Evaluation of a real-time quantitative PCR assay for diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients – a pilot study

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be held in April-2016

CERTIFICATE

This is to certify that the dissertation entitled, "**Evaluation of a real-time quantitative PCR assay for diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients – a pilot study"** is the bonafide work of Dr.S.Dhanalakshmi toward the M.D **(Branch – IV Microbiology)** Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be conducted in **April-2016.**

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I hereby declare that this M.D Dissertation entitled "Evaluation of a real-time quantitative PCR for diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients – a pilot study" is the bonafide work done by me under the guidance of Dr. Joy Sarojini Michael, Professor, Department of Clinical Microbiology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

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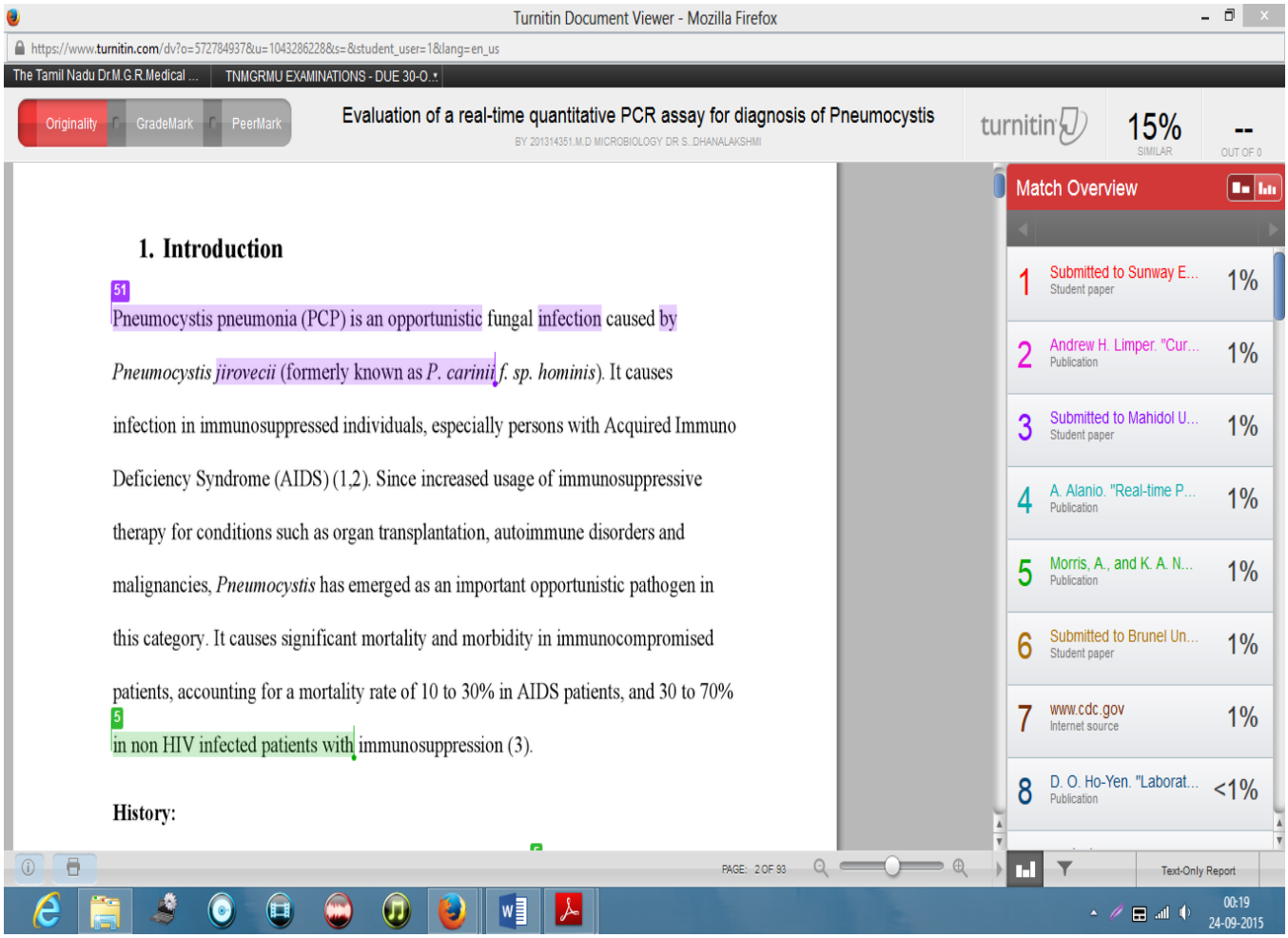
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1. Introduction
Pneumocystis pneumonia (PCP) is an opportunistic fungal infection caused by Pneumocystis jirovecii (formerly known as P. carinii f. sp. hominis). It causes infection in immunosuppressed individuals, especially persons with Acquired Immuno Deficiency Syndrome (AIDS) (1,2). Since increased usage of immunosuppressive therapy for conditions such as organ transplantation, autoimmune disorders and malignancies, Pneumocystis has emerged as an important opportunistic pathogen in this category. It causes significant mortality and morbidity in immunocompromised patients, accounting for a mortality rate of 10 to 30% in AIDS patients, and 30 to 70% in non HIV infected patients with immunosuppression (3).
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Contents

1. Introduction

Pneumocystis pneumonia (PCP) is an opportunistic fungal infection caused by *Pneumocystis jirovecii* (formerly known as *P. carinii f. sp. hominis*). It causes infection in immunosuppressed individuals, especially persons with Acquired Immuno Deficiency Syndrome (AIDS) (1,2). Since increased usage of immunosuppressive therapy for conditions such as organ transplantation, autoimmune disorders and malignancies, *Pneumocystis* has emerged as an important opportunistic pathogen in this category. It causes significant mortality and morbidity in immunocompromised patients, accounting for a mortality rate of 10 to 30% in AIDS patients, and 30 to 70% in non HIV infected patients with immunosuppression (3).

History:

Pneumocystis was first identified by Carlos Chagas in the lungs of rats and guinea pigs in 1909. He thought it could be a form of *Trypanosoma cruzi*, since he identified this organism in lungs of patients who had died of *Trypanosoma* infection. Antonio Carini also noted these cysts in rat lungs with trypanosomiasis, but he thought it might be a different organism. Three years later, in 1912 Pierre Delanoe and Marie Delanoe classified this organism under separate genus *Pneumocystis*, which was descriptive of the small but highly refractive and densely staining spherical cyst form of the microbe with predilection for lung and named the organism in honour of Dr.Carini as *Pneumocystis carinii*, but they believed that it was a parasite. In 1942, it was reported

as the cause of pneumonitis in three infants in Netherland. Soon after Vanek and Otto Jirovec identified *Pneumocystis* as a cause of interstitial plasma cell pneumonia in malnourished infants during World War II and this pneumonia was referred to as Pneumocystis carinii pneumonia (PCP). Even though the causative organism was identified in early 19th century, only 80 cases were reported by 1973, as *Pneumocystis* was an uncommon cause of disease. But this scenario changed during the AIDS epidemic in the 1980s. Thereafter PCP became one of the common clinical diseases associated with immunocompromised patients (4–6).

Pnuemocystis was believed to be a protozoan for a period of 80 years from its discovery until 1988. Analysis of 16SrRNA revealed that *Pneumocystis* was a fungus not a protozoa (5).

It was found that *Pneumocystis* infects only mammals and historically all the forms were referred to as *P.carinii.* Since it is species specific, the human form was renamed *P.jirovecii* in honour of Otto Jirovec in 1999, and the name *P.carinii* was reserved for rat form. Genetic sequence analysis of 18SrRNA of *P.jirovecii* (human derived) and *P.carinii* (rat derived) showed 5% difference between these two species. So species infecting different mammals are dissimilar. Even though there was a change in nomenclature, the abbreviation PCP remains to be used and the disease referred to as Pneumocystis pneumonia (7).

There are many challenges for laboratory diagnosis of PCP. Even though this is a fungus, this cannot be cultured in vitro in the laboratory. Therefore the laboratory diagnosis is based on visualisation of organism by microscopy in respiratory samples like induced sputum, broncho alveolar lavage (BAL), endo tracheal aspirate (ETA) and lung tissue. Stains commonly used to demonstrate this organism in the laboratory are Giemsa, toluidine blue-O, Gomori-Grocott methenamine silver, Diff-Quick and direct immunofluorescence assay (8). But sensitivity of the microscopic methods vary from 60 to 92% for BAL samples and 35 to 78% for endo tracheal aspirates and induced sputum samples (9).

Moreover following the introduction of highly active anti-retroviral therapy (HAART) for AIDS and co-trimoxazole prophylaxis for PCP, the incidence of PCP has come down. Thus, samples from suspected cases will have low burden of organisms which makes the diagnosis even more difficult by conventional microscopic methods (10).

Molecular diagnosis by Polymerase chain reaction (PCR) with its low detection limits may be a good technique to detect the presence of *P.jirovecii* in the lung (11). There are many target genes for PCR such as dihydropteroate synthase (DHPS), dihydrofolate reductase (DHFR), internal transcribed spacer regions of the rRNA (ITS), mitochondrial large subunit rRNA (mtLSU rRNA), major surface glycoprotein (MSG), 5S rRNA, 18S rRNA, and cdc2, which were evaluated in many studies (12). Among these mtLSU rRNA target is being widely used for diagnosis as this gene has

multiple copies and is a highly conserved nucleotide sequence which gives high sensitivity and specificity (11).

Colonization is an another important entity caused by *Pneumocystis* in immunocompromised patients, which means presence of low number of organisms without causing disease (4). Even though conventional PCR can detect low burden of the organism, it is not quantitative, as it cannot differentiate active PCP infection from colonization. A real time quantitative PCR (qPCR) can differentiate these two scenarios and can guide clinicians in diagnose PCP appropriately, especially in non HIV infected immunocompromised patients who are likely to have low burden of organisms (13).

2. Aim and Objectives of the Study:

Aim:

This study aims to evaluate a real-time quantitative PCR assay for diagnosing *Pneumocystis jirovecii* pneumonia in immunocompromised patients, compare these results with conventional microscopic methods and to correlate with clinical classification (composite standard) of PCP.

Objectives:

i. To evaluate a real-time quantitative PCR (qPCR) assay for diagnosis of Pneumocystis pneumonia (PCP) in immunocompromised patients.

ii. To compare qPCR assay with conventional microscopic methods such as Giemsa staining and direct immunofluorescence assay (DFA) for diagnosis of PCP.

iii. To evaluate the Cost effectiveness of this new assay when compared to the conventional microscopic methods currently used for routine diagnostics.

3. Review of literature

3.1. Epidemiology:

3.1.1 Global burden:

Before the AIDS epidemic in 1980, PCP was a rare disease among the immuno-compromised patients, accounting to 5%–25% in transplant patients, 2%–6% in patients with collagen vascular disease, and 1%–25% in patients with cancer (14). After 1980, *P.jirovecii* became the most common opportunistic infection among HIV infected persons, affecting 20 per 100 person-years for those with CD4+ cell counts <200 cells/μL. With the introduction of anti-pneumocystis prophylaxis in 1989, there was a decline in the incidence of PCP.

In USA, the percentage of AIDS cases with PCP declined from 53% in 1989 to 42% in 1992. With the introduction of HAART, the incidence of PCP further decreased to 3.4% per year from 1992 through 1995. The rate of decline was further declined to 21.5% per year from 1996 through 1998 (14).

In Europe, the EuroSIDA study showed that incidence declined from 4.9 cases per 100 person-years before March 1995 to 0.3 cases per 100 person-years after March 1998 (14).

In developing countries, the incidence of PCP has declined with the advent of interventions such as anti-pneumocystis prophylaxis and HAART. These interventions are limited for developing regions like Sub Saharan Africa, where 23 million people are living with HIV infection. High PCP prevalence of about 27% reported from certain African countries (15).

3.1.2 The burden in India:

There are very few studies/case reports published from India. One of the explanations could be the low sensitivity of available microscopic methods routinely used to diagnose *Pneumocystis* in most centres in India (16).

In 1993, three cases of *Pneumocystis* were reported in AIDS patients from India. A prospective study from North India done in August 1993 to December 1998 showed that prevalence of *Pneumocystis* in HIV infected and non HIV infected immunocompromised patients was 6.1% and 1.5% respectively (17). One cross sectional study done from January 2010 to October 2011in AIIMS, New Delhi showed a 15% prevalence of PCP in immunocompromised children, among these 45% children had renal disorders and were on immunosuppressants, though not on PCP prophylaxis (18).

3.2. The *Pneumocystis jirovecii*

3.2.1 Taxonomy:

Pneumocystis jirovecii belongs to the phylum Ascomycota, class Pneumocystidomycetes, order Pneumocystidales, the family Pneumocystidacae, and to the genus *Pneumocystis.* The genus is descriptive of the small but highly refractive and densely staining spherical cyst form of the microbe that are extracellular, host obligate, host specific and typically restricted to the lung tissues of mammals.

Initially it was classified as a protozoan based on following criteria (7):

i. Its morphology and host pathology.
ii. Absence of chitin in cell wall which is a characteristic of fungus.
iii. Presence of morphological forms which is characteristic of protozoa such as cyst and trophozoites.
iv. Non response to anti-fungal drugs.
v. Response to the drugs which is commonly used to treat protozoan infections such as co-trimoxazole and pentamidine.

16srRNA sequencing of rat derived *P.carinii* in 1988 changed the history of *Pneumocystis,* reclassifying it into Kingdom Fungi. Other cloned genes of *P.carinii* which was helpful in this evolutionary relationship are dihydrofolate reductase (DHFR), thymidylate synthase (TS), β tubulin, α tubulin, the TATA-binding protein, P type cation- translocating ATPase, and the actin gene. Analysis of the above molecules placed *P.carinii* on its own branch between the ascomycetes and basidiomycetes of an evolutionary tree, but definitely within the fungal kingdom (5).

3.2.1.1. Other species (19)**:**

i. *Pneumocystis carinii* – found in rats
ii. *Pneumocystis wakefieldiae* – found in rats
iii. *Pneumocystis murina* – found in mice
iv. *Pneumocystis oryctolagi* – found in rabbits

3.2.2 Structure:

Pneumocystis exists in three forms, the cyst, precyst (sporocyte), and trophozoite (trophic form) (19,20).

Cyst: thick walled, round to ovoid or cub shaped structure, measures about 6 to 8µm size and contains up to eight pleomorphic sporozoites or spore.

Precyst: smaller than mature cyst, measures 5 to 6µm size and oval in shape, contains rigid cell wall with 2 to 8 nuclei at varying levels of nuclear division.

Trophozoite: extra cystic form, representing an excysted sporozoite and it is thin walled measures about 1 to 4µm size, commonly exists in clusters.

3.2.2.1 Antigenic structure:

Pneumocystis has many surface antigens that are glycosylated with mannose glycoproteins. These antigens are called glycoprotein A (gp A) or major surface glycoprotein (MSG) which has molecular masses ranging from 95 to 120 KDa and has an important role in pathogenesis by taking part in integration of *Pneumocystis* into host alveolar epithelial cells and evasion of the host defence mechanism. It is highly immunogenic. It has the ability to undergo antigenic variation by which it evades host immune response (6,21).

A second family of surface antigens i.e. subtilisin like serine proteases encoded by PRT1 (protease), also known as KEX multigene family was identified on the surface of *Pneumocystis carinii.* But only a single gene of KEX family was identified in *P.jirovecii* and *P.murina* and it is believed to take part in antigenic variation.

3.2.2.2. Cell wall:

The fungal cell wall is made up of complex carbohydrates and structural proteins. β-1, 3-glucans are a major structural component of the *Pneumocystis* cell wall in both cystic and trophic forms. However there is little or no chitin in its cell wall. These glucan gives structural stability to the organisms in the infected lung as well as induce inflammatory response. Cholesterol is the dominant sterol present in *Pneumocystis*. Instead of ergosterol, the organism synthesizes distinct Δ7,C-24 alkylated sterols. Coenzyme Q10 (CoQ10) is the major ubiquinone homologue synthesized by the organism (6,21).

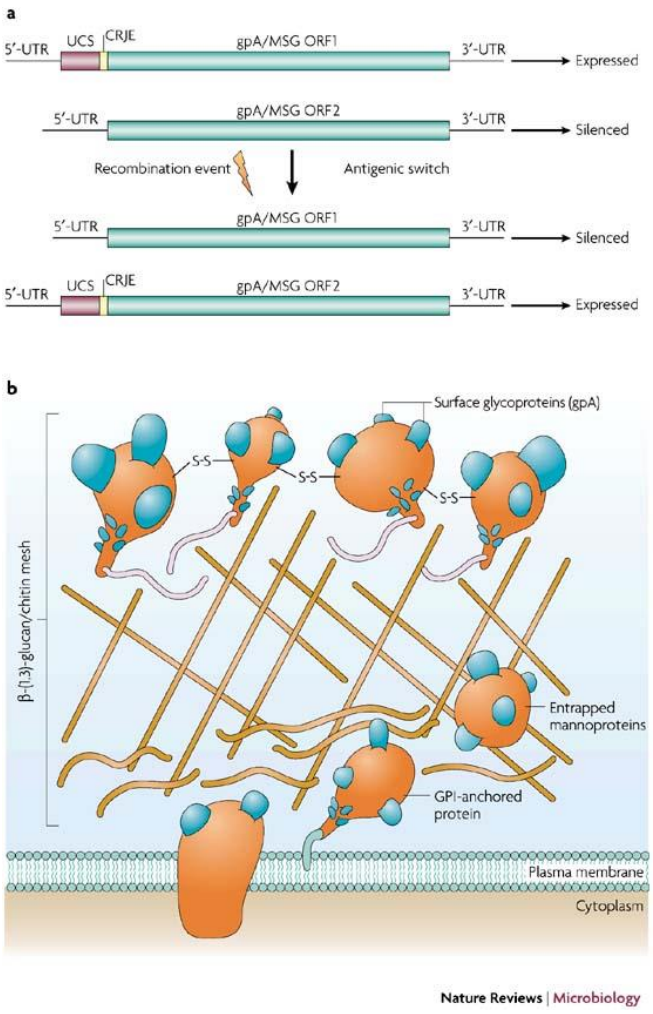


Fig. 3.1 Showing fungal cell wall and antigenic structure (21)

3.2.3. Life cycle:

Man acquires infection by inhalation of *Pneumocystis* from an individual with active PCP or transient subclinical colonization. There are two phases in the life cycle, asexual and sexual. In the **asexual phase**, multiplication is by simple binary fission of trophic form. In the **sexual phase**, haploid trophozoites conjugate together and form a diploid zygote or precyst which undergoes meiosis first, then mitosis to produce mature cyst or spore case which contains eight haploid intracystic bodies or a spore. Spores have different shapes being, either spherical or elongated and are released from the cyst through rents in the cyst wall (6,22).

