DIAGNOSIS AND EPIDEMIOLOGY OF *ASPERGILLUS FUMIGATUS* AND *ASPERGILLUS FLAVUS* INFECTIONS BY MOLECULAR TECHNIQUES IN HAEMATOLOGY PATIENTS.

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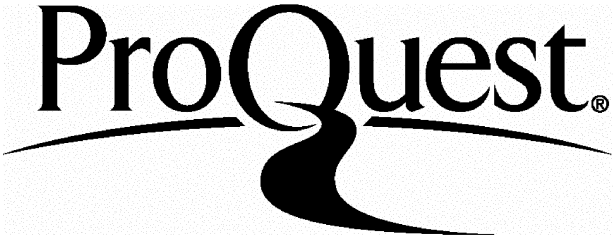

ABSTRACT

Invasive pulmonary aspergillosis (IPA) remains difficult to diagnose. Two polymerase chain reactions (PCR) for *A. fumigatus* introduced in 1993 were potentially powerful new diagnostic tools in the management of IPA.

The clinical management of a group of 87 haematology patients with IPA was analysed restrospectively, while *Aspergillus* isolates and bronchoalveolar lavage (BAL) fluid from these patients and others were studied using the new PCR methodology.

The radiological detection of LISA (lesion with imaging suggestive of aspergillosis) had a 90% positive predictive value for IPA in a subgroup of 39 patients who underwent lung resections based on the presence of LISA.

Analysis of the survival of these 87 patients by Cox's proportional hazard model showed a significant negative association between the presence of LISA and death, and a significant association of relapsed haematological disease and death, while other factors, particularly surgical treatment, had no independent effect.

The sensitivity of a PCR using primers based on the alkaline protease gene (2 pg) was superior to that of one based on the 26S/intergenic spacer region of the rDNA complex (200 pg), and the former assay was used for subsequent experiments.

DNA recovery experiments from BAL fluid demonstrated a 3 log DNA loss during the extraction process. A variable DNA loss of up to 50 fold from suspensions of conidia in water was demonstrated, and the overall sensitivity of the extraction/PCR was equivalent to that of culture.

PCR was performed on 68 *Aspergillus* culture negative BAL's, including 39 from haematology patients pulmonary infiltrates. Only 16 positive results were obtained from 228 validated reactions, and none were reliably reproducible, even in patients with subsequently proven IPA. The method cannot be recommended for clinical use.

PCR, followed by SSCP of the products, with or without prior restriction, was insufficiently discriminatory as a typing method for *A. fumigatus* strains.

TABLE OF CONTENTS

ABSTRACT **2**

TABLE OF CONTENTS **4**

LIST OF TABLES **12**

LIST OF FIGURES **13**

ACKNOWLEDGEMENTS **16**

DEDICATION **17**

ABBREVIATIONS **18**

CHAPTER 1: INTRODUCTION. **21**

1.1. BRIEF HISTORICAL INTRODUCTION **21**

1.2. OVERVIEW OF CLASSIFICATION WITHIN MEDICALLY IMPORTANT FUNGI **21**

1.3. TAXONOMY OF ASPERGILLUS **23**

1.4. MORPHOLOGICAL AND CULTURE CHARACTERISTICS OF *ASPERGILLUS* **26**

1.4.1. Macroscopic and culture characteristics 26

1.4.2. Microscopic characteristics 26

1.4.3. Growth characteristics 28

1.4.4. Histology 30

1.5. LIFE CYCLE OF *ASPERGILLUS* **30**

1.6. ENVIRONMENTAL DISTRIBUTION OF *ASPERGILLUS* SPECIES 34

1.7. OVERVIEW OF DISEASES CAUSED BY *ASPERGILLI* 34

1.8. HOST/PARASITE RELATIONSHIPS 35

1.8.1. Mechanisms of resistance against host defences by *Aspergillus* 35

1.8.2. Host defence against conidia 37

1.8.3. Host defence against hyphae 38

1.8.4. Other host defence mechanisms including humoral 38

1.9. PULMONARY ASPERGILLOSIS 39

1.9.1. Classification of pulmonary aspergillosis 39

1.9.2. Saprophytic aspergillosis 41

1.9.2.1. Airway colonisation 41

1.9.2.2. Aspergilloma (also known as fungus ball or mycetoma) 41

1.9.2.3. Invasion of necrotic tissue 44

1.9.3. Allergic aspergillosis 44

1.9.3.1. Hypersensitivity pneumonitis (extrinsic allergic alveolitis) 44

1.9.3.2. Loeffler's syndrome 45

1.9.3.3. Extrinsic asthma 46

1.9.3.4. Allergic bronchopulmonary aspergillosis (ABPA) 46

1.9.4. Invasive aspergillosis 50

1.9.4.1. Predisposing factors to invasive aspergillosis. 50

1.9.4.2. Acute *Aspergillus* tracheobronchitis 51

1.9.4.3. Pleural aspergillosis 52

1.9.4.4. Chronic necrotising aspergillosis 52

1.9.4.5. *Aspergillus* bronchopneumonia 53

1.9.4.6. Angioinvasive aspergillosis 54

1.9.4.7. Miliary aspergillosis 56

1.10. METHODS OF DIAGNOSIS 56

1.10.1. Reasons for diagnostic difficulties in IPA 56

1.10.2. Clinical features 59

1.10.3. Radiology 59

1.10.4. Sputum cytology and culture 66

1.10.5. Nasal culture 67

1.10.6. Cytology and culture of BAL, bronchial aspiration and brushings 67

1.10.7. Histological techniques and use of transbronchial, transcutaneous and open lung biopsies 70

1.10.8. Combination of investigations 72

1.10.9. *Aspergillus* antigen and antibody studies. 72

1.10.9.1. Antibodies 72

1.10.9.2. Antigens 74

1.10.10. Polymerase Chain Reaction (PCR) technology for *Aspergillus* 86

1.11. TYPING OF *ASPERGILLUS FUMIGATUS* STRAINS 106

1.11.1. General considerations 106

1.11.2. Antigenic bands 107

1.11.3. Phenotypic methods (enzymes and susceptibility to toxins). 108

1.11.4. DNA methods 108

1.11.4.1. RFLPs 109

1.11.4.2. Molecular probes 110

1.11.4.3. RAPD 111

1.11.5. Combinations of typing methods 112

1.11.6. Drawbacks and discussion 114

1.11.7. Possible use of single-stranded conformation polymorphism (SSCP) as a typing method for *Aspergillus fumigatus.* 115

1.12. INCIDENCE 117

1.13. TREATMENT 120

1.13.1. Amphotericin B 120

1.13.2. Azoles 123

1.13.3. Combination therapy 124

1.14. Surgery 125

1.15. SURVIVAL 128

1.16. BASIS AND AIMS OF THIS STUDY 128

CHAPTER 2: MATERIALS AND METHODS 131

2.1. CLINICAL SUBJECTS 131

2.2. ANALYSIS OF SURVIVAL DATA 132

2.3. ISOLATES 134

2.4. BRONCHOALVEOLAR LAVAGE FLUID (BAL) COLLECTION AND STORAGE 136

2.5. LUNG TISSUE 141

2.6. ENZYMATIC EXTRACTION OF DNA FROM ASPERGILLUS SPECIES (DENNING et al 1990) 141

2.7. QUICK ASPERGILLUS DNA EXTRACTION METHOD (MODIFIED FROM AUFAUVRE-BROWN et al 1992) 145

2.8. METHOD FOR DNA EXTRACTION FROM BAL (ACCORDING TO TANG ET al 1993). 147

2.9. MEASUREMENT OF DNA CONCENTRATION 148

2.10. PCR METHOD 1: ASPERGILLUS FUMIGATUS RIBOSOMAL DNA PCR (Spreadbury et al 1993)..... 148

2.11. PCR METHOD 2: ASPERGILLUS FUMIGATUS AND FLAVUS ALKALINE PROTEASE GENE DNA PCR (Tang et al 1993)..... 149

2.12. DNA PURIFICATION..... 150

2.13. RESTRICTION ENDONUCLEASE DIGESTION OF GENOMIC DNA BY XhoI AND SalI..... 151

2.14. RESTRICTION ENDONUCLEASE DIGESTS OF PCR PRODUCTS OBTAINED BY METHOD 2 BY DdeI AND StyI..... 152

2.15. ELECTROPHORESIS OF DNA, VISUALISATION AND PHOTOGRAPHY..... 153

2.16. SSCP METHOD..... 154

2.17. PROMEGA SILVER STAINING METHOD FOR MDE GELS..... 155

2.18. PHOTOGRAPHY OF MDE GEL..... 156

CHAPTER 3: CLINICAL DATA..... 157

3.1. BACKGROUND..... 157

3.2. DEMOGRAPHY AND PREVIOUS HAEMATOLOGICAL TREATMENT.. 160

3.3. POSITIVE PREDICTIVE VALUES OF RADIOLOGICAL DIAGNOSIS OF IPA BASED ON HISTOLOGICAL/ MICROBIOLOGICAL FINDINGS IN RESECTED LUNG TISSUE..... 161

3.4. BACKGROUND AND OPERATIVE DETAILS OF PATIENTS WITH HISTOLOGICALLY DOCUMENTED IPA POST RESECTION..... 165

3.5. COMPLICATIONS AND RECURRENCE OF IPA FOLLOWING SURGERY .. 168

3.6. REASONS FOR NOT OPERATING ON PATIENTS WITH RADIOLOGICAL AND MICROBIOLOGICAL DIAGNOSIS 169

3.7. SURVIVAL 170

3.8. *ASPERGILLUS* ISOLATES FROM RESECTED LUNG AND CORRELATION WITH DURATION OF AMPHOTERICIN B PRE RESECTION..... 177

3.9. CONCLUSIONS 178

CHAPTER 4: SENSITIVITY OF TWO POLYMERASE CHAIN REACTIONS AND *ASPERGILLUS* DNA RECOVERY FOLLOWING EXTRACTION AND PCR COMBINED 181

4.1. BACKGROUND 181

4.2. HYPOTHESES 182

4.3. DNA MATERIALS 183

4.4. LOWER LIMIT OF DNA DETECTION BY PCR METHOD 1 185

4.5. LOWER LIMIT OF DNA DETECTION BY PCR METHOD 2 187

4.6. COMPARISON OF METHOD 1 AND METHOD 2 FOR *ASPERGILLUS* PCR.. 189

4.7. RECOVERY OF *A. FUMIGATUS* DNA DILUTED IN BAL POST EXTRACTION AND SUBJECTED TO PCR..... 189

4.8. DNA EXTRACTION/PCR OF DILUTIONS OF..... 192

A. FUMIGATUS AND *A. FLAVUS* CONIDIA IN WATER 192

4.9. CONIDIAL CONCENTRATIONS REQUIRED TO PRODUCE *A. FUMIGATUS* GROWTH ON A SABOURAUD PLATE. 196

4.10. SUMMARY AND CONCLUSIONS 196

4.10.1. Comparative sensitivity of the two PCR methods 196

4.10.2. Possible reasons for poor DNA recovery from BAL fluid 197

4.10.3. Discussion of results of DNA extraction from conidia 198

4.10.4. General discussion and implications for future work 198

CHAPTER 5: APPLICATION OF THE ALKALINE PROTEASE BASED POLYMERASE CHAIN REACTION TO CLINICAL SAMPLES 200

5.1. RANDOM *ASPERGILLUS* CULTURE NEGATIVE BAL SAMPLES FROM PATIENTS OUTSIDE THE HAEMATOLOGY UNIT 200

5.2. BAL SAMPLES FROM PATIENTS WITH 206

HAEMATOLOGICAL AND PULMONARY DISEASE 206

5.3. BAL SAMPLES FROM ASYMPTOMATIC HIV POSITIVE VOLUNTEERS.... 215

5.4. RESECTED LUNG TISSUE 216

5.5. CONCLUSIONS & DISCUSSION 219

CHAPTER 6: DNA EXTRACTION AND MOLECULAR EPIDEMIOLOGY OF 30 *ASPERGILLUS* ISOLATES 221

6.1. ENZYMATIC DNA EXTRACTION OF 30 *ASPERGILLUS* STRAINS AND XhoI/SalI RESTRICTION ANALYSIS ON WHOLE GENOMIC DNA 221

6.1.1. DNA extraction (general) 221

6.1.2. Strain variability in ease of DNA extraction 225

6.1.3. *SalI* and *XhoI* restriction digests 227

6.2. PCR OF EXTRACTED *ASPERGILLUS* STRAINS BY METHODS 1 AND 2 229

6.3. DdeI AND StyI RESTRiCTION ANALYSIS OF PRODUCTS BY PCR METHOD 2 231

6.4. SSCP ANALYSIS OF RESTRICTED AND UNRESTRICTED PCR PRODUCTS 234

6.5. DISCUSSION OF EPIDEMIOLOGICAL STUDIES & CONCLUSIONS 241

CHAPTER 7: CONCLUSIONS 243

REFERENCES 248

APPENDICES 282

APPENDIX I: ACCESS IPA DATABASE 282

APPENDIX II: BUFFERS, ETC 286

APPENDIX III: METHOD FOR PROMEGA WIZARD™ PCR PREPS DNA PURIFICATION SYSTEM 290

APPENDIX IV: PUBLICATIONS ARISING FROM THIS THESIS 292

LIST OF TABLES

TABLE 1-2: SUMMARY OF *ASPERGILLUS* PCRS 90

TABLE 2-1: SUMMARY OF *ASPERGILLUS* ISOLATES FROM PATIENTS IN THE HAEMATOLOGY UNIT. 135

TABLE 2-2: BAL SPECIMENS WITH THEIR BATCH/LETTER DESIGNATION 139

TABLE 3-1: DEMOGRAPHY AND PREVIOUS TREATMENT 161

TABLE 3-2: SURGERY, HISTOLOGY, AND MICROBIOLOGY IN 59 PATIENTS WITH LISA 164

TABLE 3-3: DEMOGRAPHY AND PREVIOUS HAEMATOLOGICAL TREATMENT ACCORDING TO DIAGNOSTIC CATEGORY AND WHETHER RESECTED 166

TABLE 3-4: SURVIVAL BY DIAGNOSTIC CATEGORY 171

TABLE 3-5: IMPACT OF INDIVIDUAL FACTORS ON DEATHS FROM ALL CAUSES EXPRESSED AS UNADJUSTED RELATIVE HAZARDS (RH). 172

TABLE 3-6: ADJUSTMENT OF SEVERAL FACTORS SIMULTANEOUSLY ON DEATH FROM ALL CAUSES EXPRESSED AS ADJUSTED RELATIVE HAZARDS (RH). 173

TABLE 5-1: SUMMARY OF PCR RESULTS ON RANDOM *ASPERGILLUS* CULTURE NEGATIVE BAL'S FROM 13 PATIENTS OUTSIDE THE HAEMATOLOGY UNIT. 202

TABLE 5-2: SUMMARY OF PCR RESULTS OF PATIENTS WITH HAEMATOLOGICAL AND PULMONARY DISEASE 209

LIST OF FIGURES

FIGURE 1-1 THE MICROSCOPICAL STRUCTURE OF *ASPERGILLUS* SPECIES 28

FIGURE 1-2: MYCOTIC LUNG SEQUESTRUM ON CHEST X-RAY 64

FIGURE 1-3: MYCOTIC LUNG SEQUESTRUM ON CHEST X-RAY 64

FIGURE 1-4: MYCOTIC LUNG SEQUESTRUM ON CT SCAN 64

FIGURE 1-5: CAVITATING LESION ON CT SCAN 64

FIGURE 1-6: HALO SIGN LESION ON CT SCAN 65

FIGURE 1-7: CHEST X-RAY OF DISSEMINATED ASPERGILLOSIS 65

FIGURE 3-1: SURVIVAL ACCORDING TO METHOD OF DIAGNOSIS, AND TO WHETHER IPA WAS TREATED SURGICALLY 174

FIGURE 3-2: KAPLAN – MEIER CURVES REPRESENTING THE IMPACT OF VARIOUS INDIVIDUAL FACTORS ON DEATH FROM ALL CAUSES 175

FIGURE 4-1: PCR BY METHOD 1 OF KNOWN AMOUNTS OF *A.FUMIGATUS* DNA.... 186

FIGURE 4-2: PCR BY METHOD 1 OF KNOWN SMALL AMOUNTS OF *A.FUMIGATUS* DNA IN TRIPLICATE..... 186

FIGURE 4-3: PCR BY METHOD 2 OF KNOWN AMOUNTS OF *A.FUMIGATUS* DNA.... 188

FIGURE 4-4: PCR BY METHOD 2 OF KNOWN SMALL AMOUNTS OF *A.FUMIGATUS* DNA IN TRIPLICATE..... 188

FIGURE 4-5: PCR BY METHOD 2 OF KNOWN AMOUNTS OF *A.FLAVUS* DNA 190

FIGURE 4-6: PCR BY METHOD 2 OF KNOWN SMALL AMOUNTS OF *A.FLAVUS* DNA 4 OF EACH 190

FIGURE 4-7: RECOVERY OF KNOWN AMOUNTS OF *A.FUMIGATUS* DNA FROM BAL AFTER EXTRACTION AND PCR..... 191

FIGURE 4-8: RECOVERY OF KNOWN AMOUNTS OF *A.FUMIGATUS* DNA FROM BAL OR WATER AFTER EXTRACTION AND PCR 191

FIGURE 4-9: EXTRACTION, THEN PCR BY METHOD 2 OF KNOWN NUMBERS OF CONIDIA OF *A.FUMIGATUS* STRAIN 238 IN WATER..... 194

FIGURE 4-10: EXTRACTION, THEN PCR BY METHOD 2 OF KNOWN NUMBERS OF CONIDIA OF *A.FUMIGATUS* STRAIN 239 IN WATER..... 194

FIGURE 4-11: EXTRACTION, THEN PCR BY METHOD 2 OF KNOWN NUMBERS OF CONIDIA OF *A.FUMIGATUS* STRAIN 2109 IN WATER..... 195

FIGURE 4-12: EXTRACTION, THEN PCR BY METHOD 2 OF KNOWN NUMBERS OF CONIDIA OF *A.FLAVUS* STRAIN 30 IN WATER..... 195

FIGURE 5-1: PCR BY METHOD 2 OF *ASPERGILLUS* CULTURE NEGATIVE BAL'S FROM PATIENTS OUTSIDE THE HAEMATOLOGY UNIT..... 203

FIGURE 5-2: PCR BY METHOD 2 OF *ASPERGILLUS* CULTURE NEGATIVE BAL'S FROM PATIENTS OUTSIDE THE HAEMATOLOGY UNIT..... 203

FIGURE 5-3: DNA EXTRACTED FROM BATCH 1 OF *ASPERGILLUS* CULTURE NEGATIVE BAL'S FROM PATIENTS WITH PULMONARY DISEASE..... 207

FIGURE 5-4: PCR BY METHOD 2 OF BATCH 1 OF *ASPERGILLUS* CULTURE NEGATIVE BAL'S FROM HAEMATOLOGY PATIENTS WITH PULMONARY DISEASE 211

FIGURE 5-6: PCR BY METHOD 2 OF BATCH 4 OF *ASPERGILLUS* CULTURE NEGATIVE BAL'S FROM HAEMATOLOGY PATIENTS WITH PULMONARY DISEASE 214

FIGURE 5-7: PCR BY METHOD 2 OF TWO SAMPLES OF *ASPERGILLUS* CULTURE NEGATIVE LUNG RESECTED FROM HAEMATOLOGY PATIENTS WITH HISTOLOGICAL IPA..... 218

FIGURE 6-2: COMPARATIVE QUALITY OF DNA EXTRACTED IN MANCHESTER AND AT RFH. 226

FIGURE 6-3: COMPARATIVE QUALITY OF DNA EXTRACTED IN MANCHESTER AND AT RFH. 226

FIGURE 6-4: *SAL I* RESTRICTION DIGESTS OF DNA FROM *A. FUMIGATUS* STRAINS 21 AND 22. 228

FIGURE 6-5: *XHOL* DIGEST OF MANCHESTER DNA..... 230

FIGURE 6-8: SSCP OF PCR PRODUCTS BY METHOD 1 OF VARIOUS *A.FUMIGATUS* STRAINS..... 235

FIGURE 6-9: SSCP OF PCR PRODUCTS BY METHOD 2 OF VARIOUS *A.FUMIGATUS* AND *A.FLAVUS* STRAINS 236

FIGURE 6-10: SSCP OF PCR PRODUCTS BY METHOD 2 OF 15 *A.FUMIGATUS* STRAINS 238

FIGURE 6-11: SSCP OF *DDE*I RESTRICTIONS OF PCR PRODUCTS BY METHOD 2 FROM VARIOUS *A.FUMIGATUS* AND *A.FLAVUS* STRAINS 239

FIGURE 6-12: SSCP OF *DDE*I RESTRICTIONS OF PCR PRODUCTS BY METHOD 2 OF 17 *A.FUMIGATUS* STRAINS 240

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DEDICATION

To my husband, John Clapham, whose tireless patience and support made this work possible.

ABBREVIATIONS

ABPA	Allergic Broncho Pulmonary Aspergillosis
Asp.	*Aspergillus*
AIDS	Acquired Immunodeficiency Syndrome
ALL	Acute Lymphoblastic Leukaemia
ALLO	Allogeneic bone marrow transplant
AML	Acute Myeloid Leukaemia
ANCA	Anti Neutrophil Cytoplasmic Antibody
AUTO	Autologous bone marrow transplant
APS	Ammonium Persulphate
A+W	Alive and Well
BAL	Bronchoalveolar Lavage
BALISA	Biotin Avidin Linked Immunosorbant Assay
BMT	Bone Marrow Transplant
CFU	Colony Forming Unit
CGL-ACC	Chronic Granulocytic Leukaemia in Accelerated Phase
CGL-CP	Chronic Granulocytic Leukaemia in Chronic Phase
CGL-Blast	Chronic Granulocytic Leukaemia in Blast crisis
CI	Confidence Interval
CLL	Chronic Lymphocytic Leukaemia
CMV	Cytomegalovirus
CT	Computerised Tomography
df	Degrees of freedom
DNA	Deoxyribonucleic Acid
EBGA	European Research Group of the Biotype and Genotype of *Aspergillus fumigatus*
EIA	Enzyme Immuno-Assay

EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immunosorbant Assay
EMBL	European Molecular Biology Laboratory
EORTC	European Organisation for Research and Treatment of Cancer
FSO	Filter Sterilise Only
GCG	Genetics Computer Incorporated
G-CSF	Granulocyte Growth Stimulating Factor
GM-CSF	Granulocyte/Monocyte Growth Stimulating Factor
GYEP	Glucose, Yeast, Peptone
HIV	Human Immunodeficiency Virus
HCL	Hairy Cell Leukaemia
HSV	Herpes Simplex Virus
ICBN	International Code of Botanical Nomenclature
IPA	Invasive Pulmonary Aspergillosis
LISA	Lesion with Imaging Suggestive of Aspergillosis
LFU	Lost to Follow-Up
LRS	Likelihood Ratio Statistic
MAI	Mycobacterium Avium Intracellulare
MDE	Mutation Detection Enhancement
MDS	Myelodysplastic Syndrome
MLS	Mycotic Lung Sequestrum
MRI	Magnetic Resonance Imaging
MSG	Mycoses Study Group
MRSA	Methicillin Resistant *Staphylococcus Aureus*
MUD	Matched Unrelated Donor Transplant
N/A	Not Applicable
NCPF	National Collection of Pathogenic Fungi
NHL	Non-Hodgkins Lymphoma

NHS	National Health Service
OD	Optical Density
PAS	Periodic Acid Shiff
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCP	*Pneumocystis carinii* Pneumonia
PM	Post Mortem
RAPD	Random Amplification of Polymorphic DNA
rDNA	ribosomal DNA
RIA	Radio Immuno Assay
RIP	Rest In Peace (patient died).
RFH	Royal Free Hospital
RFLP	Restriction Fragment Length Polymorphism
RH	Relative Hazard
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
SAA	Severe Aplastic Anaemia
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Sp.	Species or sputum
SPSS	Statistical Package for the Social Sciences
SSCP	Single Stranded Conformational Polymorphism
TBE	Tris Borate EDTA
TE	Tris EDTA
TMED	N, N, N', N'-TetraMethylEthyleneDiamine
TPE	Tris Phosphate EDTA
UV	Ultra Violet
VUD	Volunteer Unrelated Donor

CHAPTER 1: INTRODUCTION.

1.1. BRIEF HISTORICAL INTRODUCTION

Aspergillus was first described in the XVIIIth century (Micheli 1729) and so named because of the microscopic appearances of its spore bearing structure. The first infection in man, recognised to be due to a fungus of this genus, was described in 1842 in Scotland (Bennett 1842). Virchow reviewed the early literature (Virchow 1856) and presented four autopsy cases of aspergillosis in which secondary invasion of pre-existing lung lesions had occurred. The first case of invasive pulmonary aspergillosis as an opportunistic infection was described in Britain (Rankin 1953).

Since then, a large body of literature has been published; describing a wide variety of pathology (particularly pulmonary) caused by the organism in humans and animals. Such diseases still present a diagnostic and therapeutic challenge in spite of advances in imaging and laboratory techniques and the advent of new antifungal agents.

1.2. OVERVIEW OF CLASSIFICATION WITHIN MEDICALLY IMPORTANT FUNGI

Fungi were initially classified as plants, but today occupy their own kingdom (*Mycetae* or *Fungi*). All fungi share the common characteristics of being eukaryotic and chemoheterotrophic, and of having chitin or cellulose or both in their cell walls. Much of the identification of fungi is based on their reproductive structures. These are called spores and may be a result of either asexual

(involving mitosis only) or sexual (involving meiosis and fusion of the nuclei of two cells) reproduction. If only asexual structures are present, then the fungus is called an anamorph, if sexual structures are present, then the fungus is called a teleomorph (Warren 1996).

Although some fungi have lost the ability to reproduce sexually under laboratory conditions, most mycologists believe that every fungus has a sexual phase (or teleomorph) that may manifest itself if the correct environmental conditions and nutritional requirements are provided. Confusion in nomenclature arises partly because much of the traditional (and broadly used) classification of pathogenic fungi is based on whether or not a sexual phase is recognised for a particular fungus. As a result, the anamorph and teleomorph forms of the same fungus have tended to find themselves allocated to different classes altogether until the demonstration, in a fungus classified as a deuteromycete, of a sexual form identical to an already named ascomycete. Examples include *Arthroderma benhamiae* which now refers to the sexual form of *Trichophyton mentagrophytes* and *Pseudallescheria boydii* whose anamorph is *Scedosporium apiospermum* (Koneman *et al* 1992). Because correct taxonomy according to the International Code of Botanical Nomenclature (ICBN) stipulates that precedence should be given to teleomorph names when known, this has required re-naming and re-classifying many fungi including some that are still commonly known as *Aspergillus* (Pitt 1994).

1.3. TAXONOMY OF ASPERGILLUS

Traditionally, the genus *Aspergillus* belongs to the class Deuteromycetes (from the Latin *deutero*: second). The Deuteromycetes are also known as "fungi imperfecti", that is, fungi in which only an asexual phase of reproduction (anamorph) has been observed (Koneman *et al*, Samson 1992). *Aspergillus* belongs to the subclass Hyphomycetes (from Greek *hyphe*: "web") and the order *Moniliales* (from the Latin *monile*: "necklace") in which asexual spores (conidia) are borne directly off hyphae and do not aggregate within fruiting bodies. This order includes most of the important hyaline and dematiaceous moulds.

Several species traditionally included in the genus *Aspergillus* have recognised teleomorphs (Samson 1992), however, and this would require them to be re-named and to be classified as Ascomycetes (order Eurotiales, a term derived from the Greek *euros,* meaning "mould"). Ascomycetes, until recently, comprised only the teleomorphs of *Aspergillus* species and are characterised by large, closed, bag like structures called cleistothecia, which contain smaller bag like structures named asci, each of which contains four ascospores, the products of meiotic division. Examples of species traditionally classified as *Aspergillus*, and now renamed include *Eurotium* in the section typified by *A. glaucus*, *Neosartorya* in the section typified by *A. fumigatus*, although not for *A. fumigatus* itself, and *Emericella* in the section typified by *A. nidulans* (Samson 1992).

The taxonomy within the *Aspergillus* genus has also been confused by other terminology issues, but is still based on the monograph by

Raper and Fennell (Raper and Fennell 1965), who described 132 species subdivided into 18 "groups". The term "group", however, has no standing in the botanical code. So in 1985, in the first *Penicillium* and *Aspergillus* Workshop, Gams *et al.* replaced the "groups" by sections, in turn comprised within subgenera (Samson 1990). They reviewed their classification in 1990, and many new species have been added, of which 43 are recognised by Samson (Samson 1990).

Further taxonomic difficulty arises from the fact that classifications based on Raper and Fennell, although very broadly used, do not ascribe priority (referring to a species by the very first name used to describe it), as required by the ICBN. However implementing the ICBN recommendations would mean abandoning many well-known names such as *A. niger* and *A. ochraceus.* A solution is currently being sought by producing lists of "names in current use", and seeking to sanction those at a botanical congress (Pitt 1994). This concept is yet to be implemented (Samson 1999) but the process has been taken one step further by providing a list of synonyms for these species (Samson and Pitt 1999). Although the list will have no formal status in *Aspergillus* nomenclature, it should be useful for practical purposes.

Furthermore, type material of some insufficiently described *Aspergillus* taxa is lacking, and work is in progress to remove such organisms from current classification (Samson and Gams 1984).

The role of molecular studies in the classification of fungi is not well established, and, in the current state of knowledge, re-classification

based on such studies may be detrimental to the food industry, as exemplified by the very close molecular relationship between *A. flavus* and *A.oryzae*, of which only *A.oryzae* is useful and safe in fermentation cultures (Samson 1991).

In spite of the intricacy of *Aspergillus* taxonomy, matters remain relatively simple for clinical purposes, where the majority of infections are caused by *A.fumigatus* (85% of cases) and *A. flavus* (5% to 10% of cases), while *A.niger* (2% to 3% of cases) and *A.terreus* (2% to 3% of cases) are involved less frequently, and other species only very occasionally (Denning 1996).

1.4. MORPHOLOGICAL AND CULTURE CHARACTERISTICS OF *ASPERGILLUS*

1.4.1. Macroscopic and culture characteristics

Aspergillus species can be suspected in culture if a rapidly (within 3 to 5 days) growing granular colony, usually yellow, yellow-green, yellow-brown, or green, with a distinct margin and a white peripheral apron is observed on an agar plate containing a fungal culture medium. The appearance of the colonial morphology can vary considerably; depending on the culture medium being used (Koneman *et al* 1992). The classical morphology, as described by Raper and Fennel (Raper and Fennel 1965, Samson 1992) can be observed reliably only on Czapek's agar. Certain environmental species may appear as white, cottony colonies. *A. niger*, as the name indicates, produces a black, peppered surface when the colony reaches maturity.

1.4.2. Microscopic characteristics

Microscopically, *Aspergillus* species are characterised by the aspergillum, arising from a foot cell, and consisting of a conidiophore terminating in a vesicular tip, which in turn bears one or two layers of specialised cells (phialides and metulae), from which a string of conidia is formed. An aspergillum with phialides borne directly on the vesicle is called a uniseriate aspergillum (a typical example is *A. fumigatus*). If a second layer of specialised cells is present on the vesicle, the aspergillum is referred to as biseriate (examples are *A. nidulans* and *A. terreus*). The cells of the

second layer, located between the phialides and the vesicle, are called metulae (Samson 1992, Timberlake 1990). Figure 1-1 shows these features in diagram form.

The length and width of the conidiophores, the size and contour of the conidiophore tip, the arrangement of the phialides, and the colour, size, and length of chains of the conidia are the main features used in making species identification (Koneman *et al*, Samson 1992) within the *Aspergillus* genus, particularly when Czapek agar is used. Other criteria, such growth on specialised media, ability to obtain a teleomorph, metabolite profiles, enzyme profiles, serology, and molecular biology are also used to differentiate between *Aspergillus* species (Samson 1992). In most instances however, morphological criteria were simply supported by the newer techniques. In addition, enzyme profiles and molecular biology studies have been used for epidemiological purposes to differentiate further between *Aspergilli* of a given species, and will be discussed in more detail later (see sections 1.11.3 and 1.11.4.

