Pneumocystis Pneumonia in Liver Transplant Recipients: Clinical Course, Epidemiological Analysis and Risk Factors

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Research

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Abstract

Background: We aimed to investigate the clinical course, possible transmission routes and the potential risk factors of Pneumocystis pneumonia in liver transplant recipients.

Methods: The study was performed by collecting and analyzing the clinical, epidemiological, and molecular data from patients with Pneumocystis pneumonia as well as from matched controls.

Results: There were a total of ten patients diagnosed with Pneumocystis pneumonia containing prospectively included six patients and retrospectively collected four patients, of which seven were transferred to the surgical intensive care unit and four died. The transmission map revealed inter-patient transmission of Pneumocystis jirovecii was impossible. Pneumocystis jirovecii detection was negative in all air samples. It was positive only in one sample from the twelve healthcare workers with close contact to diseased patients. Five out of 79 liver transplant recipients during the outbreak were colonized with Pneumocystis pneumonia had totally different genotypes based on multilocus sequence typing. Additionally, we found an unreported mutation at the cytochrome b gene (566 C/T and C838C/T). The absolute CD19+ B-cell counts (odds ratio: 1.028; 95% confidence interval: 1.000-1.057; P = 0.049) was defined to be the only significant independent risk factor using multivariable conditional logistic regression.

Conclusions: Pneumocystis pneumonia is a severe complication following liver transplantation. The outbreak may not be caused by nosocomial transmission. A decrease in absolute CD19+ B-cell counts may play an important role in the development of Pneumocystis pneumonia.

Introduction

Pneumocystis jirovecii (P. jirovecii), an opportunistic fungal microorganism, can cause Pneumocystis pneumonia (PCP) resulting in significant mortality (1-5). PCP used to occur mainly in patients diagnosed with human immunodeficiency virus (HIV) due to lymphocyte deficiency (1, 2). PCP is now a growing concern in non-HIV patients, especially in patients who have undergone solid organ transplantation because post-transplant PCP generally presents as severe pneumonia and may cause nosocomial outbreaks (3, 4, 6–8). Risk factors for PCP have been suggested among transplant recipients, mostly in renal transplant recipients (9-11). However, due to its lower incidence, there are few reports with respect to predictors for PCP in liver transplant recipients, and the predictive value of immune cells has not been further discussed in this cohort.

Moreover, as it is not possible to culture P. jirovecii with routine methods, PCP diagnosis depends on molecular detection of P. jirovecii deoxyribonucleic acid (DNA) by quantitative polymerase chain reaction (qPCR) (12, 13); nevertheless, this approach is not suitable for an epidemiological investigation of a PCP outbreak. Exploring an outbreak transmission route relies on spatiotemporal and strain-typing measures because genotyping based on multilocus sequence typing has been proposed as the standard tool for strain characterization in virtue of excellent discriminatory power and reproducibility (12). Presently, the exact route of transmission among PCP patients is not clearly understood. In PCP outbreaks, epidemiological research with genotyping has suggested an inter-human transmission route or an inter-human airborne transmission route in patients with different primary diseases (7, 8, 14-16). However, a systematical study of all the potential transmission routes has not been reported in liver transplant recipients.

In this study, we retrospectively collected data from a cluster of four patients infected with PCP between July 2015 and July 2016, and performed a prospective study on the new onset of PCP patients from April 2018. The clinical, epidemiological, and molecular characteristics of the PCP patients and controls were systematically analyzed to investigate the clinical course, potential possible transmission routes and risk factors.

Materials And Methods

Study design

The Beijing Chaoyang Hospital has a total of 1,900 beds and performs more than 100 liver transplantations per year. No patients with PCP had been observed during the previous ten years. Between July 2015 and July 2016 a cluster of four patients were diagnosed with PCP. We retrospectively collected their data, and performed a prospective controlled study on PCP patients from the first onset in April 2018 (patient no.5) to determine the clinical course, transmission routes and the risk factors. If there had been no new onset of PCP patients for more than six months after the diagnosis of the last PCP patient, we considered it an end of an outbreak due to a lack of a definition. The study
was approved by the Institutional Review Board of Beijing Chaoyang Hospital (No.2016-2-19-38) in accordance with the Helsinki declaration of 1975, as revised in 1983. Written informed consent was obtained from all participants.