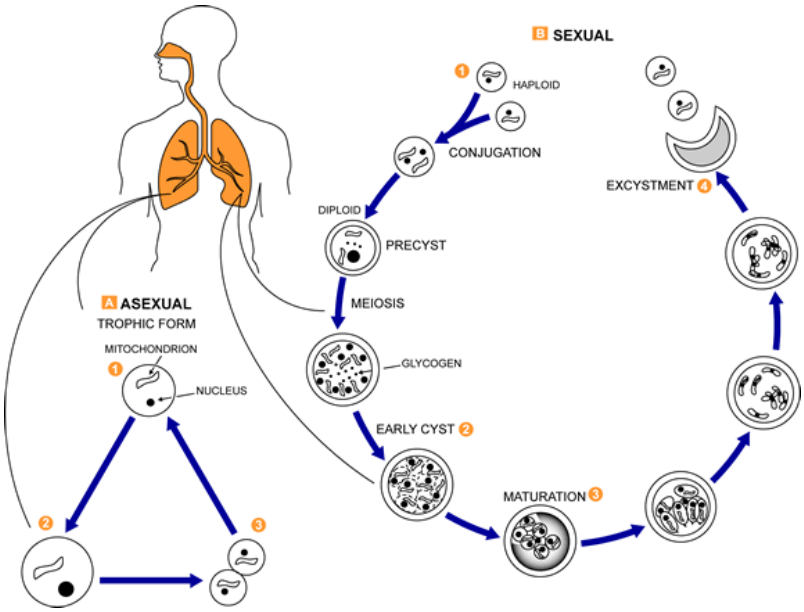


Fig. 3.2 Showing life cycle of *Pneumocystis* (Adapted from CDC DPDx – reference 22

3.3. Pathogenesis of PCP:

There is an abundant inflammation involving neutrophils and CD8 cells by *Pneumocystis* infection. This is the primary event in the pathogenesis of PCP which causes pulmonary injury characterised by diffuse alveolar damage and impaired gas exchange leading to respiratory failure which is the hallmark of the disease (21).

Due to its small size, inhaled *Pneumocystis* escapes from upper respiratory tract and is deposited in the alveoli. The trophic form attaches tightly to type 1 alveolar epithelial cells and to a lesser extent to type 2 alveolar epithelial cells and initiates infection. The adherence of the organism produces lung damage and host response which leads to a massive influx of CD8 cells, neutrophils and macrophages along with local and systemic pro inflammatory cytokines (6,21).

3.3.1 Host response to *Pneumocystis* infection:

Host defences against *Pneumocystis* infection includes both innate and adaptive immunity. The innate immune system is comprised of alveolar macrophages, surfactant protein (SP)-A and SP-D (6).

3.3.1.1 Macrophage activity:

Pneumocystis is taken up by alveolar macrophages, primary resident phagocyte, through mannose receptors which interact with gpA/MSG on the surface of the organisms. There are also interactions between *Pneumocystis* β-glucans and the dectin-1 receptor. β-glucans in the cell wall of *Pneumocystis* stimulate macrophages to produce TNF, IL-8, and macrophage inflammatory protein 2 (MIP2). TNF recruits a massive amount of neutrophils, lymphocytes and monocytes for clearance of the

organisms. But neutrophils induce lung injury through the production of proteases, oxidants and cationic proteins. Even though TNF causes lung injury, it induces the generation of IL-8 from epithelial cells and interferon γ (IFN-γ) from lymphocytes, which further promotes inflammatory cell activation and recruitment during PCP. IL-8 production is directly correlated with lymphocyte infiltration and decreased gas exchange in severe PCP. IL-8 level in broncho alveolar lavage fluid (BAL) could be a predictor of respiratory failure and death in PCP. IL-8 levels in BAL fluid in non HIV infected immunocompromised patients with PCP is higher compared to HIV infected patients with PCP and this level directly proportionate to the oxygenation index.

Impaired macrophage function in patients with AIDS and patients with malignancies suppresses *Pneumocystis* clearance (21).

3.3.1.2. Surfactant proteins activity:

SP-A accelerates the interaction between alveolar macrophages and *Pneumocystis*, thus phagocytosis also. By contrast the organisms can also escape the host defence mechanism as SP-D mediates aggregation of the organisms into large conglomerates which is poorly phagocytosed by macrophages. During PCP, production of surfactant phospholipids is reduced which leads to stiffer lung which is difficult to ventilate (21).

3.3.1.3. Lymphocyte response during PCP:

Both CD4 and CD8 T lymphocytes play important role in pathogenesis. CD4 cells have crucial role in host defence mechanism by providing memory function which recruit additional effector cells like monocytes and macrophages which are

responsible for elimination of the organism. So there is an increased risk of infection if CD4 cell count are less than 200/µl (21).

3.4. Clinical manifestations of PCP:

3.4.1. Risk factors:

PCP is an opportunistic fungal infection occurs in immunosuppressed individuals. Immunosuppression is mainly due to either underlying diseases/conditions or its treatment. Risk groups can be classified into HIV infected and non HIV infected patients since clinical features and risk factors are quite different for both groups (23).

3.4.1.1. Risk factor for HIV infected patients:

The most common and only one risk factor in HIV infected patients are CD4 T lymphocyte counts. PCP is common if CD4 count is less than 200 cells/µl.

3.4.1.2. Risk factors for non HIV infected patients:

Diseases or conditions which increases the susceptibility for PCP in non HIV infected patient groups are following (23);

i. Haematological malignancies
ii. Solid organ tumour
iii. Organ transplantation or haematopoietic stem cell transplantation (HSCT)
iv. Connective tissue disorders on immunosuppression.

Treatment related risk factors are use of following (23);

i. Corticosteroids
ii. Purine analogues

iii. Anti-CD52 and anti-CD20 monoclonal antibodies

iv. Calcineurin inhibitors

v. TNF-α antagonists

3.4.2. Clinical manifestations in HIV infected patients:

In HIV infected patients, PCP has a slow progressive course of about 2 weeks to 2 months. Clinical manifestations include subtle onset of dyspnoea, fever, non-productive cough and chest discomfort. Respiratory examination in acute cases reveal tachypnoea, tachycardia, and diffuse dry rales. Oral thrush is a commonly observed condition in these group of patients.

Bilateral diffuse symmetrical lung infiltrates extending from the perihilar region as butterfly pattern is a classic finding in Chest radiography. This pneumonia is referred to as interstitial plasma cell pneumonia.

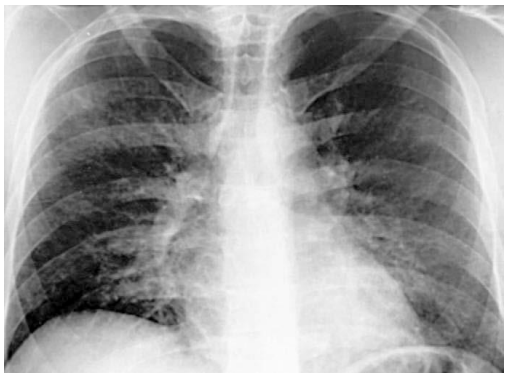


Fig. 3.3 Chest X-ray showing bilateral lung infiltrates (Adapted from reference 6)

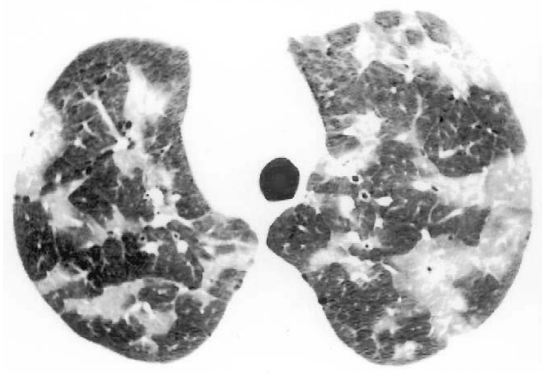


Fig. 3.4 HRCT showing typical ground glass opacity (Adapted from reference 6)

In mild cases of PCP Chest radiography may be normal. Atypical radiographic presentations such as nodules, blebs, cysts, unilateral infiltrates, lymphadenopathy, effusion and pneumothorax may also occur. In such cases high resolution computed tomography (HRCT) will be helpful. It shows ground glass opacity (GGO). Impaired oxygenation or hypoxaemia is a common abnormality seen in PCP patients. Severity of disease can be classified into mild, moderate, and severe, based on alveolar-arterial oxygen difference. Serum lactate dehydrogenase (LDH) levels are often high, reflecting lung injury, though nonspecific (24).

3.4.3. Clinical manifestations of PCP in non-HIV patients:

In contrast to HIV infected patients, PCP in non-HIV infected immunocompromised presents with an abrupt onset of respiratory insufficiency, despite harbouring low levels of the organism. This difference is mainly due to aberrant immune response seen in this group of patients. Mortality rate is high (30% to 60%) in non HIV infected patients compared to 10 to 20% in HIV infected patients (23).

3.5. *Pneumocystis* colonization:

The detection of *Pneumocystis* organisms or their DNA in respiratory samples of individuals without any signs and symptoms of PCP, is referred to as *Pneumocystis* colonization or carriage or subclinical infection (25,26).

3.5.1. Epidemiology of *Pneumocystis* colonization:

Exposure to *Pneumocystis* happens in early childhood. This was supported by a Spanish seroprevalence study in 233 healthy children, which showed overall

seroprevalence rate of 73%, with an age-related increase from 52% at 6 years to 66% at 10 years and 80% at 13 years (27).

Prevalence of *Pneumocystis* colonization in HIV infected individuals varies from 14% to 69%. This wide variation is may be due to several reasons - difference in samples used/the population tested or the sensitivity of test used in various studies (26).

In non HIV infected immunosuppressed individuals, prevalence rate accounts for 35% to 60% (25). *Pneumocystis* colonization can also occur in patients with chronic lung diseases like COPD, chronic bronchitis, cystic fibrosis, lung cancer, interstitial lung disease of about 2.6% to 35% (26).

3.5.2. Consequences of *Pneumocystis* colonization:

The true clinical significance of *Pneumocystis* colonization is not known. However some authors have suggested that it can lead to active PCP in susceptible hosts as well as acts as a reservoir for transmission of disease. It may induce pulmonary inflammation and mild lung injury as suggested by association with chronic lung disease and could be an important cofactor for progression of lung disease (26).

3.6. Laboratory diagnosis:

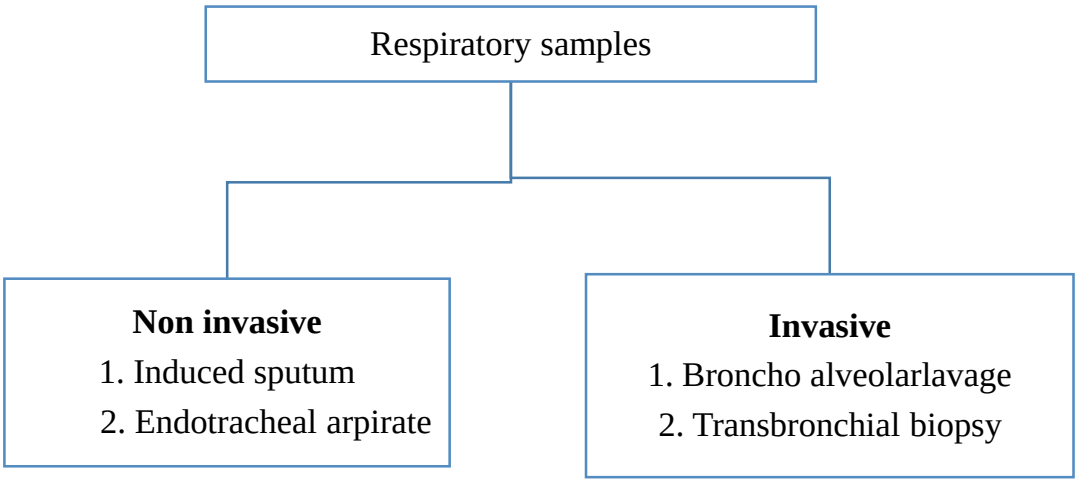
The role of the laboratory in the diagnosis of any infection depends on the clinical condition of the patients. In patients with HIV infection presenting late in their illness with pneumonia, the clinical presentation alone can be diagnostic and laboratory diagnosis of PCP may be unnecessary. However, in patients with early disease, including those receiving prophylactic chemotherapy, or those who are not obviously

immunocompromised, the laboratory diagnosis of PCP is critical for correct management.

The laboratory diagnosis of PCP is challenging as in vitro cultivation is not successful and the diagnosis is mainly dependent on direct demonstration of the organism in respiratory specimen.

3.6.1. Samples:

Respiratory samples which are routinely used for diagnosis of PCP are classified as follows:



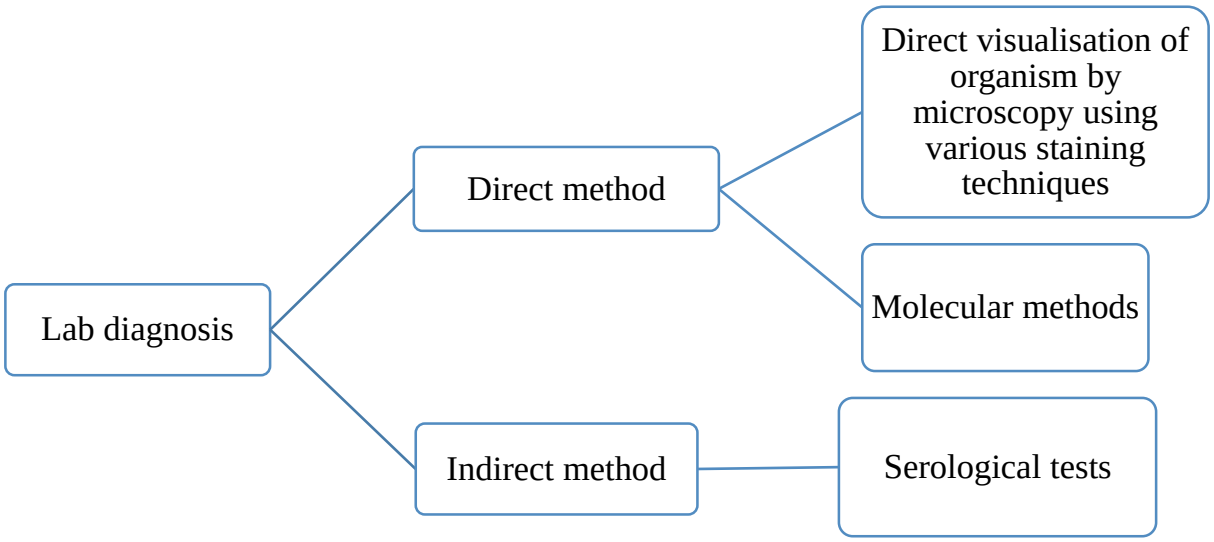
- **Induced sputum** is obtained after nebulization with 3 to 5% hypertonic saline for 10 to 20 minutes and the material is mainly from central proximal airway.
    - The advantage of induced sputum is that it is non-invasive, repeatable and inexpensive (28).

- Disadvantage is that it has only a yield of 55%, when compared to other invasive samples like BAL and trans bronchial biopsy which have a yield of 79% and 90% respectively (29).

- **Broncho alveolar lavage (BAL)** is obtained through bronchoscopy and the material is from the peripheral airways and alveolar compartment (29).

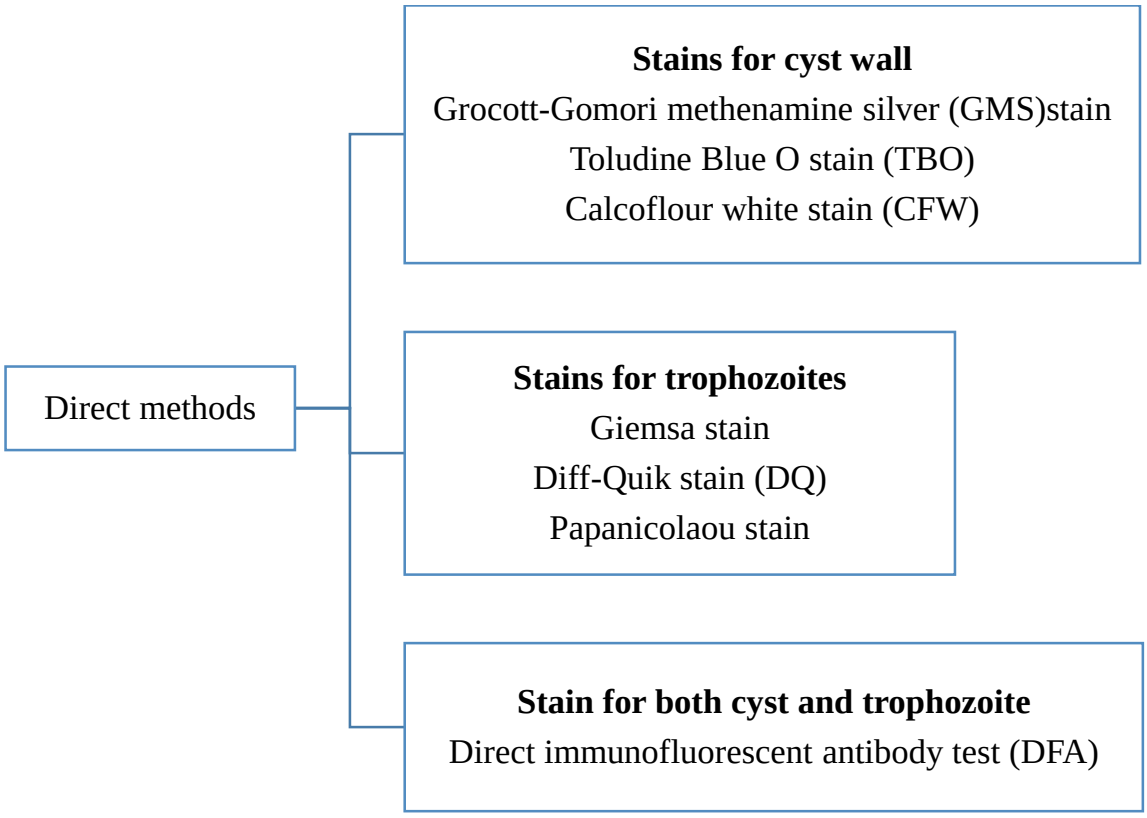
3.6.2. Classification:

Laboratory diagnosis of *Pneumocystis jirovecii* can be classified as follows:



3.6.2.1. Direct methods:

Direct method comprises different staining techniques which stain different morphological forms of *P.jirovecii,* and classified as follows:



Grocott-Gomori methenamine silver (GMS) staining:

Principle:

It is a silver staining method which stains the cyst wall. The principle of this stain is that the mucopolysaccharide components of the fungal cell wall are oxidized to release aldehyde groups. The aldehyde groups then react with the silver nitrate, reducing it to a metallic silver, rendering them visible. Gold salt stabilizes the complex and sodium thiosulfate wash will remove the excess silver.