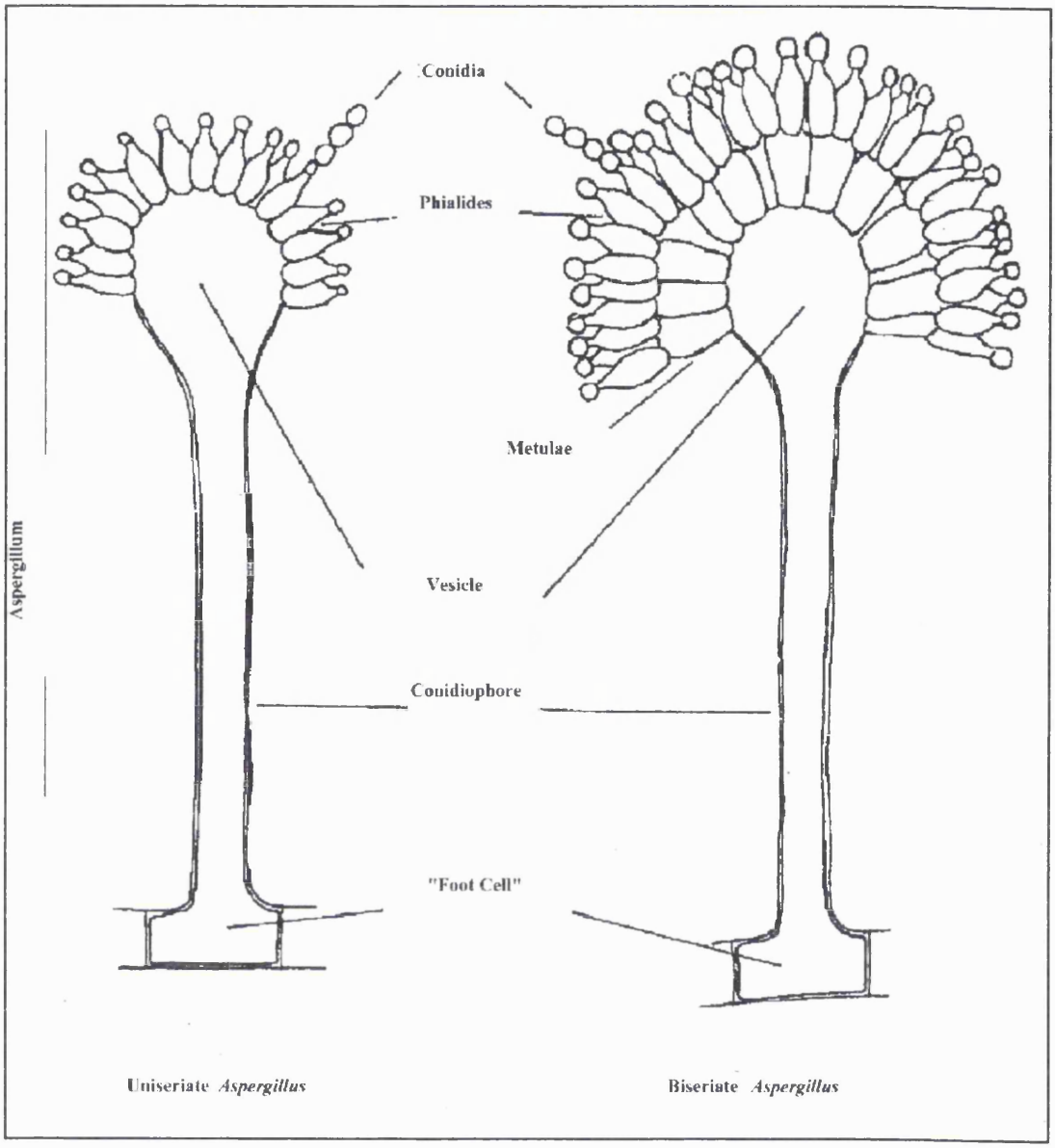


Figure 1-1 The Microscopical Structure of *Aspergillus* Species

1.4.3. Growth characteristics

Like in most fungi, the vegetative cells of *Aspergillus* are haploid, multinucleate hyphae. These hyphae are uniform, 4-6 μm wide, hyaline and septate with parallel walls. They grow by apical extension and multiply by branching (Timberlake 1990). Vegetative growth typically occurs when conidia are inoculated in liquid growth

medium with agitation, and follows germination of the conidia (Saxena and Sinha 1973).

Under conditions of nitrogen and carbon source sufficiency, *Aspergilli* grow vegetatively (Martinelli 1976). Conidiophores and conidia (the hallmarks of asexual reproduction) are not usually formed unless the hyphae are exposed directly to an air interface (Law and Timberlake 1980, Champe *et al* 1981, Yager *et al* 1982). This may explain the typical histological appearances of invasive aspergillosis in diseased tissue sections, where the characteristic aspergillum appearances are very seldom seen. Instead, vegetative growth alone occurs, and septate hyphae with dichotomous branching at 45^{o} are the norm. This feature can usually be seen in haematoxilin and eosin preparations, although periodic acid Schiff or silver stains demonstrate hyphae best (Koneman *et al* 1992).

1.4.4. Histology

The histological appearance of septate hyphae with dichotomous branching at 45° are very suggestive of *Aspergillus* invasion, but cannot be considered diagnostic: other fungi such as *Pseudallescheria boydii* are very similar to *Aspergillus* in tissue section; on the other hand degenerate *Aspergillus* hyphae may swell, become quite irregular in shape, and resemble fungi of the order *Mucorales*, or be mistaken for the pseudohyphae of *Candida* (Fraser 1993). In the absence of culture or immunological investigation, definitive histological identification or exclusion of *Aspergillus* as the cause of the disease must therefore be made with care.

One histological feature that can help with the diagnosis of aspergillosis is the presence of conidiophores, which, although seen only rarely, are pathognomonic. They tend to be seen when the site of infection is exposed to air, for example in tracheobronchitis (Fraser 1993). Another such feature is the presence of irregularly shaped, angulated, strongly refractile calcium oxalate crystals. These are associated with certain *Aspergillus* species, particularly *A. niger*, and are believed to be the result of oxalic acid produced by the organism and calcium derived by the host (Nime 1973, Kurrein 1975).

1.5. LIFE CYCLE OF *ASPERGILLUS*

Aspergillus species, by definition, do not undergo sexual reproduction (see section 1.3). The account below of the conidiation

of *Aspergillus* is based mainly on a study using electron microscopy of freeze substituted *A. nidulans* (Mims *et al* 1988), which describes five stages in this process.

The asexual reproduction cycle begins when a conidiophore stalk initial arises from the foot cell, a specialised hyphal compartment. This anchors the mature conidiophore in the growth substratum and provides cytoplasmic connection with the rest of the thallus or mycelium, defined as the entertwined mass of hyphae forming the mat-like fungal growth. During the first stage of development, the stalk grows as a thick walled aerial hypha that elongates by apical growth. Elongation continues until the stalk reaches a height of about 100μm.

According to studies of *Aspergillus nidulans* and various other filamentous fungi (Oliver 1972, Gooday 1983), apical vesicles, which are thought to contain cell wall precursors and cell wall polymerising enzymes, are abundant near the tip of the growing conidiophore stalk where they fuse with the plasma membrane. When growth stops, signalling entrance into the second stage of development, vesicles decrease rapidly in numbers and become dispersed into the periphery of the conidiophore tip. This subsequently swells to about 10μm. The height of the coniophore from the base of the stalk to the tip of the vesicle is 98.9 +/- 4.0μm. This value is species specific and varies little in response to different growth conditions, implying that it is genetically determined (Clutterbuck 1969).

In the third stage of conidiophore development, numerous buds are produced synchronously on the surface of its tip, giving rise to metulae. Repeated mitotic divisions occur in the conidiophore tip, and a single nucleus enters each metula, although the exact timing of this with respect to metula maturation is uncertain. Other organelles also enter the differentiating metulae before these cells become delimited by formation of septa produced by centripetal deposition of cell wall material near their bases. These septa, like those in hyphae, possess central pores providing cytoplasmic continuity between the cells. The pores are guarded by Woronin bodies on both sides, which have been proposed to serve as emergency safety plugs that can seal off cells following breakage to prevent protoplasmic loss (Reichle and Alexander 1965, Collinge and Markham 1989).

The distal tips of metulae then produce buds that develop into uninucleate, sporogenous phialides, which become delimited by incomplete septa providing cytoplasmic connection to the metulae. This constitutes the fourth stage of conidiophore development.

Conidia are formed during the fifth stage of conidiophore development by repeated division of the phialide nucleus. A conidium initial develops as a cytoplasmic protrusion from the tip of a phialide. The phialide nucleus enters the neck region and divides mitotically. One daughter nucleus enters the differentiating conidium, whereas the other nucleus moves back down the neck of the phialide. Once the conidium initial is fully expanded, a septum develops in the phialide neck. This septum initially possesses a central pore that closes as the septum thickens. As the conidium becomes delineated, the phialide nucleus again undergoes division to

produce another spore, and the process continues to form long clonal chains of spores.

Conidia undergo maturation, which is defined as differentiation occurring after septum formation and closure has delimited the conidium from the phialide. The maturation process consists mainly of cell wall condensation, and formation of two new cell wall layers, making four in total. These final stages of conidial maturation result in impermeable spores, which are sealed off, from the environment. This is thought to be required for the establishment of spore dormancy (Sewall *et al* 1990).

Subsequent germination of the conidia initiates further vegetative growth and/or conidiation cycles.

1.6. ENVIRONMENTAL DISTRIBUTION OF *ASPERGILLUS* SPECIES

Aspergillus species are ubiquitous in nature, being commonly found in soil (Summerbell *et al* 1989), water and organic debris. They are said to be more common in the tropics (Pitt 1994). They are found in virtually any environment that contains decomposing organic material (Elstead 1991). Pepper (De Bock *et al* 1989), spices and marijuana cigarettes (Birch *et al* 1995) are well recognised sources. The atmosphere of hospital wards has also been found to contain conidia on many occasions, particularly in connection with renovation works (Arnow *et al* 1978, Sarrubi *et al* 1981, Opal *et al* 1986) or bird excreta near ventilation systems (Nolard 1996).

1.7. OVERVIEW OF DISEASES CAUSED BY *ASPERGILLI*

Not surprisingly, diseases caused by *Aspergillus* species are of worldwide distribution, and affect both humans and animals. Particular species of *Aspergillus* appear to have a predilection for certain animals or sites, for example *A. terreus* causes fatal systemic illnesses in German Shepherd dogs, while *A. niger* causes mainly aural infections (Pitt 1994). The organism is easily spread by aerosols of conidia (the number of which varies with geographical location and season), and although it can cause disease in most organs, the lungs are by far the most commonly affected. Extrathoracic dissemination occurs in 25 to 50% of cases (Gefter 1992). Within the lungs, the tissue reaction to the presence of fungus and the associated clinical and radiological manifestations are more

varied than those associated with any other infectious agent, with the possible exception of *M. tuberculosis* (Fraser 1993).

As with other pulmonary infections, the risk of developing disease after contact with *Aspergillus* depends on the interplay between virulence and inoculating dose of the organism, type of exposure, and the ability of the host to resist infection, as influenced by the competence of local and systemic host defences.

1.8. HOST/PARASITE RELATIONSHIPS

1.8.1. Mechanisms of resistance against host defences by *Aspergillus*

Autopsy studies have shown that *A. fumigatus* spores are present and viable in human lungs more frequently than would be expected on the basis of their prevalence among fungal spores in the air (Mullins and Seaton 1978). This suggests that *A. fumigatus* has properties that protect it from normal lung defences.

Factors that enhance the pathogenicity of *Aspergilli* include their ability to withstand a broad range of temperatures, and their small, aerodynamic size that allows them to penetrate easily into the alveolar spaces (Elstead 1991). The conidial diameter itself also may be a factor influencing the risk of disease. For example, conidia of *A. fumigatus* measure 2 to 3μm across, a size ideal for inhalation and deposition in the distal respiratory tract. By contrast, conidia of the relatively less common pathogens *A.niger* and *A. flavus* are 4 to 5 μ m in diameter, a size more likely to be associated with proximal

airway deposition, and, possibly, more rapid mucociliary clearance. This, however, cannot be the sole explanation for variation in species pathogenicity, as some species that are rare causes of human disease have a conidial diameter similar to that of *A. fumigatus* (Fraser 1993).

Proteolytic destruction of lung tissue by enzymes such as elastase play a role in the pathogenesis of IPA, as demonstrated by the fact that elastase production by *A. fumigatus* correlates with its ability to cause lung disease and death in mice (Kothary *et al* 1984). *A. fumigatus* also produces a substance that interferes with the alternative complement pathway and decreases the binding of C3b (an opsonin) to the fungal surface. Furthermore, conidia do not cause the generation of the chemoattractant C3a unless they have germinated. Thus both the opsonic and the chemoattractant functions of complement are inefficient in response to *A. fumigatus* conidia (Washburn *et al* 1986). In addition, gliotoxin, a metabolite of *A. fumigatus*, inhibits the phagocytic function of macrophages, and also inhibits the induction of alloreactive cytotoxic T cells in vitro (Mullbacher *et al* 1984).

Reactive oxygen species are important mediators of *Aspergillus* killing by phagocytes (Washburn *et al* 1987) but there is evidence that *Aspergillus* conidia are relatively resistant to cell free killing by oxidants (Levitz and Diamond 1985). They are also inhibitory to oxygen radical production in neutrophils exposed to phorbol myristate acetate, and relatively poor stimulants of superoxide anion and hydrogen peroxide production by mouse peritoneal exudate cells (Robertson *et al* 1987). The significance of these potential

pathogenic mechanisms to pulmonary disease in humans is poorly understood.

1.8.2. Host defence against conidia

In mice, conidia have been shown to be phagocytosed by alveolar macrophages within 15 minutes of exposure to aerosolised *A. flavus* and such phagocytosis prevented subsequent germination and invasion of lung tissue, even in neutropenic or athymic mice (Merkow *et al* 1971, Schaffner *et al* 1982). The binding of *A. fumigatus* conidia to murine macrophages in vitro is not enhanced by opsonisation (Kurup 1984), but is inhibited by soluble polysaccharides that block the mannose receptor on the surface of macrophages, suggesting that attachment is mediated by the interaction of a carbohydrate component on the fungal wall with specific receptors on the macrophage (Kan and Bennett 1988).

Following phagocytosis, macrophages kill microorganisms largely by oxidative killing. The addition of catalase (which degrades hydrogen peroxide) has been shown to reduce fungal killing by normal macrophages in vitro (Washburn *et al* 1987).

In summary, alveolar macrophages and are the first line of defence against *Aspergillus*. Failure of macrophages to phagocytose or destroy inhaled spores results in germination and extracellular growth of hyphae.

1.8.3. Host defence against hyphae

Clinical observation demonstrates that neutropenia is a major risk factor for the development of aspergillosis, and it appears that the neutrophil is involved in defence against *Aspergillus* hyphae. Diamond *et al*, using both light and electron microscopy (Diamond *et al* 1978) demonstrated that neutrophils attached to and spread over the surfaces of hyphae in vitro, and that shortly thereafter, morphological changes in the fungus suggested severe damage and death. Metabolic inhibitor studies suggested that oxidative mechanisms were responsible for neutrophil mediated killing of the extracellular hyphae. Monocytes may also be involved in killing *Aspergillus* hyphae (Diamond *et al* 1983). The importance of oxidative killing by phagocytes in the defence against *Aspergillus* is evidenced further by the susceptibility of chronic granulomatous disease patients (who fail to produce reactive oxygen species in response to phagocytosis) to *Aspergillus* infections (Johnson and Baehner 1971, Cohen *et al* 1981, Ezekowitz *et al* 1988).

1.8.4. Other host defence mechanisms including humoral

Aspergillus pneumonia is not common in patients with the acquired immunodeficiency syndrome (Broaddus *et al* 1985, Rankin and Daniele 1988, Murray and Mills 1990), implying that lymphocyte directed cell mediated immunity is unlikely to be of major importance in defence against *Aspergillus*. Similarly, immunosupressed patients with invasive aspergillosis tend not to have a detectable antibody response (Young and Bennett 1971, see

also section 1.10.9.1.), although there does not appear to be a high rate of *Aspergillus* infection in patients with hypogammaglobulinaemia.

The major defence mechanisms of the central airways include mucociliary clearance, cough, bronchoconstriction, and local secretion of inflammatory mediators by airway cells such as mast cells, eosinophils, neutrophils, lymphocytes and intraluminal macrophages.

Diseases that are primary disorders of the conducting airways (namely asthma and cystic fibrosis) predispose to allergic bronchopulmonary aspergillosis, the development of which requires colonisation by *Aspergillus*. It is frequently stated that such colonisation is promoted in airway disorders by alteration in mucociliary clearance and/ or characteristics of the mucus. A hyperactive immune response is likely to be involved in the subsequent reaction to *Aspergillus* colonisation that results in allergic bronchopulmonary aspergillosis (see section 1.9.3.4).

Excessive immunity also results in *Aspergillus* induced asthma and hypersensitivity pneumonitis (see sections 1.9.3.1 and 1.9.3.4).

1.9. PULMONARY ASPERGILLOSIS

1.9.1. Classification of pulmonary aspergillosis

Traditionally, pulmonary aspergillosis is considered to comprise three general forms of disease (Fraser 1993) :

1. Saprophytic, in which there is fungal growth without invasion of viable tissue. Airway colonisation, aspergilloma (fungal ball) and invasion of necrotic tissue are all subtypes of saprophytic aspergillosis.

2. Allergic, characterised by the presence of hypersensitivity reaction to fungal hyphae or conidia. Certain types of asthma, hypersensitivity pneumonitis, eosinophilic pneumonia (Loeffler's syndrome), and allergic bronchopulmonary aspergillosis(ABPA) are all forms of allergic aspergillosis.

3. Invasive, in which there is extension of the fungus into viable pulmonary tissue. *Aspergillus* tracheobronchitis, bronchopneumonia, pleuritis and empyema are all forms of invasive pulmonary aspergillosis (IPA), as are angioinvasive aspergillosis (including mycotic lung sequestra (Kibbler *et al* 1988)), miliary aspergillosis and chronic necrotising aspergillosis.

These three varieties are not mutually exclusive, and may in fact constitute a continuous spectrum of disorders that is dependent on the host's immune status, underlying lung morphology, and to a certain extent the type and degree of fungal spore exposure (Gefter 1992). Occasional cases of saprophytic or allergic disease progress to invasive disease, and allergic bronchopulmonary aspergillosis sometimes is associated with an aspergilloma. As a rule, however, each form tends to occur alone and is associated with significantly different pathogenesis, pathology and clinical features.

Each of the pathological entities will now be discussed in turn below (adapted from Fraser (Fraser 1993) unless stated otherwise)

1.9.2. Saprophytic aspergillosis

1.9.2.1. Airway colonisation

This usually occurs in the presence of chronic airway disease such as asthma, bronchiectasis and chronic bronchitis. In these conditions, the organism can be cultured in the sputum or bronchial washings of 2 to 3% of patients, while it is found in the sputum of 10 to 57% of individuals with cystic fibrosis. Some of these patients will have allergic bronchopulmonary aspergillosis, but the majority have no disease state attributable to the fungus.

1.9.2.2. Aspergilloma (also known as fungus ball or mycetoma)

Although this disease can be caused by a variety of fungi, *Aspergillus fumigatus*, and more rarely *A.nidulans* are by far the commonest, and all three terms are used synonymously. An aspergilloma consists of a grossly identifiable cluster of intertwined hyphae matted together with a variable amount of mucus and cellular debris. Microscopy reveals that many of the hyphae show degenerative changes or frank necrosis, particularly in the central portion. Polymorphonuclear leucocytes are frequently clustered at the periphery of the fungal mass, but the remainder is usually acellular. The surrounding cavity wall is composed of a layer of fibrous tissue in which there is a variable number of mononuclear inflammatory cells and small blood vessels. These are derived from

the bronchial circulation, and when numerous, lead to haemoptysis. In ectatic airways and in some pulmonary parenchymal cavities, there is frequently an epithelial lining (respiratory or squamous in type), with foci of damage, separating the aspergilloma from the fibrous tissue.

An aspergilloma is most often identified radiographically as a roughly spherical nodule or mass separated by a crescent-shaped lucency from an adjacent cavity wall.

Patients with aspergilloma usually have precipitins to *Aspergillus* in their serum (Glimp and Bayer 1983). This is of clinical importance as there are many causes of intracavitary mass other than aspergilloma.

The most common condition predisposing to aspergilloma formation is chronic cavitary tuberculosis, and a quarter of all patients with aspergilloma has a history of this disease. Conversely, 17% of patients with healed tuberculous cavities had developed aspergillomas at 3 years in one study (Research Committee of British Thoracic and Tuberculosis Association, 1970). The second most common condition associated with aspergilloma is sarcoidosis, in which 10% of patients develop this complication.

Many other abnormalities can predispose to aspergilloma formation, including bronchiectasis of any aetiology, chronic abscess, cavities related to chronic fungal infection such as coccidioidomycosis, bullae associated with emphysema, congenital abnormalities such as

bronchogenic cysts and intralobar sequestration, and bronchial stumps remaining after pneumonectomy.

The natural history of aspergilloma is variable. Haemoptysis will occur in 50 to 70% of cases, and cause death in 5%. When arising from cavity wall vessels derived from the bronchial circulation, it is amenable to bronchial artery embolisation. In other cases, haemorrhage occurs from a ruptured pulmonary artery branch situated in close proximity to the cavity wall, when bronchial artery embolisation will be of no benefit.

Complete resolution without intervention is said to occur in 7 to 10% of cases.

Patients with aspergilloma have a high mortality of 31% at 3 years and 56% at 10 years, with only a third of deaths from respiratory causes directly attributable to the aspergilloma or its treatment, and the rest from pneumonia or respiratory failure reflecting the poor respiratory function in patients predisposed to aspergillomas (Jewkes *et al* 1983). In this retrospective study, patients treated with surgical resection of the aspergilloma did better than those managed medically, although the better survival of the surgical group may have been due to the selection of patients with better lung function and more localised pulmonary disease.

1.9.2.3. Invasion of necrotic tissue

This is the least common type of saprophytic aspergillosis, and consists of invasion and colonisation of necrotic tissue, usually infarcted lung or necrotic tumour. The distinction between this and true invasive aspergillosis can be difficult, and the clinical context is the key to differentiation.

1.9.3. Allergic aspergillosis

1.9.3.1. Hypersensitivity pneumonitis (extrinsic allergic alveolitis)

This is a diffuse granulomatous inflammation of the terminal airways and alveolar walls, resulting from repeated inhalation of organic dusts (e.g. the conidia and mycelia of *Aspergillus* species) or low molecular weight chemicals (Elstead 1991). At least three distinct forms of occupational hypersensitivity pneumonitis are caused by *Aspergillus* species: malt worker's lung by *A. clavatus* in whisky maltings, farmer's lung by *A. fumigatus* (as well as by thermophilic actinomycetes) in vegetable compost, and dog house disease by *A. versicolor* in the straw of dog bedding (Hendrick 1996).

Following a sensitizing period of exposure which may vary from weeks to years, the affected subject experiences repeated episodes of malaise, fever, chills, with widespread aches and pains accompanied by cough and undue breathlessness 3 to 9 hours after exposure. The severity and duration of symptoms depends on exposure dose, and

the main physical signs are respiratory distress at rest with fever and gravity dependent crackles. Spontaneous recovery occurs within 12 to 24 hours, and can be accelerated with corticosteroids. Repeated low dose exposure can result in permanent fibrotic lung damage with pulmonary hypertension and right heart failure (Hendrick 1996).

Until recently (Elstead 1991), extrinsic allergic alveolitis was considered to be a type III immune response. This has been questioned on the basis that IgG or IgM antibodies to the causative agent are not uniformly found in the serum of affected subjects, and that they are frequently found in the serum of similarly exposed but unaffected individuals.

Instead, the non-caseating granulomatous appearance of biopsied lung, and the consistent finding of an acute T-lymphocyte response in the bronchial alveolar lavage (BAL) fluid of patients with the disease support the current consensus opinion that cell mediated hypersensitivity plays a dominant role in extrinsic allergic alveolitis. The flu-like symptoms associated with the disease are thought to be due to cytokine release (Hendrick 1996).

1.9.3.2. Loeffler's syndrome

Aspergillus is listed as a cause of Loeffler's syndrome, defined as eosinophilic pneumonia with transient pulmonary opacities, blood eosinophilia, fever and cough (Lane 1996a), in some publications (Fraser 1993), and may indeed be implicated in some cases. Loeffler syndrome-like manifestations are cited in other publications (Elstead

1991, Lane 1996b) as part of the disease spectrum of allergic bronchopulmonary aspergillosis (see section 1.9.3.4). The treatment of Loeffler's syndrome is removal of the precipitant, and corticosteroids.

1.9.3.3. Extrinsic asthma

This type I allergic reaction is associated with high IgE levels and consists in reversible airway narrowing with wheezing in response to challenge with the relevant causative agent. *Aspergillus fumigatus* is a well recognised cause of seasonal (usually autumnal) asthma. The initial symptoms of straightforward asthma progress to allergic bronchopulmonary aspergillosis in a proportion of cases (also see section 1.9.3.4)(Lane 1996b).

Up to a third of patients with extrinsic asthma have positive skin tests and precipitins to *A. fumigatus*, although only a minority will develop bronchoconstriction in response to the organism (Elstead 1991).

1.9.3.4. Allergic bronchopulmonary aspergillosis (ABPA)

This is the commonest of the allergic manifestations attributable to Aspergillus and usually occurs on a background of asthma.

ABPA is characterised by airway colonisation by *Aspergillus* with asthma, bronchial impaction by mucus plugs containing the

organism, peripheral blood eosinophilia, fleeting pulmonary infiltrates with bronchial wall damage leading to proximal bronchiectasis (in itself confirmatory of the diagnosis in the absence of cystic fibrosis) and cutaneous/serological evidence of sensitisation to *Aspergillus* organisms (Fraser 1993, Lane 1996b).

Zhaoming and Lockey (Zhaoming and Lockey 1996) recently summarised the ABPA diagnostic criteria as defined by Greenberger (Greenberger 1994), and introduced the useful acronym "ARTEPICS": A: Asthma; R: Roentgenographic infiltrates; T: Tests for *A. fumigatus* positive on skin; E: Eosinophilia; P: Precipitating antibody to *A. fumigatus*; I: IgE in serum elevated; C: central bronchiectasis and S: Serum specific IgG and IgE anti-*A. fumigatus* antibodies elevated. The latter criterion is difficult to apply, as the assays required are not broadly available. It becomes essential in establishing the presence of ABPA in cases of cystic fibrosis, or when proximal bronchiectasis is absent. Whenever investigated, the IgG and IgE antibody responses are characteristic of ABPA and differentiate it from other forms of allergic aspergillosis (Trompelt *et al* 1994).

Patients with ABPA may have recurrent acute episodes of cough (sometimes productive of brown pellets or bronchial casts), dyspnoea (usually non wheezing except in the early stages), haemoptysis and fever. They may also be asymptomatic. Many have allergic manifestations in addition to asthma, including rhinitis, eczema, and food and drug hypersensitivity.

Chest X-rays characteristically show parallel and often branched opacities representing mucous plugging of proximal bronchi. These may be transient (if the patient coughs up the plugs) or persistent (in which case irreversible bronchiectasis often is present). There has also been an interest in the use of CT scanning for the early detection of bronchiectasis in asthmatic patients suspected of having ABPA (Neeld *et al* 1990, Angus *et al* 1994). Both studies demonstrates that bronchiectasis is considerably more common in patients with clinical and serological evidence of ABPA than in those with asthma and positive skin test for *A. fumigatus* without other evidence of ABPA (43/102 versus 3/66 and 41% versus 15% respectively). The presence of bronchiectasis in some of the asthmatic patients would point either to earlier detection of ABPA by CT scanning than by other methods or to previously unrecognised irreversible lung damage in asthmatic patients. Both studies were considered to support the diagnostic use of CT scanning in asthmatic patients suspected of having ABPA.

Airway colonisation by *Aspergillus* is necessary for the development of ABPA, and Robertson et al observed that macrophage killing of *Aspergillus* spores was inhibited by serum (Robertson *et al* 1987) thus providing an explanation for the propensity of this fungus to colonise the airways of patients with protein rich bronchial exudates. ABPA is indeed a frequent complication of asthma (1-2% of asthmatic patients are affected), but will also occur in 10% of patients with cystic fibrosis. Once established, *Aspergillus* colonisation may alter the quality of the mucus, and result in the characteristic plugs.

The mucus plugs of ABPA have a typical laminated appearance caused by alternating bands of eosinophils and mucus. Fungal hyphae (often fragmented, and sometimes degenerating) can be readily seen with a silver stain, and *Aspergillus fumigatus* may be cultured from the plugs (Greenberger 1994).

Whenever patients with ABPA undergo lung biopsy, proximal airways containing the mucus plugs are typically ectatic and inflamed, with a predominance of plasma cells and eosinophils. It is thought that toxins derived from hyphae (viable or degenerating) or enzymes/mediators released from host inflammatory cells are capable of injuring the adjacent airway wall, eventually resulting in bronchiectasis. Fungal invasion of the bronchial wall is almost never seen, in spite of the fact that steroids (sometimes with antifungal agents such as itraconazole) are the main treatment modality for ABPA.

Another histological abnormality not uncommonly seen in ABPA is bronchocentric granulomatosis, where a layer of palissaded epithelioid histiocytes replaces the airway epithelium of small bronchi and bronchioles, and more or less completely surrounds intraluminal necrotic debris. In fact, this tissue reaction is most frequently identified in cases of ABPA, and when seen, should suggest the diagnosis, particularly on samples which do not contain more proximal airways with characteristic appearances.

1.9.4. Invasive aspergillosis

1.9.4.1. Predisposing factors to invasive aspergillosis.

The defence mechanisms against *Aspergillus* species have been outlined above. Most cases of invasive pulmonary aspergillosis are thought to develop following the inhalation of conidia present in the atmosphere, although sometimes infection occurs by haematogenous spread of infection elsewhere in the body, or, rarely, by direct spread from a focus of saprophytic or allergic disease.

Some degree of impairment in host defence is almost invariable, and is often due to a combination of pre-existing disease and its therapy. The most common predisposing condition is neoplasia; particularly acute leukaemia and lymphoma, while patients with solid tumours are less predisposed. Bone marrow transplantation, particularly allogeneic, is a strong risk factor for the development of IPA. Cases are also seen following organ transplantation, viral infections (especially influenza), diabetes melitus, and in association with renal or hepatic failure. In addition to these underlying diseases, a history of previous therapy with antineoplastic drugs, corticosteroids and/or antibiotics is common. IPA is relatively uncommon in individuals with AIDS. Rare cases of IPA have been described in individuals without any of these predisposing causes, usually as a result of occupational exposure to an overwhelming number of *Aspergillus* spores. This form of infection has been referred to as primary invasive aspergillosis (Clancy and Nguyen 1998).

IPA in haematological practise tends to present as *Aspergillus* bronchopneumonia, angioinvasive pulmonary aspergillosis, or miliary aspergillosis, although these varieties of aspergillosis are not exclusive to such patients. These diseases will be considered in detail below. The other forms of IPA, namely acute tracheobronchitis, pleural aspergillosis, and chronic necrotising aspergillosis are now briefly described.

1.9.4.2. Acute *Aspergillus* tracheobronchitis

Acute *Aspergillus* tracheobronchitis is limited completely or predominantly to the tracheobronchial tree, and accounts for about 5% of cases of IPA. Three forms are recognised:

1. Intraluminal continuous involvement by fungal growth with pseudomembranes lining/ partially obstructing the airway lumen. Microscopically, inflammation and necrosis are often confined to the mucosa only, with little or no extension beyond the airway wall. The disease is often overlooked in life due to lack of radiological signs.

2. Patchy involvement of the airway by one or more discrete plaques of *Aspergillus* growth. Such infection may grow into the airway lumen, forming an obstructive mass, or invade into the tracheal and bronchial wall, then extend into adjacent tissue. Complications at this stage include haemorrhage, fistula or abscess formation, pneumonia and parenchymal infarction.

3. Involvement of the smaller bronchi or bronchioles, which can be diffuse (and present with progressive dyspnoea) or localised to one lobe (when it presents with productive cough). Histologically, the abnormality is characterised by bronchocentric granulomatosis resembling that seen in ABPA, with the important differences that eosinophils are virtually absent in adjacent airways, and that densely packed hyphae are seen rather than scattered fragments. This rare variant of *Aspergillus* tracheobronchitis progresses rapidly to parenchymal disease in severely immunosupressed patients, but has also been seen in apparently immunocompetent patients, where it progresses slowly.

