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Minireview

MINIREVIEW

Pathobiology of *Pneumocystis* pneumonia: life cycle, cell wall and cell signal transduction

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One sentence summary: Review of the life cycle, cell wall components and cell signaling of *Pneumocystis*, an important cause of pneumonia in immunosuppressed individuals.

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ABSTRACT

Pneumocystis is a genus of ascomycetous fungi that are highly morbid pathogens in immunosuppressed humans and other mammals. *Pneumocystis* cannot easily be propagated in culture, which has greatly hindered understanding of its pathobiology. The *Pneumocystis* life cycle is intimately associated with its mammalian host lung environment, and life cycle progression is dependent on complex interactions with host alveolar epithelial cells and the extracellular matrix. The *Pneumocystis* cell wall is a varied and dynamic structure containing a dominant major surface glycoprotein, β-glucans and chitins that are important for evasion of host defenses and stimulation of the host immune system. Understanding of *Pneumocystis* cell signaling pathways is incomplete, but much has been deduced by comparison of the *Pneumocystis* genome with homologous genes and proteins in related fungi. In this mini-review, the pathobiology of *Pneumocystis* is reviewed, with particular focus on the life cycle, cell wall components and cell signal transduction.

Keywords: opportunistic infection; acquired immunodeficiency syndrome (AIDS); cell wall; life cycle; cell signaling

INTRODUCTION

Pneumocystis is among the most important fungal pathogens in immunosuppressed humans. Historically, *Pneumocystis* has been closely associated with the AIDS epidemic. Mortality due to *Pneumocystis* in HIV patients has declined in recent years thanks in large part to antiretrovirals and use of effective prophylactic medications in the developed world. However, with the continued global AIDS epidemic, *Pneumocystis* remains an important pathogen worldwide. *Pneumocystis* is also an important pathogen in individuals without HIV taking immunosuppressive medication. Efforts to study *Pneumocystis* have been greatly limited by the inability to maintain continuous *ex vivo* axenic culture of the organism. Nonetheless, study of organisms isolated directly from the infected lung of immunosuppressed research animals has allowed for greater insight into the biology of *Pneumocystis*. This article will review the pathobiology of *Pneumocystis* pneumonia with particular focus on the life cycle, cell wall biology and cell signal transduction.

HISTORY AND TAXONOMY

Pneumocystis was first described in 1909 by Carlos Chagas. He initially misidentified it as a schizogonic form of *Trypanosoma cruzi*, but, in 1912, husband and wife researchers Delanoë and Delanoë at the Institut Pasteur in Paris observed the organism in *Trypanosoma*-free rats and concluded that it was a new organism, proposing the name *Pneumocystis carinii* (Delanoë and Delanoë 1912; Calderon-Sandubete *et al.* 2002; Chabe *et al.* 2011). The

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organism was initially classified as a protozoan, but there was ongoing controversy in the subsequent decades about whether to classify it as a protozoan or fungus (Chabe *et al.* 2011). The advent of gene sequencing techniques in the late 1980s finally settled the debate. In 1988, sequencing of the rRNA and subsequent analysis led to overwhelming evidence for assignment of *Pneumocystis* to the kingdom Fungi (Edman *et al.* 1988). *Pneumocystis* is included in the phylum Ascomycota and is related to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Edman *et al.* 1988).

Pneumocystis has been described in the lungs of numerous mammalian species (Yoshida *et al.* 1981; Shiota *et al.* 1990; Dei-Cas *et al.* 1998). Originally, *Pneumocystis* was felt to be a single species that subsists across mammals, causing zoonotic infection in humans (Kucera 1967). However, it has since been discovered that the different *Pneumocystis* species or strains that infect different mammals are phenotypically (Gigliotti *et al.* 1986) and genetically (Sinclair *et al.* 1991) distinct. A unique *Pneumocystis* species has been identified for nearly all mammal species studied, and each has stringent host specificity such that they do not cross-infect other mammalian species (Gigliotti *et al.* 1993; Durand-Joly *et al.* 2002). In primates, genetic divergence among *Pneumocystis* species has been shown to be proportional to the phylogenetic divergence of the host species (Demanche *et al.* 2001). This suggests that *Pneumocystis* is an ancient organism that has co-evolved with mammals. Historically, all *Pneumocystis* strains were identified as *P. carinii*. However, in 2001, the International Workshop on Opportunistic Protists proposed to designate the human *Pneumocystis* as a separate species, named *Pneumocystis jirovecii* (Stringer, Cushion and Wakefield 2001, Stringer *et al.* 2002; Stringer, Beard and Miller 2009). The original name *P. carinii* was retained for a *Pneumocystis* species that co-exists with rats. Although it is likely that each *Pneumocystis* strain associated with a different mammalian species may represent a distinct species within the genus *Pneumocystis*, at the time of writing only three other species have been named and described, i.e. *Pneumocystis murina* (mouse) (Keely *et al.* 2004), *Pneumocystis wakefieldiae* (rat) (Cushion, Keely and Stringer 2004) and *Pneumocystis oryctolagi* (rabbit) (Dei-Cas *et al.* 2006).

Pneumocystis was first recognized as a human pathogen in the 1940s after it was observed in the lungs of malnourished infants afflicted with plasma cell pneumonitis (van der Meer and Brug 1942; Catherinot *et al.* 2010). The AIDS epidemic in the 1980s led to markedly increased interest in *Pneumocystis* pneumonia due to high incidence of the disease in patients with AIDS. *Pneumocystis* was among the first reported AIDS-associated illnesses (Masur *et al.* 1981), and, in the 1980s, *Pneumocystis* was the AIDS-defining illness for nearly half of all adults diagnosed with AIDS, with associated high mortality (Jaffe, Bregman and Selik 1983). Fortunately, with the advent of antiretroviral therapies and efficient use of prophylactic medications, there has been a substantial decline in the incidence of *Pneumocystis* pneumonia in HIV-positive patients in the developed world (Ives, Gazzard and Easterbrook 2001). However, with the continued global AIDS epidemic, *Pneumocystis* remains an important pathogen in the developing world, particularly in areas without widespread access to antiretroviral therapy or accurate diagnostic testing for *Pneumocystis* (Malin *et al.* 1995; Russian and Kovacs 1995; Chakaya *et al.* 2003; Bates *et al.* 2013; Saeed, Farid and Jamsheer 2015). In the developed world, the widespread use of immunosuppressive medications for chronic disease has now led to increased incidence of *Pneumocystis* pneumonia in adults and children without HIV, particularly individuals taking corticosteroid therapy (Yale and Limper 1996; Roux *et al.* 2014; Stern *et al.* 2014).

LIFE CYCLE

A complete understanding of the life cycle of *Pneumocystis* is difficult because the organism has yet to be grown in continuous *ex vivo* axenic culture, although growth of *Pneumocystis* in co-culture with host cells has been described (Schildgen *et al.* 2014). *Pneumocystis* has many features to suggest that it is probably an obligate biotroph, obtaining essential nutrition from living host cells. A genomic analysis comparing *Pneumocystis* with the reconstructed genome of an ancestral fungus suggests that it evolved into its current state through loss of genes from multiple pathways essential for independent growth and reproduction (Cisse, Pagni and Hauser 2014). *Pneumocystis* is an ascomycetous fungus, and much of the understanding of its life cycle has been generated from microscopic observation with extrapolation from the life cycle of other ascomycetous fungi (Fig. 1). *Pneumocystis* organisms have a high tropism for the lung. All life cycle stages have been observed within the lungs, and *Pneumocystis* exists almost exclusively within the lung of infected hosts. *Pneumocystis* has rarely been described in extrapulmonary sites and generally only in host mammals with very heavy organism burden and with structural damage to the lungs (Guttler *et al.* 1993; Chary-Reddy and Graves 1996). *Pneumocystis* resides extracellularly, predominantly in the lung alveoli. Three major life cycle stages have been identified by morphology: the cyst (also known as the spore case or ascus), the precystic form and the trophic form stages (Walzer *et al.* 1976; Matsumoto and Yoshida 1984). These stages were initially described at a time when *Pneumocystis* was felt to be a protozoan, which is why the nomenclature of the life cycle forms are over time evolving to be more consistent with other fungal organisms (Cushion 1998).

The trophic form of *Pneumocystis* is mononuclear and thin walled. The trophic form predominates during active infection and fills the alveolar space, with only a few cystic and precystic forms present (Walzer *et al.* 1976). Trophic forms have a highly irregular pleomorphic shape and are variable in length, ranging from 1 to 8 μm long, with multiple pseudohyphal structures that may help to anchor to host cells (Walzer *et al.* 1976; Filice *et al.* 1985; Ruffolo, Cushion and Walzer 1986; Shiota, Yamada and Yoshida 1986; Dei-Cas *et al.* 1991). The trophic forms preferentially anchor to type I alveolar epithelial cells in host alveoli (Shiota, Yamada and Yoshida 1986). Ultrastructural analysis of the trophic form shows a single nucleus surrounded by a thin nuclear membrane, rough and smooth endoplasmic reticulum, glycogen granules and usually a single mitochondrion (Cushion 1998). The majority of trophic forms observed in an infected organism are haploid, but some are diploid (Martinez *et al.* 2011). It is not certain whether the diploid trophic organisms are the result of mating between two trophic forms or represent a step towards asexual division of a single organism; however, there is increasing evidence that *Pneumocystis* does have a sexual life cycle phase. The trophic form expresses a pheromone receptor (Vohra *et al.* 2004), and in other actinomycete fungi such as *S. cerevisiae* a similar pheromone receptor binds peptide pheromones released from cells of compatible mating types, triggering conjugation of the two cells to form a zygote. Additionally, mating type (MAT) genes have been described in multiple *Pneumocystis* species that are homologous to the MAT genes in *S. pombe* involved in sexual reproduction (Almeida *et al.* 2015). The function of these genes in *Pneumocystis* have not been fully characterized, but the