Procedure (30)

- Slide is microwaved for 40 seconds in a 10% chromic acid solution.
- It is then rinsed with water followed by 1% sodium metsbisulfite for 30 seconds.
- The slide is kept in a Coplin jar containing 50ml of working methenamine solution and microwaved for 65 seconds.
- The slide is rinsed with water and treated with 1% gold chloride for 2 to 5 seconds.
- It is then rinsed with water and treated with 5% sodium thiosulfate.
- Counter staining is carried out with light green working solution, following which it is rinsed in xylene
- Coverslip is applied and examined under light microscopy.

Appearance:

Cysts are distinctly stained with black or brown colour with a typical cup shaped morphology against a green background. In some instances, cyst wall thickening gives "double comma" appearance. Dark brown to black staining of the folds gives a "crinkled raisin-like" appearance. The intra cystic daughter form cannot be visualised, thus empty cysts appear similar to cysts with all eight spores (19).

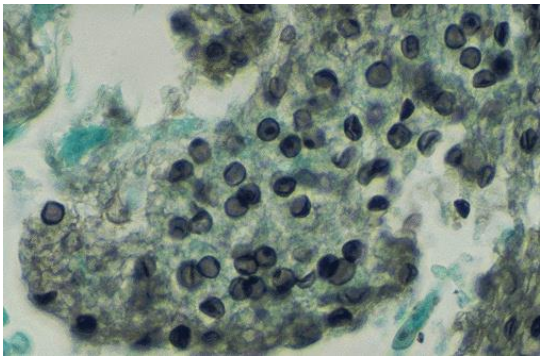


Fig. 3.5 showing cysts of *Pneumocystis jirovecii* in BAL, stained with GMS stain (adapted from reference 30)

Advantages (19)

i. Cysts can be easily detected
ii. Host cells are not stained

Disadvantages (29,31)

i. Trophozoites cannot be identified
ii. Expensive
iii. Time consuming
iv. Requires skilled personnel
v. High background staining

Toludine blue O (TBO) staining:

Principle:

This is a cyst wall staining method, based on the principle of "metachromasia". Toludine blue is an acidophilic metachromatic dye, has affinity for tissues with high content of DNA and RNA, and DNA stains purple with a blue background (32).

Procedure (33)

- The smear is treated with sulfation reagent (combination of glacial acetic acid and sulphuric acid) in a Coplin jar for 10 minutes.
- It is then rinsed in cold running tap water for 5 minutes.
- Slide is kept in toluidine blue O stain for 3 minutes.
- The smear is decolourised with 95% ethyl alcohol and absolute alcohol for 10 seconds each.
- Coverslip is applied with permount.
- The slide is examined under 20X, and 40X objective in light microscopy.

Appearance:

Here, cysts appear similar to those stained by the GMS stain, but that the colour of the cyst is light purple or lavender in colour against blue background. This will stain other yeasts and fungal elements also.

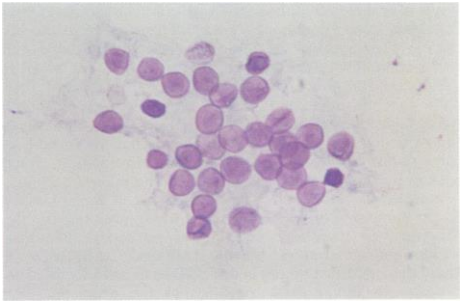


Fig. 3.6 showing cysts of *Pneumocystis jirovecii* in BAL, stained with TBO stain (adapted from reference 31)

Advantages (19)

i. Cysts can be easily detected

ii. Host cells are not stained

iii. Faster than silver staining method (20 minutes)

Disadvantages (19,29)

i. Mixture of sulphuric acid and glacial acetic acid is required for the procedure. As both are noxious substances, requires a fume hood
ii. Yeasts can be mistaken for *Pneumocystis* cyst
iii. Stains only cysts, other forms cannot be identified

Calcofluor white (CFW) staining:

Principle:

Calcofluor white is a fluorescent whitener or fluorescent brightener. It has an active ingredient, Cellufluor, which has the ability to bind non-specifically with cellulose and chitin of the fungal cell wall. When exposed to UV light, it fluoresces.

Procedure (30)

- One drop of Calcofluor stain is added to the sample on a clean glass slide.
- Coverslip is applied and is kept in dark humidifying chamber at room temperature for 10 minutes
- Examined under a fluorescence microscope

Appearance:

The cyst is stained brightly against a dark background, often with characteristic "double parenthesis" or "body within the cyst" appearance. It produces a yellow green or apple green fluorescence at 420 to 490 nm with suppression filter of 515 nm, and a fluorescent blue colour at 340 to 380 nm with suppression filter of 430 nm (19,34).

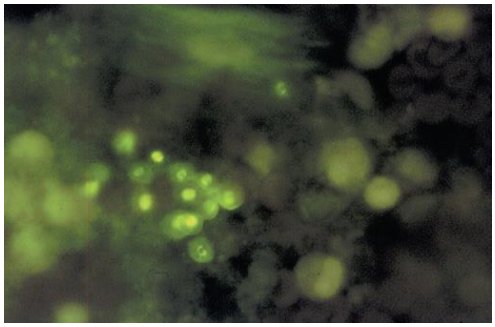


Fig. 3.7 showing cysts of *Pneumocystis jirovecii* in BAL, stained with Calcofluor white (adapted from reference 34)

Advantages:

i. Technically simple procedure
ii. Inexpensive
iii. Cysts fluoresce brightly.

Disadvantages:

i. Fluorescence microscope is required
ii. It will stain other fungal elements also and requires expertise
iii. Forms other than cysts cannot be visualised.

Giemsa staining:

Principle:

Giemsa stain is a type of Romanowsky stain, containing both eosin Y (acidic dye) and oxidised methylene blue, azure B (basic dye). Eosin Y stains basic organelles of the cell such as cytoplasm, whereas azure B stains acidic organelles such as nucleus.

Procedure:

- The smear is fixed with methanol
- The slide is immersed in a Petri dish containing Giemsa stain for 30 minutes.
- The slide is washed with tap water
- Examined under an oil immersion objective.

Appearance:

This does not stain the cyst wall, but stains nuclei of all morphological forms in the life cycle a reddish purple, while the cytoplasm stains light blue. Clusters of trophic forms give "mat appearance". The cyst wall appears as a clear circumscribed zone around a reddish purple nuclei. This will stain lung cells also, but the nuclei appears larger and deep reddish purple (19).

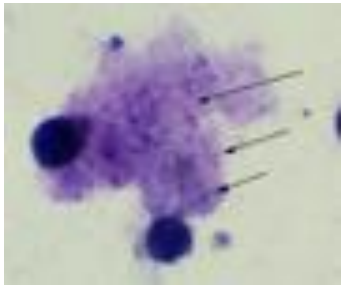


Fig. 3.8. Showing trophic forms of *Pneumocystis jirovecii* in BAL, stained with Giemsa stain (adapted from CDC DPDx)

Advantages:

i. Simple technique, that can be adapted in all kind of laboratories
ii. Inexpenisve
iii. Stains all stages of *Pneumocystis* life cycle
iv. Sensitivity is expected to be high because of trophic forms which are ten times more than cysts, while other stains do not detect the former.

Disadvantages:

i. Requires an experienced person to distinguish between *Pneumocystis* trophic cluster from host cells
ii. High background staining

Diff-Quik staining:

It is a modifed Wright Giemsa stain or rapid Giemsa like stain. It is a rapid staining technique that takes very few minutes to perform. The principle is same as the Giemsa stain (35).

Procedure:

- The smear is air dried, and dipped in fixative (Fast green in methanol) for 30 seconds
- It is then dipped in Diff-Quik stain I (Eosin G in phosphate buffer) for 30 seconds
- Counterstaining is carried out with Diff-Quik stain II (Thiazine dye in phosphate buffer) for 30 seconds.
- It is then rinsed with tap water and dehydrated in absolute alcohol.
- Mounted under a light microscope.

Appearance:

The Diff-Quik staining is a very good method for detection of alveolar casts. The Presence of alveolar casts in BAL indicates active *Pneumocystis* infection. Thus, Diff-Quick staining is useful particularly for this sample.

Fig. 3.9 Showing alveolar casts of amphophilic, amorphous material in honeycomb pattern of *Pneumocystis jirovecii* stained by Diff-Quik stain,

Advantage and disadvantages are same as Giemsa staining, except that it takes less time i.e. less than five minutes to perform staining.

Papanicolaou (Pap) staining:

It is a routine cytopathological staining method. Since it can stain the trophic form, it is used to stain *Pneumocystis.*

Appearance:

It stains clusters of extra cellular organisms a green colour, but thick clusters of organisms appear bicolored.

Advantage:

i. Stain is commonly available in all cytopathological lab

Disadvantages:

i. Trophic forms are stained faintly
ii. Better stains are available

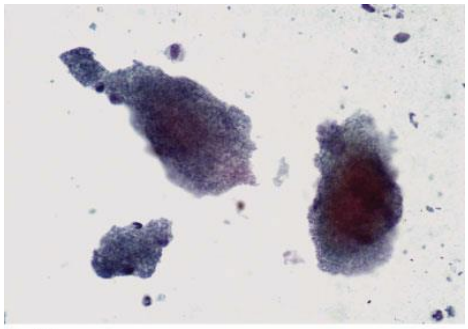


Fig. C-2

Fig.3.10 Showing trophic form of *Pneumocystis jirovecii* in BAL, stained with Pap stain (adapted from reference 30)

6.2.1.7. Direct immunofluorescent antibody test:

Principle:

It is an antigen detection method by direct immunofluorescence technique. Here fluorescein isothiocyanate (FITC) labelled monoclonal antibodies are used against a family of surface glycoprotein antigen present on cyst wall, trophozoite and sporozoite to identify all forms of *Pneumocystis.*

Procedure:

- The smear is fixed with acetone
- Detection reagent containing FITC labelled monoclonal antibodies are added to sample well.
- The slide is incubated at 37°C in moist chamber for 30 minutes.
- It is then rinsed with water.
- Mounting fluid is added and cover slip is applied
- Examined under a fluorescence microscope under 20X and 40X magnification.

Appearance:

It stains the cyst, trophozoite and other forms also and fluoresces apple green. Contents of the cyst are unstained and stand out black or dull. Some times folds of cyst wall are prominent and gives a crinkled, raisin like appearance. Staining of clusters gives a diffuse green glow, within which trophic forms appear as polygons or small spheres (19).

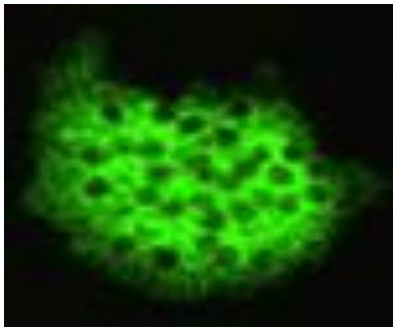


Fig.3.11 Showing typical honey comb pattern stained with DFA (adapted from CDC DPDx)

Advantages:

i. Highly sensitive and specific, since it can detect all forms
ii. Very simple and rapid technique

Disadvantages:

i. Requires fluorescence microscope
ii. Reagents are costly, cannot be implemented in resource poor laboratories
iii. Shelf life of reagent is short.

3.6.2.2. Molecular methods:

Microscopic methods have many challenges. Their sensitivity and specificity is dependent on various factors like, type of specimen either invasive or non-invasive, burden of organisms in the specimen examined, staining methodology, use of

prophylactic treatment before collection of specimen and the number of specimens examined from an individual (36).

Induced sputum is routinely employed as it is non-invasive and involves a simple procedure. It has low number of organisms compared to other invasive samples like BAL and trans-bronchial biopsy.

The low burden of organisms seen in patients who had taken prophylactic or therapeutic treatment before collection of sample. This further reduces the sensitivity of microscopic methods.

Many studies compared the different staining techniques giving variable rate of sensitivity and specificity for each staining as well as each type of samples. So we cannot rely on a single microscopic staining method for diagnosis.

Molecular methods using polymerase chain reaction (PCR) might be an alternative technique to overcome the disadvantages of staining techniques. The first conventional PCR for *Pneumocystis* was developed by Wakefield *et al* in 1990 (37). Many PCR assays have been developed in the past two decades, using different targets such as mtLSU rRNA, DHPS, DHFR, ITS, 5S rRNA, 18S rRNA, and cdc2. Among these, the PCR assays targeting mitochondrial 23SrRNA region in mtLSU rRNA gene, showed high sensitivity and specificity (38).

Further, sensitivity of conventional PCR was increased by nested PCR. Contamination is a significant problem with nested PCR, as double runs are performed in an open system. Thus, high false positivity rate and low specificity is seen with this technique (39).

Although conventional PCR was able to detect low burden of organisms, its inability to discriminate colonization from active infection made researchers move towards the real time quantitative PCR (qPCR). The first quantitative PCR for *Pneumocystis* was developed by Larsen *et al.* in 2002 (40). After that many studies done on qPCR showed that colonization has significantly lower number of organisms than active PCP infection (41–44).

Advantages of real time PCR (39):

i. Rapid: When compared to conventional PCR, as there is no post PCR processing step.
ii. Less contamination: Since amplification and detection occurs in a closed tube.
iii. Quantification of the amplified product: As the analysis is performed in the early log phase of product accumulation.

6.2.3. Serological methods:

Serological tests available for diagnosis of *Pneumocystis* are;

i. Beta-D glucan assay
ii. Serum lactate dehydrogenase (LDH) level
iii. S-adenosylmethionine level
iv. KL-6 level
v. Antibody detection by ELISA

Beta-D glucan assay:

1, 3 β-D glucan (BG) is a major structural component of cell wall of all pathogenic fungi including *P.jirovecii.* There are four kits available to perform serum β-D glucan level. The Fungitell BG assay is a chromogenic kinetic test (Associates of Cape Cod, East Falmouth, MA) was approved by the U.S. Food and Drug Administration in 2003 for presumptive identification of invasive fungal infection (IFI) (45). These studies showed that serum β-D glucan levels are elevated in active PCP cases as well as decreased with effective anti-pneumocystis treatment. BG assay has very good sensitivity and specificity of 92% and 86% respectively with 31.1pg/ml as a cut off (46). Specificity is moderate, as β-D glucan is present in other fungi also. Onishi A *et al* in a systematic review recommends that (47);

i. This BG assay can be used as a screening test for PCP because it has high sensitivity for PCP rather than IFI, which will avoid the need of invasive samples for microscopy.
ii. As this assay has moderate specificity, if test comes positive, factors like other fungal infections, use of intravenous amoxicillin clavulanic acid, treatment of patients with immunological preparations (albumins or globulins), use of cellulose membranes and filters made from cellulose in haemodialysis, and use of cotton gauze swabs/packs/pads and sponges during surgery should be ruled before taken it as a clinically significant result.
iii. It is useful in non-HIV infected patients also as there are no significant difference in the results between HIV positive and negative patients.

Serum lactate dehydrogenase (LDH) level:

LDH is a non-specific marker for the diagnosis of PCP. It is a cytoplasmic enzyme, present in all organs. Extracellular appearance of LDH indicates organ damage. In PCP it is well correlated with the degree of lung damage.

Though it has 100% sensitivity in HIV positive PCP patients, it has a limited specificity of 45%, as LDH can be elevated in other disorders like hepatic, hematologic, and neoplastic disorders. As it has low sensitivity of 67% in non-HIV infected immunocompromised patients, serum LDH level cannot be used as a screening test (48,49).

Serum S-adenosylmethionine level:

S-adenosylmethionine (AdoMet) is a universal methyl donor, synthesised from methionine and ATP by S-adenosylmethionine synthetase enzyme. It is an important intermediate involved in methylation reaction and polyamine synthesis.

Pneumocystis obtain AdoMet from host cell as they lack enzymes to synthesise it. Hence there is a decreased level of serum AdoMet in PCP patients. (50, 51). Though it is a sensitive and specific marker for PCP in HIV infected individuals, it has limited value in non-HIV infected immunocompromised patients (51).

Serum KL-6 level:

KL-6 is a mucin like glycoprotein present on type II pneumocytes and bronchiolar epithelial cells. Serum KL-6 levels are elevated in interstitial lung disease and severe lung disease. Many authors have suggested that elevated KL-6 level could be a serological marker for PCP. But these levels are elevated in other non-fungal respiratory infections like *Legionella* pneumonia, severe pulmonary tuberculosis, and RSV bronchiolitis also. Therefore it cannot be used as a specific marker for *Pneumocystis* as β-D glucan (52).

Antibody detection by ELISA:

In general serum antibody detection has limited value in immunocompromised patients, especially in HIV positive patients (53). *Pneumocystis* specific IgG can be detected by ELISA using recombinant major surface glycoprotein antigen. As healthy individuals also have significant levels of antibody, this cannot be used for routine diagnosis, but may be used for epidemiological studies (54).

3.7 Treatment:

Treatment of patient is based on the clinical severity of the disease.

The PCP is classified into mild, moderate and severe based on following criteria (Table 3.1) (55):

Table. 3.1 Classification of PCP

	Mild	Moderate	Severe
Symptoms and signs	Dyspnoea on exertion with or without cough and sweats	Dyspnoea on minimal exertion and occasionally at rest with cough and fever	Dyspnoea and tachypnoea at rest with persistent cough and fever
Arterial oxygen tension at rest (PaO2)	>82.7 mmHg	60 – 82.7 mmHg	< 60 mm Hg
Arterial oxygen saturation at rest	>96%	91 – 96%	<91%
Chest radiography	Normal or minor perihilar shadowing	Diffuse interstitial infiltration	Extensive interstitial infiltration with or without diffuse alveolar shadowing

Table 3.2 shows the summary of first and second line anti-PCP treatment (for 21 days) (56).

