

Pneumocystis jiroveci

Applied molecular microbiology, epidemiology and diagnosis

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1. INTRODUCTION

Pneumocystis jiroveci (previously known as *Pneumocystis carinii*) is an unusual opportunistic organism, which causes a severe and often fatal pneumonia in immunocompromised individuals. Until 1980 Pneumocystis pneumonia (PCP) was uncommon and primarily observed in association with syndromes of immunodeficiency or intensive immunosuppression, in particular cancer chemotherapy. With the HIV-1 pandemic, however, PCP emerged as the most common AIDS defining disease in industrialized countries. Before systematic PCP prophylaxis was introduced (33), PCP was observed as the AIDS defining event in 60% of HIV-1 infected patients and it was estimated that up to 80% of patients with CD4 counts less than 200 would eventually develop PCP (179). Following the introduction of primary and secondary PCP prophylaxis in the early nineties a decline in the incidence of AIDS related PCP was observed, which further declined after the introduction of highly active antiretroviral therapy (HAART) in the mid-nineties (147, 165). Nevertheless, PCP continues to be a common disease with a substantial morbidity and mortality. In the industrialized world PCP remains one of the most common AIDS defining events (88, 147), especially in patients unaware of being HIV-infected or for other reasons denied proper medical care. Because PCP can be difficult to diagnose, the infection was previously underdiagnosed in developing countries. There is now an increased recognition that the global burden of infection is very large, and that PCP may be one of the most important cause of pneumonia in HIV-1 infected African children (129, 174).

Three major difficulties have affected the study of PCP. First, in spite of extensive efforts, a reliable in vitro system for cultivation of *P. jiroveci* does not exist and though animal models may be used, the infection is species specific with considerable genetic difference between *Pneumocystis* infecting humans and *Pneumocystis* infecting other species. Second, the organism has never been detected outside its hosts. Third, samples of *P. jiroveci* organisms are not easily obtained; only few organisms are present in upper respiratory tract secretions

and sampling of the lower airways is usually required to isolate the organism and establish a definite histological diagnosis. As a consequence, the study of the infection has previously been difficult with little known of the primary reservoir, transmission and epidemiology of *P. jiroveci*. However, developments in molecular analysis, in particular the ability to amplify specific *Pneumocystis* genes by PCR, have now enabled genotyping and detection of the organism non-invasively. The focus of the present work was the application of molecular methods to the study of the infection in order to improve diagnosis and to advance our knowledge of the molecular epidemiology.

A key to this research has been the availability of clinical samples and data. Since 1989 prospective data on demographics, therapy and outcome among all consecutive HIV-infected patients diagnosed with PCP at Hvidovre Hospital has been collected (19).

2. PNEUMOCYSTIS, THE ORGANISM

Pneumocystis organisms were first reported and named *Pneumocystis carinii* in the beginning of last century (44). After recognition that pneumocystis organisms in different mammals are different, in 1994 an interim trinomial name change was adopted with the name

P. carinii f.sp. *hominis* for pneumocystis infecting humans and *P. carinii* f.sp. *carinii* for one of the two species infecting rats (2). Finally, in 2002, by the recognition of its genetic and functional distinctness, human pneumocystis organism was renamed *Pneumocystis jiroveci*, in honour of Otto Jirovec, who is credited for describing the microbe in humans (62, 190).

Pneumocystis was long considered as a protozoa based on morphologic features and the resistance to classical antifungal agents. However, in the late eighties, *P. carinii* was placed in the fungal kingdom based on phylogenetic analysis of ribosomal RNA (rRNA) sequences and observations of genome size (116), a position that was substantiated by functional and phylogenetic comparisons of several other genes (52, 192). Construction of phylogenetic trees based on nuclear 16S-like RNA have not identified any close relatives; fungal organisms on neighbouring branches include the fission yeast, *Schizosaccharomyces pombe*, and *Taphrina* deformans. In contrast to most other fungi, however, *Pneumocystis* possesses only 1 copy of the nuclear ribosomal RNA locus, has a fragile cell wall and contains little to no ergosterol (189). It has been suggested that the *Pneumocystis* species represent an early divergent line in the fungal kingdom, which may have branched coincident with the bifurcation of the Basidiomycete and Ascomycete lineages, and the organism has recently been placed in a group of fungi entitled the Archiascomycetes (69).

In the lung, two major forms of the organism can be identified by microscopy (Figure 2): They are traditionally called cystic form (cysts) and trophic form (trophozoites), although they more properly, in view of the fungal reclassification, could be referred to as sporangium and yeast cells (178). The cystic form (sporangium) is thick-walled oval, approximately 5 to 8 μ m in diameter and contain up to eight daughter forms (spores or endospores, formerly known as intracystic bodies or sporozoites), which will become trophic forms after excystation. The trophic form (yeast, formerly trophozoite) is small (2 to 5 μ m), thin-walled, pleomorphic and often has an eccentric nucleus. The trophic forms are often seen in clusters. A

Figure 1. Light microscopy of bronchoalveolar smears. Left: Immunofluorescence of trophozoites. Right: Giemsa stain of cysts and trophozoites.

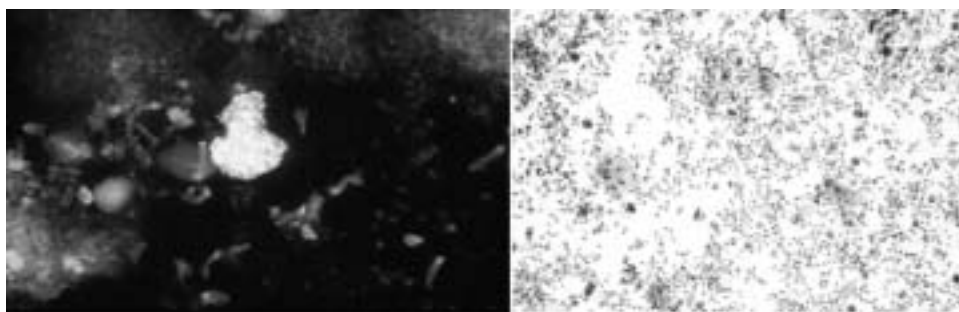
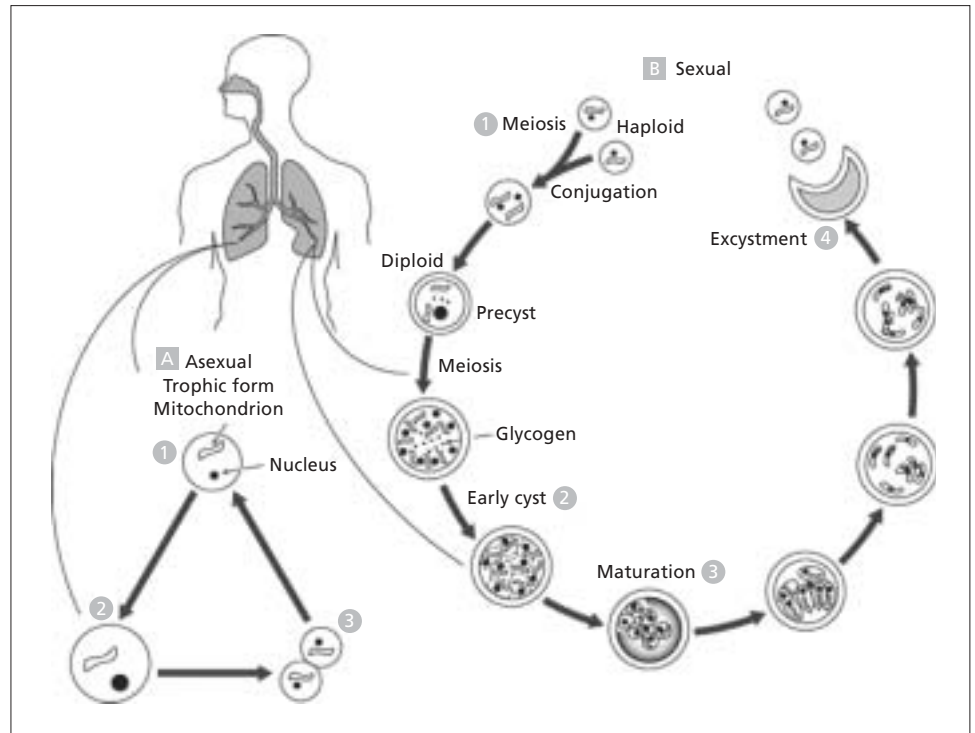


Figure 2. Proposed lifecycle of *Pneumocystis*. Reproduced with permission from CDC (1), based on the drawing by John J. Ruffolo in Cushion M. *Pneumocystis carinii*, Topley and Wilson's Microbiology and Microbial Infections: 9th ed. New York: Arnold Publishing; 1998.



third form, the precyst, is rarely encountered, but is thought to represent an intermediate stage. There is no detailed knowledge of the lifecycle and the mode of replication has not been definitely established, but both asexual and sexual life cycles have been proposed based on electromicrographic observations of synaptonemal complexes (40, 137). *Pneumocystis* proximity to ascomycetes makes it probable that a complex pattern of differentiation exists. Trophic forms are thought to undergo asexual replication by binary fission. Another cycle leads to the formation of the cyst form. By conjugation a diploid zygote is produced, which undergoes meiosis and subsequent mitosis, resulting in the subsequent formation of precyst, early cyst and finally mature cyst. As the mature cyst ruptures, 8 intracystic spores are released and develop into trophic forms. The recent demonstration of genes in *Pneumocystis*, which in other fungi are involved in mating and pheromone responsiveness also suggest a sexual replication cycle (187).

After inhalation, the organism does not enter the host cell, but attaches tightly to the surface of type I alveolar cells. Adherence is primarily mediated by the 95-140 kDa major surface glycoprotein (MSG) (119, 140). This protein is the most abundant antigen on the surface of *Pneumocystis* and has a crucial role in interaction with the host and elicitation of an immuneresponse. MSG represents a family of proteins encoded by highly polymorphic genes, repeated and distributed among all chromosomes of *Pneumocystis*. MSG shows high-level of antigenic variation by switching the expression of multiple MSG genes, with a system that resembles the antigenic system used for antigenic variation in *Trypanozoma cruzi* (9, 191). The precise purpose of MSG variation is not definitely established, but it is likely that it serves for evasion of the host immune response by use of antigenic variability.

3. PHYLOGENETICS

Genetic and antigenic analyses have shown that *Pneumocystis* includes a broad family of organisms, with species specificity among its mammalian hosts (15, 67, 189). Phylogenetic comparisons show high level of nucleotide divergence between organisms in different animal hosts (209). Heterogeneity among different *Pneumocystis* organisms has been shown at the mitochondrial large subunit ribosomal RNA (183), the mitochondrial small subunit rRNA gene (83), the gene encoding thymidylate synthase (138), the *arom* locus (12), β -tubulin (51) and in the gene encoding dihydropteroate synthase

(DHPS) (207). Recent sequence analysis, which compared mitochondrial large subunit rRNA and DHPS sequence from 18 different nonhuman primate species have demonstrated specific sequences associated with each host. The sequence divergence correlated to the phylogenetic difference between the host species, suggesting coevolution (45).

Three levels of genetic divergence have been suggested based on the degree of nucleotide divergence (Table 1). Class III divergence range from 15 to 50% sequence divergence and is observed between *pneumocystis* isolates obtained from different animal species. Class II divergence ranges from 4 to 30% and have been observed within *Pneumocystis* infecting the ferret and rat, but so far this level of divergence has only been found in these species.

Class I sequences differ by 0 to 4% and have been shown between *Pneumocystis* populations found in humans, monkeys and rats (41, 45, 210). Of the genes investigated, the highest divergence is observed in the internal transcribed spacer regions of the rRNA operon, in which up to 40-50% nucleotide divergence is observed. Remarkably, this level of genetic divergence is actually greater than the degree of divergence observed between certain fungi classified as distinct species. The genomes of *Pneumocystis* species infecting mouse, human, rat and ferret have been estimated to be 6.5, 7.0, 7.7, and 11 Mb of DNA, respectively, which are relatively small genome sizes for a fungus, when compared to e.g. the 16 Mb *S. cerevisiae* genome. Chromosome organization has not been definitely determined, since extensive chromosome polymorphism has been characterized among different strains of rat *P. carinii* (39, 116).

4. MOLECULAR TYPING OF *P. JIROVECI*

Development of a typing system for *P. jiroveci* is critical for investigating the transmission and epidemiology of PCP. Presently, it is not possible to distinguish strains by morphology and no reliable in vitro culture exists. Because clinical samples contain only relatively

Table 1. Divergence of gene sequences in % of different *Pneumocystis* isolates. Updated from reference 189.

Genes	Class I	Class II	Class III
mtrRNA, <i>arom</i> , β -tubulin, TS, <i>arom</i> , DHPS, DHFR	0-0.8	4-7	15-34
ITS	2-4	20-30	40-50

Table 2. Genes used in the typing of *P. jiroveci*. ITS nomenclature typing according to Lee (104).

Gene	Size	Number of different sequences types	Polymorphic nucleotide position	Sequence types
Mitochondrial large-subunit rRNA (105,200)	356 bp	5	81 85 248	C/T T/C/A C/T
Mitochondrial small subunit rRNA (200)	300 bp	2	160 196	C/A T/G
5S rRNA (100)	120 bp	6	23 63 79 82 88	T/G A/T T/C A/G T/C
Arom (12)	237 bp	4	121 208	T/C A/G/C
ITS1	161 bp	21	See table 3	
ITS2	192 bp	29	See table 4	
ITS1+2 combinations (77,104,159, V)		65		
DHPS (98, III)	834 bp	5	Wildtype Codon 55 Codon 57 Codon 60	
Cytochrome b (215)	1038 bp	7	279 362 369 516 1032	C/T C/T G/T C/T T/A
SSCP (72,73)		35	Not applicable	Number of patterns:
ITS1	204 bp			3
26S	426 bp			6
mt26S	340 bp			4
β -tubulin	309 bp			3
Tandem repeats in the MSG gene (128)	123 bp	5	6 tandem 10-nucleotide repeats	5 patterns: Number of repeats from 2-6

small amounts of organisms mixed with host DNA, pure extractions of the organism are difficult or impossible to obtain and standard genetic methods like karyotyping or random amplification of polymorphic DNA cannot be performed. Investigation of the transmission and epidemiology of infection has therefore relied on PCR-based DNA sequence analysis.