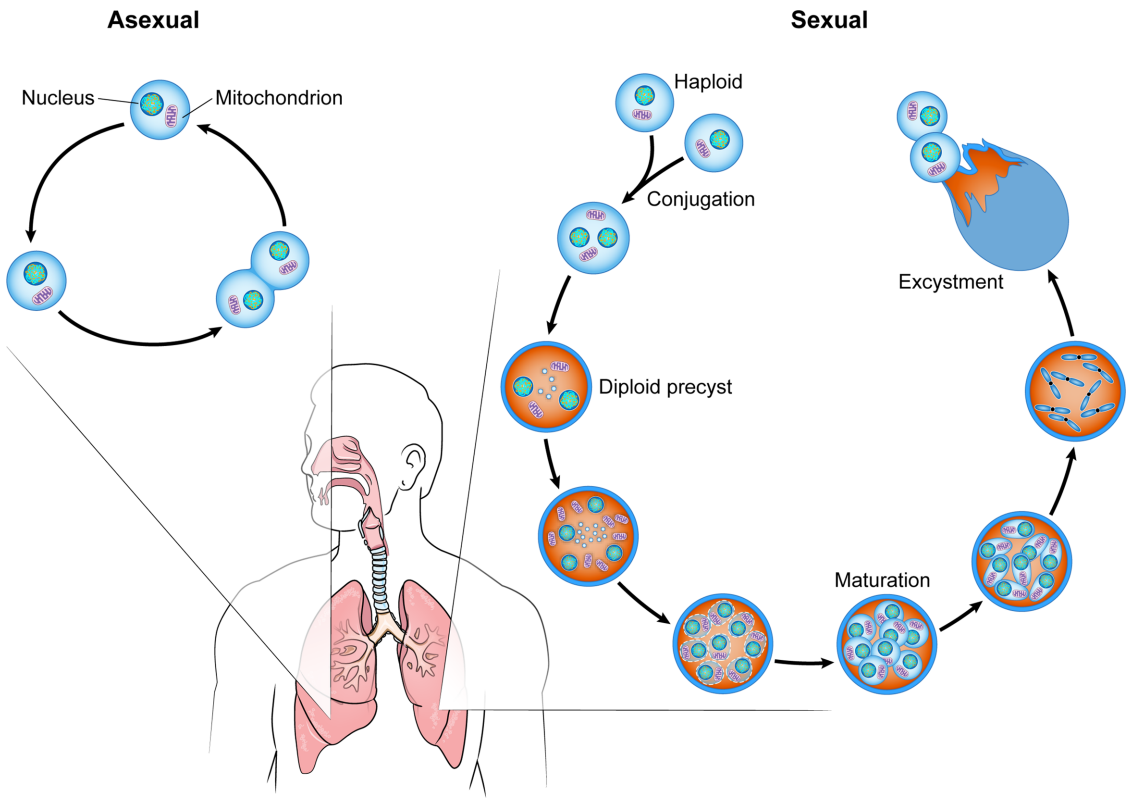

Figure 1. Proposed *Pneumocystis* life cycle. Asexual reproduction of trophic forms probably occurs during acute infection. Sexual reproduction probably occurs when haploid trophic forms conjugate to form a diploid precyst. The precyst matures through the early, intermediate and late phase to form a mature cyst which ultimately releases young trophic forms.

number of MAT genes and location on a single DNA molecule suggest that *Pneumocystis* may reproduce by primary homothallism, meaning that there is a single self-compatible mating type rather than two opposite mating types (Almeida *et al.* 2015).

The next phase in the life cycle after the trophic form is the precyst, which may be an intermediate stage in sexual reproduction. The precyst is larger than the trophozoite, ~4–8 μm, and more spherical in shape (Cushion 1998). There are three precyst phases that probably occur in progression leading to formation of the cyst (Yoshida 1989). The early precyst is round and mononuclear with cell wall composition similar to the trophic form except that pseudohyphae are mostly absent (Chabe *et al.* 2011). Synaptonemal complexes, which are protein structures associated with meiotic division, have been observed in the trophic and early precyst forms, suggesting sexual reproduction (Matsumoto and Yoshida 1984). The intermediate precyst phase is defined by thickening of the cell wall from 40 nm to ~100 nm and also nuclear division with resultant increase to 2–8 nuclei in each organism (Walzer *et al.* 1976). The late precyst phase is characterized by a mature cystic appearance with eight well-defined nuclei. It is distinguished from the final cystic form in that it does not yet have eight well-defined intracystic bodies with associated organelles; rather, it simply has eight nuclei each of which will ultimately be contained in an individual spore (Walzer *et al.* 1976).

The cystic form is an ascus-like structure or spore case that is characterized by a thick cell wall with an outer membrane (De Stefano *et al.* 1990). It is 4–8 μm in length (Walzer and Cushion 2005). A mature cyst typically contains eight intracystic bodies within its cell wall, but can also contain smaller numbers such as two or four spores (Martinez *et al.* 2011). Each intracystic body is ~1 μm in diameter and consists of a double membrane, a single nucleus and a mitochondrion. Young trophic forms are released from mature cysts. Budding has not been observed in *Pneumocystis*. Formation of a pore in the cyst wall allows for release of the eight haploid ascospores, which then attach to type I alveolar epithelial cells of the host mammal (Itatani 1994). The cystic form is probably the means by which *Pneumocystis* is able to survive in the environment outside of the lungs (Chin *et al.* 1999). *Pneumocystis*-infected mice treated with anidulafungin, an echinocandin drug which inhibits β-1,3-glucan synthesis, are unable to transmit the infection to other mice (Cushion *et al.* 2010). This suggests that the cyst form is essential for transmission of infection.

Adhesion of *Pneumocystis* trophic forms to the host alveolar epithelial cell and matrix proteins is a key component of its life cycle. Smaller, presumably young, trophic forms without pseudopodal structures are ultrastructurally attached to host type I alveolar epithelial cells by adhesion (Walzer *et al.* 1976). Larger, presumably mature, trophic forms attach with a complex infolding between the *Pneumocystis* pseudopodal structures and the host cell membrane (Dei-Cas *et al.* 1991). The organism anchors itself to the host cell without disrupting the host cell plasma membrane. The lung alveolar epithelial cells reside within a complex collection of extracellular matrix proteins, and adhesion of *Pneumocystis* to these extracellular matrix proteins such as fibronectin and vitronectin is likely to be the first step in attachment of organisms to the host cells and propagation of infection (Pottratz *et al.* 1991; Limper *et al.* 1993). Binding of *Pneumocystis* to fibronectin and other cell matrix proteins triggers altered gene expression that results in increased *Pneumocystis* growth and proliferation. Specifically, binding of trophic forms

to collagen 1, fibronectin and vitronectin triggers upregulation of genes including *Pcste20* that promotes morphological changes, mating and organism proliferation (Kottom and Limper 2002; Kottom *et al*. 2003). This response occurs almost exclusively in the trophic form rather than the cystic form (Kottom and Limper 2002). The cell surface molecules that mediate this response have not been fully characterized. Major surface glycoprotein (MSG) expressed in the *Pneumocystis* cell wall has been shown to interact with fibronectin (Pottratz *et al*. 1991), but this is probably not the primary mechanism behind the cell signaling pathways that trigger proliferation and growth as MSG is expressed in both the cystic and trophic form. More recently, an integrin-like *Pneumocystis* extracellular matrix adhesion receptor, PcInt1, has been described which mediates *Pneumocystis* attachment to fibronectin (Kottom, Kennedy and Limper 2008). This receptor is expressed almost exclusively by the trophic form and has been hypothesized to be important for triggering growth and life cycle progression in response to *Pneumocystis* binding to host cells (Kottom, Kennedy and Limper 2008).

CELL WALL

The *Pneumocystis* cell wall is a dynamic carbohydrate-rich structure consisting of β-glucans, chitins and other carbohydrate polymers (Roth *et al*. 1997; De Stefano *et al*. 1998). These cell wall components are important for cell growth and integrity, as well as mediating host immune response to *Pneumocystis* infection. The components of the cell wall vary across the different life cycle states of *Pneumocystis* such as the trophic and cystic forms.

A key component of the *Pneumocystis* cell wall is MSG, also known as glycoprotein A (gpA). MSG from *P. carinii* is a glycoprotein weighing ~120 kDa that is heavily carboxylated with mannose residues (Gigliotti *et al*. 1988; Radding *et al*. 1989; Kovacs *et al*. 1993). MSG is the among the most abundant cell surface proteins in *Pneumocystis*. MSG is important for pathogenesis as it is involved in adhesion to lung alveolar epithelial cells and evasion of host defenses. The MSG is also the target for host immune defenses as it is a major antigen recognized by antisera of exposed hosts (Haidaris *et al*. 1992). Antigenic variation of MSG expression has been proposed as a mechanism for evasion of host defenses by *Pneumocystis* (Kovacs *et al*. 1993).

