Detection of *Pneumocystis jirovecii* Copy Number in BronchoAlveolar Lavage and Nasal swab Fluids of Immunocompromised Patients

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Abstract: The current study was tried to identification the of Pneumocystis jirovecii copy number in immunocompromised patients suffering from pulmonary disease, known as (PCP) Pneumocystis pneumonia by using real-time PCR (qPCR) and DNA sequencing for Phylogenetic tree analysis study of local Pneumocystis jirovecii confirmative detection based mitochondrial large subunit ribosomal RNA gene method by used PCR. The present study was conducted on 93 samples 53 samples were bronchoaleveolarlavage BAL were collected from patients whom suffer from (Pneumonia, chronic obstructive Pulmonary disease, Lung cancer, Tuberculosis, and HIV, Transplantation, Asthma, Hepatitis, Diabetes mellitus of both types with chest infection). And 40 sample nasal swabs were taken from infants suffer from pneumonia as diagnostic by pediatrician , BAL samples collected from Baghdad Medical City hospital and from Educational Al-Diwaniyah hospital while nasal swabs samples collected from Maternity and childhood teaching hospital in Al-Diwaniyah city at the period from the beginning of November 2015 to the end of May 2016.

The result of real-time PCR for detect target genes (qpcr mtLSU rRNA gene, PCR-ssrRNA gene) was showed positive samples were 72 and negative samples were 21, the rate of detection of pneumocystis jirovecii by qPCR method were 77.4%. DNA sequencing technique was used for genetic confirmation and phylogenetic tree analysis of Pneumocystis jirovecii as well as direct submission of some local Pneumocystis jirovecii isolates in NCBI-gene bank data base.

Key words : Pneumocystis jirovecii , BronchoAlveolar , Immunocompromised Patients

1. Introduction

Pneumocystis jirovecii is one of the most recurrent and severe opportunistic infections in immunocompromised patients it is the causative agent and responsible for a severe pulmonary disease, known as (PCP) *Pneumocystis* pneumonia (Calderón-Sandubete

et al ., 2002; Cushion ,2004). It's also regarded as one of the most frequent and serious complications, affecting immunocompromised patients such as those with HIV infection *(*Aliouat-Denis *et al.,* 2009). And it is an important cause of morbidity and mortality among them (Elvin *et al.*, 1994; Morris and Norris , 2012). In chronic immunosuppressive medications and are thus at a risk for PCP is rapidly growing such as malignancies, solid organ transplant recipients and patients treated by immunosuppressive agents are susceptible to this infection (Sulieman *et al.*, 2014; Sassi *et al.*,2012). And some other conditions such as diabetes and severe malnutrition increase the risk of PCP (Sanno *et al.*, 2007; Morrow *et al.,* 2014 and Morris *et al.*, 2004). Cough is generally non-productive but may be productive cough in up to a third of patients, exertional dyspnea, Fever, tachypnea, chest pain, there may be signs of AIDS such as thrush, oral hairy leukoplakia, kaposi's sarcoma , scattered crackles and wheeze may be present, or (rarely) signs of focal consolidation, extrapulmonary disease may manifest as hepatosplenomegaly,

lymphadenopathy or ocular disease (Cisse *et al*., 2012). In the diagnosis of *Pneumocystis jirovecii* culturing is not dependable yet , so confirmation of the diagnosis requires identification of organisms in bronchoalveolarlavage and staining of the cyst wall or trophozoite (Bowling *et al*., 1973). Monoclonal antibodies for detecting *P. jiroveci* are available and have a sensitivity greater than 90% for detecting *P. jiroveci* in induced sputum from HIV-infected patients, immunological diagnostic method of *Pneumocystis jiroveci* pneumonia (PJP) can be difficult in patients without HIV infection (Catherinot *et al* .,2010). Real-time PCR SYBR green-based real-time PCR technique(Espy,2006).Was conducted for rapid detection of *Pneumocystis jirovecii* according to method described by (Huggett *et al*.,2015). The current study was aimed to highlight the role of *Pneumocystis jirovecii* in patients who suffering from pneumonia with some risk factors, to achieve this goal, the following objectives were conducted :

1- Bronchoalveolarlavage (BAL) fluid were collected from patients who suffer from pneumonia with some risk factors (immunocompromised patients) .

