STUDY OF GENOTYPIC VARIATION OF *PNEUMOCYSTIS JIROVECII* IN THAI PATIENTS BY USING REAL-TIME PCR WITH HIGH RESOLUTION MELT ANALYSIS AND DNA SEQUENCING METHOD

WATCHARAPORN JONGRATANAVANICH

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Lay the pipette aside, I promised I would make this part shorter than the rest of the thesis. That will be a challenge, but not because I am so expressive; rather, and extraordinary number of people have significantly improved the quality of my life and dissertation work during the part four years. I cannot possibly acknowledge all the individuals who have contributed to my life here in Mahidol University. I apologize to those who I fail to mention.

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ABSTRACT

Pneumocystis jirovecii (*P. jirovecii*) is the second most opportunistic fungal infection in immunocompromised patients in Thailand. However, little information regarding epidemiological data and genotypic variation in mitochondrial large-subunit rRNA (mt LSU rRNA) of this pathogen had been available. The objective of this research was to study genotypes of this organism in immunocompromised Thai patients by DNA sequence analysis and high resolution melt analysis of mt LSU rRNA gene. The sixty-four clinical specimens consisted of bronchoalveolar lavage fluid (BALF), sputum (SP), and gastric wash (GW) from immunocompromised Thai patients suspected of having *Pneumocystis jirovecii* pneumonia (PcP) were used to analyze the genotypes. The criteria of type classification were based on a typing score described firstly by Wakefield *et al.* From all clinical specimens studied, two new types were detected. Two unique and different dominant types of *P. jirovecii* were observed in immunocompromised Thai patients which originated from coinfection. These differences can be used as genetic markers for studying the epidemiology, transmission patterns, and probable antimicrobial resistance research.

KEY WORDS: *PNEUMOCYSTIS JIROVECII*/ GENOTYPIC VARIATION/ mitochondrial large-subunit rRNA

111 pages

การศึกษาความหลากหลายทางพันธุกรรมของเชื้อนิวโมซีสทีส จิโรเวคซิไอ ในผู้ป่วยคนไทยโดยใช้เทคนิคการตรวจวัดปริมาณกรดนิวคลิอิกในสภาพจริงร่วมกับการวิเคราะห์ High Resolution Melt และวิธีการเรียงลำดับเบสบนดีเอ็นเอ

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บทคัดย่อ

เชื้อนิวโมซีสทีส จิโรเวคซิไอจัดเป็นเชื้อราฉวยโอกาสที่พบบ่อยเป็นอันดับสองที่ทำให้ผู้ป่วยที่มีภูมิคุ้มกันบกพร่องเกิดโรคปอดอักเสบ *Pneumocystis jirovecii* pneumonia (PcP) อย่างไรก็ตามความรู้ทางด้านความหลากหลายทางพันธุกรรมและระบาดวิทยาของเชื้อราชนิดนี้ ที่แยกจากผู้ป่วยชาวไทยยังมีน้อย จุดประสงค์ของการศึกษานี้ เพื่อทำการศึกษารูปแบบความแตกต่างของลำดับเบสบนดีเอ็นเอบริเวณตำแหน่งยีน mitochondrial large subunit ribosomal RNA (mt LSU rRNA) จากตัวอย่างน้ำล้างปอด (BALF) เสมหะ (SP) และน้ำล้างกระเพาะอาหาร (GW) ซึ่งเก็บจากผู้ป่วยชาวไทย โดยใช้วิธีการเปรียบเทียบระหว่างวิธีการเรียงลำดับเบสบนดีเอ็นเอและเทคนิคการตรวจวัดปริมาณกรดนิวคลิอิกในสภาพจริงร่วมกับการวิเคราะห์ high resolution melt โดยการศึกษาครั้งนี้ใช้เกณฑ์การคัดแยกสายพันธุ์ของ Wakefield และคณะ ซึ่งทำการเปรียบเทียบกับนิวคลีโอไทด์ที่ใช้เทียบเพื่อคัดแยกสายพันธุ์ (consensus sequence) ของ mt LSU rRNA ของเชื้อนิวโมซีสทีส จิโรเวคซิไอ จากสิ่งส่งตรวจจำนวน 64 ตัวอย่าง ทั้งนี้เป็นการศึกษายีน mt LSU rRNA ของเชื้อนิวโมซีสทีส จิโรเวคซิไอ ครั้งแรกในผู้ป่วยคนไทย ผลการศึกษาครั้งนี้พบว่าลักษณะทางพันธุกรรมของเชื้อนิวโมซีสทีส จิโรเวคซิไอ มีการผันแปรเมื่อเทียบกับลักษณะพันธุกรรมที่มีการรายงานก่อนหน้านี้ จากประเทศต่างๆ ข้อมูลดังกล่าวอาจนำมาใช้ในการศึกษาทางด้านระบาดวิทยา กระบวนการติดต่อของเชื้อชนิดนี้ รวมทั้งการศึกษาการดื้อยาเฉพาะในกลุ่มผู้ป่วยคนไทยในอนาคตได้

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LIST OF ABBREVIATIONS

/	per
%	Percentage
bp	Base Pair
°C	Degree Celsius
cm	Centimeter
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxytyrosine-5'-triphosphate
DNA	Deoxyribonucleic acid
et al.	*et alii* (Latin), and others
e.g.	*exempli gratia* (Latin), for example
Fig.	Figure
G	Gram
g	Acceleration gravity
hr	Hour
i.e.	*id est* (Latin), that is
Ig	Immunoglobulin
IU	International unit
K	Kilo
kbp	Kilobase pair

LIST OF ABBREVIATIONS (cont.)

l	Liter
M	Molar
Mbp	Megabase pair
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mol	Mole
MW	Molecular weight
µl	Microlitre
ng	Nanogram
nm	Nanometer
OD	Optical density
PcP	*Pneumocystis jirovecii* pneumonia
pH	Power of hydrogen
rpm	Round per minute
sec	Second
SMZ	Sulfamethoxazole
TMP	Trimethoprim
U	Unit
UV	Ultra violet
V	Volts
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

Pneumocystis jirovecii **(***P. jirovecii***)** is an opportunistic fungus pathogen which causes *Pneumocystis jirovecii* pneumonia **(**PcP**)** in immunocompromised individuals (1, 2). *Pneumocystis* was first identified in 1909 by Carlos Chagas in his guinea pig model of trypanosome infection. Chagas thought this pathogen was identified as a new trypanosomal life form (3). Antonio Carinii identified similar organisms in infected rat lungs in 1910, and likewise thought they were a new type of trypanosome (4). Until 1912 that the Delanoës, recognized that the organism identified by both Chagas and Carinii was a separate species (5). These investigators named the organism *Pneumocystis carinii* (*P. carinii*), highlighting the unique tropism of the parasite for the lung and giving credit for the discovery to Carinii rather than Chagas (5). *P. carinii* was initially classified as a protozoan based on the histological characteristics of the two identified life cycle forms, the small trophozoite form (trophic stage) and the larger cyst (spore case), and the response of the infection to treatment with the anti-protozoan medication, trimethoprim-sulfamethoxazole (TMP-SMX) and pentamidine. An important taxonomy shift came in 1988 when *P. carinii* was placed phylogenetically in the fungal kingdom after sequencing of the small subunit ribosomal RNA (mt SSU rRNA) (6). *Pneumocystis* species are currently classified within the phylum Ascomycota, in a unique class, order and family (Pneumocystidomycetes, Pneumocystidales and Pneumocystidaceae, respectively). The various forms of *P. carinii* that infect hosts were historically designated with special form or forma specialis (f. sp.), so for example *P. carinii* f. sp. *murina* was the form that infects mice, whereas *P. carinii* f. sp. *hominis* was the form that infects human. In a recent nomenclature change, the form that infects humans has been

renamed as *P. jirovecii*, after the pathologist Jirovec who first reported the organism in humans (7). The primary symptoms of which include progressive dyspnea, nonproductive cough, and low-grade fever (8). These patients are often profoundly hypoxemic, and chest radiographs typically show bilateral and diffuse interstitial pneumonia (9, 10).

P. jirovecii has been reported as a fungus which is one of the most common opportunistic pathogen in Human Immunodeficiency Virus (HIV)-infected patients (11). In Thailand, *P. jirovecii* is ranked as the second most common opportunistic pathogen in HIV-infected patients and has become a public health problem (12). This fungus pathogen is also primarily associated with pneumonia in the other profoundly immunocompromised conditions e.g., those patients undergoing chemotherapy for malignant disease and organ transplantation (13, 14, 15). Among renal transplant recipients, PcP can still occur several months after transplantation and late after prophylaxis discontinuation. Graft rejection appears to be the major risk factor for PcP in these patients. PcP prophylaxis should be therefore maintained or resumed, especially when lymphocytopenia is present as it could increase further the risk of PcP (14). During the treatment of cancer, patients showing respiratory symptoms with interstitial shadow frequently showed positive polymerase chain reaction (PCR) results for *Pneumocystis* deoxyribonucleic acid (DNA) which related to systemic corticosteroid and immunosuppressive agents (15).

There are two major difficulties have affected the study of *P. jirovecii*. First, in spite of extensive efforts, a reliable *in vitro* system for cultivation of *P. jirovecii* does not exist, the infection is not species specific with considerable genetic difference between *Pneumocystis* infecting humans and other species (16). Second, samples of *P. jirovecii* organisms are not easily obtained; only few organisms are present in the upper respiratory tract secretions and sampling of the lower airways is usually required to isolate the organism and establish a definite histological diagnosis (17). Nowadays, there is an effective anti-pneumocystosis chemoprophylaxis, primarily with, drug combination of TMP-SMX (18, 19). However, PcP is still the most common life-threatening acquired immunodeficiency syndrome (AIDS) (20) indicator condition in the world in patients whose cluster of differentiation 4 **(**CD4**)** T

lymphocyte cell count has declined to <200 cells/µl for the first time. Unfortunately, highly active antiretroviral therapy (HAART), which is the most effective for HIV-infected patients has not been wildly used in Thailand because cost remains high (21).

Epidemiological data is important for understanding patterns of transmission and developing diagnostic methods (22). Despite the frequency of PcP, tremendous gaps exist in understanding the epidemiology of *P. jirovecii*. Recently, a substantial number of genes and gene fragments have been identified for potential uses in analysis and characterization of *P. jirovecii* strains. Molecular techniques have shown diversity among isolates of human derived *P. jirovecii* by comparison of DNA sequence variations at a number of different genetic loci (23). There has been molecular typing based on nucleotide sequence variation of *P. jirovecii* genome including the mitochondrial large subunit ribosomal RNA (mt LSU rRNA) gene (24). Based on the variation of mt LSU rRNA gene, Beard *et al*. reported sequence types which can identify four types and their genetic variation of *P. jirovecii* among 191 clinical specimens from five United State cities (25). Additionally, the following study of thirty HIV-infected patients with PcP showed that the severity, prognosis, and the frequency of recurrent episodes of the disease depended on the specific type. This data may also suggest that in addition to the host factor, the severity of PcP may also be associated with mt LSU rRNA gene mutation (26).

The question of latency, reactivation and recent acquisition are greatly important as they relate to treatment and prevention of pneumocystosis. The analysis of variable gene loci in organisms associated with recurrent PcP can address these questions (27). Several studies demonstrated different *P. jirovecii* sequences in separate disease episodes. This data ruled out the possibility of reactivation in recurrent pneumocystosis (28). Other data showed that genotype frequency distribution patterns varied by patients' place of diagnosis rather than their place of birth (29). This possibility indicates that the infection is acquired later than the first few months or years of life and that any latency has natural limits. Sources of infection, both environment and person-to-person have been proposed as modes of transmission of *P. jirovecii*. Recently, environment sampling provided evidence that

the airborne presence of *P. jirovecii* which from variation of genetic material is plausible (29).

Even though *P. jirovecii* is presently gaining more consideration, its life cycle, metabolism and even molecular biology are still poorly understood. Remarkably few studies of genetic diversity and molecular biology have been reported so far. The lack of knowledge regarding of this microorganism in Thailand has lead to the scarcity of experimental approaches to rational genotypic study and other research areas.

OBJECTIVES

1. To investigate genotypic variation of *P. jirovecii* in clinical specimens collected from patients at Faculty of Medicine Siriraj Hospital, Mahidol University by conventional PCR and sequencing method

2. To evaluate a reliable method for detection and study genotypes of *P. jirovecii*

CHAPTER II

LITERATURE REVIEW

2.1 *Pneumocystis jirovecii* (*P. jirovecii*)

Pneumocystis jirovecii, the causative agent of PcP, is a unicellular eukaryotic microorganism that appears to be ubiquitous in geographic distribution and harmlessly present in the lungs of a wide variety of homeothermic terrestrial vertebrates (30, 31). This microorganism is an opportunistic pathogen causing pneumonia in immunocompromised individuals (13). Historically, it was associated with disease in children in Poland after World War II and subsequently with patients whose receiving chemotherapy for the treatment of malignant disease or undergoing organ transplantation but the highest incidence of PcP was found in HIV-infected patients (1, 11). PcP ultimately develops on one or more occasions in at least 80% of AIDS patients, and is reported to be the major identifiable cause of death in at least 25% of AIDS patients. Moreover, a radiographically diffuse bilateral pneumonia, which is characterized by an eosinophilic intra-alveolar exudates and a mild plasma cell interstitial pneumonitis previously considered rare, is being recognized increasing (32).

Even though *P. jirovecii* is presently gaining more consideration, the metabolism, molecular biology and even a life cycle of this microorganism are still poorly understood (33). Remarkably few studies of its biochemistry and metabolic activity have been reported so far. The lack of such knowledge on this microorganism lead to scarcity of experimental approaches to rational chemotherapy and extent other research areas.

2.1.1 Taxonomy

The taxonomy of *P. jirovecii* has long been uncertain. Developing from a small unicellular "trophic form" into a "spore case" containing eight "spores", its life cycle superficially resembles those seen both in the Protozoa and Fungi (34). Morphological and ultra structural observation has led some investigators to conclude that the organism is a protozoan while others have suggested that it more closely resembles a fungus (35). Using phylogenic analysis of *Pneumocystis* SSU rRNA, it has recently been shown that *P. jirovecii* is probably fungus (36). The *Pneumocystis* SSU rRNA were shown the closer structural distances to fungi (*Neurospora crassa* and *Saccharomyces cerevisiae*) than to protozoa (*Dictyostelium discoidium* and *Trypanosoma brucei*). Analysis of other ribosomal RNA genes (5S, 5.8S, 26S) supported the microorganism closely related to fungi. Furthermore, sequence analysis of genes coding for thymidylate synthase (TS) (37), dihydrofolate reductase (DHFR) (38) and mitochondrial proteins all revealed a closer relationship to fungi (39). The *P. jirovecii* TS and DHFR genes are found on separated genes, as in fungi, in contrast to protozoa, where the coding sequences for these proteins are present as a single gene. However, the structure and antimicrobial susceptibility patterns of *P. jirovecii* differ a great deal from those other pathogenic fungi. Thus, whether this organism comprises a unique genus or family of fungi or is merely a unique pathogen within a known family or genus remains to be determined.

The taxonomic classification of *P. jirovecii* has been the object of considerable debate, with the majority of investigators favoring in protozoa over a fungal classification. Elucidation of its taxonomic status has been hindered by the inability to grow the organism in non-animal systems. It can be grown on several tissue culture cell lines but is limited to a ten to fifteen-fold increase, and continuous passage has not been achieved (40). Recently, *P. jirovecii* has been propagated on an axenic culture medium, but only to levels similar to those attained in tissue culture (41). Moreover, this culture method cannot be reproducible by other group. Further development of cell-free culture systems may eventually aid in the classification of *P. jirovecii*.

2.1.2 Life cycle

The life cycle of *P. jirovecii* is still somewhat unclear which all information on the life cycle has come from the studies using electron microscopy (Figure 1). It is generally accepted to include four basic stages: trophic form, sporocyst, spore case, and spores. trophic form is 1-5 μm long, uninucleate, ameboid structures with a double layered wall. This form adheres to alveolar walls and is the most metabolically active form. Sporocysts are approximately 5 μm long, oval, smooth and have a thicker cell wall. Spore case is 4-6 μm long, with a three layered cell wall. Spore case is thought to be the major immunogenic stage, although shared epitopes with trophic form have been demonstrated. Spores usually eight in number are found within sporocyst and spore case. Environment is normally a reservoir source of this pathogen (42).

Based on these forms, Filice *et al*. have proposed a complex life cycle with both sexual and asexual phases (35). In the sexual phase, spore cases were inhaled by a susceptible host, then rupture and release spores. At least some of these spores are believed to be haploid, based on demonstration of structures indicative of meiotic division in early sporocyst. These haploid forms theoretically represent isogametes capable of copulation. Diploid trophic form resulting from this copulation then completing the sexual phase by develop into spore cases. About the asexual phase, which involves binary fission of trophic form, is thought to account for the majority of *P. jirovecii* multiplication within the lung. The fate of *P. jirovecii* outside of the host is unknown and the possibility of an environmental phase must also considered.

Most studies of the cell structure of *Pneumocystis* spp. have been carried out on human and rat infections or on short-term cultures of *P. jirovecii* and *P. carinii* f. sp. *carinii* isolated from infected lung. No morphological differences have been detected between human and rat *Pneumocystis* spp., nor among the forms described in various other animals.