An interesting aspect of MSG is that, although there are genes for ~80 isoforms in the *Pneumocystis* genome (Stringer and Keely 2001), only a single isoform of MSG is expressed on the cell surface at any one time. Isoforms differ by *Pneumocystis* species and may confer some species specificity and facilitate evasion of host defenses (Stringer *et al*. 1993). Isoforms can also vary in sequence by as much as 35% (Stringer and Keely 2001). Although each individual *Pneumocystis* organism only expresses a single MSG isoform, a population of *Pneumocystis* in a single host will express multiple MSG isoforms during infection (Linke *et al*. 1994). Transcription and transport to the cell surface of MSG is restricted to a single isoform by the following method: each *Pneumocystis* organism contains a library of ~80 MSG genes (Kutty *et al*. 2008). The MSG genes are clustered near the telomeres, with 2–4 MSG genes at the ends of each of the 15 chromosomes (Wright *et al*. 1995). Only a single MSG gene is expressed at any one time, with the remainder being transcriptionally silent. A promoter region called the upstream conserved sequence (UCS) appears only a single time in each *Pneumocystis* genome, immediately upstream of the MSG isoform that is expressed by the particular organism (Sunkin and Stringer 1997; Haidaris *et al*. 1998; Kutty, Ma and Kovacs 2001; Schaffzin and Stringer 2004). The UCS encodes a peptide that is responsible for directing MSG to the endoplasmic reticulum where it undergoes glycosylation (Sunkin and Stringer 1997). The UCS peptide is subsequently cleaved off the MSG and the MSG is directed to the cell surface (Sunkin and Stringer 1997). It is unclear how *Pneumocystis* switches between different MSG isoforms. The location of the UCS at the telomere may promote recombination events that facilitate switching of the UCS to a different MSG gene, resulting in expression of a different MSG isoform (Edman *et al*. 1996; Stringer and Keely 2001).

Members of two other gene families, MSG-related (MSR) and PRT1, are also clustered with MSG near the telomere in *P. carinii*, and may also be important for cell surface antigenic variation (Keely *et al*. 2005). Expression of these genes is not dependent on the UCS, and a *P. carinii* population that predominantly expresses a single MSG isoform will express a variety of PRT1 and MSR isoforms (Keely and Stringer 2003; Ambrose *et al*. 2004). MSR genes closely resemble MSG and express a protein found on the cell surface (Huang *et al*. 1999; Keely *et al*. 2005). The PRT1 genes encode a family of proteases (Ambrose *et al*. 2004). Interestingly, >40 PRT1 genes have been described in *P. carinii*, but only a single PRT1 gene has been described in other *Pneumocystis* species, including *P. jirovecii* (Kutty and Kovacs 2003; Keely *et al*. 2005). In *P. jirovecii*, a single PRT1 gene has been described which encodes Kex1, a kexin-like protease that is probably responsible for processing proteins in the Golgi apparatus (Kutty and Kovacs 2003). This protein may be involved in proteolytic processing of MSG prior to transport to the cell surface. In *P. carinii*, multiple PRT1-encoded proteases are expressed on the cell surface and have unknown function, but may be responsible for degradation of exogenous host proteins or facilitating antigenic variation by modification of other cell surface proteins (Russian *et al*. 1999; Stringer 2007).

β-Glucan is another key component of the *Pneumocystis* cell wall. Fungal cell wall β-glucans are comprised of D-glucose polymers linked together via β-1,3 linkages to form a backbone with variable attachment of side chains formed by β-1,6 linkages (Douglas 2001). β-Glucans are assembled by a complex of enzymes located within the cell membrane. Many of the genes involved in synthesis, maintenance and degradation of β-glucans in the *Pneumocystis* cell wall have been characterized, primarily by comparison with homologous genes and proteins in other ascomyceteous yeasts. The enzyme PcGsc-1 glucan synthetase is responsible for polymerizing uridine-5′-diphosphoglucose into β-1,3-glucan polymers, thus forming the glucan backbone of the *Pneumocystis* cell wall (Kottom and Limper 2000). The cystic and trophic forms of *Pneumocystis* have distinct cell wall components, with β-1,3-glucan being abundant in the cystic form but undetectable in the trophic form (Nollstadt *et al*. 1994), and the enzyme PcGsc-1 glucan synthetase is expressed almost exclusively in the cystic form (Kottom and Limper 2000). The cystic form has also been shown to contain β-1,6-glucan, and a putative β-1,6-glucan synthase gene, *Pckre6*, has recently been described in *Pneumocystis* (Kottom *et al*. 2015). β-Glucans are essential for stability of the cyst form of *Pneumocystis*, and administration of a β-1,3-glucan synthesis inhibitor has been shown to prevent the development of viable cysts (Matsumoto, Yamada and Amagai 1991). An important regulator of glucan cross-linking is the *Pcphr1* gene. The *Pneumocystis Pcphr1* gene, which is homologous to *PHR1/PHR2* genes in *Candida albicans*, encodes a protein that probably functions to cross-link β-1,6-glucans to the β-1,3 backbone (Fonzi 1999; Kottom, Thomas and Limper 2001). The *Pcphr1* gene is pH responsive, with optimal expression

at pH 7.0–7.5 (Kottom, Thomas and Limper 2001). This may allow for adaptation of the cell wall to changing environmental conditions, such as preparing the cell for life cycle progression when at the physiological pH of the host mammal. *Pceng2* is a single-copy gene encoding an endo-β-1,3-glucanase that is expressed exclusively in the cystic form of *Pneumocystis* (Villegas, Kottom and Limper 2010). Endo-β-1,3-glucanase degrades the β-1,3-glucan backbone, resulting in solubilization and release of MSG from the cell wall (Kutty *et al.* 2015). PcEng2 expression has been hypothesized to facilitate switching the MSG isoform expressed by *Pneumocystis* to allow for antigenic variation (Kutty *et al.* 2015).

β-Glucans of *Pneumocystis* and other fungi are highly immunostimulatory to host cells including alveolar epithelial cells, dendritic cells and macrophages (Hoffman, Standing and Limper 1993; Vassallo, Standing and Limper 1999; Limper *et al.* 2003; Steele *et al.* 2003; Evans *et al.* 2005; Carmona *et al.* 2006). β-Glucans stimulate via the innate immune system by binding to pattern recognition receptors such as dectin-1 and other C-type lectin receptors (Ricks *et al.* 2013). The relative importance of each cell wall component in stimulating innate immune activation is unknown. β-1,3-Glucan is the dominant carbohydrate in the *Pneumocystis* cell wall, but other glucans such as β-1,6 polymers have been shown to stimulate a vigorous immune response in vitro and may be equally or more important for host immunity (Kottom *et al.* 2015). Activation of dectin-1 occurs via spleen tyrosine kinase (syk) and leads to nuclear factor-κB (NF-κB) translocation to the nucleus, resulting in production of inflammatory cytokines by the host cell (Dennehy *et al.* 2008). This vigorous host inflammatory response to *Pneumocystis* β-glucans paradoxically is likely to be a major contributor to respiratory failure in hosts with *Pneumocystis* pneumonia.

The *Pneumocystis* cell wall also probably contains chitins, but the mechanisms of chitin production and regulation in *Pneumocystis* are not as well characterized as those of other cell wall components. Chitin is an *N*-acetylglucosamine carbohydrate polymer that is used to provide structural strength in the fungal cell wall and also arthropod exoskeletons (Merzendorfer 2011). Studies using antisera probes for chitin and *N*-acetyl-D-glucosamine oligosaccharides have suggested that chitin is present in the *Pneumocystis* cell wall at all life cycle phases, including both trophic and cystic forms (Walker, Garner and Horst 1990; Garner, Walker and Horst 1991). The mechanism for synthesis of chitin has not been fully described, but the *Pneumocystis* genome contains at least one gene, *Pcchs5*, that is probably a chitin synthesis chaperone, as suggested by strong homology to known genes in *S. cerevisiae* and *S. pombe* (Villegas, Kottom and Limper 2012). Other ascomycetous fungi have between two and 20 chitin synthetase genes so there are probably other *Pneumocystis* genes involved in chitin synthesis (Merzendorfer 2011). Chitinase activity is also necessary for *Pneumocystis* cell wall regulation. The cell wall biosynthesis kinase PcCbk1 is expressed in both cystic and trophic forms, and has multiple regulatory functions including cell growth, separation and mating (Kottom and Limper 2004). Expression of PcCbk1 induces expression of yeast chitinases, which may be necessary to facilitate the physical separation of proliferating cells (Kottom and Limper 2004). Research into host immune response to *Pneumocystis* cell wall components has largely focused on MSG and β-glucans. However, chitin may also be important during the host immune response, as chitinases are expressed by mammals and induced in the lungs during Th2 inflammation (Elias *et al.* 2005; Villegas, Kottom and Limper 2012).

CELL SIGNALING AND CELL CYCLE CONTROL

Cell signal transduction

Sophisticated cell signaling pathways allow *Pneumocystis* to change cell physiology in response to environmental cues including regulation of reproduction, life cycle events, cell wall maintenance, changes in organism morphology (pseudopodal extensions), and proliferation. Understanding of *Pneumocystis* signaling pathways remains incomplete, but much has been learned by comparison of *Pneumocystis* genes with homologous genes and proteins found in other ascomycetous fungi.

One of the earliest signal transduction elements described in *Pneumocystis* was the gene *Pcg1*, which encodes a G-protein α (Smulian *et al.* 1996). Guanine nucleotide-binding proteins (G-proteins) are highly conserved signal transduction proteins, which in other fungi have been shown to regulate cell growth, mating and nutritional responses (Obara *et al.* 1991; Baasiri *et al.* 1997; Neves, Ram and Iyengar 2002; Segers and Nuss 2003). The role of *Pcg1* in *Pneumocystis* is uncertain, but deletion of a similar gene in *Neurospora crassa* suggests that it may be involved in virulence and reproduction (Baasiri *et al.* 1997). Other *Pneumocystis* G-proteins have subsequently been reported. The small G-protein, PcCdc42, may be important for facilitating the gene expression changes that are triggered when the trophic form of *Pneumocystis* binds to the host cell and extracellular matrix. This is among the most important life cycle events in *Pneumocystis* as it triggers upregulated expression of *Pcste20* and *Pccbk1*, other genes responsible for mating and proliferation. PcCdc42 is probably the upstream mechanism of activation of PcSte20 during this process (Krajicek *et al.* 2010).