2- Nasal swabs were taken from infants suffer from pneumonia as diagnostic by pediatrician.

3- Extraction of DNA and amplified with specific primers of target genes (qpcr mtLSU rRNA gene, PCR-ssrRNA gene).

4- Determination of target genes copy number of Bronchoalveolar lavage (BAL) and nasal swabs using quantitative -PCR method.

5- DNA sequencing technique was used for genetic confirmation and phylogenetic tree analysis of *Pneumocystis jirovecii* as well as direct submission of some local *Pneumocystis jirovecii* isolates in NCBI-gene bank data base.

2. Sample Collection and Diagnosis Method

Patients: BAL were collected from 53 patients whom suffer from (Pneumonia, chronic obstructive Pulmonary disease, Lung cancer, Tuberculosis, and HIV, Transplantation, Asthma, Hepatitis, Diabetes mellitus of both types with chest infection). And 40 nasal swabs were taken from infants suffer from pneumonia as diagnostic by pediatrician. BAL is a saline wash of the airways bronchi and air sacs alveolar was down the fluid were aspirate and collected in sterile container were taken from patients were stored at -4°c in refrigerator nasal swab after diagnosis of pneumonia by pediatrician taken nasal swabs and place in the culture medium provided . Molecular technique for

detection *Pneumocystis jirovecii* using qPCR, in both BAL and nasal swab samples . DNA extraction for qPCR DNA extraction from BAL and nasal swab samples was using a commercial Genomic DNA extraction kit (Reagent Genomic DNA extraction kit, Geneaid. USA) .The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) .The Real-Time PCR primers based mitochondrial large subunit ribosomal RNA gene were used for specific detection of *Pneumocystis jirovecii* as well as PCR primers based small subunit ribosomal RNA gene were used for phylogenetic tree analysis study, these primers were designed in this study using NCBI-Gene bank data base and primer3 plus and these primers provided by (Bioneer company, Korea).as table 1.

Primer	Sequence		Amplicon
qpcr mtLSU rRNA gene	F	AGATAGTCGAAAGGGAAACAGC	114bp
	R	GCTGTTTCCAAGCCCACTTC	
PCR-ssrRNA gene		GGACAAAGAGAGGGACGACC	248bp
		ATATCTTCGTGCTGCCCTCC	

Real Time PCR was performed for detection of *Pneumocystis jirovecii* mitochondrial large subunit ribosomal RNA gene and this technique was carried out according to method described by (Huggett *et al.*,2015).Real-Time PCR master mix was prepared by using (AccuPower® GreenStar™ qPCR PreMix kit Bioneer. Korea).

Table 2: qPCR master mix components:

RT-PCR Master Mix	Volume
DNA template	5µL
Forward gene primer (20pmol)	2µL
Reverse gene primer (20pmol)	2µL

DEPC water	11µL
Total	**20µL**

Real-Time PCR thermocycler conditions was set according to primer annealing temperature and kit instructions using Real-time PCR thermocycler system.

Table 3: qPCR thermocycler conditions

qPCR Step	Temperature	Time	Repeat cycle
Initial denaturation	95 °C	5min	1
Denaturation	95 °C	15 sec	45
Annealing\Extension Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

DNA sequencing method was performed for phylogenetic tree analysis study of local *Pneumocystis carinii* confirmative detection

based mitochondrial large subunit ribosomal RNA gene. The sequencing of the PCR product of ribosomal RNA gene, where the 248bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada).

3. Statistical Analysis

Data were analyzed using statical package for social science (spss) version (22), T-test chi-square test, and Mann Whitney test were used For compression between the cases groups. Numeric data were presented as mean and standard deviation were as categorical data were presented as number and percentage the level of significance was considered at p-value as ≤ 0.05.

4.Results

The rate of detection of *pneumocystis jirovecii* by qPCR method was estimated to be 77.4%.