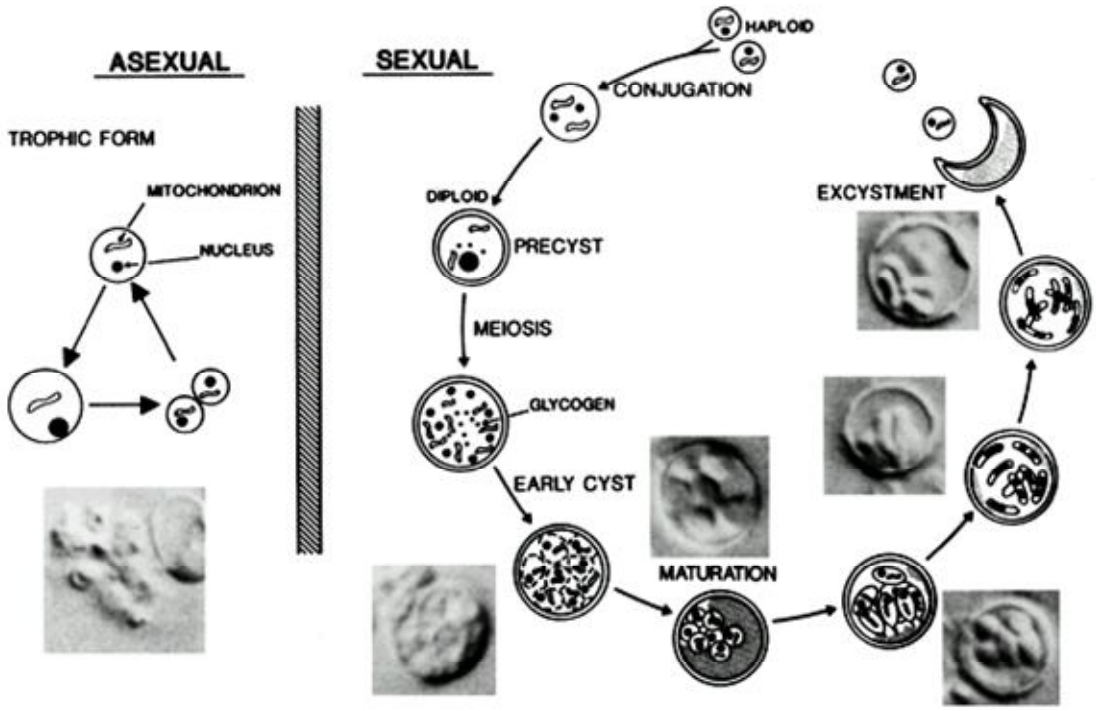


Figure 1. Proposed life cycle of *P. jirovecii*. Asexual phase: trophic forms replicate by mitosis sporocyte to spore. Sexual phase: haploid trophic forms conjugate and produce a zygote or sporocyte. The zygote undergoes meiosis and subsequent mitosis to produce eight haploid nuclei spores which exhibit different shapes (such as, spherical and elongated forms). It is postulated that elongation of the spores precedes release from the spore case. It is believed that the release occurs through a rent in the cell wall. After release, the empty spore case usually collapses, but retains some residual cytoplasm. A trophic stage, where the organisms probably multiply by binary fission is also recognized to exist (42, 71, 244).

2.1.2.1 Trophic stages (Trophic form)

The primary proliferative life cycle of *P. jirovecii* consists of small, haploid trophic forms that grow into large, diploid trophic forms and reproduce asexually by cell division (42-44). There is evidence for a process of binary fission from electron microscopy (35, 44-46), but no one has reported observation on cell division in living specimens. There is no clear evidence for a yeast-like budding process, from either light microscopic or ultrastructural studies.

When describing the morphology and ultrastructure of the trophic form of *P. jirovecii* that most investigators have not distinguished between small and large forms. Because relatively few investigators have specificially described the small trophic form (17, 43-45, 47-49). The detailed consideration of the cell structure of trophic forms will be given in the section on the large trophic form.

2.1.2.1.1 Small trophic form

The small trophic form is a very small cell (1.5-2 μm) when living cells are viewed by light microscopy in fresh preparations (45, 59, 50). It is round to ellipsoid in shape that probably a haploid progeny cell derived asexually by binary fission from a large parent cell. Additionally this form is likely the product of sexual reproduction and excystation. Morphologically and ultrastructurally, the small trophic form closely resembles the round form of spore. Compared with the large trophic form, the small trophic form has few or no tubular extensions of the cell surface. It has a small nucleus about 0.5 μm and its cytoplasm has less glycogen and fewer other inclusions.

2.1.2.1.2 Large trophic form

The large trophic form is about 3-5 μm in size and when living cells are viewed by light microscopy in fresh preparations (17, 45, 50). It is more irregular in shape than the small trophic form. Perhaps plasticity of shape depends on a combination of the cell size, the degree of cell turgor and thickness of the cell wall. Ultrastructural studies, many investigators have described the shape of

trophic forms as “ameboid” or “pleomorphic”, and frequently the shape of large trophic forms appears very irregular in electron micrographs. However, it is clear that the large trophic form of *P. jirovecii* is difficult to fix the cell wall. Usually, mitochondria are swollen and cristae are not preserved, and often the nuclear envelope is not well preserved. Frequently the cytoplasm appears extracted. These signs of poor fixation result from inadequate cross-linking and large fluxes of water and solutes which, in turn, lead to other artifacts during clinical specimen dehydration and polymerization of the plastic embedding material. Efforts to improve specimen preparation and the application of a variety of staining methods may yield additional information about the native cell structure of the large trophic form.

2.1.2.2 Sporocyst (Precyst)

The sporocyst is an intermediate stage of sexual reproduction during which a large parent cell (zygote) undergoes encystment (sporogenesis) (43, 46, 51-60). The parent cell is about 4-5 μm in diameter and has an ovoid shape and a large nucleus (about 2 μm). The cytoplasm and its complement of organelles resemble that of a large trophic form (61). Synaptonemal complexs, indicative of meiotic prophase, have been observed in this stage (17). As development progresses, the cell becomes more spherical and the cell wall becomes thicker by formation of an inner electron-lucent layer beneath the outer electron-dense layer. The surface shows few tubular expansions. The four haploid nuclei that are produced by meiosis probably divide by mitosis to form eight nuclei in the cytoplasm. As the nuclear divisions near completion, mitochondria become aggregated. The final stage of sporogenesis is the formation of eight spores. The cytoplasm becomes partitioned by infoldings of the cell membrane to enclose each nucleus plus a mitochondrion, some cytoplasmic to form progeny cells, and some endoplasmic reticulum. During this process not all of the cytoplasm of the parent cell is incorporated into progeny cells. The unincorporated residual cytoplasm remains at the periphery of the spore case against the thickened wall.

2.1.2.3 Spore case (Cyst)

At the completion of encystment, the mature spore case is round (about 5 μm in diameter), smooth (rarely shows tubular expansions), thick-walled (Figure 2, 4) and contain eight spores. The ultrastructure of this stage is well characterized and extensively documented because it tends to be well preserved by various fixation techniques. The spore case wall is thick (about 120-160 μm) owing to the development stages. Spore Case commonly show a focal thickening of the wall caused by a patch of increased development of the electron-lucent layer. The focal thickening is visible by light microscope in Gomori Methenamine Silver (GMS) stained specimens or by interference contrast microscope of unstained clinical specimens (17). There is considerable evidence that the thick inner layer is rich in glucan (62-66). De Stefano *et al*. found evidence for a lipid membrane-like component between the inner and outer layers of the wall (63, 67, 68). The outer dense layer probably contains some chitin (51, 69), and the outermost surface may consist of an irregular layer or fuzzy coat. In mature spore case, after formation of the progeny cells, residual cytoplasm, including some organelles and inclusions, remains as a peripheral layer inside the spore case wall.

2.1.2.4 Spores (Intracystic bodies)

The progeny cells within a mature, thick-walled reproductive spore case can display a variety of morphologies that can be distinguished by light microscope (70, 71). They may be round (resembling small trophic forms), or ellipsoid, or large (irregular in shape and tightly packed), or elongated (banana shaped) with very dense cytoplasm, perhaps caused by the loss of water (Figure 3). All these forms have been well documented at the ultrastructural level (70, 72-76). The significance of these variation is not understood. It has been suggested that the cytoplasmic densification of elongated from might indicate dormancy, making this form suited for transmission between hosts. The nucleus, cytoplasmic components, and cell surface of spores are essentially the same as trophic forms. There is a single report of apparent microtubules in a spore (70).

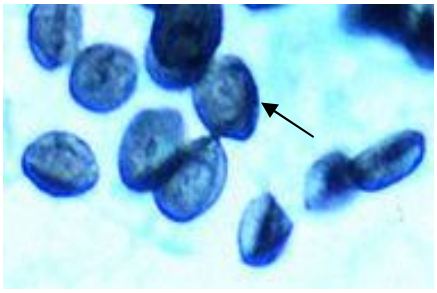


Figure 2. Human sputum with spore cases of *P. jirovecii*, the pathogen that causes lethal pneumonia in an immunocompromised patients. GMS stained, (Light Microscope, 1000X)

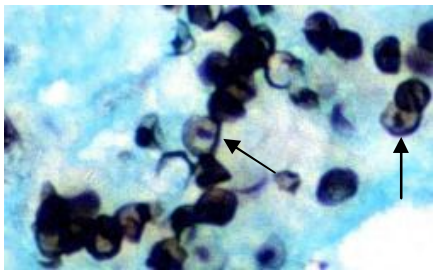


Figure 3. Representation morphology spore cases of *P. jirovecii*. Wall of spores were indicated by arrows. GMS stained, (Light Microscope, 400X)

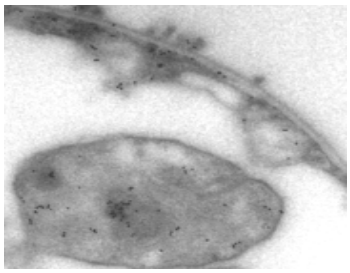


Figure 4. The spore case was appeared dark on the white background. The original magnification was X1500. *P. jirovecii* spore case with eight spores (Transmission Electron Microscope (TEM)), (From Wessel (75))

2.1.3 Biochemistry and molecular biology of *P. jirovecii*

Although *P. jirovecii* has been known to be a significant human pathogen for more than ninety years, very little is known about its basic genetic properties. Due to the inability to obtain the organism in large quantity, remarkably few studies of its biochemistry and metabolic activity have been reported so far. The very first work on this area was published by Pesanti and Cox (77). They found that *P. jirovecii* trophic form obtained by alveolar lavage of rats were able to metabolize [^{14}C]-glucose into $^{14}CO_2$ and inhibitable by excess unlabeled various sugar and iodoacetate which indicated the presence of complete glycolytic pathway. They also were shown that the organism has protein and ribonucleic acid (RNA) synthetic activities (77). More recently, Pesanti shown that the organism consumes oxygen by cyanide-sensitive cytochrome-mediated pathway and possesses a modest level of superoxide dismutase (SOD) but he failed to indicate the presence of other antioxidant enzymes: catalase and glutathione peroxidase. These studies have been ambiguous, however, in that case there is no certainly that the parameters measures truly reflect properties of *Pneumocystis* spp. rather than of other microbial components of the lavage sample studied (78). In 1987, Mazer and colleagues, by using histoenzymology method, had demonstrated the presence of lactate dehydrogenase (LDH), glutamate dehydrogenase and succinate dehydrogenase but not glucose-6-phosphate dehydrogenase in spore case of *P. jirovecii*. These results comfirms the presence of glycolysis pathway and probably functioning Krebs cycle in this organism (79).

Lipschik and colleagues (80) had tried to study polyamine metabolism in this organism but failed to demonstrate the ornithine decarboxylase (ODC) activity. However, they reported the incorporation of ornithine and arginine into putrescine and spermidine but not spermine and the incorporation could be inhibited by α-difluoromethyl ornithine (DMFO), an inhibitor of ODC (80). Furthermore, DMFO has been used in clinical trial on PcP with some success but still need lot more investigation before it can be approved as the main choice for chemotherapeutic treatment. There is increasing evidence that *P. jirovecii* cannot only transport and synthesize lipids from the simple precursors, but can also synthesize small amounts of

unique lipids for its own cellular functions. Besides, *P. jirovecii* has been found to possess 3-hydroxy-3-methylglutamyl co-enzyme A (HMG CoA) reductase. Interestingly but not surprisingly, the organism have also been shown to possess glucan synthesis activity, which is normally present in fungi (81).

One of the key metabolic pathways that have been gaining most interest is the folate pathway. The effectiveness of antifolate compounds such as TMP-SMX suggested the presence of target enzymes (82), DHFR and dihydropteroate synthase (DHPS). However, *P. jirovecii* has been shown to be capable to incorporate radiolabelled p-aminobenzoic (PABA) into reduced folates which indicates the presence of the *de novo* folate synthesis but not be able to salvage reduced form of folate from the environment (83). In order to obtain more knowledge on the folate metabolism and to enhance drug design and testing, *P. jirovecii* DHFR has been partially purified directly from the organism harvested from rat lungs and also cloned, expressed in *E. coli*, purified and characterized (38). There are few other enzymes involving the folate metabolism that have already been cloned and expressed as active enzymes. These are TS, DHPS, dihydroneopterin aldolase (DHPA) and 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase (HPPK). If these enzymes were really active in cells and the presence of two other enzymes (dihydroneopterintriphosphate pyrophosphohydrolase and GTP cyclohydrolase) could be demonstrated, it will indicate the existence of a complete *de novo* folate synthesis pathway in this organism. Other genes have been identified are cation transporting ATPase, calmodulin, transcription factor IID, α-tubulin, β-tubulin, DNA polymerase, RNA polymerase I,II and III (84, 85).

2.1.4 Types of *P. jirovecii* microorganisms

In first time which *P. carinii* was described, it was thought that there was only one type of organism that was capable of infecting many different mammalian host species. Using molecular techniques, it has been shown that many different types of *P. carinii* organisms comprising a very heterogeneous group and has changed its name from *P. carinii* f. sp. *hominis* to *P. jirovecii*. The study of the host species-

related parasite genetic diversity raises the notion of host species non-specificity, except in *P. jirovecii* that can transmit only in human.

The genetic and functional differences between *Pneumocystis* spp. organisms from different host species are substantial, but many investigators are reluctant to assign new species name to these organisms at present. This conservative approach to nomenclature changes is understandable. Although designating new *Pneumocystis* species is currently unpopular, many researchers in the field have agreed that the single name *P. jirovecii* is inadequate at this juncture, and a trinomial nomenclature system which includes the Latin name of the host species were adopted for distinguishes the types of *Pneumocystis* spp. organisms such as *P. carinii* f. sp. *ratti* and *P. carinii* f. sp. *carinii* from rat, *P. carinii* f. sp. *mustelae* from ferret, *P. carinii* f. sp. *equi* from horse, *P. carinii* f. sp. *suis* from pig, *P. carinii* f. sp. *muris* from mouse and *P. carinii* f. sp. *oryctolagi* from rabbit (86, 87). Consequently, in keeping with the International Code of Botanical Nomenclature (ICBN), it is no longer correct, either biologically or taxonomically, to refer to the human *Pneumocystis* organism as *P. jirovecii* instead of *P. carinii* f. sp. *hominis*. In the broadcast database of *P. carinii* by DNA sequencing has shown that *P. carinii* gene sequence is for a 300 bp segment of mt LSU rRNA , which has been detected in organisms from nine host species (shrew, rabbit, ferret, mouse, rat, horse, pig, monkey and human) (88, 89). The sequence variation at this locus ranges between 4 to 27%. The most extensively studied pair of *Pneumocystis* spp. are those from human and rat, in which eight different loci have been compared at the sequence level (6, 37, 90-94). While characterization of chromosome sizes and gene sequences has demonstrated wildspread genetic variation within the genus *Pneumocystis*, the significance of these differences in terms of species identification is difficult to assess (95-101).

2.2 *P. jirovecii* infection/ pneumocystosis/ *Pneumocystis jirovecii* pneumonia

2.2.1 Clinical manifestations

Clinical manifestations of *P. jirovecii* infection in human were first described by Szmigiel *et al.* (101), PcP is characterized by increasing dyspnea that may occur only with exertion initially, but may progress to the point it occurs with talking or at rest (101). The mechanism of dyspnea is probably related to hypoxemia produced when alveoli are filled with *P. jirovecii* organisms and their related exudates. Fevers and cough are usually nonproductive, but some patients have sputum. Presenting symptoms also can include chest tightness or pain may related to a pneumothorax and hemoptysis (102). However, there are differences in the clinical presentation of PcP, depending on the patient's HIV status. In non HIV-infected population, the onset of symptoms may be followed within days by severe dyspnea and hypoxemia (103). In adults with immunosuppression unrelated to HIV, the median duration from onset of symptoms until diagnosis is in the range of five to ten days, compared with a median of twenty five or more days in HIV-infected patients. Whereas HIV-infected patients have more prolonged and insidious development of clinical disease, they have better oxygenation and smaller alveolar-to-arterial oxygen differences at the time of diagnosis than patients with other causes of immunosuppression (104-109).

2.2.2 Pathology and pathogenesis

Like other fungi, *P. jirovecii* is postulated to be acquired from the environmental sources, in this case, via air-bourne route (inhalation) (110). Most children are exposed to *P. jirovecii* by the age of three or four. Attempts in the past to infect rats with isolated organisms have failed suggesting that neither the spore case nor trophic form is the infective stage. However, some evidence on ultrastructural studies reveals that *P. jirovecii* attaches tenaciously to lung epithelial cells during development of pneumonitis with a preference for type I alveolar epithelial cells (55, 111). The mode of attachment of this organism still remains an enigma.

As in any infectious disease, the pathogenesis of PcP involved a complex interaction between the organism and its mammalian host. The specific processes involved are poorly understood. The pathogenesis of *P. jirovecii* pneumonia can be divided into five stages.

2.2.2.1 Establishment of the infection

The portal of entry for *P. jirovecii* is the respiratory tract. Many experimental studies have shown that *P. jirovecii* can be transmitted from person to another by the airborne route and can also be transmitted by intratracheal inoculation of infected lung homogenates (112).

Once inhaled, *P. jirovecii* does not appear to colonize in the upper airways, but is deposited within the alveolus. The precise steps in initiation of infection were unknown. The first step in this process is attachment to a specific host cell. It is unclear what *P. jirovecii* inoculum size is needed to establish the infection or after the infection has been established, how long the organism remains in the host (113).

2.2.2.2 Organism proliferation

The specific host immune defects that permit proliferation of *P. jirovecii* in the lung have not been elucidated. Its low virulence and the lack of a reliable *in vitro* cultivation system have hindered direct organism challenge experiment or cytotoxicity assays. Impaired cellular immunity is generally considered to be the major predisposing factor, based on the following lines of evidence: the underlying disease in *P. jirovecii* patients. Proliferation appears to involve asexual and sexual phases of the life cycle (114). Trophic forms multiply by binary fission, whereas spore case development involves the fusion of gametes and a series of stages that culminate in the formation of spores; when these spores were released from the spore case, they develop into trophic forms. Little is known about the factors that influence encystations or excystation. Although the major site of *P. jirovecii* proliferation is the lung, the rising numbers of cases of disseminated pneumocystosis being reported in AIDS patients raise the intriguing possibility of parts of the

organism's life cycle occurring at extrapulmonary sites (115). Investigation of this phenomenon might provide clues about *P. jirovecii* growth characteristics.