4.1 GENOTYPING OF *P. JIROVECI*

Genetic diversity in human pneumocystosis was first observed in the large subunit mitochondrial large subunit rRNA operon in which single base polymorphisms were detected at three nucleotide positions (105, 111, 183). Since then different sequence types of *P. jiroveci* have been identified in a number of genetic loci (Table 2). As shown in Table 2, the discriminatory power of typing based on nucleotide sequence variations of genes encoding the small and large subunit rRNA (mtSSU and mtLSU rRNA), arom, and DHPS are relatively limited with, respectively, two, five, four, and five types described. Because of the relatively low discriminatory power of these loci, alternatives were sought. Since DNA sequences that encode rRNA have been central to the study of genomic variations and taxonomic relationships in fungi and other species, this locus was chosen for investigation (27, 111, 113). Like other organisms, *Pneumocystis* has three species of nuclear rRNA transcripts: 18S, 5.8S and 26S. Between these genes are two regions called internal transcribed spacer (ITS) regions ITS1 and ITS2 (Figure 3).

In many parasites and fungi, primary ITS sequence analysis are in accordance with known taxonomic subfamilies and have improved distinction between separate strains (65, 66, 168). In contrast to most other organisms, *Pneumocystis* has only a single copy of the nuclear rRNA genes including ITS (154, 197). Studies, which have

compared ITS genotyping of specimens to other loci, such as mtLSU, mtSSU and DHPS have found that this locus is the most informative as more sequence types are detected by ITS (100, 200, VI).

4.2 ITS TYPING OF *P. JIROVECI*

ITS typing of *P. jiroveci* was first reported by Lee and co-workers in 1993 (110, 113). Based on single nucleotide polymorphisms or deletions at several positions of the ITS1 and ITS2 genes, the initial typing scheme designated ITS1 types as A, B and C and ITS2 sequence types a, b and c, with four different combination types (Ac, Bb, Ba and Bc) observed among 15 samples. Subsequently, Wakefield and co-workers reported 10 different ITS types in samples obtained from 24 episodes of AIDS related pneumonia, designating these types as subtypes according to proximity to the original types reported by Lee (e.g. A₁b₂) (201) (Table 3). When more ITS types were found, this typing scheme was extended to include 11 ITS1 and 17 ITS2 types (Wakefield, personal communication, Table 3 and Table 4). However, an alternative nomenclature of typing was proposed when several new ITS sequence variations were found in a comprehensive study of samples from 9 countries (104). In this revised typing scheme, ITS1 is classified into 15 genotypes, designated as type A through O, and ITS2 is classified in 14 different types, designated a through n (Table 3 and 4). Additional ITS1 genotypes and ITS 2 genotypes have since then been reported in specimens from US, Italy and Japan, which brings the total number of reported geno-



Figure 3. Illustration of ITS 1 and ITS 2 regions in relation to the genes encoding rRNA of *Pneumocystis*.

Table 3 and 4. ITS sequence types, nomenclature according to Lee (104) and Wakefield (200). Nucleotide position in bold are used as scoring positions by both typing schemes to classify different ITS types. The first sequence is the consensus type based on the score reported by Lee (104), representing a (artificial) common consensus type which can be used for comparison to type and report new ITS types. To be considered as a distinct type, a sequence has to be observed in at least two different specimens. The two different score systems of Lee and Wakefield are compared. Nucleotide positions, which are used in both typing systems, are indicated in bold. Bases that are different from the consensus sequence are shown, hyphens indicate missing bases.

Table 3. ITS1 sequence types

ITS1 type		Nucleotide position (bp)										
Nucleotide		6	12	15	21	23-24	28	34	42	53-54	80-81	116-118
Consensus (Lee)		T	T	A	T	TT	T	T	T	AT	AG	TTA
Lee	Wakefield											
A	A ₁	C	T	A	T	--	T	T	T	--	--	TTA
B	A ₂	C	-	A	T	--	T	T	T	--	--	TTA
C	B ₃	C	-	A	T	--	T	T	T	--	AG	TTA
D		C	T	A	T	--	T	T	T	--	AG	TTA
E	B ₁	T	-	A	T	--	T	T	T	--	AG	TTA
F	C	T	-	A	T	--	T	T	T	--	AG	---
G	A ₄	T	-	A	T	--	T	T	T	--	--	TTA
H		T	-	A	T	TT	C	T	T	--	AG	TTA
I		T	-	A	T	--	C	T	T	AT	AG	TTA
J		T	-	C	T	--	C	T	T	--	AG	TTA
K		T	-	C	T	--	C	T	T	--	AG	TTA
L		T	-	A	T	--	T	C	T	--	AG	TTA
M		T	-	A	T	--	T	T	C	--	AG	TTA
N	B ₂	T	-	A	A	--	T	T	T	--	AG	TTA
O		T	T	A	T	--	T	T	T	--	AG	TTA
	A ₃	T	-		A						--	TTA
	B ₄	T	-		G						AG	TTA
	B ₅	T	-		T						TG	TTA
	B ₆	T	A		T						AG	TTA

Table 4. ITS2 sequence types.

ITS2 type		Nucleotide position (bp)								
Nucleotide		52-57	61-65	68-74	76	122	160	166-171	173	177-183
Consensus (Lee)		TAATAA	AAATA	AATATT	T	C	G	ATATAT	G	CAAAATA
Lee	Wakefield									
a	d ₁	TAA---	AA-TA	AATATTT	-	-		ATATAT	G	-----
b	b ₁	TAA---	AA-TA	AATATTT	-	-		--ATAT	G	-----
c	b ₂	TAA---	AA-TA	AATATTT	-	C	T	--ATAT	G	-----
d	b ₂	TAA---	AA-TA	AATATTT	-	C		--ATAT	G	-----
e	a ₁	TAA---	AA-TA	AATATTT	-	-		---AT	G	-----
f	a ₄	TAA---	AA-TA	AATATTT	T	-		---AT	A	-----
g	a ₃	TAA---	AA-TA	AATAAAT	-	-		---AT	A	-----
h	a ₄	TAA---	AA-TA	AATATTT	-	-		---AT	A	-----
i/l	c ₁	-----	AAATA	-----	-	-		---AT	A	-----
j		-----	-----	AATAAAT	-	-		--ATAT	G	-----
k		-----	-----	AATAAAT	-	-		---AT	A	CAAAATA
m		TAATAA	AA-TA	AATAAAT	-	-		---AT	A	-----
n		TAA-AT	AA-TA		-	-		-----	A	-----
	a ₂	TAA---		AATAAAT	-	-		---AT	G	-----
	a ₅	TAA---		AATATAT	-	-		---AT	G	-----
	a ₆	TAG---		AATATTT	-	-		---AT	A	-----
	b ₃	TAA---		AATGTTT	-	C		--ATAT	G	-----
	c ₂	-----	AAATA	-----	-	-		---AT	G	-----
	d ₂	TAA---		AACATTT	-	-	A	TATAT	G	-----
	e ₁	TAATAA		AATAAAT	-	-		---AT	A	CAAAATA
	e ₂	TAA---		AATATTT	-	-		---AT	A	CAAAATA
	e ₃	AAATAA		AATAAAT	-	-		---AT	A	CAAAATA
	f	TAA---		AATAAAT	-	-		---AT	A	---ATA

types to approximately 27 different ITS 1 genotypes and 35 different ITS2 genotypes (77, 159).

ITS genotyping is performed by single or nested PCR amplification of the rRNA operon with primers spanning the ITS1, 5.8S and ITS2 region (II, V). Subsequently the nucleotide sequence can be analyzed by hybridization and/or DNA sequencing. In most early studies the genotypes were determined by direct sequencing; however this method may preclude the detection of multiple types in specimens with more than one genotype. In order to detect multiple ITS types in a specimen, the primary PCR product is usually cloned and the clones sequenced.

In our studies, mixed infections (more than a single ITS genotype) have been detected in 23% of all specimens, in other reports mixed infection have accounted for 17-77% of samples (17, 72, 100, 201, V). Because ITS in *Pneumocystis* is a single copy gene, mixed infection indicates that a specimen contains more than one genotype.

In our first study of ITS typing we used a combination of hybridi-

zation and cloning to analyze ITS sequences (V). The hybridization was done by type-specific probing with oligonucleotide probes ITS1A, ITS1E, ITS2b, ITS2e, and ITS2m to determine whether the specimen contained a single or multiple types of *P. jiroveci* (114). The specimens that contained a single type of *P. jiroveci* were sequenced and those that contained multiple types were cloned and then sequenced. Screening of recombinant clones that contained the correct insert was achieved by colony hybridization (86). A coinfection was assumed if, for one locus, the PCR product from a specimen hybridized to more than one oligonucleotide. During this work, we observed that probing or hybridization was ambiguous in a large part of the samples and in the end most samples had to be analyzed by cloning of PCR products and DNA sequencing. Following the recognition of an increasing number of new ITS sequence types and coinfections with multiple different ITS types, we have since this report performed ITS typing by cloning and DNA sequencing (VI).

Table 5. Occurrence of *Pneumocystis jiroveci* internal transcribed spacer (ITS) types in 130 Danish AIDS patients. Reproduced from table 1 in paper V.

ITS type	N
Eg	42
Ne	27
Eb	18
Ai	16
Bi, Ec, Ee	9
Bb	8
Ei	7
Eh	6
Jf, Me	4
Ac, Ad, Bg, Gg, Gi, Ni	3
Ba, Cg, Dg, Ea, Ej	2
Ab, Be, Bh, Bk, Bm, De, Di, Ed, Ef, Em, Fg, Fp, Gb, He, Ie, Ih, Ii, In, Kf, Ng, Nn, Oe, Og, Oh, On, E-dk313, E-dk029, N-dk323, A-dk594	1

Although potential ITS1 and ITS2 combinations are numerous, the number of ITS combination types observed is more limited. Presently, approximately 65 different ITS genotypes have been reported worldwide (Table 2) and in Denmark 52 different types (V, VI). Table 3-4 illustrate the difficulties in assigning an observed genotype to both classifications, because certain genotypes have only been reported by a single nomenclature system and because the classification by Wakefield has fewer nucleotide scoring positions than the Lee classification.

4.3 OTHER TYPING METHODS: SSCP AND UCS REPEATS

An alternative typing method is single strand conformation polymorphism (SSCP) analysis. In SSCP, nucleotide polymorphisms in PCR fragments are detected by the resulting changes in secondary structure by nondenaturing gel electrophoresis and silver staining or radiolabelling. SSCP analysis of *P. jiroveci* has primarily been investigated by Hauser and co-workers (72, 73, 153). Four different genes are analyzed by SSCP (Table 2), with identification of different SSCP patterns for each gene. A total of 35 different SSCP patterns were reported in samples obtained from 212 European PCP patients, with 77% of isolates being infected with two or more and 23% with a single SSCP type (72). The suggested advantages of the SSCP method are a high discriminatory power and the ability to detect a high number of coinfections. Limitations of the method are that only relatively short PCR regions can be assessed, that the sensitivity of detecting single nucleotide polymorphisms is lower than sequencing, and that the SSCP patterns generated are dependant on the individual setup (e.g. temperature) in each laboratory. So far, the use of SSCP by other research groups have been limited (125).

More recently a novel typing method has been described based on variation in tandem repeats in upstream conserved sequence (UCS) of the MSG expression site (128). Using this typing method 5 different pattern of UCS repeats was reported among 147 isolates, of

which 43% of isolates were coinfecting with multiple UCS repeat patterns (Table 2).

5. MOLECULAR EPIDEMIOLOGY

5.1 EPIDEMIOLOGY OF *P. JIROVECI* GENOTYPES

The finding of geographical clustering and differences in frequency of PCP between different countries has been debated. Differences in reported PCP incidences are difficult to interpret because of geographic variations in access to diagnostic procedures (e.g. BAL verified PCP versus tentative diagnosis), the number of patients at risk (i.e. CD4+ lymphocyte count <200 cells/ μ L) and the epidemiology of other opportunistic infections (i.e. TB). In a European cohort study of more than 5000 patients with AIDS, the adjusted relative risk of developing PCP was lower (RR: 0.77, 95% CI: 0.6-0.99) in patients from southern Europe compared to Northern Europe and in intravenous drug abusers compared with homosexuals (RR: 0.71, 95% CI: 0.6-0.9) (122). In two retrospective studies, differences in the risk of PCP were found in different zip code areas of San Francisco and Cincinnati, suggesting geographical clustering (48, 151). In a study of *Pneumocystis* specimens obtained from five US cities, the genotype patterns of mtLSU and DHPS genotypes varied by patients' place of diagnosis but not by place of birth (17). In contrast, only low genetic diversity was observed in a study of mtLSU genotypes among samples obtained from United Kingdom, United States, Zimbabwe and Brazil (210).

To investigate the molecular epidemiology of infection we genotyped samples obtained from Danish HIV-1 infected patients during a 7 year period (V). ITS 1 and 2 genotypes were determined in 162 bronchoalveolar lavage samples obtained from 130 patients between 1989 and 1996. By type specific hybridization and sequencing of subclones, we observed a remarkably complex picture of ITS types. In 23% of samples mixed infection with more than one ITS subtype was present and as many as 7 different types were detected in a single sample. A total of 49 different ITS genotypes was detected, however 4 types (Eg, Ne, Eb and Ai) accounted for almost half of the sequence types detected (Table 5, 6). We found no specific overall changes in occurrence of ITS genotypes during the study period, no evidence of clustering of specific genotypes, and no link between ITS genotypes and demographic variables or season of the year (V).

Quite similar patterns of genotype distributions have been found in most studies published regardless of geographical origin (Table 6). The majority have reported that the same genotypes, in particular ITS type Eg, Ne and Eb are common, whereas the remaining ITS types are detected more infrequent (Table 6). In most studies, 5 or fewer ITS type's accounts for more than half of all subtypes. These findings suggest that certain strains of *P. jiroveci* across the world may be relatively conserved. Furthermore, there is additional evidence, which support an absence of genotype changes over time: Very similar genotypes were found at the ITS, mtLSU rRNA, arom, and the mitochondrial small subunit rRNA loci, when archival PCP

Table 6. Reports of ITS types.

