis jirovecii* pneumonia diagnosed with DNA sequencing in post-renal transplant patients in Sri Lanka

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Sri Lankan Journal of Infectious Diseases 2021 Vol.11(1):27-31

DOI: http://dx.doi.org/10.4038/sljid.v11i1.8320

Abstract

Pneumocystis pneumonia (PCP) caused by the fungus *Pneumocystis jirovecii*, is an opportunistic infection mainly in immunocompromised patients. Among renal transplant patients, PCP is a serious complication associated with a high mortality rate.

PCP was clinically diagnosed in a 53-year-old female and 35-year-old male post kidney transplant patients admitted to the National Hospital, Kandy, Sri Lanka. Confirmation of the diagnosis by precise molecular and phylogenetic analysis is reported for the first time in Sri Lanka. The molecular data generated from the specimens obtained from these two patients can be further used for diagnostic and phylogenetic studies.

Keywords: *Pneumocystis jirovecii, pneumonia, kidney transplant, Sri Lanka, molecular diagnosis*

Introduction

Pneumocystis pneumonia (PCP) caused by the fungus *Pneumocystis jirovecii* is an opportunistic infection that causes major infectious complications in immunocompromised patients.[1] Kidney transplant patients have shown mortality rates from 29% to 50% due to PCP.[2] The risk for PCP development appears to be higher during the early period after transplantation.[2] A previous study in Sri Lanka stated that there was a lack of available data on PCP as it is diagnosed using histological staining procedures.[3] These are the first two patients in whom precise molecular diagnosis with DNA sequencing and phylogenetic analysis of *P. jirovecii* was carried out in Sri Lanka. Molecular diagnosis and species identification was carried out using polymerase chain reaction (PCR), DNA sequence alignment, and phylogenetic analysis.

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Received 25 August 2020 and revised version accepted 9 December 2020.

Patient 1 was a 53-year-old female post-kidney transplant patient who was admitted with pneumonia to the surgical intensive-care unit (SICU) of the National Hospital, Kandy, Sri Lanka. She had a cough and difficulty in breathing for four days. Initial management included intravenous meropenem, piperacillin, vancomycin and valganciclovir. She required ventilation and endotracheal tube (ET) secretions were sent for PCR one day after admission to the ICU. PCR results and confirmation of the diagnosis was available within 24h of receiving the sample, two days after the patient was put on the ventilator.

Patient 2 was a 35-year-old male admitted to the nephrology ICU, National Hospital, Kandy, Sri Lanka with fever and pneumonia. He had undergone renal transplantation two years previously. The severity of his clinical condition warranted mechanical ventilation on admission. A bronchoalveolar-lavage (BAL) fluid sample was obtained and sent for bacterial, fungal and viral studies. PCR results and confirmation of the diagnosis of *Pneumocystis* pneumonia was informed to the ICU within 24h of receiving the sample. Although co-trimoxazole treatment was started, this patient died two weeks after his admission to the ICU.

Molecular diagnosis

Genomic DNA was extracted using a commercial DNA extraction kit (Invitrogen™PureLink™ Genomic DNA Mini Kit, Thermo Fisher Scientific, USA) following the manufacturer's protocol. The PCR was carried out with pAZ102E and pAZ102H primer pair to amplify the 346 bp long mitochondrial large subunit rRNA gene (mLSUrRNA) of *P. jirovecii*.[4] The PCR was carried out in a final volume of 25 µl containing a 10xPCR buffer, 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate (dNTP), 10 pmol primers, Taq DNA polymerase (5u/µl) and 5 µl of template DNA. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min and the final extension at 72 °C for 5 min. Electrophoresis of 10 µl of PCR product through a 1.5% agarose gel was carried out to confirm successful amplification and for a rough quantification. Both samples gave positive results for *Pneumocystis jiroveceii*. PCR products were subjected to direct DNA sequencing at Macrogen Corporation, Korea using PCR primers as sequencing primers.

