Pneumocystis carinii

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Pneumocystis carinii is an atypical fungus that causes pneumonia in immuno compromised individuals. *P. carinii* comprises a heterogeneous group of organisms that have been isolated from a wide range of mammalian host species. *P. carinii* infection is host species specific, the *P. carinii* organisms that infect humans have only been found in humans. This review discusses the application of molecular techniques to the study of the biology and epidemiology of *P. carinii* infection. It addresses the use of DNA amplification for the detection and diagnosis of *P. carinii* pneumonia. Studies investigating the reservoir of infectious *P. carinii* organisms, the routes of transmission of the infection, and the emergence of drug resistant strains of *P. carinii* are also discussed.

*Pneumocystis carinii* causes pneumonia in immunocompromised individuals. Historically, it has been associated with disease in children in orphanages in Poland after World War II, and subsequently with patients undergoing organ transplantation, or receiving chemotherapy for the treatment of malignant disease. More recently, by far the highest incidence of *P. carinii* pneumonia (PCP) is in patients with HIV infection. *P. carinii* infection causes a radiographically diffuse bilateral pneumonia, which is characterised by an eosinophilic intra-alveolar exudate and a mild plasma cell interstitial pneumonitis. *P. carinii* is an extracellular organism that is found in the alveolar spaces of the lung. Other pathological sites have been occasionally observed, but these reports have been restricted to the profoundly immunocompromised such as those in the late stages of AIDS.

One of the major problems in *Pneumocystis* research is the absence of an *in vitro* culture system. Although many researchers have attempted to propagate the organism, both with and without feeder cells, the prolonged propagation of *Pneumocystis* is still not possible, nor the production of clonally derived stocks. Consequently, many fundamental aspects of the organism remain unknown, including its life-cycle. To date, all information on the life-cycle has come from studies using electron microscopy. Two distinct morphological forms have been identified: the single nucleated, thin-walled trophic form, and the cystic form which possesses a thick cell wall containing up to eight intracyclic bodies. Another morphological form, termed the procyst, has been observed which has a range of
morphological features and is thought to develop from the trophic form, with thickening of the cell wall and an increase in the number of nuclei.

**Pneumocystis is a fungus**

*P. carinii* was originally thought to be a protozoon, when first described in the early 1900s. The advent of molecular techniques has now firmly established *P. carinii* as a member of the fungal kingdom, although its exact position has not yet been elucidated. As an alternative to classical methods such as morphological characterisation, DNA sequence analysis has been used, initially with the determination of the sequence of the gene encoding the 18s rRNA. Subsequently, the DNA sequence of eight *P. carinii* mitochondrial genes was determined and this supported the fungal assignment. Since this original work, the sequences of many other genes have been elucidated and have been used to attempt to place *P. carinii* within the fungal kingdom. The data suggest that *P. carinii* is a very ancient organism, and provisionally place the organism in the fungal kingdom at the branch point between the Ascomycota and the Basidiomycota.

**There are many different types of Pneumocystis organisms**

When *Pneumocystis* was first 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 there are many different types of *P. carinii* organisms comprising a very heterogeneous group. *Pneumocystis* organisms have been isolated from a large number of different mammalian species, including laboratory animals, domestic animals, wild animals and also animals from zoological parks. Laboratory animals, and in particular the rat and mouse model of *P. carinii* infection, have been widely used for research purposes, for the propagation of large numbers of *P. carinii* organisms and for *in vivo* studies. *P. carinii* organisms from this range of mammals have been shown to be different using DNA sequence analysis. *P. carinii* infection is now recognised to be host species specific, each mammalian host species harbours one or more distinct genetic variety of *P. carinii*. This has been demonstrated by DNA sequence analysis of *P. carinii* organisms from different host species and confirmed by cross-species inoculation studies.

**Human-derived *P. carinii* is different from all other types of *P. carinii* organisms**

Although human-derived *P. carinii* is morphologically very similar to other types of *P. carinii*, and the pathology of the disease in humans is similar to that in immunosuppressed animals, such as rats and mice, human-derived
Pneumocystis carinii is very different at a molecular level. The organisms that cause PCP in humans have only been found in humans. Human-derived P. carinii is similar, but not identical, to organisms isolated from other primates. P. carinii organisms have been isolated from 18 different non-human primate species obtained from zoological parks, and these are significantly different from each other and from human derived P. carinii.