1.9.4.3. Pleural aspergillosis

Pleural aspergillosis is usually secondary to other forms of pulmonary aspergillosis, and is common in *Aspergillus* bronchopneumonia.

1.9.4.4. Chronic necrotising aspergillosis

Chronic necrotising aspergillosis is a rare form of IPA with poorly understood pathogenesis. The disease has an indolent clinical course with a history of fever and productive cough over a period of months or years, and radiological changes resembling those of fibrocaseous tuberculosis, seen most often in the upper lobes. Pathological changes include fibrosis, cavitation (which may be followed by the development of an intracavitary mycetoma (Gefter 1992)), and necrotising granulomatous inflammation, again resembling tuberculosis, and distinguished from it by the presence of hyphae

within tissue in direct relation to foci of granulomatous inflammation.

Affected patients invariably have no history of intensively immunosuppressive disease or treatment, but some have a degree of derangement of their defence mechanisms in the form of diabetes mellitus, malnutrition, connective tissue disorders or a recent course of low dose steroids. Many affected patients have an underlying pulmonary abnormality such as long standing infarct, inactive tuberculosis, pulmonary fibrosis, pneumoconiosis or prior lobectomy (Binder *et al* 1982).

1.9.4.5. *Aspergillus* bronchopneumonia

This occurs when there is fungal proliferation and invasion primarily in relation to small membranous and proximal respiratory bronchioles and shows many of the pathological characteristics of bacterial bronchopneumonia. Typically, there are multiple foci of disease in the early stages, hence the macroscopic fine nodular appearance. Progression to (a) circumscribed area(s) of segmental or lobar consolidation occurs as infection spreads from the airway lumen to the adjacent parenchyma, with visible small foci of cavitation/ necrosis. The whole of one or both lungs may eventually become involved.

Microscopically, there is necrosis of the airway wall and adjacent pulmonary parenchyma, with an intense polymorphonuclear leucocyte reaction in the non-neutropenic patient. Fungal hyphae are

confined to the necrotic material, which liquefies and drains via the involved airway, resulting in cavitation.

Sometimes confluent disease is associated with pulmonary vascular invasion and secondary haemorrhage. Rarely, the area of bronchopneumonia is limited in extent and relatively well controlled by host defences, when it is best described as an abscess.

1.9.4.6. Angioinvasive aspergillosis

Although vascular invasion can occur in all types of invasive aspergillosis, it is invariable in two forms of IPA, best referred to as infarctive (when vessel permeation leads to the observed pathology) and nodular (when it probably does not).

The infarctive form of angioinvasive aspergillosis may complicate any form of IPA and is consistently related to vascular damage. It appears grossly as an area of clear-cut infarction. As with infarcts occurring with straightforward thromboemboli, those associated with *Aspergillus* invasion of a blood vessel are located adjacent to the pleura, and have a triangular shape. Haemorrhage may occur into such necrotic tissue.

In nodular invasive aspergillosis, affected lung appears as a well circumscribed nodule 0.5 to 3 cm across, with a pale centre and a peripheral haemorrhagic rim. Histologically, the pale area consists of lung parenchyma showing necrosis and intensive permeation by fungal hyphae, but absence of inflammation. Vascular infiltration is prominent, and tends to be in a relatively large vessel at the centre of

the lesion. The lumen of the vessel appears occluded by fungal hyphae, and its wall transgressed by them, although thrombosis and vasculitis are rarely seen. The peripheral rim of the lesion consists of a zone of haemorrhagic and congested lung parenchyma, often without inflammation.

Several considerations lead to the conclusion that the areas of necrosis in nodular invasive aspergillosis represent coagulative necrosis by toxins or enzymes secreted by the fungus rather than true infarction. First the spherical shape of the lesions is quite unlike the typical wedge shape of pulmonary infarct. Second, unlike infarcts, these lesions have no relationship to the pleura. Third, nodules are not infrequently found to encompass a fissure, which is unlikely to be due to simultaneous infarction of two vessels positioned symmetrically across the fissure. Finally, similar round necrotic areas are found in invasive *Aspergillus* tracheobronchitis when no vascular invasion is demonstrable.

The mycotic lung sequestrum (MLS) is an important variant of nodular invasive aspergillosis, of which it probably represents a later stage. It is identified radiologically as an aspergilloma-like focus of parenchymal consolidation with a cavity containing a roughly spherical opacity. The appearance of such lesions often coincides with neutrophil recovery in a previously neutropenic patient. Histologically, they consist of necrotic lung with numerous hyphae, which do not extend into viable tissue. The cavity wall consists of organisation tissue whose inner aspect is sometimes lined by a layer of polymorphonuclear leucocytes, leading to the speculation that the necrotic lung becomes separated from viable tissue by the action of

proteolytic enzymes derived from polymorphs. This, however, cannot be the only explanation for the separation of necrotic and viable tissue, as mycotic lung sequestra can occur in neutropenic patients, and not all cavity walls of MLS are lined with neutrophils.

1.9.4.7. Miliary aspergillosis

This occurs in about 12% of cases of IPA. It represents widespread haematogenous dissemination of *Aspergillus* to all parts of the lung. Histologically, minute colonies of the organism are distributed randomly within the lung parenchyma, which is itself consolidated by variable amounts of oedema and haemorrhage.

The classification of IPA into the categories outlined is important as each has a different method of spread, which is reflected in different clinical and radiological appearances, and may in turn require different diagnostic methods, be amenable to different treatments and carry different prognoses.

1.10.METHODS OF DIAGNOSIS

1.10.1. Reasons for diagnostic difficulties in IPA

An accurate diagnosis of IPA is difficult and reasons for this include the protean and often non-specific radiological manifestations of the disease, the poor clinical/coagulation status of affected patients which often precludes lung biopsies, and the ubiquity of *Aspergillus* in the environment causing uncertainty as to whether an isolate from a non sterile site is a contaminant, a colonizer or a pathogen.

Because of this, positive *Aspergillus* cultures found on one occasion only are generally classified as due to colonisation or environmental contamination. The diagnostic status of patients with such cultures can be controversial and only cases satisfying a set of rigorous but variable criteria (Denning and Stevens 1990) tend to be reported in the literature.

For example, criteria for "Definite Aspergillosis" according to current published definitions by the Mycoses Study Group (MSG) include (Denning *et al* 1994): histological diagnosis with or without positive culture, or a positive culture from tissue obtained by an invasive procedure such as transbronchial biopsy or percutaneous needle aspiration. "Probable Aspergillosis", according to the same definitions, is only applicable to pulmonary disease, and includes the radiological appearance of new nodules or new cavities in a host predisposed to IPA, who must also have a minimum of two cultures from sputum or one from bronchoalveolar lavage (BAL), or positive cytology on BAL. Several of these criteria require the use of invasive procedures often avoided in clinical practice.

The same group also recognises the category of "Possible Aspergillosis", which includes a positive sputum culture or typical radiological appearance, or less typical radiological appearances but suggestive symptoms/signs in a susceptible host. This category is not recommended for use in clinical trials.

Matters are complicated further because, notwithstanding its status as a notable culture contaminant, *Aspergillus* often fails to be isolated from the respiratory secretions of patients with

histologically proven IPA. On the other hand, when a positive *Aspergillus* culture does manifest itself, this can be two weeks or more after inoculation, a delay too considerable to allow timely clinical action. Furthermore, histological appearances of IPA, although reasonably characteristic (see above), are not pathognomonic and can be confused with those of other filamentous fungi (Yeghen *et al* 1996), particularly when no fungus is isolated from the tissue section. Finally, the incentive for aggressive investigation of individuals with suspected IPA is often lacking because most will be receiving amphotericin B (the standard treatment for the disease) on an empirical basis for fever of unknown aetiology in a neutropenic patient.

For a number of years, efforts have been made to estimate the value of various types of lung biopsy (open, trans-bronchial, trans-cutaneous) together with that of less/non invasive clinical tests (radiology, sputum culture, nasal culture, broncho-alveolar lavage [BAL], and more recently, immunological or molecular studies). Attempts have been made at establishing methodology, and also sensitivity and specificity values for the more generally available diagnostic methods by comparison with histological or autopsy confirmation. A review of the literature on these matters is presented below. Particular emphasis has been placed on the role of antigen/antibody studies, and of the polymerase chain reaction.

1.10.2. Clinical features

Several authors have emphasised the importance of the clinical syndrome of fever, cough and thoracic pain as an important pointer to IPA in the neutropenic patient, especially when accompanied by blood stained sputum or frank haemoptysis (Young *et al* 1970, Gefter1992, Caillot *et al* 1995 and 1997, Nucci *et al* 1995). Caillot states that 92% of his IPA cases have cough, 76% chest pains and 54% haemoptysis.

1.10.3. Radiology

The radiological appearances of IPA have attracted a great deal of interest as the ultimate non invasive diagnostic test, and particularly so since high resolution CT scanning and MRI scanning have become widely available. The chest X-ray is said to be abnormal in 75-100% of patients early in the IPA syndrome (Herbert and Bayer 1981), the earliest lesions consisting of single or multiple nodules (corresponding to the histopathological target lesions), and progressing to cavitation of nodules, coalescence of nodules to form diffuse bilateral pumonary consolidation, or development of large, wedge shaped, pleural based areas of consolidation mimicking pulmonary infarction. The distribution of chest X-ray appearances, in a review of 190 patients with documented IPA, (Herbert and Bayer 1981) was as follows:

- Consolidations, including wedge shaped: 22%.
- Patchy infiltrates: 21%.
- Nodular infiltrates: 11%.

- Cavitary lesions: 38%.
- Normal: 8%.

Many of these appearances could have a number of aetiologies, but presentation as a wedge shaped area of consolidation, which may or may not accompanied by cavitation, is highly typical of IPA in the neutropenic or BMT patient although relatively uncommon (Denning *et al* 1997). The nodular form of angioinvasive pulmonary aspergillosis, however, remains the main presentation of the disease with radiological characteristics sufficiently typical to be of diagnostic value.

Radiological descriptions of the MLS variety of nodular aspergillosis started to be published in the early 1980's, although the term MLS was not suggested until 1988 when Kibbler *et al* reviewed the literature (Kibbler *et al* 1988). Cavities containing a rounded opacity and exhibiting the air crescent sign on X-ray were seen in the lung fields of IPA patients with leukaemia and normal chest radiology prior to the onset of neutropenia, thus making true mycetoma formation unlikely. The neutrophil count had recovered to greater than 0.5 x 10^9/l in 15/18 of patients included in Kibbler's review (comprising patients with neutrophils of less than 1 x 10^9/l) when the air crescent sign appeared, while 3/18 were still neutropenic at less than 0.5 x 10^9/l at that time. Five patients in that review were "neutropenic" or "leucopenic" at the time of MLS identification, although their absolute neutrophils count was not available.

The relationship between neutrophil count and radiological recognition of MLS was studied in detail (Albeida *et al* 1985) in a

series of 11 patients included in Kibbler's review. The authors demonstrate radiological evidence of pseudo- mycetoma on average two days after the neutrophil count exceeded 0.5 x 10^9/l. The same group (Gefter *et al* 1985) pointed out the limitations of the appearance of lesions with the air crescent sign in supplying an early diagnosis of IPA: the median interval between cavitation and the initial appearance of consolidation was approximately two weeks, all patients (Jewkes *et al* 1983) had a white cell count of at least 1 x 10^9/l at the time of cavitation, and radiographic signs of improving consolidation were consistently observed within 2-3 days before cavitation, in keeping with the theory that MLS formation is a manifestation of the healing stages of nodular IPA.

A typical sequence of radiological events is documented in patients with nodular IPA (Gefter 1992). The initial abnormality often consists of one or more subtle nodular opacities that may be overlooked for several days on chest X-ray while CT scanning detects intrathoracic complications in as many as 57% of BMT patients in whom the chest X-ray was negative (Graham *et al* 1991).

On CT scanning, the nodular opacities may show a characteristic "halo sign" consisting of a circular area of ground glass attenuation attributed by different authors to the rim of coagulative necrosis around the central invaded blood vessel (Gefter 1992), to local haemorrhage surrounding the infarction (Logan and Muller 1996) or to haemorrhagic infarction (Broderick *et al* 1996). In addition to the perifocal halo, these early IPA lesions often demonstrate peripheral enhancement on contrast enhanced CT-scans. Typically, the nodular areas coaslesce to form single or multiple areas of consolidation, and

evidence of cavitation appears upon recovery of the neutrophil count leading to the air crescent sign.

The current opinion, irrespective of what the peripheral halo represents, is that it is the precursor of the peripheral cavitation which leads to the air crescent sign, and that both radiological signs are highly indicative of IPA only if in the correct context of neutropenia/ immunosupression with fever (Kaiser and Rochat 1996). In the context of bone marrow transplantation, it is particularly important to relate such CT scan findings to the time elapsed since BMT, as aspergillosis tends to be most likely in the 2-3 weeks of profound neutropenia that follows the procedure (Worthy *et al* 1997).

Figures 1-2 to 1-7 show a range of typical radiological appearances in IPA.

In a recent publication (Caillot *et al* 1997.), high resolution thoracic CT identified a halo sign in 16 out of 19 patients with subsequently proven aspergillosis, and an air crescent sign in the remaining 3. The authors' protocol had involved routine thoracic CT scanning in their neutropenic febrile population with radiographic infiltrates since 1991, whereas previously CT scanning was reserved for patients with an established diagnosis achieved by other means. They demonstrated a significant reduction in the time from first clinical sign to diagnosis from 7 +/- 5.5 days pre 1991 to 1.9 +/- 1.5 days post 1991, which was in turn associated with an improved prognosis.

At the Royal Free Hospital (Berger 1998), the chest is routinely scanned after 48 hours of fever unless a focus of infection has been identified elsewhere. If fever persists and the first scan is normal, it is repeated after 4-5 days irrespective of chest X-ray findings. When the first scan is abnormal but non-specific, a repeat scan is performed, as the nature of the infection is frequently revealed by studying its evolution. The lung is scanned with 10 mm thick slices at 10 mm intervals. The images are then carefully scrutinised and selected areas re-scanned with 1 mm thick slices at 1 mm intervals.

A further refinement which may show a lesion with exquisite detail is to scan suspect areas using a small field of view, approximately 15 cm in diameter, centred on the lesion in question.

CT scanning is also helpful in guiding further invasive diagnostic procedures, such as the best location for needle biopsy and open lung biopsy (Denning *et al* 1997), or on defining whether bronchoscopy is the best modality for confirming diagnosis (Heussel *et al* 1997). A diffuse picture on CT scanning correlates with the best yield from bronchoalveolar lavage, whereas solitary, and particularly peripheral nodules are unlikely to be amenable to microbiological diagnosis by broncoscopy (McWhinney *et al* 1993, Janzen *et al* 1993). These issues will be discussed further below.

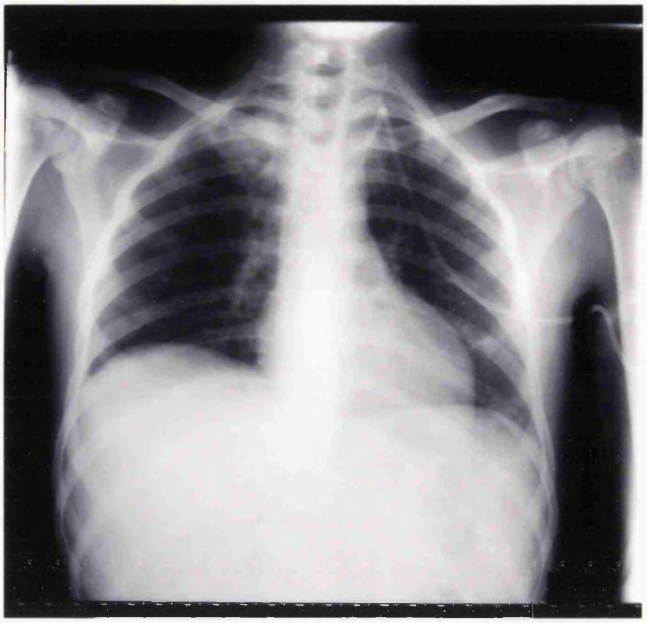


Figure1-2: Mycotic lung sequestrum on chest x-ray

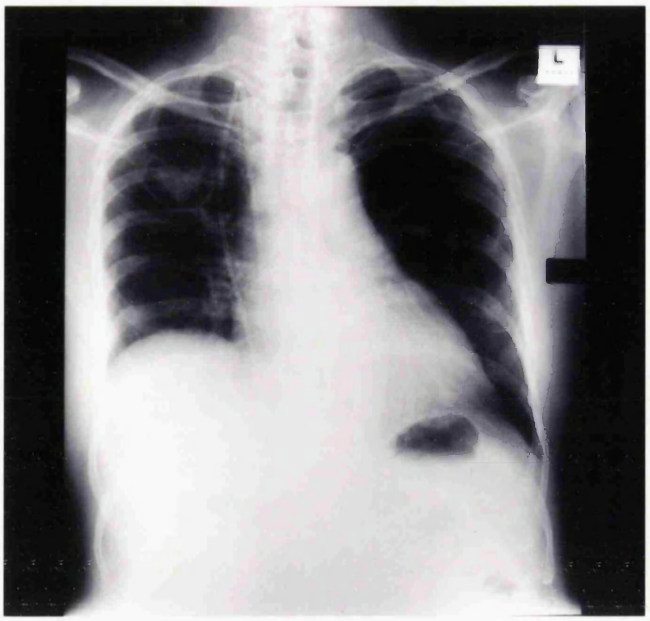


Figure1-3: Mycotic lung sequestrum on chest x-ray

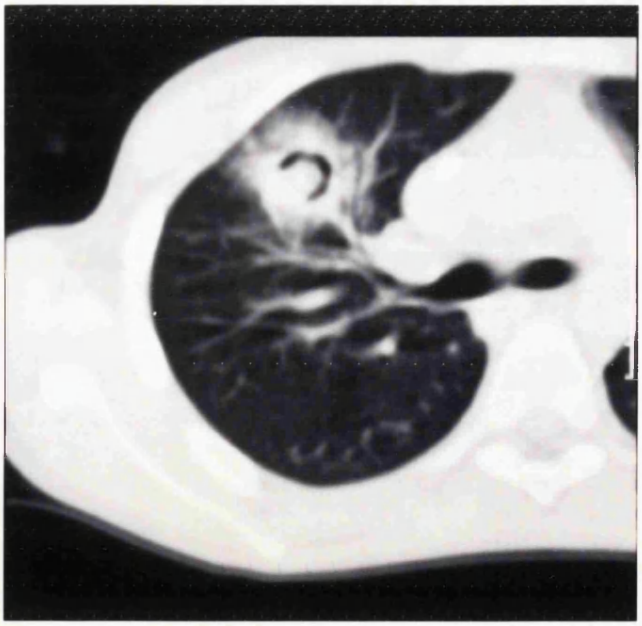


Figure1-4: Mycotic lung sequestrum on CT scan

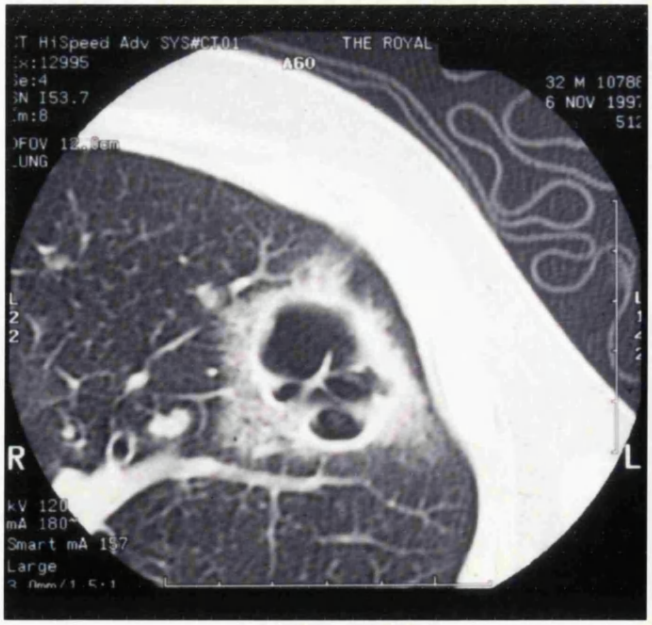


Figure1-5: Cavitating lesion on CT scan

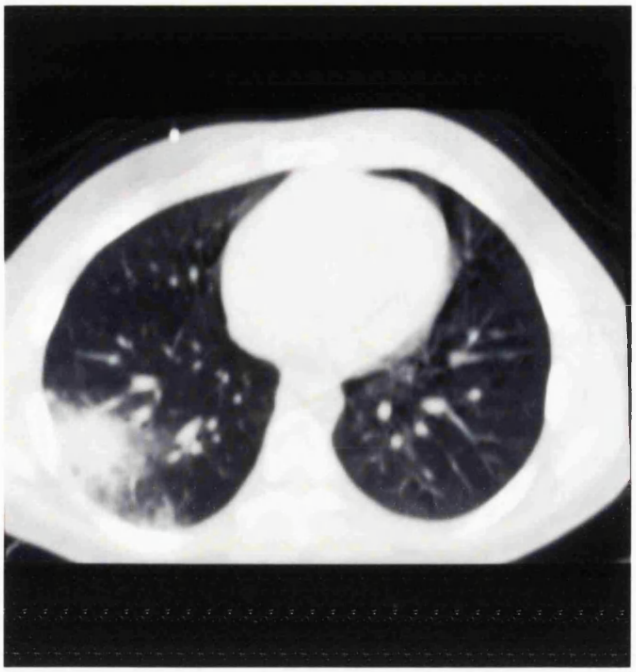


Figure1-6: Halo sign lesion on CT scan

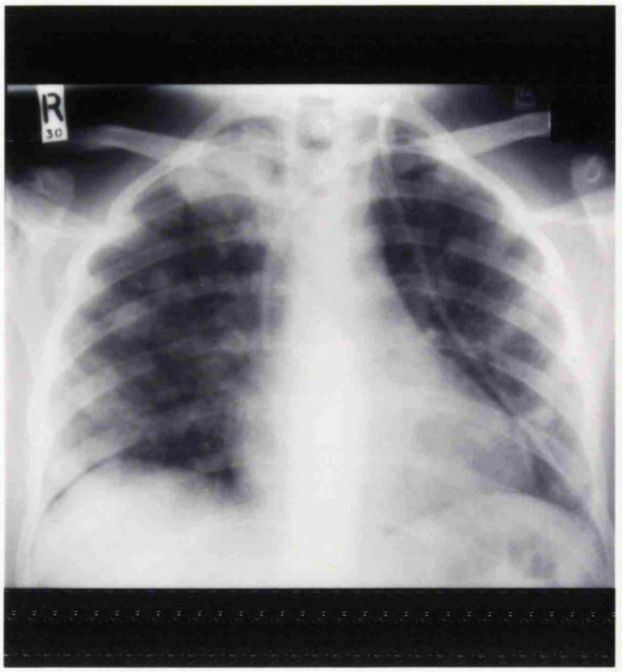


Figure1-7: Chest x-ray of disseminated aspergillosis

The MRI characteristics of the early lesions of nodular IPA have also been described (Herold *et al* 1989): they have a target like appearance consisting of hypointense centres with isointense or hyperintense rims on T1-weighted scans. These lesions demonstrate Gadolinium enhancement. No work has been published, as yet, which compares the relative merits of CT and MRI scanning in the early diagnosis of IPA.

1.10.4. Sputum cytology and culture

Aspergillus organisms are isolated from sputum in fewer than 10% of cases (Gefter 1992) subsequently found to have IPA, but the sensitivity of this is increased by submitting at least three samples (this may be difficult as these patients often have no sputum production), and ensuring that they are appropriately processed for fungal culture as described below.

Microscopic examination of pulmonary samples is optimised by the use of the fluorescent dye Calcofluor White (originally used in the textile industry), which preferentially stains cellulose, one of the components of fungal cell walls (Warren 1996). Staining a sample (after digestion and centrifugation if appropriate) with Calcofluor White added to an equal volume of potassium hydroxide improves the sensitivity of detection of fungal elements (Denning *et al* 1997). The authors recommend that the deposit should be cultured on various media, including Sabouraud agar at 30°C and 37°C for up to three weeks.

However, a positive culture of *Aspergillus* from the sputum of healthy people occurs in 1% to 16% (Denning 1996). Studies have shown a false positive rate of more than 20% for *Aspergillus* sputum culture in bone marrow transplant recipients (Denning *et al* 1997), although repeated isolation of *A.fumigatus* or *A.flavus* from multiple samples is strongly associated with invasive disease (Treger *et al* 1985). On the other hand, the isolation of an *Aspergillus* species (in particular *A. fumigatus* and *A. flavus*) from the sputum of a high risk patient, even on a single occasion, is often indicative of invasive infection (Yu *et al* 1986) and Warnock (Warnock 1995) advises that it should never be dismissed.

1.10.5. Nasal culture

Isolation of *Aspergillus* species from nasal surveillance cultures in leukaemic patients has been shown to correlate with the presence of invasive aspergillosis, especially in the context of an apparent outbreak (Aisner *et al* 1979, Sandford *et al* 1980). However, the utility of such surveillance varies with the frequency of isolation, and current recommendations (Denning *et al* 1997) are that individual units should establish its value based on their own experience.

1.10.6. Cytology and culture of BAL, bronchial aspiration and brushings

As for sputum, microscopy of BAL fluid substantially increases the sensitivity of the investigation, and some culture negative samples

are microscopy positive. Bronchoscopic techniques have the advantage that lower respiratory tract samples can be obtained from patients who do not produce sputum, and that the specimens are less likely to be contaminated by oropharyngeal flora than sputum.

However, the study of non- invasive bronchoscopic procedures as diagnostic tools for IPA is compounded by the fact that *Aspergillus* isolation is influenced by the extent and nature of pulmonary disease. This was demonstrated convincingly in a small series where *Aspergillus* was isolated from BAL in all of 7 patients with diffuse disease, but in none of 7 patients with focal disease (Mc Whinney *et al* 1993). Unfortunately, most bronchoscopy series in IPA, while aimed at establishing the sensitivity and specificity of the technique, do not describe systematically the radiological appearances of the cases. Results are very likely to be influenced by the case mix within the series, which in turn is not evident, so that difficulty in translating the data into clinical practice ensues.

In a group of 32 patients with proven or highly probable IPA who underwent bronchoscopy, 69% were *Aspergillus* positive on BAL (Caillot *et al* 1997), and the sensitivity of BAL was improved further by processing for *Aspergillus* antigen (see below). Most of these patients had multiple and bilateral focal pulmonary lesions. Another group (von Eiff *et al* 1994), based on 11 cases of IPA (9 haematological malignancies, 3 only with focal lesions), showed a sensitivity of 38% for BAL, 64% for aspiration of bronchial secretions, and 100% for protected specimen brushing. The specificity in each case was 100%.

In a series of 353 consecutive bronchoscopies on 300 patients, with an autopsy rate of 58% on 115 deaths, positive *Aspergillus* culture (n=10) or cytology (n=4) was detected in 14 of 21 patients (66.7 %) with subsequently documented IPA (Levy *et al* 1992), all of whom had haematological malignancies. Of the 14 *Aspergillus* positive patients with documented IPA, 9 can be assumed to have diffuse lung disease on the basis of concomitant PCP, HSV or CMV pneumonia, and the radiology of the rest is not described. On the other hand, 41 patients had positive *Aspergillus* cultures, but IPA was refuted by PM examination in 8 who died, and documented in only 10 (24.4%). The authors report cytology sensitivity of 64% and specificity of 99.1% with culture sensitivity of 40% and specificity of 90.3%.

Doubts have always existed amongst clinicians as to the safety of even relatively non-invasive procedures such as bronchoscopies in seriously ill patients with coagulation abnormalities and this risk has now recently been quantified (Dunagan *et al* 1997). In a series of 71 bronchoscopies in BMT patients, a complication rate of 27% and mortality rate of 3% have been demonstrated. Major complications were more common in patients with a prolonged prothrombin time and were more common in protected specimen brushings than in BAL alone.

In summary, the usefulness of BAL for the diagnosis of IPA remains controversial. Current guidelines for the investigation of fungal infections (Denning *et al* 1997) recommend the use of BAL for diffuse shadowing, but of needle or open biopsy for focal lesions, with the additional recommendation that whenever BAL is

performed, specific fungal cytology should be undertaken as it improves the diagnostic yield.

1.10.7. Histological techniques and use of transbronchial, transcutaneous and open lung biopsies

Histological examination of lung tissue, particularly if obtained by open lung biopsy or by resection of whole diseased areas, is the gold standard for the diagnosis of IPA, although not practised widely for fear of complications. There are no formal studies of the diagnostic yield of open lung biopsy in the diagnosis of IPA.

The appearances of different types of IPA are discussed in detail above, and stains in current use are haematoxilin and eosin, PAS and silver stains. Identification of a fungus as *Aspergillus* in tissue section is generally straightforward, and aided by concomitant microbiological culture of the specimen. In cases where difficulty arises, and where a more specific diagnosis is important, immunohistological techniques have been described, based on immunofluorescence and immunoperoxidase reagents (Kaufman 1992). These techniques are not widely available; they are also said to be compounded by cross-reactivity with other species, and by non-reactivity with certain *Aspergillus* species, although common species are detected.

Resection of whole diseased areas has therapeutic as well as diagnostic implications, and will be discussed fully in the "Treatment" section.

Transbronchial biopsy has a false negative rate of approximately 50% (Gefter 1992). This is attributable to sampling errors, and to the fact that the procedure is only suitable for central lung lesions or diffuse disease. Bearing in mind that the diagnostic yield of BAL alone is high in the latter, and that transbronchial biopsy is complicated by pneumothorax in 5% of patients, and by significant bleeding in 1 to 2%, with a risk of bronchopneumonia as well (McCabe 1988), there is probably little place for the procedure in the investigation of suspected IPA. It is not mentioned in the current guidelines for investigation of fungal infection (Denning *et al* 1997).

Percutaneous needle biopsy of focal lesions will yield the diagnosis in approximately 50 to 80% of cases of opportunistic infection in immunocompromised patients (Denning *et al* 1997, Pan-Chyr Yang 1992). Large (18 gauge) needles yield 91% samples suitable for histological examination (Haramati 1995). The procedure is done under radiological guidance and the samples processed for cytology, culture, and histology. Bleeding occurs rarely if coagulopathy and thrombocytopenia are adequately corrected. Approximately 10 % to 20% of patients develop an air leak, and 2% will require drainage.

Interestingly, different authors report different rates of *Aspergillus* isolation from resected, biopsied or autopsy tissue which is histologically invaded. For example, Temeck (Temeck *et al* 1994) reports 94% positive culture rate in miscellaneous fungal infections (of which 63% IPA), Young (Young *et al* 1970) 70%, Weinberger (Weinberger *et al* 1992) 47% and Caillot (Caillot *et al* 1995) 100%. Although these rates of positive culture are high, the fact that up to

53% of invaded tissue is sterile is surprising. This may be due to treatment, or hyphal damage by neutrophils or macrophages.

1.10.8. Combination of investigations

One group was able to make a pre-operative diagnosis in 36% of patients with subsequent open lung biopsy/resection confirmation of invasive fungal lung disease (63% IPA) by using a combination of radiology and culture methods (BAL, sputum/empyema fluid) (Temeck *et al* 1994)

1.10.9. *Aspergillus* antigen and antibody studies.

1.10.9.1. Antibodies.

In spite of ubiquitous exposure to *Aspergilli*, antibodies to these organisms are not commonly found in the sera of healthy persons (Rinaldi 1983). Immunodiffusion and counterimmune electrophoresis are the most widely used assays for the measurement of antibodies to *Aspergilli*, and are mainly of clinical value for the diagnosis of allergic bronchopulmonary aspergillosis and pulmonary aspergilloma (Andriole 1996) (see also above under description of individual diseases). ABPA patients have *A. fumigatus* specific serum antibodies of IgE and IgG classes. They have similar levels of IgG1 and IgG2, which frequently react against protein antigens of *A. fumigatus*. On the other hand, the sera of aspergilloma patients tend to react against the carbohydrate and glycoprotein antigens of the organism, and to contain more antibodies of the IgG1 subclass (Kurup and Kumar 1991). A recent detailed study of differential

antibody responses to a variety of *A. fumigatus* antigens in a case of aspergilloma followed over 9 years (Tomee *et al* 1995) indicates that therapeutic response and destructive microinvasion during symptomatic episodes may be monitored by measuring the antibody response to a 40-kD metalloprotease.