2.2.2.3 Changes in the alveolar microenvironment

The development of pneumocystosis is accompanied by anatomical and physiological or biochemical changes in the host but it has been difficult to establish a definite cause and effect relation. There is little evidence that *P. jirovecii* is invasive, has an intracellular phase in its developmental cycle or secretes cytotoxic factors. The underlying conditions that predispose to pneumocystosis may produce broad changes in the host and other opportunistic infections may also be present. However, with the use of animal models and careful controls, alteration of the host attributable to *P. jirovecii* have been delineated.

2.2.2.4 Anatomical changes

The principal finding on hamatoxylin-eosin (H&E)-stained lung sections is the foamy, vacuolated alveolar exudate. In corticosteroid-treated rats, this exudate develops with progressive *P. jirovecii* replication and consists of organisms, degenerative cell membranes, surfactant, and host protein (115). Alveolar macrophages are present but not conspicuous. The alveolar septa become thickened with hypertrophy and hyperplasia of the type II cells, interstitial edema and a mild mononuclear cell infiltrate.

2.2.2.5 Physiological and biochemical changes

The major finding of pneumocystosis have been impaired gas exchange, altered lung compliance and mechanics, and a decline in surfactant phospholipids in bronchoalveolar lavage fluid (BALF). Among the phospholipid constituents, there appears to be a fall in phostatidylcholine and a rise in sphingomyelin (116-119). The physiological and biochemical changes in humans with pneumocystosis are generally similar to those in the rat model, but the types of studies have been different. Emphasis in human studies has been placed on arterial blood gas measurements because they are easy to perform and have important clinical significance. Pneumocystosis patient leads to damage of respiratory cell; deficiency of

surfactant phospholipids, which results in changes in alveolar surface tension, lung distensibility, and ventilation-perfusion abnormalities. The degree of hypoxemia has been the most widely used marker for disease severity and prognosis (117).

2.3 Epidemiology and transmission of *P. jirovecii*

2.3.1 Reactivation of latent infection

The reactivation of latent infection theory postulates that individuals are exposed to *P. jirovecii* early in childhood. Although the immune system is able to ward off clinical infection, the organism remains latent within the host. Subsequently, if the host's immune system fails, the latent *P. jirovecii* can be reactivate and cause clinical infection. Several lines of evidence including the seroprevalence of anti-*Pneumocystis* antibodies, the high rate of PcP among HIV-infected infants, the demonstration of *P. jirovecii* in respiratory specimens fron immunocompetent hosts, and the characteristics of the organism itself have been considered to be evidence in support of this theory. *P. jirovecii* appears to be a ubiquitous organism. Evidence that *P. jirovecii* is common derives from the demonstration that a majority of children develop anti-*Pneumocystis* antibodies early in life, and the prevalence of these antibodies appears to increase with age (120-124). One series found that healthy children with no detectable anti-*Pneumocystis* antibodies at birth or in the first three months of life began to demonstrate a positive antibody titer at seven months of age (125-128).

In contrast to the studies described above, multiple studies have failed to find the organism on autopsy, in lung tissue, or in respiratory clinical specimens from non-immunocopmpromised individuals. Wakefield was unable to find evidence of *P. jirovecii* in BALF fluid from ten healthy subjects using PCR (129). Peters replicated this result in post-mortem lung tissue from fifteen immunocompetent adults (130). Contini *et al.* studied a group of children with normal immune systems and found that

none of fifteen subjects had *P. jirovecii* detected by PCR of nasopharyngeal aspirates (131). Studies using PCR to detect *P. jirovecii* DNA in non-immunocompromised animals have been similarly unsuccessful. Although small numbers of subjects may have resulted in sampling error, if long-term carriage of *P. jirovecii* occurred in the majority of people, then one would expect studies using these sensitive methods to detect the organism's DNA in at least some proportion of the population.

2.3.2. Active acquisition of infection (Environmental exposure and person-to-person transmission)

If latent infection is not the sole mechanism for infection with *P. jirovecii,* then the question of how new infection arises needs to be answered. Two possibilities exist: either patients become infected after exposure to an environmental source of the organism or they become infected after exposure to another person carrying the organism.

Several studies have detected *P. jirovecii* DNA in environmental specimens. One study in the United States reported sequencing the DNA of human *P. jirovecii* from pond water specimens (132). Specimens taken from puddles and from a river did not contain *P. jirovecii* sequences. Wakefield was set up air filters in both rural and urban settings in an attempt to find airborne both rat- and human-specific forms of *Pneumocystis* spp. DNA (133). Another possible source of *P. jirovecii* in the environment is the soil. *P. jirovecii* is classified as a fungus based on biochemical data and genetic analysis, and fungi commonly inhabit in the soil. Outbreaks of fungal infections such as blastomycosis, coccidioidomycosis and histoplasmosis have been linked to places of soil disturbance. Navin *et al.* reported that patients with documented PcP were much more likely to have a history of recent camping, traveling, swimming or gardening and CD4 cell count-matched control (134). Potentially, these variations may be related to different degrees of soil exposure within urban area. Future studies performing environmental sampling and tracing outbreaks through genetic patterns of *P. jirovecii* are necessary to elaborate on environmental risks for PcP. The possibility that *P. jirovecii* can be transmitted from person to person has been raised since the first reports of outbreak in transplant units and oncology.

Singer originally reported a series of eleven patients with various malignancies, all of whom were diagnosed with PcP over a three month period at a New York hospital (135). Many of these patients had contact with each other, suggesting that they might have transmitted the disease to one another. Alternatively, they might have shared a common environmental exposure. Other reports have shown similar clustering of PcP cases among patients with hematological malignancies and transplant patients. One report documented of PcP in the non-immunocompromised wife of an immunocompromised man with PcP. Possibly, they shared either a common exposure to *P. jirovecii* or the husband transmitted the organism to his wife (29). A series of PcP cases among renal transplant patients is of particular interest because the transplant patients shared a waiting room and clinic with HIV-infected patients, and there was some suggestion that the transplant patients with PcP had spent more time at the clinic than the transplant patients without PcP (14). Molecular genotyping of *P. jirovecii* specimens has been employed to find links between patients in clusters of PcP. Helweg-Larsen *et al.* described clusters of HIV-infected patients with PcP and patients with hematological malignancies and PcP (136). Genotypic analysis of the *P. jirovecii* from these patients demonstrate that four of fourteen patients shared the same type and had also been housed in the same room or hospital ward (136). Although this study raises the possibility of person-to-person transmission, many of the patients studied did not shared the same genotype, indicating that person-to-person transmission may not be the only or even the most important route of transmission of PcP (136). Recently, report shown that nasopharyngeal aspirates taken from three healthy contacts of pediatric patients with PcP transiently contained *P. jirovecii* DNA, although genotyping was not performed on these samples.

In case of human transmission is occurring, it probably takes place via an airborne route. Multiple human studies have shown that *P. jirovecii* DNA can be recovered from air samples taken from patient rooms. Olsson *et al.* reported that four out of five specimens taken from patient rooms had genotypes identical to the patients (137). The correlation of air specimens with infection is not absolute, however, as studies have also found *P. jirovecii* DNA in air specimens collected in areas remote from infected individuals (138). Hospital air specimens from non-patient-care areas

may contain *P. jirovecii* DNA. It is unclear if *P. jirovecii* in hospital air specimens is derived from environmental sources or from infected persons.

Additional evidence of host-to-host transmission via an airborne route is provided by animal studies. Air filters containing *P. carinii* can transmit the agent where placed in animal rooms, and exposure to non-sterile air is essential for the development of the disease (139-140). Immunosuppressed rats maintained in an environment with sterile are does not develop PcP, but those rats placed in a room with unfiltered air develop PcP. Other studies have found that immunosuppressed mice and rats develop PcP only after being housed with infected animals (141-142). Similar results have been reported in macaques with simian immunodeficiency virus (SIV). These animals do not become infected with *P. carinii* while in isolator cages, but develop PcP if housed with other macaques (143). Further evidence of airborne transmission is found in studies of normal rats that transiently become colonized with *P. carinii* when placed in rooms with infected and immunosuppressed rats. The healthy rats rapidly clear the organism from their lungs when removed from infected colonies (144). Whether these animal models accurately reflect human infection is currently unknown.

Exposure to *P. jirovecii* can also be documented by assessing serological response to the organism. However, the serum examination of anti-*P. jirovecii* antibodies has yielded conflicting results about the possibilities of person-to-person transmission. Lundgren *et al.* found no difference in serum antibody titers between health care workers who were exposed to patients who infected *P. jirovecii* and those who were not exposed (145). This result debated with the earlier work done by Leigh *et al*. that found advanced *P. jirovecii* titers were found in health care workers exposed to patients who infected *P. jirovecii*, but not in workers without similar exposure (146). The overall prevalence of positive antibodies was 96% in the exposed population. Differences in degree of patients exposure and in timing of specimens collection may explain the contradictory results reported by these authors. Example, if the health care workers in one study spent more time in the patients' rooms, they

might be more likely to develop an antibody response than workers who were in the room for a brief time.

2.4 Immunity response to *P. jirovecii*

It had long been thought that the host inflammatory or immune response plays little or no role in the pathogenesis of PcP (147). This assumption was based on the fact that immunosuppressive agents are used to induce pneumocystosis and the host inflammatory changes on histological examination are inconspicuous. It was only after withdrawal of corticosteroid in experimental pneumocystosis that the rats developed a vigorous response and cleared the organism from the lung. The process included phagocytosis of *P. jirovecii* by alveolar macrophages, the development lymphocytic infiltrates in peribronchial and perivascular areas and systemic lymphocytosis which continued proliferation of type II cells, a rise in local and systemic antibodies to the organism. Interstitial fibrosis also developed, thereby supporting the findings in humans that recovery was accompanied by evidence of lung damage. However, in recent years, the occurrence of pneumocystosis in HIV-infected patients has raised the possibility that the host immune response or inflammatory may be deleterious. This hypothesis is supported by the deterioration in blood oxygenation soon after starting anti-*P. jirovecii* drugs; the effectiveness of corticosteroids in preventing this deterioration and in improving survival when given with anti-*P. jirovecii* drugs; and the association of increased neutrophils in BALF of patients with poor prognosis. Insights into the cells mediating this host response have been gained from studies of immunodeficient mice that develop PcP without the need for corticosteroids. Severe Combined Immunodeficiency (SCID) mice with pneumocystosis, which were immunologically reconstituted by bone marrow transplantation or infusion of CD4 cells, experienced early deaths from a hyperinflammatory reaction and then cleared *P. jirovecii* from their lungs (148). Other investigators have found that CD4 cell-depleted mice could also mount an

inflammatory response to *P. jirovecii*. The histological picture in these studies resembled that found in rats when steroids were withdrawn. These data illustrate the helpful and harmful effects of the immune response or host inflammatory. CD4 cells and other as yet undefined cell types can mediate this response, that these cells tend to congregate at specific regions of the lungs (i.e., peribronchial and perivascular areas) suggests that they may exert their effects by cytokines. The practical application of this work is that immunomodulators, which have far more specific effects on the host than corticosteroids, may play an important role in the treatment of pneumocystosis (147-149).

2.4.1 Humoral immunity

Although the exact role of antibodies in the host response to *P. jirovecii* infection is unknown, a specific humoral response has been documented in association with this infection. Antibodies to *P. jirovecii* are present in most of the population (121-123, 150-151). Anti-*P. jirovecii* antibodies increase in frequency in early childhood, suggesting exposure to the organism at an early age. Specific anti-*P. jirovecii* antibody responses were investigated by indirect immunofluorescent assay (IFA) in twenty three cases of proven or clinically suspected pneumocystosis among patients of a British oncology unit (152). In this group, ten patients demonstrated titers higher than 1:32, five patients shown a fivefold rise in antibody titer, three converted from negative to a 1:8 titer, and one proven case remained negative. Among ninety one controls, 56% demonstrated antibodies, with the highest titer being 1:32. A subsequent study, using the same infected human lung sections as antigen, detected antibodies at a titer of 1:8, or higher, in 48% of nonimmunosuppressed children (153). A trend to more of the older children having higher levels of antibody was noted. A report in the United States examined serum antibodies of children and adults in low- and high-risk groups for PcP by IFA and immunoblotting (121). Antibodies to the 40-kDa antigen were the most common antibody response detected in all groups. Antibodies to any *P. jirovecii* antigen were found in 86% of children by thirty months of age. Antibodies to *P. jirovecii* were found in 74% of adults, but were detected more frequently in healthy controls than in immunosuppressed patients. HIV-infected

patients who experienced one or more episodes of PcP were studied sequentially for changes in the antibody response. Active serum IgG or IgM antibody responses, as defined by the appearance of a new band or an increase in staining intensity of an existing band, were detected in 12-93% of patients, depending on the antigen examined. Another study investigated serological (IgG) responses to purified gp95, the major surface antigen of human *P. jirovecii* (154). The frequency of serum antibodies to this antigen was significantly higher in HIV-infected patients with pneumocystosis than in HIV-infected patients without the disease or in healthy controls. Sequential analysis of the *P. jirovecii* patients demonstrated that 43% of these individuals mounted an antibody response to gp95. Thus, although the significance of the humoral response is unknown in human disease, some patients mount an active response in the presence of infection. The antibody response in the presence of acute disease can include both IgG and IgM antibodies. Characterization of the response to specific purified or recombinant antigens may serve to enhance our understanding of the pathobiology of the disease and may be of prognostic or diagnostic value.

2.4.2 Cellular immunity

In most of the cases of human pneumocystosis, the disease occurs in hosts with underlying conditions that depress the humoral and cellular immune responses (155). The cell-mediated branch of the immune system is now considered the major component in the host's ability to resist PcP. Studies in an animal models of pneumocystosis have provided inferential as well as direct experimental data supporting this conclusion. Some of the more pertinent aspect of these studies has been presented in the following sections. *In vitro* proliferative responses of lymphocytes from normal and immunocompromised human subjects to *P. carinii* have been conducted to gain insight into the host's cellular immune response to this organism (155-157). *P. carinii* was obtained from rats and grown in tissue culture were used to test proliferative responses of peripheral blood mononuclear cells (PBMC) of normal individuals and patients at different stages of HIV infection (158). The PBMC from the normal adults shown a good response to the culture *P. carinii*; HIV class II (asymptomatic) and class III (lymphadenopathy) patients shown a lower

response; and class IV (HIV-infected patients) demonstrated the lowest response. In fact that PBMC from normal individuals responsed suggests that most humans have been sensitized to *P. carinii* antigens. The observation is consistent with serological studies that show that most normal adults have detectable antibody against the organism. A significant proliferative response was observed in PBMC from 83% of normal individuals when *P. carinii* antigen preparation was obtained from infected human lung tissue and used in a blastogenic assay. No response was seen in PBMC from any of the HIV-infected patients tested in this study, even though a high percentage of these individuals were diagnosed as having PcP. Studies in which levels of serum antibodies were tested, no correlation between the serum antibody titers and the degree of proliferative responses to *P. carinii* in individual patients was found, suggesting that the humoral and cellular responsive elements may be directed toward different epitopes of the organism. In another study, using human and rat alveolar macrophages and rat *P. carinii*, it was shown that the macrophage mannose receptor is important to the cell's ability to bind and uptake *P. carinii.* It is possible that in the diseased state, the mannose-rich surface proteins on *P. carinii* are inaccessible; this could be due to blocking components (i.e., surfactant, antibodies), or the proteins could be lost by shedding. Instead of antibodies to *P. carinii* acting as opsonins (and facilitating *P. carinii* ingestion), they could interfere with the binding of the mannose receptor of the macrophages to this organism (159). Furthermore, potential shedding of the major protein could down-regulate the expression and activity of the macrophage mannose receptor, causing inhibition of the parasite-host interaction. Thus, the interaction between macrophages and *P. carinii* is complex, and additional in vitro as well as in vivo studies are needed to better define the factors that play an important role in the ultimate outcome of this interaction. The roles of specific cytokines, such as tumor necrotic factor (TNF) and interferon gamma (IFN-γ), have on the *P. carinii*-killing activity of macrophages. Information about the role of polymorphonuclear leukocytes in the host defenses against *P. carinii* is limited (158, 159). There have been a few cases reported in which PcP has been seen in patients with granulomatous disease. In yet another report, leukocyte transfusion resulted in recovery from the PcP. On the other hand, an increase in neutrophils in BALF of some

P. carinii-infected patients is often associated with advanced disease and poor prognosis. It is obvious that further studies are needed to determine what function, if any, polymorphonuclear leukocytes have in the host response to PcP (160).

The role that natural killer cell (NK cell) in defending against PcP is also poor understood (161). A case of severe PcP was reported in a patient with depressed NK cell cytotoxicity and hypogammaglobulinemia (decreased levels of IgA and IgG). From these findings, suggested that NK cell may be important in defense against *P. jirovecii* infections. The fact that NK cell have role in killing pathogens, including fungi, such as *Cryptococcus neofermans*, and also in the regulation of the immune response, indicate that more studies are needed on the contribution of these cells to the innate resistance to pneumocystosis (162, 163).

2.5 Treatment of pneumocystosis

Standard treatment regimens for PcP include either TMP-SMX, administered orally or intravenously, or parenteral pentamidine isethionate. Both agents appear to have similar efficacy in both HIV-infected and HIV-uninfected patients, although the spectrum of adverse effects is quite different. In addition, successful therapy for PcP in AIDS generally requires up to twenty one days of either agent, rather than the standard fourteen days regimen used in other immunosuppressed patients.