Table 4: Results of qPCR

qPCR	**N**	**%**
Positive	72	77.4
Negative	21	22.6
Total	93	100
Specificity	71.4	
PPV	89.5	

NPV	66.7

* PPV: positive predictive values

* NPV: negative predictive values

The present study showed higher mean log in HIV (11.91), while low mean log in Pneumonia (4.63),and mean log of the Lung cancer, TB, DM, COPD, Asthma, Transplantation, Hepatitis (7.90, 7.26, 6.71, 5.67, 5.10, 5.08, 4.93), (table 5).

Table 5: Association between infection detected by qPCR and risk factor

Risk	**qPCR positive cases**		**qPCR copy number (log)**	
	N	**%**	**Mean**	**SD**
Pneumonia	43	76.79	4.63	1.15
COPD	1	100.00	5.67	---
Lung cancer	6	85.71	7.90	2.36
TB	6	66.67	7.26	1.69

HIV	1	100.00	11.91	---
Transplantation	3	60.00	5.08	0.48
Asthma	3	100.00	5.10	0.46
Hepatitis	3	60.00	4.93	0.47
DM	6	100.00	6.71	1.66

* COPD: Chronic obstructive pulmonary disease.

* TB: Tuberculosis.

* HIV: Human immunodeficiency virus.

* DM: Diabetes mellitus.

DNA sequencing result

Multiple sequence alignment analysis of *Pneumocystis carinii* large subunit ribosomal RNA gene mitochondrial partial sequence based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tools). That show the multiple alignment analysis similarity (*) and differences in *Pneumocystis carinii* large subunit ribosomal RNA gene nucleotide sequences with NCBI-BLAST Pneumocystis large subunit ribosomal RNA gene mitochondrial sequence.