Study (reference)	Country	Occurrence of selected ITS types (#)				Three most common ITS types	No of unique ITS types/ No of episodes/ No of specimens examined	No of samples with more than one ITS types
		Eg	Ne	Eb	Ai			
Lu (113)	US	0	0	7	5	Eb, Ai	4/15/15	3/15
Tsolaki (201)	UK	3	14	1	3	Ne, Ed, Eg	10/22/24	8/24
Latouche (100)	F+I	2	1	1	2	Eg, Ai, Ec,	10/13/?	0/13
Latouche (101)	F	4	7	1	2	Ne, Eg, Ai	9 /23/?	0/23
Helweg-Larsen (II)	DK+UK	2	5	1	1	Ne, Eg, Ed	12/15/15	4/13
Lee (104)	7 countries	59	43	25	12	Eg, Ne, Eb	59/np/207	52/207
Tsolaki (202)	UK	12	8	5	0	Eg, Ne, Eb	15/12/35	7/35
Hosoya (77)	J	8	6	10	1	Eb, Eg; Ne	30/24/24	16/24
Helweg-Larsen (V)	DK	42	27	18	16	Eg, Ne, Eb	49/141/162	37/162
Nimri (159)	US	27	16	np	np	Eg, Ne,	21 ITS1-20 ITS2 /60/60	36/60
Atzori (11)	I	9	1	5	1	Eg,Kf	78/115/115	37/115

Details of the most common ITS types observed. Number of ITS types was counted according to the occurrence in cloned sequences. The occurrence of some of the most common ITS types are shown in column 3-6. ITS types are described according to the Lee nomenclature. np: not provided.

specimens obtained before the HIV pandemic from 1968 to 1981 were compared with current specimens from AIDS patients (200). A number of the infrequently detected ITS types have only been reported in single studies and could be specific for geographical location. However, in view of the high number of different ITS types reported and the problems of interpretation of mixed infections it remains to be established whether certain sequence types are characteristic of a certain geographical location or just coincidental.

Our findings are in line with the largest study of SSCP genotypes. Hauser et al studied SSCP genotypes in 212 European patients and found no association between SSCP type or number of co-infecting types per patient and geographical location, year of collection, sex, age, or HIV status (72). In agreement with our findings only few patients were infected with the same genotype during a 6 month period.

5.2 ITS GENOTYPES AND PROGNOSIS

The association between strains and morbidity of PCP has been studied by ITS genotyping of *P. jiroveci*. A connection between ITS genotypes and severity of PCP was initially suggested by Miller and Wakefield (145). They compared 11 HIV-positive PCP patients with mild disease with 15 patients with moderate or severe disease based on a baseline PaO₂ cut-off value of 9.3 kPa. The ITS sequence type A2c1 (corresponding to type Ai) was more often found among patients with moderate to severe disease whereas B2a1 (type Ne) was more prevalent among patients with mild disease. Further type B2a1 was the most frequently type identified in samples from second and third episodes of PCP, whereas B1a3 (type Eg) was found in first episodes but never in subsequent episodes of PCP. This result suggested that B2a1 (type Ne) is more persistent or transmissible.

To investigate the possibility of virulence associated with specific ITS genotypes, we analyzed clinical data in relation to ITS genotyping in our cohort of 130 patients (V). In contrast to the findings summarized above, we were unable to show any correlation between specific ITS types and clinical outcome, specifically there was no association between ITS type and the severity of PCP by using the same grouping based on PaO₂. We found no evidence of a link between specific ITS types and primary or recurrent episodes of PCP.

5.3 MODE OF INFECTION: REACTIVATION VS. REINFECTION

For many years, it was believed that clinical disease was a result of reactivation of latent infection carried from childhood. This view was primarily based on serologic data. The majority of children develop anti-*pneumocystis* antibodies during childhood, and seroconvert before four years of age, with an uniformly high prevalence of antibodies to the infection among adult populations in different countries (118, 120, 166, 169, 214).

From the early nineties, however, several data opposing this theory emerged. In animal experiments, airborne transmission of *P. carinii* was demonstrated in the rat model of the infection with susceptible, immunosuppressed animals acquiring the infection from infected animals, either housed in the same cage or in an adjacent cage in the same isolator unit (80). In addition, immunocompetent mice were transiently colonized with *P. carinii* after close contact with *P. carinii*-infected SCID mice and were then able to transmit the infection to *P. carinii*-free SCID mice that develop PCP (49). In both rats and SCID mice with PCP there was no persistence of latent organisms after treatment of pneumonia and immunoreconstitution (34, 203).

Among children with perinatally acquired HIV, the incidence of PCP is highest between 3 and 6 months of age (174, 182). In contrast, there is little evidence of clinical illness or specific symptoms in connection with the primary infection in immunocompetent children. In a prospective study of 74 immunocompetent children less than two years old, Vargas et al were able to detect *P. jiroveci* DNA in nasopharyngeal aspirates of 32% by nested PCR, even though most of the children were healthy at time of detection (203).

In this study, the maximum length in which DNA could be detected was 6 months, with 85% of children developing antibodies at the age of 20 month. Autopsy studies suggest that the clearance of organisms is complete, with no detectable organisms by microscopy or PCR in post-mortem lungs from immunocompetent patients (142, 167).

In HIV-infected PCP patients, the clearance of organisms is slow but appears also to be complete with proper treatment and chemoprophylaxis. Residual cysts can be detected by microscopy in 63-90% at the completion of 3 weeks of therapy, but has no correlation with subsequent risk of relapse or death in patients receiving chemoprophylaxis after treatment (160, 173, 181). At four to six weeks after therapy organisms can be detected in 25% of patients, but are eventually cleared 3-4 months after the completion of therapy (56). Further, discontinuation of secondary PCP chemoprophylaxis in patients who responds to HAART therapy is safe, without evidence of recurrent PCP (103).

5.4 TRANSMISSION OF PCP

Although animal studies suggest that the principal infection route is by air (32,80), the exact mode of transmission remains unknown. Two major modes have been proposed, either transmission from (a yet unknown) environmental source by airborne spores or transmission directly person to person.

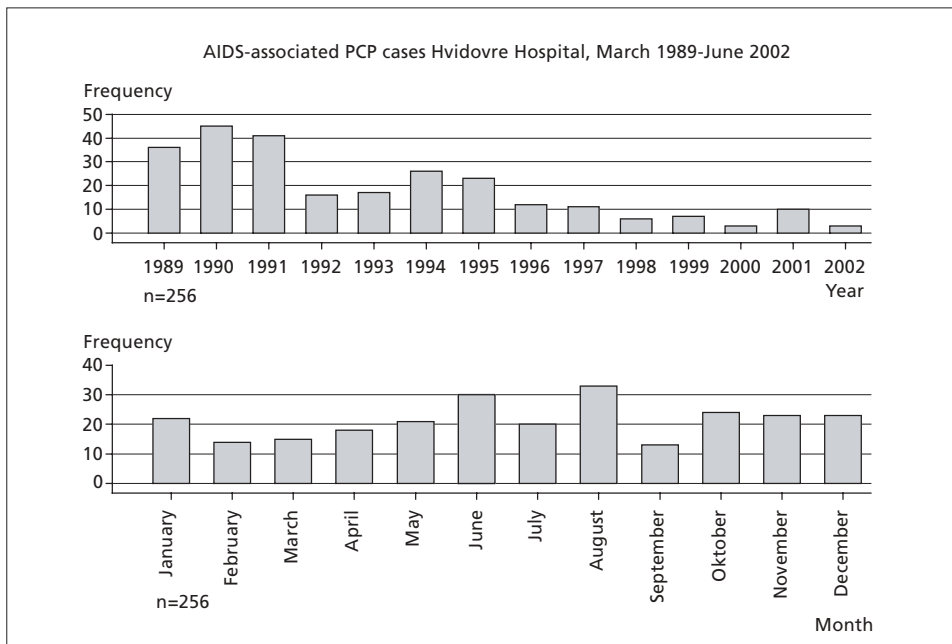
A possible environmental source of infection is supported by detection of *P. jiroveci* DNA in air filters from hospital, urban and rural settings (14, 162, 208) and in pond water (30). In one case-control study, recent gardening, hiking or camping was associated with a greater risk of PCP (157), which hypothetically could support soil as a source of infection, equivalent to other airborne fungal infections from soil like *Coccidioides immitis*, *Histoplasma capsulatum* or *Blastomyces hominis*. Further support comes from other studies. 1) In a *P. carinii* rat model, airfilters carried an infective form of the organism, which retained infectivity after several months at -80°C to room temperature, suggesting the presence of a long term viable spore-like form (88). 2) During the eighties, the relative proportion of AIDS patients with PCP remained relatively constant whereas the overall number of AIDS patients increased rapidly (123). Further, no significant monthly variation in cases of PCP occurred in our cohort from 1989-2002 (Figure 4). 3) No difference in risk of contacting PCP has been found associated with living in cities compared to rural areas (123). These observations all suggest an environmental source of infection, because a more epidemic spread of PCP would have been expected if direct transmission between patients were the predominant mode of infection.

5.5 CASE CLUSTERS OF PCP

One observation that may support a direct mode of transmission is reports of PCP "outbreaks". Apparent clusters of PCP were first observed in malnourished children during World War II (63). Since then there has been sporadic observations of mini-epidemics over the last 30 years. Most reports of possible outbreaks have been reported in immunosuppressed children with malignancies and transplant recipients with the number of patients in each cluster ranging from 2 to 19 (II).

We studied three apparent outbreaks of PCP among patients with haematological malignancies and with HIV-infection by genotyping of the ITS locus (II). Eight patients were diagnosed with PCP during a three-month period at a haematological unit in Copenhagen. Six patients were from two independent clusters of PCP observed in a HIV/AIDS unit in London. Although a common ITS sequence type was observed in two patients who shared a room in the haematological unit and also in two of the HIV patients, there was no indication of a particular ITS type being responsible for either of the clusters. Nine different ITS sequence types were observed in samples from the patients with haematological malignancies, and two and three different sequence types, respectively, were found among the pa-

Figure 4. Variations in the occurrence of AIDS associated PCP cases at Hvidovre Hospital.



tients with HIV. In four patients more than one ITS genotype was detected. Similar observations were made in a Swedish study of three PCP outbreaks, in which no evidence of transmission of mtLSU genotypes were found and in a French study of three different couples diagnosed with PCP (102, 161). In contrast, similar genotypes at the mtLSU, DHPS and ITS locus was observed in a case report of a HIV-1 infected mother and 5 week old infant who developed PCP at the same time (143).

The interpretation of case-clusters is not straightforward. First, there are inherent difficulties in assessing exposure retrospectively. Second, the incubation period of infection is not definitively known. In most case-clusters the presumed incubation period of infection have ranged from two to 12 weeks, which is in accordance with observations in rodent models (23, 32). However, recent findings in SIV infected monkeys (22) suggest that the time of incubation may be much longer. Third, there is a risk of publication bias. Although the majority of PCP cases are seen in HIV infected patients, almost all case-clusters have been described in transplant centres among non-HIV patients in which the "outbreaks" may merely represent random fluctuations in number of PCP cases.

Indeed, a recent case control study among HIV patients found no association between exposure to persons with PCP and subsequent development of PCP (222). In conclusion, although apparent outbreaks have been described, this evidence of direct transmission is so far not conclusive.

5.6 EXPOSURE TO PCP IN HEALTH CARE SETTINGS

The possibility of person to person transmission has been examined by serological and PCR studies of health care workers (HCW) exposed to patients with PCP. Serological studies are conflicting; although Leigh and colleagues observed higher antibody titres in HCW exposed to AIDS patients compared to workers without such exposure, no evidence of elevated titres or of detectable DNA was found in a similar study of HCW by Lundgren et al (107, 117). In contrast, recent PCR studies do provide some evidence of person to person transmission. Vargas et al identified *P. jiroveci* DNA in three healthy contacts to a child with PCP (204) and Miller et al's observed an elevated risk of *P. jiroveci* DNA carriage in HCW with close contact to patients with PCP (144).

5.7 RECURRENT PCP

The possibility that reinfection rather than reactivation of latent disease is the cause of PCP has been supported by a number of studies on recurrent PCP (Table 7). They observed a change of genotype in

approximately half of patients with recurrent separate episodes of PCP. We found a similar rate of genotype change in our study of ITS types (6 of 10 patients, who had recurrent PCP, Table 7). However, we also found almost the same rate of genotype switch when two consecutive BAL specimens from the same episode were compared (10 of 19 patients, 53%). The high rate of genotype change during single episodes observed in our study is in contrast to previous reports. Keely and colleagues (91-93) found the same mtLSU rRNA genotypes in four patients, from whom specimens were taken at the initiation and completion of therapy. ITS genotyping for these specimens was not reported. Latouche et al (100, 101) observed the same mtLSU and ITS genotype of *P. jiroveci* in 10 patients, in whom sequential samples from the same episode of pneumonia were typed by direct sequencing. The reason for the discrepancy between the reported results is unclear, but might be explained by differences in sample size or related to differences in methods.

The significance of genotype switches in patients who suffer multiple episodes of PCP is therefore unclear. One possibility is that patients are eradicated of *P. jiroveci*, but are re-infected. An alternative explanation is that treatment does not eliminate the infection, but that *P. jiroveci* remains in a latent state, emerging later to cause disease when the immune system or prophylactic treatment fails. The principal evidence for re-infection is the observation of different genotypes during different disease episodes. Although on the face of it, such genotype switching would seem to indicate re-infection; several factors could conspire to generate apparent switches in a patient whose infection was caused by the activation of latent organisms. Among these factors, the prerequisite and dominant one is the presence of multiple genotypes in a patient. If mixed infections occur, different genotypes might be detected during different episodes as a result of sampling from different parts of the lung during different episodes (VI).

Table 7. Comparison of *Pneumocystis jiroveci* genotypes from the first and second episode in patients (N) with recurrent PCP.

Loci	N	Same type	Different type	Study
MtLSU rRNA	12	6	6	Keely (91)
ITS	7	3	4	Tsolaki (116)
ITS	6	1	5	Latouche (101)
ITS	5	3	2	Margutti (132)
ITS	10	4	6	Helweg-Larsen (V)
SSCP	13	7	6	Nahimana (155)
Total (%)	53	24 (45%)	29 (55%)	

5.8 HETEROGENEITY AND COMPARTMENTALIZATION OF *P. JIROVECI* GENOTYPES

To investigate the influence of mixed infections and variability of genotypes we undertook a study of genotype heterogeneity in autopsy lungs (VI). Polymorphisms at three genetic loci were investigated by analysis of DNA obtained from different segments of the lungs. Of these loci, mtLSU and ITS were chosen to provide high discrimination. The third locus, DHPS, was chosen because of its association with sulpha resistance (III). From each lung segment five recombinant PCR clones were sequenced to detect coinfections with different strains.