Phosphate transfer catalyzed by mitogen-activated protein (MAP) kinases and other kinases is a highly important mechanism of cell signal transduction in many fungi (Gustin *et al.* 1998; Johnson and Lapadat 2002). Fungal MAP kinases have been shown to regulate growth, reproduction, cell wall maintenance and response to environmental changes (Thomas and Limper 2007). The first MAP kinase described in *Pneumocystis* was Pcm, which is a MAP kinase that appears to mediate pheromone signaling (Thomas *et al.* 1998a). Pcm in *Pneumocystis* is similar to Fus3, a MAP kinase in *S. cerevisiae* that is activated during low nutrient conditions to trigger production of peptide pheromones (Thomas *et al.* 1998a). These pheromone peptides bind to pheromone receptors on *S. cerevisiae* cells of opposite mating type and trigger a series of events that leads to conjugation of the cells and formation of an ascus (Bardwell *et al.* 1994; Gustin *et al.* 1998). There is evidence to suggest that a similar mating process mediated by Pcm takes place between the haploid trophic forms of *Pneumocystis*. *Pcm* is predominantly found in the trophic form of *Pneumocystis* rather than the cystic form (Vohra, Puri and Thomas 2003), and *Pneumocystis* trophic forms have been shown to express a pheromone receptor (Vohra *et al.* 2004). Furthermore, *Pcm* restores pheromone signaling to *S. cerevisiae* strains in which Fus3 has been deleted (Vohra, Puri and Thomas 2003).

The best-characterized kinase cascade in *Pneumocystis* is the cell wall integrity pathway. The model for understanding this pathway has been well described. The cell integrity kinase cascade in *S. cerevisiae* protects the cell wall in response to environmental stressors such as heat, osmolar stress or nutrient limitations (Gustin *et al.* 1998; Xu 2000). In *S. cerevisiae*, increased environmental temperature triggers a cascade that progresses through upstream MAP kinase *Bck1* to *Mkk1/Mkk2* to downstream MAP kinase *Slt2* which ultimately results in increased expression of Fks2, a β-1,3-glucan synthetase that preserves cell

wall integrity and prevents cell death (Gustin *et al.* 1998; Xu 2000). Multiple homologous elements of this pathway have been described in *Pneumocystis*. A *Pneumocystis Pccbk1* gene has been identified, and expression of this gene in *S. cerevisiae bck1*Δ yeast corrects their temperature-sensitive cell lysis defect (Thomas *et al.* 2003). A gene with high homology to *S. cerevisiae Mkk1* has also been identified in the *Pneumocystis* genome (Walzer *et al.* 1976). Finally, the *Pneumocystis* gene *Pcmkp1* has been described which encodes a MAP kinase that is highly homologous to *slt2*. Expression of *Pcmkp1* in *slt2*Δ *S. cerevisiae* strains has been shown to restore cell integrity pathway function (Fox and Smulian 1999). In *Pneumocystis*, *Pcmkp1* is upregulated in response to oxidative stress, and *Mkp1* has been hypothesized to be the terminal kinase in a pathway analogous to the *S. cerevisiae* cell integrity pathway (Fox and Smulian 1999).

Cell cycle

The cell cycle of *Pneumocystis* is controlled by the expression and activation of cell division cycle (cdc) molecules. The components and phases of the cell division cycle are highly conserved across eukaryotic organisms. Cdc2 is a serine-threonine kinase that has been shown in other ascomycetous fungi to be essential for progression from G_1 phase to S phase and from G_2 phase to mitosis. *Pneumocystis* contains a Cdc2 molecule that appears to have similar function (Thomas *et al.* 1998b). *Pneumocystis* PcCdc2 has greater activity in the trophic compared with the cystic form, consistent with the idea that trophic forms have more rapid cell cycling (Thomas *et al.* 1998b). Cdc13 is another cell division cycle kinase that is closely associated with Cdc2. Cdc13 has been shown in other fungal organisms to bind to Cdc2, forming an activated Cdc2–Cdc13 complex that is essential for completion of meiosis (Fisher and Nurse 1995). Cdc13 levels vary during the cell cycle and peak in late G_2 and early M phase (Fisher and Nurse 1995). In *Pneumocystis*, a PcCdc13 homolog has been identified that restores function to Cdc13-deficient *S. pombe* strains (Kottom *et al.* 2000). Interestingly, *Pneumocystis* PcCdc13 is present at higher levels in the cystic form compared with the trophic form (Kottom *et al.* 2000). This may suggest that Cdc2 interacts with different cyclins during different *Pneumocystis* life cycle stages, specifically that another cyclin partner for Cdc2 besides Cdc13 may be expressed by the trophic form. A third cell cycle kinase, PcCdc25, has been identified in *Pneumocystis*. In other fungi, Cdc25 has multiple functions that include activation of Cdc2 and DNA checkpoint arrest (Gustafson, Lumper and Leon 1999). Expression of *Pneumocystis* PcCdc25 by Cdc25-deficient *S. pombe* strains restores cell growth and restores the DNA damage checkpoint, but does not restore the DNA replication checkpoint (Gustafson *et al.* 2001). This suggests differences in the role of Cdc25 between *Pneumocystis* and *S. pombe*.

Some of the proteins involved in DNA replication have also been described in *Pneumocystis*, with particular attention devoted to histone acetyl transferase pathways. Histone proteins form the core of the nucleosome, the basic package of DNA in eukaryotic organisms, and modifications to histones are important in the regulation of DNA transcription and replication in all eukaryotic organisms. One particular histone modification, the acetylation of histone H3 Lys56, has been shown to be essential for DNA repair after checkpoint activation (Chen *et al.* 2008). A novel histone acetyl transferase, Rtt109, has been identified which performs the H3 Lys56 acetylation in fungi (Han *et al.* 2007). Rtt109 has been found in many yeast, but it has not been identified in mammals or other higher eukaryotic organisms (Han *et al.* 2007). This makes it a potentially attractive drug target for anti-*Pneumocystis* therapy. In *Pneumocystis*, PcRtt109 and PjRtt109 have been demonstrated which restore function to rtt109Δ *S. cerevisiae* (Kottom *et al.* 2011; Dahlin *et al.* 2014). Chaperone proteins PcAsf1 and PcVps75, essential for Rtt109 function, have also been characterized in *Pneumocystis* (Pupaibool *et al.* 2013). Further research is needed to determine whether anti-Rtt109 pharmacotherapy may be an effective treatment for *Pneumocystis* infection.

CLINICAL DISEASE

Epidemiology and transmission

The reservoir for *Pneumocystis jirovecii* pneumonia (PJP) infection is not fully understood. Although *Pneumocystis* DNA has been detected in sites such as orchards and pond water (Wakefield 1994; Casanova-Cardiel and Leibowitz 1997), no reservoir of viable organisms has been found outside mammalian hosts. PJP is primarily transmitted via the airborne route and is widely prevalent. Transplacental vertical transmission from mother to child probably may also occur in humans and some other host species (Cere *et al.* 1997; Hong *et al.* 1999; Montes-Cano *et al.* 2009). The primary infection with PJP in an immune-competent host is usually asymptomatic and occurs early in life. By the age of 20 months, up to 85% of individuals display antibodies to *Pneumocystis*, most of which have not displayed any evidence of clinical disease (Vargas *et al.* 2001). PJP in humans may be spread from person to person causing subclinical infection until it is transmitted to an immunocompromised host, where fulminant pneumonia occurs. Transmission of PJP is probably airborne. In an air sampling study, *Pneumocystis* organisms were detected in the air as far as 8 m away from infected individuals, with the burden of organisms decreasing proportional to the distance from the patient (Choukri *et al.* 2010). In populations of immunocompromised hosts who reside in close proximity, such as a hospital ward, transmission of PJP from person to person has been documented to occur by molecular typing (Rabodonirina *et al.* 2004).

There is evidence that PJP in immunosuppressed humans is, in most cases, due to acquisition of new infection rather than reactivation of latent infection acquired in youth. A normal immune response appears to be sufficient to clear *Pneumocystis* infection entirely in most people without apparently leaving behind a state of latent infection. Rats with prior *Pneumocystis carinii* pneumonia (PcP) do not show evidence of reactivation of PcP infection when immune suppression is induced unless they are exposed to other infected rats (Vargas *et al.* 1995). Multiple serial PJP infections in human are generally caused by genetically distinct strains of PJP, suggesting that reactivation of latent infection does not occur (Keely *et al.* 1995). Furthermore, the PJP infection strain is also associated with an individual's current country of residence rather than their birthplace, again supporting that reactivation of a latent infection acquired in youth does not occur (Beard *et al.* 2000). However, epidemiological analyses of the PJP strain from respiratory samples as in these studies must be interpreted with caution because analysis of respiratory samples does not identify all of the PJP strains carried by the patient when compared with autopsy analysis (Helweg-Larsen, Lundgren and Lundgren 2001).

Colonization by PJP, defined as an asymptomatic carrier state where PJP organisms can be isolated from the respiratory system but there is no clinical evidence of infection, has been described in populations of individuals with structural lung disease or immunosuppression (Morris and Norris 2012). Studies

investigating whether PJP can colonize immune-competent humans without lung disease have produced variable results. Some studies have failed to find any evidence of PJP colonization in humans at all (Peters *et al.* 1992; Leigh *et al.* 1993; Nevez *et al.* 2006). while others have reported an incidence as high as 65% (Medrano *et al.* 2005; Ponce *et al.* 2010; Vargas *et al.* 2010). If it does occur, colonization in healthy hosts may occur at very low organism burden, as the studies reporting high incidence have generally analyzed a large volume of lung tissue obtained at autopsy. In particular, the 65% rate of colonization was reported by a study from Chile of otherwise healthy individuals killed by trauma or violence where an autopsy sample of 3–7% of total weight of the right upper lobe of the lung was analyzed for evidence of *Pneumocystis jirovecii* DNA by PCR (Ponce *et al.* 2010). This suggests that a colonization state for *Pneumocystis* in healthy humans may exist but at a very low organism burden that is not detectable by traditional methods.