Antibody detection assays are of little practical value for diagnosis in immunosupressed patients because many are incapable of mounting an antibody response (particularly those with lymphoid malignancies, or bone marrow transplant recipients). There is also a delay between the onset of infection and the development of an antibody response, reducing the value of these tests even in those capable of making a response.

Early studies of *Aspergillus* antibodies in invasive aspergillosis confirmed no elevation at the time of clinical onset of the disease, but demonstrated significant rises starting at day 14 at the earliest (Mara *et al* 1979). In solid organ transplant patients, a raised *Aspergillus* specific IgG was detected in 84% of recipients with invasive aspergillosis, although the titres had fallen to within the normal range in certain cases by the time the infection was clinically apparent (Trill *et al* 1985). In both studies, multiple samples needed to be collected over time for the investigation to be of any benefit, and the information obtained was either untimely or difficult to interpret. The presence of *Aspergillus* antibodies does however assume technical importance in patients undergoing investigation for invasive aspergillosis when detection of *Aspergillus* antigen is sought, as this requires prior dissociation of any immune complexes using an acid/heat method.

1.10.9.2. Antigens.

Although *Aspergillus* antigen detection, since its introduction in the late 1970's, was thought to have great potential in the management of aspergillosis, it still has not attained the status of a routine diagnostic test. Theoretical advantages are speed of diagnosis, non invasive methodology, independence from host immune response, and scope for quantification of fungus load with prognostic and therapeutic implications. Over the last 20 years, much experimental and clinical work has aimed to detect *Aspergillus* antigen (as undefined polysaccharide, glycoprotein or purified galactomannan) using radioimmunoassays (RIA), then ELISA's of increasing sensitivity on both blood and urine, and also BAL. Both polyclonal and monoclonal antibodies have been used. In spite of the expected advances of these antigen detection tests, most have been disappointing because of low sensitivity, in turn attributable to characteristics of the assays in use, and to the transient nature of the antigenaemia. Key studies are outlined briefly below and summarised in Table 1-1.

The first report of *Aspergillus* polysaccharide antigen detection in the serum and urine of experimentally infected rabbits with invasive aspergillosis used counterimmunoelectrophoresis and was published in 1978 (Lehman and Reiss 1978). Next, Shaffer *et al* described a RIA (Shaffer *et al* 1979a, Shaffer *et al* 1979b) which detected an undefined polysaccharide antigen (with initial minimum sensitivity of 500ng/ml, subsequently improved) in a rabbit model of aspergillosis as well as in 3 patients with the disease.

Over the next five years, extensive studies by Weiner's and colleagues (Weiner *et al* 1979,1980,1985,1986) evaluated a competitive RIA based on a purified cell wall carbohydrate antigen of *A. fumigatus* and rabbit antisera to polyvalent homogenate of *A. fumigatus*. Validation of their method by testing sera from 79 haematology patients with documented aspergillosis status and an average of 1.9 admissions and 7.8 sera each was published in 1987 (Talbot *et al* 1987). In this important study, the authors express the *Aspergillus* antigen results as the ratio of radiolabelled counts bound in unknown specimens to those in a standard containing no unlabelled antigen. The diagnostic sensitivity of the RIA was 74%, the specificity 90%, the positive predictive value 82% and the negative predictive value 85%. Antigen was detected before invasive aspergillosis was suspected in 30% of admissions, and before microbiological or pathological evidence of disease in 46%. The RIA would have been the first positive diagnostic test in 77% of episodes of IPA. Consistent correlations were found between clinical course and level of antigenaemia in most patients. The limitations of the study were a false negative rate of ≈25%, and a false positive rate of 10%. The authors also perceived the use of radiolabelled materials as a drawback liable to preclude widespread use.

Author + date	Technique	Antigen detected	Sample(s) used	Sensitivity (minimal concentration detected)	Clinical data	Other comments
Lehman + Reiss 1978	Counterimmune electrophoresis	Undefined polysaccharide antigen from serum of infected rabbit	Serum + urine of infected rabbits	0.5 mg/ml	Antigen detected in serum and urine of 8/8 lethally infected rabbits and of post-mortem serum of 1 patient with histologically proven aspergillosis	First report of diagnostic test based on Aspergillus antigen detection
Shaffer et al 1979 a,b	RIA	2 different undefined polysaccharide antigens	Serum of infected rabbits + 20 patients	500 ng/ml 10-100 μ g/ml	Highest levels of antigenaemia in 2 patients with proven aspergillosis. One patient with high antigen levels treated early as IPA on basis of this result alone and survived.	Assay used for clinical study less sensitive and uses Staph. aureus as solid phase
Weiner et al 1979, 1980,1985, 1986	Competitive RIA using rabbit antisera to A.fumigatus homogenate	Purified cell wall carbohydrate antigen from crude Aspergillus extract	Serum, BAL, BAL pellets +CSF of infected patients, rabbits and dogs	110 ng/ml	Validated (Talbot et al 1987) in 79 patients with documented aspergillosis status. Sensitivity for aspergillosis 74%, specificity 90%, positive predictive value 82%, negative predictive value 85%. Antigen detected in 30% before clinical suspicion of aspergillosis, and in 46% before any laboratory evidence	25% false negatives 10% false positives Uses radioactivity Acidification and heating needed to dissociate bound antigen and increase sensitivity

Table 1-1: Summary of Aspergillus antigen detection

Author + date	Technique	Antigen detected	Sample(s) used	Sensitivity (minimal concentration detected)	Clinical data	Other comments
Sabetta et al 1985	ELISA	Carbohydrate antigen	Serum of 19 patients with aspergillosis +42 controls. Serum of 6 infected rabbits	10 ng/ml	Antigen detected in 11/19 aspergillosis patients and none of controls. Antigen detected in 5/6 infected rabbits.	Tests on single serum samples often negative, multiple specimens increase detection
Dupont et al 1987	RIA ELISA	Galactomannan	Serum + urine of patients and infected rabbits	10-40 ng/ml	Galactomannan in serum of 4/12 infected rabbits and 2/12 infected patients. Galactomannan in urine of all infected rabbits and 54% infected patients.	Rapid clearance of inoculum from plasma with delayed urinary excretion

Table 1-1: Summary of Aspergillus antigen detection (Continued)

RIA= radioimmunoassay

ELISA= enzyme linked immunosorbant assay

Author + date	Technique	Antigen detected	Sample (s) used	Sensitivity (minimal concentration detected)	Clinical data	Other comments
Le Pape and Deunf 1988	ELISA	Glycoprotein antigen	28 sera from 10 patients with invasive aspergillosis	10-20 ng	Antigen detected in 9/10 patients	No indication given of how many of the sera from patients were positive but in the "negative" infected patient, low level of antigenaemia doubled over the course of the disease. All control sera negative
Johnson et al 1989	BALISA	Glycoprotein antigen	Serum of 45 BMT patients	68 ng/ml	Antigen detected in 3/4 (75%) of patients with proven aspergillosis and in 1/1 patient with suspected aspergillosis	Tests on single serum samples often negative, multiple specimens increase detection

Table 1-1: Summary of Aspergillus antigen detection (Continued)

BALISA: biotin-avidin linked immunosorbant assay

Author + date	Technique	Antigen detected	Sample (s) used	Sensitivity (minimal concentration detected)	Clinical data	Other comments
Rogers et al 1990	Polyclonal human serum Rat monoclonal antibody	Galactomannan	Urine and serum of neutropenic haematology patients	14 ng/ml in serum, 13 ng/ml in urine, 7ng/ml in serum, 10ng/ml in urine	Antigen detected in serum or urine of 16/19 patients with proven infections and 11/13 patients with clinically suspected infections but only 13% sera and 20% urines tested from these patients positive over the course of the disease.	Some samples positive with polyclonal serum but not monoclonal antibody. "Pastorex" and "Platelia" now based on this monoclonal antibody.
Patterson et al 1995	Inhibition ELISA with polyclonal rabbit antiserum	Uncharacterised carbohydrate antigen	Serum	<10ng/ml	54 imunosuppressed patients with serum antigenaemia >50 ng/ml studied over 65 months. 19 had proven aspergillosis, 16 probable aspergillosis, 14 indeterminate, 5 false positives.	Only 41% of serum samples from patients positive on at least one occasion positive. Higher antigen levels in disseminated aspergillosis and non-survivors.
Caillot et al 1995	Pastorex	Galactomannan	Serum and BAL of patients diagnosed by CT scan	15 ng/ml	59% patients with IPA Pastorex negative. 75% tests done on IPA patients Pastorex negative. Pastorex on BAL identifies 83% cases of IPA compared to 69% by direct microscopy or culture.	Only 12% patients serum antigen positive on day of IPA diagnosis by other methods. Increased number of tests per patient increases pick-up rate.

Table 1-1: Summary of Aspergillus antigen detection (Continued)

Author + date	Technique	Antigen detected	Sample (s) used	Sensitivity (minimal concentration detected)	Clinical data	Other comments
Verweij et al 1995	Sandwich EIA (Sanofi)	Galactomannan	Serum of 2 BMT patients with IPA	0.5-1 ng/ml	Galactomannan detected 4 and 28 days before appearance of pulmonary infiltrates	Course of antigen titer corresponded with outcome.
Rohrlich et al 1996	Sandwich EIA (Sanofi)	Galactomannan	Prospective study of serial sera from 37 high risk patients	0.5-1 ng/ml	Positive predictive value 83%	Antigen detection precedes clinical and radiological onset of aspergillosis and reverses with clinical improvement. No antigen detection in 13 colonised patients with cystic fibrosis.
Sulahian et al 1996	Sandwich EIA (Sanofi) Pastorex	Galactomannan	Serum of 215 BMT patients	0.5-1 ng/ml 15 ng/ml	Loss of specificity to 81% within 30 days of BMT with 19% false positive rate by Platelia	Possible interference with assay of cytotoxics or their metabolites. Platelia more sensitive and detects infection earlier than Pastorex

Table 1-1: Summary of Aspergillus antigen detection (Continued)

Author + date	Technique	Antigen detected	Sample (s) used	Sensitivity (minimal concentration detected)	Clinical data	Other comments
Swanick et al 1997	Sandwich EIA (Sanofi)	Galactomannan	Serum of 40 febrile neutropenic patients + fungal culture supernatants	0.5-1 ng/ml	Loss of specificity in febrile neutropenic patients with 15% false positive rate	Possible interference with assay of cytotoxics or their metabolites. Cross reactivity with exo-antigens of Penicillium chrysogenum, Penicillium digitatum, Paecilomyces variotii and Alternaria species.
Maertens et al 1999	Sandwich EIA (Sanofi)	Galactomannan	Prospectively collected sera of 71 patients with autopsy proven aspergillosis	0.5-1 ng/ml	92.6% sensitivity, 95.4% specificity, 93% positive predictive value, 95% negative predictive value.	8% false positive rate, possibly overestimated.

Table 1-1: Summary of Aspergillus antigen detection (Continued)

EIA: enzyme immunoassay

In contrast to these results, early assays designed to detect purified galactomannan, a major antigenic component of *Aspergillus*, were disappointing. One group (Dupont *et al* 1987), using both a RIA and an enzyme immunoassay, detected galactomannan in the serum of only 4 out of 12 rabbits infected with a lethal dose of *A. fumigatus* and of only 2 of 12 patients with invasive aspergillosis, whereas urinary galactomannan was found in all lethally infected rabbits, and in 54% of patients with invasive aspergillosis. These results are explained (de Repentigny 1992) by animal experimental data whereby radiolabelled galactomannan is rapidly cleared from plasma by hepatic Kupffer cells, with a delay of 24 hours in urinary excretion of 35% of the inoculum

In 1990, another group (Rogers *et al* 1990) published the evaluation of a polyclonal human serum and of a monoclonal antibody for the detection of galactomannan in the serum and urine of 121 neutropenic patients after leukaemia or bone marrow transplantation. Although antigen was detected in the serum or urine of 16 of 19 patients early in the course of IPA and in 11 of 13 clinically suspected fungal infections, only 13% of 482 serum samples and 20% of 173 urine samples from these patients were positive over the course of their disease. Also some samples were positive with the polyclonal serum but not the monoclonal antibody, which suggests the presence of additional circulating antigens. The fact that galactomannan was detected in a significantly greater number of urine samples than serum samples from patients with IPA confirms Dupont's findings.

Several groups using ELISA or related techniques for the detection in serum of *Aspergillus* glycoprotein antigen (Le Pape and Deunf 1988, Johnson *et al* 1989) or carbohydrate antigen (Sabetta *et al* 1985), had already confirmed the observation that tests on single serum samples were often negative, whereas multiple specimens from the same patient increased the frequency of detection.

In a more recent study (Patterson *et al* 1995) returned to using an uncharacterised carbohydrate antigen of *Aspergillus* together with a polyclonal rabbit antiserum in an inhibition ELISA with minimal antigen detection of <10 ng/ml, as an attempt to overcome the perceived low sensitivity of galactomannan based systems. They studied the clinical characteristics of all patients with at least one serum antigen level greater than 50ng/ml by this ELISA over a 65 month period in order to determine the utility of the test. Fifty-four immunosuppressed patients fulfilling this criterion were identified. Nineteen of them had proven invasive aspergillosis (histopathologically proven disease with growth of *Aspergillus* species on culture), and their maximum antigen values were 70-3500 ng/ml. Sixteen patients had probable invasive aspergillosis (a positive culture for *Aspergillus* and a compatible clinical illness but no histology) with maximum antigen level of 52-800ng/ml. The fourteen remaining patients had an indeterminate diagnosis (clinical evidence of infection, and persistent fever unresponsive to antibiotics without growth of pathogens) and maximum antigen values of 56-400 ng/ml. The authors also demonstrated higher antigen values in patients with disseminated aspergillosis than in those with IPA, higher median antigen levels in those who died than in those who survived, and a course of antigenaemia correlating with

clinical outcome. However, there were 5 false-positives with antigen level 100-500 ng/ml, and the study design precluded the detection of false negatives. In addition, only 41% of all serum samples from patients positive on at least one occasion were antigen positive, and the initial serum sample was negative in the majority of cases subsequently shown to have proven or probable aspergillosis although no data is available as to the clinical circumstances of this initial sample. This important study demonstrates a satisfactory positive predictive value of *Aspergillus* antigen detection for the diagnosis of aspergillosis, although the issues of sensitivity and fitness for use as an early diagnostic tool remain unaddressed.

A latex agglutination test based on the monoclonal antibody used by Rogers (Rogers *et al* 1990), and subsequently a sandwich ELISA (Pastorex *Aspergillus* and Platelia *Aspergillus*, Sanofi Diagnostics Pasteur) have now become available commercially, and will hopefully encourage data collection on a larger scale and clarification of the role of *Aspergillus* antigen detection in the clinical management of aspergillosis. The main advantage of the latex agglutination method is simplicity of use, but results obtained with it so far do not compare well with those using the better of the RIA's and ELISA's described above.

For example, the Pastorex test was used on the serum of 33 out of 36 patient with proven or highly probable IPA (Caillot *et al* 1995). This was positive on at least one occasion in only 1 of 8 patients tested less than three times, and in half of the remaining 26 patients who were tested between 5 and 35 times. On the whole, 59% of patients with IPA remained *Aspergillus* antigen negative by the Pastorex

method, as did 75% of all tests done on these patients. Only 12% of patients were antigen positive on the day of IPA diagnosis by other methods, and none before this, so that *Aspergillus* antigenaemia did not contribute to early diagnosis in any of the cases in this series. This could be because of the authors' intensive and timely use of early high resolution CT scanning caused them to reach a diagnosis of IPA before the onset of antigenaemia, or because the sensitivity of the Pastorex method is suboptimal. When performed directly on BAL fluid in the same group of patients however, the Pastorex test identified 83% of cases of IPA, whereas diagnosis by direct microscopy or culture could only be made in 69%, and the test may find a clinical role in this context.

The Sanofi Diagnostics sandwich ELISA kit using the rat monoclonal antibody EB-A2 as both captor and detector for galactomannan has a lower detection limit of only 0.5-1 ng/ml of galactomannan; the lowest achieved so far. It yielded a positive predictive value of 83% (Rohrlich *et al* 1996), with antigen detection preceding clinical and radiological onset of aspergillosis. Furthermore, no antigenaemia was detected in the serum of 13 patients with cystic fibrosis known to have airway colonisation by *Aspergillus*. A very recent autopsy controlled prospective evaluation of serial screening for circulating galactomannan using the same assay (Maertens *et al* 1999) and based on the analysis of 71 patients found a sensitivity of 92.6%, specificity of 95.4%, positive predictive value of 93% and negative predictive value of 95%. The false positive rate was 8%, but the authors consider that this figure may have been overestimated.

The improved sensitivity may unfortunately be accompanied by some loss of specificity, particularly within 30 days of BMT (Sulahian *et al* 1996) and in febrile neutropenic patients (Swanink *et al* 1997), in whom the false positive rate is as high as 15%. One possible explanation for this problem, namely interference of cytotoxic drugs or their metabolites with the assay, is suggested by the demonstration (Hashigushi *et al* 1994) of false positive Pastorex *Aspergillus* latex agglutination tests in the urine of rats treated with cyclophosphamide. The loss of specificity can be partly compensated by repeat sampling and early introduction of another sensitive diagnostic method such as high resolution CT scanning. There is preliminary data supporting the use of the sandwich ELISA for the early diagnosis of aspergillosis and for the monitoring of response to antifungal treatment (Verweij *et al* 1995). Future clinical data concerning the outcome and economic implications when fungal infections are treated very early and monitored with a sensitive method are awaited with great interest.

1.10.10. Polymerase Chain Reaction (PCR) technology for *Aspergillus*

The concept of using a rapid and early diagnostic method for *Aspergillus* infections by DNA amplification is immediately attractive because of the difficulty in achieving microbiological, or indeed any documentation of these infections. While conventional microbiological diagnosis of *Aspergillus* infections is usually slow, and tends to detect advanced disease mostly, histological diagnosis is often unacceptable, and antigen detection methods have been

disappointing. Furthermore, empirical treatment with systemic antifungals is costly and carries the risk of serious side effects. Finally, the patient population at risk is increasing due to expansion in specialities using immunosuppressive therapies.

These considerations prompted scientists interested in aspergillosis to design and evaluate PCR methods capable of amplifying *Aspergillus* DNA. This was particularly relevant in the context of early successes of PCR in detecting a number of pathogens eluding standard diagnostic techniques, for example HIV-1 (Ou *et al* 1988) and Cytomegalovirus (Shibata *et al* 1988) in 1988, *Toxoplasma gondii* (Burg *et al* 1989) in 1989, and *Pneumocystis carinii* (Wakefield et al 1991) in 1991. The literature to date on PCR methods for the detection of *Aspergillus* is discussed below and summarised in Table 1-2.

The first report of detection of *A. fumigatus* by PCR was published by Spreadbury *et al.* in 1993 (Spreadbury *et al*, 1993,1994). The assay was based on parts of the 26S/intergenic spacer region of the organism, and yielded a 401 bp product highly specific for *A. fumigatus,* although products of different sizes were obtained for other *Aspergillus* species, and for two *Penicillium* species. An internal probe specific for *A. fumigatus* was also designed, for use in Southern hybridization, with a view to confirming specificity and increasing sensitivity. A DNA sequence encoding rRNA was chosen because of high number of copies per organism, and therefore likely high sensitivity of amplification. The sequence unfortunately has a high G+C content of 71%, requiring the addition of glycerol to the PCR mixture in order to stabilise the product.

This first *A. fumigatus* PCR was capable of detecting 1 pg of *A. fumigatus* DNA by UV visualisation, and 100 fg following Southern analysis. In a mouse model of IPA, there were no positive PCR's by UV visualisation from lung tissue of 8 healthy animals, of which one was positive after Southern hybridisation, or 3 experimentally inoculated but culture and histology negative mice, of which two were positive after Southern hybridisation. One out of six mice with positive lung culture +/- histology was PCR negative, and the rest positive. A 93% correlation between culture and PCR was thus reported, with one clear false negative PCR.

Respiratory specimens from 22 patients were also tested by this technique. PCR was positive in 3 of 3 with culture positive invasive aspergillosis, in only 1 of 3 patients with positive sputum culture due to colonisation (the 2 others were faintly positive by Southern hybridization), as well as in 2 of 10 (3 more by Southern hybridization) immunosuppressed patients without aspergillosis and in 2 of 7 patients not at risk of IPA. The 4 culture negative/PCR positive samples were attributed to probable heavy colonisation of the patients' airway. Although the 2 culture positive/PCR negative results raise concerns about the sensitivity of the assay, the authors appeared reassured by this result and interpreted it as colonisation of insufficient extent to cause a positive PCR, with the possibility that the PCR may be useful in discriminating between invasive infection and colonisation. In the same context, they considered the Southern hybridization step too sensitive for clinical use, and more likely to give rise to false positives results than to detect low level infection. Finally, the likelihood of atmospheric contamination causing false

positive PCR's was addressed by showing that as many as 10^5 *Aspergillus* conidia in the PCR mix are required to render it positive, although the conidia had not undergone the DNA extraction process.

Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for *A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridization (*A. fumigatus*)	Clinical data	Other comments
Spreadbury *et al* 1993	PCR specific for A. fumigatus	26S/ intergenic spacer region	Lungs of experimentally infected mice + clinical respiratory specimens	401	*A. fumigatus*. Products of other sizes for some other *Aspergillus* sp. + 2 *Penicillium* sp.	1 pg	100 fg	93% correlation between culture and PCR in lungs of experimentally infected mice, with 1 false negative. Positive in 4/6 culture positive sputa, including all 3 patients with proven IPA.	High G+C contents in sequence. Glycerol needed to stabilise product.
Tang *et al* 1993	PCR specific for *A. fumigatus* + *A. flavus*	Alkaline protease gene	BAL	747	*A. fumigatus* *A. flavus*	5 pg	500 fg	94.4% specificity for IPA in a group of 23 immunocompromised patients, with sensitivity of 100%.	All positive results without Southern hybridization step.

Table 1-2: Summary of *Aspergillus* PCRs

Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for *A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridization (*A. fumigatus*)	Clinical data	Other comments
Reddy *et al* 1993	PCR specific for *A. fumigatus* + *A. restrictus*	IgE binding protein	Urine	315	*A. fumigatus* *A. restrictus*	20 pg	600 fg	2/13 urines from BMT patients positive, including that from the only patient with proven invasive aspergillosis (cerebral).	Method compared with that of Tang *et al* and found to be preferable (Urata *et al*, 1997)
Hopper *et al* 1993	PCR amplifies 42 species of fungi. *Hae III* restriction analysis refines diagnosis but not as much as to genus level.	Ribosomal DNA	Mainly fungal isolates. Assorted clinical samples	310 for all 42 species	Yeasts Septate moulds Zygomycetes Dimorphic fungi	Not known for *Aspergillus*	Not known for *Aspergillus*	One BAL culture positive for *A.fumigatus* also PCR positive	SSCP (Walsh *et al* 1995) at 2 temperatures can differentiate between *Aspergillus* species

Table 1.2: Summary of *Aspergillus* PCRs (continued)

Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for *A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridization (*A. fumigatus*)	Clinical data	Other comments
Melchers *et al* 1994	Hot start PCR targeting all common pathogenic *Aspergillus* sp. with optional reverse transcriptase phase	18 S ribosomal DNA	Lungs of experimentally infected mice + clinical BAL	263	*A. fumigatus*, *A. flavus*, *A.terreus*, *A.niger*, *A. nidulans*. *Paecilomyces* +*P. marneffei* are excluded by hybridization + restriction.	1 pg without RT-PCR 10 fg with RT-PCR	1fg	PCR positive in only 5/6 culture positive mouse lungs. PCR positive in 6/6 patients with proven or suspected IPA (only 1 culture positive). Possible cross-reaction with unspecified *Penicillium* sp. Method compared with antigen detection by ELISA in haematology patients with fever (Verweig *et al*, 1995). More false positives by PCR.	All positive results without hybridization or RT-PCR step.

Table 1.2: Summary of *Aspergillus* PCRs (continued)

sp. = species

.Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for*A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridazation (*A. fumigatus*)	Clinical data	Other comments
Makimura *et al* 1994	PCR specific for common *Aspergillus* and some *Penicillium* species	18 S ribosomal DNA	Sputum	385	*A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, *A. oryzae*, *A. versicolor* & 5 *Penicillium* sp.	1pg	not tested	3/3 sputum samples from IPA patients PCR positive but only one culture positive.	*P.marneffei*, a serious pathogen in AIDS patients in Asia, not amplified.
Bretagne *et al* 1995	PCR amplifies all 4 common *Aspergillus* sp. Product size differentiates sp. Internal competitive template detects amplification failure.	Mitochon -drial DNA	BAL	135	*A. fumigatus*, *A. flavus*, *A.terreus*, *A.niger*.	Detects at least 5 conidia in water.	not tested	Amplification failure in 5.5%. False positive or colonisation in 25%.	Poor predictor of IPA in 41 AIDS patients (Bart-Delabesse *et al*, 1996)

Table 1.2: Summary of *Aspergillus* PCRs (continued)

.Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for *A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridazation (*A. fumigatus*)	Clinical data	Other comments
Katz *et al* 1996	2 PCRs specific for *A. fumigatus.*	Alkaline protease gene	Isolates from farmed ostriches in Australia.	704 1216	*A. fumigatus*	1pg	1pg	Not applicable	One strain positive by one assay, negative by the other
Yamakami *et al* 1996	Nested PCR specific for all 4 common pathogenic *Aspergillus* sp. and *A. nidulans.*	18 S ribosomal DNA	serum (mice and humans)	357	*A. fumigatus*, *A. flavus*, *A.terreus*, *A.niger*, *A. nidulans.*	50 fg	5 fg	Positive in 70-71% of known infections, compared to 43-60% by antigen detection (Pastorex)	N/A

Table 1.2: Summary of *Aspergillus* PCRs (continued)

.Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for *A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridazation (*A. fumigatus*)	Clinical data	Other comments
Einsele *et al* 1997	PCR amplifies all important pathogenic fungi. Species differentiation by species specific probes	18 S ribosomal DNA	Whole blood	482-503	All important pathogenic fungi	100 fg	50 fg	Sensitivity 100% and specificity 98% for fungal infection.	Positive PCR preceded radiological signs in 12/17 patients Subsequent PCR negativity reflects response to treatment.

Table 1-2: Summary of *Aspergillus* PCRs (continued)

.Author + date	Technique	Region amplified	Sample (s) used	Product size (bp) for *A. fumigatus*	organisms amplified	Sensitivity by UV visualization (*A. fumigatus*)	Sensitivity by Southern hybridazation (*A. fumigatus*)	Clinical data	Other comments
Loffler *et al* 1998	PCR-ELISA based on the primers described by Einsele *et al* and using *C.albicans* or *A.fumigatus* probes to capture amplicons	18 S ribosomal DNA	Fungal cells added to whole blood from volunteers. Blood from known patients	N/A	*C. albicans* *A. fumigatus*	N/A	5 cfu/ml	All patients with proven or probable fungal infection were PCR positive.	Digoxigenin/biotin/ streptavidin method. Can be performed using commercial kits exclusively

Table 1-2: Summary of *Aspergillus* PCRs (continued)

sp. = species

CFU = colony forming unit

Another *Aspergillus* PCR method was published later that year by the same institution (Tang *et al* 1994). The second assay was based on the alkaline protease (a possible virulence determinant) gene of *A. fumigatus* and *A. flavus*, fragments of which had been cloned and sequenced by the authors in the course of previous experiments (Tang *et al* 1992). It was capable of detecting 5pg of A. *fumigatus* DNA by UV visualisation in the form of a 747 bp product, and 500 fg by Southern hybridization. The sensitivity of *A. flavus* detection was 10 times less for both UV visualisation and Southern hybridization, and the corresponding product was 690 bp. Assuming the genome size of *A. fumigatus*, which is haploid, to approximate that of *A. nidulans* at 50 fg, this level of sensitivity corresponds to 100 genome equivalents.

BAL fluid from all 4 culture positive patients (*A. fumigatus* in all , and one with *A. flavus* as well) with proven or probable IPA was PCR positive by Tang's method, as was that from one cytology positive, culture negative probable case. *A. fumigatus* only was detected by PCR in the patient with dual infection, which was attributed to the lower sensitivity of the assay to *A. flavus* together with the possibility that *A. fumigatus* DNA was acting as a competitive template. One more of the remaining 18 immunocompromised patients was PCR positive in the absence of *Aspergillus* culture or evidence of IPA. Five of 28 non-immunosuppressed controls were PCR positive, of which two, including a known case of ABPA, were culture positive. All positive PCR results were obtained without the additional use of Southern Hybridization, and a sensitivity of 100% with specificity of 94.4% for IPA in immunosuppressed patients is reported for the assay. The

"false positives" are attributed to airway colonisation after careful exclusion of experimental contamination. An assay with greater sensitivity than culture was thus first described for the detection of the 2 common pathogenic *Aspergillus* species, with the logical implication that it will also detect airway colonisation with greater frequency than culture would.

A third PCR was published in 1993 (Reddy *et al* 1993), based on an IgE binding protein of *A. fumigatus* homologous with cytotoxins of other Aspergilli. The product was 315 bp, and obtained from *A. fumigatus* and *A. restrictus* only. The sensitivity for *A. fumigatus* was 20pg on ethidium bromide gel, and 0.6 pg by Southern analysis. Preliminary analysis of 13 urine samples from bone marrow transplant patients showed a positive result in two, including the only one who had invasive aspergillosis (cerebral).

A new PCR derived from the genes encoding the 18S rRNA of *Aspergillus* species was published in 1994 (Melchers *et al* 1994), and had the advantage that it could amplify nucleic acids from all common pathogenic *Aspergilli* (*A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*), as well as *A. nidulans* and *Paecilomyces* (this could be differentiated from *Aspergilli* by hybridization of the 363 bp product to a specific probe) and *Penicillium marneffei*, which could be differentiated from the rest by restriction analysis. The assay included an optional reverse transcriptase step and a hot start. The sensitivity was 1 pg of *Aspergillus* DNA without the reverse transcriptase step, which increased it to 10 fg of nucleic acids. The maximal sensitivity was 1 fg of nucleic acids after Southern analysis.

The PCR was first tested in an experimental mouse model of invasive aspergillosis (*A. fumigatus*). All control mice were negative by culture, histology and PCR. The fungus was cultured from multiple foci in the lungs of all six infected animals, but PCR was only positive in five. Hyphae were found on histological examination in all the livers, although *A. fumigatus* was not recovered by culture from any and PCR was positive in all six. The authors explain these findings by the absence of conidia during the growth of *A. fumigatus* in liver tissue, whereas air contained in the lungs induces sporulation of growing hyphae. The implication, although not formally stated by the authors, was that conidia grow better than hyphae on culture media, but are less well detected in PCR. Such a phenomenon would explain some of Spreadbury's findings as well.

Melchers group also tested the PCR on the BAL's of 14 controls, and 14 neutropenic patients, 4 of who had proven, and 2 suspected IPA. Only one case was *Aspergillus* culture positive. A positive PCR was obtained from all 6 patients with proven or presumed IPA. It was also positive in 3 other neutropenic patients with pulmonary infiltrates, including twice on a patient whose BAL grew an unspecified *Penicillium* on both occasions, raising the issue of specificity of the PCR in the presence of a *Penicillium* other than *marneffei* or *chrysogenum*. Of interest is the fact that no additional positive results were obtained from these samples by repeating the PCR's in the presence of the reverse transcriptase step or by Southern analysis. The authors concluded that the sensitivity of 1 pg of DNA is sufficient for clinical purposes, particularly as a genome of *A. nidulans* is estimated to contain 50 fg of DNA, so that the basic

PCR will detect as few as 20 organisms. They also pointed out that nucleic acid amplification from a site subject to colonisation such as the airway will only give information as to whether *Aspergillus* is present, which in turn would make the patient at risk of IPA, but that the presence or absence of IPA would have to be ascertained in the light of the overall clinical picture.