Table. 3.2 Anti-PCP treatment

Disease classification	First line drug	Second line drugs	TMP-SMX failure
Moderate to severe	Trimethoprim-sulfamethoxazole (TMP-SMX) intravenously four times daily	1. Clindamycin-Primaquine orally 2. Pentamidine intravenously	1. Substitute or add intravenous Pentamidine 2. Substitute Trimetrexate + leucovorin
Mild to moderate	TMP-SMX orally four times daily	1. Nebulised Pentamidine 2. Clindamycin-Primaquine orally 3. Atovoquone orally 4. Dapsone + TMP orally	1. TMP-SMX intravenously four times daily 2. Pentamidine intravenously

Prophylaxis:

Prophylactic treatment is recommended in HIV positive patients with CD4 counts less than 200/$mm^{3.}$ The drug of choice is Trimethoprim Sulphomethoxazole (TMP-SMX) one tablet daily (TMP 80 mg/ SMX 400 mg) or two tablets thrice weekly, which will give protection for toxoplasmosis also. Alternatives are nebulised Pentamidine, Atovoquone, and Dapsone-TMP combination (24).

In non HIV immunocompromised patients, prophylaxis with TMP-SMX should be considered carefully, as it causes hepatotoxicity and bone marrow depression. Prophylaxis is recommended for renal transplantation patients because 5 to 33 % of mortality rate was reported due to PCP without prophylaxis. It is also recommended in Wegener's granulomatosis patients as incidence of PCP is more in these group of patients (23).

Emerging resistance

TMP-SMX is an effective drug for treatment as well as prophylaxis. But resistance is reported due to mutation in the *Pneumocystis* DHFR, DHPS gene which is the target for TMP-SMX and leads to treatment failure (21). High prevalence rate of DHPS gene mutation is reported in developed countries than in developing countries i.e 72% in USA, 56% in Africa, 6.2% in India (57).

4. Materials and methods

4.1. Study design:

This was a prospective study of diagnostic test accuracy for a period of one year, conducted in the Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore.

In this study, respiratory samples were collected from adult immunocompromised patients with respiratory illness and PCP as one of the differential diagnosis. All samples were subjected to conventional microscopic methods such as Giemsa staining, direct fluorescent antibody test and the molecular diagnostic method - real time quantitative PCR assay.

According to EORTC (European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group) clinical criteria, patients were categorized into definite PCP, probable PCP and non PCP based on symptoms, signs, radiological features, and response to anti-pneumocystis treatment. Results of the molecular test was compared against the EORTC clinical criteria.

4.2. Ethics approval:

The approval for the study was obtained from the Institutional Review Board, CMC, Vellore (IRB Min No: 8824 [DIAGNOSE] dated 07.04.2014).

4.3. Study duration:

The study was conducted over a period of one year from June 2014 to May 2015.

4.4. Study samples:

Respiratory samples like induced sputum, endotracheal aspirate (ETA), and Broncho alveolar lavage (BAL) sent for routine diagnosis of PCP by DFA test were taken for this study.

The Department of Clinical Microbiology is an NABL Accredited laboratory. It has different sections handling different kinds of samples. More than 6 lakhs samples are processed per year. Mycology section receives more than 6000 samples a year, of which 150 to 200 samples per year are received are for *Pneumocystis jirovecii* DFA test.

The Department of Infectious Diseases and Research Centre (IDRC) in CMC Vellore conducts HIV clinic thrice a week. They see more than 4000 patients in a year. Voluntary Counselling and Testing Centre (VCTC) which is part of their department does HIV testing and pre and post-test counselling to 1400 to 1500 patients a year of which around 170 to 200 patients are found to be HIV positive.

The Department of Haematology in CMC, Vellore sees 1200 new out patients every year and admits about 700 patients every year for inpatient care. These patients come from all over India and cover the whole spectrum of blood diseases. They perform 110 allogenic bone marrow transplantation per year.

4.5. Sample size calculation:

Due to the paucity of data on the prevalence of PCP in India, sample size could not be calculated, and a pilot study was conducted.

Thirty (30) HIV infected and seventy (70) non-HIV infected immunocompromised patients with suspected PCP were enrolled in this study as per the inclusion criteria**.**

4.6. Inclusion criteria:

i. Respiratory samples from adult immunocompromised patients with symptoms and signs suggestive of PCP.
ii. Only one sample from each patient was included, if multiple samples were sent from the same patient.

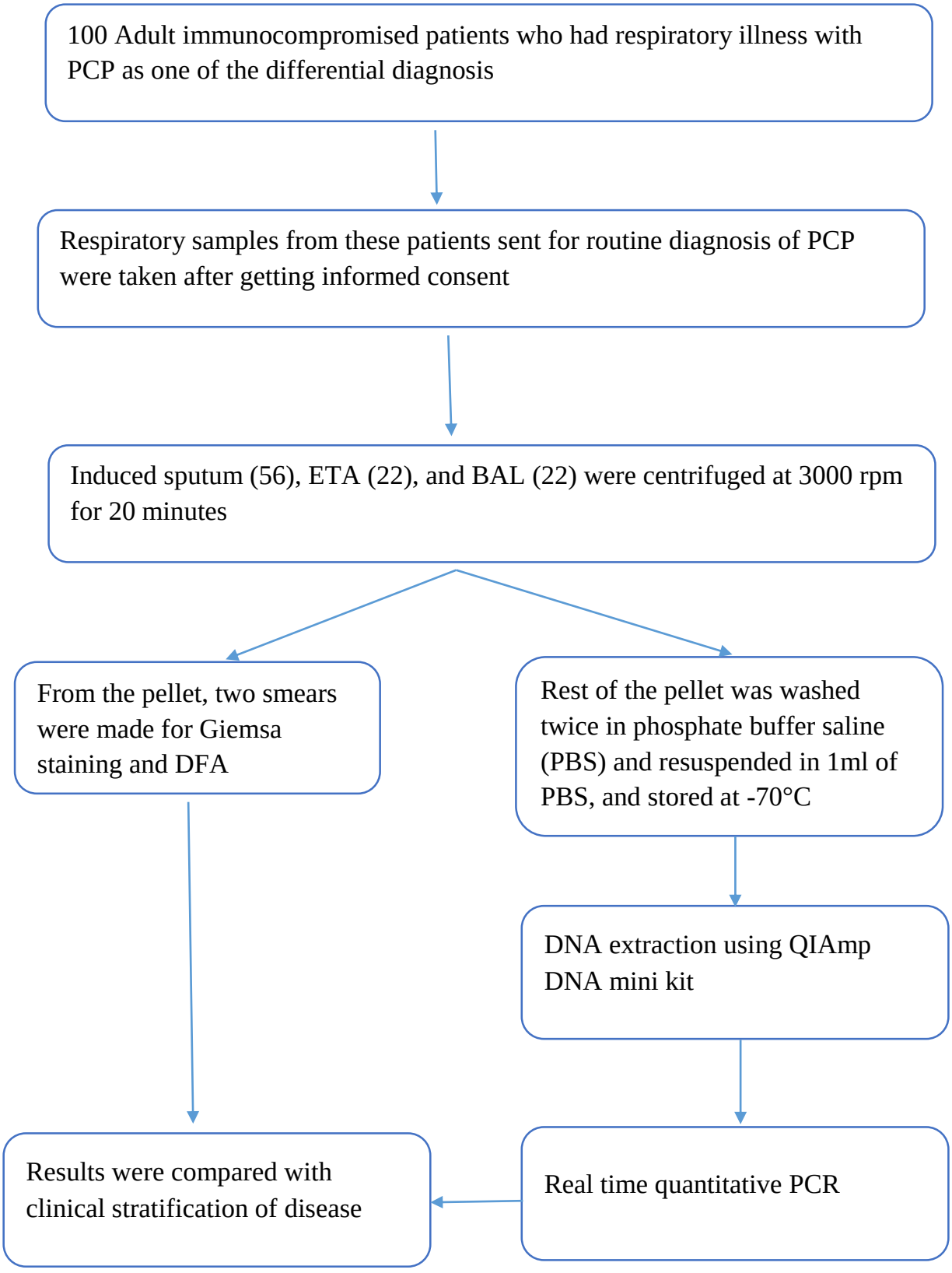
4.7. Exclusion criteria:

i. Samples from non-immunocompromised patients were excluded.
ii. Unsatisfactory samples such as samples with excessive salivary contamination were excluded.
iii. Samples from paediatric patients who were less than 18 years were also excluded.

4.8. Data source:

Relevant clinical information like age, sex, admitted unit, HIV status, CD4 counts if HIV positive, cause for immunosuppression, cardinal symptoms like fever, dry cough, progressive dyspnoea, PaO2 value, radiological features, and treatment history were collected from patient's clinical record. Testing of the study samples were done in the department of Clinical Microbiology.

4.9. Study algorithm:



4.10. Processing of study samples:

A. Induced sputum:

Purulent material from the induced sputum was taken into a test tube and equal amount of freshly prepared 1% dithiothreitol (DTT) was added and incubated at room temperature for 30 to 45 minutes with intermittent vortexing. Then centrifuged at 3000rpm for 20 minutes. The supernatant was discarded and two smears were made from the deposit, one for Giemsa staining and one for DFA.

B. BAL and ETA:

BAL and ETA were centrifuged at 3000 rpm for 20 minutes and the supernatant was discarded. Two smears were made from the deposit, one for Giemsa staining and one for DFA.

C. For real time quantitative PCR (qPCR):

For qPCR assay, the pellet (deposit) was washed twice in phosphate buffer saline (PBS) and resuspended in 1ml of PBS. This was aliquoted into two separate vials and stored at -70°C.

4.11. Giemsa staining procedure:

i. Smear was fixed by methanol.

ii. Slide was immersed into a Petri dish containing Giemsa stain for 30 minutes.

iii. Then the slide was washed under slow running tap water.

iv. The smear was screened under oil immersion objective.

4.12. Direct fluorescent antibody test procedure:

i. Smear was fixed by acetone.

ii. 50µl of detection reagent (MERIFLUOR) containing FITC labelled monoclonal antibodies against cell wall and matrix antigens of *P. jirovecii* cysts, sporozoites and trophozoites was added to sample well.

iii. Slide was incubated at 37ºC in a moist chamber for 30 minutes.

iv. Stain was removed by washing under slow running tap water.

v. The slide was immersed in a Coplin jar containing tap water, and agitated in between for 7-10 minutes.

vi. Mounting fluid was added to the smear and cover slip applied.

vii. The slide was screened under fluorescence microscope at 20X and 40X magnification**.**

4.13. Real time quantitative PCR assay:

Principle:

Polymerase chain reaction amplifies a specific target region of the template DNA strand. In real time quantitative PCR assay, PCR product is measured at each cycle in real time by fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of amplicons produced.

4.13.1. DNA extraction:

A. Materials required:

- QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) containing:

  Proteinase K

  Lysis buffer AL

  Wash buffer 1 AW1

  Wash buffer 2 AW2

  Elution buffer AE

  QIAamp Mini Spin Columns

  Collection Tubes (2 ml)

- Ethanol
- Nuclease free water
- Dry bath (temperature to be set at 56°C)

B. Procedure:

DNA extraction was performed in the 'PCR dirty' room as per manufacturer's instructions.

i. 20µl Qiagen Proteinase K was taken in the bottom of a 1.5 ml micro centrifuge tube.

ii. 200µl resuspended pellet was added to the micro centrifuge tube.

iii. Then, 200µl Buffer AL (lysis buffer) was added to the sample in the same tube.

iv. These substances were mixed well by pulse vortexing for 15 seconds.

v. The tube was incubated at 56°C for 10 minutes.

vi. After this, the tube was briefly centrifuged to remove the drops from inside the lid.

vii. 200µl of absolute alcohol was added to the tube.

viii. Again, it was mixed well by pulse vortexing for 15 seconds.

ix. Then, the tube was briefly centrifuged to remove the drops from inside the lid.

x. The mixture was placed in the QIAamp spin column with a 2 ml collection tube without wetting the rim and the cap was closed.

xi. The spin column was centrifuged at 8000 rpm for 1 minute.

xii. The QIAamp spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.

xiii. The QIAamp spin column was carefully opened and 500µl Buffer AW1 (Wash buffer) was added to it without wetting the rim and the cap was closed.

xiv. It was centrifuged at 8000 rpm for 1 minute.

xv. The QIAamp spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.

xvi. The QIAamp spin column was carefully opened and 500µl Buffer AW2 (Wash buffer) was added without wetting the rim and the cap was closed.

xvii. It was centrifuged at full speed of 14,000 rpm for 3 minutes.

xviii. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded.

xix. The QIAamp spin column was carefully opened and 100µl Buffer AE (eluting buffer) was added to it.

xx. The tube was incubated at room temperature for 1 minute.

xxi. It was then centrifuged at 8000 rpm for 1 minute and the spin column was discarded.

xxii. The 1.5 ml centrifuge tube contained DNA which was labelled and stored at -70°C in four aliquots.

4.13.2. Quantification of *Pneumocystis*:

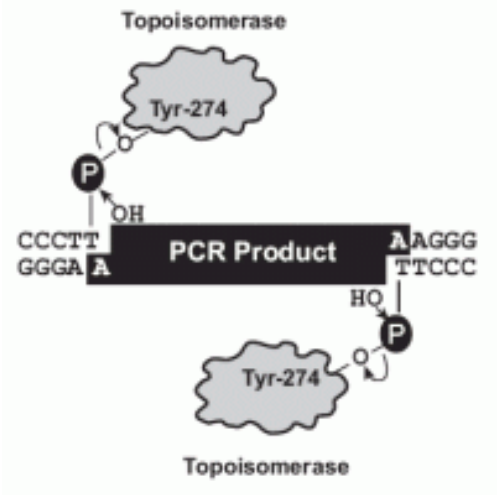
Plasmids used for quantification were produced by cloning the PCR amplified product into the PCR-TOPO TA vector (TOPO® TA Cloning® kit, Invitrogen, Carlsbad, CA, USA). Nano drop method used for quantification of plasmids. Standard curve was generated by the amplification of a tenfold dilution series of a plasmid standard (2×10^9 to 2×10^5 copies/reaction). Quantification was performed using ABI (Applied Bio system) 7500 Fast PCR system software by extrapolation of data to standard curves.

4.13.2.1. Cloning:

Cloning was done with TOPO TA Cloning® Kit (with pCR®2.1-TOPO® and PureLink™ Quick Plasmid Miniprep Kit)

Principle:

Taq polymerase used in PCR has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in TOPO TA Cloning® kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.



Primers for cloning:

Primers for cloning were designed by online Primer 3 software (Version 0.4.0). These primers amplifies 700-bP fragment of *Pneumocystis jirovecii* mitochondrial large subunit rRNA (mtLSU rRNA) gene which contained the segment (120-bp) used for qPCR assay.

The lyophilised primers used for the cloning were reconstituted in 1X Tris EDTA (TE) buffer and stored as single use aliquots at -20°C. The primer sequences were as follows:

Primers	Sequence
PjcF	5’-TCGGCGAATAGGATTTTCAC-3’
PjcR	5’-TTGCATAATGGGTCAGCAAG-3’

Cloning procedure:

A. **Production of PCR products:**

This was done with two known DFA positive samples.

The concentrations of the different components of the PCR mix for one reaction are given below:

Reagents	**For one reaction**
Hot start mix	12.5µl
PjcF primer	0.5 µl (20µM concentration)
PjcR primer	0.5 µl (20µM concentration)
Water	9.0 µl
Extracted DNA	2.5 µl
Total volume	25 µl

A conventional PCR method was carried out in Gene-AMP PCR system 9700 (Applied Biosystem).

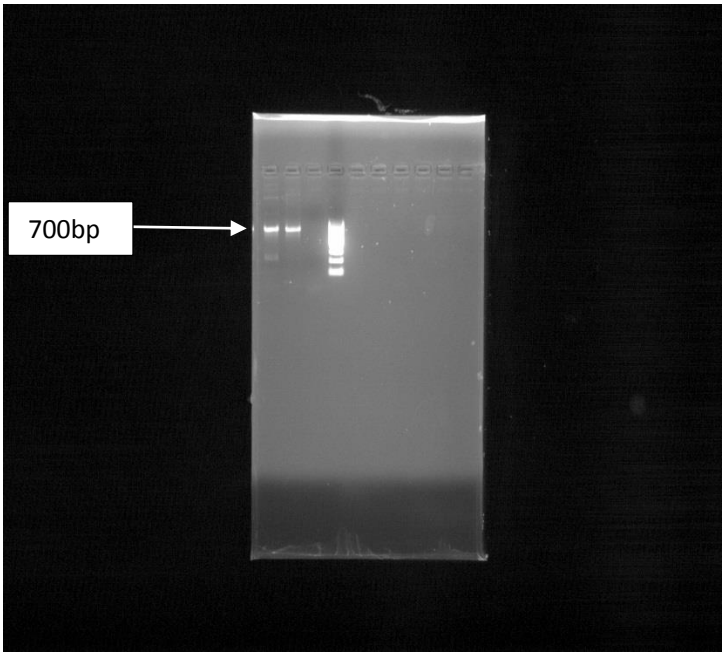
The cycling conditions were as follows;

Initial holding at 95°C for 15 minutes

Denaturation at 94°C for 30 seconds
Annealing at 52°C for 30 seconds
Extension at 72°C for 1 minute
} - 35 cycles

Final extension at 72°C for 1 minute

Amplicons were checked by 2% agarose gel electrophoresis and it showed a single discrete band at 700bp.



B. **Per formation of TOPO Cloning reaction:**

TOPO cloning reaction was set up with following reaction mix:

Reagent	**Volume**
Fresh PCR product	4 µl
Salt solution	1 µl
TOPO vector	1 µl
Final volume	6 µl

Reaction was gently mixed and incubated at room temperature for 5 minutes.

C. **Transformation of one shot competent cells:**

Requirements for transformation:

LB (Luria-Bertani) plates containing 50 µg/mL ampicillin

S.O.C. medium (supplied with the kit)

40 mg/ml X-gal in dimethylformamide (DMF)

42°C water bath

37°C shaking and non-shaking incubator

LB medium preparation:

Ingredients:

Agar - 1.5g

Tryptone - 1.0g

Yeast extract - 5.0g

Sodium chloride - 1.0g

De-ionized water - 95ml

pH - 7 (adjusted by using 1N NaOH)

Ingredients were mixed well by heating, autoclaved and cooled to 55°C. To this ampicillin 50 µg/ml was added.

100 ml of LB broth was prepared without agar.