Clinical presentation and treatment

Clinical disease caused by *Pneumocystis* almost universally presents as infection of the lung in an immunosuppressed host. *Pneumocystis* has only rarely been described to infect other organ systems (Guttler *et al.* 1993; Chary-Reddy and Graves 1996). *Pneumocystis* pneumonia is closely associated with the AIDS epidemic, but can affect many other populations of immunosuppressed patients. In particular, patients receiving chronic corticosteroid therapy or other immunosuppressive medications are at risk of *Pneumocystis* pneumonia (Yale and Limper 1996; Fillatre *et al.* 2014; Grubbs and Baddley 2014; Roux *et al.* 2014). As more immunosuppressive medications are used as therapy for a variety of chronic diseases, the incidence of *Pneumocystis* pneumonia in non-AIDS patients may be rising (Morris and Norris 2012). The mortality associated with *Pneumocystis* pneumonia remains high, and is generally higher in non-AIDS compared with AIDS patients. In AIDS patients, *Pneumocystis* pneumonia has a mortality of 10–30% and in non-AIDS patients the mortality is 40–70% (Mansharamani *et al.* 2000; Festic *et al.* 2005; Monnet *et al.* 2008; Fei *et al.* 2009). This difference may be due to a more robust host inflammatory response in non-AIDS patients resulting in greater lung damage. The aforementioned high mortality figures are described in patients who usually are hospitalized and receiving maximal treatment with current standard of care medications including antibiotics. This underscores the need for further research to identify better therapies for *Pneumocystis* pneumonia.

The current first-line antibiotic for treatment of *Pneumocystis* pneumonia is trimethoprim–sulfamethoxazole (TMP-SMX) (Limper *et al.* 2011). This has been the standard of care since the 1980s. Other second-line agents include pentamidine, clindamycin–primaquine and atovaquone (reserved for mild to moderate disease) (Limper *et al.* 2011). TMP-SMX in *Pneumocystis* works by inhibiting the enzyme dihydropteroate synthase (DHPS) to impair folate synthesis. In other pathogenic organisms, mutations of the DHPS gene can result in resistance to TMP-SMX therapy. In *Pneumocystis*, strains with mutant DHPS have been reported, and the frequency of this mutation in PJP infection may be increasing, possibly due to selection pressure from the widespread use of TMP-SMX therapy for *Pneumocystis* prophylaxis and treatment (Huang *et al.* 2004; Dini *et al.* 2010; Yoon *et al.* 2013; Sheikholeslami *et al.* 2015). However, studies examining whether mutant DHPS in *Pneumocystis* actually results in clinical resistance to TMP-SMX *in vivo* have not been clear cut, and fortunately no strains of PJP that are entirely resistant to TMP-SMX therapy have yet been described (Huang *et al.* 2004; Crothers *et al.* 2005; Yoon *et al.* 2013).

Antifungal medications have also been studied in *Pneumocystis* pneumonia, with mixed results. Many available antifungals target sterol components of the organism. Cholesterol is the most abundant sterol in *Pneumocystis*, accounting for ~75% of its sterols. *Pneumocystis* resembles rust fungi in that it lacks the major fungal sterol, ergosterol, the binding site for amphotericin (Kaneshiro 2002). This renders amphotericin, a commonly used antifungal drug, ineffective in the treatment of *Pneumocystis* infection (Limper *et al.* 2011). Azole antifungals are a class of widely used antifungal medications that target sterol synthesis. Azoles inhibit the cytochrome P450 enzyme 14α-lanosterol demethylase which facilitates a key step in the conversion of lanosterol to ergosterol (Porollo *et al.* 2012). *Pneumocystis* has been shown to be resistant to triazole antifungals such as itraconazole and fluconazole (Bartlett *et al.* 1994). *Pneumocystis* probably does synthesize sterols *de novo* and also modifies cholesterol scavenged from its mammalian host (Kaneshiro 2004). Therefore, therapies targeted at sterol synthesis and modification pathways may ultimately be found that are effective. However, identifying antifungals that target non-sterol synthesis pathways may show more promise. Echinocandin antibiotics such as caspofungin inhibit β-1,3-glucan synthetases to disrupt the integrity of the cell wall. Echinocandins have been shown in animal models to be highly effective in eliminating cyst forms in *Pneumocystis* infection, but their impact on trophic forms is far less effective (Cushion *et al.* 2010; Sun and Tong 2014). Furthermore, no large-scale clinical trials of caspofungin in human *Pneumocystis* pneumonia have yet been performed, and this agent should not be considered as stand-alone therapy for PJP. Further understanding of the pathobiology of *Pneumocystis* will hopefully lead to development of novel antifungals to improve clinical outcomes in this deadly infection.

CONCLUSION

The last decade has seen a tremendous growth in understanding of the pathobiology of *Pneumocystis*. Cell wall components and their synthesis mechanisms have been characterized along with their complex interaction with the host lung and immune system. Some important cell signaling pathways have been identified by comparison with closely related fungi. However, despite our increased understanding of *Pneumocystis*, mortality due to *Pneumocystis* pneumonia remains high and the first-line therapy has not changed since the 1980s. Further research is needed to better characterize the pathobiology of *Pneumocystis* so that more effective targeted therapies can be developed.

Conflict of interest. None declared.

REFERENCES

Almeida JM, Cisse OH, Fonseca A, *et al.* Comparative genomics suggests primary homothallism of Pneumocystis species. *mBio* 2015;**6**:1.

Ambrose HE, Keely SP, Aliouat EM, *et al.* Expression and complexity of the PRT1 multigene family of Pneumocystis carinii. *Microbiology* 2004;**150**:293–300.

Baasiri RA, Lu X, Rowley PS, *et al.* Overlapping functions for two G protein alpha subunits in Neurospora crassa. *Genetics* 1997;**147**:137–45.

Bardwell L, Cook JG, Inouye CJ, *et al.* Signal propagation and regulation in the mating pheromone response pathway of the yeast Saccharomyces cerevisiae. *Dev Biol* 1994;**166**:363–79.

Bartlett MS, Queener SF, Shaw MM, *et al.* Pneumocystis carinii is resistant to imidazole antifungal agents. *Antimicrob Agents Chemother* 1994;**38**:1859–61.

Bates M, Mudenda V, Mwaba P, *et al.* Deaths due to respiratory tract infections in Africa: a review of autopsy studies. *Curr Opin Pulm Med* 2013;**19**:229–37.

Beard CB, Carter JL, Keely SP, *et al.* Genetic variation in Pneumocystis carinii isolates from different geographic regions: implications for transmission. *Emerg Infect Dis* 2000;**6**:265–72.

Calderon-Sandubete EJ, Varela-Aguilar JM, Medrano-Ortega FJ, *et al.* Historical perspective on Pneumocystis carinii infection. *Protist* 2002;**153**:303–10.

Carmona EM, Vassallo R, Vuk-Pavlovic Z, *et al.* Pneumocystis cell wall beta-glucans induce dendritic cell costimulatory molecule expression and inflammatory activation through a Fas–Fas ligand mechanism. *J Immunol* 2006;**177**:459–67.

Casanova-Cardiel L, Leibowitz MJ. Presence of Pneumocystis carinii DNA in pond water. *J Eukaryot Microbiol* 1997;**44**:28S.

Catherinot E, Lanternier F, Bougnoux ME, *et al.* Pneumocystis jirovecii pneumonia. *Infect Dis Clin North Am* 2010;**24**: 107–38.

Cere N, Drouet-Viard F, Dei-Cas E, *et al.* In utero transmission of Pneumocystis carinii sp. f. oryctolagi. *Parasite* 1997;**4**:325–30.

Chabe M, Aliouat-Denis CM, Delhaes L, *et al.* Pneumocystis: from a doubtful unique entity to a group of highly diversified fungal species. *FEMS Yeast Res* 2011;**11**:2–17.

Chakaya JM, Bii C, Ng'ang'a L, *et al.* Pneumocystis carinii pneumonia in HIV/AIDS patients at an urban district hospital in Kenya. *East Afr Med J* 2003;**80**:30–5.

Chary-Reddy S, Graves DC. Identification of extrapulmonary Pneumocystis carinii in immunocompromised rats by PCR. *J Clin Microbiol* 1996;**34**:1660–5.

Chen CC, Carson JJ, Feser J, *et al.* Acetylated lysine 56 on histone H3 drives chromatin assembly after repair and signals for the completion of repair. *Cell* 2008;**134**:231–43.

Chin K, Luttrell TD, Roe JD, *et al.* Putative Pneumocystis dormant forms outside the mammalian host, and long-term culture derived from them: initial characterizations. *J Eukaryot Microbiol* 1999;**46**:95S–9S.

Choukri F, Menotti J, Sarfati C, *et al.* Quantification and spread of Pneumocystis jirovecii in the surrounding air of patients with Pneumocystis pneumonia. *Clin Infect Dis* 2010;**51**:259–65.

Cisse OH, Pagni M, Hauser PM. Comparative genomics suggests that the human pathogenic fungus Pneumocystis jirovecii acquired obligate biotrophy through gene loss. *Genome Biol Evol* 2014;**6**:1938–48.

Crothers K, Beard CB, Turner J, *et al.* Severity and outcome of HIV-associated Pneumocystis pneumonia containing Pneumocystis jirovecii dihydropteroate synthase gene mutations. *AIDS* 2005;**19**:801–5.

Cushion MT. Taxonomy, genetic organization, and life cycle of Pneumocystis carinii. *Semin Respir Infect* 1998;**13**:304–12.

Cushion MT, Keely SP, Stringer JR. Molecular and phenotypic description of Pneumocystis wakefieldiae sp. Nov., a new species in rats. *Mycologia* 2004;**96**:429–38.