In two of three patients a mixture of different mtLSU and ITS genotypes was observed in BAL samples and in the autopsy sub-clones. In the lungs, compartmentalization of specific ITS and mtLSU sequence types were observed in different lung segments. Although the most predominant genotypes detected in the diagnostic respiratory samples were also the most commonly detected in the lung segments, minor populations of ITS genotypes were detected in the lung but not in the respiratory samples of two patients.

The results of this study were controversial when published. In previous studies, a tacit assumption was that genotypes detected in respiratory samples reflected the global infection within the lung. However, recent data have confirmed the existence of genotype heterogeneity. In samples taken from different site of a *P. jiroveci* infected human lung the sterol profiles had large variations in pneumocysteroles (8). These intrapersonal variations were similar to differences observed between different patients, suggesting distinct phenotypic populations. Uneven distribution of two ITS types by analysis of different segments from a child autopsy lung was observed by Ambrose et al (144). They were unable to detect both genotypes in every lung segments. Ma et al observed genotype differences in UCS repeat patterns between sputum and BAL samples as well as between right and left BAL samples obtained from the same patient (128). Furthermore, studies which have employed rigorous and detailed subcloning of loci with high variability like ITS or used a combination of typing methods, have generally reported a higher incidence of mixed infection compared to studies using direct sequencing of relatively invariant loci.

5.9 INTERPRETATION OF MOLECULAR TYPING

The demonstration of cluster cases and genotypic switching in recurrent episodes of PCP can be interpreted to support the hypothesis of nosocomial transmission. Based on these findings, some authors have recommended prevention of PCP by isolation of PCP patients to limit the exposure of immunocompromised patients (31, 91, 216). Presently, however the exact mode of transmission remains unknown. In our genotype study of two presumed outbreaks of PCP, we found no evidence of direct transmission being a primary cause of infection. Although experimental and epidemiological observations suggest that pneumonia may sometimes be caused by transmission from one patient to another, this mode of transmission does not seem to play a definite part in the majority of cases. Genotype data supports that infection with *P. jiroveci* is not clonal, that coinfections with multiple genotypes may be common and that in some cases distinct populations of organisms may reside within the lung. These observations have important implications for interpretation of genotyping and suggest that single locus typing cannot reliably distinguish between reactivation of latent infection and de novo infection.

The interpretation of genotype data and in particular ITS sequence types, should be cautious since genotyping performed on respiratory samples cannot a priori be assumed to represent all genotypes present within the lung (as discussed in section 5.8). The broad diversity of types and the absence of genotype clusters could seem to indicate that *P. jiroveci* is ubiquitous and that multiple sources of the pathogen may co-exist. In view of the specificity of *P. jiroveci* for humans, the data suggest a complex picture of transmis-

sion. It is likely that healthy carriers, in particular infants, may constitute an important natural reservoir of *P. jiroveci* organisms. Transmission may happen both as a result of transmission of airborne organisms between immunocompromised patients and as a result of transmission from transient low level asymptomatic carriage in immunocompetent hosts. Further, both reactivation and re-infection remain possible sources of infection. The empirical rationale for isolating PCP patients is therefore limited. To interpret DNA typing of the organism properly, more knowledge of the life cycle, infectivity and reproductive mode is required. In future, the development of new genotyping methods will have to examine the degree of genotype variation within human lungs and should preferably be based on multilocus sequencing in order to reliably distinguish strain differences.

6. DETECTION OF *P. JIROVECI*

It is a matter of debate whether PCP should be treated without diagnostic confirmation of the infection. A high likelihood of PCP among HIV-1-infected patients with CD4 cell-counts below 200/ μ l, hypoxia, and diffuse interstitial infiltrates may favour empirical treatment. However, because of the inadequate specificity of clinical signs and the risk of toxicity with treatment, confirmation of PCP is essential an important information for proper evidence-based patient management.

6.1 CONVENTIONAL METHODS

The standard method for diagnosis of PCP relies on the microscopic visualization of *P. jiroveci* organisms in respiratory samples. Because very few organisms are present within the upper respiratory tract, lower respiratory secretions are usually necessary for definitive diagnosis. Bronchoalveolar lavage (BAL) combined with colorimetric and an immunofluorescent stain of BAL fluid is considered the method of choice with sensitivity and specificity in excess of 95%. An alternative is examination of material obtained by induced sputum. However, the sensitivity of this method is more dependent on the experience of the personnel performing the procedure and evaluating the samples, with high variation in the diagnostic sensitivity reported (between 50 to 90%) (55, 79, 97, 141). Staining of lung biopsy specimens is sometimes needed to definitively diagnose PCP pneumonia in non-HIV infected immunocompromised patients, due to the fact that these patients in contrast to AIDS patients have smaller number of organisms.

6.2 PCR METHODS

Because BAL is an invasive and uncomfortable procedure, which is not without risk, alternative non-invasive test have been evaluated. PCR diagnosis of *P. jiroveci* was introduced experimentally in 1990 when Wakefield et al first described DNA amplification of the multicopy mitochondrial ribosomal RNA gene (211,213). Since then, several studies have investigated PCR detection of *Pneumocystis* DNA in respiratory samples from HIV-1 positive and negative patients. To increase the diagnostic sensitivity of non-BAL specimens, different PCR methods have been evaluated, including different gene targets and the use of nested PCR, in which a second round of PCR reamplifies the primary PCR product.

6.3 NON-INVASIVE DIAGNOSIS

Upper respiratory specimens usually present insufficient numbers of organisms to enable diagnosis by microscopy, but the sensitivity of PCR analysis permit the detection of organisms below the threshold of microscopy. Even oral washes, which are easily obtained by rinsing and gargling the mouth with normal saline, are suitable for PCR diagnosis. Wakefield et al were the first to study the diagnostic ability of oral wash combined with PCR; A diagnostic sensitivity and specificity of 78% and 100% was observed by using single round PCR in a study of 31 HIV-1 infected patients (185, 212).

We tested an improved PCR method for diagnosis on oral wash

Table 8. Detection of *P. jiroveci* by TD-PCR in 61 paired oral wash and BAL specimens obtained from HIV-infected patients. Adapted from data in paper I.

	BAL microscopy positive	BAL microscopy negative	Likelihood ratio of a positive test/ negative test
BAL PCR positive	28	3	9.6/0.02
BAL PCR negative	0	30	
Oral wash PCR positive	25	2	14.7/0.1
Oral wash PCR negative	3	31	

and BAL fluid in a prospective study among HIV-positive patients (I). PCR on oral wash specimens, PCR on BAL and microscopic examination of BAL was compared by the use of two different PCR protocols. The principal objective of the study was to develop a routine PCR method that could generate a diagnostic answer within one working day. A single-round Touchdown PCR (TD-PCR) protocol with the ability to detect PCR inhibition was developed. The TD-PCR was evaluated in a routine diagnostic laboratory and was compared to a previous PCR protocol, run in a research laboratory (211).

Sixty-one paired oral wash and BAL specimens were evaluated by TD-PCR. To detect PCR-inhibition, which often is a problem in respiratory specimens, a lambda DNA internal process control was added to the same reaction tube as the clinical specimen. The main findings are shown in Table 8. Using the same method, we found equivalent diagnostic accuracy in a study of 26 patients with haematological malignancy (76). Oral wash specimens were easy to obtain even among severely ill patients with high risk of haemorrhage and *Pneumocystis* DNA could be detected in all 8 patients with PCP and in none without PCP.

Since then other investigators have investigated oral wash for PCP diagnosis (section 6.4, Table 13 page 29). In the largest published study, in which 175 paired samples were investigated, sensitivity and specificity similar to our results were reported with a positive and negative likelihood ratio of 14.4 and 0.1, respectively (60).

6.4 PCR DIAGNOSIS OF PCP, SYSTEMATIC REVIEW

More than 20 studies which investigated the diagnostic use of PCR have now been published. Comparisons of these studies are difficult because of differences between studies in patient groups, PCR methods and study design.

To summarize the current literature a systematic review of diagnostic accuracy was done. The results are shown in Figure 5, Figure 6, Figure 7, Figure 8, Table 9 and Table 10 (unpublished). Studies were identified through Medline and data extracted to diagnostic 2x2 tables (47). When possible, the diagnostic accuracy was calculated according to patients with clinical PCP and a systematic review of likelihood ratios was performed (25, 43). Likelihood ratios were chosen for comparisons as these values have advantages to traditional predictive values (Box 1) (64). They are defined as the likelihood that a particular test result would be found in a patient with the target disorder, relative to the likelihood of the same result occurring in a patient without the target disorder. As shown, differences were observed between the studies according to respiratory specimen used for diagnosis (BAL vs. sputum/oral wash) and PCR methods (single PCR vs. nested PCR). In most studies, which assessed PCR analysis of BAL fluid, sensitivity and specificity ranged from 90 to 100%. In studies using BAL fluid, although there is significant heterogeneity among studies, the overall pooled estimate (random effects model using the method of DerSimonian and Laird with a continuity correction of 0.5) of the positive likelihood ratio of a positive test is 9.9 (95% CI, 6.4-15.1) and the overall likelihood ratio of a negative test 0.06 (95% CI, 0.03-0.11).

In comparison, even greater variations in diagnostic accuracy have been reported when upper respiratory specimens are used for PCR diagnosis. Although the likelihood ratio of a positive test PCR

The likelihood ratio of a positive test (sensitivity/(1-specificity)) is the ratio of the true positive rate to the false positive rate and the likelihood ratio of a negative test ((1-sensitivity)/specificity) is the ratio of the false negative rate to the true negative rate. A suggested interpretation is that convincing diagnostic evidence can be taken from positive likelihood ratios greater than 10 or negative likelihood ratios less than 0.1. Strong diagnostic evidence is derived from positive likelihood ratios above 5 and negative likelihood ratios below 0.2 – although this depends on the pre-test probability (85).

when used on upper respiratory samples is overall acceptable (13.9, 95% CI: 8.2-23.7) and has a sensitivity comparable to microscopy of induced sputum (87%, 95% CI: 81-94%), the overall likelihood of a negative PCR test in these samples is not optimal (0.17, 95% CI: 0.10-0.28). The significant heterogeneity among studies (range: 0.03-0.53), reflect a relatively high rate of false positive results in some studies, in particularly those in which nested PCR were used. Similar conclusions can be drawn if only PCR studies examining oral wash are analysed. For these studies, the overall likelihood of a positive test is 14 (95% CI 8.2-23.7) and the overall likelihood of a negative test is 0.23 (95% CI 0.13-0.41).

6.5 SOURCES OF HETEROGENEITY IN DIAGNOSTIC PCR STUDIES

Figure 5 to 8 visualises the remarkable heterogeneity in the reported diagnostic performance of PCR for diagnosis of PCP. There may be several reasons for the variability between studies, but from the literature and our own experience it is apparent that important sources of heterogeneity are:

Characteristics of patient population:

1. Higher diagnostic value of PCR studies among HIV-positive compared to HIV-negative patients.
2. Higher prevalence of *P. jiroveci* carriage among immunosuppressed patients, in particular HIV-negative, compared to HIV-1 infected and immunocompetent individuals.
3. Chronic pulmonary comorbidity e.g. COPD may enhance carriage of *P. jiroveci*.
4. Differences in use of PCP prophylaxis or treatment before obtaining the diagnostic specimens, in one study 82% of patients with discrepancy between PCR and staining had received prophylaxis prior to the diagnostic procedure (171).

PCR method performance:

1. Quantity of DNA recovered. Less *P. jiroveci* DNA is present in upper compared to lower airway samples.
2. PCR inhibition. Respiratory specimens often contain inhibitors of PCR, in a Portuguese study of oral wash, PCR inhibition was noted in 17% of PCR negative samples (136).
3. PCR target. Higher sensitivity of using multicopy compared to single copy genes (60).
4. Single versus nested PCR. Overall, nested PCR has higher sensitivity, but also higher risk of false positives findings (185).

Study methodology:

1. Differences in the definition of PCP. Although most studies have used microscopy to define PCP, the definition of "gold standard" was in some studies more ambiguous and included clinical signs or response to treatment. Such discrepant analysis may be biased with overestimation of sensitivity and specificity (146).

6.6 FALSE POSITIVE PCR RESULTS: COLONISATION?

Discrepant results between PCR and microscopy with "false positive" PCR results were already noted in the first studies investigating PCR for diagnosis of PCP. At this period of time it was believed that asymptomatic colonisation was rare. Later, several PCR based

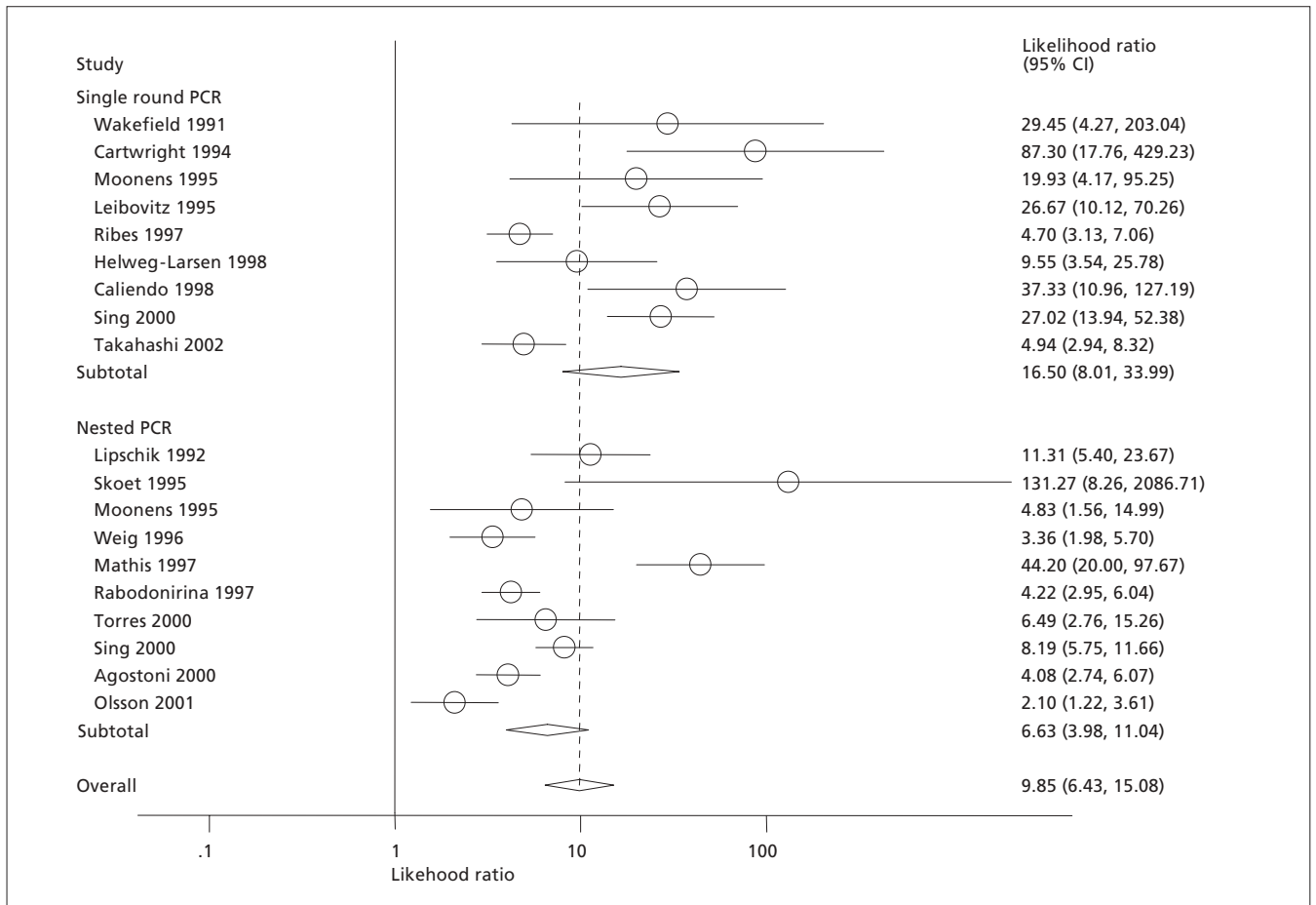


Figure 5. Likelihood ratio of a positive PCR test using BAL.