Transformation Procedure:

i. 2µl TOPO cloning reaction was added into a vial of one shot chemically competent *E.coli* and mixed gently (not by pipetting up and down).
ii. Reaction was incubated on ice for about 30 minutes.
iii. The cells were heat shocked for 30 seconds at 42°C without shaking.
iv. The tube was transferred immediately to ice.
v. 250 µl of S.O.C medium was added (The medium was brought to room temperature before the procedure)

vi. The tube was capped tightly and kept in the shaker at 200 rpm speed in 37°C incubator for 1 hour.

vii. 40 µl of X-gal was overlayed on LB plate (containing 50µg/ml ampicillin) to differentiate transformed (white colonies) from non-transformed, self-ligated plasmid colonies (Blue colour).

viii. 50 µl of transformation was spread on prewarmed (37°C) LB agar.

ix. LB agar plate was incubated at 37°C for 24 hours.

D. **Analysis of transformants:**

Approximately two to four white colonies were taken from the plate and inoculated into LB broth containing ampicillin 50 µg/ml and incubated at 37°C overnight. Extraction of plasmid was carried out from the LB broth using PureLink Quick Plasmid DNA Mini Prep Kit (given along with TOPO TA Cloning® Kit).

Plasmid extraction:

The PureLink Quick Plasmid DNA Mini Prep Kit was designed to isolate high quality plasmid DNA up to 30 µg from *E.coli* cells in 30 to 45 minutes.

Principle:

Cells are lysed by alkaline substances and the lysate applied to silica membrane column that selectively binds plasmid DNA. Contaminants are removed by wash buffers and plasmid DNA is eluted in TE buffer.

The PureLink Quick Plasmid DNA Mini Prep Kit containing

Resuspension buffer

RNase A

Lysis buffer

Precipitation buffer

Wash buffer W9 and W10

TE buffer

Wash and recovery tubes

Spin column

Procedure:

i. 5 ml of overnight LB broth was centrifuged to sediment the cells
ii. 250 µl of resuspension buffer with RNase A was added to the cell pellet and the pellet was resuspended until it was homogeneous
iii. To it 250 µl of lysis buffer was added and mixed gently by inverting the capped tube five times. This was incubated at room temperature for 5 minutes.
iv. 350 µl of precipitation buffer was added and mixed immediately by inverting the tube until the mixture was homogeneous. The lysate was centrifuged at 14000 rpm for 10 minutes
v. Supernatant from the tube was added to the spin column in a 2 ml wash tube and the column was centrifuged at 14000 rpm for 1 minute
vi. 500 µl of wash buffer (W10) was added to the column and incubated at room

temperature for 1 minute, then the column was centrifuged at 14000 rpm for 1 minute. Flow through was discarded and the spin column was placed in a new wash tube

vii. 700 µl of wash buffer (W9) was added and the column was centrifuged at 14000 rpm for 1 minute. Flow through was discarded and the spin column was placed in a new wash tube. The column was centrifuged at 14000 rpm for 1 minute. Wash tube with flow through was discarded.

viii. Spin column was placed in a clean 1.5ml recovery tube. 75 µl of TE buffer was added to the centre of the column and incubated at room temperature for 1 minute.

ix. The spin column was centrifuged at 14000 rpm for 2 minutes and the column was discarded. The recovery tube, which contained purified plasmid DNA, was aliquoted and stored at -20°C

E. **Analysis of plasmid:**

Extracted plasmid DNA was analysed by two methods.

i. The first method was by conventional PCR using cloning primers, with same reaction mix formula and cycling condition as described above. The PCR product was analysed by 2% agarose gel electrophoresis and there was a discrete band at 700 bp.

ii. The second method was another conventional PCR using M13 Primers provided with the kit for sequencing.

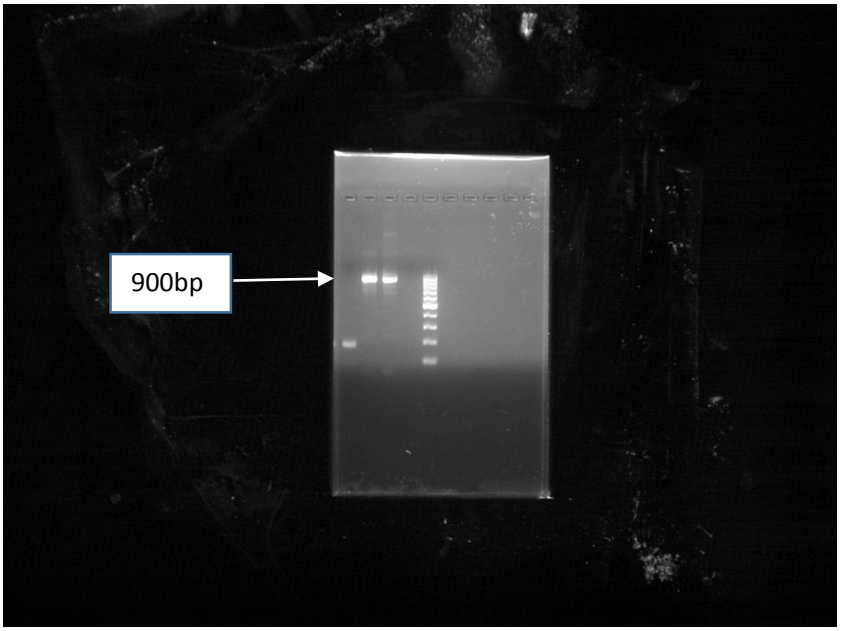
The primer sequences were as follows:

Primer	**Sequences**
M13 Forward	5’-GTAAAACGACGGCCAG-3’
M13 Reverse	5’-CAGGAAACAGCTATGAC-3’

The concentrations of the different components of the PCR mix for one reaction are given below:

Reagents	**For one reaction**
Hot start mix	25 µl
M13 Forward primer	1 µl
M13 Reverse primer	1 µl
Water	18 µl
1/10 diluted plasmid DNA	5 µl
Total volume	50 µl

The cycling conditions were as described above for cloning and the PCR product was analysed by 2% gel electrophoresis. There was a discrete band at 900bp.



F. **Quantification of plasmid DNA:**

Quantification of plasmid DNA was done by Nano drop method.

Undiluted plasmid was containing 114.3 ng/µl of DNA.

Copy numbers were calculated by "URI genomics & sequencing centre calculator for determining the number of copies of a template" from "cels.ur.edu/gsc/cndna.html" website.

The formula used is:

number of copies = (amount * 6.022x1023) / (length * 1x109 * 650)

Copy numbers in the undiluted plasmid was found to be **2.29×10^{10} copies.**

4.13.3. Standardization of real time quantitative PCR assay:

The assay was standardised by running PCR assay several times with different concentration of probe i.e. 0.2 µM, 0.1 µM and different temperature annealing and extension such as 50°C, 52°C, 54°C, 56°C and 60°C. We got a good sigmoidal curve at 54°C with 5 µM concentration of probe. So we finalised that temperature and concentration.

4.13.4.Real time quantitative PCR assay procedure for *Pneumocystis jirovecii*:

Primers for qPCR:

The lyophilised primers and probes used for the qPCR were reconstituted in 1X Tris EDTA (TE) buffer and stored as single use aliquots at -20°C. The primer and probe sequences were as follows:

Primers	**Sequence**
PjF1	5′-CTGTTTCCCTTTCGACTATCTACCTT-3′
PjR1	5′-CACTGAATATCTCGAGGGAGTATGAA-3′
PjP1	5′- FAM-TCGCACATAGTCTGATTAT-NFQMGB-3′

The primers target 121-bp fragment of *P.jirovecii* mitochondrial large subunit rRNA (mtLSUrRNA) gene (13).

Master Mix preparation:

The concentrations of the different components of the PCR mix for one reaction are given below:

Reagents	**For one reaction**
TaqMan Universal Master mix (Applied Biosystems)	12.5 µl
PjF1	1.0 µl (0.4µM concentration)
PjR1	1.0 µl (0.4µM concentration)
TaqMan-MGB probe PjSL	0.5 µl (0.2µM concentration)
Extracted DNA	10 µl
Total volume of reaction	25 µl

Procedure for amplification:

i. The master mix was prepared in the clean room or 'DNA-free' room for the appropriate number of reactions with the above template.
ii. A 96 well PCR reaction plate was taken, 15 µl master mix was added to each well.
iii. DNA extracts were removed from the storage area, brought to room temperature.
iv. 10 µl of DNA was added to the well according to the template made and the final volume was 25 µl. Addition of DNA was done in the 'dirty room'.
v. Nuclease free water was used as negative control after every three samples.

vi. In each cycle five standards (1×10^8 to 10^4) and one positive control were used.

vii. Amplification reactions were carried out in ABI 7500 Fast PCR system.

viii. The cycling conditions were:

Initial holding temperature at 95^0C for 10 minutes.

Denaturation at 95^0C for 15 seconds

Annealing and extension at 54^0C for 1 minute

Total number of cycles are 45.

PCR analysis:

PCR analysis was done with ABI 7500 Fast PCR system software.

Determination of the limit of detection of *Pneumocystis jirovecii* qPCR:

The lower limit of detection of the assay was determined by testing of serial dilutions of the plasmid standards which used for quantitation. The undiluted plasmid standard contained 2×10^{10} copies/ml. The standards were serially diluted so as to obtain 1×10^{-1} to 1×10^{-10} dilutions of undiluted plasmid. The copies number in the corresponding dilutions were 2×10^9 to 2 copies/ml. The dilutions were then tested in triplicates. Copies number in the lowest dilutions which were detected by qPCR assay was determined as limit of detection of the assay. The lower limit of detection of the assay was determined to be 20 to 200 copies/ml.

Specificity of the qPCR:

The specificity of qPCR assay was tested with three sputum samples which were high positive for *Mycobacterium tuberculosis*, *Candida albicans*, and *Streptococcus pneumoniae* (heavy growth in culture).

4.13.5. Internal control:

In order to search for integrity of DNA as well as PCR inhibitors, each clinical sample was tested in a second reaction mixture containing primers and probes for Human RNase P gene (designed at CDC). Human RNase P gene is a single-copy gene encoding the RNA moiety for the RNase P enzyme.

The primer and probe sequences were as follows;

Primers	**Sequences**
Forward (HURNASE-P-F)	5'-AGA TTT GGA CCT GCG AGC G -3'
Reverse (HURNASE-P-R)	5'-GAG CGG CTG TCT CCA CAA GT- 3'
Probe (BHQ1HURNASE-P)	5'FAM-TTC TGA CCT GAA GGC TCT GCG CGBHQ-3'

The concentration of different components of the PCR mix for one reaction are given below:

Reagents	For one reaction
TaqMan Master Mix (Invitrogen)	12.5 µl
PCR water	2.45 µl
MgCl2	1.5 µl
Forward primer	2.0 µl
Reverse primer	2.0 µl
Probe	2.0 µl
Rox	0.05 µl
DNA extract	2.5 µl
Total volume	25 µl

PCR was carried in Applied Bio Systems 7500 Fast Real time PCR system.

The cycling conditions were as follows;

Initial holding temperature at 95°C for 10 minutes

Denaturation at 95°C for 15 seconds

Annealing and extension at 65°C for 1 minute

Total number of cycles are 50

PCR analysis:

CT (cycle threshold) value below 35 was taken as satisfactory.

4.14. Categorization of patients:

Recruited patients were categorized into definite PCP, probable PCP, and non PCP based on following criteria (11):

Definite PCP

- Patients presented with typical symptoms of PCP such as fever, dry cough, and progressive dyspnea
- Bilateral interstitial infiltration on chest X-ray or ground glass opacity on high resolution CT thorax.
- Clinical response to anti-pneumocystis treatment and
- DFA test positive in respiratory samples.

Probable PCP

- Patients presented with typical symptoms of PCP and meet all the criteria for “definite PCP” except that DFA give negative results.

Non-PCP

- Patients who presented with atypical symptoms
- Variant lung infiltrations on chest X-ray
- Clinical response to other antimicrobial agents
- Have other definite diagnosis.

4.15. Statistical methods:

All the patient parameters and test results were entered in EpiData. The distribution of age among the categories was analysed using one way ANOVA. This has been presented with mean and standard deviation. Categorical variables were presented with frequency and percentage while their association with the diagnosis was assessed using chi-square test or fisher's exact test. AUC (Area under Receiver Operating Characteristic (ROC) curve) was given for the copy numbers comparing with DFA. For all the analyses done using statistical software STATA 13.1, p-value of less than 0.05 was considered as statistically significant.

5. Results:

5.1. Demographic data:

During the period of study from June 2014 to May 2015, 189 samples were received for routine DFA test, in which five samples were positive by DFA.

After considering the inclusion and exclusion criteria as mentioned in materials and methods section, 100 respiratory samples were included in the study.

All the samples included in the study were from adult immunocompromised patients only.

Fig. 5.1 shows the patient distribution.

30 (30%) samples were from HIV infected patients and 70 (70%) samples i.e. two third were from non-HIV infected immunocompromised patients.

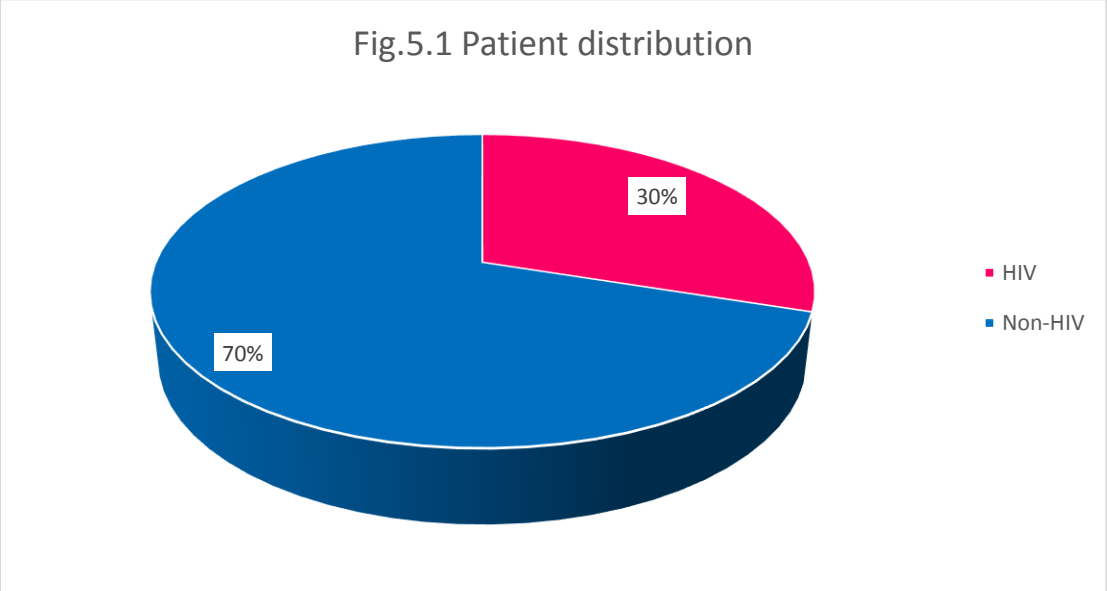


Fig. 5.2 shows the percentage distribution of non-HIV immunocompromised patients.

Among the seventy non-HIV infected immunosuppressed patients, 27 (38.6%) had haematological malignancies, 29 patients (41.4%) were on immune suppressive therapy for auto immune disorders, eight had (11.4%) solid organ malignancies and six patients (8.6%) were post renal transplantation.

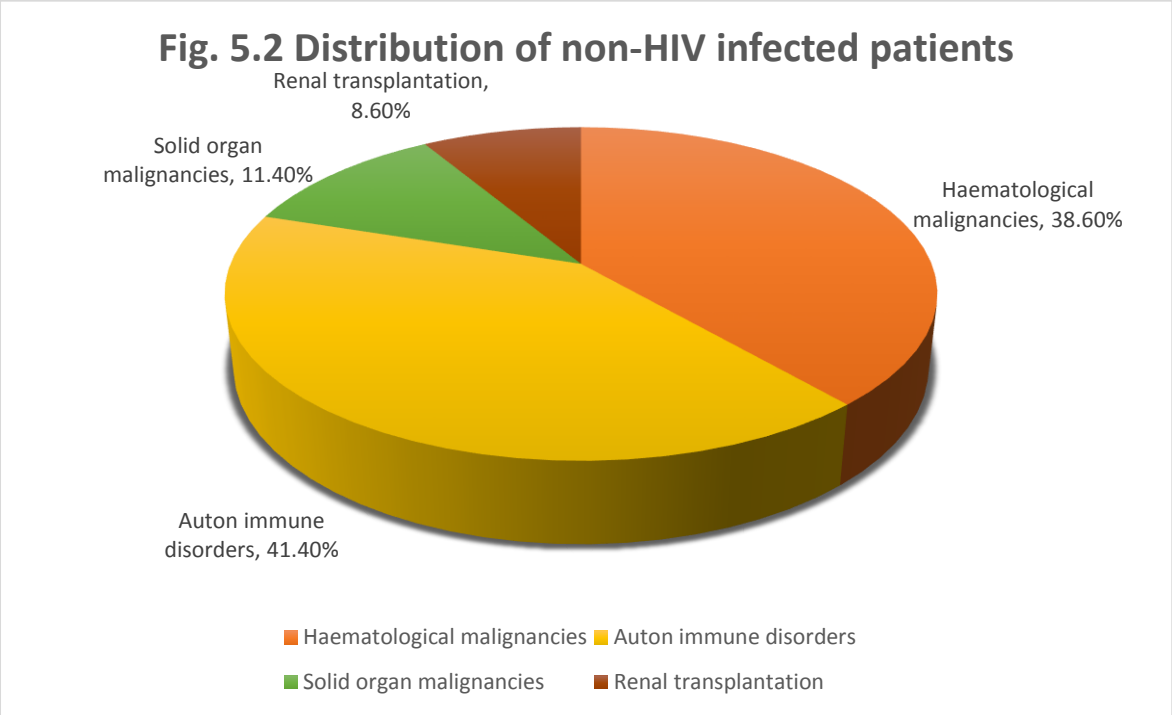


Fig. 5.3 shows the patient distribution in haematological malignancies. In this group (n=27), patients had different diseases like multiple myeloma (n=7), lymphoma (n=7), myelodysplastic syndrome (n=3), auto immune lympho proliferative syndrome (n=3), chronic lymphoid leukaemia (CLL) (n=3), acute myeloid leukaemia (AML) (n=2), acute lymphoid leukaemia (ALL) (n=1), and chronic myeloid leukaemia (CML) (n=1).