PCP patients were defined when the following criteria were met: (i) clinical symptoms including fever, cough, and dyspnea; (ii) radiographic findings of a new alveolar or interstitial infiltrate on a chest computerized tomography; (iii) either sputum or bronchoalveolar lavage fluid (BALF) positive for P. jirovecii. (13)

Immunosuppressive management

No changes in routine immunosuppressive regimens have been implemented for nearly 10 years. Immunosuppressive therapy consisted of induction with basiliximab (20 mg on day 0 and 4) and maintenance, which was based on steroids, mycophenolate mofetil, and calcineurin inhibitor (tacrolimus or cyclosporine A). Methylprednisolone (500 mg) was intravenously infused during operation. After surgery, it was given by 240 mg/day, and daily reduced by 40 mg till the 7th postoperative day. Then it was changed to prednisolone (20 mg/day). Prednisolone was gradually withdrawn within one month in cases of malignant liver diseases or 3 months afterward except in autoimmune diseases. Sirolimus was used in selected patients with impaired renal function or for its antitumor effects one month following liver transplantation.

Transmission map

We prospectively collected and analyzed data concerning PCP patients’ interactions within the hospital from the first admission of the fifth patient until the diagnosis of PCP in the last patient to determine the possible patient-patient transmission. The outpatient clinic, the hepatology ward, and the surgical intensive care unit (SICU) were located at different positions of the hospital. Outpatients had no direct contact with inpatients. The hepatology wards for pre-transplant and post-liver transplant recipients were on the same floor but in different corridors. Patients had to share a ward before liver transplantation and were transferred to a single ward from the SICU following surgery.

Air sampling

Due to a lack of an air sampling device, we used the traditional passive sedimentation method according to the Hospital Sanitary Disinfection Standard (GB15982–2012) to collect the specimen with a medium plate about 9 cm in diameter (17, 18). Only the SICU had laminar airflow with high-efficiency particulate air filtration. The windows and doors of the wards were closed and the patients stayed still for at least 10 min before sampling. The medium plates filled with sterile saline were placed at different sites. If the room was \( \leq 30 \text{ m}^2 \), three sites (inner, middle and outer) were selected along a diagonal line. Otherwise, five sites were chosen including four corners and the middle point. All sites were one meter away from the floor and the walls. During sampling, the plates were opened for 30 min.

Screening for colonization in healthcare workers and patients

Healthcare workers (HCW), who had been working for more than two years at our center, were prospectively sampled for P. jirovecii detection. HCW were divided into two groups, consisting of those who had direct contact with PCP patients and those who had no occupational contact with PCP patients as a negative control. Meanwhile, liver transplant recipients upon admission were prospectively sampled to determine the frequency of colonization with P. jirovecii during the outbreak and six months after the outbreak for consecutive six month. For HCW and liver transplant recipients with respiratory symptoms, a BALF sample or a sputum sample was taken. Otherwise, a nasal sample was taken.

DNA extraction and P. jirovecii detection

For each sample, the collection liquid was centrifuged at 10,000 rpm for 3 min, then the supernatant was carefully removed to leave a 0.5 mL pellet for qPCR processing. DNA was extracted (Smart LabAssist-32) by using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer’s recommendations. Four µL of DNA extract, negative control, and positive control were added to 36 µL of reaction separately. Cycling conditions were as follows: 1 cycle of 2 min at 37 °C; 2 min at 94 °C; 40 cycles of 15 s at 93 °C and 60 s at 60 °C; single point fluorescence detected at 60 °C (ABI7500 PCR System). Positive DNA samples of P. jirovecii were subsequently stored at -20 °C for typing.

Pneumocystis genotyping

Positive DNA samples of P. jirovecii from immunocompromised patients with different primary diseases were included as a control group during the same period at Beijing Chaoyang Hospital, in addition to samples from PCP cases, HCW and colonized patients. Samples were sequenced blindly by a commercial company. A combination of three loci belonging to the superoxide dismutase (SOD), the mitochondrial large subunit of ribosomal ribonucleic acid (mtLSU rRNA), and the cytochrome b (CYB) genes were chosen (12, 13, 19–21). Nested PCR was used for amplification (ETC811, Eastwin Life Sciences), and bands of expected sizes were purified, and sequenced (Table 1). Cycling
conditions were as follows: 20 s at 96 °C, 30 s at 62 °C; 10 cycles of 20 s at 96 °C, 30 s at 52 °C, and 1 min at 72 °C; followed by a 5-min hold at 72 °C. Then, sequencing was performed on an ABI 3730XL DNA sequencer (Applied Biosystems) using a sequencing ready reaction kit (ABI). Consensus sequences were aligned with reference sequences (GenBank accession numbers AF146753.1 for SOD, M58605.1 for mtLSUrRNA, and AF320344.1 for CYB). P. jirovecii isolates with one or more nucleotide differences were considered to be different strains.