Cushion MT, Linke MJ, Ashbaugh A, *et al.* Echinocandin treatment of pneumocystis pneumonia in rodent models depletes cysts leaving trophic burdens that cannot transmit the infection. *PLoS One* 2010;**5**:e8524.

Dahlin JL, Kottom T, Han J, *et al.* Pneumocystis jirovecii Rtt109, a novel drug target for Pneumocystis pneumonia in immunosuppressed humans. *Antimicrob Agents Chemother* 2014;**58**:3650–9.

De Stefano JA, Cushion MT, Sleight RG, *et al.* Analysis of Pneumocystis carinii cyst wall. I. Evidence for an outer surface membrane. *J Protozool* 1990;**37**:428–35.

De Stefano JA, Myers JD, Du Pont D, *et al.* Cell wall antigens of Pneumocystis carinii trophozoites and cysts: purification and carbohydrate analysis of these glycoproteins. *J Eukaryot Microbiol* 1998;**45**:334–43.

Dei-Cas E, Brun-Pascaud M, Bille-Hansen V, *et al.* Animal models of pneumocystosis. *FEMS Immunol Med Microbiol* 1998;**22**: 163–8.

Dei-Cas E, Chabe M, Moukhlis R, *et al.* Pneumocystis oryctolagi sp. nov., an uncultured fungus causing pneumonia in rabbits at weaning: review of current knowledge, and description of a new taxon on genotypic, phylogenetic and phenotypic bases. *FEMS Microbiol Rev* 2006;**30**:853–71.

Dei-Cas E, Jackson H, Palluault F, *et al.* Ultrastructural observations on the attachment of Pneumocystis carinii in vitro. *J Protozool* 1991;**38**:205S–7S.

Delanoë P, Delanoë E. Sur les rapports des kystes de carinii du poumon des rats avec le Trypanosoma lewisi. *CR Acad Sci* 1912;**155**:658–60.

Demanche C, Berthelemy M, Petit T, *et al.* Phylogeny of Pneumocystis carinii from 18 primate species confirms host specificity and suggests coevolution. *J Clin Microbiol* 2001;**39**:2126–33.

Dennehy KM, Ferwerda G, Faro-Trindade I, *et al.* Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur J Immunol* 2008;**38**:500–6.

Dini L, du Plessis M, Frean J, *et al.* High prevalence of dihydropteroate synthase mutations in Pneumocystis jirovecii isolated from patients with Pneumocystis pneumonia in South Africa. *J Clin Microbiol* 2010;**48**:2016–21.

Douglas CM. Fungal beta(1,3)-D-glucan synthesis. *Med Mycol* 2001;**39**(Suppl 1):55–66.

Durand-Joly I, el Aliouat M, Recourt C, *et al.* Pneumocystis carinii f. sp. hominis is not infectious for SCID mice. *J Clin Microbiol* 2002;**40**:1862–5.

Edman JC, Hatton TW, Nam M, *et al.* A single expression site with a conserved leader sequence regulates variation of expression of the Pneumocystis carinii family of major surface glycoprotein genes. *DNA Cell Biol* 1996;**15**:989–99.

Edman JC, Kovacs JA, Masur H, *et al.* Ribosomal RNA sequence shows Pneumocystis carinii to be a member of the fungi. *Nature* 1988;**334**:519–22.

Elias JA, Homer RJ, Hamid Q, *et al.* Chitinases and chitinase-like proteins in T(H)2 inflammation and asthma. *J Allergy Clin Immunol* 2005;**116**:497–500.

Evans SE, Hahn PY, McCann F, *et al.* Pneumocystis cell wall beta-glucans stimulate alveolar epithelial cell chemokine generation through nuclear factor-kappaB-dependent mechanisms. *Am J Respir Cell Mol Biol* 2005;**32**:490–7.

Fei MW, Kim EJ, Sant CA, *et al.* Predicting mortality from HIV-associated Pneumocystis pneumonia at illness presentation: an observational cohort study. *Thorax* 2009;**64**:1070–6.

Festic E, Gajic O, Limper AH, *et al.* Acute respiratory failure due to pneumocystis pneumonia in patients without human immunodeficiency virus infection: outcome and associated features. *Chest* 2005;**128**:573–9.

Filice G, Carnevale G, Lanzarini P, *et al.* Life cycle of P. carinii in the lung of immuno-compromised hosts: an ultrastructural study. *Microbiologica* 1985;**8**:319–28.

Fillatre P, Decaux O, Jouneau S, *et al.* Incidence of Pneumocystis jiroveci pneumonia among groups at risk in HIV-negative patients. *Am J Med* 2014;**127**:1242 e1211–47.

Fisher D, Nurse P. Cyclins of the fission yeast Schizosaccharomyces pombe. *Semin Cell Biol* 1995;**6**:73–8.

Fonzi WA. PHR1 and PHR2 of Candida albicans encode putative glycosidases required for proper cross-linking of beta-1,3- and beta-1,6-glucans. *J Bacteriol* 1999;**181**:7070–9.

Fox D, Smulian AG. Mitogen-activated protein kinase Mkp1 of Pneumocystis carinii complements the slt2Delta defect in the cell integrity pathway of Saccharomyces cerevisiae. *Mol Microbiol* 1999;**34**:451–62.

Garner RE, Walker AN, Horst MN. Morphologic and biochemical studies of chitin expression in Pneumocystis carinii. *J Protozool* 1991;**38**:12S–4S.

Gigliotti F, Ballou LR, Hughes WT, *et al.* Purification and initial characterization of a ferret Pneumocystis carinii surface antigen. *J Infect Dis* 1988;**158**:848–54.

Gigliotti F, Harmsen AG, Haidaris CG, *et al.* Pneumocystis carinii is not universally transmissible between mammalian species. *Infect Immun* 1993;**61**:2886–90.

Gigliotti F, Stokes DC, Cheatham AB, *et al.* Development of murine monoclonal antibodies to Pneumocystis carinii. *J Infect Dis* 1986;**154**:315–22.

Grubbs JA, Baddley JW. Pneumocystis jirovecii pneumonia in patients receiving tumor-necrosis-factor-inhibitor therapy: implications for chemoprophylaxis. *Curr Rheumatol Rep* 2014;**16**:445.

Gustafson MP, Limper AH, Leof EB. Characterization of the Cdc25 phosphatase in Pneumocystis carinii. *J Eukaryot Microbiol* 1999;**46**:129S.

Gustafson MP, Thomas CF, Jr, Rusnak F, *et al.* Differential regulation of growth and checkpoint control mediated by a Cdc25 mitotic phosphatase from Pneumocystis carinii. *J Biol Chem* 2001;**276**:835–43.

Gustin MC, Albertyn J, Alexander M, *et al.* MAP kinase pathways in the yeast Saccharomyces cerevisiae. *Microbiol Mol Biol Rev* 1998;**62**:1264–300.

Guttler R, Singer PA, Axline SG, *et al.* Pneumocystis carinii thyroiditis. Report of three cases and review of the literature. *Arch Intern Med* 1993;**153**:393–6.

Haidaris CG, Medzihradsky OF, Gigliotti F, *et al.* Molecular characterization of mouse Pneumocystis carinii surface glycoprotein A. *DNA Res* 1998;**5**:77–85.

Haidaris PJ, Wright TW, Gigliotti F, *et al.* Expression and characterization of a cDNA clone encoding an immunodominant surface glycoprotein of Pneumocystis carinii. *J Infect Dis* 1992;**166**:1113–23.

Han J, Zhou H, Horazdovsky B, *et al.* Rtt109 acetylates histone H3 lysine 56 and functions in DNA replication. *Science* 2007a;**315**:653–5

Han J, Zhou H, Li Z, *et al.* Acetylation of lysine 56 of histone H3 catalyzed by RTT109 and regulated by ASF1 is required for replisome integrity. *J Biol Chem* 2007b;**282**:28587–96.

Helweg-Larsen J, Lundgren B, Lundgren JD. Heterogeneity and compartmentalization of Pneumocystis carinii f. sp. hominis genotypes in autopsy lungs. *J Clin Microbiol* 2001;**39**:3789–92.

Hoffman OA, Standing JE, Limper AH. Pneumocystis carinii stimulates tumor necrosis factor-alpha release from alveolar macrophages through a beta-glucan-mediated mechanism. *J Immunol* 1993;**150**:3932–40.

Hong ST, Park YK, Kim J, *et al.* Is Pneumocystis carinii vertically transmitted to neonatal rats? *Korean J Parasitol* 1999;**37**:149–56.

Huang L, Crothers K, Atzori C, *et al.* Dihydropteroate synthase gene mutations in Pneumocystis and sulfa resistance. *Emerg Infect Dis* 2004;**10**:1721–8.

Huang SN, Angus CW, Turner RE, *et al.* Identification and characterization of novel variant major surface glycoprotein gene families in rat Pneumocystis carinii. *J Infect Dis* 1999;**179**:192–200.

Itatani CA. Ultrastructural demonstration of a pore in the cyst wall of Pneumocystis carinii. *J Parasitol* 1994;**80**:644–8.

Ives NJ, Gazzard BG, Easterbrook PJ. The changing pattern of AIDS-defining illnesses with the introduction of highly active antiretroviral therapy (HAART)in a London clinic. *J Infect* 2001;**42**:134–9.

Jaffe HW, Bregman DJ, Selik RM. Acquired immune deficiency syndrome in the United States: the first 1,000 cases. *J Infect Dis* 1983;**148**:339–45.

Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;**298**:1911–2.

Kaneshiro ES. Sterol biosynthesis in Pneumocystis: unique steps that define unique targets. *Drug Resist Updat* 2002;**5**:259–68.