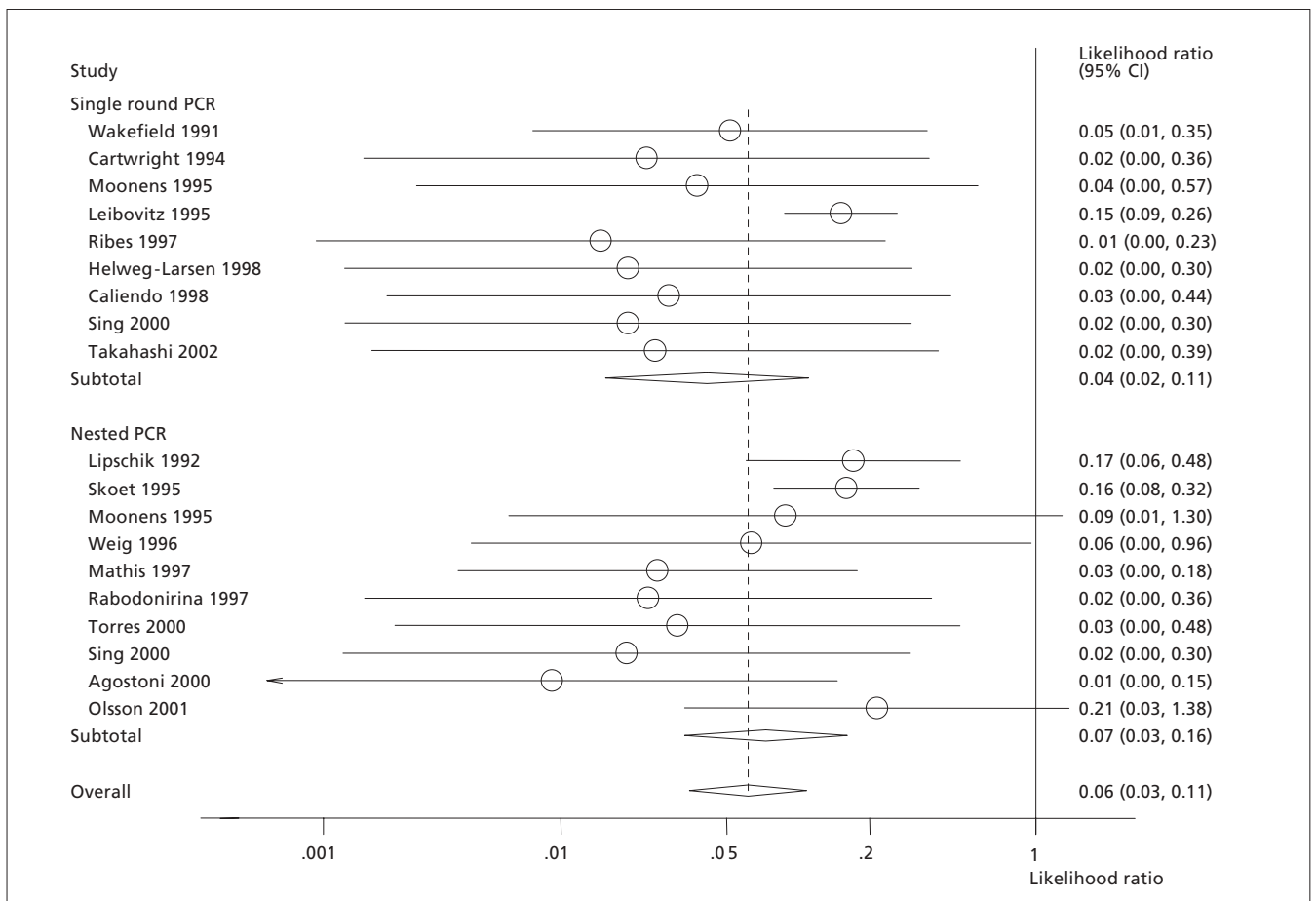


Figure 6. Likelihood ratio of a PCR negative test using BAL.

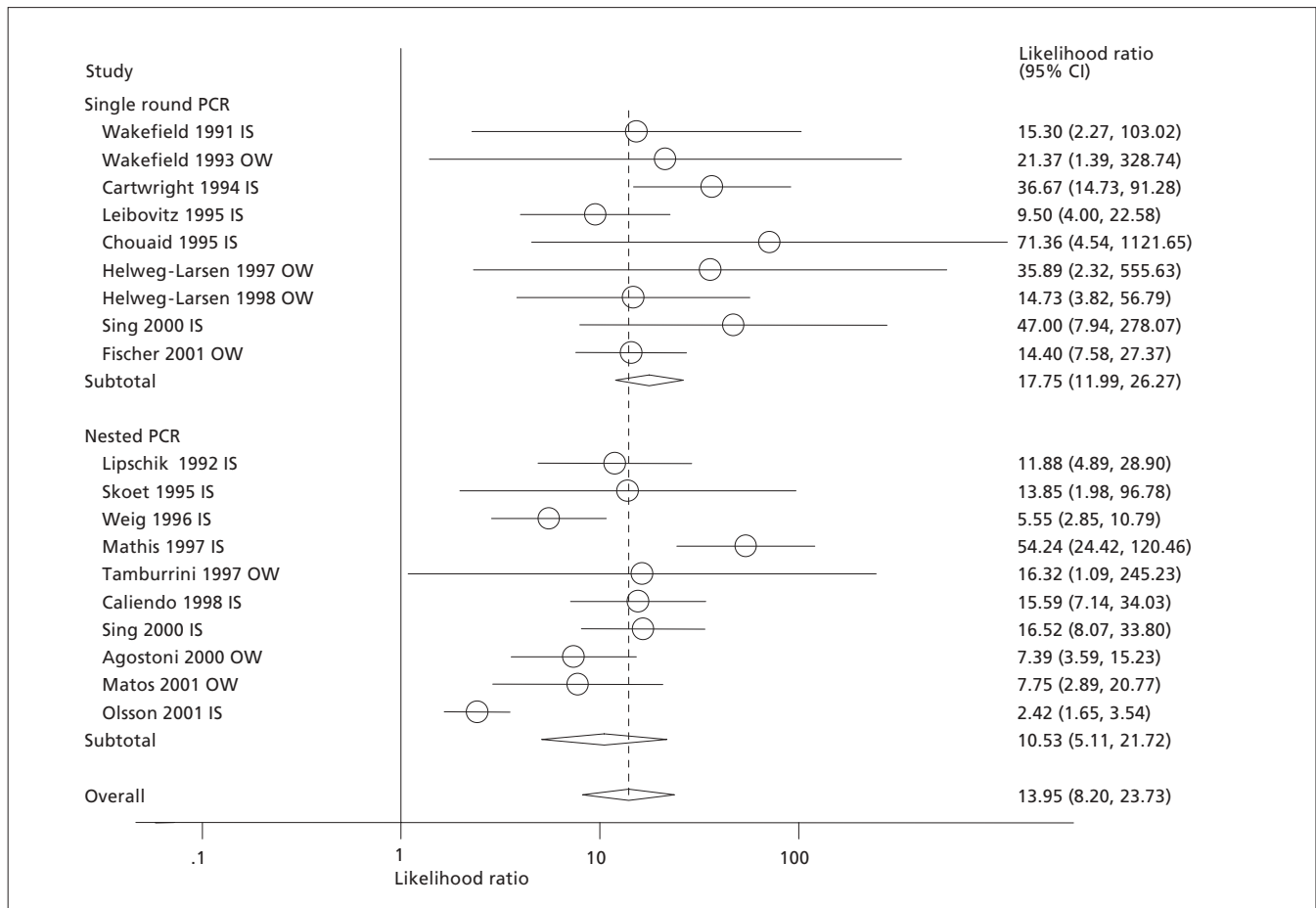


Figure 7. Likelihood ratios of a PCR positive test in studies using Sputum (IS) and oral wash (OW).

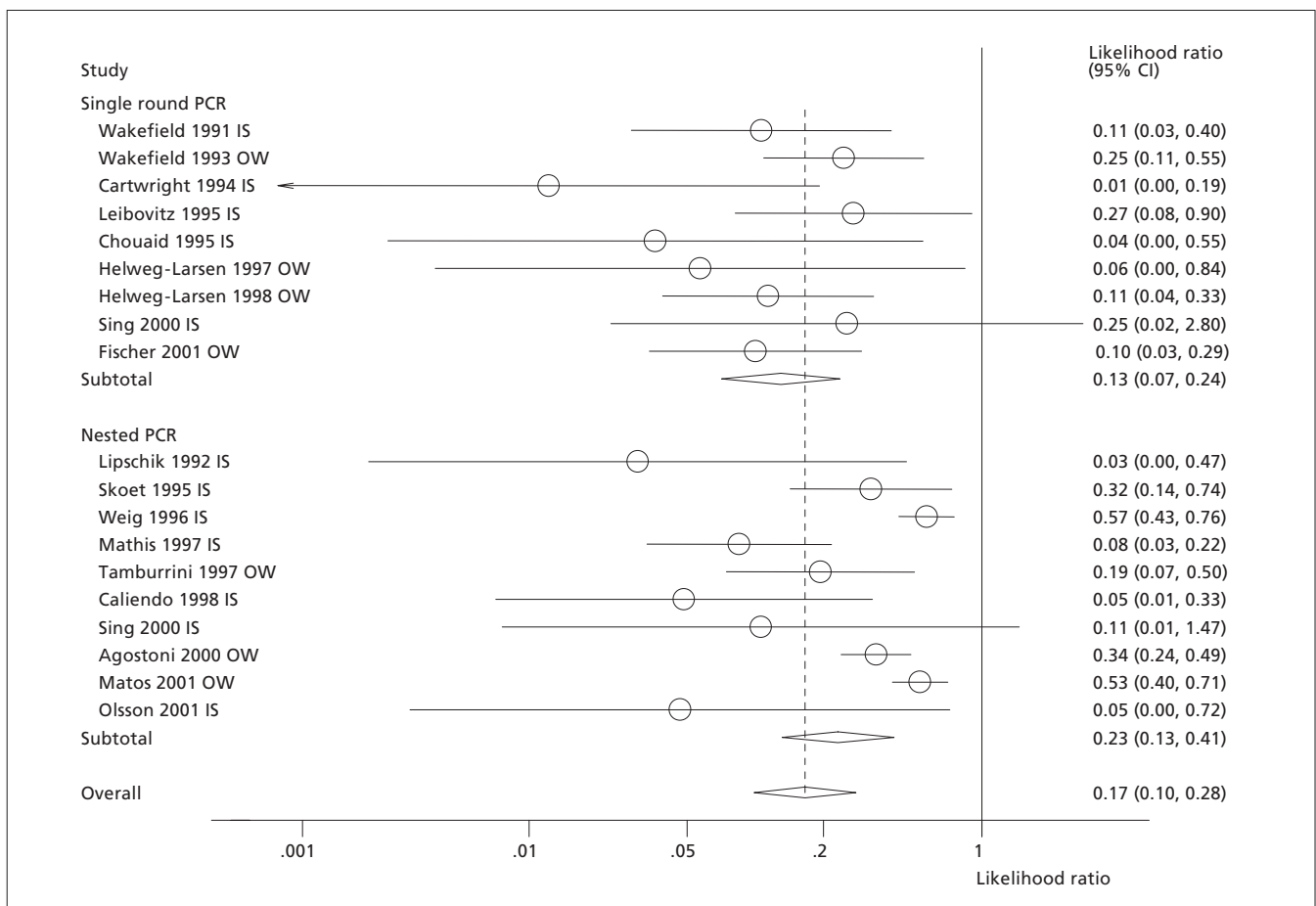


Figure 8. Likelihood ratios of a PCR negative test in studies examining Sputum (IS) and oral wash (OW).

Table 9. Diagnostic value of PCR when used on BAL.

Study	Year	n	PCR Method	Sensitivity (%)	Specificity (%)
Wakefield (211)	1991	51	Single	95.0	96.8
Cartwright (29)	1994	154	Single	100	99.3
Moonens (149)	1995	43	Single	100	96.7
Leibovitz (106)	1995	200	Single	85.3	96.8
Ribes (172)	1997	129	Single	100	79.3
Helweg-Larsen (I)	1998	61	Single	100	90.9
Caliendo (28)	1998	112	Single	100	97.9
Sing (185)	2000	259	Single	100	96.6
Takahashi (194)	2002	81	Single	100	80.7
Lipschik (109)	1992	113	Nested	84.2	92.6
Skoet (186)	1995	117	Nested	85.0	100
Moonens (149)	1995	18	Nested	100	83.3
Weig (220,221)	1996	46	Nested	100	72.2
Rabodonirina (171)	1997	127	Nested	100	77.0
Mathis (135)	1997	312	Nested	97.5	97.8
Torres (198)	2000	47	Nested	100	86.2
Agostoni (6)	2000	146	Nested	100	76.0
Sing (185)	2000	257	Nested	100	88.2
Olsson (163)	2001	32	Nested	87.5	58.3
Meta-analysis pooled estimate-random method (95%CI)				99.1 (97.5-100)	92.6 (90-95.3)

Table 10. Diagnostic value of PCR when using sputum or oropharyngeal washings.