Table 1

| Locus      | Primers (specific amplification) | Amplicon size | Polymorphisms |
|------------|----------------------------------|---------------|---------------|
| SOD        | F: AATAAGGTITTAATTAGTCTT         | 762 bp        | 110, 191, 215 |
|            | R: CTGACCATCCATATATTTCA          |               |               |
| mtLSU rRNA | F: GTGAAACGGCCAGAGTCCC           | 1216 bp       | 54–57, 80, 85, 248, 288 |
|            | R: CCGAGTTCTCTCGGAATA            |               |               |
| CYB        | F: TATGGTTCATTACAGGACTG          | 1016 bp       | 279, 299, 348, 362, 369, 516, 547, 566, 675, 742, 832–833, 838 |
|            | R: ACCAGAGGAATAAACAATAAAG         |               |               |

†SOD, the superoxide dismutase; mtLSU rRNA, mitochondrial large subunit of ribosomal RNA; CYB, cytochrome b

Antibodies and flow cytometric measurement

Peripheral blood from PCP patients within three days after diagnosis and from matched controls with an equal follow-up time was stained with multitest antibodies Anti-Human CD45-FITC/CD3-PC5/CD4-RD1/CD8-ECD for T-cells, and Anti-Human CD45-PC5 and Anti-Human CD19 PC-7 for B-cells (Beckman Coulter) at room temperature in the dark for 15 min, respectively, then lysed with OptiLyse C (Beckman Coulter) at room temperature in the dark for 10 min. After that, 0.2 µL Isoton III Diluent was added to the mixture. Finally, Flow-Count Fluorospheres (50 µL) was added prior to collection on a flow cytometer. A fluorescence activated cell sorter Cytomics ™ FC500 Flow Cytometer from Beckman Coulter was used for measurement, and data were analyzed using CXP Analysis (Beckman Coulter).

Risk factor analysis

For risk factors analysis, each PCP case was matched to 3 controls who had not developed PCP following liver transplantation. They were matched by gender, age (± 3 years), primary diseases for transplantation (malignant or benign), and model for end-stage liver disease score (± 3, one day before transplantation). Preoperative parameters, operative parameters and postoperative parameters of these patients were collected and analyzed.

Statistical analysis

Data analyses were carried out by using SPSS 19.0 computer software (IBM Corp., Armonk, NY, USA). Values were expressed as mean ± standard deviation if possible. The Fisher’s exact test was used for categorical variables while the independent samples t-test and Mann-Whitney U test were employed for normal quantitative variables and non-normal quantitative variables, respectively. Variables with a P-value < 0.1 on univariate analysis were included in the multivariable model. Multivariable conditional logistic regression was performed to determine independent risk factors for PCP. Results of the multivariable conditional logistic regression are presented as odds ratios and 95% confidence intervals. A P-value < 0.05 was considered statistically significant. A receiver operating characteristic curve was built to assess the diagnostic performance of the predictor(s) associated with PCP patients.

Results

Clinical course

A cluster of ten patients were diagnosed with PCP following liver transplantation between July 2015 and January 2019 according to the criteria described above at our center at Beijing Chaoyang Hospital. Among these patients, four cases were retrospectively collected (from July 2015 to July 2016) and six cases prospectively included, which we considered an outbreak cluster (from April 2018 to January 2019). As there had been no new onset of PCP patients for more than six months since February 2019, we defined it an end of the outbreak cluster.

The characteristics of PCP patients are summarized in Table 2. The overall incidence rate for the whole period was 2.67% (10/375). There were nine males and one female with a mean age of 55.5 years. Six patients were diagnosed with hepatocellular carcinoma and four
patients with cirrhosis. Tacrolimus based immunosuppressive regimens were applied to nine patients in comparison to cyclosporin A to one patient. The period between transplantation and PCP diagnosis ranged from 1 week to 51 weeks. None of the patients were submitted to PCP prophylaxis at the time of diagnosis. Nine patients (9/10) with post-transplantation PCP were readmitted to hospital, of which seven (7/9) were transferred to SICU with 85.7% (6/7) in need of mechanical ventilation, and 42.9% (3/7) in need of extracorporeal membrane oxygenation support. All patients were treated with Trimethoprim/Sulfamethoxazole. Four patients died from respiratory failure. The overall in-hospital mortality was 40% (4/10). The mortality was 57.1% (4/7) in patients requiring ICU treatment.