2.5.1 Trimethoprim-sulfamethoxazole

The treatment for PcP, TMP-SMX is the generally agent of choice in patients without a history of life-threatening intolerance, because of its comparable efficacy but lower incidence of severe adverse effects (163). The efficacy of this agent for treating PcP was first reported in 1975, in a subsequent prospective, randomized, pediatric study comparing TMP-SMX had equivalent efficacy and the advantages of oral administration, improved tolerance, and fewer adverse effects (164). TMP-SMX

is a combination of two antimicrobial agents which act at different sites to inhibit folate synthesis. Trimethoprim inhibits DHFR, which converts dihydrofolate to tetrahydrofolate. The sulfa component inhibits the enzyme DHPS, which catalyzes the conversion of PABA to dihydropteroate. The most important effect of this sequential inhibition is the interruption of thymidine synthesis in susceptible organisms (163). A combination of agents with differing sites of action has the theoretical potential to produce synergistic efficacy while reducing the likelihood of the emergence of resistant organisms. Thus, TMP-SMX possesses several pharmacological features that make it an attractive choice of therapy. A series of prospective and retrospective studies of patients with PcP, both HIV-infected and HIV-uninfected patients, demonstrates a response rate for TMP-SMX ranging from 60% to 90% (165-168). Some of the variability in these response rates may due to differences in patients selection, drug administration and extent of illness.

2.5.2 Pentamidine

Pentamidine belongs to the diamidine class of compounds and has broad-spectrum antimicrobial activity. It was first used to treat PcP in the late of 1950s when, in one study of 212 premature infants with PcP, pentamidinereduced the case fatality rate from 50% to 3.5% (169). Subsequent reports have confirmed the efficacy of pentamidine in immunosuppressed children and adults with documented PcP and in newborns (170). Pentamidine administered either intravenously or intramuscularly is associated with minor adverse effects in virtually 100% of patients and with major toxicity requiring discontinuation of therapy in up to 47% of patients (166, 171, 172). In one retrospective chart review comparing PcP in HIV-infected patients with PcP in patients with other immunodeficiencies. 11% of HIV-infected patients who received pentamidine as initial therapy required institution of an alternative agent because of adverse effects, compared with none of the patients with other cause of immunodeficiency. The most severe, dose limiting toxicities associated with pentamidine include pancreatitis, azotemia, dysglycemia, leucopenia and hypocalcemia, as well as those less frequently observed, such as thrombocytopenia, nausea, vomiting, orthostatic hypotension and ventricular tachyarrhythmias. Some of

these side effects, such as azotemia, may remit promptly after dose reduction or discontinuation. Other, however, such as pancreatic dysfunction, may be profound, life-threatening and irreversible (173). When administered intramuscularly, pentamidine may cause local adverse effects, such as inflammation, sterile abscesses, pain in up to 20% of patients. In some instances these abscesses may progress to ulceration and require skin grafting. Since the safety of slow intravenous administration is now well established, pentamidine should be given intramuscularly only in unusual circumstances.

2.5.3 Dapsone

Even though pentamidine and TMP-SMX are the only drugs licensed for the treatment of PcP, other agents appear to be safe and are reasonable second-line agents in patients failing or intolerant of standard therapy. Dapsone alone and in combination with trimethoprim has been used to treat PcP in HIV-infected patients. In one uncontrolled study of oral dapsone used to treat eighteen HIV-infected patients with documented PcP. Eleven patients responded within three to ten days of instituting therapy and successfully completed the twenty one day course. The non-responders had more severe pneumonitis and switched to alternative therapy after a mean of 5.8 days. A more recent study of dapsone monotherapy in patients with mild to moderate PcP found that none of the seven patients enrolled successfully completed a full course of therapy (174). After receiving five or more days of dapsone, orally, three patients had worsening respiratory failure, requiring mechanical ventilation in two, and four patients developed a severe toxic reaction and required alternative therapy.

2.6 Laboratory diagnosis of pneumocystosis

2.6.1 Microscopic examination

There are several major issues when discussing the "best" stain of human specimens. One has to consider ease of performance, specificity, sensitivity, rapidity of the stain and cost.

In the years before, the gold standard of diagnosis has been the GMS. This reagent, originally developed to stain fungi, selectively stains the *P. carinii* spore case wall (175). Characteristics of *P. carinii* include being about the size of red blood cell, the appearance resembling and a central dimpling that of a crushed table tennis ball (176). Usually the *P. carinii* spore cases are found in clusters, often with associated foamy material. Although considered the standard test for many years, it is not 100% sensitivity. GMS staining have been several studies showing false-negative results. In one comparative study, seven of sixty three (11%) BALF clinical specimens from patients with PcP had negative silver stains, but positive foamy material seen on Papanicolaou's stain (177). Although, the traditional silver stain usually is done overnight, modifications of the stain have led to stains being completed in two hours (178). The use of the silver stain permits the absolute counting of individual spore cases, and has been used to provide quantitation of *P. carinii* in BALF (179).

The Wright-Giemsa stain and its modification (Diff-Quik) have been used for several years to identify *P. jirovecii*. This stain does not stain the spore case wall, but in fact stain the nuclei of trophic form and spore stages. The stain has been used to quantitate the number of organisms present in BALF specimens by counting the number of clusters present (180). This has been used to follow a patient's response to therapy, since successful therapy for *P. jirovecii* is associated with more than 50% drop in cluster count (181). The stain can be performed on an air-dried specimen. Usually it is performed on a cytocentrifuge prepared specimen such as BALF or an air-dried smear such as sputum. Giemsa stain technique has also been used to characterize the inflammatory response in BALF of many diseases including idiopathic pulmonary fibrosis (182). The interpretation of the BALF specimen requires

some expertise in differentiating the various inflammatory cells, especially differentiation of the lymphocyte from the immature, monocyte-like macrophage often encountered in acute inflammation. With standard criteria, one is able to produce a reliable differential cell count of the cells retrieved by BALF. This differential cell count has been used to characterize the inflammatory response around *P. jirovecii*. Several authors have found the neutrophils in BALF are associated with more severe hypoxemia and a worse overall prognosis from the PcP (183, 184).

IFA stains have become wildly available for the specific diagnosis of *P. jirovecii* and used in most studies on humoral responses to this pathogen (185). This technique, which is dependent on the expertise of the operator in recognizing *P. jirovecii* organisms, has good specificity if fluorescence is recorded to spore case forms only. An initial study by Brzosko and Nowoslawski used formalin-fixed section of infected lung, as the antigen, in studies of children with the epidemic form of pneumocystosis (186). This, and later by the same authors, concluded that IFA was as reliable as complement fixation (187). Later work demonstrated that IFA was superior in immunocompromised individuals, since complement-fixing antibodies were often not detectable. In the Polish studies, all forty three infants with clinical suspected or documented demonstrated to *P. carinii.* When these cases were segregated by age, IgM antibodies alone were found in those under eight weeks, combinations of IgM and IgG antibodies in some children three to six months old, and only IgG responses were detected those children older than six months of age. Antibodies to *P. carinii* were detected in only two of thirty seven asymptomatic controls younger than two years and three of thirteen infants with mild respiratory symptoms. For the less-than-optimal specimens of BALF, bronchial wash and sputum, the fluorescent stains have increased sensitivity and specificity over the GMS and Wright-Giemsa stain (188-192). There are several different stains, involving both direct immunofluorescent assay (DFA) and IFA techniques. The antibodies used are against major cell wall and surface antigen of *P. jirovecii*. Many of the kits use the monoclonal antibodies developed by Kovacs *et al* (189). Two reports directly compared the DFA versus IFA techniques. In each study, the DFA was more sensitive, but the difference was not significant (185).

2.6.2 Molecular detection of *P. jirovecii*

The detection of *P. jirovecii* for diagnostic purposes has utilized histochemical staining of respiratory clinical specimens with GMS staining (193-195). In order to obtain sufficient organisms, this has required invasive specimens from the alveoli, originally transbronchial biopsy specimens, and subsequently BALF. IFA staining with anti-*Pneumocystis* antibodies has also been used successfully on this type of specimen (196-199). Recently, Molecular techniques have been developed for detection of *P. jirovecii.* Since *P. jirovecii* cannot be cultured *in vitro***,** pure isolates of the parasite cannot be obtained. Only relatively small amounts of the organism are recovered from clinical specimens and many conventional methods of genotyping cannot be applied to these specimens. The technique of DNA amplification using the PCR has overcome the problem of low numbers of organisms. Amplification of *P. jirovecii* DNA using the PCR technique which most widely used locus for the detection of *P. jirovecii* DNA is the gene encoding the mt LSU rRNA which has been shown to be a sensitive and specific means of detection using PCR (90, 199). A number of other genes have used as a target for a PCR assay but some of these primers are not entirely specific for *P. jirovecii* (200).

Since the original work on the detection of *P. jirovecii* in BALF, the PCR assay has been applied to other respiratory clinical specimens both invasive and non-invasive specimens such as induced sputum, bronchial wash, nasopharyngeal aspirates and oropharyngeal washes. In these respiratory tract clinical specimens, where the number of *P. jirovecii* organisms is low, PCR methods have been successfully developed. Oropharyngeal washes can be carried out on severely ill patients who are unable to sustain more invasive diagnostic procedures and nasopharyngeal aspirates can be easily obtained from paediatric patients. Both types of clinical specimens have been successfully use for the diagnosis of PcP with the PCR assay (201-205). Studies in control groups of both immunocompromised and immunocompetent individuals without PcP have been carried out to test the utility of these non-invasive upper respiratory tract clinical specimens for the diagnosis of PcP. *P. jirovecii* DNA was not detected in the clinical specimens from these control groups (206). PCR detection of

P. jirovecii DNA in blood or serum clinical specimens has shown conflicting results. The use of these specimens for diagnosis of PcP remains to be established, although in rare cases of disseminated *P. jirovecii* infection, PCR on blood clinical specimens may show *P. jirovecii* DNA (207-209). Although PCR detection of *P. jirovecii* DNA in respiratory tract specimens has proved to be very high diagnostic value and successful for detection *P. jirovecii* DNA, it is present primarily restricted to research laboratories, and is not frequently used in diagnostic services. However, PCR has also been found to be a highly specific and sensitive technique for the detection of *P. jirovecii* DNA for epidemiological studies, including the detection of this organism in environmental samples (210). Real-time Polymerase Chain Reaction (Real-time PCR) is a real-time and can be on-line analysis of PCR products produced during amplification in a closed system. There are many fluorescence formats for on-line PCR monitoring, such as hybridization probes (LightCycler^{TM}, Roche Diagnostics) (211). The LightCycler is an ultra-rapid thermal cycler, which its advantages to monitor the amplification of PCR product in real-time and on-line analysis. Furthermore, the LightCycler system also provides an innovation and unique approach perfectly result for detection and study of genetic diversity by using the melting curve analysis feature. A fast and post PCR high throughput technique to scan for sequence variation in a target gene. Single base changes in the target amplicons are detected by their altered melting temperature which is monitored through the release of fluorescent dsDNA binding dye (212). These altered melting properties give rise to changes in the shape of melting curve when compared with a reference sample. High resolution melt (HRM) analysis is an extension of previous DNA dissociation (or "melting") analyses. It is used to characterize DNA samples according to their dissociation behavior as they transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperature. A HRM instrument collects fluorescent signals with much greater optical and thermal precision than previous methods to create new application possibilities (213). HRM analysis is a post PCR technique for study of high throughput mutation scanning and genotyping analysis that a new and attractive gene scan tool for quickly performs the PCR and identifies sequence alterations without requiring post-PCR treatment. Several HRM methods for mutation detection of human genetic

disease and polymorphism have been further reported (214). The technique are involves a standard PCR reaction and use of a double stranded DNA binding dye such as Light Cycler® 480 ResoLight Dye that have low toxicity in an amplification reaction and therefore be use at higher concentrations for greater saturation of the dsDNA sample. Greater dye saturation means measured fluorescent signals have higher fidelity, apparently because there is less dynamic dye redistribution to non-denatured regions of the nucleic strand during melting and because dyes do not favor higher melting temperature products (215). When PCR product was melted each PCR product will be exhibited a characteristic melting or disassociated behavior (216). DNA sequence analysis is particularly adapted to and has been widely used for the study of microorganisms at the evolutionary level because nucleotide sequence allows direct measurement of genetic distances. Sequencing of the same genomic regions of *P. carinii* organisms infecting different hosts, in particular of the variable region of the mitochondrial 26S rRNA (mt 26S) has revealed the divergence at the nucleotide sequence level between f. sp. of different hosts and types within the same host. The results are in agreement with those of karyotyping and confirm the existence of one *P. carinii* f. sp. in each of the following host such as human (217), mice (218, 219), ferret (220) and of other f. sp. specific to horses (90), pigs (221), rabbits (219) and shrews (89, 222, 223). In addition sequence analysis revealed that two different special forms can be found not only in rats but also in ferrets (220-224). The results suggest that the special forms are on different evolutive branches. Comparison of gene sequences was used to investigate *P. jirovecii* (*P. carinii* f. sp. *hominis*) phylogeny, and suggested that *P. jirovecii* organisms constitute a unique branch between the ascomycetes and basidiomycetes in the fungal kingdom. This reason was clearly distinguished *P. jirovecii* (*P. carinii* f. sp. *hominis*) from other special forms of this pathogen. DNA sequence analysis of PCR products carrying variable region of the genome have been developed to type *P. jirovecii* because of their ability to detect polymorphisms with a high sensitivity. Five of the seven genomic loci have been investigated for *P. jirovecii* were reported to be polymorphic. Out of these five loci, two internal transcribed spacers of the nuclear rRNA genes operon (ITS1 and ITS2) (225-228), mt LSU rRNA (229, 230) and mt 26S gene (231-235) proved to be the most useful for study of

genetic polymorphisms. The variability at these positions allowed the definition of different alleles.

Typing of *P. jirovecii* is complicated by the fact that co-infections with two or more types probably occur. This phenomenon has been demonstrated in human, rat and ferret. Co-infections in humans were suggested by the presence of two or sometimes three alleles of a genomic region in clinical specimen from single patients. However, there are other possible explanations for these finding: presence of two or more copies of the same region per genome with variation between the copies or heterozygosity of diploid organisms. In the absence of data, these alternative explanations cannot be firmly excluded (236). Direct sequencing of PCR products has also been used. Co-infections were assessed when double peaks were observed. This was found in 10-30% of the clinical specimens analyzed (228-237).

Conclusively, DNA sequencing is the most discriminative technique available. Disadvantages of this method are the criteria of fastidiousness and high cost.

2.7 Ribosomal RNA of *P. jirovecii*

The first step in expression of a gene, the transcription of DNA to RNA, is the primary level at which gene expression is regulated in both prokaryotic and eukaryotic cells. RNAs in eukaryotic cells are then modified in various ways for example; introns are removed by splicing process convert the primary transcript into its functional form. Different types of RNA play an important roles in cells thus messenger RNAs (mRNAs) serve as templates for protein synthesis; ribosomal RNA (rRNAs) and transfer RNAs (tRNAs) function in mRNA translation. rRNA is the RNA component of the ribosome, the organelle that is the site of protein synthesis in all living cells. rRNA provides a mechanism for decoding mRNA into amino acids and interacts with tRNAs during translation by providing peptidyl transferase activity. The tRNAs bring the necessary amino acids corresponding to the appropriate mRNA

codon. The rRNAs form two subunits, the LSU and SSU. mRNA is sandwiched between the small and large subunits and the ribosome catalyzes the formation of a peptide bond between the 2 amino acids that are contained in the rRNA.

A ribosome also has 3 binding sites called A, P, and E. The A site in the ribosome binds to an aminoacyl-tRNA (a tRNA bound to an amino acid). The amino (NH2) group of the aminoacyl-tRNA, which contains the new amino acid, attacks the ester linkage of peptidyl-tRNA (contained within the P site), which contains the last amino acid of the growing chain, forming a new peptide bond. This reaction is catalyzed by peptidyl transferase. The tRNA that was holding on the last amino acid is moved to the E site, and what used to be the aminoacyl-tRNA is the peptidyl-tRNA. A single mRNA can be translated simultaneously by multiple ribosomes.

2.7.1 Ribosomal RNA genes

In prokaryotes a small 30S ribosomal subunit contains the 16S rRNA. The large 50S ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon. There may be one or more copies of the operon dispersed in the genome (for example, *Escherichia coli* has seven). Archaea contains either a single rDNA operon or multiple copies of the operon. The 3' end of the 16S rRNA (in a ribosome) binds to a sequence on the 5' end of mRNA called the Shine-Dalgarno sequence. In contrast, eukaryotic ribosomes contain four types of RNA, specified the 5S, 5.8S, 18S and 28S rRNAs. The organized within the nucleolus are 5.8S, 18S and 28S rRNAs which is the site of rRNA transcription, subsequently process and ribosome assembly. This organization is transcribed as a single unit by RNA polymerase I, yielding a 45S ribosomal precursor RNA, called pre-rRNA. The 45S pre-rRNA is processed to the 18S rRNA of the 40S small ribosomal subunit and to the 5.8S and 28S rRNAs of the 60S large ribosomal subunit. Transcription of the 5S rRNA, which is also found in the 60S ribosomal subunit, takes place outside of the nucleolus and is catalyzed by RNA polymerase III. The important of ribosome production is particularly evident in developing cells, in which the rRNA genes are amplified to support the synthesis of the large amount of ribosomes required for early cell development. Following each

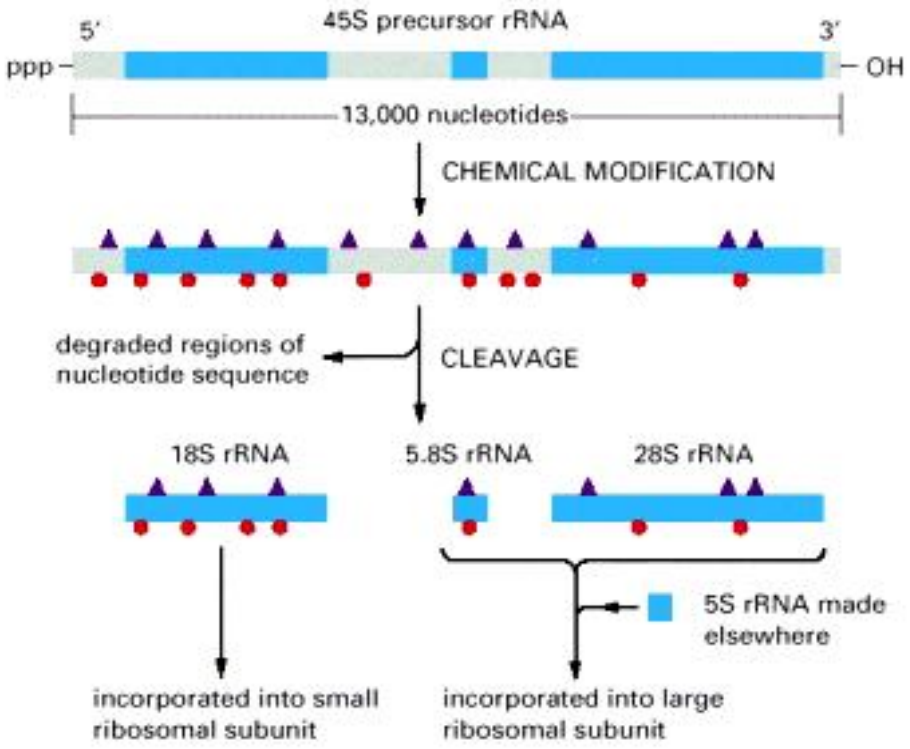
cell division, nucleoli form around the chromosomal regions that contain the5.8S, 18S and 28S rRNA genes, which are therefore called nucleolar organizing region. The formation of nucleoli requires the transcription of 45S pre-rRNA, which appears to fusion of small pre-nucleolar bodies that contain processing factors and other components of the nucleolus.