Study	Year	N	Specimen	PCR	Sensitivity (%)	Specificity (%)
Wakefield (211)	1991	37	Induced sputum	Single	90.0	94.1
Wakefield (212)	1993	31	Oral wash	Single	77.8	100
Cartwright (29)	1994	207	Induced sputum	Single	100.0	97.59
Leibovitz (106)	1995	84	Induced sputum	Single	75.0	92.1
Chouaid (35)	1995	49	Induced sputum	Single	100	100
Helweg-Larsen (76)	1997	26	Oral wash	Single	100	100
Helweg-Larsen (I)	1998	61	Oral wash	Single	89.3	93.9
Sing (185)	2000	94	Induced sputum	Single	100	98.9
Fischer (60)	2001	175	Oral wash	Single	90.6	93.7
Lipschik (109)	1992	71	Induced sputum	Nested	100	92.6
Skoet (186)	1995	33	Induced sputum	Nested	69.2	95.0
Weig (220,221)	1996	162	Induced sputum	Nested	47.8	91.4
Mathis (135)	1997	403	Induced sputum	Nested	91.7	98.3
Tamburrini (196)	1997	27	Oral wash	Nested	83.3	100
Caliendo (28)	1998	120	Induced sputum	Nested	95.5	93.9
Sing (185)	2000	159	Induced sputum	Nested	100	94.8
Agostoni (6)	2000	146	Oral wash	Nested	69.0	90.7
Matos (136)	2001	114	Oral wash	Nested	50.0	93.6
Olsson (163)	2001	57	Induced sputum	Nested	100	60.0
Meta-analysis pooled estimate-random method (95%CI)					87 (81-94)	96 (94-98)

studies confirmed the existence of subclinical infection in both immunosuppressed and immunocompetent individuals. However, the extent of colonisation has been debated with marked variation in the rates of *P. jiroveci* carriage reported. In a study preceding the use of effective PCP prophylaxis, *P. jiroveci* DNA was detected in induced sputum by nested PCR from eight asymptomatic HIV-infected patients with CD4 counts less than 200 cells/ μ l, of which five later developed PCP 164 to 352 days after (54). Nevez et al. reported that 20% of 169 HIV-negative patients with moderate or severe immunosuppression were positive by nested PCR; none of these patients developed PCP (158). Olsson et al and Takahashi et al reported a PCR positive rates of 28% and 20%, respectively, among HIV-negative immunosuppressed patients without definite or probable PCP (163, 194). Armbruster et al noted that 6% of 77 immunocompetent HIV-negative patients were colonized (10) and Sing et al. found by nested PCR that 17-20% of immunocompetent patients with primary pulmonary disorders were nested PCR positive (185). None of these patients developed PCP (184). These observations suggest that colonization with *P. jiroveci* is more common than previously thought, even among immunocompetent individuals.

In view of the conflicting reports regarding the rate of subclinical infections, we wished to evaluate the detection of *P. jiroveci* DNA in patients not primarily suspected of PCP (VII). In a case control study, respiratory samples from 367 patients were analyzed by PCR. For each PCR-positive case, four PCR-negative controls, randomly

chosen from the PCR-negative patients, were matched by sex and date of birth. *P. jiroveci* DNA was detected in 16 (4.4%) of patients. Main findings were a higher rate of chronic or severe concomitant illness (94% vs. 50%, OR: 15, 95% CI: 2-651) in the PCR positive patients compared to controls and a higher rate of corticosteroid exposure in cases compared to controls (75% vs. 13%, OR: 21, 95% CI: 5-106). Detection of *Pneumocystis*-DNA was associated with a worse prognosis. Seven (44%) of the patients with positive PCR died within one month compared to nine (14%) of the controls ($p=0.01$). None of the nine PCR-positive patients who survived had developed PCP at one year of follow-up. However, because of the retrospective study design, in which autopsies were not done, it is unknown if the detection of *P. jiroveci* is an independent predictor of death or merely a marker of severe illness.

Whether the differences in reported rates of *P. jiroveci* detection result from population based differences in *P. jiroveci* carrier rates or stems from differences in PCR methods is unknown. PCR is extremely sensitive and will detect both living and dead organisms. In our study, the risk of false positive reactions was reduced by a strict definition of PCR positives. Samples were only considered true PCR positive if three different *P. jiroveci* gene targets could be amplified. Other studies (158, 184, 185) only requested amplification of a single gene with nested PCR, which may have increased the risk of false positive results.

The low rate of PCR positivity we observed is comparable to other

observation studies. Visconti et al (205) reported that 2.5% of 78 immunocompetent patients were positive by nested PCR. In a Danish study, 4.7% of 1762 consecutive lung biopsies at autopsy were microscopically positive for *P. jiroveci* (180). Oz et al (164) found no *P. jiroveci* DNA by single round PCR in any of 258 upper respiratory tract specimens from 86 immunocompetent individuals in the absence of microscopy verified PCP. Furthermore, Lidman et al's (108) found no evidence of *P. jiroveci* DNA in IS samples from 19 health care workers.

6.7 INTERPRETATION OF PCR RESULTS

Similar to other diagnostic tests, it is essential that the diagnostic accuracy of an applied PCR test is well defined not only in terms of technical performance but also in terms of diagnostic performance in different patient populations. The available data from several PCR studies suggest that while a negative PCR test on both invasive and non-invasive samples has reasonable power to rule out PCP, a positive PCR test must be carefully interpreted in the context of clinical findings. Single round PCR is to be preferred for diagnostic purposes, since nested PCR has increased risk of false positives results and only a limited increase in diagnostic sensitivity. As shown, comparison of the data from different clinical studies is complicated due to differences in patient populations, and PCR methods. In addition, test performance obtained in research studies may not be achieved in real-life routine settings. In the absence of clinical features typical of the pneumonia, the presence of *P. jiroveci* DNA in upper respiratory tract specimens may indicate harmless colonization of the respiratory tract rather than PCP. A single PCR result must always be viewed with caution and interpreted in the context of the patient's symptoms, the clinical findings and the results of other procedures for detection of other pathogens. Continuous education of clinicians in the proper use and interpretation of PCR-generated results, as well as in the limitations of the tests, is therefore of importance. Currently, our PCR method (I) is available as a routine test at Statens Serum Institute, Copenhagen.

A potential and serious drawback of PCR diagnosis is the risk of missing the diagnosis of other respiratory diseases, if BAL is not performed. In many immunocompromised patients a wide range of infectious or malignant diseases present with symptoms and signs, which are indistinguishable from PCP. Although clinical and laboratory findings such as exertional hypoxemia, interstitial infiltrates, oral candidiasis or elevated LDH may suggest PCP in an HIV infected patient, the specificity of these signs is low as the same signs may result from other infections. Differential diagnosis therefore usually demands microscopic examination and culture of respiratory specimens. Importantly, even when PCP is confirmed, concomitant pulmonary infections may be present. In our cohort; bacterial coinfections were detected in 20% of AIDS-associated PCP (19). Hence, PCR diagnosis should be viewed as a supplement and not an alternative to standard tests.

Oral washes are quickly and noninvasively obtained, which is of particularly importance in patients unable to sustain BAL. Compared to nasopharyngeal aspirates or induced sputum, oral washes have equivalent diagnostic sensitivity and are much less unpleasant. Although similar sensitivity may be achieved by experienced microscopic testing of induced sputum, PCR test on oral washes offer a sensitive test to hospitals without immediate access to experienced histological evaluation. Furthermore, PCR may be used to confirm a diagnosis of PCP in cases of ambiguous microscopic findings.

The development of sensitive and specific, standardized, and commercially available amplified nucleic acid tests for diagnosis of PCP would be highly desirable. Further developments are needed to distinguish between colonisation and clinical disease. Potentially promising methods are real-time quantitative PCR and detection of *P. jiroveci* mRNA. Recent research suggest that organism load as assessed by quantitative PCR is higher in cases of PCP compared to cases of colonisation (99). If these findings are confirmed and the

method further developed, quantitative real-time PCR could well be a rapid and specific test for PCP. In addition, viability of *P. jiroveci* organism may be tested by study of *Pneumocystis* mRNA, which could offer a potential method for monitoring of treatment (130).

7. DHPS MUTATIONS AND SULFONAMIDE RESISTANCE

7.1 SURVIVAL

Untreated PCP among HIV patients is almost always fatal. In the beginning of the HIV epidemic the mortality rate of PCP was reported to be 30-40% (26, 87), increasing to 70% among patients with progression to respiratory failure (152). Following improvements in the care of PCP patients, including earlier recognition of the infection and in 1990 the introduction of adjuvant corticosteroids, mortality rates fell to 10-20% (16, 21, 36, 57, 121). In the US, an 18% one-month mortality was observed among 4412 PCP cases from 1992 to 1998 (50). From 1989 to 2002, we observed an overall one-month mortality of 15% among 241 HIV infected patients (unpublished results). By multivariate Cox regression analysis, low PaO₂ at admission, age, initial antimicrobial therapy other than TMP-SMZ, and a positive BAL CMV culture were independent factors associated with a decreased 3-month survival (19,74). In addition, the degree of lung inflammation is an important prognostic factor with BAL neutrophilia and elevated immune markers such as IL-8 associated with an increased risk of death (18, 20).

7.2 TREATMENT OF PCP

Three weeks of sulfamethoxazole in combination with trimethoprim (cotrimoxazole, TMP-SMX) was introduced for treatment of PCP in 1978 and has since remained the standard of treatment (75, 82). Although toxicity from this regimen is common, comparisons with alternatives such as pentamidine, dapsone-trimethoprim, clindamycin-primaquine or atovaquone have shown superior or equivalent effect with equivalent or less toxicity (75). Among AIDS patients, failure of responding to primary treatment with cotrimoxazole has been observed in 10 to 40% of cases (24, 70, 96). In our cohort, primary anti-PCP treatment was changed in 60 (24%) of 256 consecutive episodes of AIDS-associated PCP between 1989 and June 2002 (74). Toxicity was the cause of treatment change in 27 (11%) patients, which occurred after a median of 10 (range 1-17) days. Suspected treatment failure was the reason for change in 33 (13%) patients, with treatment change after a median of 18 (range 5-34) days. Although variations in the reported rates of treatment failures may reflect differences in the definition of treatment failure or threshold for changing therapy, it is evident that a substantial number of PCP cases fail to improve, even under optimal therapy. Indeed, the mortality of PCP related respiratory failure which require ventilatory support has not changed during the last 10 years (13).

7.3 PCP PROPHYLAXIS AND FAILURE OF PROPHYLAXIS

In the beginning of the nineties, chemoprophylaxis with low dosage of cotrimoxazole was shown effective in reducing the risk of PCP among patients at risk (177). Among both HIV-infected and HIV-negative patients the risk of developing PCP is closely correlated to level of CD4 count, with most cases of HIV-associated PCP occurring at CD4 counts less than 200 cells/ μ L, and non-HIV associated PCP cases at less than 300 cells/ μ L (131). After a episode of PCP, the rate of relapse is 50% within six months if no prophylaxis is instituted (148). Primary and secondary PCP prophylaxis is recommended for all HIV-1 infected patients with CD4+ lymphocyte counts less than 200 cells/ μ L and can be safely interrupted if stable CD4 counts over this level are reached as a result of combination antiretroviral therapy (94,103). Use of cotrimoxazole is associated with a number of adverse effects (24, 71, 176, 177). Before combination antiretroviral therapy became available, it was noticed that after 13 months of cotrimoxazole prophylaxis half of the patients that had started would have experienced an adverse event, in particular rash,

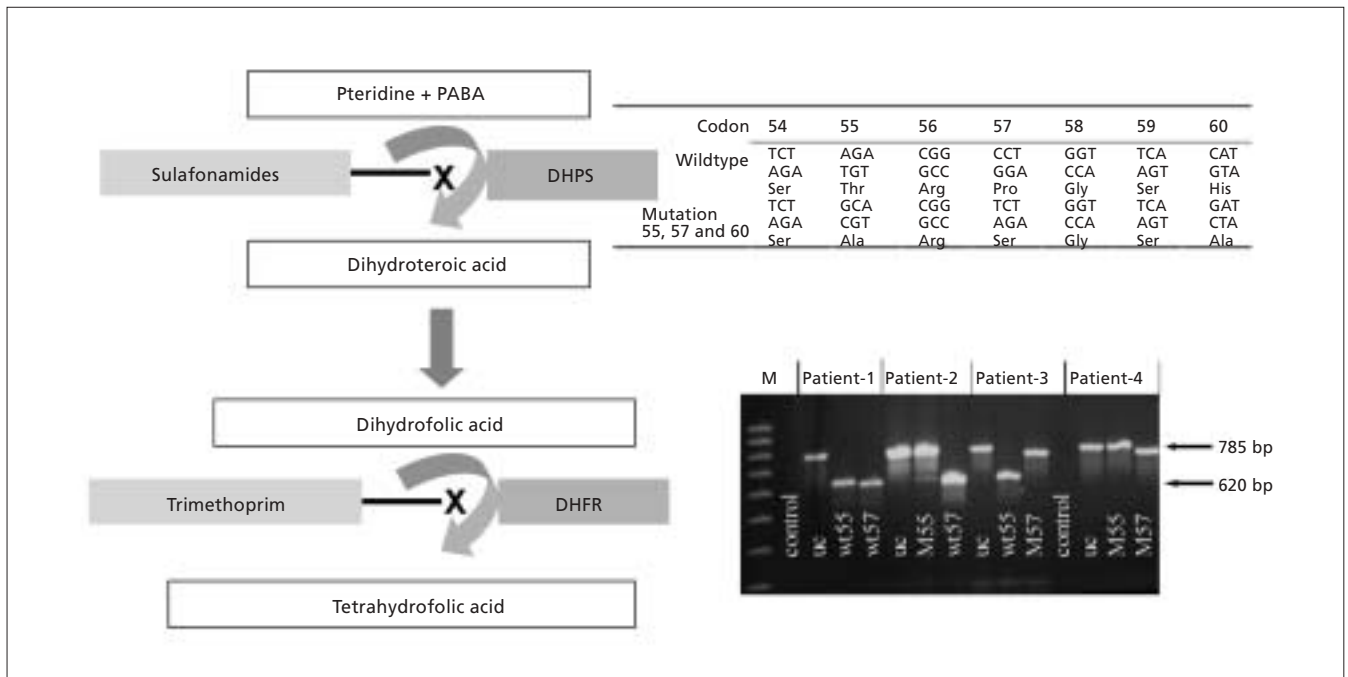


Figure 9. Inhibition of folate synthesis by sulfonamides and trimethoprim. PABA, paraaminobenzoic acid; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase. *Top right:* Alignment of DHPS wild type and mutations at codon 55-70 in *P. jiroveci*. *Bottom right:* Gel of DHPS mutation RFLP-assay. From each sample, 3 aliquots were loaded, with from left to right: uncut control, digestion with *AccI* and digestion with *HaeIII*. uc: uncut; wt55: wildtype at codon 55; wt57: wildtype at codon 57. Control: PCR negative control, adapted from IV.

and after 3 years half would have switched to other types of prophylaxis (24). For patients who are intolerant to cotrimoxazole, dapsone, another sulfone drug, is a common choice of therapy (170). The major source of prophylaxis failure is non-compliance. Although there has been considerable variation in the reported rates of cotrimoxazole failures, breakthrough in a patient who is compliant with cotrimoxazole prophylaxis is uncommon (95, 115, 177). However, documented cases of breakthroughs have been reported in patients apparently compliant with cotrimoxazole prophylaxis (7, 53, 58). In a recent African study, 33% of infants developed PCP in spite of prescribed prophylaxis (129). In addition, failure of non-co-trimoxazole prophylaxis is well documented, even in compliant patients. In open-label, randomized studies among HIV patients failure rates for dapsone and inhaled pentamidine ranged from 0.32 cases to 2 per 100 patient months of prophylaxis (68, 81, 134, 188, 219). Further, among bone marrow transplant recipients the relative risk of developing PCP was 18.8 with dapsone compared to cotrimoxazole (188).