| Patients | Age range | Primary diseases | Date of diagnosis | Time since transplant (w) | Sample type | Outcome |
|----------|-----------|------------------|-------------------|---------------------------|-------------|---------|
| P1       | 66–70     | HCC              | 7.19.2015         | 9                         | sputum      | Dead    |
| P2       | 61–65     | HCC              | 9.9.2015          | 8                         | sputum      | Dead    |
| P3       | 51–55     | HCC              | 3.24.2016         | 21                        | sputum      | Alive   |
| P4       | 61–65     | Cirrhosis        | 7.26.2016         | 15                        | BALF        | Dead    |
| P5       | 36–40     | Cirrhosis        | 4.1.2018          | 51                        | sputum      | Alive   |
| P6       | 26–30     | Cirrhosis        | 6.21.2018         | 21                        | BALF        | Dead    |
| P7       | 51–55     | Cirrhosis        | 8.17.2018         | 1                         | sputum      | Alive   |
| P8       | 56–60     | HCC              | 10.3.2018         | 19                        | sputum      | Alive   |
| P9       | 61–65     | HCC              | 12.15.2018        | 17                        | sputum      | Alive   |
| P10      | 61–65     | HCC              | 1.14.2019         | 10                        | sputum      | Alive   |

†PCP, Pneumocystis pneumonia; HCC, hepatic cell carcinoma; BALF, bronchoalveolar lavage uid

Transmission map

Based on visual investigation, no previous PCP patients had a chance to contact the following PCP patients before diagnosis during hospitalization. Both patient no.8 and no.9, and patient no.9 and no.10 might meet at outpatient clinic, respectively; however, they had an appointment on different dates after we checked their records. Moreover, PCP patients lived in different areas of China and did not know each other before admission. Therefore, the transmission map revealed that inter-patient transmission of P. jirovecii was impossible among these patients (Fig. 1).

P. jirovecii detection of air samples and HCW

First, we wanted to check whether P. jirovecii could be transmitted through air. After the diagnosis of PCP, air samples were collected in each ward as soon as possible, as well as in a room not used for patient care as a negative control. A total of 21 air samples from six wards and a negative control (three samples for each) were collected using the traditional passive sedimentation method. However, P. jirovecii detection was negative in all air samples.

Then, HCW were investigated as a potential source of P. Jirovecii infection. All HCW had an annual check-up and were in good condition. Since none of them had any respiratory symptoms during the period, HCW had to provide a nasal sample with a moist sterile cotton-swab (no BALF samples or sputum samples). P. Jirovecii detection was done in samples from twenty HCW (one sample for each), of which twelve were from those HCW who had close contact with PCP patients, and eight from the negative group. P. jirovecii detection was positive only in one sample from HCW encountering PCP patients (1/12).

Frequency of colonization with P. Jirovecii in liver transplant recipients

Finally, we decided to determine the frequency of colonization with P. Jirovecii in liver transplant recipients. Samples from liver transplant recipients (79 v.s 94) upon admission were prospectively collected for six consecutive months since the diagnosis of the No.6 PCP patients and August 2019 (six months after the diagnosis of the tenth PCP patient), respectively. There were no new onset of PCP patients between August 2019 and April 2020. The characteristics of liver transplant recipients are summarized in Table 3. BALF samples (n = 7 and 11), sputum samples (n = 24 and 32) and nasal samples (n = 48 and 51) were taken from these patients during and after the outbreak,
respectively. Five patients sampled during the outbreak were positive for *P. jirovecii* detection and classified as colonized in comparison to two patients sampled after the outbreak (*P* > 0.05).

| Characteristics                  | During the outbreak | After the outbreak | P value |
|----------------------------------|---------------------|--------------------|---------|
| Number                           | 79                  | 94                 |         |
| Sex                              | 0.260               |                    |         |
| Male                             | 63                  | 81                 |         |
| Female                           | 16                  | 13                 |         |
| Age                              | 53.09 ± 10.24       | 53.33 ± 9.33       | 0.871   |
| Primary diseases                 | 0.757               |                    |         |
| Benign                           | 38                  | 43                 |         |
| Malignant                        | 41                  | 51                 |         |
| Sample type                      | 0.661               |                    |         |
| BALF samples                     | 7                   | 11                 |         |
| Sputum samples                   | 24                  | 32                 |         |
| Nasal samples                    | 48                  | 51                 |         |
| *P. jirovecii* detection         | 0.313               |                    |         |
| Positive                         | 5                   | 2                  |         |
| Negative                         | 74                  | 92                 |         |