2.7.2 Transcription and processing of rRNA

Each nucleolar organizing region contains a cluster of tandemly repeated rRNA that are separated from each other by nontranscribed spacer DNA. The primary transcript of the rRNA is the large 45S pre-rRNA, which contains the 18S, 5.8S and 28S rRNA as well as transcribed spacer regions (Figure 5). External transcribed spacer (ETS) are present at both of 5' and 3' ends of the pre-rRNAs, and two ITS lie between the 18S, 5.8S and 28S rRNA sequences. The initial processing step is a cleavage within the external transcribed spacer near the 5' end of the pre-rRNA, which takes place during the early stages of transcription. Once transcription is complete, the external transcribed spacer at the 3' end of the molecule is removed. This step is followed by a cleavage at the 5' end of the 5.8S region, yielding separate precursors to the 18S and 5.8+28S rRNAs. Additional cleavage then resulted in formation of the mature rRNAs (Figure 6).

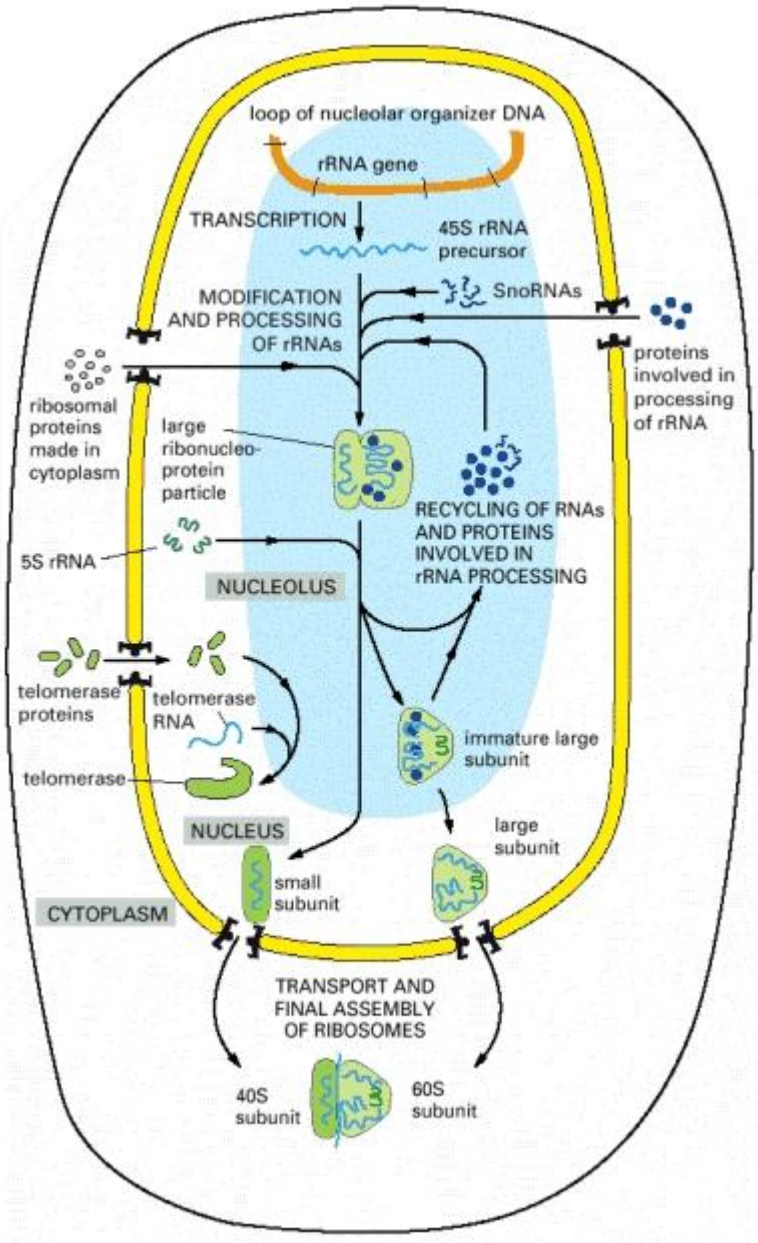
2.7.3 Ribosomal RNA gene studies

The DNA sequences that encode rRNAs have been extensively used to study the taxonomic relationships and genetic variation in fungi (31). The rRNA gene cluster is found in both mitochondria and nuclei, consists of highly conserved and variable regions, which include the genes for the small 5.8S, and LSU rRNA (242). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved sequences found in the SSU and LSU genes have been exploited to study the many relationships among distantly related fungi (243-245).



(From Molecular Biology of the cell, 1994 (244))

Figure 5. The processing of a 45S rRNA precursor molecule into three separate ribosomal RNAs. Nearly half of the nucleotide sequences in the primary RNA transcript are degraded in the nucleus.



(From Molecular Biology of the cell, 1994 (244))

Figure 6. The diagrammatic shows the processing of rRNA

2.8 Genetic heterogeneity of human-derived *P. jirovecii*

The *P. jirovecii* organisms which opportunistic infected the human host have been shown to be significantly different from those infecting other mammals, and have been assigned the name *P. jirovecii* instead *P. carinii* f. sp. *hominis* (86). High levels of genetic heterogeneity have been detected within this group of organisms and these have been exploited in the tool development for studies epidemiological data of *P. jirovecii* organisms which infect the human host have been shown to be significantly different from those infecting other mammals.

2.8.1 Variation at the mitochondrial small subunit rRNA gene (mt SSU rRNA)

Variation of *P. jirovecii* mt SSU rRNA gene has been observed in a 300-bp portion. Two nucleotide positions have been found at which a single nucleotide polymorphism (SNP) has been observed at position of 160 and 196. The base A or C and T or G has been found at position 160 and 196, respectively. Four possible sequence types, only two have been recorded to date, A160/G196 and C160/T196 (238).

2.8.2 Variation at the mitochondrial large subunit rRNA gene (mt LSU rRNA)

Sequence polymorphisms have been observed among isolates of *P. jirovecii* in a 346-bp portion of the mt LSU rRNA gene (24, 230, 238-241). A total of three nucleotide positions have been reported to date, at which a single polymorphism has been observed at position 81, 85 and 248 (Table 1.). Variation at position 85 has been observed at a relatively high frequency, the bases thymine (T), cytosine (C), and adenine (A) have been found at this position but not guanine (G). The typing of isolates of *P. jirovecii* using the variation at this position has been utilized in a number of epidemiological studies. Variation at position 81 appears to be very infrequent and has only been reported in one sample to date, in which T was found rather than C (238). A low frequency of variation has also been observed at position 248 where C is found in the majority of samples and T in the others.

Table 1. Nucleotide sequence types of *P. jirovecii*

Locus	**Sequence type**		
	Nucleotide position		
	81	**85**	**248**
mt LSU rRNA	C	T	C
	C	C	C
	C	A	C
	C	C	T
	C	A	T
	T	C	C

(From Wakefield (23, 233))

CHAPTER III

MATERIALS AND MATHODS

3.1 Chemicals and reagents

All chemicals and reagents used in these studies are analytical grade available.

Monoclonal mouse anti-*P. carinii* antibodies (clone 3F6) were obtained from DAKO AS, Glostrup, Denmark. Agarose was obtained from Promega (U.S.A.). Absolute ethanol (C_2H_6O) was purchased from BDH Laboratory supplied (England). QIAamp® DNA mini kit (QIAGEN Inc, U.S.A.) and QIAquick® DNA purification kit (QIAGEN, Germany) were purchased for extraction genomic DNA and recovery DNA from agarose gel, respectively. FastStart PCR Master, FastStart Universal Probe Master (ROX) and Light Cycler® 480 ResoLight Dye were purchased from Roche Molecular Biochemicals (U.S.A.). The methods for preparation of all chemical reagents were shown in the Appendix.

3.2 Ethical consideration

This study was approved by Siriraj Institutional Review Board (SIRB) on research involving human subjects, by the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Date of allowance on March, 2010 (160/2553(EC2)). Data management must keep with confidentially data.

3.3 Clinical specimens

Sixty-four clinical specimens were obtained from clinician who suspected that their patients may infected with *P. jirovecii*. Patients have symptoms such as fever, non-productive cough, shortness of breath, progressive exertional dyspnea, hemoptysis and weight loss. All clinical specimens consisted of forty-six BALF, twelve of sputum (SP) and five of gastric wash (GW) were analyzed in this study. The criteria for inclusion of clinical specimens were suspected PcP, sent to diagnoses of PcP during 2006 to 2009 at the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. The exclusion criteria of clinical specimens consisted of clinical specimens which less than 1,000 µl because it could not diagnose of *P. jirovecii* by staining with Giemsa, GMS stain, IFA technique, conventional PCR and Real-time PCR with HRM analysis. All clinical specimens were stored at -40 ºC. The gender and age of each clinical specimens were shown in Table 3. The number of female (N=34) was slightly more than that of male (N=30). The entire study while the age range extended from less than a year to ninety years.

3.4 Microscopic examination detection of *P. jirovecii*

3.4.1 Giemsa stain

Giemsa staining has been routinely used for the detection of trophic form and spore in smear of clinical specimens, it normally unstains the sporocyte wall. For detection of the sporocyte, other stains such as GMS must be used instead (246). Sulphation of smears before staining with Giemsa allows sporocyte to be visualised, thus enabling a single stain to be used to show all the stages of BALF, SP and GW clinical specimens, which is particularly useful.

Clinical specimens were smeared on glass slides by spreader then kept at room temperature for 1 min. Stained with giemsa dye for 20 min. To wash by buffer

water for 10 to 15 sec. Kept at room temperature for 3 min and examined by using light microscopy.

3.4.2 Gomori methenamine silver stain

GMS stain has been advocated commonly to highlight sporocyst wall of *P. jirovecii* in clinical specimens (247). Unfortunately, GMS staining is time-consuming and expensive technique, which requires well-trained laboratory personnel. Clinical specimens were smeared on glass slides by spreader then kept at room temperature. Fixed with methanol (75%) for 5 min and chromic acid (75%) at 65°C for 1 min. Washed by flown distilled water 3 times then dip into 1% potassium metabisulfite for 1 min. To washed by flown distilled water 3 times, then dip into MSN-DMSO at 90-95°C for 3 min, then washed by flown distilled water 2 times, then dip into 0.2% gold chloride for 2 min. To washed by flown distilled water then dip into 2% sodium thiosulfate for 1 min. To washed by flown distilled water then dip into 0.02% light green for 1 min, then washed by flown distilled water 3 times then dip into absolute ethanol for 1 min twice a time and unclouded by xylene for 1 min and mounted by permount (slide mounting solution) with cover glass, examined by using light microscopy (248).

3.4.3 Indirect immunofluorescent assay

IFA was used to detect *P. carinii*. IFA using monoclonal mouse anti-*P. carinii* antibodies (clone 3F6; DAKO AS, Glostrup, Denmark) was performed with commercially available murine monoclonal antibody labeled with fluorescein isothiocyanate that reacts with an antigenic epitope highly specific with *P. carinii* parasites. Briefly, specimens were mixed with 0.25% trypsin in phosphate buffered saline (PBS) and were incubated at room temperature for 5 min. Then, centrifuge preparations of trypsinized fluids were made. Indirect immunofluorescence with acetone-fixed smears was performed by using monoclonal mouse anti-*P. carinii* antibodies followed by rabbit anti-mouse IgM-FITC labeled, according to the instructions provided by the manufacturer then examined by using fluorescent microscope (Axiolab, Carl Zeiss, Germany) (249).

3.5 Molecular detection of *P. carinii*

3.5.1 Extraction of DNA

Extraction of DNA by using QIAamp® DNA Mini Kit (QIAGEN Inc, U.S.A.). The QIAamp® protocol (DNA purification from body fluids (spin protocol)) was performed according to the manufacturer's instructions. A 200 µl of clinical specimens were applied into the 1.5 ml-microcentrifuge tube. Each specimen was denatured by 20 µl QIAGEN Protease (or proteinase K), 200 µl Buffer AL to the sample then mixed by pulse-vortexing for 15 sec. In order to ensure efficient lysis, DNA yield reaches a maximum after lysis by incubate 10 min at 56°C. Briefly centrifuge for remove drops from the inside of the lid, then added 200 µl absolute ethanol into the same 1.5 ml-microcentrifuge tube. Applied the mixture to the QIAamp® spin column and centrifuged at 8,000 rpm for 1 min. Placed the QIAamp® spin column into a clean 2-ml collection tube. Opened the QIAamp spin column and added 500 µl Buffer AW1, then centrifuged at 8,000 rpm for 1 min. Placed the QIAamp spin column into a clean 2-ml collection tube and added 500 µl Buffer AW2 then centrifuged at 14,000 rpm for 3 min, centrifuged at full speed again for 1 min. Placed the QIAamp spin column into a clean 1.5 ml-microcentrifuge tube and added 100 µl Buffer AE for first elution, incubated at room temperature for 1 min, and then centrifuged at 8,000 rpm for 1 min. Second and third elutions step with a further 50, 25 µl Buffer AE, respectively. Centrifuged at 8,000 rpm for 1 min. The genomic DNA was kept at -40°C in case that the repeated experiment is required (250, 251).

3.5.2 Synthetic oligonucleotide primers

Two primers used in this study were synthesized by Bio Basic Inc. (Canada) and QIAgen operon (U.S.A.). All primers were derived from the transcribed region of *P. jirovecii* rRNA gene (252). These primers were used to perform conventional PCR; PCR reaction was performed with primer pAZ102-H and pAZ102-E were shown in Table 2. Location on mt LSU rRNA of PCR primers was demonstrated in Figure 7. These primers were diluted with sterilized deionized distilled water, aliquoted and kept at -40°C until used.

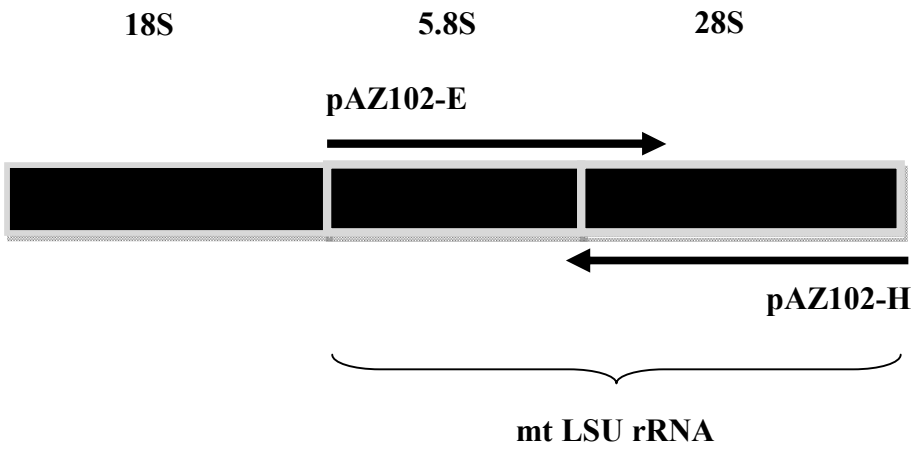


Figure 7. Location on mitochondrial large subunit ribosomal RNA of PCR primers

1	TTGTGGTAAGTAGTGAAATACAAATCGGACTAGGATATAGCTGGTTTTCT	50
51	GCGAAAATTGTTTTGGCAAATTGTTTATTCCTCTCAAAAATAGTAGGTAT	100
101	AGCACTGAATATCTCGAGGGAGTATGAAAATATTTATCTCAGATATTTAA	150
151	TCTCAAAATAACTATTTCTTAAAATAAATAATCAGACTATGTGCGATAAG	200
201	GTAGATAGTCGAAAGGGAAACAGCCCAGAACAGTAATTAAAGCTCCCCAA	250
251	TTAATATTAAGTGAAATAAAAGTTGTTGGATATCTAAGACAGTTAAGAAG	300

Figure 8. The sequences show a consensus sequence of mitochondrial large subunit ribosomal RNA regions of *P. jirovecii* provided by Wakefield *et al*., 1990 (23)

3.5.3 PCR amplification of mt LSU rRNA region

To amplify the portion of mt LSU rRNA gene region, conventional PCR was performed. To prevent the problem of carry-over with contaminated DNA in the PCR reaction mixture, certain working areas and sets of automatic pipettes (SOCOREX® CALIBRA 822, Socorex Isba S.A., Switzerland) which specifically kept for PCR work only. There were three completely separated areas (i.e., sample preparation, pre-PCR and post-PCR areas) and three sets of automatic pipettes were allocated specifically for each area. Furthermore, all reagents needed in proceeding PCR were aliquoted into several tubes and one tube was used each time for each experiment and discarded after use. In each experiment, positive (stock specimen from Parasitology laboratory) and negative controls (sterile distilled water) were also included.

3.5.4 PCR amplification

The PCR reaction was performed in a 0.2 ml thin wall PCR tube (Axygen® Scientific, U.S.A.). In a final volume of 50 µl, the reaction mixture contain 25 µl of FastStart PCR Master, 2.0 µl of both pAZ102-H and pAZ102-E primers, 10 µl of DNA template and 11 µl of sterilized deionized distilled water. The reaction mixture was processed in a Perkin Elmer GeneAmp 2400. The program consisted of one cycle of 94°C for 5 min to denaturing the genomic DNA, then 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. At the last cycle, the extension step at 72°C was performed for 7 min to complete partial polymerization. The 10 µl of sterilized deionized distilled water was used as a template of negative control. The PCR products were then stored at -20°C until future analyzed (252, 253).