This PCR was used by the same group the following year (Verweij *et al* 1995) in a comparison with *Aspergillus* galactomannan detection by sandwich ELISA on the BAL fluid of 19 Haematology patients with fever and pulmonary infiltrates and 35 non-neutropenic controls. The ELISA was also performed on serum samples. PCR was positive in 5 of 7 patients with radiology suggestive of IPA, 3 of who were culture positive. Four were also ELISA positive, and one culture positive patient was ELISA negative. A sixth patient was positive by ELISA only. One of two patients with possible IPA was ELISA and PCR positive, the other negative by both methods. Three of 10 neutropenic patients thought unlikely to have IPA were positive by PCR (n=2) or ELISA (n=1), but not both. Five of 35 non-neutropenic patients had positive PCR, while none had positive ELISA. All patients with positive BAL by ELISA also had positive serum ELISA, first detectable 4 to 30 days before the onset of radiological signs in 4 patients. The authors point out that a positive PCR result, unlike galactomannan detection, can be due to the presence of contaminating conidia without mycelial development. They advocate very close monitoring of patients with positive serum galactomannan, and pre-emptive treatment with systemic antifungals at the earliest symptoms or sign of infection.

A PCR reported from Japan in 1994 (Makimura *et al* 1994), also based on 18-S ribosomal DNA, was capable of detecting *A. versicolor* and *A. Oryzae* in addition to the five *Aspergillus* species amplified by Melchers assay, together with five *Penicilium* species, although *P. marneffei,* a serious pathogen in AIDS patients in South Asia, was not specifically tested. The product was 385 bp and 1pg of *A. fumigatus* DNA could be detected on ethidium bromide. Three sputum samples from patients with proven IPA, of which only one was culture positive, were tested by this method and found to be positive, thus showing the PCR to be more sensitive than culture.

The following year, another new PCR was reported (Bretagne *et al* 1995), with a 135 bp product for *A. fumigatus*, different sized products for *A. flavus*, *A. terreus* and *A. niger*, and no amplification for other organisms. It was based on mitochondrial DNA and featured an internal competitive template aimed at detecting amplification failure. The authors were able to detect fewer than 5 conidia in water using this test, but do not give a sensitivity in terms of minimal weight of DNA detected. This PCR showed amplification failure in 3 of 55 BAL's. Although it confirmed IPA in 3 out of 3 patients, it was also positive in 12 of 49 (25%) who did not develop aspergillosis. The same group used the PCR again, the following year on the BAL's of 41 AIDS patients (Bart-Delabesse et al 1996), one of which failed to amplify. PCR was negative in 78% but positive in 22% (n=9). Only one patient, who was also *Aspergillus* culture positive with a sinus infection, was infected with the organism. None of the 8 other patients developed *Aspergillus* related clinical problems over a follow-up of 1 to 24 months, in spite

of profound immunosupression. The authors concluded that PCR is a poor predictor of IPA in this population.

Further PCRs for the detection of *Aspergillus* were described in 1996. The first two, which amplify parts of the alkaline protease gene of *A.fumigatus* (Katz *et al* 1996) but use different primers from those used by Tang, were tested on isolates from farmed ostriches in Australia, on the basis that IPA is a major cause of mortality in these birds. The finding of main interest was that one of the *A. fumigatus* strains was repeatedly negative by one assay, but positive by the other, illustrating variability in genetic make-up of the organisms as a cause of false negative PCR results.

A nested PCR method using primers based on the 18S rRNA but differing from those previously described (Yamakami *et al* 1996), was published later in the year, amplifying a 357 bp sequence, and capable of detecting 50 fg of *A. fumigatus* DNA, as well as DNA from *A. nidulans*, *A. flavus*, *A. niger*, and *A.terreus*. The PCR was tested in the sera of 12 experimentally infected mice with detection in 71% (43% by Pastorex), and in the sera of 20 patients with invasive aspergillosis, with detection in 70% (60% by Pastorex). No comparison was established with the more sensitive galactomannan sandwich ELISA.

A comparison between the PCR's described by Reddy and Tang was made on DNA and clinical respiratory samples (Urata *et al* 1997). This showed sensitivities of 10pg and 100 pg respectively by Southern analysis on DNA. The cytotoxin based PCR was positive in 5 of 10 patients with aspergilloma, of which 3 were culture

positive, which compared with 6 of 10 patients with the alkaline protease based PCR. In the case of 3 patients with invasive aspergillosis, of which 2 were culture positive, the cytotoxin based PCR was positive in all, and the alkaline protease based PCR in one only. The authors express preference for Reddy's method, and consider that the length of Tang's product may lead to false negative results in cases where the sample is less well preserved.

In 1993, the year when the first *Aspergillus* specific PCR's were published, a different approach to the molecular diagnosis of these infections from clinical specimens was published (Hopfer *et al* 1993), involving amplification of a 310 bp segment of ribosomal DNA common to all 42 genera of fungi tested, and followed by restriction analysis with *HaeIII*. The restriction step allowed allocation of the fungus to one of five groups: *Candida* and related yeasts, *Cryptococcus* and *Trichosporon* species, *Aspergillus* and related septate moulds, Zygomycetes, or dimorphic fungi. Single-Strand Conformational Polymorphism (SSCP) was subsequently shown to be a useful method of distinguishing between amplicons of these categories of isolates after PCR with the same primers (Walsh *et al* 1995).

The most important development in the concept of a PCR amplifying all important pathogenic fungi was published in 1997 (Einsele *et al* 1997), where species specific oligonucleotide probes were used to distinguish between amplicons, and clinical applicability was tested on 601 whole blood samples from 121 individuals classified retrospectively by fungal infection status. The primers, again based on 18S rRNA genes, yielded products of 482 to 503 bp depending on

the pathogen tested. Sensitivity for *Aspergillus* species was 100 fg by ethidium bromide, and 50 fg by Southern blot hybridization. *Histoplasma capsulatum* was the only non-*Aspergillus* organism hybridising with the *Aspergillus* specific probe. The assay detected all of 21 proven invasive fungus infections if two samples were tested, giving 100% sensitivity. PCR was positive in none of 35 healthy controls, and in only 3 of 65 patients (189 samples) with immunosupression and no fungal infection, with (n=36) or without (n=29) fungal colonisation. Specificity was thus 98%. The positive PCR preceded radiological signs in 12 of 17 patients with IPA or hepatosplenic candidiasis by a median of 4 days. For ten patients with invasive fungal infections responding to antifungal therapy, the PCR became persistently negative after 14 days, in contrast to 11 patients who remained PCR positive while failing to respond to treatment.

This assay is clearly very important for the accurate diagnosis of invasive fungal infections. The requirement for Southern analysis in each sample, with the use of at least two probes per sample in the first instance (*Aspergillus* and *C. albicans*, with the introduction of 4 *Candida* non- albicans probes subsequently if negative), render the assay cumbersome and expensive, and put it beyond the reach of most laboratories as a rapid diagnostic test.

The same group has recently published a modification of the technique, which was applied, to *A. fumigatus* and *C. albicans* only (Loffler *et al* 1998). A PCR-ELISA was described, which had a similar sensitivity as the original assay, potential for automation and

also made maximal use of commercially available kits. Is also had capacity for simple adaptations so as to be able to detect other fungi.

Briefly, DNA was extracted using the QIamp Tissue kit (Qiagen, Hilden, Germany) and amplified using the primers described previously (Einsele *et al* 1997) with digoxigenin labelling using Dig-Labelling kit (Boehringer Mannheim, Mannheim, Germany). A commercially available PCR-ELISA kit (Boehringer Mannheim, Mannheim, Germany) used steptavidin coated microtiter plates to capture digoxigenin labelled PCR product hybridised to biotin labelled species specific probe and anti-digoxigenin conjugated with horseradish peroxidase as an indicator.

The assay was used to screen 40 patients at risk of invasive fungal infection, of whom 7 developed proven invasive aspergillosis, 7 possible invasive aspergillosis and 15 possible invasive fungal infection. The rest had no evidence of invasive fungal infection. All those with proven or probable invasive aspergillosis were PCR-ELISA positive with 63/142 positive sera (45%), while 5/48 (8%) sera from those without fungal infection were positive although showing weaker reactions. The 11 patients with possible invasive fungal infection had an intermediate result with 15/80 positive PCR samples, most patients having a single positive test.

The PCR-ELISA for *Aspergillus* still depends on at least dual sampling to achieve 100% sensitivity, but appears to have a lower false positive rate than antigen detection by sandwich ELISA (see above) on the limited number of samples tested. It is the first *Aspergillus* DNA detection test which has been standardised and is

suitable for automation and adaptation to the detection of other fungi. On this basis, it may become a powerful tool in the management of invasive aspergillosis, provided further clinical data and cost analysis, which are awaited with interest, prove satisfactory.

1.11.TYPING OF *ASPERGILLUS FUMIGATUS* STRAINS

1.11.1. General considerations

The incidence, acquisition and host-pathogen relationships in invasive aspergillosis have been discussed in detail above, with particular reference to IPA. The occurrence of clusters of disease within hospitals in connection to nearby construction work, poorly maintained ventilation systems, the presence of potted plants or the use of non-irradiated pepper, together with the almost exclusive predilection of the disease for immunocompromised, often hospitalised hosts, have lead to the general perception that invasive aspergillosis is a nosocomial infection.

The evidence for this is mainly circumstantial, and aspergillosis does occurs in units where precautions are taken to avoid patient exposure to spores and in the absence of construction works. It is possible that patients who subsequently develop aspergillosis arrive in hospital colonised with the organism, which screening cultures fail to detect, and that invasion proceeds once host defence mechanisms fail.

Much research interest has been expressed in typing strains of *Aspergillus fumigatus*, the commonest species causing aspergillosis, with a view to clarifying these epidemiological points by tracking

identical isolates, and to improving infection control measures. Additional benefits of a reliable typing system for *A. fumigatus* would be better understanding of its virulence factors, ecology and perhaps also taxonomy.

The variability in macroscopic and microscopic morphology, in speed of growth and in heat tolerance between individual *A. fumigatus* isolates, although lacking the reproducibility required of a typing method, has implied strain variation and encouraged further investigations. The earlier methods were based on phenotypic differences, and are briefly outlined.

1.11.2. Antigenic bands

Variation in antigenic profile of *A. fumigatus* was first demonstrated in 1984 (Wallenbeck *et al* 1984), using crossed immunoelectrophoresis and crossed radioimmunoelectrophoresis on 10 isolates. No attempt to assign types to the differing banding patterns using this technique was made, however. The first true antigenic typing method was an immunoblot described in 1989 (Burnie *et al* 1989) and using rabbit hyperimmune antiserum as a probe. Eleven types, based on 16 antigenic bands, were identified from 21 isolates arising from 8 cases of aspergilloma. Three types were found in one patient, and two types each in two others. Limitations of the method include marked gel to gel variation, difficulty in standardising antisera between laboratories, and concerns that the observed polymorphism could be influenced by

differential gene expression due to growth conditions and duration rather than by true interspecies differences.

1.11.3. Phenotypic methods (enzymes and susceptibility to toxins).

Multilocus enzyme electrophoresis, involving the comparison of relative mobilities of an intracellular enzyme between different strains after starch gel electrophoresis, was first used in 1992 (Matsuda *et al* 1992) to demonstrate differences between strains of *A. fumigatus*. The combined relative mobilities of lactate dehydrogenase, fumarase and malate dehydrogenase divided 34 isolates into 15 groups, while glucose-6-phosphate dehydrogenase and glutamate dehydrogenase were poorly discriminatory. Enzyme based methods are still in use, particularly as comparative tools for new technology, and in conjunction with other typing procedures to gain additional discrimination (see below).

Another phenotypic method depending on differential susceptibility of *A. fumigatus* strains to killing by yeast toxins (Fanti *et al* 1989) was also described and found 15 types amongst 32 clinical isolates. It never gained popularity.

1.11.4. DNA methods

DNA based typing techniques have the advantage that they reflect true genetic variability, rather than differences in gene expression between organisms, and are generally considered robust in terms of reproducibility. As a result, and in conjunction with the rapid

progress in molecular biology in recent years, they have gained popularity as experimental typing tools for *A. fumigatus.* The techniques available for this purpose are restriction fragment length polymorphisms (RFLP) of genomic DNA, hybridazation of digested genomic DNA with a variety of probes, and random amplification of polymorphic DNA (RAPD) together with variants on this method.

1.11.4.1. RFLPs

Denning (Denning *et al* 1990) was the first to apply DNA technology, in the form of RFLP analysis, to the typing of *A. fumigatus* isolates. Profiles of 31 isolates from *Sal*I and *Xho*I restriction enzymes revealed 24 DNA types, with 3 of them represented by 3 isolates each. *Bgl*II yielded 2 different patterns within the isolates, and other restriction enzymes were not useful in demonstrating differences. One American isolate was the same as a German isolate, and one hospital isolate was the same as a sewage isolate. This technique was used on *A. fumigatus* isolates from patients with aspergilloma (Denning *et al* 1991) to show that more than one strain may be present in the lungs of these patients. The main drawback of this technique is that it requires very good quality, high molecular weight DNA, as some of the RFLP's of interest are larger than 23kb. Furthermore, the bands require photographic enlargement techniques for good visualisation. Burnie subsequently (Burnie *et al* 1992) also used restriction analysis, with *Xba*I this time, to identify 6 types amongst 21 isolates.

1.11.4.2. Molecular probes

Hybridising digested DNA from *A. fumigatus* with a probe in order to discriminate between strains of the organism was first reported in 1990 (Spreadbury *et al* 1990). The intergenic spacer region from the ribosomal gene complex of *A. nidulans* was used as a probe against *Eco*RI digested genomic DNA to examine 11 isolates, which yielded 3 patterns. Another study used a moderately repetitive sequence from *A. fumigatus* genomic DNA to probe *Eco*RI digested DNA from the same organism (Girardin *et al* 1994) and investigated 49 isolates from 3 aspergilloma patients. Each patient was shown to be infected by a single strain. The same technique was subsequently used in a veterinary context (Sarfati *et al* 1996) to demonstrate that aspergillosis in aborted calves is due to maternally derived strains, that the portal of entry of the organism is the gastrointestinal tract, and that cattle from the same farm slaughtered on the same day harbour the same strain, suggesting a contamination from feed material. These findings led to the speculation that gastrointestinal aspergillosis may be underestimated in humans.

Another group (Tang *et al* 1993) used telomeric sequences from *Fusarium oxyporum* to probe *EcoR*I and *Hind*II digested DNA from *A. fumigatus* with discrimination as good as RAPD, first described two years beforehand by the same group (Aufauvre-Brown *et al* 1992). The latest refinement in the Southern based methods came from Denning's group (Anderson *et al* 1996), who used whole bacteriophage M13 DNA to probe *Hind*II digests of *Aspergillus fumigatus* DNA, on the basis that the probe is easily available commercially. These methods, although robust, are labour intensive.

1.11.4.3. RAPD

RAPD is a technique using single, short oligonucleotides to amplify genomic DNA using PCR with low annealing temperatures allowing template/primer interactions with one or two mismatches. A characteristic pattern is produced (dependent on probe and genome amplified) and used to assign types to isolates. The first application of RAPD to the typing of *A. fumigatus* was in 1992 (Aufauvre-Brown *et al* 1992), when a single ten base primer, R108, was shown to be discriminatory between 9 isolates, while 43 other primers were not useful for typing purposes. The following year, a variant on RAPD, called "Interrepeat PCR" because of its use of repetitive sequences as primers (van Belkum *et al* 1993), was shown to differentiate 6 types in 7 isolates when the 22 base ERIC1 (Enterobacterial repetitive intergenic consensus) was the chosen primer, whereas eukaryotic sequences were non-discriminatory. A combination of two 8 base primers was used the same year (Loudon *et al* 1993) to examine 19 isolates, and revealed 12 types, with the suggestion that some aspergillomas contain multiple *Aspergillus fumigatus* strains. RAPD was subsequently used extensively in conjunction with other techniques, as outlined below.

RAPD is relatively straightforward and does not specifically require good quality DNA, a great advantage for work in *Aspergillus* whose DNA is notoriously difficult to extract. However, because RAPD typing is intrinsically dependent on non-stringent PCR conditions, banding patterns tends to lack reproducibility when changes occur in running conditions or in reagents (particularly *Taq* polymerase). Similarly, reproducibility between laboratories is not ideal. As an

attempt to circumvent these problems, one group (Mondon *et al* 1997) cloned and sequenced discriminating RAPD patterns from different strains of *A. fumigatus*, so that sequence specific DNA primers (SSDP) characteristic of the RAPD sequences could be designed and used in high stringency PCR. Fifty-two strains were classified into 22 different types using a combination of 5 pairs of SSDP primers, three of which were derived from R108 patterns.

1.11.5. Combinations of typing methods

Much recent emphasis has been on comparing and/or combining typing methods to achieve optimal discrimination. The two 8 based primers described by Loudon were used by the authors (Loudon *et al* 1994) in conjunction with immunoblotting and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to investigate an outbreak of invasive aspergillosis. Isolates from three patients infected within a month of each other were shown to be the same type by the three methods, with the conclusion that the patients were probably infected by the same strain. Another group (Rath *et al* 1995) sought to investigate the relationships between 6 isolates from a lung transplant recipient, 1 isolate from a patient who had been on the same ward, and one reference strain. They found no differences using SDS-PAGE, immunoblot or RAPD using 3 primers including Aufauvre-Brown's R108 and one of Loudon's primers, but digestion of one primer product with *EcoR1* and RAPD using pairwise combined primers discriminated between the patient's strains and the two others. The authors point out that any single technique may fail

to detect strain differences, and that a spectrum of typing methods is required to reveal or exclude cross- infection with *A. fumigatus*.

Another group (Lin *et al* 1995, Loudon and Burnie 1995), in a comparative study using RAPD, isoenzyme analysis and restriction enzyme analysis to type 35 *A. fumigatus* isolates, also demonstrated that a combination of typing techniques has higher discriminatory power than any technique used in isolation, and that both genotypic techniques are more discriminatory than the phenotypic technique. Isoenzyme analysis (alkaline phosphatase, esterase and catalase) discriminated nine types only, while 21 types were seen with a combination of 3 RAPD primers including R108, and 22 types obtained by *XhoI* and *SalI* restriction analysis together. Taking into account results from all 3 techniques yielded 28 types, but the authors recommend the use of only the two DNA based techniques in combination for optimal economy and reproducibility.

A Hungarian group (Rinyu *et al* 1995) published similar results supporting the better discrimination obtained with RAPD than with isoenzyme analysis. Sixty-one isolates of *A. fumigatus* could be subdivided into seven strains by isoenzyme analysis (β-arylesterase and phosphatase) but into 37 strains by combining results from the 2 most discriminatory RAPD primers. *HaeIII* generated mitochondrial DNA patterns, *SmaI* digested repetitive DNA and ribosomal DNA hybridization patterns were invariable, except in one strain, which also presented unique patterns with isoenzyme analysis and RAPD, and was proposed as a new species of the section *Fumigati*.

1.11.6. Drawbacks and discussion

Thus a number of techniques for the typing of *Aspergillus fumigatus* have become available over the last ten years, although they all have drawbacks in terms of discriminatory power, reproducibility, ease of performance, or applicability to bulk work. They have been tried in the investigation of hospital outbreaks of aspergillosis, with very modest success.

In addition to the technical limitations imposed by the various typing methods available, some characteristics of aspergillosis pose major problems for their application to the study of its epidemiology. First, all of the methods require an isolate, which, not infrequently, is never found when the diagnosis is made by histology, PCR or antigen detection.

Second, "clonal" outbreaks of aspergillosis are likely to be rare (Nolard 1996) because environmental contamination tends to occur suddenly but on a massive scale, so that spores from several strains of *Aspergillus fumigatus* are released simultaneously and deposited in areas difficult to access during routine cleaning, while air sampling tends to be performed too long after the contaminating event, and often fails to detect the relevant organism.

As an attempt to resolve some of these issues, the European Research Group for the study of the Biotype and Genotype of *A. fumigatus* (EBGA network) was created. This group aims to collect a large number of isolates from patients with guaranteed follow-up

from various geographic areas, to preserve the isolates under optimal conditions, and to study them on a large scale by all the typing methods available. There is hope that better understanding of the epidemiology of outbreaks, and of the differential pathogenicity of strains will result.

1.11.7. Possible use of single-stranded conformation polymorphism (SSCP) as a typing method for *Aspergillus fumigatus.*

Single stranded conformation polymorphism (SSCP) is a relatively new technique which is proving very useful in mutation screening (Watson *et al*, 1992), although it has not been used, to date, as a tool for the molecular typing of *Aspergillus fumigatus.*

A single nucleotide difference between two short single-stranded DNA molecules, usually obtained by heat denaturation of a PCR product containing the area of interest, induces a difference in the conformations adopted by the two strands. This conformational difference is sufficient to produce changes in the electrophoretic mobilities of the molecules on neutral polyacrilamide gels.

Many papers describing the uses of SSCP in the fields of bacteriology, virology and parasitology have been published since 1994, when the technique started to assume a key role in the molecular biology literature. The typing, or otherwise classifying of closely related organisms, together with screening for drug resistance emerge as the principle utilisations of SSCP in

microbiology. The application of SSCP to human mycology, surprisingly, has been restricted to a handful of publications only.

As described in section 1.10.10, SSCP was used to distinguish between genera, and occasionally species, when performed on amplicons resulting from universal primers for pathogenic fungi (Walsh *et al*, 1995). Similarly, amplification by universal primers followed by SSCP can discriminate between various pathogenic *Aspergilli* (Rath and Ansorg, 2000), and between species of *Aspergillus* Section *Flavi* (Kumeda and Asao, 2000). SSCP can also be utilised to differentiate between *Candida* species in the context of a PCR capable of amplifying DNA from six medically important *Candida* species (Hui *et al*, 2000). SSCP has been described as a typing method for fungi only in *Pneumocystis carinii* (Mathis *et al*, 1996) and *Sporothrix schenckii* (Sugita, 2000).

From 1994, SSCP was being used in the Royal Free Hospital Microbiology Department to screen for drug resistance in *M. tuberculosis* (Billington *et al*, 1999; Davies *et al*, 2000), and for allelic variation in *Pneumococcus* autolysin (Gillespie *et al*,1997), thus providing the laboratory with good local experience with the technique. This gave us the means to screen products obtained by diagnostic PCRs from various *A.fumigatus* strains for single base pair differences, which, if informative, could be the basis for a typing method not requiring an isolate (see 1.16 and 6.4).

1.12.INCIDENCE

The issues of incidence and diagnosis are intricately linked in the case of invasive pulmonary aspergillosis, because the estimation of incidence is complicated by difficulty in making (or excluding) the diagnosis with certainty in vivo. Consequently, publications dealing with the incidence of IPA are often based on post- mortem series (thus automatically excluding any patients who survived their disease) or on patients (currently the minority) with suspected IPA who underwent lung biopsy.

Invasive aspergillosis is the second most common cause of fungal infection in immunocompromised patients. A multicentre autopsy study from 12 centres in North America and Japan from 1980 to 1988 demonstrated that 30% of all fungal infections were due to *Aspergillus* (Bodey *et al* 1992). The incidence of aspergillosis appears to be increasing. A large study of autopsies in two Frankfurt hospitals between 1978 and 1992 showed an increase in the prevalence of invasive fungal infections from 2.2% to 5.1% during the study period, and *Aspergillus* infections (often in patients with other end stage disease) accounted for most of the increase (Groll *et al* 1996). More recent necropsy data (1994/1995) showed invasive aspergillosis in 4% of cases, of which 56% were unsuspected before death (Vogeser *et al* 1997). Incidence studies based on post mortem data, although based on a firm diagnosis, have the limitation that they quantify the risk of dying of or with aspergillosis rather than the risk of developing the disease in life.

Several publications have assessed the incidence of aspergillosis in bone marrow transplantation, based on variable diagnostic criteria. This incidence was 5.6% in an allograft series published in 1993 (Saugier-Veber *et al* 1993). It reached 11.2% in a BMT study published in 1997 (Wald *et al* 1997), which quoted an incidence figure from earlier years at 5.7%. A survey of transplanted lymphoma patients (1993) showed an aspergillosis incidence of 8.7% for allo-BMT, 5.6% for autologous stem cell transplants, and 4.5% for auto-BMT (Iwen *et al* 1993). The incidence of pulmonary aspergillosis alone was 7.2% in a large bone marrow transplantation study (Pannuti *et al* 1991). It is noteworthy that in 69% of cases, aspergillosis in BMT patients is diagnosed after the recovery of the neutrophil count (Wald *et al* 1997).

Data concerning the incidence of aspergillosis in non transplanted haematology patients is more difficult to obtain, mainly because it is dependent on the duration of neutropenia, which in turn is highly variable.

Prolonged neutropenia is a major predisposing factor for aspergillosis, and the risk has been calculated to increase from 1% per day after the first three weeks of neutropenia to 4.5% per day after five weeks (Gerson *et al* 1984). Similarly, in a cohort of 158 bone marrow transplant patients diagnosed with invasive aspergillosis (Wald, 1997), neutropenia was only identified as a risk factor for aspergillosis in those developing the disease >40 days after BMT.

Other aspects of treatment also influence the incidence of aspergillosis in neutropenic subjects. For example, within a single institution, 2% of myeloablative (chemotherapy or BMT) episodes were complicated by proven or suspected IPA if performed in laminar air-flow room, but 7% developed IPA if performed in a single reverse isolation room (Caillot *et al* 1995).

An estimation of the average risk of aspergillosis is found in a recent prospective study (Nucci *et al* 1995), where *Aspergillus* infection is described in 2.5% of episodes of febrile neutropenia, and IPA in 1.6%. In a 60 bedded Oncology Unit (Krcmery *et al*, 1996), mould infections (affecting the lungs with or without other organs in all but 1 case), occurred in 2% of febrile neutropenic episodes, with *Aspergillus* accounting for just over half, and *Mucor*, *Fusarium*, *Acremonium* and *Aureobasidium* for the rest. Seventy five percent of affected patients suffered from haematological malignancies. Environmental risk factor analysis showed an association with construction work in 70%, with the presence of flowers in 75%, with the use of pepper in 70%, and with nursing in other than a single room in 65%. Such data, although useful, is clearly only applicable to institutions with similar case mix and treatment protocols as the reporting institution, and within a limited time scale in view of the changing incidence of aspergillosis.

1.13.TREATMENT

1.13.1. Amphotericin B

Conventional treatment of aspergillosis in immunocompromised patients is with amphotericin B (amphoB), which was first used in 1959 (Denning 1996a). Treatment failure rates with amphoB are said to range from 50 to 90% (Denning and Stevens 1990). New reasons for concern are in two recent publications (Weinberger *et al* 1992, Caillot *et al* 1995) which point out that 50% -64% of their cases of invasive aspergillosis respectively first developed clinical presentations suggestive of the disease after receiving amphotericin B to cumulative doses of up to 2.5g or at a daily dose of 0.93 +/- 29mg/kg/day for 16 +/-9 days. This suggests that aspergillosis is capable of developing or progressing in individuals treated with doses of amphotericin considered to be therapeutic in established cases, and raises doubts about the often repeated statement that the earlier IPA is treated, the better the prognosis (Warnock 1995).

Much of the current confidence in amphotericin B is based on work by the EORTC Group (EORTC 1989) in the context of empirical administration in neutropenic fever resistant to broad spectrum antibiotics: earlier and more frequent resolution of fever was found in patients randomised to receive amphotericin B rather than no antifungal treatment after 4 days of fever on antibacterial treatment. No difference in overall survival was reported, but four fatal fungal infections occurred in those who received no amphotericin, compared with none in those who did receive it.

Although encouraging, this data deals with a situation clearly different to that in a patient with proven aspergillosis. At the same time, trials of antifungal agents in the context of definite fungal infections are bedevilled by diagnostic uncertainties and relatively small patient numbers, so that appropriate prospective data of individual antifungal agent efficacy, and comparative data between different antifungal drugs is scanty.

Denning (Denning 1996) recognises that the standard choice of antifungal therapy in haematology patients tends to be amphotericin B, but states that itraconazole is also a reasonable first choice of therapy in patients able to take the drug orally and who are on no interacting medication. He points out that increasing the dose of amphotericin B to 1-1.25mg/kg/day for patients already on the drug when they develop IPA is often not an option in current practice, as the doses used for empirical treatment of fever tend to be of 1mg/kg, whereas previously they often were of the order of 0.5mg/kg/day. Furthermore, some of the data supporting the higher efficacy of an increased dose of amphotericin B came from patients who had also received Flucytosine, so that the relative contributions of the two therapeutic manoeuvres are unknown (Warnock 1995).

Because the higher dosage range of amphotericin B can be difficult to achieve as a result of renal toxicity and systemic side effects (including fever and rigors), lipid based formulations of the drug have been developed: these include liposomal amphotericin B (AmBisome) in which the drug is encapsulated in phospholipid containing liposomes, amphotericin B colloidal dispersion (Amphocil) in which the drug is packaged into small lipid discs, and

the ribbon- like amphotericin B lipid complex (Abelcet). These preparations, although much less nephrotoxic than conventional amphotericin B deoxycholate, and generally better tolerated by patients, are considerably more expensive than the parent compound. No randomised trial has shown that they are superior to amphotericin B in their antifungal effect, although there are several reported series of patients successfully treated with these compounds, both *de novo*, or as salvage therapy after failure of conventional amphotericin B. The largest series of patients with invasive aspergillosis treated with AmBisome was reported by Mills (Mills *et al* 1994).

Much emphasis has been placed on the fact that these lipid-based compounds allow dose escalation. This however has to be taken in the context of comparative trials between conventional amphotericin B and AmBisome. The efficacy of 1mg/kg of amphotericin B deoxycholate has been shown to be no different to that of 1mg/kg or 3mg/kg of AmBisome in a Kaplan-Meier analysis of time to defervescence in the setting of empirical therapy for fever of unknown origin in a randomised trial (Prentice *et al* 1997). In the same trial however a significantly greater number of patients responded to 3mg/kg of Ambisome than to 1mg/kg of Ambisome or amphotericin B deoxycholate, which were equivalent. Although another large trial demonstrated equivalence of 1mg/kg and 4mg/kg of Ambisome in the treatment of invasive aspergillosis (Ellis *et al* 1998), no comparison was made with conventional amphotericin B. Thus higher doses of lipid based preparation may be necessary to achieve a given therapeutic effect, so that dose escalation data with lipid based preparations is not as impressive as it seems.

1.13.2. Azoles

Response rates of 75% to 78% have been reported for IPA treated with itraconazole, a triazole compound (Caillot *et al*, 1995 and 1997). There are several reports suggesting that invasive aspergillosis resistant to amphotericin B in neutropenic patients will respond to itraconazole (Denning *et al* 1989, Dupont 1990), although data from randomised trials comparing amphotericin B and itraconazole is limited to a small study of 32 patients showing similar efficacy of the two agents, as reviewed by (Richardson and Kokki, 1998).

The use of itraconazole has been somewhat restricted by the lack of a commercially available intravenous preparation, and a number of drug interactions. The reduced absorption of itraconazole capsules in neutropenic and BMT patients, which is thought to be due to complications such as mucositis and graft versus host disease, means that effective blood concentrations of >250 ng/ml (Boogaerts *et al* 1989) may be difficult to achieve in some of those most at risk of developing aspergillosis. Furthermore, antacids and H2 blockers interfere with the absorption of itraconazole, which is best assimilated if taken with an acid drink (Denning 1996 b). The liquid oral formulation of itraconazole is absorbed more reliably and offers a potential solution to these problems (Warnock 1995).

Itraconazole is metabolised by cytochrome P450, and drugs that induce these enzymes (including commonly used agents such as anticonvulsants and rifampicin) are likely to lead to subtherapeutic

itraconazole levels, and preclude its use. Another important interaction is the elevation of cyclosporin levels observed in transplant recipients with the administration of itraconazole, and often requiring halving of the cyclosporin dose (Denning 1994).

The new triazole compound voriconazole also shows promise in the treatment of aspergillosis (Caillot *et al* 1997), although data from formal comparative studies with other antifungal agents is awaited.

1.13.3. Combination therapy

Combination therapy of invasive aspergillosis with amphotericin B and flucytosine, rifampicin or itraconazole has all been used, with good effect in some cases. However, flucytosine and amphotericin B have been shown to be synergistic against certain isolates in vitro, but antagonistic against others (Denning 1994). Similarly, there are theoretical reasons why the amphotericin B + itraconazole combination should be antagonistic although this has not been substantiated in limited in vitro experiments or a number of clinical cases. Finally, the use of rifampicin with amphotericin B, although attractive on the basis of in vitro synergy, would preclude subsequent administration of itraconazole and expose transplant patients to potentially serious drug interactions with cyclosporin. Denning emphasises that such combination therapies for aspergillosis are currently not recommended (Denning 1994).