**P. jirovecii typing**

Positive *P. jirovecii* samples from 6 PCP cases (from P5 to P10), 12 unrelated controls (renal transplantation n = 2, renal failure n = 2, multiple myeloma n = 1, severe pneumonia n = 2, Behcet's disease n = 1, dermatomyositis n = 1, antineutrophil cytoplasmic antibody-associated vasculitis n = 1 and chronic obstructive pulmonary disease n = 1), 1 colonized HCW and 7 colonized liver transplant recipients were genotyped. Among these samples, genotyping failed for 15 samples because of a weak signal. Results for four PCP cases and seven unrelated controls are reported in Table 4.
Table 4
Multilocus sequence typing analysis of samples from liver transplant recipients with PCP and PCP controls.

| Polymorphisms     | Patient number | P6 | P8 | P9 | P10 | C4 | C5 | C6 | C8 | C9 | C10 | C11 |
|-------------------|----------------|----|----|----|-----|----|----|----|----|----|-----|-----|
| SOD genotype      |                | SOD2 | SOD1 | Mixed | (SOD2/SOD1) | SOD2 |
| 110               |                | TT  | / | CC  | / | / | / | / | / | CT | TT  | / |
| 191               |                | TT  | / | TT  | / | / | / | / | / | TT | TT  | / |
| 215               |                | CC  | / | TT  | / | / | / | / | / | TC | CC  | / |
| mtLSU rRNA genotype | Allele 8       | Allele 8 | Allele 3 | Allele 8 | Allele 8 | Allele 8 |
| 54–57             |                 | 4xA/4xA | / | / | / | / | 4xA/4xA | 4xA/4xA | 4xA/4xA | 4xA/4xA | / |
| 80                |                 | CC  | CC | CC  | CC | CC | CC | CC | CC | CC  | CC  | CC |
| 85                |                 | TT  | CT | TT  | CC | TT | TT | CC | TT | TT  | TT  | CC |
| 248               |                 | CC  | CT | CC  | CC | CC | TT | CC | CC | CC  | CC  | TT |
| 288               |                 | AA  | / | / | / | / | AA | AA | AA | AA  | AA  | / |
| CYB genotype      | Mixed (CYB10/CYB11) | CYB2 | Mixed (CYB1/CYB6) | CYB1 | CYB2 | CYB2 |
| 279               |                 | CC  | / | / | / | / | CC | CC | CC | CC  | CC  | / |
| 299               |                 | CC  | / | / | / | / | CC | CC | CC | CC  | CC  | / |
| 348               |                 | AA  | / | / | / | / | AA | AA | AA | AA  | AA  | / |
| 362               |                 | CC  | / | / | / | / | CC | CC | CC | CC  | CC  | / |
| 369               |                 | GG  | / | / | / | / | GG | GG | GG | GG  | GG  | / |
| 516               |                 | CC  | / | / | / | / | CC | CT | CC | CC  | CC  | / |
| 547               |                 | CC  | / | / | / | / | CC | CC | CC | CC  | CC  | / |
| 566               |                 | CT  | / | / | / | / | CC | CC | CC | CC  | CC  | / |
| 675               |                 | AA  | / | / | / | / | AA | AA | AA | AA  | AA  | / |
| 742               |                 | CC  | / | / | / | / | CC | CC | CC | CC  | CC  | / |
| 832–833           |                 | TT/TT | / | / | / | / | TT/TT | TT/TT | TT/TT | TT/TT | TT/TT | / |
| 838               |                 | CT  | / | / | / | / | TT | TT | TT | TT  | TT  | TT |

†PCP, Pneumocystis pneumonia; SOD, superoxide dismutase; mtLSU rRNA, mitochondrial large subunit of ribosomal RNA; CYB, cytochrome b; P, transplant recipients with PCP; C, immunocompromised patients with PCP as a control group; /, sequence typing failure; _ new genotypes are underlined.
Table 5  
Risk factors for the development of PCP in liver transplant recipients  