These studies are very relevant to the epidemiology of the infection. They indicate that Pneumocystis infection in man is not a zoonotic infection, and cannot be acquired from a reservoir of organisms carried in the lungs of other mammalian host species.

The levels of heterogeneity observed between Pneumocystis organisms isolated from different hosts are sufficiently high to suggest that the genus Pneumocystis contains a large number of different species. There is, however, a reluctance to define these organisms as different species solely on the basis of DNA sequence heterogeneity. The experiments that are necessary to show the existence of biological species, namely the lack of productive mating between varieties, are not yet possible, because of the absence of in vitro culture of P. carinii. As an interim measure, a trinomial nomenclature for the naming of these organisms has been adopted by the Pneumocystis community, in which the term forma specialis is used to distinguish between the different types, for example P. carinii f. sp. carinii has been adopted for rat-derived organisms and P. carinii f. sp. hominis for human-derived organisms.

Molecular detection of Pneumocystis

For many years, the detection of Pneumocystis organisms for diagnostic purposes has utilised histochemical staining of respiratory samples with Grocott's methenamine silver stain. In order to obtain sufficient organisms, this has required invasive samples from the alveoli, originally transbronchial biopsy samples, and subsequently broncho-alveolar lavage (BAL) fluid. Immunofluorescence staining with anti-Pneumocystis antibodies has also been used successfully on this type of sample. More recently, molecular techniques have been developed for the detection of Pneumocystis, based on the amplification of P. carinii DNA using the polymerase chain reaction (PCR). The most widely used locus for the detection of P. carinii DNA is the gene encoding the mitochondrial large subunit rRNA (mtLSU rRNA), which has been shown to be a sensitive and specific means of detection using PCR. A number of other genes have been used as a target for a PCR assay, but some of these primers are not entirely specific for P. carinii.

Since the original work on the detection of P. carinii in BAL samples, the PCR assay has been applied to other respiratory samples including non-invasive samples such as induced sputum, spontaneous expectorates,
oropharyngeal washes and nasopharyngeal aspirates. In these upper respiratory tract samples, where the number of *P. carinii* organisms is low, nested 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 samples have been successfully used for the diagnosis of PCP with the PCR assay\(^{32-36}\). Studies on control groups of both immunocompromised and immunocompetent individuals without *P. carinii* pneumonia have been carried out to test the utility of these non-invasive upper respiratory tract samples for the diagnosis of *P. carinii* pneumonia. *P. carinii* DNA was not detected in the samples from these control groups\(^{37}\).

PCR detection of *P. carinii* DNA in serum or blood samples has shown conflicting results. The use of these samples for the diagnosis of PCP remains to be established, although in rare cases of disseminated *P. carinii* infection, PCR on blood samples may show *P. carinii* DNA\(^{38-40}\).

Although PCR detection of *P. carinii* DNA in respiratory tract samples has proved to be very successful and of high diagnostic value for *P. carinii* pneumonia, it is at present primarily restricted to research laboratories, and is not frequently used in diagnostic services. PCR has also been found to be a sensitive and specific method for the detection of *P. carinii* DNA for epidemiological studies, including the detection of the organism in environmental samples\(^{31}\).

**Genotyping of *P. carinii f. sp. hominis***

In order to conduct epidemiological studies of *P. carinii* infection, it is necessary to be able to distinguish between isolates of *P. carinii*. In the past, a number of conventional methods of typing organisms, such as serotyping, have been tested, and have not been successful for *P. carinii*. However, more recently, molecular techniques utilising DNA sequence polymorphisms have been successfully developed for the typing of *P. carinii f. sp. hominis* isolates. Sequence heterogeneity has been observed in a number of *P. carinii f. sp. hominis* genes, and two loci have been most widely studied, the mtLSU rRNA and the internal transcribed spacer (ITS) regions of the nuclear rRNA operon. Two nucleotides have been shown to vary at the mtLSU rRNA locus and, on the basis of this, five different genotypes have been identified. The ITS regions are far more variable. Numerous polymorphisms have been identified, and up to 59 different ITS genotypes have been reported\(^{41-43}\). Other molecular methods, such as single-strand conformational polymorphism (SSCP), have also been developed\(^{44}\).