Sequences were edited manually in MEGA 7.0.26.[5] Sequence similarity search was done using the NCBI BLAST (www.ncbi.nlm.nih.gov/blast) search program. The multiple sequence alignment was obtained with default gap penalties in the MEGA 7.0.26.[5] Phylogenetic analysis was performed with this sequence alignment. Maximum likelihood (ML) tree was obtained using the RAxML 8.2.10 in the Cyber Infrastructure for Phylogenetic Research project (CIPRES) Science Gateway v.3.3.[6] Model of evolution was the general time reversible model of sequence evolution (GTR). The rapid bootstrapping algorithm was implemented with 1000 bootstrap replicates in the RAxML version; GTR and the CAT approximation of rate heterogeneity were applied. Bayesian analysis was conducted in MrBayes, with the model as GTR.[7] Four Markov Chain Monte Carlo (MCMC) chains were run for one million generations as three heated chains and one cold chain. The four chains reached burn-in time by 200,000 generations. The frequency of clades in trees was sampled every 100 generations. The ML and Bayesian trees were rooted with *Saccharomyces cerevisiae* (GenBank acc. no. NC 027264).

Two sequences were obtained with a length of 262 bp and 250 bp. These sequences were submitted to the GenBank with accession numbers MT106898 and MT106899. Sequence similarity search identified 100% match with the *P. jirovecii* reference sequences (GenBank Acc. no. FJ357845, FJ357851, MH010439, MH010437, KJ733280). The sequence alignment contained 13 taxa, including 11 reference nucleotide sequences. The ML tree and the Bayesian tree both had the same tree topologies. The phylogenetic tree revealed a single clade of all the *P. jirovecii* reference sequences and two Sri Lankan samples (Figure 1).

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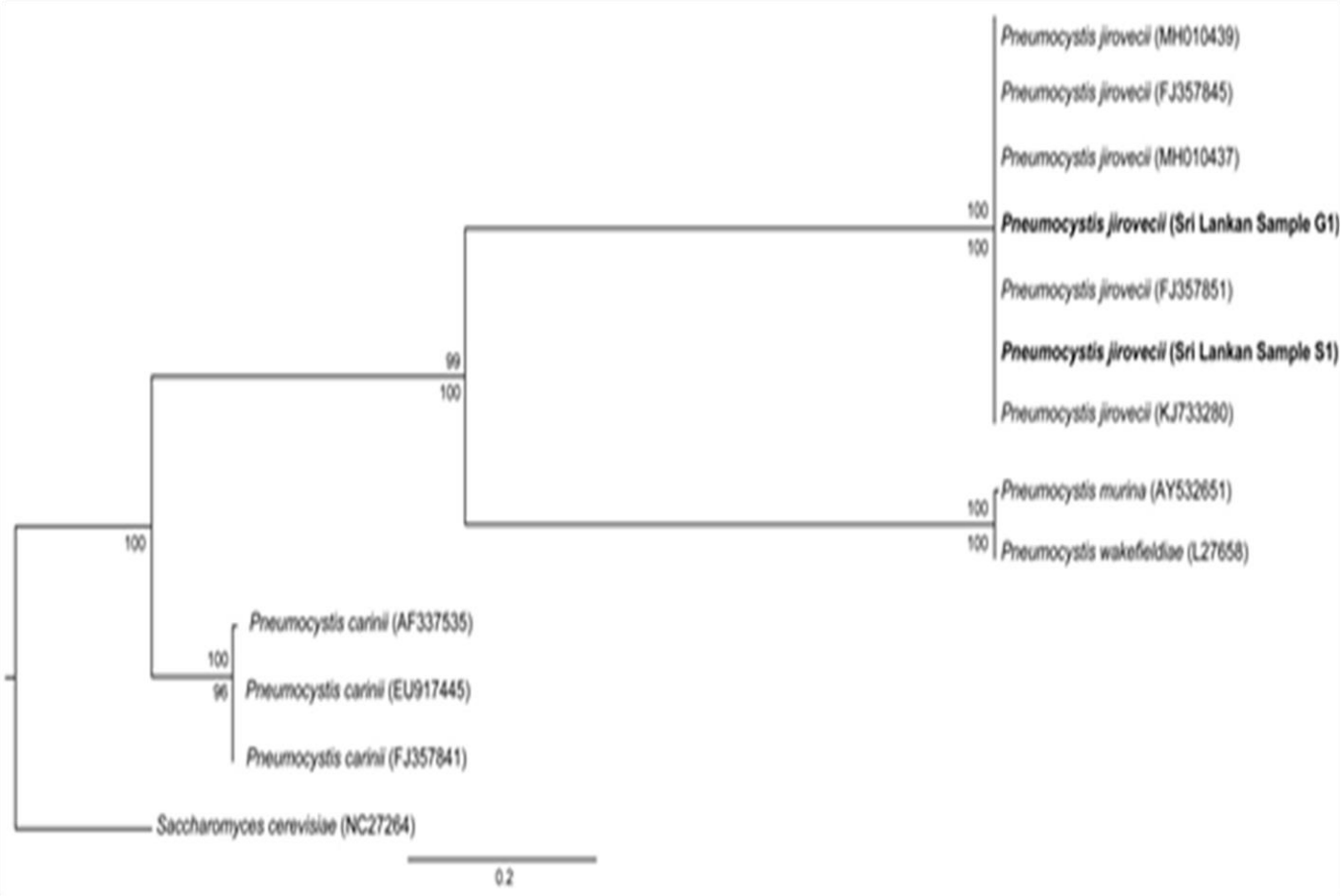