Haemopoietic growth factors (G-CSF and GM-CSF) are often used, on theoretical grounds, to reduce the period of neutropenia and/or

enhance macrophage function in patients with aspergillosis. No publication to date has demonstrated an improvement in survival with these measures.

1.14.SURGERY

The Haematology unit at the Royal Free Hospital has a long standing policy of resecting most focal lesions showing the radiological appearances of IPA (almost exclusively MLS, with occasional lesions demonstrating the "halo sign") (Kibbler *et al* 1988, Wong *et al* 1992, McWhinney *et al* 1993), in conjunction with systemic antifungal treatment, and have obtained excellent results: none of Wong's series suffered post-operative death or a relapse of their IPA, 7 of the group described by Mc Whinney were able to undergo BMT following lung resection for IPA without recurrence of their aspergillosis, and Kibbler reports cure from IPA post surgery in both his cases, even in the face of subsequent leukaemic relapse. These resections were performed in order to allow cure, to prevent the risk of massive haemoptysis, to reduce the risk of relapse following subsequent neutropenic episodes or bone marrow transplantation, and to optimise the chances of making a definite diagnosis.

Temeck (Temeck *et al* 1994), however, based on her series of 36 patients subsequently shown to have fungal infections (IPA in 23 cases) with immune compromise, points out that the key to the success of surgery in Wong's series may have been that the neutrophil count had recovered to >0.5 $x10^9/l$ in 13 out his 16 patients. Of Temeck's patients (all of whom received appropriate antifungal treatment after diagnosis), 13 underwent wedge biopsy

and 23 some form of pulmonary resection. The radiological appearances were variable, and the resections performed mainly for debulking and diagnostic reasons. Of the resected patients, 9 died (together with 2 biopsied patients) during their hospitalisation, and 6 of them had aspergillosis. Statistical analysis showed that the single most important predictor of death following open lung biopsy or resection was angioinvasive fungal disease, which in turn clustered within patients with neutropenia < 0.1 $x10^9/l$, haematological disease, and recent chemotherapy or steroids. Thus lung surgery can be associated with 39% early mortality in patients with invasive fungal infections when a substantial proportion of the group remains neutropenic.

On the other hand, a French group (Caillot *et al* 1997) undertook resections in 16 patients with subsequently proven IPA, 14 of whom had the CT halo sign often associated with IPA in the neutropenic phase, and only two an air crescent sign associated with bone marrow regeneration, while at least six were stated to have residual granulocytopenia. They recorded only 6.25% death rate in these operated patients in spite of the presence of granulocytopenia in 37.5%. The need for surgical intervention was based on the classical criteria of debulking, haemoptysis or obtension of tissue diagnosis in half the patients. The other half underwent resection for prophylaxis of massive haemoptysis based on the novel criterion of CT evidence of an aspergillar lesion adjacent to the pulmonary artery or its main branches. Twenty patients without such critical lesions were not considered at risk of haemoptysis, and were treated conservatively, with a 72% improvement or cure rate from IPA in the group of 36

patients. Unfortunately no comparison in prognosis was established based on whether surgery took place or not.

It thus appears that the neutrophil count at the time of surgery may have an influence on the outcome of the procedure, but the importance of this has not been assessed. The role of surgery in the management of IPA also remains unclear except in the context of actual or threatened haemoptysis (best assessed on the radiological basis described by Caillot), when a definite diagnosis is necessary (as in patients in whom systemic antifungals may be particularly hazardous), and perhaps also if lesions are stable or increasing in size after neutrophil regeneration when further myeloablative treatment is planned.

No controlled trial has taken place comparing medical and surgical management combined of IPA with medical management alone . Different authors report similar results with or without surgery in given situations: Mc Whinney's 94% mortality figure for IPA treated surgically post BMT is in keeping with most other data not involving surgery, while his 100% IPA free survival in patients undergoing resection pre BMT is matched by a small group treated with systemic amphotericin alone (Richard *et al* 1993). At the moment, surgical resection of IPA lesions can therefore only be recommended in selected cases until the issues of its safety in neutropenic patients, and of whether it actually improves prognosis are fully addressed.

1.15.SURVIVAL

Invasive pulmonary aspergillosis in the context of haematological disease is renowned for its grave prognosis. Although the mortality of nosocomial pneumonia in non neutropenic, non haematology patients is high and was reported as 36.6% (Celis *et al* 1988) in a series in which only 1 in 118 cases was due to *Aspergillus*, the prognosis of IPA is worse still. The mortality rate of IPA is said to reach 50% to 60% when it occurs during chemotherapy induced neutropenia, and could exceed 90% in the setting of bone marrow transplantation (BMT)(Denning and Stevens 1990). The prognosis is said to be better if the neutropenia resolves, if the aspergillosis is treated early, particularly within the first 10 days of onset of pneumonia (von Eiff *et al* 1995) and if the disease is unilateral (Caillot *et al* 1997).

1.16.BASIS AND AIMS OF THIS STUDY

Clearly, many questions remain unanswered in the diagnosis, treatment, prognosis and epidemiology of *Aspergillus* infections. The work presented here seeks to investigate some of these issues in the context of the Haematology Unit of the Royal Free Hospital, which offers an ideal study environment for a number of these problems.

This 40-bedded Haematology Unit comprises 2 wards (NHS and private) and is a tertiary referral centre. It treats Haemato-Oncology patients mainly, with particular emphasis on Bone Marrow Transplantation. In conjunction with the Microbiology, Radiology

and Cardio-Thoracic Surgery Departments, it has a special interest in *Aspergillus* infections, resulting in a prospective registry of all patients with aspergillosis.

All microbiological specimens from Haematology patients are processed on a dedicated bench, so that liaison work and data/sample collection is facilitated. *Aspergillus* isolates with details of source are stored systematically.

Until 1996, the Haematology Department at the Royal Free Hospital had a policy of treating focal IPA, whenever possible, by resection of diseased lung tissue in addition to systemic antifungal therapy, thus providing a unique opportunity to study surgery as a treatment modality for aspergillosis, and to confirm preliminary diagnoses reached by other methods.

Finally, a small outbreak of *Aspergillus* infections involving 3 cases occurred on the private ward in 1993, and was attributed to the inadvertent distribution of non-irradiated pepper to patients following a change in caterers. This afforded a new opportunity for epidemiological study.

This study, which ended case recruitment in December 1995, aims to address the following issues:

1. To determine the positive predictive values of radiological diagnosis of IPA based on cases where such a diagnosis was verified histologically.

2. To study differential survival of patients with IPA according to radiological and microbiological features, treatment of underlying disease, and whether surgery was used as a therapeutic modality.

3. To seek correlations between the ability to isolate *Aspergillus* from resected lung tissue, the duration of pre-operative amphotericin B, and survival.

4. To evaluate the sensitivity of the combined procedure of *A.fumigatus* DNA extraction and PCR, in a series of spiking experiments using conidia or purified DNA, using the methods described by Spreadbury and by Tang, and to compare them.

5. To evaluate these PCR's in a series of *Aspergillus* culture negative BAL's from Haematology patients with pulmonary infiltrates and documented follow-up, and from controls undergoing bronchoscopy for other reasons.

6. To apply RFLP technology to whole genomic DNA, using the methodology described by Denning, and seek epidemiological correlations between isolates.

7. To determine whether A. *fumigatus* and A. *flavus* can be typed by means of RFLP and SSCP on PCR products from isolates and clinical samples, as this would enable future epidemiological studies in the absence of an isolate.

CHAPTER 2: MATERIALS AND METHODS

2.1. CLINICAL SUBJECTS

Prospective registries of patients with proven or probable aspergillosis are kept in the Microbiology, Haematology and Radiology Departments of the Royal Free Hospital. All Haematology patients entered on these registries between 1982-1995 with a diagnosis of pulmonary aspergillosis were selected to include cases of isolated IPA and cases where other organs were involved as well as the lungs. The diagnosis of IPA was made according to criteria listed in Chapter 3.

87 patients were identified, and data was collected from their clinical notes or discharge summaries on the few occasions when the notes were missing. A database was constructed on Microsoft Access, and analysed using the Access database. Additional statistical analysis was performed using the SPSS Inc. system version 9 (Statistical Package for the Social Sciences, Chicago, Illinois) in the Department of Primary Care and Population Sciences at the Royal Free and University College Medical School. Relevant survival curves were derived and additional graphs were constructed using the MS Draw software.

This clinical data is fully recorded and analysed in Chapter 3, and the Access database is shown in Appendix 1.

2.2. ANALYSIS OF SURVIVAL DATA

Survival analysis methods (Collett, 1995) were used in order to examine the association of various factors (explanatory variables) with the risk of death. These methods model the time to death, allowing for right censored data. For subjects who died during the follow-up period, the time to death is the number of days from the onset of symptoms/signs of IPA to the date of death. For subjects who remained alive at the end of follow-up, the time to death is said to be "right censored" and is recorded as the time the subject remains under follow-up (from the onset of symptoms/signs of IPA until loss to follow-up or September 1996). Standard statistical techniques that were developed for right-censored data were used. Kaplan-Meier survival plots were used for a graphical display of survival time compared between sub-groups. Cox proportional hazards regression models were used to examine how the survival time depended on each of several explanatory variables. The "hazard" is the instantaneous risk of death at any one time, given survival until that time. The hazard function for the ith individual is defined by the following equation:

$H_i(t)=\exp(b_1x_{1i}+b_2x_{2i}+b_3x_{3i})h_0(t)$

Where $h_0(t)$ is the baseline hazard function (the value of the hazard for an individual whose value of all explanatory variables are zero) and $x_1+x_2+x_3$ are the explanatory variables. Estimates from proportional hazards models are presented as hazards ratios with 95% confidence intervals. Proportional hazards models do not make assumptions about the specific shape of the hazard funtion, but they do assume that, for any variable considered, the hazard ratio (which

compares different levels of the variable) does not change over time. For example, a hazard ratio of 2 relating to male gender indicates that men are at twice the risk of death as women over the follow-up period. When several explanatory variable are included simultaneously in the model, the associated hazard ratios can be interpreted as the independent effect on the risk of death of each individual factor, adjusted for all other factors. For example, a hazard ratio of 1.5 for male gender in a model including both age and sex would indicate that, after accounting for differences in age between men and women in the sample, men were at 50% increased risk of death compared to women. The hazard ratio associated with sex might be reduced from 2 to 1.5 because the men in the sample tended to be older, and it was this fact that in part explained their increased risk of death. Statistical significance was assessed using the log likelihood ratio statistic. $P<0.05$ was considered statistically significant.

2.3. ISOLATES

Aspergillus species were isolated from clinical specimens by culture on Sabouraud's agar (see Appendix 2) at 30°C and 37°C for up to 10days. The species diagnosis was made by macroscopic examination of the colonies followed by light microscopy using the adhesive tape technique and lactophenol cotton blue. Isolates of *A. fumigatus* and *A. flavus* were stored as conidial suspensions in sterile distilled water at room temperature in the dark for up to 4 years. They originated from the following sources:

- Twelve *A. fumigatus* and eight *A. flavus* isolates from patients in the Haematology Unit at the Royal Free Hospital. These isolates included 13 (8 *A. fumigatus* and 5 *A. flavus*) which had been cultured from respiratory secretions or resected lung tissue of 8 patients included in the IPA database. The source and numbering of these isolates are detailed in Table 2-1 overleaf.

- One *A. fumigatus* isolate from a sachet of non-irradiated pepper served to Haematology patients (isolate 22).

- Two National Collections of Pathogenic Fungi (NCPF) strains of *A. fumigatus*, 2109 and 2140.

- Seven isolates of *A. fumigatus* kindly donated by Dr David Denning, and numbered 210, 233, 234, AF235, 237, 238, 239.No clinical data was given with the isolates.

Isolate number(s)	*Aspergillus* species	Source of isolate	Patient in IPA database ?
5	*A. flavus*	resected lung	yes
6 & 7	*A. fumigatus*	resected lung (of same patient)	yes
8	*A. fumigatus*	resected lung	yes
29	*A. flavus*	resected lung	yes
23a & 23b	*A. fumigatus*	sputum (of same patient)	yes
26 & 27	*A. flavus*	sputum (of same patient as 23a +23b)	yes
30	*A. flavus*	sputum	yes
31	*A. fumigatus*	sputum	yes
20	*A. fumigatus*	nasal secretions of patient with MLS	yes
21	*A. fumigatus*	retinal fluid of patient with MLS	yes
9	*A. fumigatus*	external ear	no
10 & 12	*A. flavus*	external ear (of same patient as 9)	no
15	*A. flavus*	sputum	no
16	*A. fumigatus*	splenic tissue of patient with myeloproliferative disease	no
17a & 17b	*A. fumigatus*	bone biopsy	no

Table 2-1: Summary of *Aspergillus* isolates from patients in the Haematology Unit.

Stored conidia from these isolates were cultured on Sabouraud agar plates when used for general purposes (e.g. species confirmation) or Sabouraud agar in tissue culture flasks (see section 2.5) at 37°C when used for the preparation of stock conidia for DNA extraction.

2.4. BRONCHOALVEOLAR LAVAGE FLUID (BAL) COLLECTION AND STORAGE

Approximately 3 ml of BAL fluid was retrieved from specimens prior to routine processing in the microbiology laboratory at the Royal Free Hospital. Ethics Committee approval was not sought for this procedure as the bronchoscopies were performed for standard clinical indications and the amount of sample was surplus to diagnostic requirements. The BAL fluid was distributed as 250 µl aliquots, using autoclaved (see Appendix 2 for all autoclaving procedures) pipette tips, into sterile 1.5 ml microcentrifuge tubes. The aliquoting was performed in the Class I hood of the respiratory specimen and tuberculosis section of the microbiology laboratory, and the microcentrifuge tubes were stored in a -70°C freezer immediately after labelling until required. The following samples were obtained:

- 39 *Aspergillus* species culture negative BAL's from 32 Haematology patients with lung disease (7 patients underwent bronchoscopy twice). All bronchoscopies had been performed for routine clinical reasons. Each sample is designated by a letter and a batch number selected randomly, and all experiments involving these specimens were performed in batches accordingly. This system is shown in Table 2-2, which also indicates whether the

corresponding patient is included in the IPA database, and what their likelihood of IPA is, according to the MSG criteria as outlined in section 1.10.1 (Denning *et al* 1994).
A "low" probability of IPA is indicated on Table 2-2 when a diagnosis other than IPA was made or likely.

Eight culture negative BALs were obtained from 7 patients included in the IPA database. One of them had undergone bronchoscopy twice, although she had a low probability of IPA when the first BAL was performed. In 3 patients, including the latter, IPA was proven histologically. Four had probable IPA. Low probability of IPA was registered in the donors of 19 other samples and possible IPA in the donors of 9 samples. These data are summarised in Table 2-2.

- 13 consecutive *Aspergillus* culture negative BAL's from patients outside the Haematology unit undergoing BAL for any reason other than suspected aspergillosis or HIV related lung disease. These samples were used for familiarisation with the *Aspergillus* DNA extraction/PCR procedure and for contamination experiments with known numbers/quantities of *Aspergillus* conidia or DNA if confirmed *Aspergillus* PCR negative. They are referred to as BAL I to XIII.

- 17 culture negative BAL's samples from volunteer asymptomatic HIV positive patients were kindly donated by Dr Mark Lipman. They had been stored at -70^{o}C and required thawing prior to the donation of a portion of the specimen. They were transported on ice from the immunology laboratory at the Royal Free Hospital to the microbiology laboratory of the same institution, immediately

aliquoted into sterile microcentrifuge tubes as described above, and re-frozen at -70°C until required.

Number	Batch	Letter	Probability of IPA	Within IPA Database?
1	1	A	probable	yes
2	1	B	low	no
3	1	C	possible	no
4	1	D	low	no
5	1	E	low	no
6	1	F	low	no
7	1	G	low	no
8	1	H	low	no
9	2	I	possible	no
10	2	A	low	no
11	2	B	proven	yes
12	2	C	low	yes
13	2	D	low	no
14	2	E	possible	no
15	2	F	low	no
16	2	K	probable	yes
17	3	A	possible	no
18	3	B	low	no
19	3	C	possible	no
20	3	D	low	no
21	3	E	possible	no
22	3	F	possible	no

Table 2-2: BAL specimens with their batch/letter designation

Number	Batch	Letter	Probability of IPA	Within IPA Database ?
23	3	G	low	no
24	3	H	possible	no
25	3	I	low	no
26	4	A	proven	yes
27	4	B	probable	yes
28	4	C	possible	no
29	4	D	low	no
30	4	E	low	no
31	4	F	low	no
32	4/5	G	proven	yes
33	4	H	possible	no
34	5	A	low	no
35	5	B	low	no
36	5	C	low	no
37	5	D	low	no
38	5	E	low	no
39	5	F	probable	yes

Table 2-2: BAL specimens with their batch/letter designation (Continued)

2.5. LUNG TISSUE

Two lung tissue samples (approximately 2 cm^3 each) from pneumonectomy specimens were stored at -70°C. One was *Aspergillus* culture negative but had histological appearances in keeping with aspergillosis. It was resected from the patient whose sputum yielded isolates 23a, 23b, 26 and 27. The other was from the patient reported in (Yeghen *et al* 1996), and *Chaetomium globosum* only had been cultured from it. The specimens were thawed and minced when required prior to PCR.

2.6. ENZYMATIC EXTRACTION OF DNA FROM ASPERGILLUS SPECIES (DENNING ET AL 1990)

This method was used for obtaining high molecular weight unsheared DNA suitable for RFLP analysis using *XbaI* or *SalI* restriction endonucleases.

1. Preparation of stock of conidia

Isolates were streaked onto a vented tissue culture flask (Schott Glass, Stafford, UK) containing Sabouraud medium and incubated at 37°C until a thick green carpet was observed. The time taken for this to occur varied from isolate to isolate (range 2 to 14 days). Five autoclaved (see Appendix 2) 0.5 cm glass beads (Sigma, Poole, UK) were added to the mycelium containing flask with 8 mls of autoclaved PBS (see Appendix 2)/ 0.5% Tween 80. The preparation

was mixed on a Luckham Rotatest shaker R100/TW for 1 hour at 50-60/min. This procedure was performed in a class I cabinet.

The conidial suspension was then counted in a haemocytometer and adjusted to approximately $2x10^8$/ml before storage in a bijou bottle sealed with Parafilm (American National Can^{TM}, Greenwich, Connecticut, USA) at room temperature in the dark.

2. Preparation of protoplasts

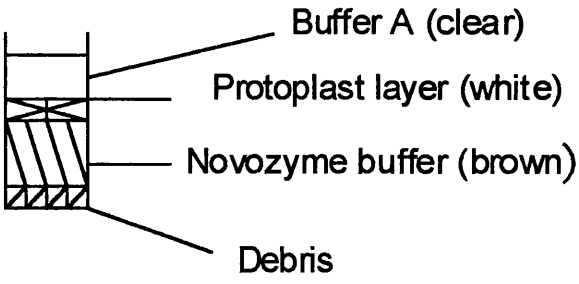
1 ml of conidial suspension was inoculated in 50 mls of Vogel's medium (see Appendix 2) contained in a sterile 250 ml conical flask, which was closed with aluminium foil, placed in a Gallenkamp Orbital incubator set at 40/min at 35°C and left for 16 hours. Non conidiating loose fungal balls were obtained. Approximately 4 such flasks were required per isolate in order to obtain 1-6 g of fungal elements for optimal DNA yield.

The contents of all cultures flasks from a single isolate were poured into a funnel lined with filter paper and emptying into a 250 ml conical flask with its side arm connected to a -15 mm Hg vacuum. The fungal elements ("pellet") were retained on the filter paper while the culture medium drained out. Each pellet was washed with a minimum of 10 mls of 0.6 M $MgSO_4$, using the same apparatus, the wash repeated once and all fluid allowed to drain out by vacuum before determining the pellet weight.

The weighed pellets were placed in sterile 30ml centrifuge tubes. For each 40mg of pellet, 900 µl of Novozyme buffer (see Appendix 2)

and 100µl of Novozyme solution (*Trichoderma harzianum* cell wall digesting enzyme [Interspex Products Incorporated, Foster City, California, USA], made up to 20mg/ml in Novozyme buffer) were added. The tubes were sealed with Parafilm and digestion allowed to proceed in a 33°C water bath for 1.5 to 5 hours, inverting tubes every 3-5 minutes, and checking for protoplast formation by microscopy before terminating incubation.

The protoplast suspension was carefully overlaid with 3ml of buffer A (see Appendix 2), and centrifuged in an MSE Centaur 2 centrifuge at 1000-1500g at room temperature for 15 minutes, to obtain the following result:



The protoplast layer was removed and transferred to a fresh tube, to which 4 ml of buffer B (see Appendix 2) was added. All transfers from this stage onward were performed using a plastic Pasteur pipette with its tip cut off to avoid shearing damage to the proplasts or DNA. The tubes were centrifuged at 800g for 10 minutes and the supernatant discarded. Pellets were resuspended gently in 10 mls buffer B, and centrifuged at 800g for 10 minutes. The supernatant was discarded and the procedure repeated once.

Each pellet was resuspended in 3 ml of buffer B and transferred to three 1.5 ml sterile microcentrifuge tubes before spinning at 800g in an MSE Micro Centaur centrifuge for 10 minutes and pouring off the buffer.

0.5 ml of buffer B was added to each tube. This preparation was either stored overnight at 4°C, or the next step started immediately. Before proceeding to DNA isolation the tubes were spun as above and the supernatant discarded.

3. DNA isolation

Lysing buffer (0.05M sodium EDTA, 2%SDS pH 8.5) was pre-heated to 70°C in a water bath. 0.5 mls of pre-heated buffer was added to each microcentrifuge tube of protoplast preparation. The contents were rapidly mixed by inversion and the tube transferred immediately to the 70°C water bath, incubated for 30 minutes, then centrifuged at 9000g for 5 minutes.

The supernatant was transferred to fresh microcentrifuge tubes and 5 µl of proteinase K (Sigma, 20mg/ml) added prior to incubation at 50°C for 1 hour.

The contents of each microcentrifuge tube were divided into two tubes containing 0.5mls of protoplast lysate each. An equal volume of TE equilibrated phenol (Camblab, Cambridge, UK) was added, the tube inverted gently then rotated slowly (10/min) on the Luckham Rotatest shaker for 10 minutes prior to centrifugation at 2000g for 10 minutes.

The aqueous phase was transferred to a fresh set of microcentrifuge tubes and an equal volume of chloroform added. The tubes were inverted gently a few times, then centrifuged at 2000g for 10 minutes.

Meanwhile, one end of a length of dialysis tubing, prepared according to the Manufacturers' instructions (see Appendix 2) then autoclaved, was double knotted and clipped. The tubing used was Sigma D0405, designed to retain most proteins of molecular weight 12,000 or greater. The aqueous phase from the microcentrifuge tubes was transferred to the dialysis tubing, which was then double knotted and clipped at the other end.

The DNA was dialysed for 4 days at room temperature against 2 litres of agitated TE buffer pH 7.5 (see Appendix 2), which was changed at least 7 times. The DNA preparations were stored at 4°C until further use.

2.7. QUICK ASPERGILLUS DNA EXTRACTION METHOD (MODIFIED FROM AUFAUVRE-BROWN ET AL 1992)

This is suitable for PCR methods; the DNA obtained may be slightly sheared.

10 drops of conidial suspension were inoculated into 5cm petri dishes containing 4mls of GYEP broth (see Appendix 2) each, and incubated for 24-48 hours at 37°C, until a thin green mycelium was

obtained. An adequate quantity of DNA was obtained by processing 2-3 mycelial mats per isolate.

The mycelia were drained on filter paper for 30 minutes, then cut roughly in quarters and put in sterile containers. They were frozen at -70°C for 30 minutes, which rendered the mycelium brittle and allowed efficient grinding in a freshly autoclaved mortar and pestle. This procedure was found to be more effective than the freezing in liquid nitrogen followed by vortexing with glass beads which is described in the original method. A thick green paste was thus obtained and divided between two 30mls Teflon centrifuge tubes (BDH Merck, Lutterworth, UK).

1.6 mls extraction buffer (200mM Tris-CL pH8.0. 0.5M NaCl, 0.01M EDTA, 1%SDS) was added per mycelium, and mixed well. Doubling the recommended amount of extraction buffer was found to enhance DNA yield.

An equal volume of phenol/chloroform/isoamyl alcohol (Camlab) was added with gentle mixing to create an emulsion and the mixture spun for 15 minutes at 8000g in an MSE Centaur 2 centrifuge. The procedure was repeated on the aqueous phase, then an equal volume of chloroform was added to the newly obtained aqueous phase and spun at 8000g for 15 minutes.

The aqueous phase was removed into a sterile Teflon tube and two volumes of 100% ethanol added. This was left for one hour at -20°C, then spun at 8000g for 30 minutes. The supernatant was discarded, the deposit rinsed with 70% ethanol, then the pellet air

dried, and resuspended in 1-1.5 mls of distilled water with 50μg/ml RNA ase before storing in a sterile microcentrifuge tube.

The preparation was refrigerated at 4°C until required and used promptly, otherwise stored at -20°C.

2.8. METHOD FOR DNA EXTRACTION FROM BAL (ACCORDING TO TANG ET AL 1993).

250μl of DNA extraction buffer (200mM Tris-CL pH8.0. 0.5M NaCl, 0.01M EDTA, 1%SDS), together with 5 μl of 20mg/ml proteinase K solution, was added to each 1.5 ml microcentrifuge tube containing 250 μl of BAL, shaken briefly, and incubated in a water bath at 65°C for 1 hour.

500 μl of Phenol/ chloroform/ isoamyl alcohol was added, mixed well, and spun at 9000g for 10 minutes. Supernatants were transferred into a fresh set of microcentrifuge tubes.

Using same procedure, each supernatant was extracted with 500 μl of chloroform, and supernatants transferred into fresh sets of microcentrifuge tubes.

1 ml of 100% ethanol was added to each tube, and left for one hour at -20°C then spun at 9000 g for 30 min, prior to discarding the supernatant, and washing with 70% ethanol.

The DNA was suspended in 15-30µl of sterile distilled water containing RNA ase 50 µg/ml before PCR. This DNA was processed for PCR both neat and at a dilution of 1 in 100.

2.9. MEASUREMENT OF DNA CONCENTRATION

The DNA concentration was estimated by comparative electrophoresis against a known amount of *HindIII* digest of phage λ DNA or by spectrophotometry of a 1 in 100 dilution at 260 nm. Absorbance at 260nm divided by absorbance at 280nm was used as an indication of the purity of the DNA preparations. The DNA purity is satisfactory if the ratio is approaching 2 (Sambrook *et al* 1989)

2.10.PCR METHOD 1: ASPERGILLUS FUMIGATUS RIBOSOMAL DNA PCR (SPREADBURY ET AL 1993)

This method utilises primers Af1 and Af2 diluted to 132µg/ml. All primers were obtained from British Bio- technology Products Ltd.

Af1: 5'-CCTTGGCTAGATTTGTTGGC-3'
Af2: 5'-TCAACCGACTCCCCTCAACC-3'

All PCR procedures were performed according to a strict 4 room strategy, where reagents and equipment were only moved from room 1 to 3 and never the reverse. The 4 rooms were as follows:

Room 1- PCR clean room used for preparation of the mastermix.
Room 2- PCR grey room for mixing of DNA template and mastermix.

Room 3- All handling of amplimers.
Room 4- DNA template preparation.

Each reaction mixture consisted of 10 µl of 10x reaction buffer (100mM Tris-Cl, pH 8.3, 500mM KCl, 15 mM MgCl, Bioline), 15 µ l glycerol, 0.25 mM each of nucleotide mixture (Promega, Southampton, UK), 2.5 units of Taq (Bioline, London, UK) and 5µl of each primer, made up to 90 µl with distilled water, and overlaid with 50µl of mineral oil.

10 µl of DNA template was added. Amplification took place in a HYBAID Omnigene III (Middlesex, UK) thermal cycler with an initial cycle of 5 minutes of denaturation at 95°C, 1minute 20s of annealing at 56°C, and 2 minutes of primer extension at 72°C, followed by 31 cycles of 40s denaturation, 1 minute 20s of annealing, and 2 minutes of primer extension with a 3 minute extension step in the final cycle.

A product of 401 bp is diagnostic of *Aspergillus fumigatus* by this method.

2.11.PCR METHOD 2: ASPERGILLUS FUMIGATUS AND FLAVUS ALKALINE PROTEASE GENE DNA PCR (TANG ET AL 1993)

The method uses primers Alp11 and Alp12 diluted to 132µg/ml (British Bio- technology Products Ltd).

Alp 11: 5'-AGCACCGACTACACTTAC-3'

Alp 12: 5'-GAGATGGTGTTGGTGGC-3'

All PCR procedures were performed according to the strict 4 room strategy described in 2.9.

Each reaction consisted of 10µl of 10x buffer (100mM Tris-Cl, pH 8.3, 500mM KCl, 15 mM MgCl, Bioline), 0.25 mM each of nucleotide mixture (Promega), 2.5 units of Taq (Bioline) and 5µl of each primer, made up to 90 µl with distilled water, and overlaid with 50µl of mineral oil.

10 µl of DNA template was added. Amplification took place in a HYBAID Omnigene III thermal cycler with 42 cycles of 30s of denaturation at 94°C, 45s of annealing at 63°C, and 2 minutes of primer extension at 72°C.

A product of 747 bp is diagnostic of *Aspergillus fumigatus* and of 690 bp of *Aspergillus flavus*.

2.12.DNA PURIFICATION

PCR products (obtained by method 1 or method 2) of available *A.fumigatus* and *A.flavus* strains were purified prior to SSCP or restriction endonuclease digestion with *DdeI*/SSCP, using Promega Wizard™ PCR Preps DNA purification System for Rapid Purification of DNA Fragments. A summary of the Manufacturers instructions is given in Appendix 3.

2.13.RESTRICTION ENDONUCLEASE DIGESTION OF GENOMIC DNA BY XHOI AND SALI

Restriction digests with *SalI* and *XhoI* (both supplied by Promega) of whole high molecular weight DNA obtained by the enzymatic technique described above were attempted for the purpose of typing isolates, according to the method described by Denning *et al.* These attempts were largely limited by failure to obtain DNA of sufficient concentration and/or molecular weight (see Chapter 6). They were performed as follows:

All the DNA available for an isolate (400-700 µl of 1-50 µg/ml DNA was mixed with 2-3 µl (24-32 units) of restriction enzyme, and with the calculated amount of x10 buffer (buffer D for both *SalI* and XhoI, see Appendix 2) supplied by manufacturers to provide an appropriate salt environment, in autoclaved microcentrifuge tubes. The mixture was incubated at 37°C for 3 hours in an incubator.

Half a volume of 7.5M ammonium acetate was added, followed by 2 volumes (DNA + ammonium acetate) of 100% ethanol kept at -20°C, dividing the DNA to be precipitated into 2 microcentrifuge tubes if necessary. The tubes were left at -20°C for 1 hour, then spun at 9 000g for 5 minutes, before discarding the supernatant, and washing with 70% alcohol.

The DNA was dried at 37°C for 1 hour and resuspended in 20 µl of distilled water (10 µl each microcentrifuge tube then combined if 2 used).

The restricted DNA was run immediately on a 0.7% agarose gel (see section 2.14).

2.14.RESTRICTION ENDONUCLEASE DIGESTS OF PCR PRODUCTS OBTAINED BY METHOD 2 BY DDEI AND STYI

The sequence of the 747 bp product obtained by PCR Method 2 on *A. fumigatus* DNA was determined by performing a nucleic acid query on the EMBL (European Molecular Biology Laboratory) database. Restriction sites were determined by sequence analysis using GCG (Genetics Computer Inc) version 7. Dr Louise Newport and Dr Vince Emery kindly performed these searches. *DdeI* and *StyI* were found to yield two fragments of similar size, and were used to determine whether restriction fragment length polymorphisms exist on the PCR products from different isolates. Both restriction endonucleases were supplied by Promega. Variability was verified both by direct visualisation of DNA under ultraviolet light on ethidium bromide stained gel, and by performing SSCP on the restricted PCR products (see below). The restriction procedure was as follows: PCR products to be restricted were purified in the first instance as outlined in section 2.11 and Appendix 3, yielding 50 µl of purified product.