| Parameters                          | Univariate analysis | Multivariate analysis |
|------------------------------------|---------------------|-----------------------|
|                                    | With PCP            | Without PCP           | P       | OR    | CI    | P       |
| Preoperative                       |                     |                       |         |       |       |         |
| Sex (male)                         | 9                   | 27                    | 1.000   |       |       |         |
| Age                                | 55.50 ± 13.00       | 55.93 ± 10.57         | 0.916   |       |       |         |
| Primary disease (benign)           | 3                   | 9                     | 1.000   |       |       |         |
| Child score                        | 7.90 ± 2.73         | 8.23 ± 2.84           | 0.747   |       |       |         |
| MELD score                         | 14.90 ± 9.77        | 15.50 ± 10.60         | 0.875   |       |       |         |
| BMI                                | 24.51 ± 3.09        | 24.70 ± 3.50          | 0.876   |       |       |         |
| Diabetes                           | 2                   | 9                     | 0.696   |       |       |         |
| Smoking                            | 3                   | 10                    | 1.000   |       |       |         |
| Drinking                           | 2                   | 10                    | 0.693   |       |       |         |
| Heart disease                      | 1                   | 2                     | 1.000   |       |       |         |
| Respiratory disease                | 1                   | 1                     | 0.442   |       |       |         |
| Albumin (g/L)                      | 37.08 ± 6.60        | 35.82 ± 7.03          | 0.620   |       |       |         |
| Creatinine (µmol/L)                | 72.72 ± 19.82       | 97.61 ± 92.67         | 0.662   |       |       |         |
| Bilirubin (µmol/L)                 | 234.43 ± 348.05     | 148.97 ± 221.42       | 0.888   |       |       |         |
| INR                                | 1.38 ± 0.55         | 1.38 ± 0.56           | 0.978   |       |       |         |
| Lymphocyte count (/L)              | 1.24 ± 0.68         | 1.11 ± 0.68           | 0.626   |       |       |         |
| Lymphocyte percentage (%)          | 22.54 ± 10.13       | 21.98 ± 8.80          | 0.868   |       |       |         |
| Operative                          |                     |                       |         |       |       |         |
| Operating time (min)               | 549.50 ± 219.15     | 500.13 ± 111.00       | 0.354   |       |       |         |
| Warm ischemia time (min)           | 2.70 ± 1.06         | 2.57 ± 0.86           | 0.802   |       |       |         |
| Cold storage time (h)              | 7.20 ± 1.03         | 7.43 ± 0.94           | 0.376   |       |       |         |
| Bleeding (ml)                      | 740.00 ± 245.85     | 790.00 ± 326.26       | 0.660   |       |       |         |
| Transfusion (ml)                   | 600.00 ± 432.05     | 693.33 ± 734.82       | 0.707   |       |       |         |
| Surgical type (piggyback)          | 10                  | 29                    | 1.000   |       |       |         |
| Anhepatic phase (min)              | 58.80 ± 30.33       | 74.00 ± 36.72         | 0.246   |       |       |         |
| Postoperative                      |                     |                       |         |       |       |         |
| Acute rejection                    | 3                   | 3                     | 0.153   |       |       |         |

†PCP, Pneumocystis pneumonia; OR, odds ratio; CI, 95% confidence interval; MELD, model for end-stage liver disease; BMI, body mass index; INR, International normalized ratio; CMV, cytomegalovirus; MMF, Mycophenolate mofetil; ICU, intensive care unit

‡PCP cases tested right after diagnosis; controls tested following the same period as PCP patients
### Parameters

| Parameters                  | Univariate analysis | Multivariate analysis |
|-----------------------------|---------------------|-----------------------|
|                             | With PCP | Without PCP | P  | OR | CI         | P        |
| CMV                         | 2        | 4          | 0.629 |    |            |          |
| Respiratory infection       | 4        | 7          | 0.418 |    |            |          |
| Bile leak                   | 0        | 1          | 1.000 |    |            |          |
| Delayed graft function      | 1        | 0          | 0.250 |    |            |          |
| Bleeding                    | 0        | 0          | -     |    |            |          |
| Abdominal infection         | 2        | 4          | 0.629 |    |            |          |
| Immunosuppressants          | 0.906    |            |       |    |            |          |
| Tacrolimus                  | 9        | 28         |       |    |            |          |
| Sirolimus                   | 4        | 9          |       |    |            |          |
| MMF                         | 4        | 14         |       |    |            |          |
| Cyclosporin A               | 1        | 2          |       |    |            |          |
| Prednisone                  | 1        | 1          |       |    |            |          |
| Tacrolimus concentration (ng/ml) | 7.57 ± 2.62 | 5.52 ± 2.14 | 0.023 | 0.770 | 0.435–1.366 | 0.372 |
| ICU stay time (h)           | 91.90 ± 34.92 | 125.23 ± 70.44 | 0.161 |    |            |          |
| ‡CD3+ T-cell counts (/µL)   | 306.81 ± 222.40 | 655.00 ± 323.44 | 0.003 | 0.996 | 0.953–1.041 | 0.854 |
| ‡CD4+ T-cell percentage (%) | 62.53 ± 16.41 | 67.58 ± 13.99 | 0.349 |    |            |          |
| ‡CD4+ T-cell counts (/µL)   | 168.15 ± 152.02 | 365.01 ± 220.06 | 0.012 | 1.004 | 0.955–1.056 | 0.872 |
| ‡CD4+ T-cell percentage (%) | 31.64 ± 11.42 | 37.28 ± 11.60 | 0.189 |    |            |          |
| ‡CD8+ T-cell counts (/µL)   | 132.32 ± 85.59 | 261.34 ± 175.18 | 0.033 | 1.009 | 0.963–1.056 | 0.710 |
| ‡CD8+ T-cell percentage (%) | 29.82 ± 15.61 | 27.66 ± 15.28 | 0.701 |    |            |          |
| ‡CD19+ B-cell counts (/µL)  | 48.09 ± 39.11 | 164.68 ± 89.50 | 0.000 | 1.028 | 1.000-1.057 | 0.049* |
| ‡CD19+ B-cell percentage (%)| 12.34 ± 10.03 | 18.54 ± 10.63 | 0.114 |    |            |          |