3.5.5 Real-time PCR with HRM analysis

Real-time PCR assay involved Light Cycler® 480 technology, which combines rapid thermocycling with online fluorescence detection of the PCR products (254). The reaction was performed in a volume of 25 µl of a mixture contained 12.5 µl of FastStart Taqman® Probe Master (Roche Molecular Biochemicals) and 1.0 µl of each oligonucleotide primers, 1.0 µl of Light Cycler® 480 ResoLight Dye. The final concentration of sterilized deionized distilled water was adjusted to 4.5 µl. 5.0 DNA templates then was placed into each hole of plate, capped, and then placed into the LightCycler ™ rotor. Amplification occurred in three steps procedure: denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The transition rate of temperature was set at 20°C/sec for denaturation to annealing, 20°C/sec for annealing to extension and 10°C/sec for from extension to denaturation. The different primer pairs have the identical optimal PCR annealing temperature. Acquisition of the fluorescent signal from the specimens was carried out at the end of the elongation step. To detect the specific product from non-specific products and primer dimers, HRM analysis was done immediately after amplification. The PCR product was heated to 95°C, annealed at 65°C (annealing temperature +10°C), and then slowly heated from 65°C to 95°C at 0.2°C/sec to obtain the melting curve. The PCR product will be detected by using TaqMan probes are hydrolysis probes that are designed to increase the specificity of real-time PCR assays. The TaqMan probe principle relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection. As in other real-time PCR methods, the resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR; however, the TaqMan probe significantly increases the specificity of the detection.

TaqMan probes consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end. Several different fluorophores (e.g. 6-carboxyfluorescein, acronym: FAM, or tetrachlorofluorescin, acronym: TET) and quenchers (e.g. tetramethylrhodamine, acronym: TAMRA, or dihydrocyclopyrroloindole tripeptide minor groove binder, acronym: MGB) are

available. The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source via FRET (Fluorescence Resonance Energy Transfer). As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals.

TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. The PCR product were subjected to analysis by agarose gel electrophoresis on a 1.5 % agarose gel for confirm the efficiency of the high resolution melt analysis. Specialized HRM instruments will be ploted the change in fluorescence that occurs when double stranded DNA amplicons melt to form single stranded DNA. Differences between homozygous samples will be distinguished by a simple shift in the melting temperature (T_m), whereas heterozygous samples will be distinguished by changes in the shape of the melt curve (255). HRM of PCR products has been employed in many biomedical research for a number of years to detect mutations in genotypic variation study and distinguish of nucleotide polymorphism. Its application in the study of *P. jirovecii* genome has not been reported. Therefore, it is interesting to apply real-time PCR with HRM analysis in studying genotypic variation in this organism.

3.5.6. Detection of amplified DNA

After PCR amplification, the PCR amplified products were analyzed. 5 µl of PCR product was combined with 1 µl of dye (at ratio 5:1) The preparation was loaded into slots of the 1.5 % agarose gel which was submerged in electrophoretic chamber filled with 0.5X Tris-acetate-EDTA (TAE) buffer.

A horizontal type gel electrophoresis chamber was routinely used. Electrophoresis was carried out at constant voltage of 100 Volt, for 30 min or until the tracking dyes had migrated to a distance which was sufficient for separation of DNA fragments, or were near the rim of the gel. 1 µl of 100 bp DNA ladder (Promega, Germany) was used as a standard marker in order to estimate Mw of DNA fragments. The gel was stained with 2 µg/ml ethidium bromide for 10 min (256). After staining with ethidium bromide and destain with distill water, the DNA was visualized under a long wavelength UV-light transilluminator FOTO UV®26 (FOTODYNE®, U.S.A.). The UV illuminated gel was photographed with POLAROID* MP-4 LAND CAMERA (FOTODYNE®, U.S.A.) using polaroid film type 667 (Polaroid Inc., England).

3.5.7 Purification of DNA fragments from PCR products

The desired DNA products were purified by using QIAquick® Purification Kit (QIAGEN, Germany) with the protocol recommended by the manufacturer. Purification of DNA fragments with this kit was based on the fact that in the presence of a high concentration of chaotropic salt the DNA is bound selectively to silica-membrane in a special centrifuge tube. The DNA bound tightly with silica-membrane during a series of rapid “wash-and-spin” steps in the presence of high salt to remove other residues. Finally, low salt elution removed DNA from silica-membrane (256). After gel electrophoresis 45 µl of PCR products were placed into a sterilized 1.5 ml microcentrifuge tube. Add 5 volumes (225 µl) of buffer PB to 1 volume of each PCR products. Place a QIAquick spin column in s 2 ml collection tube and apply the samples to the QIAquick column and centrifuge for 1 min at 13,000 rpm. Discard flow-through and place the QIAquick column back into the same collection tube. DNA bound selectively to silica-membrane in a special centrifuge tube was washed

once with 750 µl buffer PE and once again with washing buffer PE, supplying in the kit. To elute DNA, the silica-membrane was applied with 30 µl of elution buffer i.e., buffer EB (10mM Tris-HCl, pH 8.5) let the column stand for 1 min and centrifuge at 13,000 rpm for 1 min to remove DNA from the membrane (257).

3.6 Molecular typing of *P. jirovecii*

3.6.1 DNA sequencing

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. All purified DNA were sequence by ABI PRISM® 3700 sequencer to confirm this microorganism. The primers pAZ102-H (5'-GTGTACGTTGCAAAGTACTC-3') was used to perform directional sequencing from reverse strand. DNA sequencing was conducted by Tecdragon Inc., Hongkong, Republic of China and Ward Medic Ltd., Bangkok, Thailand.

3.6.2 Sequences alignment analysis

Sequence alignment analysis is a way of arranging the sequences of DNA to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns. All sequences were undergone validating. Forward and reverse strands were aligned by using bioinformatic program ClustalW version 2.0 and followed by manually edit (BioEdit software). Subsequently, the multiple alignment was performed to align the validated sequences of mt LSU rRNA region with the consensus sequences (Figure 8.) provided by Wakefield *et al*. (23) using program OMIGA 2.0.

3.6.3 *P. jirovecii* typing

Pneumocystis jirovecii typing used in this study followed the typing method described by Wakefield *et al* (23, 233, 252, 262). After all sequences were aligned with mt LSU rRNA consensus sequences, the mt LSU rRNA alleles were subsequently identified by comparing the variation of the nucleotide at the scoring position with respect to the consensus sequences. The scoring position of mt LSU rRNA consisted of three nucleotides which allocate at position 81, 85 and 248. A new sequence variation found at any position within scoring position will be accepted to make the sequence as a distinct allele. The sequences having variation found outside the scoring position will be accepted as a new scoring position and will be concluded that genetic variation can be depend on the population in each geographical area.

CHAPTER IV

RESULTS

4.1 Microscopic examination detection of *P. jirovecii* by staining methods

4.1.1 Giemsa stain

Sixty-four clinical specimens were obtained from Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. This study was blinded experiment thus impossible to contact patients. Characteristic of positive results to detected all stages of the *P. jirovecii* life cycle was demonstrated in Figure 9. Thirty-seven of the sixty-four clinical specimens were gave positive results. Giemsa stain was gave sensitivity range from 44.12% to 96.67% and specificity range from 46.15% to 76.47% when compared with other techniques.

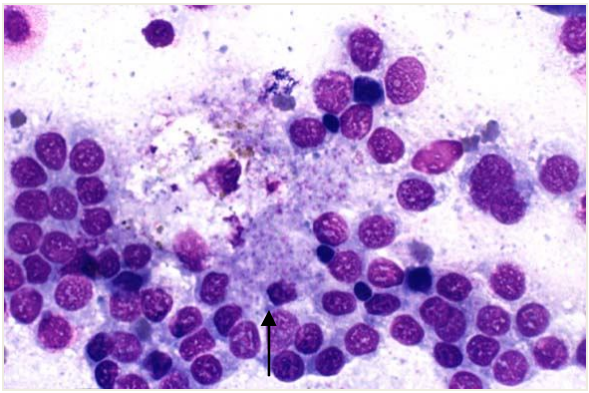


Figure 9. Giemsa stain of *P. jivovecii* sporocytes and spore cases. Fungi in a BALF clinical specimen stained with Giemsa dye (Light microscope, 1000X). Sporocyte wall was not stained by Giemsa dye, but they were appeared like a clear peripheral halo around cyst.

4.1.2. Gomori methenamine silver stain

GMS stain has been widely used as a method for fungal organisms detection which particularly useful in staining carbohydrates. GMS usually cooperate with Giemsa stain for detection of *P. jirovecii.* It also used to identify *P. jirovecii.* This study was indicated that these structures must be of sporocyst wall origin rather than of cytoplasm origin, because the thickened part of the sporocyst wall showed a dense deposition of silver particles, probably corresponding to the parentheses-like structure. Thirty of the sixty-four clinical specimens were gave positive results by GMS stain which demonstrated in Figure 10. Silver stain was considered to be a sporocyst wall-staining agent for detected this pathogen. GMS stain was gave sensitivity range from 42.37% to 78.38% and specificity range from 73.33% to 96.30% when compared with other techniques excepted IFA and Real-time PCR because IFA technique was not gave true negative result while Real-time PCR was gave all positive results.

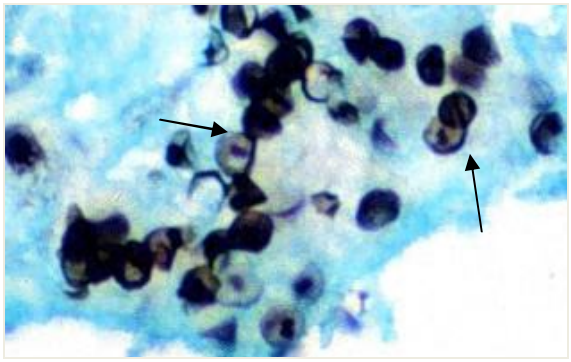


Figure 10. Gomori methenamine silver stain of *P. jirovecii* sporocyst (BALF specimen, 1000X). The sporocysts were appeared as a cup shaped, spherical, or crescent-shaped object measuring 4-8 µm in diameter. Some sporocysts are empty and collapsed, other contained dark bodies or dots in silver-stained preparations.

4.1.3. Indirect immunofluorescent assay

Monoclonal antibody was reacted with an antigenic epitope of *P. jirovecii*. Alveolar spaces Ab-1 was specific to *P. jirovecii* and *P. carinii* distinctly. No reactivity was found with other pathogens such as *Toxoplasma gondii*, *Trypanosoma cruzi*, *Plasmodium falciparum*, *Cryptosporidium sp*., *Candida albicans*, *Entamoeba histolytica*, *Aspergillus flavus* and *Aspergillus fumigatus* (193, 194, 196). Antibody was diluted by antibody diluents into 1:20 concentrated antibody. An IFA specimen was considered positive if two or three sporocysts were seen in at least three high power fields. Fifty-nine of the sixty-four clinical specimens were gave positive results by IFA which demonstrated in Figure 11. IFA assay was gave sensitivity range from 81.08% to 92.65% and specificity range from 3.33% to 22.22% when compared with other techniques excepted GMS and Real-time PCR because GMS technique was not gave true negative result while Real-time PCR was gave all positive results.

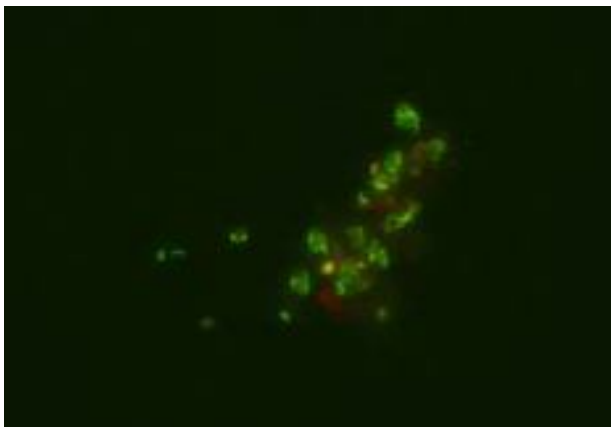


Figure 11. *P. jirovecii* in clinical specimens of sputum stained with IFA. (The staining pattern were visualized as homogeneous rings corresponding to individual sporocyte walls and free extracystic *P. jirovecii*)

4.2 Molecular typing of *P. jirovecii*

4.2.1 Amplification of mt LSU rRNA region of *P. jirovecii* from clinical specimens

The mt LSU rRNA region is the mitochondrial large subunit ribosomal RNA gene which is known as a conserve region and appears as a target for detecting biodiversity of *P. jirovecii* (23). From sequence data of mt LSU rRNA gene reported by Wakefield *et al.* (88), two sequences were selected, i.e., pAZ102-H and pAZ102-E to be used as primers for amplification of mt LSU rRNA region by PCR technique. The sequences of primers were shown in Table 2; the pAZ102-E was corresponded to the portion of 18S rRNA gene, the pAZ102-H was corresponded to the portion of 28S rRNA gene. PCR amplification was performed with one pair of oligonucleotide primers and the chromosomal DNA of *P. jirovecii* was used as DNA templates (88). PCR system with the pair of primers, i.e., primer pAZ102-H and pAZ102-E, yielded the DNA fragment of 346 bp in length. The PCR products amplified from *P. jirovecii* were shown in Figure 12.

Sixty-four clinical specimens were included in this study. The characteristic including clinical specimens no., age, sex, and type of clinical specimens of patients which suspected PcP infection were shown in Table 3. This study used sterile distilled water (PCR grade) as a negative control. The characteristic of positive results to amplified *P. jirovecii* DNA was shown in Figure 12. Thirty-four out of the sixty-four clinical specimens gave positive results. Conventional PCR was gave sensitivity range from 50.00% to 73.33% and specificity range from 20.00% to 68.00% when compared with other techniques excepted Real-time PCR because Real-time PCR was gave 100% positive results. The desired DNA products were purified by using QIAquick® Purification Kit (QIAGEN, Germany).

Table 2. PCR primers used for amplification of mt LSU rRNA region of *P. jirovecii*

Primer Name And Direction	**Primer Sequence (5'-3')**	**Product Size (bp)**	**References**
pAZ102-H	GTGTACGTTGCAAAGTACTC	346	Wakefield *et al*., 1990 (90)
pAZ102-E	GATGGCTGTTTCCAAGCCCA		

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 M

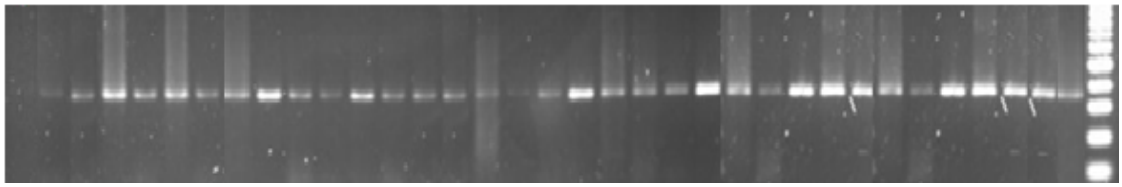


Figure 12. Ethidium bromide-stained 1.5% agarose gel of thirty-four positive PCR amplified DNA from clinical specimens using primers pAZ102-H and pAZ102-E which was specific with mt LSU rRNA region of *P. jirovecii* (346 bp in length of DNA fragment)

Lane 1: Negative control

Lane 2-35: 346 bp PCR products from thirty-four clinical specimens

Lane M: 100 bp ladder standard marker

Table 3. Clinical specimen No., age, sex, and type of clinical specimens of sixty-eight patients.

Clinical specimen No.	Age	Sex	Type of Clinical Specimens
1	-	female	BALF
2	51	male	Sputum
3[a]	7mths	male	GW
4	45	female	sputum
5	23	male	sputum
6	15	female	sputum
7	68	male	BALF
8	62	female	sputum
9	3mths	male	BALF
10	29	female	BALF
11	14	female	BALF
12	-	female	BALF
13	-	female	BALF
14	64	male	BALF
15	-	male	BALF
16	83	female	BALF
17	46	female	BALF
18	30	female	BALF
19	58	female	BALF
20	40	female	BALF
21	47	female	BALF
22	36	male	BALF
23	48	male	sputum

Table 3. Clinical specimen No., age, sex, and type of clinical specimens of sixty-eight patients (cont.).

Clinical specimen No.	Age	Sex	Type of Clinical Specimens
24[a]	83	female	BALF
25	43	female	BALF
26	68	male	BALF
27	62	female	BALF
28	69	male	BALF
29	36	female	BALF
30	30	female	BALF
31	53	male	BALF
32	35	female	BALF
33	12	female	GW
34	50	female	BALF
35	78	male	BALF
36	31	male	BALF
37	27	male	BALF
38	63	male	BALF
39	36	female	BALF
40[a]	48	male	sputum
41	41	male	BALF
42	6mths	male	GW
43	45	female	BALF
44[a]	51	female	sputum
45	41	male	BALF
46	46	female	BALF

Table 3. Clinical specimen No., age, sex, and type of clinical specimens of sixty-eight patients (cont.).