Figure 1: Maximum likelihood tree obtained by the RAxML (8.2.10) in CIPRES Science Gateway V3.3

Note: Numbers above and below the nodes indicate Rapid Bootstrap values (>90) and Bayesian posterior probabilities respectively. Sri Lankan *P. jirovecii* samples: S1 and G1 are in bold letters. The scale bar represents 0.2 nucleotide divergence

Discussion

A previous study conducted in Sri Lanka identified PCP as being responsible for 25% of deaths from infections following renal transplantation.[8] It is one of the major infections that causes death in post-renal transplant patients.[8] The two patients we report were 53 years and 35 years old respectively. The 35-year-old patient passed away 2 weeks after admission. Previously conducted multivariate analysis showed that older age at transplantation and the use of mycophenolate mofetil (MMF) were risk factors for the development of PCP.[9] However, whether the patients that we report were on MMF is not known from data available to us.

The early phase after organ transplantation shows an increased risk of PCP, especially under intensive immunosuppression. However, PCP in the second patient occurred two years after renal-transplant. Information on both patients of their immunosuppressive therapy was not available. Infections that occur more than one year after renal transplant has raised the necessity of extending the period of primary prophylaxis beyond the first year after transplantation.[10] Details of post renal transplant prophylactic regimes in these patients was also not available.

PCR has been shown to have greater sensitivity and specificity for the diagnosis of PCP compared with routine silver staining. For PCR testing, induced sputum and bronchoalveolar-lavage fluid are taken for DNA extraction and *Pneumocystis* mitochondrial large-subunit ribosomal RNA (rRNA) is amplified. In this study, the phylogenetic analysis identified two separate clades for *P. jirovecii* and *Pneumocystis carinii*. The analysis that we conducted is in agreement with previous molecular studies that differentiated *P. jirovecii* from *P. carinii*.[1] The molecular data generated from this preliminary study can be used for further molecular analysis.

Conflict of interest: Authors declare no conflict of interest.

Ethics: We obtained informed consent from the patients' relatives for publication.

Acknowledgment: We thank the patients' relatives for providing consent to publish this study.

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