‡PCP, Pneumocystis pneumonia; OR, odds ratio; CI, 95% confidence interval; MELD, model for end-stage liver disease; BMI, body mass index; INR, international normalized ratio; CMV, cytomegalovirus; MMF, Mycophenolate mofetil; ICU, intensive care unit.

SOD2, Allele 8 and mixed (CYB10/ CYB11) were all identified in one transplant recipient and SOD1 in another transplant patient. SOD2 and mixed (SOD2/SOD1) were identified in 1 patient from the control group, Allele 3 and Allele 8 in 1 and 4 patients, mixed (CYB1/CYB6), CYB1 and CYB2 in 1, 1 and 3 patients, separately. Taking into account mtLSUrRNA and SOD typing, the four liver transplant recipients had totally different genotypes from each other though the nucleotide positions were not completely sequenced at the 2 loci. Moreover, patient no.6 harbored a previously unreported combination of C566 C/T, C838C/T mutation at the CYB gene (CYB10/CYB11, Fig. 2), whereas none of the controls carried the mutation (22, 23).

### CD3 + T-cell, CD4 + T-cell, CD8 + T-cell and CD19 + B-cell detection by flow cytometry

First, we used FACS to analyze CD3 + T-cell percentage, CD4 + T-cell percentage, CD8 + T-cell percentage, and CD19 + B-cell percentage from patients with and without PCP (Fig. 3). However, the CD3 + T-cell percentage (62.53 ± 16.41% v.s 67.58 ± 13.99%), CD4 + T-cell percentage (31.64 ± 11.42% v.s 37.28 ± 11.60%), CD8 + T-cell percentage (29.82 ± 15.61% v.s 27.66 ± 15.28%), and CD19 + B-cell percentage (12.34 ± 10.03% v.s 18.54 ± 10.63%) were similar between the two groups (P > 0.05). Subsequently, absolute cell counts were analyzed. We found liver transplant recipients with PCP had a marked decrease of absolute CD3 + T-cell counts (306.81 ± 222.40/µL v.s 655.00 ± 323.44/µL, P <
patients were infected from different sources so it was reasonable to infer that HCW and transplant recipients might get infected from partially distinct from those detected in unrelated controls from other departments because of incomplete sequencing. This meant PCP sufficient to differentiate the P. jirovecii strains. To our surprise, PCP patients showed completely different P. jirovecii strains, which were suspicion.

In possible false-negative results. Invasive investigations could improve active screening of P. jirovecii infected patients in case of high patients or vice versa. Additionally, we had to admit that PCR on swabs was less sensitive than lower respiratory samples, which may result found to have higher occurrence of P. jirovecii when compared with controls. There was a possibility that they got infected from PCP each other through airborne transmission route combining transmission map analysis. Finally, colonized HCW and colonized patients were laminar airflow rooms which made it difficult to collect P. jirovecii. Nevertheless, it was still impossible for PCP patients to get infected from each other through airborne transmission route combining transmission map analysis. Finally, colonized HCW and colonized patients were found to have higher occurrence of P. jirovecii when compared with controls. There was a possibility that they got infected from PCP patients or vice versa. Additionally, we had to admit that PCR on swabs was less sensitive than lower respiratory samples, which may result in possible false-negative results. Invasive investigations could improve active screening of P. jirovecii infected patients in case of high suspicion.