Failure of prescribed PCP prophylaxis may occur because of poor adherence to treatment, insufficient drug levels or possibly as a consequence of sulfonamide resistance. In view of the high number of HIV-patients continuously exposed to low dose cotrimoxazole, there have been concerns that sulphonamide resistance may have developed in *P. jiroveci*.

7.4 SULFONAMIDE RESISTANCE AND DHPS MUTATIONS

The action of cotrimoxazole is mediated by inhibition of two key enzymes in folic acid synthesis (Figure 9). Sulfamethoxazole (SMX) and dapsone targets the enzyme dihydropteroate synthase (DHPS), which catalyzes the formation of dihydrofolate from para-aminobenzoic acid. Sulfonamide is a structural analog of p-aminobenzoic acid (PABA), the substrate of the DHPS enzyme, and inhibits it competitively. Trimethoprim inhibits dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate. The combination of trimethoprim and sulfamethoxazole is thought to have a synergistic effect on bacteria, however in experimental models of *Pneumocystis* the effect is almost entirely due to sulfamethoxazole (217).

Resistance to trimethoprim-sulfamethoxazole has been exten-

sively documented in bacterial and parasitic infections (84). In San Francisco, the use of cotrimoxazole PCP prophylaxis increased cotrimoxazole resistance among all among isolates of *Staphylococcus aureus* and 7 genera of Enterobacteriaceae from 6.3% in 1988 to 53% in 1995 among HIV infected patients (133). Similarly, widespread use of sulfa drugs for malaria and bacterial infection in Africa have produced high rates of resistance in *P. falciparum* and many bacterial species (59).

In *E. coli*, *N. meningitidis*, *S. pneumonia* and *P. falciparum*, point mutations in conserved regions of the DHPS gene confer sulfonamide resistance by decreasing the affinity for sulphonamides and sulfones (42, 193, 199). In *Pneumocystis*, DHPS is encoded by the folic acid synthesis (*fas*) gene, a multifunctional gene that encodes dihydroneopterin aldolase and hydroxymethyl-dihydropterin pyrophosphokinase (207). Lane et al were the first to report sequence polymorphisms in the DHPS gene of *P. jiroveci* in 1997 (98). They detected several sequence mutations which produced nonsynonymous amino acid changes. The mutations at position 55 and 57 are located in a region of the DHPS protein, which is highly conserved among different microbial organisms and different *Pneumocystis* species (124). Mutations at codon 55 to 60 are implicated in sulfa resistance in *E. coli*, *S. pneumoniae* and *P. falciparum* (4, 112, 199). Soon after the study by Lane et al, two small studies suggested an association of DHPS mutations with previous exposure to cotrimoxazole prophylaxis (90) and prophylaxis failure (139).

7.4.1 Methods for detection of DHPS mutations

In most studies, mutations in the DHPS gene of *P. jiroveci* have been detected by direct sequencing of PCR amplified DHPS obtained from clinical samples. However, the use of DNA sequencing for finding these mutations is rather expensive and time-consuming. Since DHPS mutations may be a potential cause of sulfa-resistance in *P. jiroveci*, the development of a simple method for the detection of these mutations was warranted. To this mean we investigated the use of a restriction fragment length polymorphism assay (RFLP) assay to detect the most common types of DHPS mutations (IV). By use of the restriction enzymes *Acc I* and *Hae III*, mutations in codon 55 and 57 can be detected. These enzymes cut in wildtype DHPS; in case of mutation at codon 55 or 57, there is no cleavage by *Acc I* or

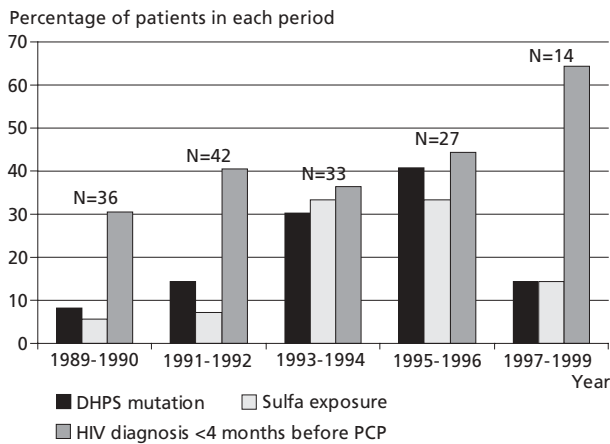


Figure 10. Percentage of PCP episodes with DHPS mutations, exposure to sulfa drugs (>1week continuous sulfa use) and recent HIV diagnosis according to calendar year of diagnosis, N indicates number of PCP episodes in each period. Chi-square for trend $p = 0.01$. Reproduced from paper III.

Hae III, respectively (Figure 9). We found that the RFLP assay correctly identified mutations when compared to direct sequencing of the DHPS gene in 27 isolates. The total turnaround time for the RFLP assay is approximately 8 h, which compares favourably with the 2 days required for sequencing. The method has been applied to study PCP samples in Portuguese HIV-patients (38), and a similar use of RFLP has been described by Roux and colleagues (175).

An alternative method for detection of DHPS mutations is SSCP. Ma et al investigated nested PCR combined with the SSCP method to detect mutations at codon 55 and 57 (126). This method had a turnaround time of 10 hours and was under optimized conditions able to detect the minority population (either wild type or mutant) if present in 10% of the total population, which suggest that SSCP is more sensitive than direct sequencing for detection of a minority population. A main limitation to the RFLP and SSCP methods are the inability to detect mutations other than those at codon 55 and 57. However, at present, mutations at codon 55 and 57 seem to account for the large majority of DHPS polymorphisms.

7.5 DHPS MUTATIONS, EPIDEMIOLOGY AND CORRELATION TO EXPOSURE

To explore the clinical importance of DHPS mutations, we undertook a study in HIV-1 infected PCP patients (III). In 152 episodes of PCP in the period 1989-1999, we analyzed DHPS by PCR amplification and subsequent direct DNA sequencing. The prognostic factors assessed included CD4 count, age, pAO_2 at admission, duration of time with AIDS and lactate dehydrogenase (LDH) levels.

Four different mutation patterns with non-synonymous nucleotide changes at codon 55, 57 and 60 of DHPS were identified. In

our study, a significant increase in the rate of DHPS mutations was seen during the study period until 1996, followed by a decrease in 1997-1999 (Figure 10). These temporal changes correlated with changes in the rate of previous or current exposure to sulfonamide drugs. Exposure to sulfa drugs and DHPS mutations were highly correlated, 18 of 29 patients who were exposed carried DHPS mutations compared to 13 of 123 patients not exposed (OR: 13.8, 95% CI: 4.9-39.7) (Table 11).

A number of studies have investigated the occurrence of DHPS mutations in different countries (Table 11). Overall the association between exposure to sulfa drugs and DHPS has been confirmed, with a strong association of mutations at codon 55 and 57 to previous exposure to cotrimoxazole or dapsone prophylaxis (17, 78, 89, 90, 123, 175). Large variations in frequencies of DHPS mutations have been reported from different geographical areas, ranging from 20 to 69% of isolates. The highest levels have been observed in San Francisco where more than 80% of patients were infected with mutant strains (156). These rates are considerably higher than in Europe and Japan (195).

7.6 MECHANISMS OF EMERGENCE OF DHPS MUTATIONS

The recent increase in DHPS mutations coincides with more widespread use of PCP prophylaxis in HIV patients. DHPS mutations seems to have arisen independently in multiple isolates, rather than as a result of dissemination of mutant isolates which developed in single or limited number of *P. jiroveci* organisms, since no associations is found between DHPS mutations and specific SSCP genotypes or ITS genotypes (125) (Helweg-Larsen, unpublished findings). Additional support for the hypothesis of sulfa drug induced DHPS mutations can be derived from observations of patients with recurrent episodes of PCP. In collaboration with Hauser we recently analyzed 13 European HIV patients with recurrent episodes of PCP (155). In five of seven cases in which both episodes were caused by the same *P. jiroveci* SSCP type, a switch to mutant DHPS strain from either wild type DHPS or a mixed wild type/mutant DHPS was observed between first and second episode of PCP. The two remaining patients had a mutant strain already at the first episode. All patients had received treatment or maintenance therapy with cotrimoxazole or dapsone. These findings suggests that DHPS mutants may be selected in vivo (within a given patient) under the pressure of co-trimoxazole or dapsone (155). The emergence of DHPS mutations appears to be specific for *P. jiroveci*, because only wild-type *Pneumocystis* DHPS have been found in other primate species (46).

7.7 DHPS MUTATIONS AND OUTCOME

In our cohort, mutations in the *P. jiroveci* DHPS gene were associated with significant lower 3-month survival after diagnosis of PCP (Figure 11) (III). Among the 24 patients who died within three months after the diagnosis of PCP, ten patients had DHPS muta-

Table 11. Prevalence of DHPS mutations and associations with sulfa exposure.

Study	Country(year)	Number of DHPS mutations/number of PCP episodes	No. with DHPS mutations/no with sulfone exposure	No. with DHPS mutations/no. with no sulfone exposure	Odds ratio (95%CI)
Helweg-Larsen (III)	DK (1989-99)	31/152 (20%)	18/29 (62%)	13/123 (11%)	13.8 (4.9-39.7)
Kazanjian (89)	USA (1991-97)	42/97 (43%)	28/37 (76%)	14/60 (23%)	10.2 (3.6-30.2)
Ma (123) (exp)	USA (1985-98)	16/37 (43%)	11/16 (69%)	3/15 (20%)	8.8 (1.4-66.2)
Kazanjian (90)	USA (1976-97)	7/27 (26%)	5/7 (71%)	2/20 (10%)	22.5 (1.8-336.4)
Takahashi (195)(exp)	J (1994-99)	6/24 (25%)	2/3 (33%)	4/21 (19%)	8.5 (0.32-529.9)
Huang* (78)	USA (1996-99)	76/111 (69%)	57/71 (80%)	19/40 (48%)	4.5 (1.8-11.6)
Beard* (17)	USA (1995-98)	152/220 (69%)	np	np	np
Santos (175)	F (1993-98)	11/20 (55%)	5/5 (100%)	3/12 (25%)	RR:4 (1.5-10.7)
Costa (37)	P (1994-2001)	24/89 (27%)	5/16 (31%)	19/73	1.3 (0.3- 4.7)
Visconti (206)	I (1992-97)	7/20	4/5	3/15	16 (0.92-851.0)
Ma (127) (exp)	I (1994-2001)	9/107 (8%)	6/31	3/76	5.4 (1.1-35.1)

Treatment according to current prophylaxis or (when indicated) previous exposure (exp) to sulfone drugs (co-trimoxazole and dapsone) at diagnosis of PCP.*: same patients included in both studies. Np: not provided. RR: relative risk. Odds ratio and exact 95% confidence intervals were recalculated using Stata 8.

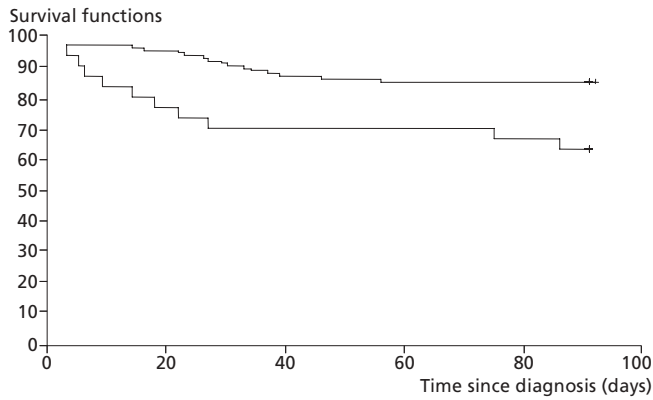


Figure 11. 3 month survival from time of diagnosis of last PCP episode. Top: wildtype, bottom: mutant DHPS. Log-rank $p=0.002$. Reproduced from paper III.

tions ($p=0.002$), of these seven were treated with co-trimoxazole and three with pentamidine. After adjustment in a Cox model for the presence of a previous AIDS-defining illness, calendar year, age, baseline CD4-cell count, and PaO_2 , the presence of *P. jiroveci* DHPS mutations remained a strong predictor of survival with an adjusted multivariate hazard ratio of 3.1 (95% CI 1.2-8.1). The survival difference remained if survival analysis was restricted to patients treated with co-trimoxazole, if the analysis was restricted to first episodes of PCP and if only samples with unambiguous mutations were considered. However, DHPS mutations were not invariably associated with failure of sulfa-drug therapy, since standard treatment with co-trimoxazole was successful in 12 of 19 PCP episodes with mutant DHPS strains.