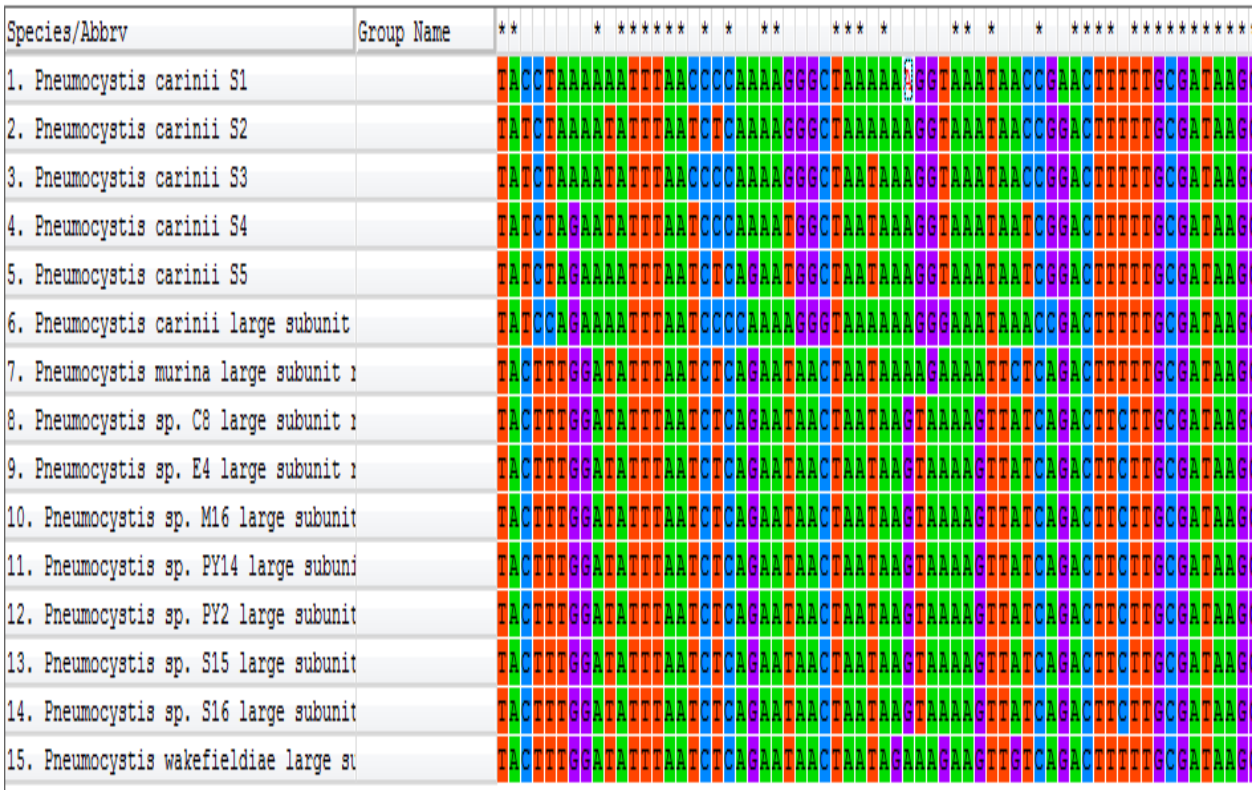

Figure 1: Multiple sequence alignment analysis of *Pneumocystis carinii* large subunit ribosomal RNA gene .

The phylogenetic tree was constructed using Maximum Likelihood tree method in (MEGA 6.0 version). The local *Pneumocystis carinii* (S1,S2,S3,S4, and S5) were show closed related to NCBI-Blast *Pneumocystis carinii* (KC494274.1) Whereas, the other NCBI-Blast *Pneumocystis* sp. were show different and out of tree.

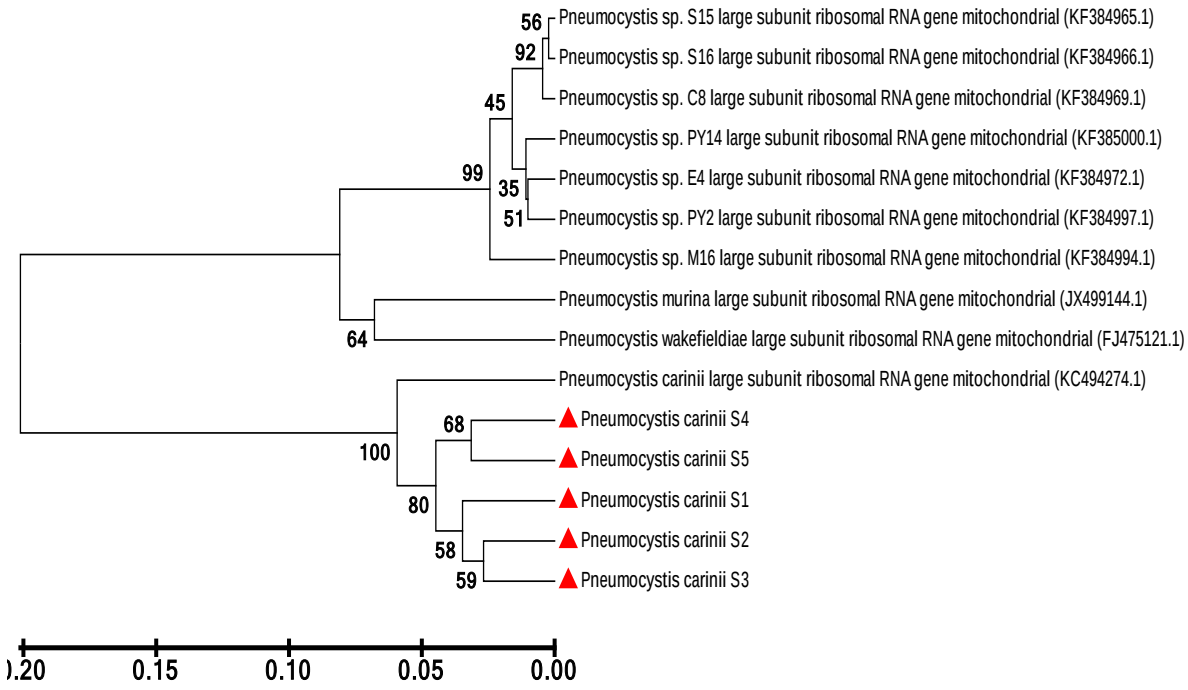

Figure 2: Phylogenetic tree analysis based on the large subunit ribosomal RNA gene mitochondrial partial sequence that used for *Pneumocystis carinii* confirmative detection.

Table 6: The NCBI-Gene bank submission

Pneumocystis carinii **isolate**	**Accession number**
Sample 1	KX 901804
Sample 2	KX 901805
Sample 3	KX 901806
Sample 4	KX 901807
Sample 5	KX 901808

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