Using these molecular typing methods, it has been shown that some patients harbour more than one strain of *P. carinii f. sp. hominis*, and that *P. carinii* infection in humans is not necessarily clonal\(^{45-47}\).
Transmission of *Pneumocystis*

*Re-activation or re-infection?*

It was thought for many years that *P. carinii* pneumonia resulted from re-activation of latent infection. Serological studies have shown that children acquire anti-*P. carinii* antibodies in childhood, and it was thought that upon immunosuppression disease ensued. There is now an increasing body of data to support the idea that in some instances *P. carinii* infection is acquired *de novo*. These data have been acquired from epidemiological studies on human-derived *P. carinii* and also from studies using animal models of the infection.

Using the rat model, it has been shown that *P. carinii* organisms were eliminated from the lungs after *P. carinii* pneumonitis, and that the persistence of latent organisms was limited. Similar results were obtained using SCID mice. In the case of human infection, a number of recent studies using sensitive and specific molecular techniques have failed to detect *P. carinii* in healthy immunocompetent individuals. Studies have been conducted on respiratory tract samples, and also on autopsy lung samples using both immunohistochemistry and DNA amplification.

The possibility that re-infection with *P. carinii* organisms is a common occurrence, rather than re-activation of latent organisms, has also been examined in HIV infected individuals with recurrent episodes of *P. carinii* pneumonia. Molecular typing was carried out on BAL samples, and in some patients, particularly those in which the episodes of *P. carinii* pneumonia were greater than 6 months apart, the sequence type detected in the second and subsequent episode was different to the first, suggesting re-infection with a different *P. carinii* organism.

In addition, population studies have shown that allelic frequency distribution patterns of *P. carinii* isolates were associated with the place of diagnosis rather than place of birth, implying that any infection acquired early in life had natural limits. All these studies suggest that *P. carinii* pneumonia is an actively acquired infection. This introduces the possibility of the development of new methods of prevention of *P. carinii* infection that rely on the identification of the specific sources of infection, rather than total dependence on chemoprophylaxis with antimicrobial agents.

Interestingly, *P. carinii* DNA has been detected in samples of airborne fungal spores collected in the countryside over a period of 3 years. This study suggests that *P. carinii* may produce stable spores as one part of the life-cycle, which can be disseminated into the environment, but are not necessarily capable of propagation. Indeed, it is not yet known whether *P. carinii* is an obligate parasite, which can only propagate within its specific host, or whether it is also capable of reproducing in an environmental niche. Recent reports demonstrating the co-evolution
of *P. carinii* with its host are strongly suggestive of its absolute requirement for the host for replication\(^2^2\).

**Person-to-person transmission**

The acquisition and transmission of *P. carinii* infection is still not clearly understood. Animal models have clearly demonstrated that the infection can be transmitted from one animal to another via the airborne route. This was first demonstrated in the rat model of the infection, in which transmission from infected rats to susceptible immunocompromised rats in close contact was shown\(^5^7\). More recently, experiments using the mouse model have investigated the transmission and carriage of *P. carinii* infection. It has been shown that immunocompetent mice, transiently parasitised by *P. carinii* organisms after close contact with *P. carinii* infected mice, were able to transmit the infection to *P. carinii*-free SCID mice\(^5^8\).

In contrast, transmission of *P. carinii* infection in humans remains unclear. It is now widely accepted that *P. carinii* infection is host-species specific, and that the *P. carinii* organisms that infect humans, *P. carinii f. sp. hominis*, are different from those infecting other mammals, and are not acquired from an animal reservoir (see above). It is hypothesised that transmission of human *P. carinii* infection may be similar to that in rats and mice, based on data from a number of studies. These include investigations on immunocompromised patients with recurrent episodes of *P. carinii* pneumonia which suggest that exposure to *P. carinii f. sp. hominis* is frequent and that re-infection with different types of *P. carinii f. sp. hominis* is common (see above)\(^4^5,^5^2\).

There have been several reports of possible transmission between patients. Apparent clusters of *P. carinii* pneumonia were first observed in malnourished children during World War II. Since then observations of mini-epidemics have been sporadic over the last 30 years. Cluster cases have primarily been described in immunosuppressed children with malignancies and in transplant recipients, the number of patients in each cluster ranging from 2–19. These clusters have been regarded as supportive of the idea that nosocomial transmission occurs, and some centres have advocated the isolation of patients with *P. carinii* pneumonia to prevent transmission to susceptible patients. However, with the inability to culture *P. carinii*, and the previous absence of a typing system for *P. carinii*, definitive proof of patient-to-patient transmission has been lacking. A recent study using genotyping at the ITS locus has examined one cluster of cases of *P. carinii* pneumonia among patients with haematological malignancies and a further two other clusters in HIV infected patients. It was suggested that person-to-person transmission of *P. carinii* may occur from an infected to a susceptible immunosuppressed patient in close contact.
within the hospital environment, but that direct transmission between patients did not account for the majority of cases within the clusters\textsuperscript{59,60}.