Clinical specimen No.	Age	Sex	Type of Clinical Specimens
47	37	male	-
48	22	male	BALF
49	32	female	sputum
50	40	male	sputum
51	40	male	sputum
52	1	male	GW
53	1	male	GW
54	26	male	BALF
55	32	male	BALF
56	37	male	BALF
57	59	male	BALF
58	44	female	BALF
59	45	male	sputum
60	44	female	sputum
61	45	female	BALF
62	37	female	BALF
63	77	female	BALF
64	90	female	sputum
65	36	male	BALF
66	52	female	BALF
67	37	female	BALF
68	-	male	BALF

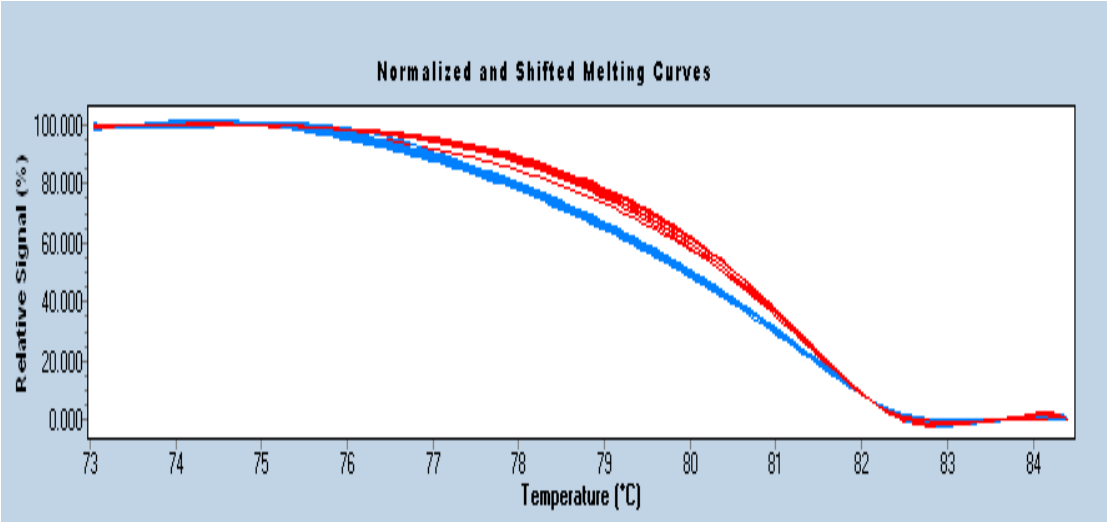
[a] The specimens that could not be amplified by PCR were shaded with grey color.

BALF; Bronchoalveolar lavage fluid, GW; Gastric wash

4.2.2 Real-time PCR with HRM analysis

Real-time PCR with HRM analysis provides a useful methodology for screening of mutation associated with genotypic variation of *P. jirovecii* in human population and thus facilitates surveillance for mutation. HRM analysis was performed on double stranded genomic DNA of clinical specimens. Typically there was used PCR technique prior to HRM analysis to amplify the desired DNA region in which their mutation of interest lies. Sixty-four clinical specimens were included in this study. All clinical specimens were gave positive results by Real-time PCR with HRM analysis which demonstrated in Figure 13. Real-time PCR was gave sensitivity 100% and could not calculated of specificity when compared with other techniques because Real-time PCR was gave 100% positive results. The desired DNA products were confirmed by gel electrophoresis and purified by using QIAquick® Purification Kit (QIAGEN, Germany). The melting temperatures of the two polymorphisms were shown in Figure 14. First group was shown the average T_m at 79.15°C (range from 78.94°C to 82.22°C) and second group was shown the average T_m at 81.06°C (range from 79.45°C to 82.18°C).

A



B

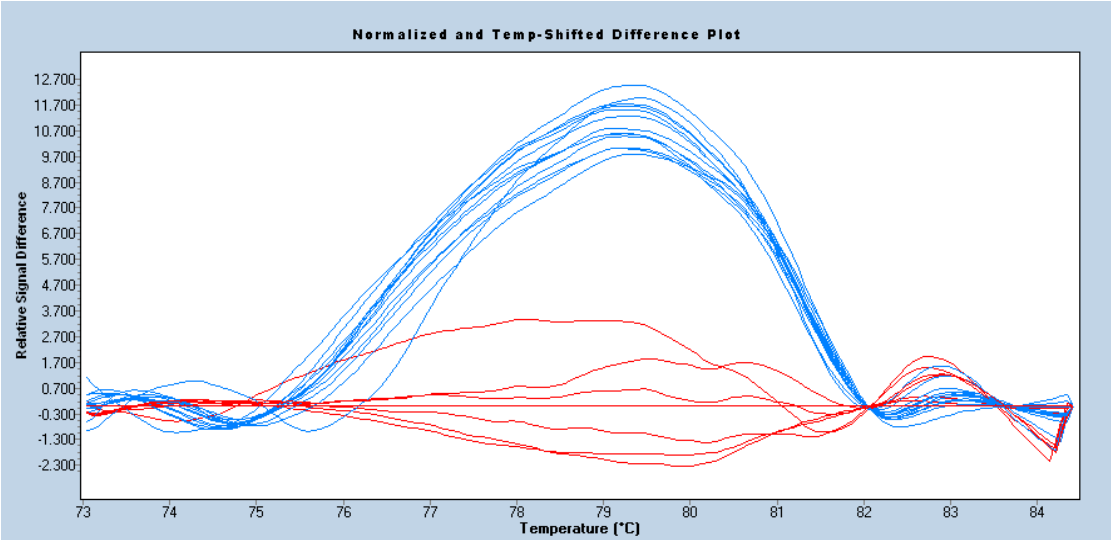


Figure 13. Results of HRM analysis from clinical specimens (BALF, SP and GW) were distinguished to first group and second group. The melting curve patterns of the mt LSU rRNA region polymorphism were shown in blue of first group and red of second group, respectively.

(A) Normalized data

(B) Difference graph

4.2.3 *P. jirovecii* typing

Mitochondrial Large Subunit Ribosomal RNA (mt LSU rRNA) region amplification were successful for sixty-four clinical specimens (from both conventional PCR and Real-time PCR) that initially diagnosed for *P. jirovecii* by giemsa staining, GMS staining and IFA observed under a light microscope and fluorescent microscope. Therefore, a total from sixty-four clinical specimens were sequenced in this study. The aligments of the mt LSU rRNA sequence with the consensus sequence were shown in Figure 15. The sequences were compared with melting curve analysis from real-time PCR with HRM analysis which were shown clearly two distinct groups of mt LSU rRNA region. The identified types of *P. jirovecii* and their frequency were shown in Table 4.

By using the scoring method which based on a number code described by Wakefield *et al*., (23) to analyze sixty-four specimens by multiple sequence alignment (ClustalW version 2.0 and OMIGA version 2.0), nucleotide substitutions and deletions were observed. This result leads to identification of two *P. jirovecii* mt LSU rRNA types. Among these six types, four were previously reported in immunosuppressed patients who were documented with PcP by Wakefield *et al*. fifth and sixth types were reported by Sinclair *et al*., (214) while the rest were considered to be the new type found in this study. When all sequences were compared with the consensus sequence which the scoring position were 81, 85 and 248, nucleotide substitutions were observed by substitution of T to A at the scoring position at the position 85. Other base changes were detected at other scoring positions. Nucleotide substitutions were observed by substitution of C to G outside the scoring position at the position 61 whereas substitution of T to C were observed at the position 62, 79, 100 and 121, respectively. Substitution of G to T at the position 63, 119, 165, 227, 295 and 315. Substitution of T to G at the position 66, 83, 95, 117, 299, 324 and 329. Substitution of T to A at the position 67, 68, 69, 71, 84, 92, 93, 97, 317 and 343. Substitution of G to C at the position 74, 119, 120, 125, 208, 231, 246 and 339. Substitution of G to A at the position 94, 116, 145, 165, 234, 237, 251, 320 and 332. Substitution of A to T at the position 76, 89, 107, 168, 172, 184, 258 and 259. Substitution of A to C at the

position 77, 91, 98, 122, 173, 245, 264, 334 and 338. Substitution of C to T at the position 88, 106, 271 and 328. Substitution of A to G at the position 90, 108, 109, 118, 132, 140 and 221. Substitution of C to A at the position 106. Substitution by deletion of T residue outside the scoring position at the position 85 and 121. Deletion of A residue at the position 122 and 134. Deletion of C residue at the position 88 and 171, respectively. All of substitutions are novel sequences are dissimilar to any sequence patterns that had been ever reported by other groups of investigators.

The frequency and distribution of *P. jirovecii* in sixty four clinical specimens which can be amplified by conventional PCR and Real-time PCR was summarized in Table 4. The first group was found in fifty one clinical specimens (79.6875%) i.e., clinical specimens No. 2, 4, 5, 8, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 21, 22, 23, 25, 27, 28, 30, 31, 32, 34, 35, 36, 37, 38, 39, 41, 42, 43, 45, 46, 47, 48, 50, 51, 53, 54, 55, 57, 59, 60, 61, 63, 64, 65, 66, 67 and 68. The second group was identified in thirteen clinical specimens (20.3125%) i.e., clinical specimens No. 1, 6, 7, 9, 16, 26, 29, 33, 49, 52, 56, 58 and 62, respectively. This finding suggests that there was the occurrence of mixed infection (coinfection with *P. jirovecii* and other species of *Pneumocystis* spp.)

Table 4. The occurrence of *P. jirovecii* mt LSU rRNA region polymorphism

Group of mt LSU rRNA region polymorphism	**Clinical Specimens No.**	**n**	**Percentage (%)**
1	2, 4, 5, 8, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 21, 22, 23, 25, 27, 28, 30, 31, 32, 34, 35, 36, 37, 38, 39, 41, 42, 43, 45, 46, 47, 48, 50, 51, 53, 54, 55, 57, 59, 60, 61, 63, 64, 65, 66, 67 and 68	51	79.69
2	1, 6, 7, 9, 16, 26, 29, 33, 49, 52, 56, 58 and 62	13	20.31

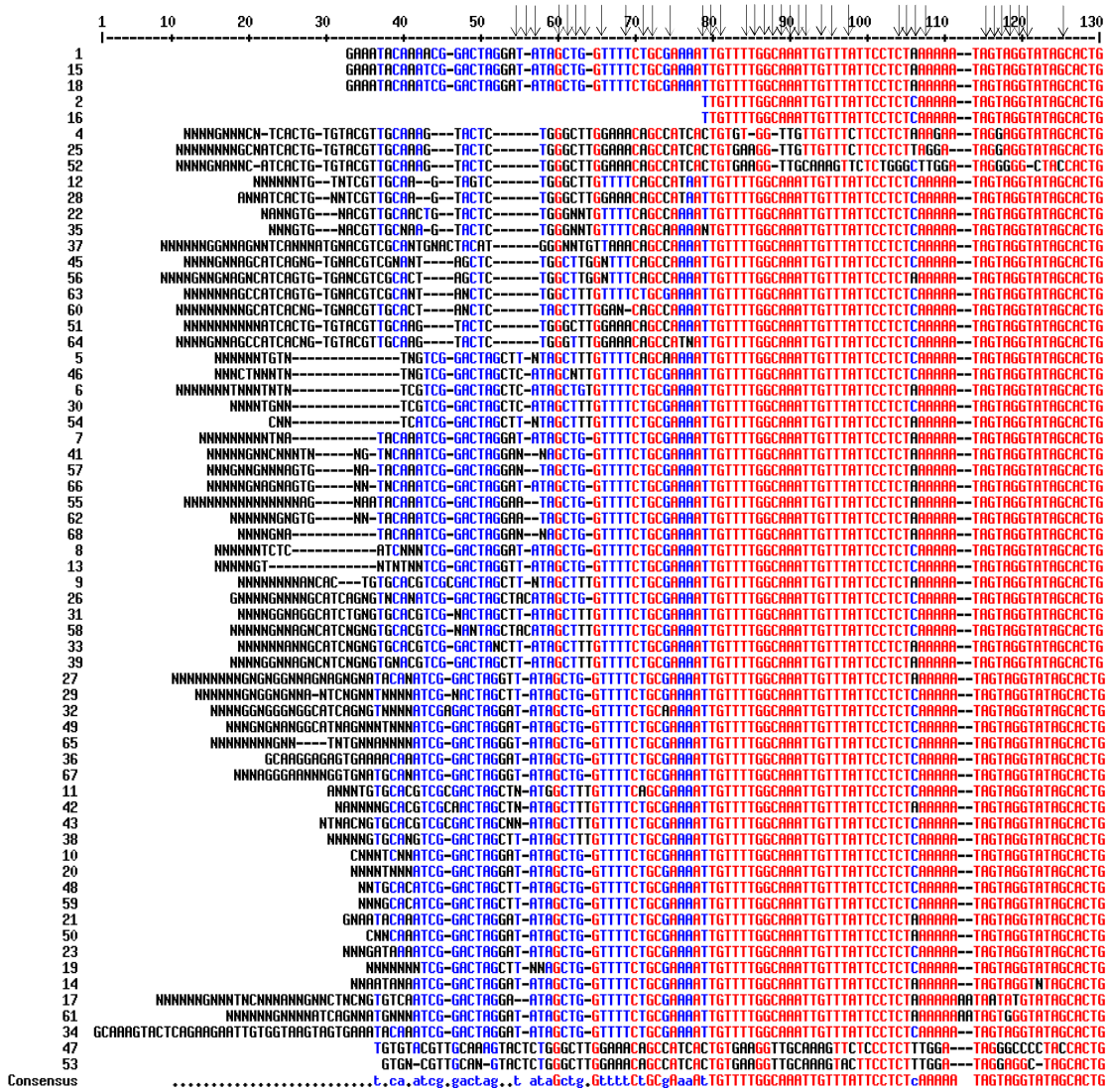


Figure 14. The sequence alignment of *P. jirovecii* mt LSU rRNA consensus sequence and all sequences. Mutations are indicated by arrows, missing bases are indicated by hyphens.

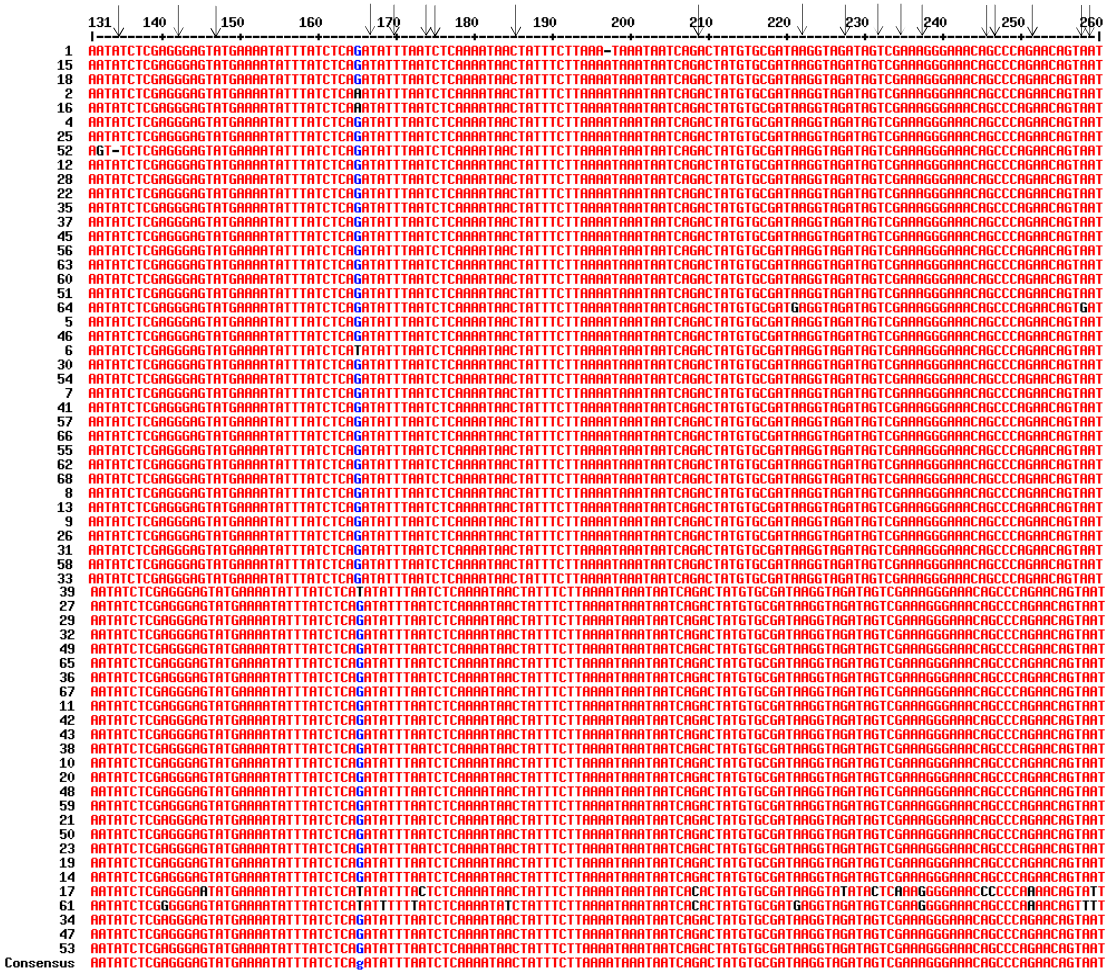


Figure 14. The sequence alignment of *P. jirovecii* mt LSU rRNA consensus sequence and all sequences. Mutations are indicated by arrows, missing bases are indicated by hyphens. (cont.)