Kaneshiro ES. Sterol metabolism in the opportunistic pathogen Pneumocystis: advances and new insights. *Lipids* 2004;**39**:753–61.

Keely SP, Fischer JM, Cushion MT, *et al.* Phylogenetic identification of Pneumocystis murina sp. Nov., a new species in laboratory mice. *Microbiology* 2004;**150**:1153–65.

Keely SP, Renauld H, Wakefield AE, *et al.* Gene arrays at Pneumocystis carinii telomeres. *Genetics* 2005;**170**:1589–600.

Keely SP, Stringer JR. Sequence diversity of transcripts from Pneumocystis carinii gene families MSR and PRT1. *J Eukaryot Microbiol* 2003;**50**(Suppl):627–8.

Keely SP, Stringer JR, Baughman RP, *et al.* Genetic variation among Pneumocystis carinii hominis isolates in recurrent pneumocystosis. *J Infect Dis* 1995;**172**:595–8.

Kottom TJ, Han J, Zhang Z, *et al.* Pneumocystis carinii expresses an active Rtt109 histone acetyltransferase. *Am J Respir Cell Mol Biol* 2011;**44**:768–76.

Kottom TJ, Hebrink DM, Jenson PE, *et al.* Evidence for proinflammatory beta-1,6 glucans in the pneumocystis cell wall. *Infect Immun* 2015;**83**:2816–26.

Kottom TJ, Kennedy CC, Limper AH. Pneumocystis PCINT1, a molecule with integrin-like features that mediates organism adhesion to fibronectin. *Mol Microbiol* 2008;**67**:747–61.

Kottom TJ, Kohler JR, Thomas CF, Jr, *et al.* Lung epithelial cells and extracellular matrix components induce expression of Pneumocystis carinii STE20, a gene complementing the mating and pseudohyphal growth defects of STE20 mutant yeast. *Infect Immun* 2003;**71**:6463–71.

Kottom TJ, Limper AH. Cell wall assembly by Pneumocystis carinii. Evidence for a unique gsc-1 subunit mediating beta-1,3-glucan deposition. *J Biol Chemistry* 2000;**275**:40628–34.

Kottom TJ, Limper AH. Subtractive hybridization analysis of Pneumocystis carinii gene activation induced by interaction with lung epithelial cells and matrix. *Chest* 2002;**121**:78S–9S.

Kottom TJ, Limper AH. Pneumocystis carinii cell wall biosynthesis kinase gene CBK1 is an environmentally responsive gene that complements cell wall defects of cbk-deficient yeast. *Infect Immun* 2004;**72**:4628–36.

Kottom TJ, Thomas CF, Jr, Limper AH. Characterization of Pneumocystis carinii PHR1, a pH-regulated gene important for cell wall Integrity. *J Bacteriol* 2001;**183**:6740–5.

Kottom TJ, Thomas CF Jr, Mubarak KK, *et al.* Pneumocystis carinii uses a functional cdc13 B-type cyclin complex during its life cycle. *Am J Respir Cell Mol Biol* 2000;**22**:722–31.

Kovacs JA, Powell F, Edman JC, *et al.* Multiple genes encode the major surface glycoprotein of Pneumocystis carinii. *J Biol Chem* 1993;**268**:6034–40.

Krajicek BJ, Kottom TJ, Villegas L, *et al.* Characterization of the PcCdc42 small G protein from Pneumocystis carinii, which interacts with the PcSte20 life cycle regulatory kinase. *Am J Physiol Lung Cell Mol Physiol* 2010;**298**: L252–60.

Kucera K. Pneumocystosis as an anthropozoonosis. *Ann Parasitol Hum Comp* 1967;**42**:465–81.

Kutty G, Davis AS, Ma L, *et al.* Pneumocystis encodes a functional endo-beta-1,3-glucanase that is expressed exclusively in cysts. *J Infect Dis* 2015;**211**:719–28.

Kutty G, Kovacs JA. A single-copy gene encodes Kex1, a serine endoprotease of Pneumocystis jiroveci. *Infect Immun* 2003;**71**:571–4.

Kutty G, Ma L, Kovacs JA. Characterization of the expression site of the major surface glycoprotein of human-derived Pneumocystis carinii. *Mol Microbiol* 2001;**42**:183–93.

Kutty G, Maldarelli F, Achaz G, *et al.* Variation in the major surface glycoprotein genes in Pneumocystis jirovecii. *J Infect Dis* 2008;**198**:741–9.

Leigh TR, Kangro HO, Gazzard BG, *et al.* DNA amplification by the polymerase chain reaction to detect sub-clinical Pneumocystis carinii colonization in HIV-positive and HIV-negative male homosexuals with and without respiratory symptoms. *Respir Med* 1993;**87**:525–9.

Limper AH, Knox KS, Sarosi GA, *et al.* An official American Thoracic Society statement: Treatment of fungal infections in adult pulmonary and critical care patients. *Am J Respir Crit Care Med* 2011;**183**:96–128.

Limper AH, Lebron F, Evans SE, *et al.* Pneumocystis carinii: cell wall beta-glucan-mediated pulmonary inflammation. *J Eukaryot Microbiol* 2003;**50**(Suppl):646.

Limper AH, Standing JE, Hoffman OA, *et al.* Vitronectin binds to Pneumocystis carinii and mediates organism attachment to cultured lung epithelial cells. *Infect Immun* 1993;**61**: 4302–9.

Linke MJ, Smulian AG, Stringer JR, *et al.* Characterization of multiple unique cDNAs encoding the major surface glycoprotein of rat-derived Pneumocystis carinii. *Parasitol Res* 1994;**80**:478–86.

Malin AS, Gwanzura LK, Klein S, *et al.* Pneumocystis carinii pneumonia in Zimbabwe. *Lancet* 1995;**346**:1258–61.

Mansharamani NG, Garland R, Delaney D, *et al.* Management and outcome patterns for adult Pneumocystis carinii pneumonia, 1985 to 1995: comparison of HIV-associated cases to other immunocompromised states. *Chest* 2000;**118**:704–11.

Martinez A, Aliouat el M, Standaert-Vitse A, *et al.* Ploidy of cell-sorted trophic and cystic forms of Pneumocystis carinii. *PLoS One* 2011;**6**:e20935.

Masur H, Michelis MA, Greene JB, *et al.* An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* 1981;**305**:1431–8.

Matsumoto Y, Yamada M, Amagai T. Yeast glucan of Pneumocystis carinii cyst wall: an excellent target for chemotherapy. *J Protozoology* 1991;**38**:6S–7S.

Matsumoto Y, Yoshida Y. Sporogony in Pneumocystis carinii: synaptonemal complexes and meiotic nuclear divisions observed in precysts. *J Protozool* 1984;**31**:420–8.

Medrano FJ, Montes-Cano M, Conde M, *et al.* Pneumocystis jirovecii in general population. *Emerg Infect Dis* 2005;**11**:245–50.

van der Meer GV, Brug S. Infection à Pneumocystis chez l'homme et chez les animaux. *Ann Soc Belge Med Trop* 1942;**22**:301–5.

Merzendorfer H. The cellular basis of chitin synthesis in fungi and insects: common principles and differences. *Eur J Cell Biol* 2011;**90**:759–69.

Monnet X, Vidal-Petiot E, Osman D, *et al.* Critical care management and outcome of severe Pneumocystis pneumonia in patients with and without HIV infection. *Crit Care* 2008;**12**:R28.

Montes-Cano MA, Chabe M, Fontillon-Alberdi M, *et al.* Vertical transmission of Pneumocystis jirovecii in humans. *Emerg Infect Dis* 2009;**15**:125–7.

Morris A, Norris KA. Colonization by Pneumocystis jirovecii and its role in disease. *Clin Microbiol Rev* 2012;**25**:297–317.

Neves SR, Ram PT, Iyengar R. G protein pathways. *Science* 2002;**296**:1636–9.

Nevez G, Magois E, Duwat H, *et al.* Apparent absence of Pneumocystis jirovecii in healthy subjects. *Clin Infect Dis* 2006;**42**:e99–101.

Nollstadt KH, Powles MA, Fujioka H, *et al.* Use of beta-1,3-glucan-specific antibody to study the cyst wall of Pneumocystis carinii and effects of pneumocandin B0 analog L-733,560. *Antimicrob Agents Chemother* 1994;**38**:2258–65.

Obara T, Nakafuku M, Yamamoto M, *et al.* Isolation and characterization of a gene encoding a G-protein alpha subunit from Schizosaccharomyces pombe: involvement in mating and sporulation pathways. *Proc Natl Acad Sci USA* 1991;**88**:5877–81.

Peters SE, Wakefield AE, Sinclair K, *et al.* A search for Pneumocystis carinii in post-mortem lungs by DNA amplification. *J Pathol* 1992;**166**:195–8.

Ponce CA, Gallo M, Bustamante R, *et al.* Pneumocystis colonization is highly prevalent in the autopsied lungs of the general population. *Clin Infect Dis* 2010;**50**:347–53.

Porollo A, Meller J, Joshi Y, *et al.* Analysis of current antifungal agents and their targets within the Pneumocystis carinii genome. *Curr Drug Targets* 2012;**13**:1575–85.

Pottratz ST, Paulsrud J, Smith JS, *et al.* Pneumocystis carinii attachment to cultured lung cells by pneumocystis gp 120, a fibronectin binding protein. *J Clin Invest* 1991;**88**:403–7.

Pupaibool J, Kottom TJ, Bouchonville K, *et al.* Characterization of the Pneumocystis carinii histone acetyltransferase chaperone proteins PcAsf1 and PcVps75. *Infect Immun* 2013;**81**:2268–75.

Rabodonirina M, Vanhems P, Couray-Targe S, *et al.* Molecular evidence of interhuman transmission of Pneumocystis pneumonia among renal transplant recipients hospitalized with HIV-infected patients. *Emerg Infect Dis* 2004;**10**:1766–73.