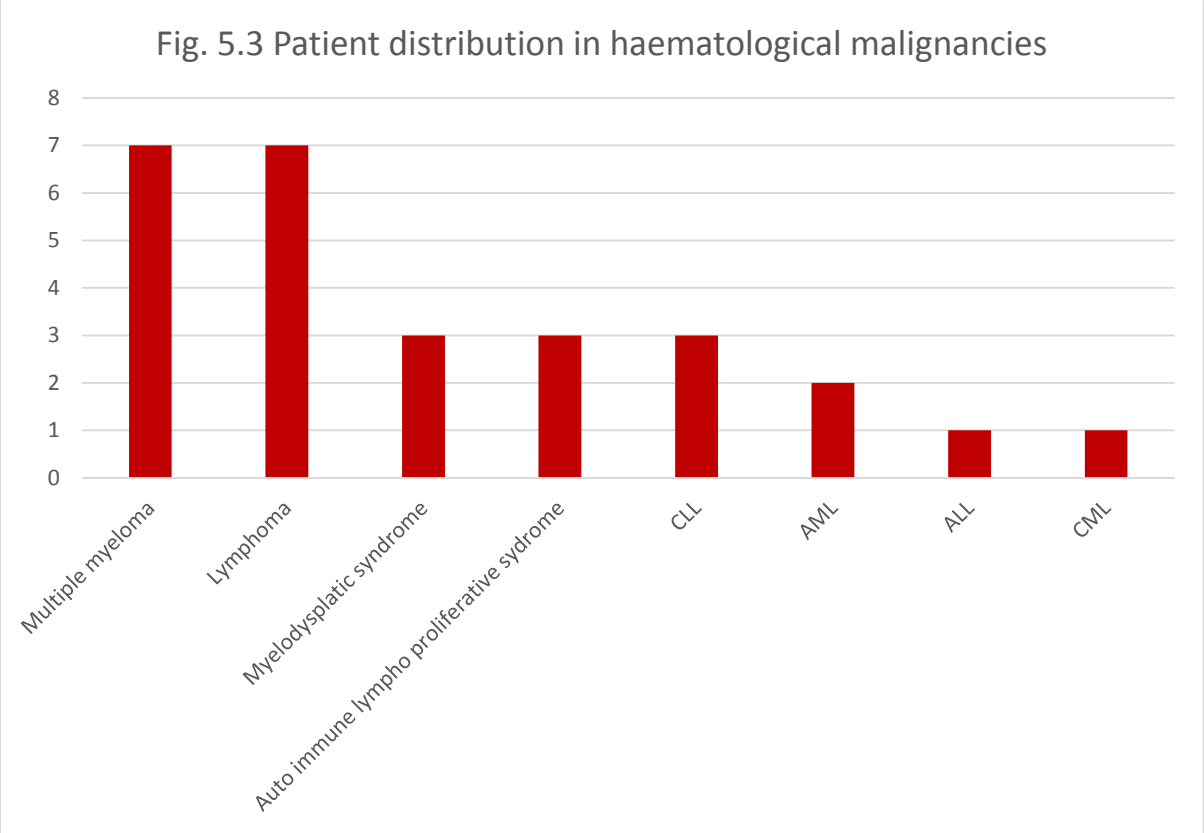


Fig. 5.4 shows the distribution of patients in auto immune disorders.

Following were the disorders found in this group of patients (n=29) i.e interstitial lung disease (ILD) (n=6), dermatomyositis (n=5), Wegener's granulomatosis (n=5), inflammatory myopathy (n=3), systemic lupus erythematosus (SLE) (n=3), auto immune glomerulo nephritis (n=2), and systemic sclerosis, diffuse scleroderma, hyper eosinophilic syndrome (HES), undifferentiated auto immune disorder were found in single patient.

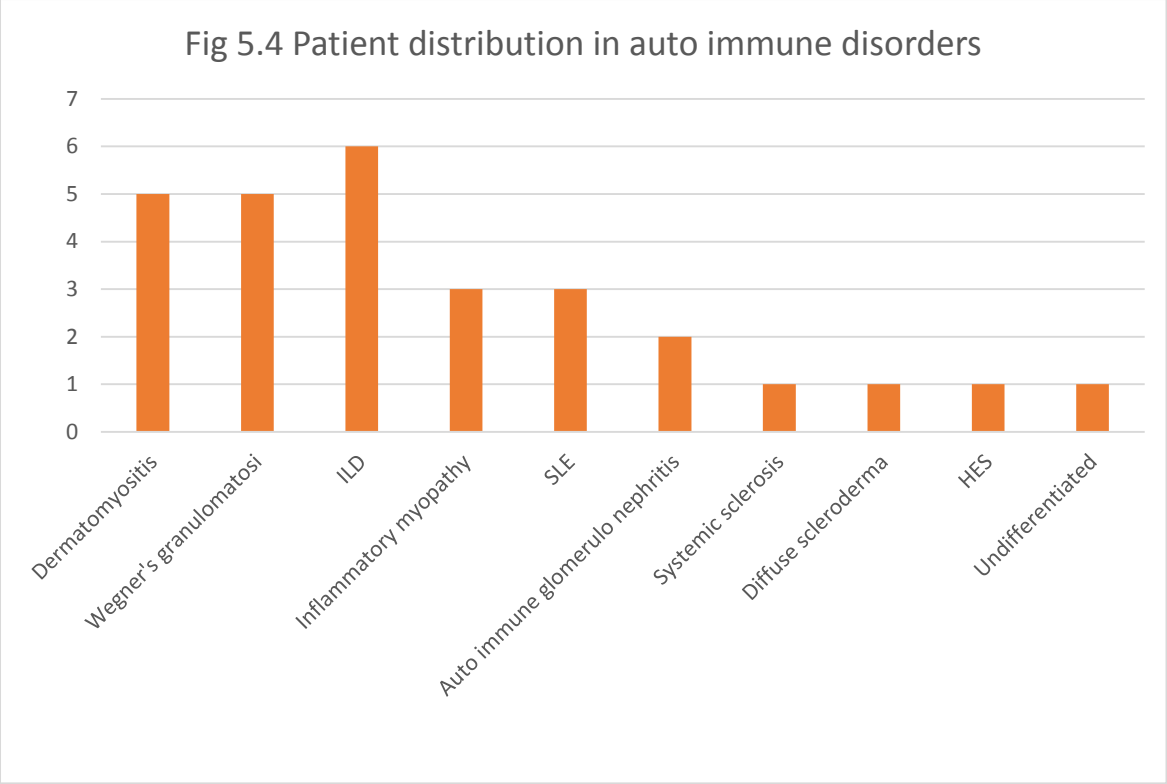


Fig. 5.5 Shows percentage distribution of the patients from various departments

Majority of samples i.e 41% were from medicine units, followed by 27% from haematology, 19% from pulmonary medicine and rest from nephrology (8%) and rheumatology units (5%).

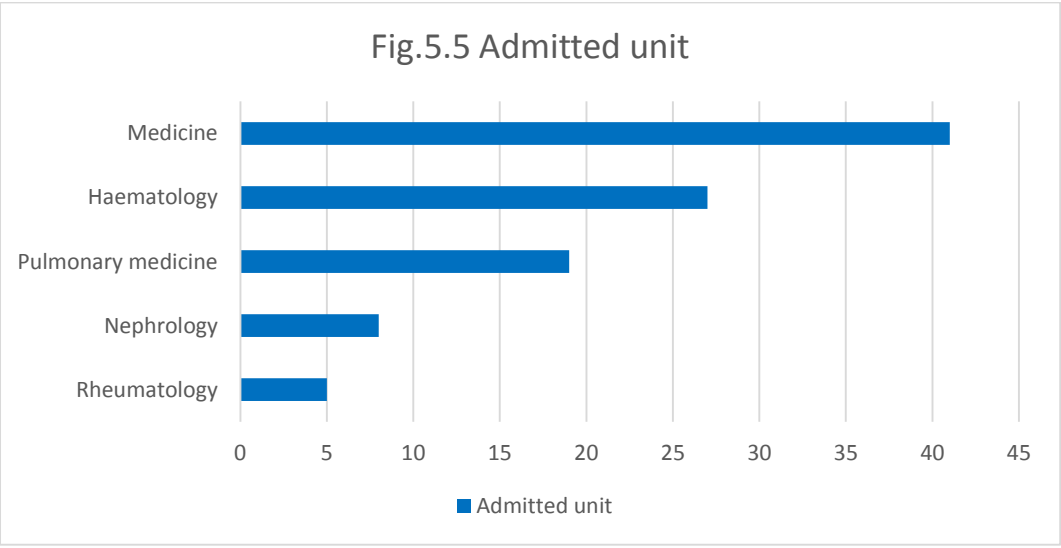


Fig. 5.6 Shows sex distribution.

Sixty three (63%) samples were from males and 37 (37%) were from females. The sex distribution was found to be slightly on the higher side for males.

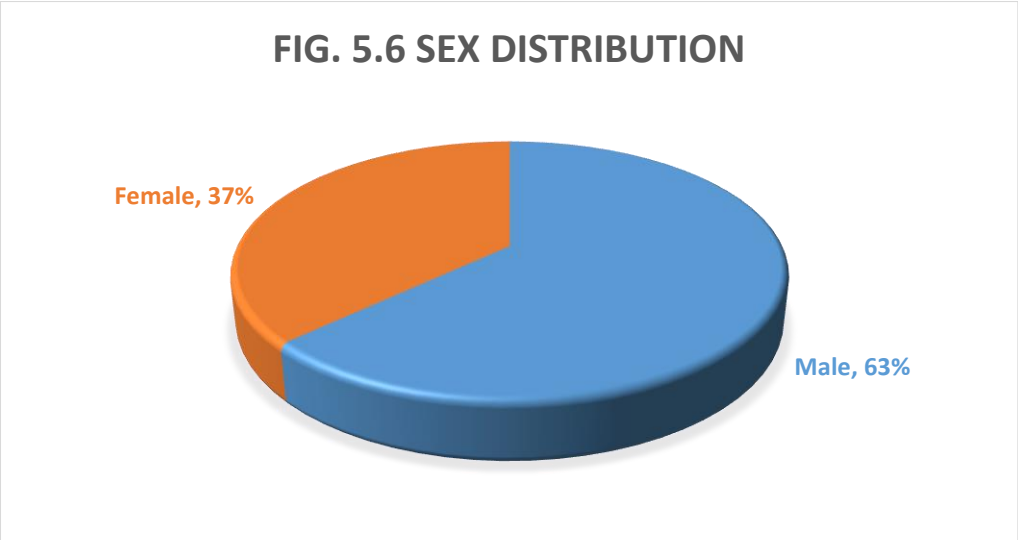
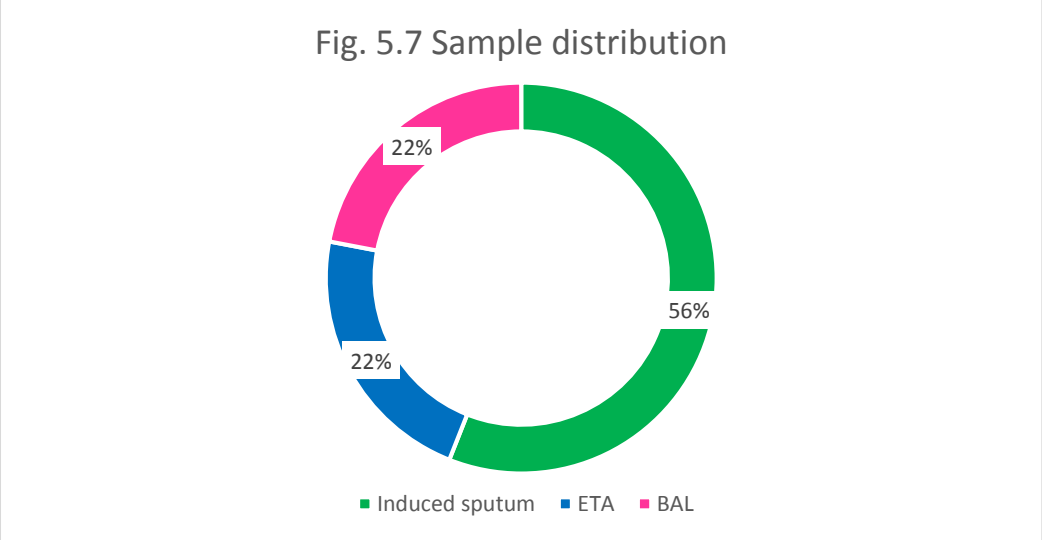


Fig. 5.7 shows the sample distribution.

Fifty six (56%) samples were induced sputum, rest were 22 each of Brochoalveolar Lavage (BAL) and Endotracheal aspirate (ETA) (22% each). Two third of the samples were non-invasive samples like induced sputum and ETA.



5.2. Patient categorization:

Fig. 5.8 shows the percentage distribution of different categories of PCP.

The patients who were recruited for this study have been categorized based on the criteria as mentioned in material and methods section. Five patients (5%) were categorized into definite PCP, 20 patients (20%) into probable PCP, and 75 patients (75%) into non-PCP groups.

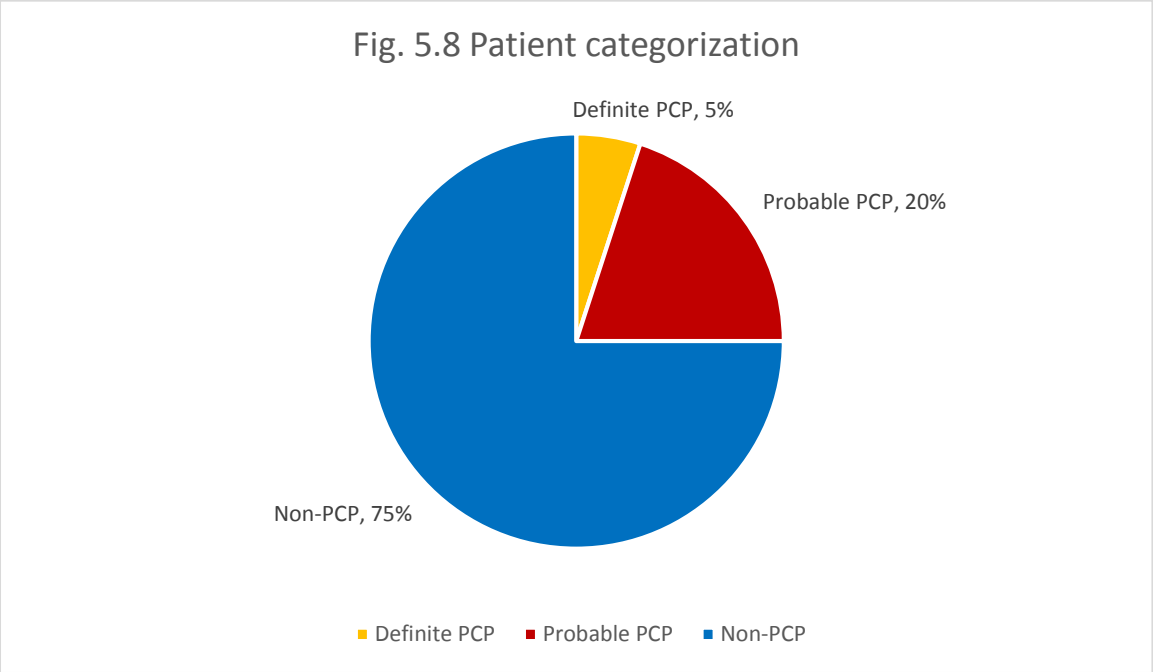
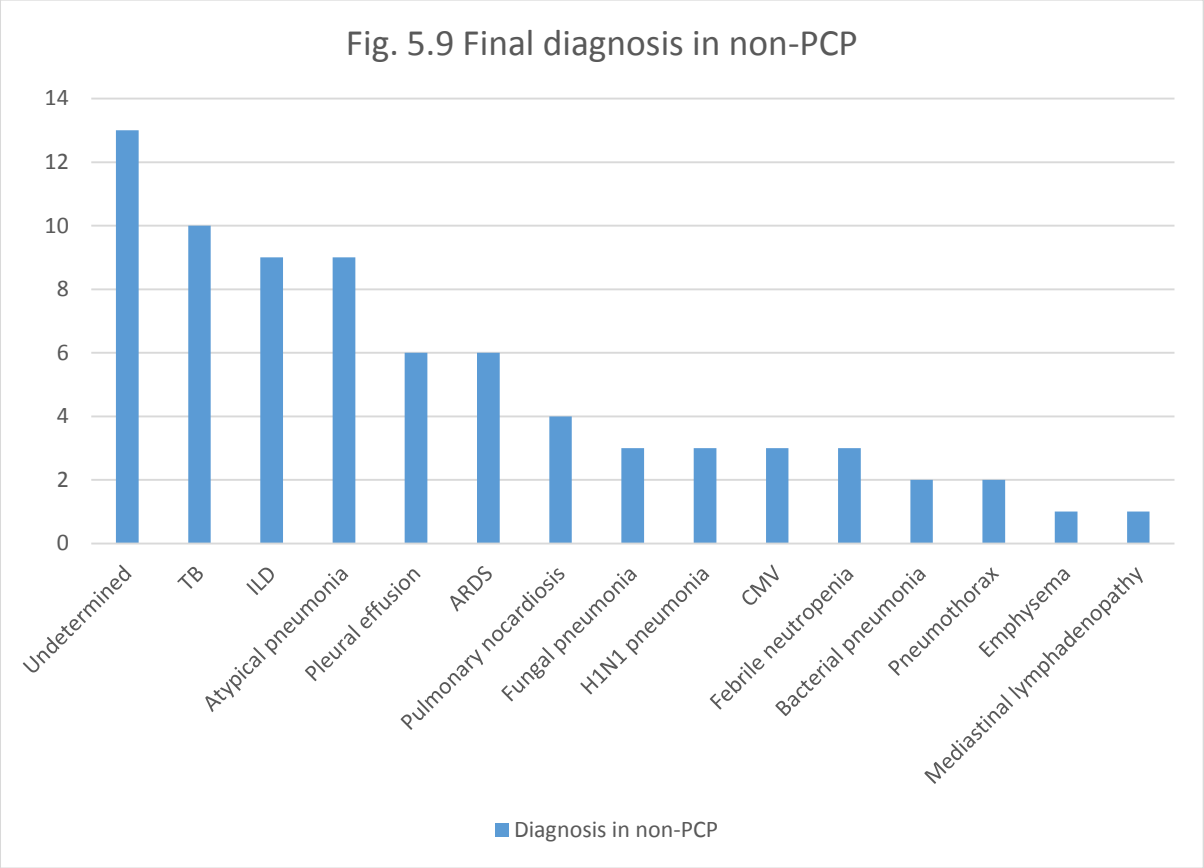


Fig. 5.9 shows final diagnosis in non-PCP group.