In other studies, hospital air has been sampled and \textit{P. carinii} DNA has been detected in air samples collected from the rooms of patients with PCP. Genotyping demonstrated that in some instances the strain of \textit{P. carinii} detected in the air was the same as that isolated from clinical samples from the patient housed in the room\textsuperscript{61–63}.

The analysis of respiratory tract samples from the uncommon clinical presentation of a mother and her infant, who both had \textit{P. carinii} pneumonia, has provided another means of investigating \textit{P. carinii} f. sp. \textit{hominis} transmission. Genotyping at 3 loci of \textit{P. carinii} organisms in BAL samples obtained from an HIV infected mother and her 4.5-week-old infant who had \textit{P. carinii} pneumonia contemporaneously, has provided additional data in support of transmission of \textit{P. carinii} f. sp. \textit{hominis} infection via the respiratory route, either from mother to infant, or to both from a common exogenous source\textsuperscript{64}.

\textbf{Emergence of drug resistant strains of \textit{P. carinii} f. sp. \textit{hominis}}

Sulpha-based drugs are used for the treatment and prophylaxis of \textit{P. carinii} infection. Sulpha resistance has been reported in a number of pathogenic micro-organisms that are treated with sulpha-based drugs. It has been postulated that resistant strains may also be developing in \textit{P. carinii}, but it has not been possible to examine this directly because of the lack of an \textit{in vitro} culture system. The enzyme dihydropteroate synthase (DHPS), in the folic acid synthesis pathway, is the target of sulpha and sulphone drugs. In \textit{P. carinii} this is encoded by the folic acid synthesis (\textit{fas}) gene, a multi-functional gene that also encodes dihydro-neopterin aldolase and hydroxymethyl-dihydropterin pyrophosphokinase\textsuperscript{65}. Comparison of the DHPS sequence from a number of micro-organisms has allowed the deliniation of the substrate binding site. Mutations in the DHPS gene which alter the structure of the enzyme may cause resistance to sulpha agents by decreasing the affinity for sulphonamides and sulphones. In other micro-organisms, for example \textit{Escherichia coli}, \textit{Neisseria meningitides} and \textit{Plasmodium falciparum}, these mutations cluster in or near highly conserved regions, close to the active site of the molecule\textsuperscript{66}.

Analysis of \textit{P. carinii} f. sp. \textit{hominis} DHPS genes sequence, by DNA amplification of respiratory tract samples from patients with \textit{P. carinii} pneumonia, demonstrated that it was highly conserved, but a number of single base polymorphisms were observed which were considered to be significant. In particular two changes were observed, at nucleotide 165, resulting in a change from A to G, and at nucleotide 171 resulting in a
change from C to T, equivalent to Thr/Ala at residue 55 and Pro/Ser at residue 57 in the amino acid sequence. On the basis of these differences, four distinct DHPS genotypes were identified. By comparison with other micro-organisms, the wild-type sequence was considered to be thr55/pro57, and these residues were also found in the homologous DHPS genes from both mouse- and rat-derived 
P. carinii
67,68. It has been postulated that the 
P. carinii f. sp. hominis
DHPS double mutant Ala55/Ser57 may affect substrate binding and may be associated with drug resistance. The mutant strain has appeared recently and in multiple institutions.69 Both of the changes in the double mutant are non-synonymous, indicative of positive evolutionary pressure. In addition, the mutant genotype has been observed in association with the failure of both sulpha treatment70 and prophylaxis68,71, suggesting that resistance may be emerging. Geographic variation in allelic frequency has been detected at the DHPS locus, and the place of diagnosis was the most significant factor influencing the frequency of the double mutant genotype, when sulpha exposure was taken into account56. These data suggest that humans, although they may not be involved in the direct infection of other humans, are nevertheless important in the transmission cycle of 
P. carinii f. sp. hominis
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**Primary 
P. carinii f. sp. hominis
infection**