30µl of purified product, 3.45 µl of appropriate buffer (x10 buffer F for *StyI*, and x10 buffer D for *DdeI*, as supplied by Promega, see Appendix 2) and 1µl of corresponding enzyme (12units) were mixed together in autoclaved microcentrifuge tubes and incubated for 3 hours at 37°C. The restriction digests were then ready to be

visualised on ethidium bromide stained agarose gel or to be used in SSCP.

2.15.ELECTROPHORESIS OF DNA, VISUALISATION AND PHOTOGRAPHY

All DNA extractions, PCR reactions and restriction enzyme digests were visualised under UV light after electrophoresis of DNA of interest on ethidium bromide stained agarose gel, and run against an appropriate molecular weight ladder standard, then photographed with a Polaroid camera. A 40% sucrose and 0.25 % bromophenol blue in water loading dye (2µl for every 10 µl of DNA unless stated otherwise) was used to allow DNA to sink into the agarose wells. Gels used were as follows:

- 100 mls of 1.5% agarose in TBE (see Appendix 2) buffer with 2 µl ethidium bromide at 80V for 1 hour, with Phage λ digested with *HindIII* (2µl containing 1 µg) as molecular weight marker. This was appropriate for most DNA preparations (unless visualisation of high molecular weight DNA is important), and for calculating DNA concentration by comparative electrophoresis.

- 100 mls of 1.5% agarose in TBE buffer with 2 µl ethidium bromide at 80V for 1 hour, with 2µl pGEM as molecular weight marker for PCR products and *StyI/DdeI* restriction digests.

- 5mm thick 0.4% agarose in TBE buffer with 2 µl ethidium bromide at 25 V for 14 hours when visualisation of high

molecular weight DNA was needed. A high molecular weight marker scale was used for this purpose (Gibco, Paisley, UK, 2 ng in 5 µl), and required heating at 65 ^{o}C for 10 minutes immediately before loading.

- 5mm thick 0.7 % agarose in TPE (see Appendix 2) buffer with 2 µl ethidium bromide at 25 V for 14 hours when visualisation of *XhoI/SalI* restriction digests was required. The Gibco high molecular weight marker scale was also used for this purpose. A coomb with wider lanes was used for this procedure, where 20 µl of DNA solution was obtained, and 5 µl of loading dye was therefore necessary.

2.16.SSCP METHOD

SSCP was evaluated as a typing technique for *A. fumigatus* isolates by performing SSCP on PCR products obtained by method 1 and method 2 for all isolates available, after purification of the products using Wizard Prep (see section 2.11 and Appendix 2). SSCP was also performed on purified *A.flavus* PCR products by method 2, and on DdeI restricts of purified *A. fumigatus* and *A. flavus* PCR products by method 2.

The glass plates of a Life Technologies S2 vertical gel rig were cleaned with alcohol. The small plate was wiped with Bind Silane (Promega, UK) mixture (see Appendix 2), then with 95% ethanol, in order to promote adhesion of the polyacrylamide gel to the plate. The large plate was wiped with Sigma Cote, then a tissue. The plates

were assembled, coated surfaces facing each other, and the sides and bottom bound with strong tape.

An MDE (Mutation Detection Enhancement) polyacrylamide analog gel was prepared as shown in Appendix 2. This was used to load the plates, a coomb was added, and the sides of the plates clamped. The gel was left to set for 1 hour, inspecting frequently for leaks, and topping up as necessary. All clamps and the coomb were removed, as was the bottom tape to allow contact with the buffer.

The plates were set vertically in the tank, and 0.6% TBE (see Appendix 2) buffer was added to the top and bottom of the tank. The current was switched on and left at 6W until required. For each lane, the following mixture was prepared in a 0.5 ml labelled microcentrifuge tube: 7 µl purified DNA, 3µl of x4 SSCP buffer (see Appendix 2), 2 µl formamide stock dye. The preparation was heated at 80^{o}C for 5 minutes in the HYBAID Omnigene III thermal cycler, transferred to an ice bucket and loaded immediately onto gel after switching off.

Only 4-6 lanes were loaded at a time to avoid diffusion, the apparatus was then switched on to 6 W until the dye was seen 1 mm below the well, after which it was switched off ready for the next set of lanes to be loaded. The gel was run at 6 W for 16 hours.

2.17.PROMEGA SILVER STAINING METHOD FOR MDE GELS

The plates were separated, and the gel immersed in stop/fix solution (see Appendix 2) and rotated for 40 minutes on a Lukham Rotatest

Shaker R100/TW at 20/min. The gel was removed from the stop/fix solution, which was reserved for future use.

The gel was rinsed 3 times, for 2 minutes at a time, in distilled water, then immersed in staining solution (see Appendix 2), rotated for 30 minutes at 20/min and rinsed briefly for 5-10 seconds.

The developer (see Appendix 2) was added, and the tray agitated gently by hand while observing closely for the appearance of bands. The developer was discarded and the stop/fix solution added immediately the required bands appeared, as the developing process proceeds rapidly from this point, and progresses further by the time the gel is adequately immersed in stop/fix solution. The gel was left in stop/fix solution for 30 minutes, then removed and dried overnight prior to photography.

2.18.PHOTOGRAPHY OF MDE GEL

A permanent image was made using APC Kodak photographic paper.

CHAPTER 3: CLINICAL DATA

3.1. BACKGROUND

The 87 patients entered into the IPA database from the institutional aspergillosis registries had pulmonary aspergillosis alone or in conjunction with other sites. One patient had severe aplastic anaemia, and the rest suffered from haematological malignancies. Operable patients with MLS and "halo sign" lesions were mostly referred for resection because of the risk of haemoptysis and the prospect of cure with excision of diseased tissue. Resections were generally performed in the absence of prior supportive culture or biopsy evidence. In order to optimise the detection of such lesions, CT scans of the thorax, previously requested sporadically, have been performed routinely since 1990 in all febrile neutropenic patients with normal chest X-ray and no response to broad spectrum antibiotics after 48 hours. The MLS, cavitating lesions and "halo sign" lesions are grouped together for convenience for the purpose of this text and referred to as Lesions with Imaging Suggestive of Aspergillosis (LISA).

The IPA database was examined, with the aim of establishing:

- The positive predictive values for IPA of radiological diagnosis LISA, based on cases with subsequent histological confirmation post-operatively.
- The operative details and complication rates of those whose IPA was treated surgically.

- A comparison of outcomes in patients with IPA according to demographic and diagnostic criteria, and to treatment modalities, with a particular view to assessing the influence of surgery on prognosis.
- The relationship between isolation of *Aspergillus* from resected lung tissue and the duration of pre-operative treatment with amphotericin B.

Whereas the criteria for IPA used in the literature are variable with preferential reporting of cases where there is a tissue diagnosis, we applied the following definitions, intended to reflect the spectrum of the disease in clinical practice:

- Patients whose lung tissue (obtained at lobectomy, biopsy, or post mortem) shows evidence of septate hyphae with dichotomous branching at 45°, whether or not *Aspergillus* is isolated from any source at any stage. These patients are said to have, respectively, a "post -operative histology diagnosis", a " biopsy diagnosis" or a "PM diagnosis".

- Patients at risk of IPA with radiological detection alone of at least one LISA in the chest and fever not responding to antibiotics. They are classified as having a "radiological diagnosis".

- Patients with clinical course and radiological appearances in keeping with IPA, and *Aspergillus* cultured from at least one sputum or BAL sample (routinely incubated on Sabouraud medium at 37°C for a minimum of 10 days) but with no subsequent histological confirmation. In otherwise convincing

pulmonary cases, positive throat or nasal swabs for *Aspergillus* are also taken as supporting evidence for IPA. These patients are classified as having a "microbiological diagnosis".

- Patients with clinical course and radiology appearances compatible with IPA, and positive cytology for *Aspergillus at* a relevant site, but no subsequent histological confirmation. They are classified as having a "cytology diagnosis".

Three of these diagnostic categories are clearly based on different clinical presentations of IPA: "post-operative histological diagnosis" represents all patients with LISA (therefore likely to be *Aspergillus* culture negative) who underwent surgery, "radiological diagnosis" represents all patients with LISA who were culture negative and did not undergo surgery, and "microbiological diagnosis" represents a group of patients unlikely to have LISA or be operable. The percentage actuarial survival at various times for these diagnostic categories, and for the biopsy and cytology categories grouped together was determined, as well as number of deaths from all causes, number of deaths from IPA, and follow-up data. Corresponding survival curves were drawn.

The IPA survival time was taken as the date of onset of symptoms /signs leading to the diagnosis of IPA until the date of death, loss to follow-up, or September 1996, whichever was the sooner.

The impact of the following factors on death from all causes was assessed using Cox's proportional hazards survival analysis models: age, sex, radiological appearances, isolation of *Aspergillus* from

respiratory secretions, previous haematological treatment (BMT or not), status of haematological disease (relapsed or not) and whether IPA was treated /treatable surgically. These models were also used to adjust for several factors simultaneously in order to determine which factors were independently predictive of death. Results are presented as relative risks with 95% confidence intervals. Statistical significance was assessed using the likelihood ratio statistic. Survival curves comparing different levels of each individual factor were constructed using the Kaplan-Meier technique.

3.2. DEMOGRAPHY AND PREVIOUS HAEMATOLOGICAL TREATMENT

35 females (F) and 52 males (M), age range 13-76 years were included in the IPA database. Thirty-two patients (11F, 21M, age range 16-62 years) had undergone allogeneic bone marrow transplantation (allo-BMT) before developing IPA. Five of the allo-BMT patients had received volunteer unrelated donor (VUD) transplants. The grafts of four patients had failed before they developed IPA. A further five allografted patients had relapse of their original disease. They were being treated again at the time of developing IPA. Four patients (2M, 2F, ages 56, 27, 49 and 50) had undergone autologous bone marrow transplantation (auto-BMT) before developing IPA. Two of these were being treated for relapsed leukaemia, post auto-BMT, when they developed IPA.

The remaining 51 patients (21 F, 30 M, ages 13-76 years) had received chemotherapy alone (n=47), or no treatment (n=4), when they developed IPA. Ten of these patients proceeded to undergo

bone marrow transplantation after their aspergillosis was treated (1VUD, 2 auto-BMT, 7 sibling allo-BMT).

These data are summarised in Table 3-1.

	Total	Males	Females	Age range	Age median
Total	87	52	35	13-76	36
Allo-BMT	32	20	11	16-62	29.5
Auto-BMT	4	2	2	27-56	49.5
Chemotherapy or no treatment	51	30	21	13-76	34

Table 3-1: Demography and Previous Treatment

3.3. POSITIVE PREDICTIVE VALUES OF RADIOLOGICAL DIAGNOSIS OF IPA BASED ON HISTOLOGICAL/ MICROBIOLOGICAL FINDINGS IN RESECTED LUNG TISSUE.

In all surgical cases, the presumptive diagnosis based on radiology was IPA, so that histological and microbiological analysis of resected samples has enabled us to establish the predictive values of our radiological diagnosis. The data is summarised in Table 3-2.

Of 59 patients with LISA, 39 underwent resection, following which aspergillosis was confirmed in 35 cases. Alternative infectious causes were found in two of the remaining cases (*Chaetomium* species in one, and *Mycobacterium Avium Intracellulare* (MAI) in the second). Although no pathology was identified in a third patient and only adhesions with loculated fluid were found in a fourth, IPA remained the likely explanation for their lung disease, thus leaving 57 patients with LISA due to proven or probable IPA.

Of note, *Aspergillus* was cultured from respiratory tract samples or nasal swabs in only eight of these 57 patients (14 %), including pre-operatively in five of the 35 cases (14%) subsequently confirmed histologically. The positive throat swab was taken from the patient with LISA found to have a sterile loculated pleural effusion at thoracotomy.

The positive predictive value of LISA detection for IPA is thus 90 %, as two out of 39 cases were "misdiagnosed", and two not confirmed. In practical terms, however, the patient with the *Chaetomium* infection had the same treatment requirements as a case of IPA and the patient with the MAI infection also benefited from lung resection.

The decortication in the patient with a loculated pleural effusion had diagnostic and therapeutic intent, but one patient did undergo an unnecessary thoracotomy, where no lesion was identified and no tissue resected. This was 12 days after the initial CT scan diagnosis. She had received amphotericin for the 24 days leading to surgery. A

repeat CT scan 11 days post surgery confirmed resolution of the lesion, in keeping with late success of amphotericin B treatment.

The negative predictive value of LISA is more difficult to establish, but is assumed to approach 100% with CT scanning, as there have been no post-mortem reports of such lesions that were not reported in life.

	Number of Patients with LISA	Supportive Culture /Biopsy at Detection of LISA	IPA Confirmed on Resected Tissue/PM	Other diagnosis Confirmed on Resected Tissue/PM
Resection	39	3 +ive sp. cultures 1 +ive nasal swab 1 +ive throat swab 1 +ive lung biopsy + sp. 1 fungal elements seen on BAL (no growth)	35	1 *Chaetomium* 1 MAI 1 loculated adhesions, decorticated, nil focal. 1 no pathology found
No Resection	20	1 +ive facial biopsy 1 +ive sub carinal biopsy 2 +ive sp. cultures 1 +ive nose swab 1 +ive pleural cytology	2 at post-mortem	1 negative lung bx
Total	59*	14	37	5

Table 3-2: Surgery, Histology, and Microbiology in 59 patients with LISA

*The two patients with *Chaetomium* and MAI are not included in Table 3.3 or in the general IPA data but are featured in Table 3.2 to demonstrate the numbers of false positives. The number of "patients with LISA " is thus 57 in Table 3.3 but 59 in Table 3.2.The two patients with LISA operated on and not found to have focal IPA at thoracotomy are both likely cases of IPA, and are included in all tables.

Sp.=sputum.

3.4. BACKGROUND AND OPERATIVE DETAILS OF PATIENTS WITH HISTOLOGICALLY DOCUMENTED IPA POST RESECTION.

The demographic details and prior haematological treatment of the 35 patients who had IPA confirmed histologically after surgery (with or without microbiological isolation of *Aspergillus*) are included in Table 3-3.

Nine of these patients had developed IPA after bone marrow transplantation, of which only two were uncomplicated sibling allo-BMT's. Twenty- five patients developed IPA after receiving chemotherapy alone, and one patient with newly diagnosed myelodysplasia had received no cytotoxics. Eight subsequently underwent bone marrow transplantation (1 auto, 7 allo) without relapse of their aspergillosis. These patients had also received additional prophylaxis with amphotericin B to cover the transplant procedure.

Four patients underwent partial lobectomies (including a patient who had a partial pleurectomy simultaneously), 11 multiple lobectomies/ segmentectomies, 12 right upper lobectomies, two right lower lobectomies, 2 left upper lobectomies (including the patient whose naso-pharyngeal aspergillosis was debrided at the same time), two left lower lobectomies and two pneumonectomies.

	TOTAL NUMBER	F	M	AGE RANGE (MEDIAN)	PRIOR ALLO-BMT	PRIOR AUTO-BMT	PRIOR CHEMOTHERAPY OR NO RX	NUMBER LISA
HISTOLOGICAL DIAGNOSIS FOLLOWING RESECTION	35	14	21	13-76 (31)	7	2	26	35
RADIOLOGICAL DIAGNOSIS ONLY	12	8	4	13-58 (37)	3	1	8	12
LISA, RESECTION, NO DIAGNOSIS.	2	1	1	41-43 (42)	2	0	0	2
MICROBIOLOGICAL DIAGNOSIS ONLY	21	7	14	15-55 (29)	10	1	10	3

Table 3-3: Demography and previous Haematological Treatment according to Diagnostic Category and whether Resected.

	TOTAL NUMBER	F	M	AGE RANGE (MEDIAN)	PRIOR ALLO-BMT	PRIOR AUTO-BMT	PRIOR CHEMOTHERAPY OR NO RX	NUMBER LISA
CYTOLOGICAL DIAGNOSIS ONLY	5	3	2	18-68 (62)	2	0	3	1
BIOPSY DIAGNOSIS ONLY	3	1	2	17-38 (33)	1	0	2	2
PM DIAGNOSIS	9	1	8	15-62 (37)	7	0	2	2
TOTAL	87	35	52	13-76 (36)	32	4	51	57

Table 3-3: Demography and previous Haematological Treatment according to Diagnostic Category and whether Resected (Continued)

3.5. COMPLICATIONS AND RECURRENCE OF IPA FOLLOWING SURGERY

Eighteen patients had an uncomplicated post-operative course, including the only one whose surgery was performed as an emergency for massive haemoptysis. Three patients had postoperative haemorrhage, and one required a repeat thoracotomy to control it. One wound, and one non-fungal chest infection were also seen post operatively. In another case, *Aspergillus* infected tissue was accidentally spilt in the thoracic cavity at operation, with no subsequent ill effects.

Two patients underwent revision pneumonectomies after initial lobectomies for progressive *Aspergillus* infection. One of them (with relapsed refractory AML) died soon afterwards and the other is a long-term survivor. Two patients died of microbiologically proven *Aspergillus* pneumonia 3 and 8 days post operatively. Another died of disseminated aspergillosis (with *A. fumigatus* grown from a vitreous biopsy) 18 days post-operatively. Three further patients died of respiratory failure 1 to 10 days post-operatively. Another died of sepsis, with no organism grown, 4 days post-operatively. All four of these early post-operative deaths were presumed due to progression of IPA.

Finally, two patients died of unclassified interstitial pneumonitis post allo-BMT/MUD, one at 27 days post-operatively, and one at 52 days. Although radiologically not typical, recurrent IPA cannot be excluded.

The post-operative complication rate was 9/26 (35%) in those who had not undergone BMT prior to developing IPA, while all but one of the BMT patients (89%) suffered from post-operative complications.

3.6. REASONS FOR NOT OPERATING ON PATIENTS WITH RADIOLOGICAL AND MICROBIOLOGICAL DIAGNOSIS

We examined the reasons for not operating in the "radiological diagnosis " patients, and also in those with a "microbiological diagnosis" who did not have diffuse lung disease.

The reasons why the 10 patients with "microbiological diagnosis" but absence of diffuse lung disease did not undergo a surgical procedure are as follows: one patient (the only one with a single LISA) improved and survived, two had disseminated aspergillosis, three had refractory haematological disease, and four deteriorated very rapidly, including one patient who had initially improved.

The 12 patients with "radiological diagnosis" did not undergo surgery for the following reasons: one had multi-organ failure, one improved, six had untreatable haematological malignancies (of whom two had stable aspergillosis until their death from other causes), and four had multiple lesions precluding operation, all but one of whom made complete recoveries.

Thus only three patients were operable but did not undergo operation.

3.7. SURVIVAL

Table 3-4 represents the survival data of patients according to diagnostic category, and is represented in the form of a survival curve in Figure 3.1. Both show a worse survival at all points in patients with "microbiological diagnosis", with a high proportion of early deaths. The survival at 2 years is only 5%, which represents the only patient with a single LISA and positive *Aspergillus* culture from the nose rather than from lower respiratory secretions. Of all the deaths in this group, 81% were due to IPA.

Table 3-4 and Figure 3-1 also shows the better survival of the two groups constituted exclusively of patients with LISA, namely the "post-operative" group with 36% actuarial survival at two years and the "radiological diagnosis" group with 21%. The percentage of deaths from IPA was very similar in these groups at 32% and 33% respectively.

Table 3-5 and Figure 3-2 show Cox's Proportional Hazards analysis of the impact of various individual factors on death from all causes, and corresponding survival curves. The adjustments for several factors simultaneously are shown in Table 3-6.

Analysis by individual risk factor reveals a non-significant trend towards increased risk of death with older age group, with haematological relapse and with BMT as a previous haematological treatment (presumably reflecting the worse immune status of transplanted patients).

	Total number	Number died	Deaths from IPA	Number LFU*	Median FU (Days)	Range FU (Days)	% Surv** 2/52	%Surv 1/12	%Surv 3/12	% Surv 12/12	% Surv 2 years
Radiological diagnosis only	12	8	4	3	77	30-1243	100	91	31	20	20
Microbiological diagnosis only	21	20	17	0	13	0-982	45	29	9	5	5
Cytology/Bx diagnosis only	8	5	4	2	106	3-1469	87	58	44	29	14
Post-resection: Histology diagnosis in 35/37#	37	22	12	10	81.5	9 - 3538	94	89	56	45	36
All patients	87	64	44	15	46	0-3538	79	67	41	26	26

Table 3-4: Survival by diagnostic category

* LFU: lost to follow-up (FU: follow-up).

** Surv: survival.

#: Excluding the patient with *Chaetomium* and the patient with MAI but including the patient with normal thoracotomy and the patient with sterile loculated effusion.

Therefore "histological diagnosis post resection" in 35/37 patients.

	Criterion	Number	Unadjusted RH (95% CI*)	LRS** χ^2(df)# and p value
Age	<30 years	41	1	1.6 (2);
	30-49 years	28	1.1 (0.6-1.9)	p=0.45
	≥50 years	18	1.5 (0.8-2.9)	
Sex	Female	35	1	6.8 (1)
	Male	52	2 (1.2-3.4)	p=0.009
Isolate grown	No	55	1	9.7 (1)
from sputum	Yes	32	2.2 (1.4-3.7)	p=0.002
BMT before	No	51	1	2.2 (1)
infection	Yes	36	1.5 (0.9-2.4)	p=0.14
Radiology findings	LISA	57	1	29.2 (2)
	No (diffuse)	12	5.2 (2.6-10.3)	p<0.001
	No (other)	18	4.0 (2.2-7.1)	
Operable	Yes	40	1	25.6 (2)
	No (bulky IPA)	12	6.3 (3.0-13.3)	p<0.001
	No (other)	35	2.9 (1.6-5.0)	
Operated	Yes	37	1	11.1 (1)
	No	50	2.4 (1.4-4.0)	p<0.001
Relapsed	No	45	1	2.0 (1) p=0.16
haematology	Yes	41	1.4 (0.9-2.4)	

Table 3-5: Impact of individual factors on deaths from all causes expressed as unadjusted relative hazards (RH).

* CI: confidence interval.

**LRS: Likelihood ratio statistic.

#df: degrees of freedom.

	Criterion	Adjusted RH (95% CI*)	LRS** χ^2(df)# and p value
Age	<30	1	
	30-49	0.8 (0.5-1.6)	3.0 (2) p=0.22
	≥50	1.6 (0.8-3.3)	
Sex	Female	1	1.7 (1) p=0.19
	Male	1.5 (0.8-2.7)	
Isolate grown from sputum	No	1	0.7 (1) p=0.38
	Yes	1.4 (0.7-2.7)	
BMT before infection	No	1	2.0 (1) p=0.15
	Yes	1.5 (0.8-2.8)	
Radiology findings	LISA	1	
	Diffuse	4.1 (1.4-12)	11.7 (2) p=0.003
	Other	4.2 (1.8-9.7)	
Relapsed haematology	No	1	7.4 (1) p=0.007
	Yes	2.3 (1.3-4.3)	
Operated	Yes	1	0.1 (1) p=0.75
	No	1.1 (0.5-2.3)	

Table 3-6: Adjustment of several factors simultaneously on death from all causes expressed as adjusted relative hazards (RH).

* CI: confidence interval.

**LRS: Likelihood ratio statistic.

#df: degrees of freedom.

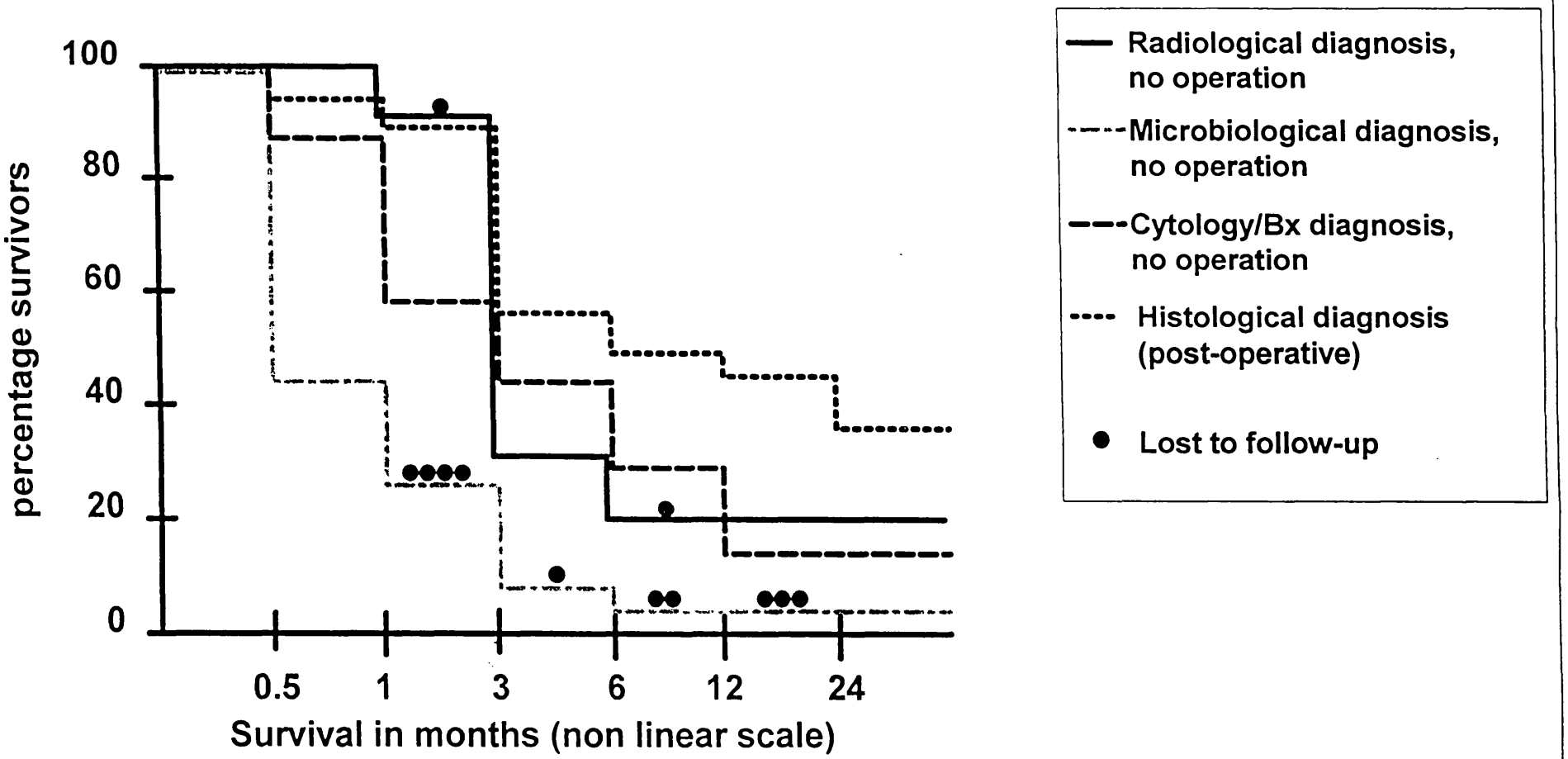


Figure 3-1 : Survival according to method of diagnosis, and to whether IPA was treated surgically

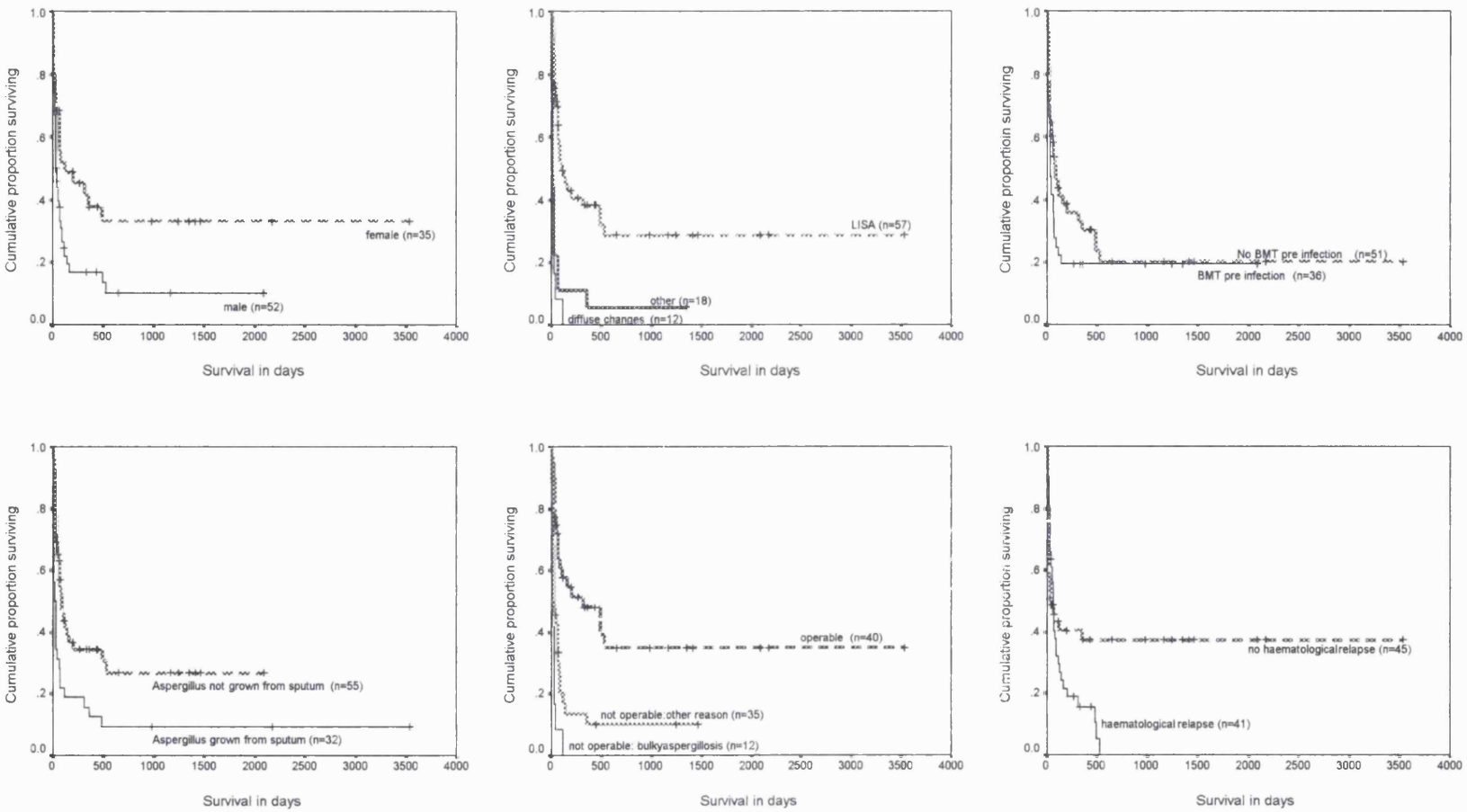


Figure 3-2: Kaplan – Meier Curves representing the impact of various individual factors on death from all causes

On the other hand, compared with radiological presentation as LISA, other radiological changes are strongly associated with increased risk of death, particularly diffuse infiltrates. Having undergone pulmonary resection for IPA and being operable (although only three patients were operable but did not undergo surgery) also considerably decreased the risk of death.

Aspergillus isolate from sputum and male sex were associated with about a twofold increased risk of death but these relative risks were attenuated and no longer significant when several factors were adjusted for simultaneously. In this adjusted model, the only factors which had a significant independent effect on risk of death were radiological presentation and haematological relapse. Haematological relapse more than doubled the risk of death but its effect was not apparent in unadjusted analysis due to the higher proportion of relapsed patients who had LISA rather than other X-ray presentation.

As radiological findings and pulmonary resection were very strongly correlated, it is not surprising that these two variables were not independent predictors of mortality. In the adjusted model, radiological appearances remained strongly predictive of death, with findings other than LISA increasing the risk by fourfold, whereas the relative risk associated with not undergoing resection was greatly reduced. This seems to suggest that the apparent favourable prognosis of patients undergoing resection is due to their underlying form of IPA rather than to the surgery itself. However it is difficult

to disentangle the effects of these two factors in this study due to the fact that nearly all operable cases underwent surgery.
The outcome in a group of eight patients whose IPA was treated with lung resection prior to bone marrow transplantation (allogeneic in seven, autologous in one) is worth noting nevertheless. Their actuarial survival was high at 71% at 2 years. Both deaths were from leukaemic relapse and none of these eight patients suffered a relapse of their aspergillosis.