The final diagnosis in non-PCP patients (n=75) were tuberculosis (TB) (n=10), atypical pneumonia (n=9), superadded infection of ILD (n=9), pleural effusion (n=6), acute respiratory distress syndrome (n=6), nocardiosis (n=4), fugal pneumonia (n=3), H1N1 pneumonia (n=3), Cytomegalo virus (CMV) pneumonia (n=3), febrile

neutropenia (n=3), bacterial pneumonia (n=2), pneumothorax (n=2), emphysema (n=1), and mediastinal lymphadenopathy (n=1). Thirteen patients who do not have any diagnosis were categorised as undetermined.



5.3. Patient demographics and clinical characteristics:

Table. 5.1 depicts different clinical characteristics of the study patients.

The age of the patients ranged from 20 years to 69 years. When observed for the age distribution, mean age for all three groups were almost same (40 and 43).

In **Definite PCP** group, HIV infection was found in two patients (40%), haematological malignancy in one (20%), and two patients (40%) on immunosuppressive therapy for auto immune disorders.

In **Probable PCP** group, nine patients (45%) were HIV infected, five (25%) had haematological malignancies, four patients (40%) were on immunosuppressive therapy for auto immune disorders, and two (20%) were post solid organ transplantation.

In **Non-PCP group**, 19 patients (25%) were HIV infected, 21 (28%) had haematological malignancies, eight (11%) had solid organ malignancies, 23 patients (31%) were on immunosuppressive therapy for auto immune disorders, and four (5%) were post solid organ transplantation.

Among the cardinal symptoms of PCP, fever was present in 80% of definite and probable group. Dry cough was found in 60% of definite and 75% of probable group. Progressive dyspnoea was seen in all (100%) definite and 95% of patients in probable group.

Radiological features suggestive of PCP were found in all (100%) patients of both definite and probable group.

Dry cough and radiological features were found to be statistically significant with p values 0.001 and 0.00 respectively.

CD4 counts were available for 27 patients. Median count in definite, probable and non-PCP group were 53, 37.5, and 70 respectively. Except five patients in non-PCP group others had CD4 counts less than 200/mm^3 and this found to be statistically insignificant.

Table. 5.1 Clinical and radiological characteristics of the study patients:

Characteristics		Definite PCP (n = 5)	Probable PCP (n = 20)	Non-PCP (n = 75)	p value
Age years (mean)		40	43	43	0.9086
Sex (Male/female)		2/3	15/5	46/29	0.292
Underlying conditions					0.586
i.	HIV infection (n = 30)	2 (40%)	9 (45%)	19 (25.33%)	
ii.	Haematological malignancies (n = 27)	1 (20%)	5 (25%)	21 (28%)	
iii.	Solid organ malignancies (n = 8)	0	0	8 (10.67%)	
iv.	Auto immune	2 (40%)	4 (20%)	23	

	disorders on immune suppressive therapy (n = 29)			(30.67%)	
v.	Solid organ transplantation (n = 6)	0	2 (10%)	4 (5.33%)	
Fever		4 (80%)	16 (80%)	50 (66.67%)	0.452
Dry cough		3 (60%)	15 (75%)	23 (30.67%)	0.001
Dyspnoea		5 (100%)	19 (95%)	57 (76%)	0.085
Radiological features suggestive of PCP		5 (100%)	20(100%)	8 (10.67%)	0.000
CD4 counts/mm^3 (median) (n=27)		53	37.5 (6-176)	70 (4-883)	0.2871
Number of patients with					
< 200/mm^3		1	8	13	
> 200/mm^3		0	0	5	

5.4. The test results:

5.4.1 Giemsa staining:

All the samples were subjected to Giemsa staining technique, which is one of the stains used to demonstrate trophic forms of *Pneumocystis jirovecii*. Here the nuclei stain reddish purple and cytoplasm light blue. None of the study samples were positive by Giemsa staining. All the smears were screened by a skilled microbiologist

5.4.2 Direct immunofluorescent antibody (DFA) test:

DFA was performed on all the samples. This method identifies both trophozoite and cyst forms of *Pneumocystis jirovecii*, where a characteristic honeycomb appearance is seen.

Table. 5.2 shows results of DFA which was positive in five patients in the definite PCP group and negative in rest of the patients.

Table. 5.2 DFA results

DFA test	**Definite PCP (n=5)**	**Probable PCP (n=20)**	**Non-PCP (n=75)**
Positive	5 (100%)	0 (0%)	0
Negative	0	20	75 (100%)
Total	5	20	75

5.4.3 Real time quantitative PCR (qPCR) assay:

Real time quantitative PCR assay was done on all the samples and positive results were given in copies/ml.

Table. 5.3 shows qPCR results, which was positive in three samples from the definite PCP group, four from the probable PCP group and one from the non-PCP group. Two samples positive by DFA test were negative by qPCR.

Table. 5.3 qPCR assay results:

qPCR assay	**Definite PCP (n=5)**	**Probable PCP (n=20)**	**Non-PCP (n=75)**
Positive	3 (60%)	4* (20%)	1 (1.33%)
Negative	2 (40%)	16 (80%)	74 (98.67%)
Total	5	20	75

* Two patients were diagnosed clinically as definite PCP

Specificity of qPCR:

Specificity of qPCR was tested with *Mycobacterium tuberculosis, Candida albicans,* and *Streptococcus pneumoniae* positive samples. All three samples were negative and no cross reactivity was found.

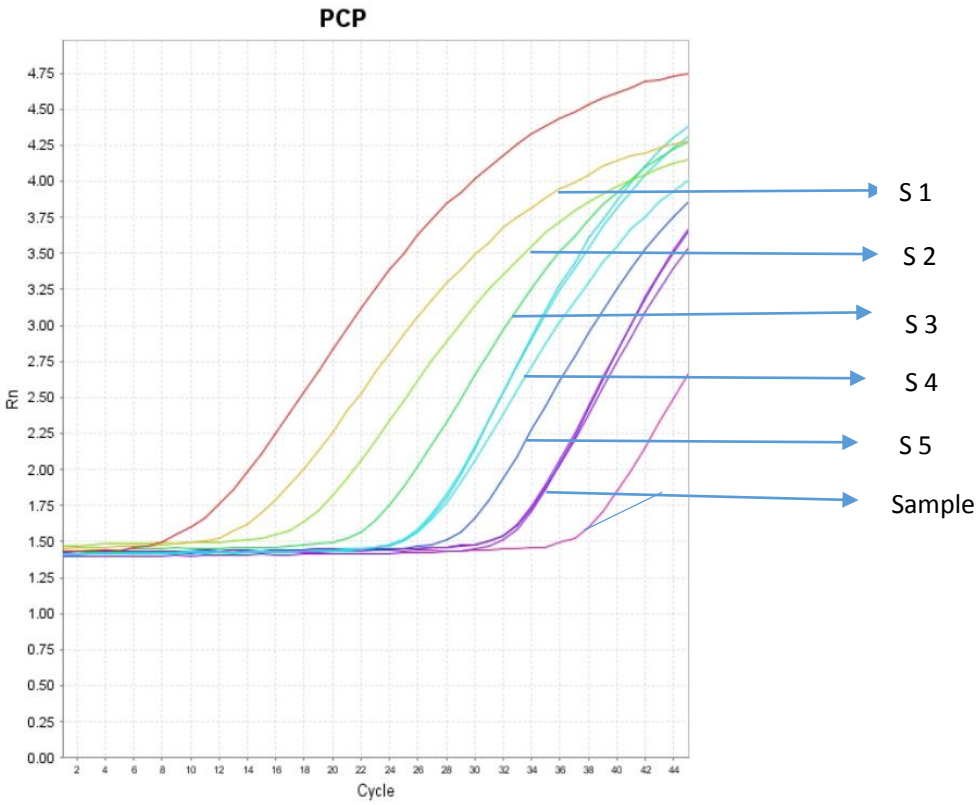


Fig. 5.10. Representative amplification plot of qPCR

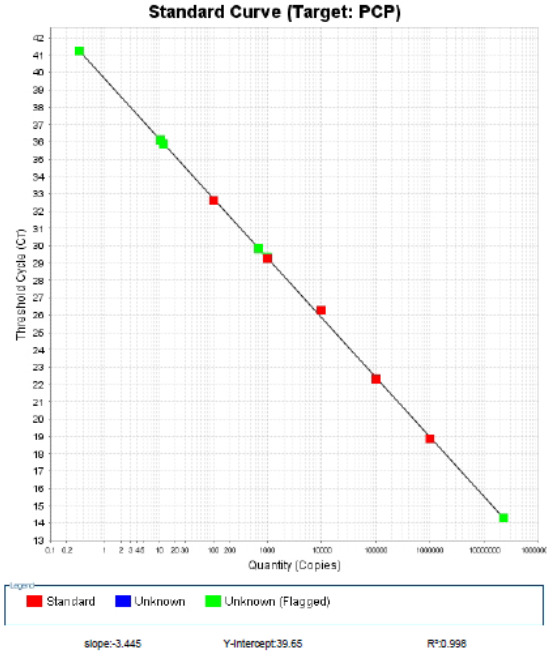


Fig. 5.11. Representative Standard curve for qPCR

Internal control:

Human RNase P gene was tested in all the study samples to look for integrity of DNA and PCR inhibitors. All samples were found to be satisfactory and PCR inhibitors were ruled out.

5.4.4 Comparison of qPCR assay and DFA test:

Table. 5.4 Shows the comparative results of qPCR and DFA test.

The comparison between these two tests showed that, DFA was able to detect *Pneumocystis jirovecii* when the copy numbers were more than 4540/ml. However, DFA failed to detect one of the samples which had 6990 copies/ml in qPCR

Table. 5.4 Comparison of qPCR and DFA test results:

qPCR positive patients	Copies/ml	Type of immunosuppression	Assigned category	DFA result
Patient 1	530	Non-HIV	Non PCP	Negative
Patient 2	1660	HIV	Probable PCP	Negative
Patient 3*	1700	HIV	Probable PCP	Negative
Patient 4	3640	HIV	Probable PCP	Negative
Patient 5*	6990	HIV	Probable PCP	Negative
Patient 6	4540	Non-HIV	Definite PCP	Positive
Patient 7	23670	HIV	Definite PCP	Positive
Patient 8	60850	Non-HIV	Definite PCP	Positive

* These patients were diagnosed clinically as definite PCP.

5.4.5 Sensitivity and specificity of each test:

Table. 5.5 Shows sensitivity, and specificity of DFA and qPCR tests. Here we have combined definite and probable PCP into a single group (diseased) and non-PCP as a separate group. It was found that qPCR assay had higher sensitivity (28%) than DFA test (20%), but statistically insignificant (p = 0.257).

Table. 5.5 Sensitivity and specificity of DFA and qPCR

Test	Sensitivity	Specificity
DFA test	20%	100%
qPCR assay	28%	98.67%

9.4.6 Receiver Operating Characteristic curve (ROC) analysis:

In a ROC curve the true positive rate (Sensitivity) of qPCR was plotted in function of the false positive rate (100-Specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal).

ROC curve analysis produced an AUC (Area under the ROC curve) of 0.78, along with a sensitivity (60%) and a specificity of 98.95% with a cut off value of 4540 copies/ml (**Fig. 5.12**).

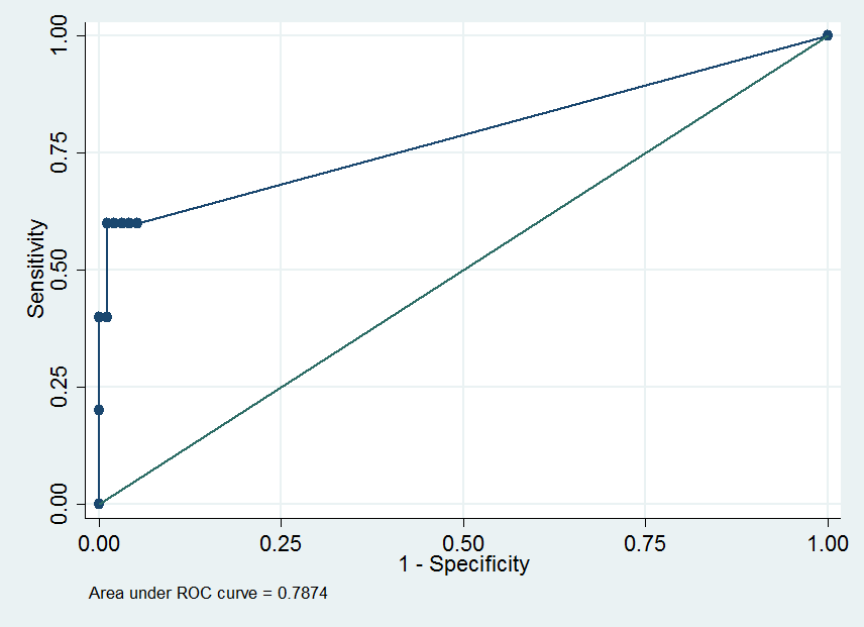


Fig. 5.12 ROC curve

Table. 5.6 Detailed report of sensitivity and specificity:

Copies number/ml	Sensitivity	Specificity	Correctly classified
≥ 0	100%	0%	5%
≥ 530	60%	94.74%	93%
≥ 1660	60%	95.79%	94%
≥ 1700	60%	96.84%	95%
≥ 3640	60%	97.89%	96%
≥ 4540	60%	98.95%	97%
≥ 6990	40%	98.95%	96%
≥ 23670	40%	100%	97%
≥ 60850	20%	100%	96%
> 60850	0%	100%	95%

5.5. Cost effectiveness analysis:

Cost effectiveness analysis for DFA and qPCR assay were done with the help of Accounts department from our institution, under following categories (**Table 5.7**) for a batch of five tests per week. Found to be qPCR assay is cheaper than DFA test.

Table. 5.7 Cost accounting details for DFA and qPCR

	DFA (Rs)	**qPCR (Rs)**
Personnel	1320	1320
Equipment	400	490
Consumables	950	1050
Chemicals and reagents	13000	7520
General	2130	2235
Total	**17800**	**12615**

6. Discussion:

Pneumocystis jirovecii is an important opportunistic pathogen causing pneumocystis pneumonia (PCP) in immunosuppressed individuals. With the introduction of highly active anti retro viral therapy (HAART) and anti-*Pneumocystis* prophylaxis, the incidence of PCP has declined in HIV infected individuals. However, this has become an emerging pathogen in non-HIV infected immunosuppressed patients and accounts for high mortality rate. PCP in this group of patients presents with an abrupt onset of symptoms and progresses rapidly to respiratory failure and death if untreated. Thus, laboratory diagnosis plays an important role in management of PCP. The diagnostic tests for PCP should be rapid as well as reliable.

In developing countries like India, the majority of PCP cases are diagnosed clinically. Since these organisms are non-cultivable, laboratory diagnosis is done by microscopic methods using Geimsa / Toludine blue O (TBO)/ Gomori methenamine silver (GMS) stains or by immunofluorescence methods. The major drawbacks of microscopy are the need for a skilled microbiologist and low sensitivity of the method (16). Currently, the molecular diagnostic methods are preferred as they are rapid and accurate with higher sensitivity.

Polymerase chain reaction (PCR) is an important diagnostic technique for diagnosis of PCP as they are non-cultivable and low sensitivity of available microscopic methods. Real time quantitative PCR (qPCR) has the advantage of quantification of the pathogen over other conventional and nested PCR techniques. This discriminates active infection from colonization.

In CMC, Vellore PCP is diagnosed by microscopic methods using direct immunofluorescent antibody test (DFA) and Giemsa staining.

In this study, qPCR assay targeting mitochondrial large subunit rRNA (mtLSUrRNA) gene of *P.jirovecii* was performed on 100 respiratory samples from HIV and non-HIV infected immunocompromised patients to evaluate this assay.

6.1 Patient's demography and clinical characteristics

In our study, 70 out of 100 (70%) patients were non-HIV infected immunosuppressed individuals, which is similar to the study done by Alanio A *et al.* and Gupta R *et al.* In their study 169/ 238 (71%) and 170/275 (62%) patients were non-HIV immunocompromised (1,13).This indicates that PCP is on the rise in this group of patients.

Presence of dry cough and radiological features suggestive of PCP such as bilateral reticular infiltration on chest X-ray or ground glass opacity in HRCT were the two features significantly associated with PCP in our study ($p < 0.05$). CD4 counts in definite and probable PCP were less than 200/$mm^{3,}$ were not significant ($p= 0.287$), as both non PCP and PCP patients had counts less than 200/mm^3. This was in contrast to the findings by Chawla *et.al* who found significant association with low CD4 counts (16).

6.2 Use of Giemsa staining

This study we found that Giemsa staining was not a useful technique for the diagnosis of PCP as none of the study samples were positive by this method. Positivity on light microscopy needs more than 10^5 organisms / ml in the clinical samples. The low copies of PCP in our samples might be the reason why we had no positives on smear microscopy using Giemsa stain. However, some studies showed higher levels of sensitivity in contrast to our experience. Chumpitazi *et al.* found that Giemsa staining had a sensitivity of 50% (9). Flori *et al*. and Jarboui *et al*. combined two staining methods such as Giemsa/GMS and Giemsa/TBO respectively. They found sensitivity of 60%, 64.3% and specificity of 100% (compared with the clinical diagnosis) (41,58). An Indian study by Chawla *et al* reported 43.7% (7/16) sensitivity for Giemsa/GMS methods (16).

6.3 Use of DFA ours vs other studies

In our study we found five out of 25 patients in the definite and probable group were positive by DFA. As we have included DFA positivity as one of the criteria for definite PCP, it is 100% positive in this group, though overall sensitivity was only 20%. Hauser *et al* reported a higher sensitivity of 93% using Merifluor kit and Jarboui *et al* reported 78.5% when compared with clinical diagnosis (58,59). The reason for low sensitivity in our study may be due to larger number of patients we had in Probable PCP group than in Definite PCP group. Jarboui et al had only one patient out of 14 definite cases and Hauser et al had no patients in the probable PCP group.

6.4 Use of Molecular methods

In our study, qPCR was positive in 60% (3/5) of definite PCP, 20% (4/20) of probable and 1.3% (1/75) of non-PCP patients. Overall sensitivity was found to be 28% in clinically suspected patients (Definite + Probable PCP) which is slightly higher than DFA. Several studies reported molecular method of any kind (qPCR, nested PCR, conventional PCR) had higher sensitivity over DFA (11,13,43,44,58,60).

Alanio *et al* developed the qPCR assay targeting mtLSU rRNA gene and the results were compared with clinical classification of high and low probable PCP. The study described two cut-off values such as 120 and 1900 TFEq/ml (Trophic equivalent/ml) to discriminate active infection from colonization in low and high probable cases respectively. Sensitivity and specificity for the diagnosis of PCP were estimated as 100% and 96.9% respectively with the lower cut-off value (120 TFEq/ml) (13).