Furthermore, positive P. jirovecii samples were genotyped. Apart from sequence typing failure in some samples or loci, the results were still sufficient to differentiate the P. jirovecii strains. To our surprise, PCP patients showed completely different P. jirovecii strains, which were partially distinct from those detected in unrelated controls from other departments because of incomplete sequencing. This meant PCP patients were infected from different sources so it was reasonable to infer that HCW and transplant recipients might get infected from
them when they had close contact. Moreover, we found an unreported mutation at the CYB gene (CYB10/CYB11) only in one PCP patient (22, 23).

Finally, risk factors for the development of PCP were analyzed. In our study, we identified decreased CD19 + B-cell counts to be the only risk factors. Nevertheless, in contrast to published studies that have reported several predictors such as age, CMV, acute rejection and decreased CD4 + T-cell counts, no such increased risk of PCP was observed in our study (9–11). Iriart X et al reported PCP occurred in elderly patients ≥ 65 (9). In our analysis, as age-matched patients were used as a control group we could not confirm age as a relevant risk factor. Nevertheless, the mean age of the cluster was 55 years old with only two patients ≥ 65. The presence of CMV viremia was associated with PCP. Basically, CMV infection may simply reflect the degree of immunosuppression. Rostved el. al. described a high rate of CMV co-infection but could not detect increased rates of CMV prior to diagnosis of PCP (24). Acute rejection implies the activation of the immune system while PCP occurred in immunocompromised patients. Therefore, acute rejection usually precedes PCP. It is the anti-rejection treatment that suppresses the immune system leading to lymphocyte deficiency, especially decreased CD4 + T-cell counts (25–27). In essence, acute rejection is a reflection of decreased CD4 + T-cell counts. Allograft dysfunction could increase the risk of PCP (28). In liver transplant recipients, immunosuppressive therapy consisted of induction and maintenance, which were all targeted at CD4 + T-cells. Decreased CD4 + T-cell counts is a clearly defined risk factor for PCP in HIV patients, while no clear threshold could be defined for CD4 + T-cell counts in liver transplant recipients (29).

A growing number of studies have stressed the key role CD19 + B-cells have played in the immune system. Rong et al found Pneumocystis burden in B cell-deficient mice progressively increased. Moreover, the clearance of Pneumocystis was delayed in B cell-activating factor receptor-deficient mice, which had few B cells and Pneumocystis-specific IgG and IgM antibodies (30). Hernandez-Novoa B et al reported CD40L-KO mice are highly susceptible to developing severe Pneumocystis pneumonia due to a decrease in CD19 + B-cell compared with immunocompetent mice (31). Therefore, the CD19 + B-cells exhibited an antifungal effect against P. jirovecii. In conjunction with these data, our study advocates the assumption that a decreased number of CD19 + B-cell in the blood may be the risk factor for PCP.

The main limitation of this study is that it represents the experience of a single center with a small number of patients. Future studies, preferably multi-center controlled clinical trials, are needed to further validate our initial report.

**Abbreviations**

Pneumocystis jirovecii, P. jirovecii; Pneumocystis pneumonia, PCP; human immunodeficiency virus, HIV; deoxyribonucleic acid, DNA; quantitative polymerase chain reaction, qPCR; bronchoalveolar lavage fluid, BALF; surgical intensive care unit, SICU; healthcare workers, HCW; superoxide dismutase, SOD; mitochondrial large subunit of ribosomal ribonucleic acid, mtLSU rRNA; cytochrome b, CYB

**Declarations**

*Ethics approval and consent to participate*

The study was approved by the Institutional Review Board of Beijing Chaoyang Hospital (No.2016-2-19-38) in accordance with the Helsinki declaration of 1975, as revised in 1983. Written informed consent was obtained from all participants.

*Consent for publication*

Not applicable

*Availability of data and materials*

All data generated or analysed during this study are included in this published article

*Competing interest*

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Authors' contributions

ZJQ and HQ contributed conception and design of the study; LXL, LZJ, LH, WQ and LZX organized the database; WB, WO, QM and XWL performed the statistical analysis; LXL, LZJ and LH wrote the first draft of the manuscript; the rest authors wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Figures

Figure 1

Transmission map of P. jirovecii among liver transplant recipients. The x-axis shows dates, and the y-axis shows patient numbers. No encounters were found among liver transplant recipients for P. jirovecii transmission. SICU, surgical intensive care unit; PCP, Pneumocystis pneumonia; P. jirovecii, Pneumocystis jirovecii
Figure 2

Molecular detection of Pneumocystis jirovecii deoxyribonucleic acid by quantitative polymerase chain reaction (A) and a previously unreported combination of C566 C/T (B), C838C/T (C) mutation at the cytochrome b gene
Figure 3

Representative flow cytometric data of the percentage of immune cells in patients with Pneumocystis pneumonia