Radding JA, Armstrong MY, Ullu E, *et al.* Identification and isolation of a major cell surface glycoprotein of Pneumocystis carinii. *Infect Immun* 1989;**57**:2149–57.

Ricks DM, Chen K, Zheng M, *et al.* Dectin immunoadhesins and pneumocystis pneumonia. *Infect Immun* 2013;**81**: 3451–62.

Roth A, Wecke J, Karsten V, *et al.* Light and electron microscopy study of carbohydrate antigens found in the electron-lucent layer of Pneumocystis carinii cysts. *Parasitol Res* 1997;**83**:177–84.

Roux A, Gonzalez F, Roux M, *et al.* Update on pulmonary Pneumocystis jirovecii infection in non-HIV patients. *Med Mal Infect* 2014;**44**:185–98.

Ruffolo JJ, Cushion MT, Walzer PD. Techniques for examining Pneumocystis carinii in fresh specimens. *J Clin Microbiol* 1986;**23**:17–21.

Russian DA, Andrawis-Sorial V, Goheen MP, *et al.* Characterization of a multicopy family of genes encoding a surface-expressed serine endoprotease in rat Pneumocystis carinii. *Proc Assoc Am Physicians* 1999;**111**:347–56.

Russian DA, Kovacs JA. Pneumocystis carinii in Africa: an emerging pathogen? *Lancet* 1995;**346**:1242–3.

Saeed NK, Farid E, Jamsheer AE. Prevalence of opportunistic infections in HIV-positive patients in Bahrain: a four-year review (2009–2013). *J Infect Dev Ctries* 2015;**9**:60–9.

Schaffzin JK, Stringer JR. Expression of the Pneumocystis carinii major surface glycoprotein epitope is correlated with linkage of the cognate gene to the upstream conserved sequence locus. *Microbiology* 2004;**150**:677–86.

Schildgen V, Mai S, Khalfaoui S, *et al.* Pneumocystis jirovecii can be productively cultured in differentiated CuFi-8 airway cells. *mBio* 2014;**5**:e01186–14.

Segers GC, Nuss DL. Constitutively activated Galpha negatively regulates virulence, reproduction and hydrophobin gene expression in the chestnut blight fungus Cryphonectria parasitica. *Fungal Genet Biol* 2003;**38**:198–208.

Sheikholeslami MF, Sadraei J, Farnia P, *et al.* Dihydropteroate synthase gene mutation rates in Pneumocystis jirovecii strains obtained from Iranian HIV-positive and non-HIV-positive patients. *Med Mycol* 2015;**53**:361–8

Shiota T, Shimada Y, Kurimoto H, *et al.* Pneumocystis carinii infection in corticosteroid-treated cats. *J Parasitol* 1990;**76**:441–5.

Shiota T, Yamada M, Yoshida Y. Morphology, development and behavior of Pneumocystis carinii observed by light-microscopy in nude mice. *Zentralbl Bakteriol Mikrobiol Hyg A* 1986;**262**:230–9.

Sinclair K, Wakefield AE, Banerji S, *et al.* Pneumocystis carinii organisms derived from rat and human hosts are genetically distinct. *Mol Biochem Parasitol* 1991;**45**:183–4.

Smulian AG, Ryan M, Staben C, *et al.* Signal transduction in Pneumocystis carinii: characterization of the genes (pcg1) encoding the alpha subunit of the G protein (PCG1) of Pneumocystis carinii carinii and Pneumocystis carinii ratti. *Infect Immun* 1996;**64**:691–701.

Steele C, Marrero L, Swain S, *et al.* Alveolar macrophage-mediated killing of Pneumocystis carinii f. sp. muris involves molecular recognition by the Dectin-1 beta-glucan receptor. *J Exp Med* 2003;**198**:1677–88.

Stern A, Green H, Paul M, *et al.* Prophylaxis for Pneumocystis pneumonia (PCP) in non-HIV immunocompromised patients. *Cochrane Database Syst Reviews* 2014;**10**:CD005590.

Stringer JR. Antigenic variation in pneumocystis. *J Eukaryot Microbiol* 2007;**54**:8–13.

Stringer JR, Beard CB, Miller RF. Spelling Pneumocystis jirovecii. *Emerg Infect Dis* 2009;**15**:506.

Stringer JR, Beard CB, Miller RF, *et al.* A new name (Pneumocystis jiroveci) for Pneumocystis from humans. *Emerg Infect Dis* 2002;**8**:891–6.

Stringer JR, Cushion MT, Wakefield AE. New nomenclature for the genus Pneumocystis. *J Eukaryot Mcrobiol* 2001(Suppl):184S–9S.

Stringer JR, Keely SP. Genetics of surface antigen expression in Pneumocystis carinii. *Infect Immun* 2001;**69**:627–39.

Stringer SL, Garbe T, Sunkin SM, *et al.* Genes encoding antigenic surface glycoproteins in Pneumocystis from humans. *J Eukaryot Microbiol* 1993;**40**:821–6.

Sun P, Tong Z. Efficacy of caspofungin, a 1,3-beta-D-glucan synthase inhibitor, on Pneumocystis carinii pneumonia in rats. *Med Mycol* 2014;**52**:798–803.

Sunkin SM, Stringer JR. Residence at the expression site is necessary and sufficient for the transcription of surface antigen genes of Pneumocystis carinii. *Mol Microbiol* 1997;**25**:147–60.

Thomas CF, Jr, Kottom TJ, Leof EB, *et al.* Characterization of a mitogen-activated protein kinase from Pneumocystis carinii. *Am J Physiol* 1998a;**275**:L193–9.

Thomas CF, Jr, Limper AH. Current insights into the biology and pathogenesis of Pneumocystis pneumonia. *Nat Rev Microbiol* 2007;**5**:298–308.

Thomas CF, Jr, Vohra PK, Park JG, *et al.* Pneumocystis carinii BCK1 functions in a mitogen-activated protein kinase cascade regulating fungal cell-wall assembly. *FEBS Lett* 2003;**548**:59–68.

Thomas CF, Anders RA, Gustafson MP, *et al.* Pneumocystis carinii contains a functional cell-division-cycle Cdc2 homologue. *Am J Respir Cell Mol Biol* 1998b;**18**:297–306.

Vargas SL, Hughes WT, Santolaya ME, *et al.* Search for primary infection by Pneumocystis carinii in a cohort of normal, healthy infants. *Clin Infect Dis* 2001;**32**:855–61.

Vargas SL, Hughes WT, Wakefield AE, *et al.* Limited persistence in and subsequent elimination of Pneumocystis carinii from the lungs after P. carinii pneumonia. *J Infect Dis* 1995;**172**:506–10.

Vargas SL, Pizarro P, Lopez-Vieyra M, *et al.* Pneumocystis colonization in older adults and diagnostic yield of single versus paired noninvasive respiratory sampling. *Clin Infect Dis* 2010;**50**:e19–21.

Vassallo R, Standing J, Limper AH. Beta-glucan from Pneumocystis carinii stimulates TNF alpha release from alveolar macrophages. *J Eukaryot Microbiol* 1999;**46**:145S.

Villegas LR, Kottom TJ, Limper AH. Characterization of PCEng2, a {beta}-1,3-endoglucanase homolog in Pneumocystis carinii with activity in cell wall regulation. *Am J Respir Cell Mol Biol* 2010;**43**:192–200.

Villegas LR, Kottom TJ, Limper AH. Chitinases in Pneumocystis carinii pneumonia. *Med Microbiol Immunol* 2012;**201**:337–48.

Vohra PK, Park JG, Sanyal B, *et al.* Expression analysis of PCSTE3, a putative pheromone receptor from the lung pathogenic fungus Pneumocystis carinii. *Biochem Biophys Res Commun* 2004;**319**:193–9.

Vohra PK, Puri V, Thomas CF Jr. Complementation and characterization of the Pneumocystis carinii MAPK, PCM. *FEBS Lett* 2003;**551**:139–46.

Wakefield AE. Detection of DNA sequences identical to Pneumocystis carinii in samples of ambient air. *J Eukaryot Microbiol* 1994;**41**:116S.

Walker AN, Garner RE, Horst MN. Immunocytochemical detection of chitin in Pneumocystis carinii. *Infect Immun* 1990;**58**:412–5.

Walzer PD, Cushion MT. *Pneumocystis pneumonia*, Vol. **194**. New York, NY: Marcel Dekker, 2005.

Walzer PD, Schultz MG, Western KA, *et al.* Pneumocystis carinii pneumonia and primary immune deficiency diseases. *Natl Cancer Inst Monogr* 1976;**43**:65–74.

Wright TW, Bissoondial TY, Haidaris CG, *et al.* Isoform diversity and tandem duplication of the glycoprotein A gene in ferret Pneumocystis carinii. *DNA Res* 1995;**2**:77–88.

Xu JR. Map kinases in fungal pathogens. *Fungal Genet Biol* 2000;**31**:137–52.

Yale SH, Limper AH. Pneumocystis carinii pneumonia in patients without acquired immunodeficiency syndrome: associated illness and prior corticosteroid therapy. *Mayo Clin Proc* 1996;**71**:5–13.

Yoon C, Subramanian A, Chi A, *et al*. Dihydropteroate synthase mutations in Pneumocystis pneumonia: impact of applying different definitions of prophylaxis, mortality endpoints and mutant in a single cohort. *Med Mycol* 2013;**51**: 568–75.

Yoshida Y. Ultrastructural studies of Pneumocystis carinii. *J Protozool* 1989;**36**:53–60.

Yoshida Y, Yamada M, Shiota T, *et al*. Provocation experiment: Pneumocystis carinii in several kinds of animals. *Zentralbl Bakteriol Mikrobiol Hyg A* 1981;**250**:206–12.