The primers and probes described by Alanio *et al* were used in our study. Highest sensitivity and specificity of 60% and 98.95% were obtained with the cut-off value of 4540 copies/ml. ROC curve was made by true positivity of qPCR compared with DFA results. This huge difference of sensitivity between our study and Alanio *et al* could be due to difference in clinical criteria used in both studies. In their study patients were categorized into high probable (16/238) and low probable (222/238) cases based on clinical and radiological presentation. In our study we used the European Organization for Research and Treatment of Cancer/Invasive Fungal

Infections Cooperative Group (EORTC) Criteria and categorised patients in definite group if patients had typical symptoms like fever, dry cough, progressive dyspnoea with radiological features suggestive of PCP and shown clinical response to anti-pneumocystis treatment, as well as positive DFA result. Patients who had all criteria of definite PCP except gave negative DFA result were categorised in Probable group. Non PCP were assigned to those who had atypical symptoms, variant lung infiltrations and other definite diagnosis.

Alvarez-Martinez MJ *et al* compared qPCR and nested PCR targeting DHPS gene in microscopy-positive stored samples. The study found that sensitivity (94%) was same for both, but specificity of qPCR (96%) was significantly higher than nested PCR (81%) (61).

Huggett JF *et al* described a qPCR method targeting HSP 70 (Heat shock protein) gene and compared with a conventional PCR targeting mtLSUrRNA gene of *P.jirovecii* and microscopy as gold standard. Estimated sensitivity, specificity for qPCR and conventional PCR were 98%, 96% and 97%, 68% respectively (42). Our study also showed the sensitivity and specificity of 60% and 94.74%, if qPCR was compared with DFA test.

Direct comparison with other studies were difficult, as there are marked difference in target chosen, type of samples, group of patients studied and Gold standard test used to compare results (59,62).

6.4.1 DFA positive qPCR negative results:

In our study, we observed that two out of five samples were negative by qPCR, but positive by DFA.

One of the discrepant samples was collected from a newly diagnosed HIV positive treatment naive patient. The patient presented with fever for two months, cough with minimal mucoid expectoration and breathlessness for a month. A clinical diagnosis of PCP was made and the patient started on curative dose of co-trimoxazole five days before the collection of sample. This could be a false negative result and may be due to empty cyst without DNA after treatment as suggested by Caliendo *et al.* (63).

The second discrepant sample was obtained from a patient with chronic myeloid leukaemia in blast transformation phase. This patient presented with acute onset of fever, dry cough and worsening dyspnoea. Treatment for PCP with therapeutic dose of co-trimoxazole and steroid was started as induced sputum was positive by DFA test. However, the patient did not improve and needed mechanical ventilation for respiratory failure. Endotracheal aspirate had grown carbapenem resistant *Klebsiella* spp. The patient's condition improved after treatment with colistin. We came to the conclusion that this might be a false positive DFA result. This false positivity could be due to misidentification of background fluorescence as trophic forms of *P.jirovecii* (11).

6.4.2 DFA negative qPCR positive results:

i. In Probable PCP group:

In probable PCP group, qPCR was positive in four out of 20 patients. All the four patients were HIV infected. Three of them had typical presentation of PCP and showed clinical improvement after treatment. One was a newly diagnosed HIV patient, with classic symptoms of PCP, who improved after intravenous co-trimoxazole. However he expired three days later due to multi organ dysfunction. Therefore these patients could be considered as definite PCP.

ii. In Non-PCP group:

In our study, one sample from non-PCP group showed positive result by qPCR with a copy number of 530/ml. This sample was collected from a nephrology patient who was on methyl prednisolone Rapid Progressive Glomerulo Nephritis (RPGN). The patient had fever, cough with minimal expectoration and no dyspnoea. High Resolution Computed Tomography (HRCT) findings were suggestive of PCP or Cytomegalo virus (CMV) and his BAL was positive for CMV. The patient improved with anti-viral therapy alone. We concluded that this could be a colonization, as there are no classic signs and symptoms of PCP as well as that low copy numbers were detected by qPCR.

6.5 Overall sensitivity and specificity of qPCR against EORTC criteria:

We have categorized the patients into three groups based on EORTC criteria. Hence the sensitivity, specificity were calculated by combining definite and probable PCP as a diseased group and non-PCP as a non-diseased group. In our study, qPCR

has sensitivity and specificity of 28%, and 98.67% respectively in the diseased group. As we have included 'probable PCP' into the diseased group, we observed low sensitivity compared to other studies (16,58,59,62)

Among nine HIV infected patients in the probable PCP group, four of them were positive by qPCR. Rest of the patients were on HAART as well as their CD4 counts are less than 200/mm^3. One patient was on therapeutic doses of co-trimoxazole for two days before the collection of sample. Other patients were on co-trimoxazole prophylaxis, which might be the reason for negative result.

Among eleven non-HIV infected patients in probable PCP group, six patients had classic symptoms and improved with co-trimoxazole treatment. Rest of the five patients were treated with multiple antibiotics as well as co-trimoxazole for atypical pneumonia. Two among them improved and two patient died due to acute respiratory distress syndrome. One patient was discharged against medical advice for worsening condition. As PCP was one of the differentials in these five patients we have included them in probable PCP group.

The factors which reduce the sensitivity of PCP diagnostic tests are type of sample (invasive or non-invasive), and Co-trimoxazole prophylaxis or treatment before collection of sample. As suggested by many authors, induced sputum has low number of organisms compared to BAL. But Alanio *et al* found that there are no significant difference in the yield in different samples i.e both induced sputum and BAL had same yield by their qPCR assay.

6.6 Cost effectiveness analysis:

The cost effectiveness analysis done with the help of Accounts department in our Institution, revealed that qPCR assay is cheaper than DFA. These findings are similar to other studies done by Mani Revathy *et al* and Harris *et al* (60,64).

Mani Revathy *et al* compared three techniques i.e microscopy using GMS staining, qPCR targeting KEX-1 gene and a conventional PCR targeting mtLSUrRNA followed by sequencing. They found that qPCR is a simple, highly sensitive and cost effective technique (60).

Harris *et al* did a cost effective analysis for all the diagnostic options for PCP such as, chest X-ray, Diff-Quik, Toludine blue O (TBO), Calcofluor white, GMS, DFA, PCR, nested PCR, and qPCR. They observed PCR methodologies are most sensitive and cost effective diagnostic option for PCP (64).

7. Limitations of the study:

The cut off value to discriminate active PCP infection from colonization was not estimated since the sample size was low. Moreover in our study only one patient was identified to have colonization (530 copies/ml). We need to perform a larger study in asymptomatic immunocompromised patients to differentiate between active infections from colonisation.

The sensitivity of our microscopy and DFA is low compared to other studies. We used only a single sample for testing. Narasimha *et al* study showed increased sensitivity of microscopy when three consecutive induced sputum samples were tested and compared to a single sample (70% vs 15%) (65). This could be applied to improve sensitivity for non-invasive samples such as induced sputum and endo tracheal aspirate (ETA). But the same principle cannot be applied for invasive sample such as Broncho alveolar lavage (BAL).

Moreover Doucette S *et al* described that BAL had a better sensitivity of 90% when compared to induced sputum in HIV patients (66). Since the sample size was low as well as low incidence of HIV in our centre, we could not ascertain the best sample for PCP diagnosis in our study.

8. Conclusion:

- This study has evaluated a real-time quantitative PCR assay (qPCR) for routine diagnosis of Pneumocystis pneumonia (PCP) in a microbiology laboratory.
- When compared with clinical classification by EORTC criteria, we observed qPCR had 60% positivity in definite PCP and 20% positivity in probable PCP.
- Overall sensitivity and specificity of DFA and qPCR in clinically suspected cases were 20%, 100% and 28%, 98.67% respectively. Though qPCR sensitivity is higher than DFA, it was not statistically significant.
- The sensitivity and specificity of qPCR when compared to DFA was 60% and 94.74%.
- The qPCR was negative in two of the five DFA positive samples, where one of the sample was found to be false positive by clinical correlation. In addition to three DFA positive samples, qPCR also detected four cases in probable group which proves that qPCR is superior to DFA.
- Cost effective analysis revealed that qPCR is cheaper than DFA test.
- We conclude that real time quantitative PCR assay (qPCR) can replace conventional microscopy as a routine diagnostic test for PCP.
- Larger studies need to be done to evaluate whether single or multiple non-invasive samples or BAL would yield a better result on qPCR.

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10. Annexure

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Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

April 14, 2014

Dr. S. Dhanalakshmi
Department of Clinical Microbiology
Christian Medical College
Vellore 632 004

Sub: **Fluid Research grant project:**
Evaluation of a real-time quantitative PCR assay for diagnosis of Pneumocystis jirovecii pneumonia in immunocompromised patients - a pilot study.
Dr. S. Dhanalakshmi, Clinical Microbiology, Dr. Joy Sarojini Michael, Clinical Microbiology, Dr. Promila Mohan Raj, Microbiology, Dr. George M Varghese, Medicine I & Infectious diseases, Dr. Priscilla Rupali, Medicine I & Infectious diseases, Dr. Balamugesh, Pulmonary Medicine, CMC, Vellore.

Ref: IRB Min No. 8824 dated 07.04.2014

Dear Dr. S. Dhanalakshmi,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Evaluation of a real-time quantitative PCR assay for diagnosis of Pneumocystis jirovecii pneumonia in immunocompromised patients - a pilot study." on April 7th 2014. I am quoting below the minutes of the meeting.

The Committee raised the following queries:

a) For the DFA test, which protein is the monoclonal antibody detecting?
b) Do you plan to run a standard curve on the titers and express results as trophic equivalents?
c) There is a minor discrepancy in budget calculation.
d) Your exclusion criteria and "no PCP" in the diagnosis is the same. Please address this.
e) As this is a quantitative test, will you be doing an "area under the curve" to get a cut-off. Please mention this.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prabhantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
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f) What is the gold standard that you are going to use, since you are using sensitivity and specificity indices? If you are using clinical with stain positive as gold standard, it may lead to high false positivity. ie. If you are stain positive and PCR negative what would you do?
g) The cost effectiveness and cost analysis has not been mentioned lucidly in the proposal.
h) Please reword the information sheet.

Dr. S. Dhanalakshmi and Dr. Joy Sarojini Michael were present during the presentation of the proposal and satisfactorily responded to the queries raised by the Members. After discussion, it was resolved to **ACCEPT the proposal AFTER receiving the suggested modifications and answers to the queries.**

Note:
1. Kindly **HIGHLIGHT** the modifications in the revised proposal.
2. Keep a **covering letter and point out the answer to the queries.**
3. Reply to the queries should be submitted within **3 months** duration from the time of the thesis/ protocol presentation, if not the thesis/protocol have to be resubmitted to the IRB.
4. The **checklist** has to be sent along with the responses.

Email the details to research@cmcvellore.ac.in and send a hard copy through internal dispatch to Dr. Nihal Thomas, Addl. Vice-Principal (Research), Principal's Office, CMC.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
M.D.,MNAMS.,DNB(Endo),FRACP(Endo) FRCPI(Edin),FRCPI(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Joy Sarojini Michael, Clinical Microbiology, CMC, Vellore

IRB Min No: 8824 dated 07.04.2014

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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

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June 20, 2014

Dr. S. Dhanalakshmi
PG Registrar
Department of Clinical Microbiology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research grant project:**
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Ref: IRB Min No: 8824 [DIAGNOSE] dated 07.04.2014

Dear Dr. S. Dhanalakshmi,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS DNB(Endo),FRACP(Endo),FAMS,P.Edin, FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Joy Sarojini Michael, Clinical Microbiology, CMC, Vellore.

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Ref: IRB Min No: 8824 [DIAGNOSE] dated 07.04.2014

Dear Dr. S. Dhanalakshmi,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Evaluation of a real-time quantitative PCR assay for diagnosis of Pneumocystis jirovecii pneumonia in immunocompromised patients - a pilot study." on February 19, 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. Dhanalakshmi, Joy Sarojini Michael, Promila Mohan Raj, George M Varghese, Priscilla Rupali, Balamugesh.
3. Proforma
4. Informed Consent form (English, Tamil & Hindi)
5. Consent form (English, Tamil & Hindi)
6. No of documents 1-5

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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on February 19, 2014 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Paul Ravindran	PhD, Dip RP, FCCPM	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. J. Visalakshi	MPH, PhD	Lecturer, Dept. of Biostatistics, CMC.	Internal, Statistician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC	Internal, Clinician
Dr. Anup Ramachandran	Ph. D	The Wellcome Trust Research Laboratory Gastrointestinal Sciences, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Jacob John	MBBS, MD	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Dr. Vivek Mathew	MD (Gen. Med.) D.M (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr. Anand Zachariah	MBBS, PhD	Professor, Medicine, CMC, Vellore	Internal, Clinician
Dr. Chandra Singh	MS, MCH, DMB	Professor, Urology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	B. Sc, DSSA	Social Worker, Vellore	External, Lay person
Mr. C. Sampath	B. Sc, BL	Legal Expert, Vellore	External, Legal Expert

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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Denise H. Fleming	B. Sc (Hons), PhD	Honorary Professor, Clinical Pharmacology, CMC, Vellore	Internal, Scientist & Pharmacologist
Dr. Anuradha Rose	MBBS, MD	Assistant Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Jayaprakash Muliyil	B. Sc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist & Epidemiologist
Mrs. Sheela Durai	M Sc Nursing	Addl. Deputy Nursing Superintendent, Professor of Nursing in Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert
Dr. Vathsala Sadan	M.Sc, PhD	Professor, Community Health Nursing, CMC, Vellore	Internal, Nurse
Dr. Nihal Thomas,	MD, MNAMS, DNB(Endo), FRACP(Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

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The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent.** On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of Rs. 1,00,000/- (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/Annual report and subsequent submission of it to the IRB.

Yours sincerely

CHRISTIAN MEDICAL COLLEGE
VELLORE
INDIA

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS DNB-Endo,FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
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Cc: Dr. Joy Sarojini Michael, Clinical Microbiology, CMC, Vellore.

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PATIENT INFORMATION SHEET

Study title: Evaluation of a real-time quantitative PCR for diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients.

Please read this carefully. It tells you important information about the study. A member of the research team will explain to you about taking part in this study. If you have any questions about the research or about this form, please ask us. If you decide to take part in this study, you must sign or provide your thumb impression in this form to show that you want to take part.

Why is this study being done?

Pneumocystis pneumonia is a disease commonly seen in patients with low immunity; E.g. patients with HIV infection and cancer patients on treatment. The tests currently available for diagnosing this organism causing this lung infection is not very good in picking up all patients with this disease . So treatment is given to these patients based on clinical presentation, not on laboratory tests. This study will help us to diagnose the disease more accurately. The respiratory sample that has been sent for routine diagnosis of your disease will also be used to evaluate this new test.

What will happen in this study?

The respiratory sample that you have provided for testing will also be used for this new test.

Will I be paid to take part in this study?

We will not pay you for providing your sample for research.

What are the risks and possible discomforts from being in this study?

There are no extra risks as your sample is already being collected to make a diagnosis. We are simply using the sample to evaluate the new test.

What are the possible benefits from being in this study?

You will not benefit personally from taking part in this study but you will help us to develop an accurate diagnostic test for Pneumocystis pneumonia which may benefit you or other patients in the future.

If I take part in this research study, how will you protect my privacy?

Information about you collected for this research study will be stored in the Investigator's research files and will be identified only by a number. Your name and other information that might identify you will be recorded with a code number. This means that no one will be able to tell which sample is yours. The research consent form you sign may be inspected by regulatory agencies or the Institutional Review Board in the course of carrying their duties. If

the signed research consent form is inspected or copied, the Hospital will use reasonable efforts to protect your privacy.

If I have questions or concerns about this research study, whom can I call?

You can call us with your questions or concerns and the details are as below

1. Dr.S.Dhanalakshmi,
   PG Registrar,
   Department of Microbiology,
   Christian Medical College, Vellore - 632 004,
   Phone: 0416 2282588, 98650 66752.

Informed Consent Form for Subjects

Informed Consent form to participate in a research study

Study Title: Evaluation of a real-time quantitative PCR for diagnosis of *Pneumocystis jirovecii* pneumonia in immunocompromised patients.

Study Number: _____

Subject's Name: _____

Date of Birth / Age: _____

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions.

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

(iii) I understand that the Sponsor of the clinical trial, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published.

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

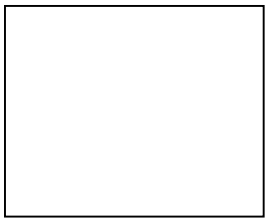
(v) I agree to take part in the above study.

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: _____/_____/_____

Signatory's Name: _____ Signature:

Or



Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature of the Witness: _____

Date: _____/_____/_____

Name and Address of the Witness: _____

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Date: _____/_____/_____

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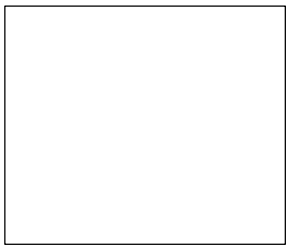

Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature of the Witness: _____

Date: _____/_____/_____

Name and Address of the Witness: _____

PCP STUDY PROFORMA

Patient Information

<table>
<tr><td colspan="2">Study ID:</td><td colspan="2">Name:</td></tr>
<tr><td>Hosp No:</td><td>Age:</td><td>Sex</td><td>M / F</td></tr>
<tr><td colspan="2">Date of sample collected:</td><td colspan="2" rowspan="3">Contact number
Ph:

Mob:</td></tr>
<tr><td>Unit:</td><td>Ward:</td></tr>
<tr><td colspan="2">HIV STATUS: POSITIVE / NEGATIVE</td></tr>
<tr><td colspan="4">Address:</td></tr>
</table>

Clinical profile:

SYMPTOMS	SIGNS
Fever: Yes / No	Pulse: _____ / minute
Dry cough: Yes / No	Blood pressure: _____/_____ mmHg
Shortness of breath: Yes / No	RR: ____ / minute
Others:	Temp: _____
	PO2: _____

Radiology

X-ray	Suggestive of PCP	Not PCP
Comments		
HRCT	Suggestive of PCP	Not PCP
Comments		

Laboratory

No	Test	Result	
1	Giemsa stain	Positive	Negative
2	IFA	Positive	Negative
3	qPCR assay	Positive	Negative
4	CD4 count		

Treatment History

Drug used			
Prophylactic / Curative			
Improvement after treatment		Yes	No
Diagnosis at discharge	**PCP**	**Probable PCP**	
Non-PCP	**Others**		