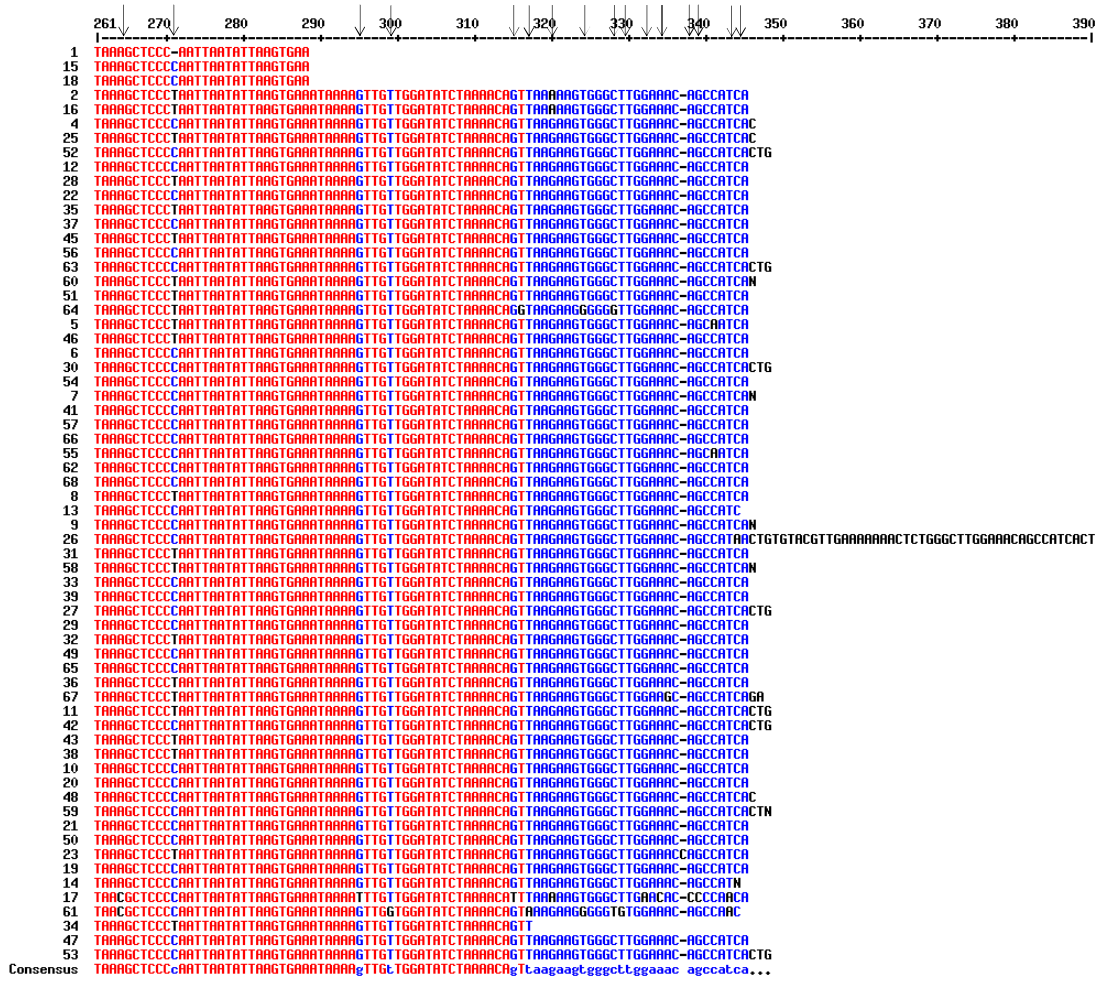


Figure 14. The sequence alignment of *P. jirovecii* mt LSU rRNA consensus sequence and all sequences. Mutations are indicated by arrows, missing bases are indicated by hyphens. (cont.)

CHAPTER V

DISCUSSION

Pneumocystis jirovecii (*P. jirovecii*) is a common opportunistic pathogen causing pneumonia in immunocompromised patients (1, 11, 12). Although pneumocystosis has been studied for decades, the organism associated with this disease is only rudimentarity understood, and numerous basic questions regarding the epidemiology have not been answered.

In this study, the genotypic variation data concerning *P. jirovecii* mt LSU rRNA types from immunocompromised Thai patients were reported. mt LSU rRNA loci provided were chosen for typing because the results obtained by using these loci provided more information than those obtained by using the other loci i.e., 5S rRNA gene and TS gene (258). This loci has not been reported before in Thai patients. However, the amplification of mt LSU rRNA region of sixty-four clinical specimens (BALF, SP and GW) that were initially tested for *P. jirovecii* by staining with Giemsa, GMS and IFA techniques then examined under the light microscope and fluorescent microscope, respectively. The specimens that were excluded to mt LSU rRNA amplification were clinical specimens No. 3, 24, 40 and 44, because it could not diagnosed of *P. jirovecii* by all diagnostic methods. However, there were still other reasons that might be responsible for such a failure. Firstly, the low amount of the organism in the clinical specimen might result in decreasing PCR sensitivity. Because the fungus primarily infects the alveolar spaces at the pneumocyte type I and noninvasive sample collection essentially recovers cells from the upper respiratory tract therefore, the amount of *P. jirovecii* organisms present in a SP is usually low. Secondly, the lower sensitivity of PCR amplification at mt LSU rRNA region as compared with the microscopic visualization may be due to the characteristic of the mt LSU rRNA region themselves. Finally, the presence of PCR inhibitors in

respiratory-tract specimen may inhibit the PCR reaction (259). There was a report that a high concentration of DNA present in the specimens (e.g., host DNA or bacterial) can inhibit the amplification reaction (259). Tang *et al.* (260) reported that the copy number of rRNA genes in *P. jirovecii* by using α-^{35}S-dCTP as a radioisotope marker and counting the radioactivity and found that the rRNA gene is present at one copy per *P. jirovecii* genome. Based on this evidence, it is not surprising that the amplification of mt LSU rRNA region provides less sensitivity than conventionally staining methods (260). The mt LSU rRNA PCR amplification was interfered by DNA extraction method used in this study (259). In the present study, the protocol using QIAamp DNA Mini Kit for DNA template preparation from body fluids (BALF, SP and GW) containing *P. jirovecii*. QIAGEN Protease (or proteinase K) which is impregnated with denaturant, free-radical traps, chelating agents will lyse most cell types on the contact and sequesters DNA within the matrix. From the protocol an ethanol (96–100%) has been reported to have an effect on the activity of some enzymatic reaction (261). When applying an ethanol into the clinical specimens, it could be possible that the ethanol in the microcentrifuge tube may act as an inhibitor for some reagents i.e., buffer AW1, buffer AW2 and buffer AE, the chemical reagents component used in digesting cells and immobilizing the DNA which are not exactly known. This proposed mechanism may interfere at the step of cell digesting and DNA immobilization.

In 1990, Wakefield *et al.* described the finding of three types of *P. jirovecii* based on nucleotide sequences variation in mt LSU rRNA region (262). Subsequently, six sequences were found to be identical to the prototype sequence reported by Sinclair *et al* (217). In this study, analysis of genotypic variation in Thai patients by sequence data was based on scoring positions previously described by Wakefield *et al* (262). Scoring was carried out at a total of three nucleotide positions in the mt LSU rRNA region (position of 81, 85 and 248). A total of two groups were identified among the sixty-four clinical specimens. The techniques used in this study included amplification and direct to DNA sequencing which PCR products from conventional PCR and Real-time PCR. The sequence types determined from the clinical specimens in this study were compared with previously published sequences

and some differences in sequence types were detected. They were observed at nucleotide positions outside the scoring positions that have not been used for typing.

The result of sequencing of the mt LSU rRNA region revealed that a number of changes were observed in lengths of short homopolymeric tracts. In the mt LSU rRNA region, these were detected in the adenine tract at nucleotide position 76, 77, 89, 90, 91, 98, 107, 108, 109, 118, 122, 132, 140, 168, 173, 221, 245, 264, 334 and 338. These were detected in the thymine tract at nucleotide position 62, 66, 67, 68, 69, 71, 79, 83, 84, 92, 93, 97, 100, 117, 121, 299, 317, 324, 329 and 343. These were detected in the cysteine tract at nucleotide position 61, 88, 106, 271,328 and 346. These were detected in the guanine tract at nucleotide position 63, 74, 94, 116, 119, 120, 125, 145, 165, 231, 234, 237, 246, 251, 295, 315, 320, 332 and 339. In their study, the sporadic variation and the changes within the homopolymeric tracts were also observed mt LSU rRNA region at the various positions. The occurrence of the new alleles resulted in establishing the new scoring positions. However, these new scoring positions will probably have to be taken into account to define a new scoring system if their presence is confirmed by using more clinical specimens in the future. Comparison between DNA sequencing and high resolution melt analysis were gave significant agreement.

Real-time PCR with HRM analysis has many advantages over conventional PCR technique (212, 213). For instance, flexible detection formats, real-time fluorescence monitoring, high speed thermal cycling, accurate quantification and qualification, rapid turn-around time, elimination of contamination risk and laborsaving. Study of mutation, real-time PCR with HRM analysis followed by melting curve analysis, using hybridization probe, are highly sensitive and an efficient approach to mutation detection with reliable result. Likewise, LightCycler® 480 instrument is able to perform dual color analysis, which is suitably distinguished by a simple shift in the T_m, whereas heterozygous clinical specimens will be distinguished by changes in the shape of the melt curve (214-216). Thirty-seven and sixty-four of the sixty-four clinical specimens were gave positive results from conventional PCR and real-time PCR techniques. Because of real-time PCR have DNA lower limit

detection lower than conventional PCR. In fact, conventional PCR can amplify DNA template like real-time PCR but in process of detection, conventional PCR product will be detected by gel electrophoresis differ from real-time PCR that will be detected by fluorescence emission each round of an amplification process. For this reason could be summarized that real-time PCR have sensitivity higher than conventional PCR.

In this study, the spectrums of polymorphism of the *P. jirovecii* mt LSU rRNA types found in immunocompromised Thai patients were similar to previous report. Evidences obtained from these studies were clear in an agreement to indicated a diversity of *P. jirovecii* mt LSU rRNA genotypes.

By applying the scoring method of Sinclair *et al.,* two main groups of *P. jirovecii* were detected which may be considered as new mt LSU rRNA types (217). Among the two groups of *P. jirovecii* mt LSU rRNA region found in this study, the first group is present at the 79.69% and the second group is present at the 20.31%. This observation was different from previous reported by Sinclair *et al* (217). The first group was found in fifty-one clinical specimens from Faculty of Medicine Siriraj Hospital, Thailand composed of BALF, SP and GW from immunocompromised patients and the second group was found in thirteen clinical specimens (BALF, SP and GW) from immunocompromised patients.

New variations of *P. jirovecii* were discovered from this study (GenBank: EF439814.1) and have the same sequence as those previously considered as sporadic variations was also found (230). Some of these sporadic variations are now classified as new alleles, therefore these new groups may be used as a genotypic marker for studying the pathology, transmission patterns and epidemiology of *P. jirovecii* in Thai population. However, it is a premature to conclude that these types are unique to certain region, since the sample size was relatively small in this present study. A considerably greater number of specimens are required to be typed before any conclusion could be drawn.

From this study, variations of clinical specimens were found to be coinfection of *P. jirovecii* and other *Pneumocystis* spp. This observation is consistent

with the report of Tsolaki *et al* (226). They concluded that PcP in immunocompromised patients was not necessarily clonal (226). There were another studies which confirmed this situation of coinfection at 25-82% (263). Although coinfection was frequently reported and following possibility should also be taken into the consideration. The mixed types may be derived from different copies of rRNA genes in the same *P. jirovecii* genome, since multiple copies of rRNA genes are commonly found in eukaryotic organisms. Additionally, it is not exactly known whether *P. jirovecii* has more than one copy of the rRNA gene, since the complete genome sequence of this organism has not already been completed.

This study was reported of *P. jirovecii* genotypes in immunocompromised Thai patients, by considering the present data with those previous reports from HIV-infected patients with PcP. It was conceived that; (i) different main types could be observed among different patient populations, and (ii) uniques *P. jirovecii* mt LSU rRNA types can be found in different groups of patient populations. These different features of *P. jirovecii* mt LSU rRNA types detected in different patient populations were presumably resulted from acquisition of fungal organism from different geographical area.

The results of this study indicated that nucleotide sequences of *P. jirovecii* from different patients may be different which, implying the existence of multiple strains of *P. jirovecii*. The observation a sequence of the mt LSU rRNA region was a hybrid between of the rat (*P. carinii* f. sp. *carinii*) and human *P. jirovecii* sequences suggested that coinfection of rat and human strains in the same host was possible. This recombination would imply that human *Pneumocystis* may infect from animals strains to humans. This notion is also suggested by the study of Sethi *et al*., were shown that *P. jirovecii* from a human bronchoalveolar lavage specimen could proliferate in mice (264). However, this study was unable to show any correlation between specific mt LSU rRNA types and clinical outcome, since the data regarding the patients background was not enough. Airborne transmission of the fungus from host to host has been demonstrated in rodent models (167). Several observations suggest that inter-individual transmission occurs in human (165). Although an environmental reservoir

remains possible, these data argue in favor of the fact that human PcP is an arthroponosis, with humans as the reservoir for *P. jirovecii*.

New detection tools such as real-time PCR assay have revealed that humans infected with *P. jirovecii* can have a large spectrum of clinical presentations, of which PcP in immunocompromised patients may represent only a small part of colonization, while other clinical presentations may constitute the major part (265). Actually, it has been shown the pulmonary colonization with *P. jirovecii* occuring frequently in immunocompromised patients (266) and less frequently in persons who are apparently immunocompetent but in those who are suffering from lung disease (267). Recently, *Miller et al.* reported that the severity of PcP might be associated with the genotypic variation of *P. jirovecii* types (26). For example, the first group was associated with a mild disease. , and the second group was associated with a moderate to severe disease. Furthermore, the first group was found in the first episodes but has been never found in subsequent episodes of PcP. The mt LSU rRNA type newly described in this study may be served as valuable data regarding to pathogenicity aspect of this organism if more data of these type is achieved. Additionally, employing the classical as well as molecular epidemiological study will aid in the understanding of this complicate organism. Information of the transmission and epidemiology of *P. jirovecii* may lead to future recommendations for immunosuppressed persons or hospitalized patients with PcP in respiratory isolation to avoid specific exposures. Moreover, these valuable data can definitely play role in controlling the incidence of this significantly opportunistic infection.

CHAPTER VI

CONCLUSION

The present study focused on the investigation of mt LSU rRNA genotypes of *P. jirovecii* from clinical specimens from immunocompromised Thai patients. Genotypic study of *P. jirovecii* types was carried out using conventional PCR, Real-time PCR with HRM analysis and DNA sequencing analysis. The results from this study are summarized as follows:

1. QIAamp DNA Mini Kit can be used as a protocol for extracting the chromosomal DNA of *P. jirovecii*
2. The amplification of mt LSU rRNA region of *P. jirovecii* by conventional PCR was successful for thirty-four out of sixty-four clinical specimens.
3. DNA amplification of mt LSU rRNA region using primers pAZ102-H and pAZ102-E provided PCR products of expected size of a 346 bp.
4. Two *P. jirovecii* mt LSU rRNA groups were identified in this study by using DNA sequencing method. Among them are the new types.
5. The first group of *P. jirovecii* typing showed the average T_m at 79.15°C (range from 78.94°C to 82.22°C) whereas the second group showed the average T_m at 81.06°C (range from 79.45°C to 82.18°C).
6. The occurrence of *P. jirovecii* mt LSU rRNA region were 79.69% and 20.31% in the first and second groups, respectively.
7. The analysis of PCR-induced sequence errors in mt LSU rRNA region showed a small number of nucleotide changes indicating that the sporadic

nucleotide variation in mt LSU rRNA region were possible, especially at the homopolymeric tracts.

8. Genetic variations were identified in this study which might be coinfection (with more than one genotype in a clinical specimen) with other *Pneumocystis* spp.

9. Clinical specimen No. 1278 has a same sequence with *P. jirovecii* isolate 71f large subunit ribosomal RNA gene, partial sequence; mitochondrial (GenBank: EF439814.1) which reported by Gupta R., *et al.* (230).

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APPENDIX

APPENDIX
CHEMICAL AND INSTRUMENT

GRAM STAIN

Crystal violet stain, Gram's iodine, Acetone and Safranin were prepared commercially.

GOMORI METHENAMINE SILVER STAIN

4% aq Chromic Acid

Chromium trioxide	4	g
Distilled water	100	ml

Chromium trioxide was dissolved in distilled water. Bottle of these solutions was stored at 4°C.

Silver solution

3% methanamine	23	ml
5% silver nitrate	1.25	ml
5% borax	3	ml
Distilled water	25	ml

3% methanamine, 5% silver nitrate and 5% borax were dissolved in distilled water. Bottle of these solutions was stored at 4°C.

0.2% aq Sodium chloroaurate

Gold Chloride	1	g
Distilled water	500	ml

Gold Chloride was dissolved in distilled water. Bottle of these solutions was stored at 4°C.

2% aq Sodium thiosulphate

Sodium thiosulphate	2	g
Distilled water	100	ml

Sodium thiosulphate was dissolved in distilled water. Bottle of these solutions was stored at 4°C.

Working light green

1% light green (CI 42095) in 1% acetic acid	10	ml
Distilled water	40	ml

1% light green (CI 42095) in 1% acetic acid was dissolved in distilled water. Bottle of these solutions was stored at 4°C.

REAGENTS FOR MOLECULAR STUDY

Ethidium Bromide (10 mg/ml)

Ethidium bromide	50	mg
Distilled water	5	ml

Distilled water was added to dissolved Ethidium bromide powder. Bottle of these solutions was stored at room temperature in silver foil wrapped container.

6X loading Dye

Bromophenol blue	2.5	g
Sucrose	40	g
Distilled water	100	ml

Bromophenol blue and sucrose were dissolved in distilled water. Dye preparation by dispensed into aliquots and stored at 4°C.

50X TAE Buffer

Tris base	242	g
Glacial acetic acid	57.1	ml
$Na_2EDTA.\ 2H_2O$	37.2	g
Distilled water to 1 Liter		

Tris base, Glacial acetic acid and $Na_2EDTA.\ 2H_2O$ were dissolved in distilled water, mixed and stored at room temperature.

0.5X TAE Buffer (Working Solution)

50X TAE Buffer	20	ml
Distilled water	1980	ml

50X TAE Buffer was dissolved in distilled water, mixed and stored at room temperature.

INSTRUMENT

- Count clock
- Microwave
- Refrigerator
- PCR cabinet (LIO LAB LTD, PART)
- Water bath (Momert, Thermology Co.,Ltd)
- Vortex mixer (Vortex Genie 2)
- Centrifuge (EBA 21, Hettich)
- Micro centrifuge (SD 220, Clover Laboratories, Taiwan)
- Adjustable volume pipettes (0.2-2.0 µl, 20.200 µl and 200-1000 µl, Gilson, Germany)

- Mini Gel Migration Trough (Cosmo Bio Co., Ltd.)
- i-MyRun.N Electrophoresis Chamber for Nucleic Acid (Cosmo Bio Co., Ltd.)
- Perkin Elmer GeneAmp 2400 Thermal Cycler PCR
- LightCycler ® 480 Real-Time PCR System (Roche Applied Science, Germany)
- Laminar Flow Cabinet Biosafety Class II (BTR, Thai Tnterfil Co.,Ltd)
- Autoclave (SANYO LABO AUTOCLAVE)
- Hot Air Oven (Momert, Thermology Co.,Ltd)

CONSUMABLE SUPPLIES

- 96-well plate (Roche Applied Science, Germany)
- Microcentrifuge tubes (1.5 ml), (Treff AG, Degersheim, Switzerland)
- Light tip eppendorf (Gilson, type 0-10 µl)
- Yellow tip eppendorf (Gilson, type 0-200 µl)
- Light blue tip eppendorf (Gilson, type 200-1000 µl)
- Microscope slide and Cover grass (Sail Brand, China)

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