A number of serological studies have indicated that healthy children acquire antibodies to 
P. carinii
early in childhood, and that 94% of normal immunocompetent children have detectable antibodies by 4 years of age72. This primary infection in immunocompetent infants is thought to be asymptomatic, and indicates that exposure to 
P. carinii
occurs early in life. More recently, DNA amplification has been used to detect 
P. carinii
DNA in non-invasive respiratory samples from healthy infants, providing direct evidence of primary 
P. carinii
infection73. It is possible that healthy children constitute an important natural reservoir of 
P. carinii f. sp. hominis
organisms, and play a role in the circulation and transmission of 
P. carinii f. sp. hominis
infection in the community.

Interestingly, an association between primary 
P. carinii f. sp. hominis
infection and sudden infant death syndrome (SIDS) has been reported, in studies in which autopsy lung samples were examined using histochemical staining74,75. A mild infection was observed, with the quantity of 
P. carinii
organisms being much lower than the numbers seen in immunocompromised individuals with 
P. carinii
pneumonia. This mild infection resembled that observed in rabbits and piglets, which spontaneously acquire and subsequently clear 
P. carinii
infection at the time of weaning76,77. More research is needed to investigate the significance of this association, for example to establish whether 
P. carinii
plays a pathogenic role in some
infants with SIDS, or whether the presence of *P. carinii* organisms indicates an as yet unrecognised underlying immune deficiency in these infants.

**Genome of Pneumocystis**

Most information about the characterisation of the genome has been obtained from rat-derived *P. carinii*, *P. carinii f. sp. carinii*, since these organisms are more readily available and better preserved than human-derived *P. carinii*. The chromosomes have been analysed by electrophoretic separation using pulsed field gel electrophoresis. The genome consists of 16 linear chromosomes that range from 300 kb to 700 kb in size, with a total genome size of 7.7 Mbp. The composition of the *P. carinii f. sp. carinii* genome is A/T rich, with a typical gene sequence having 60–65% A/T. The coding regions may contain one or more short introns, of approximately 50 bp in length, and a higher A/T content than the coding regions. It is thought that both the trophic and cystic forms contain a haploid number of chromosomes for the majority of the time, although reports of rare observations of synaptonemal complexes using electron microscopy have suggested that the organism may have a brief diploid phase.

The ends of the *P. carinii* chromosomes are of particular interest because they have been shown to contain genes that encode the surface antigens of the organism. To date, three multigene families encoding surface antigens have been identified in the subtelomeric regions of the chromosomes, the major surface glycoprotein (MSG), the major surface glycoprotein-related antigen (MSR), and the PRT1 protease. These subtelomeric gene families are important because they contain genes that play a major role in the parasite/host interaction. *P. carinii* is an unusual fungus, in that antigenic switching of MSG has been demonstrated. Regulation of expression of MSG genes is mediated by an expression site, which is characterised by a sequence known as the upstream conserved sequence (UCS), and is present in one copy per genome, in the subtelomeric region of chromosome 9. Differential expression is achieved by the installation of different copies of MSG at the expression site, by recombination between the expressed copy and another silent copy. In this way, the isoform of MSG which is expressed on the surface of an organism is changed by switching the MSG gene which occupies the expression site. It is possible that co-ordinated expression of the MSG, MSR and PRT1 gene families takes place.

Recently, an international *Pneumocystis* genome project has been initiated to determine the complete genome sequence of the *P. carinii f. sp. carinii* genome initially, and then the *P. carinii f. sp. hominis* genome. It is being undertaken by a consortium of academic institutions. Cosmid libraries have been constructed, and the cosmids are being ordered and...
assigned to specific chromosomes (http://biology.uky.edu/Pc/). In addition, an expressed sequence tag (EST) database has been created to provide an inventory of genes expressed in *P. carinii f. sp. carinii*.

The elucidation of the complete sequence of the *P. carinii* genome will increase our understanding of many aspects of this organism, including the structure and organisation of its genome. It may result in the development of efficient methods of *in vitro* culture. It will lead to the identification of every protein encoded by the genome, and yield insight into metabolic processes, and whether the organism possesses the genes necessary for a sexual replication cycle. It will facilitate the analysis of the regulation of the expression of these genes, including those encoding surface antigens, and ultimately may contribute to the identification of new drug targets for the prevention and treatment of *P. carinii* pneumonia.

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