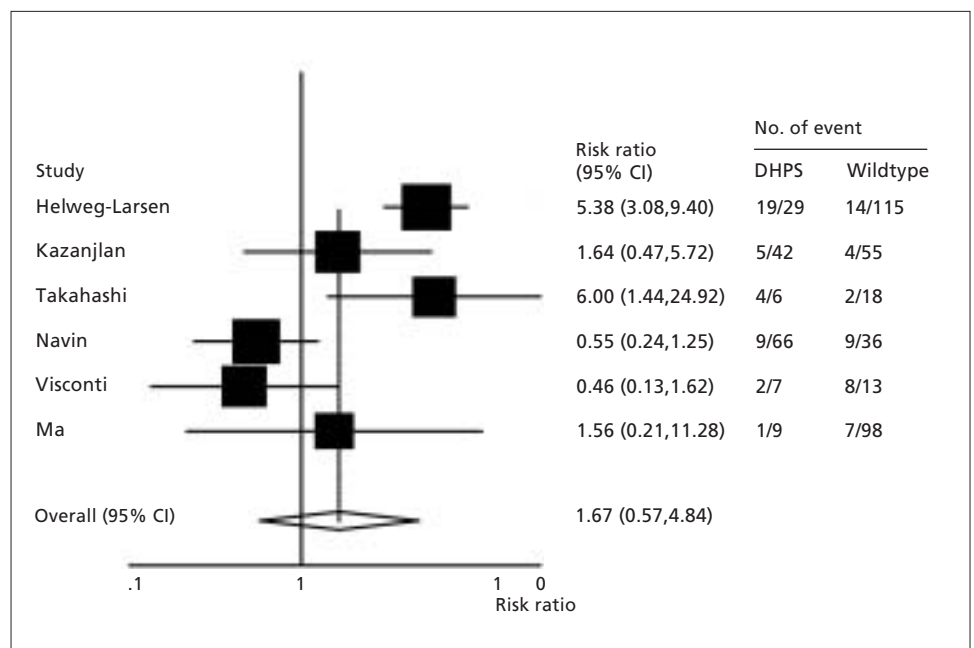
The connection between DHPS mutations and treatment failure or mortality has been studied in other reports (Figure 12). Kazanjian et al observed significantly more treatment failure of sulfone therapy among 97 PCP patients with mutant genotypes compared to wildtype, but found no difference in death rates. Navin and colleagues reported that DHPS mutations had no effect on response to treatment or survival among 130 HIV-infected patients (156). In this study an overall 71% rate of DHPS mutations were observed among 130 HIV-1 PCP patients followed from 1995 to 1999 at two hospitals in San Francisco and Atlanta. A higher rate of mutations (85% of 71 patients) was observed at San Francisco General Hospital compared to Atlanta (45% of 130) with no evidence for a con-

nection between treatment failure and mutations. On the contrary, a trend for lower death rate was found in patients treated with cotrimoxazole who carried DHPS mutation compared to wildtype (16/74 vs. 9/36). Post-hoc subanalysis even suggested an increased virulence from wildtype in patients older than 40 years. However, a potential limitation of this study is the possibility of selection bias. Within the time frame of the study more than 260 PCP patients were diagnosed with PCP at San Francisco General Hospital (150), but only 71 patients enrolled in the study. Further, only 11% of patients discharged with a diagnosis of PCP from the Hospital in Atlanta had their diagnosis verified by microscopy during the study period. A key reason was the exclusion of outpatients and patients unable to provide informed consent (Laurence Huang, personal communication).

7.8 CONCLUSION

In summary, DHPS mutations in *P. jiroveci* may have emerged recently as a consequence of selective pressure by widespread sulfonamide use. Although previous exposure to sulfone drugs have been implicated in the majority of cases described, mutant DHPS strains is now increasingly being detected in patients seemingly unexposed to sulfone drugs, particularly in the US, where remarkable high rates of DHPS mutations are being reported. These observations suggest transmission of mutant DHPS *P. jiroveci* from treated patients to others at risk. Available evidence suggests that DHPS mutations are associated with failure of low-dose sulfone prophylaxis, in particular dapsone prophylaxis. We have shown that in Danish HIV-patients, DHPS mutations are associated with a poor prognosis, however conflicting results from other studies have been published and presently it remains unclear whether the presence of *Pneumocystis* DHPS gene mutations confers clinical resistance to high dose cotrimoxazole or dapsone plus trimethoprim for PCP treatment. Several reasons for the discrepant results are possible. Among these are geographical or time dependent differences between patients in the studies and the existence of – yet undefined – drug resistance related to the DHPS mutations. Since our study on DHPS mutations only comprised 144 patients from a 10 year calendar period we cannot exclude residual confounding such as differences in severity of PCP or changes in the management of PCP during the study period. As some patients who carry mutant genotypes can be treated successfully with cotrimoxazole, it is likely that DHPS mutations are markers of other sulfa-resistance factors. In *E. coli*, DHPS mutations confer only low-level resistance to sulfathiazole, and secondary muta-

Figure 12. Risk of deaths with DHPS mutation compared to wildtype in published observation studies. Forest plot of DHPS mutations and survival in HIV-positive patients. DerSimonian random effects analysis. A tentative pooled risk estimate is shown at bottom, but should be interpreted cautiously, since metaanalysis of observation studies is subject to bias.



tions outside DHPS are required for high-level resistance. Presently, resistance to trimethoprim appears to be an unlikely cause; the efficacy of trimethoprim monotherapy is limited (218) and only wildtype DHFR gene sequences were found in one study, in which 43% of isolates carried DHPS mutations (123).

Whether DHPS mutations will impact the overall clinical efficacy of sulfonamides is unknown. As a consequence of combination antiretroviral therapy and the marked decline in AIDS associated PCP, fewer HIV patients is now exposed to sulfone drugs in Europe and USA. Thus there may now be less selective pressure on *P. jiroveci*. The situation in the developed world is different. The overwhelming majority of HIV-infected persons worldwide reside in sub-Saharan Africa, Southeast Asia, and Latin America, places where access to antiretroviral therapy is currently limited. PCP is increasingly being recognized as a significant cause of morbidity in these regions where treatment is often limited to cotrimoxazole with no alternatives (5, 61). In Africa, cotrimoxazole is widely used as first line therapy for childhood infections and is increasing being used after provisional WHO guidelines have recommended its general use to prevent bacterial and opportunistic infections in HIV infected patients. Because of the huge HIV epidemic and low cost of cotrimoxazole, there is ample risk for development of clinical sulfa resistance in *P. jiroveci*. Hence further surveillance of DHPS mutations and investigations into other possible mechanisms of sulfa resistance is warranted. Presently, however, there remains no clear clinical use for the detection of DHPS mutations, since presence of mutations does not clearly predict response to therapy.

8. SUMMARY

P. jiroveci is an unusual fungal opportunist that remains a frequent cause of a severe pneumonia in immunocompromised patients, especially HIV patients, in which PCP remains one of the most common AIDS defining illnesses. *P. jiroveci* has been difficult to study, because the organism cannot be cultured in vitro and because only relatively small amounts of the organisms can be recovered from clinical samples. Many aspects of the infection are not fully understood. However, recent advances in DNA analysis have enabled the study of the infection. In the present studies PCR based methods, were used to investigate *P. jiroveci*.

Genetic variations in the internal transcribed spacer region (ITS) of the nuclear rRNA operon of *P. jiroveci* were found in samples from two presumed case-clusters of PCP (II) and a prospective cohort of HIV-positive patients with PCP (V). In the case-cluster study, we found no indication of a particular ITS type being responsible for either of the clusters. In the cohort study, we observed a complex picture of ITS types, with a high rate of different ITS genotypes and multiple genotypes in 23% of patients. A limited number of ITS sequence types accounted for the majority of types. These common types are not substantially different from sequence types reported from other countries, suggesting that *P. jiroveci* strains in Denmark are not unique. We found no specific temporal changes in occurrence of ITS genotypes, no evidence of clustering of specific genotypes, and no link between ITS genotypes and the severity of pneumonia, demographic variables or season of the year. To investigate the influence of mixed infections and variability of genotypes we undertook a study of genotype heterogeneity in autopsy lungs (VI). We found that the infection is not clonal, that coinfections with multiple genotypes are common and that in some cases distinct populations of organisms may reside within the lung. Although these observations show that interpretation of genotype data should be cautious, the broad diversity of sequence types and the absence of temporal and clinical clusters of genotypes suggest that *P. jiroveci* may be ubiquitous, and that direct transmission from patient to patient may not be the predominant mode of infection.

The high sensitivity of PCR can be used to detect *P. jiroveci* DNA in upper respiratory samples. By using an improved single round PCR protocol we found that the PCR detection of *P. jiroveci* in oral

wash samples had high sensitivity for diagnosing PCP patients among HIV-positive patients, but that some patients are PCR positive without evidence of clinical PCP (I). In a nested case-control study we detected *P. jiroveci* DNA by PCR in 4.4% of 367 patients with non-HIV associated pneumonia, and found that cases that were PCR positive were more sick from concomitant illness and had a higher rate of corticosteroid exposure (VII). Although carriage of *P. jiroveci* DNA was associated with a higher rate of mortality compared to controls, most of patients in whom *P. jiroveci* was detected never developed clinical PCP. Together with other reports, our findings suggest that while a negative PCR test has reasonable power to rule out PCP, a positive PCR test must be carefully interpreted in the context of clinical findings. However, if properly used, PCR on oral washings is an easy, quick and sensitive method for the diagnosis of PCP, particularly in patients unwilling or unable to sustain BAL. The described PCR method is now available as a routine test from Statens Serum Institute.

In HIV-1 infected patients there has been concern if sulfa resistance might develop in *P. jiroveci*, following the widespread use of sulfonamides for prophylaxis and treatment of PCP. In the absence of a culture system, we searched for evidence of mutations in the dihydropteroate (DHPS) locus of *P. jiroveci*, the target of sulfone drugs (III). Among 152 episodes of AIDS associated PCP from 1989 to 1999 we found four different mutation patterns with non-synonymous nucleotide changes at codon 55, 57 and 60 of the DHPS locus. A statistically significant increase in the rate of DHPS mutations was seen during the study period until 1996, followed by a decrease in 1997-1999 which correlated with changes in the rate of previous or current exposure to sulfonamide drugs. DHPS mutations were highly correlated to previous exposure to sulfa drugs and were associated with failure of sulfonamide prophylaxis. Mutations were also detected in patients without known exposure to sulfa drugs. After adjustment for other prognostic variables, presence of DHPS mutations remained an independent predictor of mortality. However, conflicting results from other studies have been published and presently it remains unclear whether the presence of *Pneumocystis* DHPS gene mutations confers clinical resistance to sulfa treatment. As several patients with DHPS mutations have been successfully treated with high dose cotrimoxazole it is probable that DHPS mutations are indicators of low-level sulfa resistance which together with yet unknown sulfa resistance mechanisms may contribute to failure of sulfonamide treatment.

Together these studies support a complex picture of transmission and infection of *P. jiroveci*. The infection is cleared after primary infection in childhood; however transient colonisation without clinical disease may happen. Although interpretation of genotype studies is difficult because infection is not clonal with some patients carrying more than one strain of *P. jiroveci*, there is so far little evidence that direct transmission of *P. jiroveci* is crucial for the risk of acquiring PCP. However, the finding that DHPS mutations are increasingly detected in patients without sulfa exposure suggests that indirect human transmission is nevertheless important.

9. PERSPECTIVES

Combination antiretroviral therapy has markedly declined the incidence of PCP among HIV-1 infected in Denmark as well as in other Western countries and PCP is now again relatively rare in these regions. Hence, the epidemiological studies needed to further investigate the human infection are difficult to conduct and may perhaps also seem less imperative. However, PCP is increasing in the developing countries and although HAART is currently effective, the cumulative number of patients with HIV-1 drug resistance is also increasing, and will eventually result in more persons at risk of acquiring PCP. In this perspective, continued research will require international collaboration to ensure adequate clinical and epidemiological studies.

Advances in DNA analysis technology have provided important

insights to the infection with *P. jiroveci*, but also raise new questions. While PCR based studies have shown limited carriage after primary infection and clinical disease, there is also increasing evidence of low level asymptomatic carriage. However, the precise kinetics and incubation period of the infection and the extent to which asymptomatic carriers may contribute to the spread of infection remains unknown. A promising tool for future studies is quantitative PCR which may help to define diagnostic thresholds between clinical infection and colonisation as well as enabling studies of the kinetics and viability during treatment. Hopefully, advances in technology could also permit the detection of possible environmental forms of the organisms.

DNA typing has enabled strain characterization of *P. jiroveci* and suggest emergence of DHPS mutations and sulfa resistance under the pressure of sulfa drugs. However, in the continued absence of a reliable culture system, definitive conclusions on drug susceptibility and strain typing are difficult. Infection is not necessarily clonal and there is no recognized standard method for genotyping. An optimal multilocus typing system remains to be determined.

A major advance will be the completion of the *Pneumocystis* Genome Project, which was initiated in 1997. Currently, complete physical maps and gene sequences are being determined for the genomes of *Pneumocystis carinii* f. sp. *carinii* and *P. jiroveci* (3). These data will be crucial for further understanding of the infection and will enable identification of new polymorphic regions and drug targets and may eventually also lead to the development of a culture system.

ABBREVIATIONS

AIDS: acquired immunodeficiency syndrome
 BAL: broncho alveolar lavage
 CD4 cell: CD4 receptor positive T lymphocyte cell (plasma level)
 CI: confidence interval
 CMV: Cytomegalovirus
 COPD: chronic obstructive pulmonary disease
 DHFR: dihydrofolate reductase
 DHPS: dihydropteroate synthase
 HAART: highly active antiretroviral therapy
 HCW: health care workers
 HIV-1: human immunodeficiency virus type 1
 ITS: Internal transcribed spacer regions of the nuclear rRNA operon.
 MSG: Major surface glycoprotein
 Mt26S: mitochondrial 26S subunit
 mtLSU rRNA: mitochondrial large subunit ribosomal RNA
 mtSSU rRNA: mitochondrial small subunit ribosomal RNA
 OR: odds ratio
 PCP: pneumocystis pneumonia
 PCR: polymerase chain reaction
 RFLP: restriction fragment length polymorphism
 RR: relative risk
 rRNA: ribosomal RNA
 SCID: severe combined immunodeficiency
 SSCP: single strand conformation polymorphism
 TB: tuberculosis
 TD-PCR: touch down PCR
 UCS: upstream conserved sequence

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