3.8. *ASPERGILLUS* ISOLATES FROM RESECTED LUNG AND CORRELATION WITH DURATION OF AMPHOTERICIN B PRE RESECTION

Aspergillus was isolated from lung tissue obtained at operation in 14 out of 26 patients (54%) with a "histological diagnosis post resection" and no positive *Aspergillus* culture preoperatively whose tissue was submitted to the microbiology laboratory. The operative lung samples were not submitted for microbiology in four patients, and cultures from the remaining 12 were sterile (46%).

In addition, five patients with "histological diagnosis post resection" had *Aspergillus* isolated from sputum or nose swab prior to operation. The resected lung was culture positive for *Aspergillus* in four of these patients, and culture negative for the fifth.

In the five cases with pre-operative isolates, three (60%) were *A.fumigatus,* one (20%) *A. flavus,* and one (20%) a combination of *A. fumigatus* and *A. flavus.*

In the 14 patients with no pre-operative *Aspergillus* isolate, but positive *Aspergillus* cultures from resected lung, *A. fumigatus* was cultured from 12 (80%), and A. *flavus* from three (20%).

The 14 patients with no pre-operative *Aspergillus* isolate but *Aspergillus* growth from resected lung samples received Amphotericin B for 0-29 days (median 15 days, mean 13.7 days) before surgery. The 12 patients without *Aspergillus* isolates from resected lungs received Amphotericin B for 0-45 days (median 12.5 days, mean 16 days).

There is no statistical difference in the duration of amphotericin B treatment between these two groups ($p>0.5$, Student's t test).

3.9. CONCLUSIONS

Thus radiological detection of LISA with high resolution CT scanning has a positive predictive value of 90% for IPA in cases where histological verification took place. This suggests that cases of IPA diagnosed in this manner could usefully be included in epidemiological studies, and in trials of antifungal agents.

Surgical resection of LISA is both feasible and safe in a population of patients with haematological malignancies. The procedure, in conjunction with prophylactic amphotericin B, prevented relapse of IPA in 100% of cases operated prior to BMT, with actuarial survival of 71% at two years.

All but 3 of our patients eligible for surgery underwent pulmonary resections. The actuarial survival of patients having surgery for LISA is 89% at one month and 36% at two years, whereas that of patients with "radiological diagnosis", i.e. with LISA but inoperable, was 91% at one month, and 20% at two years. Although the patients treated surgically appear to have a somewhat better prognosis, analysis of the data with adjustment for several factors simultaneously demonstrates that it is likely that presentation of IPA as LISA is the most influential factor.

IPA presenting as LISA has in itself a good prognosis, and this, rather than any benefit conveyed by the operation itself, may be the reason why surgical resection of such lesions appears to improve survival. A randomised trial would be necessary to determine the respective advantages of conservative treatment versus surgical plus systemic antifungal treatment in operable cases of IPA.

Aspergillus is only occasionally isolated from respiratory secretions of patients with LISA (14%). Also isolation of *Aspergillus* from sputum is significantly associated with death. This significance is lost when analysis is adjusted for other factors, and radiological appearances different from LISA become most significantly associated with death. The isolation of *Aspergillus* from secretions thus only appears to be a poor prognostic indicator because of its association with cases not presenting as LISA.

Finally, *Aspergillus* is isolated from 54% of lung tissue of patients with histological aspergillosis, where the organism had survived despite a mean of 13.7 days of amphotericin B treatment. The

duration of pre-operative treatment with amphotericin B is not significantly different in those whose resected lung with histological aspergillosis was sterile. Poor penetration of amphotericin B into tissues or partial resistance of the organism to the drug are possible explanations.

Resection of LISA is an accepted treatment when haemoptysis threatens or occurs, and whenever a definite diagnosis is needed, as when anti-fungal drugs may be hazardous. Based on the findings presented here, it also appears beneficial prior to bone marrow transplantation. The merits of surgery in other circumstances are not clear.

CHAPTER 4: SENSITIVITY OF TWO POLYMERASE CHAIN REACTIONS AND *ASPERGILLUS* DNA RECOVERY FOLLOWING EXTRACTION AND PCR COMBINED

4.1. BACKGROUND

When the work described in the next two chapters started in September 1993, only two *Aspergillus* specific PCR's had been described, namely the method using primers based on the 26S/intergeneic spacer region of the rDNA complex (Spreadbury *et al* 1993) and the first method using primers based on the alkaline protease gene (Tang *et al* 1993). These two assays were used exclusively for the experiments to follow. They are referred to as Method 1 and Method 2 respectively, and were said to have respective sensitivities of 1pg (equivalent to 20 genomes based on the size of *A. nidulans*) and 5pg (100 genomes) by UV visualisation for *A. fumigatus* in the original publications.

The sensitivity of these methods could be increased further to 100 fg and 500 fg (the equivalents of 2 and 10 genomes) by adding a Southern blot and oligonucleotide hybridisation step. However, because this procedure doubles laboratory processing time and also appears to increase the likelihood of false positives, a decision was made not to make use of it in the present experiments.

Because 1 pg of *A. fumigatus* DNA was the lower limit of detection by UV visualisation of most subsequent *Aspergillus* specific PCR assays described up to the end of 1995, when the work described

here stopped, no attempt was made to use any of them. Assays based on amplification of nucleic assay sequences common to several genera of fungi followed by molecular speciation were not chosen either, both because they require additional processing beyond amplification, and because the amplification of yeast DNA from the respiratory specimens that were used was considered undesirable.

Prior to applying the two chosen PCR methods to clinical samples, their lower limit of DNA detection in the setting of the Royal Free Hospital Microbiology Laboratory was tested comparatively, with a view to selecting the more sensitive method for the processing of clinical specimens. This was done by adding serial 1:10 dilutions of known amounts of *Aspergillus* DNA in sterile distilled water to the PCR mix until a negative result was obtained.

4.2. HYPOTHESES

In addition, two new hypotheses were formulated based on the demonstration (Spreadbury *et al* 1993) that the adding up to 10^4 unextracted *A. fumigatus* conidia to the PCR mix produces a negative result, and on the findings (Melchers *et al* 1994) that *Aspergillus* culture negative liver tissue containing hyphae was PCR positive in all cases, while *Aspergillus* culture positive lung was only PCR positive in 83% of cases.

These hypotheses are:

(i) That conidial DNA is particularly difficult to extract

(ii) That the overall diagnostic sensitivity of *Aspergillus* PCR's is dictated by the combined sensitivity of the extraction and PCR procedures combined.

These hypotheses were tested as follows:
(i) By diluting known amounts of *Aspergillus* DNA in BAL fluid negative for *Aspergillus* by both culture and PCR before subjecting the mixture to DNA extraction (using the method applied to BALs), then to PCR, in order to record the smallest amount of recoverable DNA.
(ii) By extracting DNA, using the method applied to BALs, from serial 1:10 dilutions of known numbers of conidia in sterile distilled water prior to PCR, and finding the lower limit of detection. These experiments were initially attempted using dilutions of conidia in *Aspergillus* culture negative BAL fluid. However, they required to be repeated many times due to contamination problems, so that availability of suitable BAL fluid became limited and water had to be used instead.
(iii) By comparing the above results with the lower limit of *Aspergillus* DNA detection as measured by adding known amounts of DNA directly to the PCR mix.

4.3. DNA MATERIALS

Aspergillus DNA used in the experiments reported here was obtained using the enzymatic method described by Denning *et al* (Denning *et al* 1990), and quantified by visual comparison with known amounts of DNA. This relatively expensive and time consuming DNA extraction method was chosen after preliminary

experiments with a physical method (Aufauvre-Brown *et al* 1992) failed to yield sufficient amounts of DNA reliably, even after modifications (see Chapter 2). The choice of method was justified further by the fact that a stock of good quality, high molecular weight DNA was required for subsequent molecular epidemiology experiments (see Chapter 6).

DNA from all available strains of *A. fumigatus* yielded a PCR product of appropriate size with both Method 1 and Method 2, and DNA from all available strains of *A. flavus* yielded a PCR product of appropriate size with Method 2, as will be described in more detail in Chapter 6.

For the purpose of the sensitivity and DNA recovery experiments described here, only *A.fumigatus* strain 21, and *A. flavus* strain 10 were used. The concentration of the DNA extracted from *A. fumigatus* strain 21 was 200 ng/10µl, and that of DNA extracted from *A. flavus* strain 10 was 50 ng/10µl.

The BAL fluid used in the DNA recovery experiment was from two of the 13 random *Aspergillus* culture negative patients outside the Haematology unit (samples III and IV) and had repeatedly been shown to be *Aspergillus* PCR negative by Method 2 (Method 1 not attempted).

Convenient dilutions of conidia from *A. fumigatus* (strains 238 and 239) or *A. flavus* (strain 30) were counted in a haemocytometer and the concentration of spores in the remaining dilutions was derived prior to extraction and PCR.

4.4. LOWER LIMIT OF DNA DETECTION BY PCR METHOD 1

A.flavus DNA did not amplify by this method, and Figures 4-1 and 4-2 show that the lowest amount of *A. fumigatus* DNA detectable by UV light post amplification is 200pg (equivalent to 10^4 genomes). This was easily detectable on 5 different occasions, whereas 20pg and 2pg failed to produce a visible band upon amplification on at least 4 separate occasions.

This result compares poorly with the sensitivity of 1 pg reported by the authors of the method in their original paper, although the amplification failure of 10^4 unextracted conidia in the PCR mix which they report is less surprising in this light.

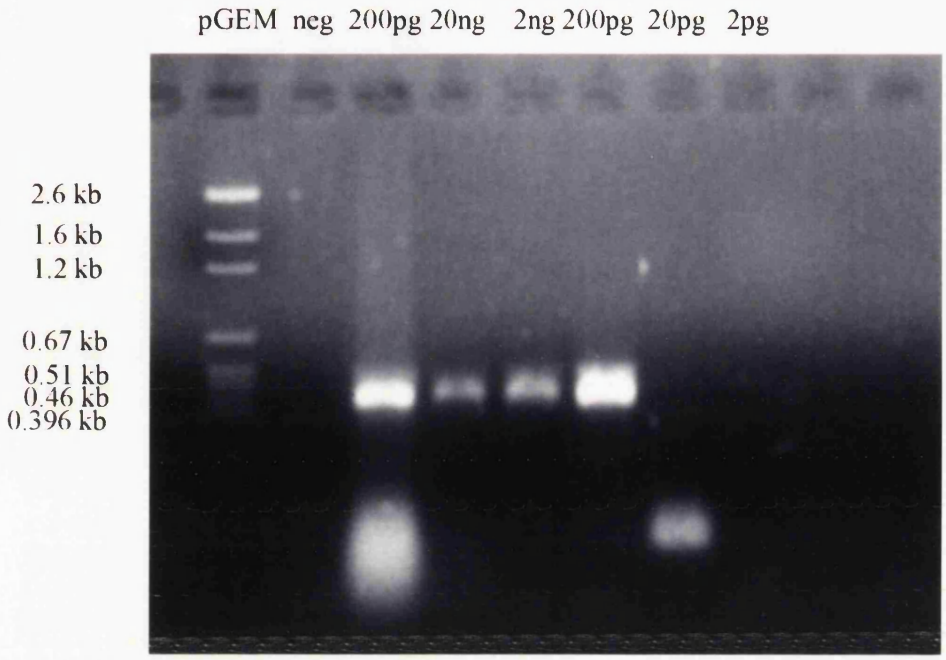


FIGURE 4-I: PCR by Method 1 of known amounts of *A.fumigatus* DNA

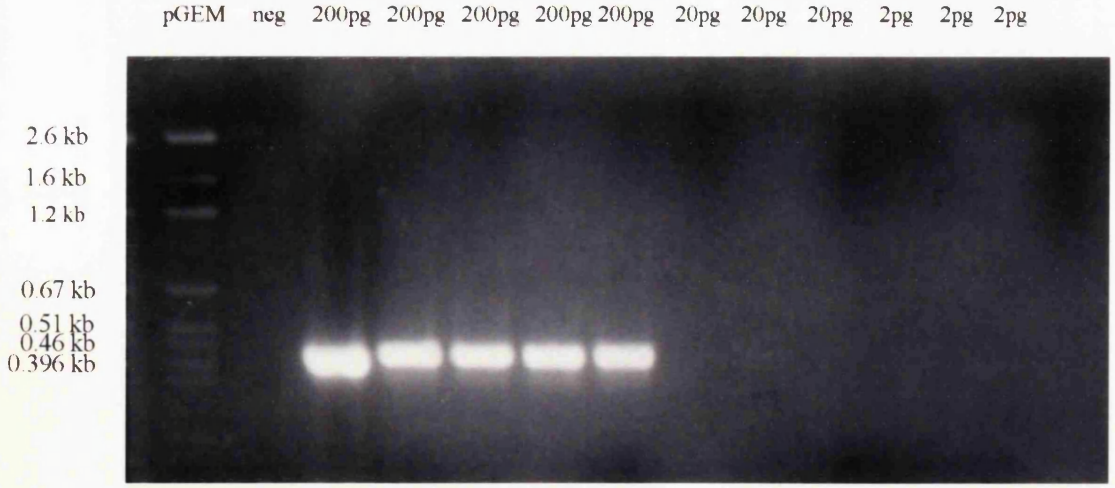


FIGURE 4-2: PCR by Method 1 of known small amounts of *A.fumigatus* DNA in triplicate

4.5. LOWER LIMIT OF DNA DETECTION BY PCR METHOD 2

Figures 4-3 and 4-4 show that 2 pg of *A. fumigatus* DNA is detectable by UV visualisation post amplification on 4/4 occasions, in keeping with the sensitivity of 5 pg reported by the authors of the method. Furthermore, 200 fg of *A. fumigatus* DNA was detectable on 4/5 occasions, 20 fg on 2/4 occasions while 2 fg was undetectable on 2 PCR attempts.

These results compare favourably with those reported in the original publication. The lack of reproducibility of results at DNA levels of 200 fg or less could be explained by unequal distribution of DNA within such low dilutions or by unreliable amplification at these levels.

Figures 4-5 and 4-6 show that the smallest amount of *A. flavus* DNA detectable by UV visualisation is 50 pg on 5/5 occasions, also in keeping with reported results. Failure of detectable amplification was observed on 5/5 occasions with 5 pg DNA.

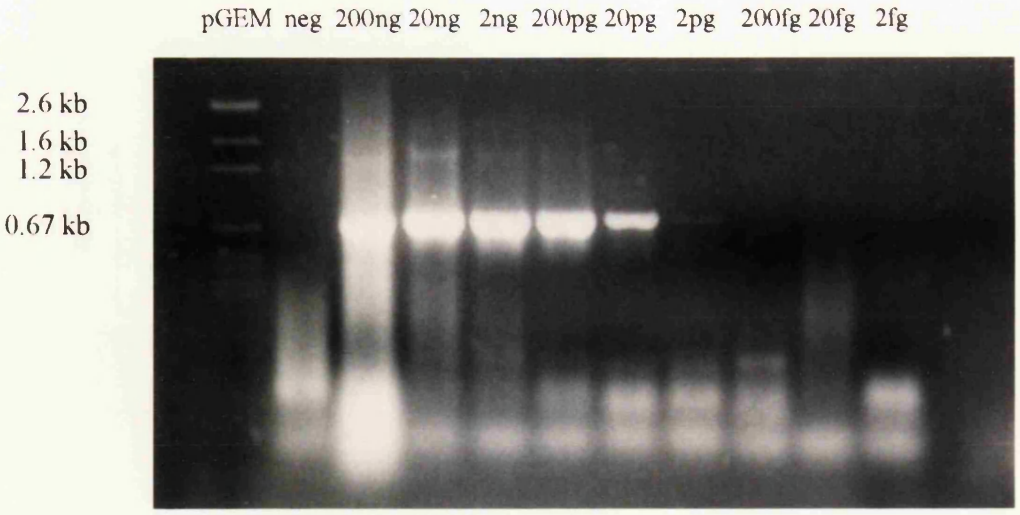


FIGURE 4-3: PCR by Method 2 of known amounts of *A. fumigatus* DNA

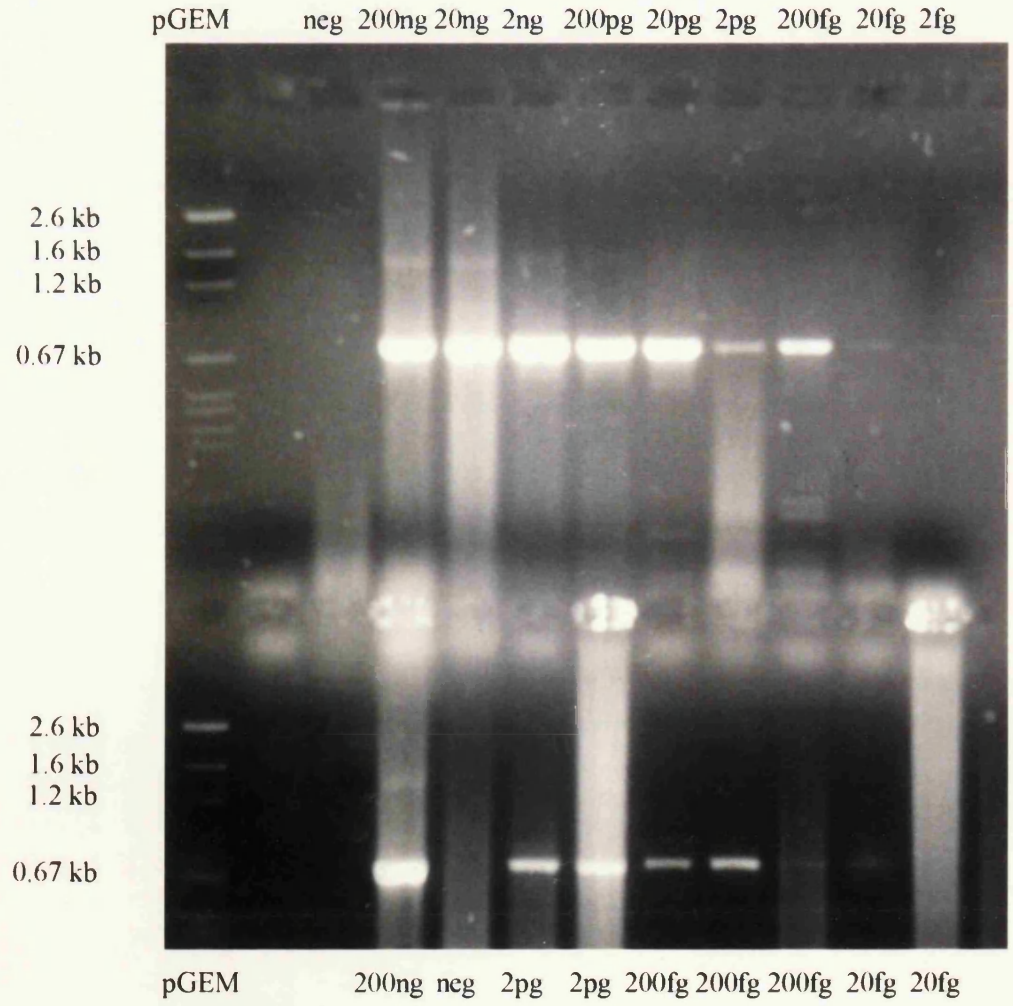


FIGURE 4-4: PCR by Method 2 known small amounts of *A.fumigatus* DNA at least in triplicate.

4.6. COMPARISON OF METHOD 1 AND METHOD 2 FOR *ASPERGILLUS* PCR

Method 2 clearly has two advantages over Method 1: unlike Method 1, it enables amplification of A. *flavus* DNA, while being 100 fold more sensitive for the purpose *A. fumigatus* DNA detection. In addition, the requirement of Method 1 for glycerol in the PCR mix to stabilise the product makes it less convenient to use. For these reasons, Method 2 alone was chosen for the processing of clinical samples (see Chapter 5) and for experiments concerned with DNA recovery or conidial extraction/PCR combined (see below). Method 1 was used for epidemiological purposes in Chapter 6, in addition to Method 2.

4.7. RECOVERY OF *A. FUMIGATUS* DNA DILUTED IN BAL POST EXTRACTION AND SUBJECTED TO PCR

10 µl amounts of serial 1:10 dilutions in water of DNA from *A. fumigatus* strain 21 (200 ng/10 µl undiluted) were added to 240 µl aliquots of the selected *Aspergillus* negative BAL, and mixed well. DNA from the resulting spiked BAL's was extracted using the standard BAL extraction method then subjected to PCR. Each DNA concentration was tested at least in duplicate. Unspiked BAL and sterile distilled water were processed in parallel. The results are shown in Figures 4-7 and 4-8.

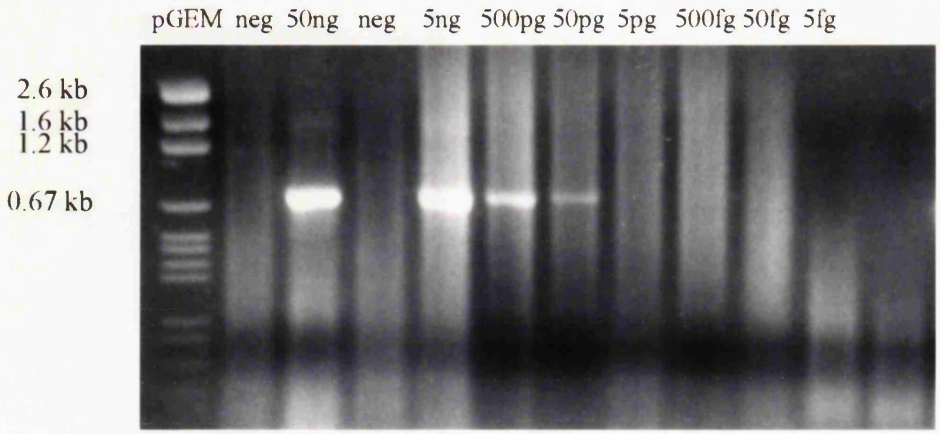


FIGURE 4-5: PCR by Method 2 of known amounts of *A.flavus* DNA

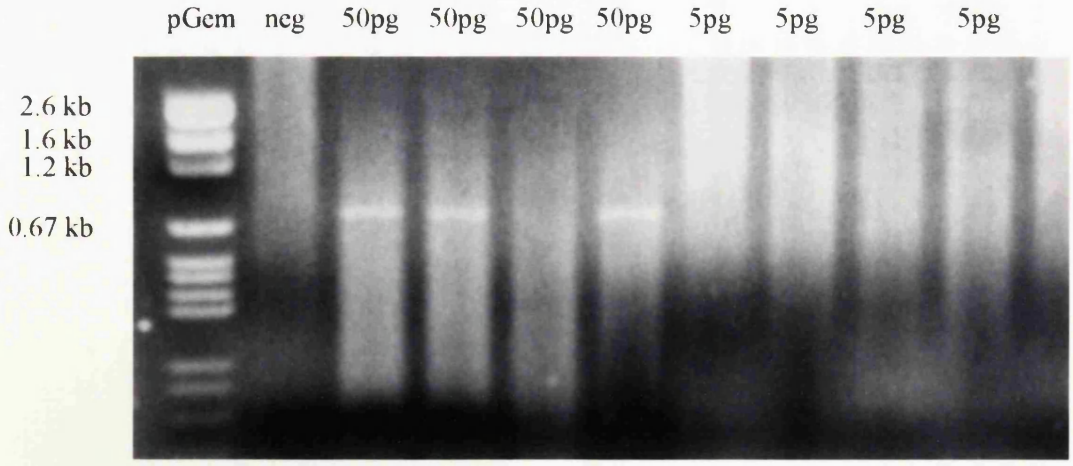


FIGURE 4-6: PCR by Method 2 of known small amounts of *A. flavus* DNA x4 of each

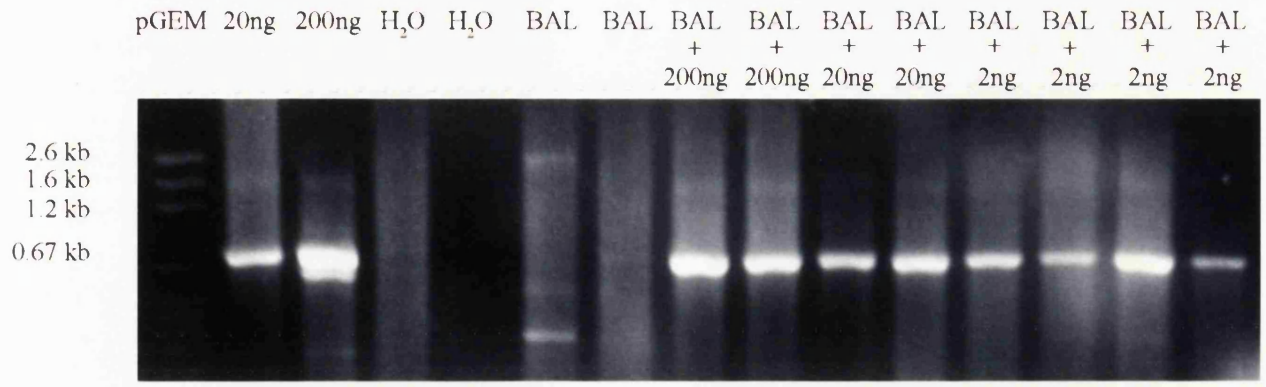


FIGURE 4-7: recovery of known amounts of *A. fumigatus* DNA from BAL after extraction and PCR

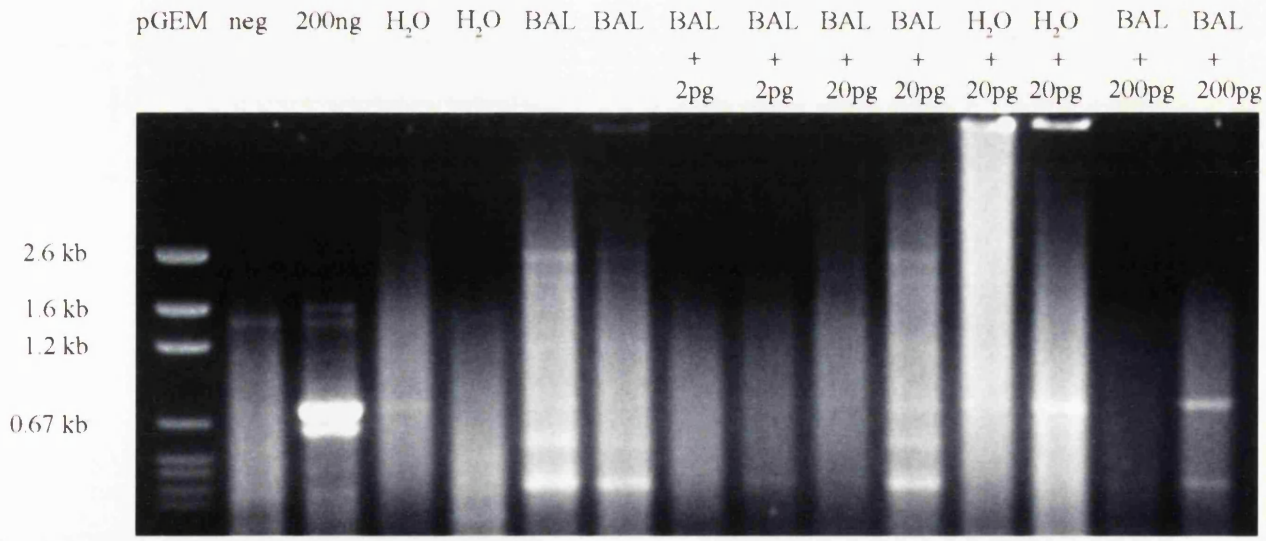


FIGURE 4-8: recovery of known amounts of *A. fumigatus* DNA from BAL or water after extraction and PCR

2 ng of *A. fumigatus* DNA is shown to be reliably detected from BAL in duplicate, while 200 pg is detected on 1/2 occasions, 20pg in 1/2 occasions, and 2 pg not at all. Figure 4-8 also shows that 20pg of DNA is recovered from water on 2/2 occasions.

Compared with the invariable detection of 2 pg of *A. fumigatus* DNA when added directly to the PCR mix, the reliable recovery of only 2 ng of such DNA from BAL demonstrates the loss of up to 3 log of DNA in the course of the extraction/PCR procedure combined.

4.8. DNA EXTRACTION/PCR OF DILUTIONS OF *A. FUMIGATUS* AND *A. FLAVUS* CONIDIA IN WATER

This was performed by subjecting 250 μl of 1:10 serial dilutions of counted conidia in sterile distilled water to the DNA extraction procedure for BAL, then to PCR by Method 2. Sterile distilled water was processed in duplicate and in parallel.

These experiments proved extremely difficult to perform initially, when heavy conidial suspensions were processed with others, and resulted in invariable contamination of the water controls, and of weak conidial suspensions. At the time, the conidia were diluted in *Aspergillus* culture negative BAL, but much BAL was wasted while trying to address the cross- contamination problem, and eventually water had to be used instead as a diluent.

Strategies adopted to avoid cross- contamination during the procedure included thorough cleaning of all hoods and water baths,

autoclaving of all equipment including racks, very frequent changes of gloves, the use of separate rooms for spiking, DNA extraction and template addition to PCR mix, as well as the processing of solutions with low or no conidial contamination before more heavily contaminated samples for each step in the procedure. The most effective step, however, was to avoid processing the first three dilutions of any conidial suspension in this type of experiment.

Figures 4-9, 4-10 and 4-11 show, respectively, the numbers A. *fumigatus* conidia from strains 238, 239 and 2109 that were detectable by UV visualisation after DNA extraction and PCR combined, and Figure 4-12 shows the same information for conidia from *A. flavus* strain 30. The smallest number of A. *fumigatus* conidia detected was 70 for strain 2109 (containing 3.5 pg of DNA), of the order of 2000 for both strains 238 and 239 (containing 100pg of DNA), and 500 for *A. flavus* (containing 25 pg of DNA).

These results suggest that DNA from *A. flavus* conidia is fully recovered during the combined extraction/PCR procedure (but only one valid experiment available), while a DNA loss of up to 50 fold from *A. fumigatus* conidia is observed.

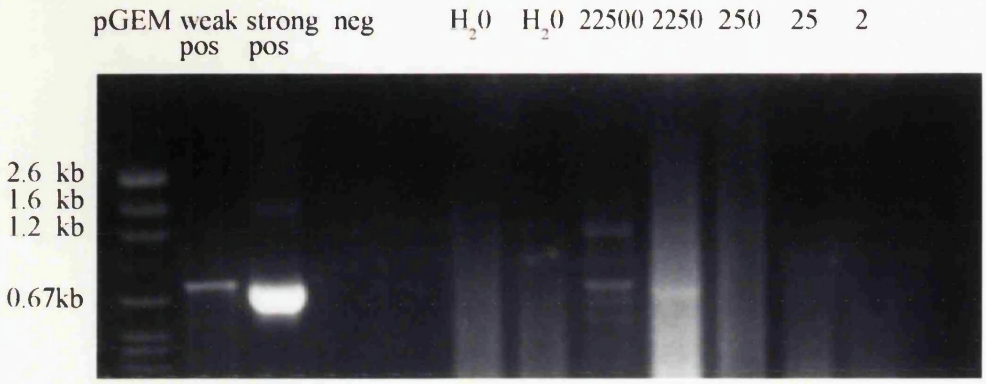


FIGURE 4-9: extraction, then PCR by Method 2 of known numbers of conidia of *A. fumigatus* strain 238 in water

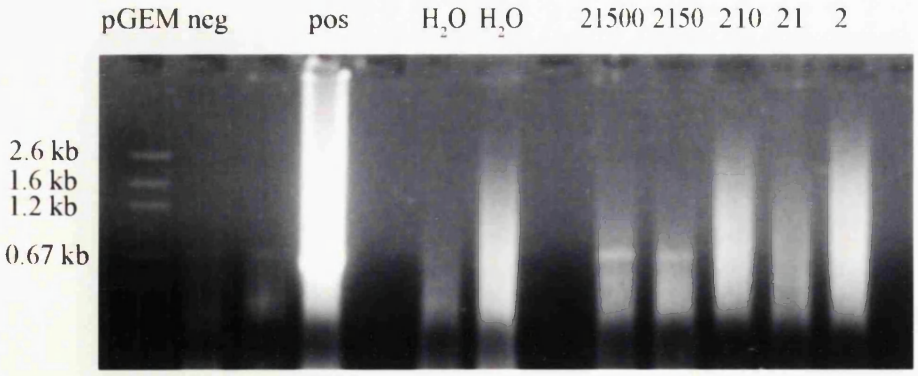


FIGURE 4-10: extraction, then PCR by Method 2 of known numbers of conidia from *A. fumigatus* strain 239 in water

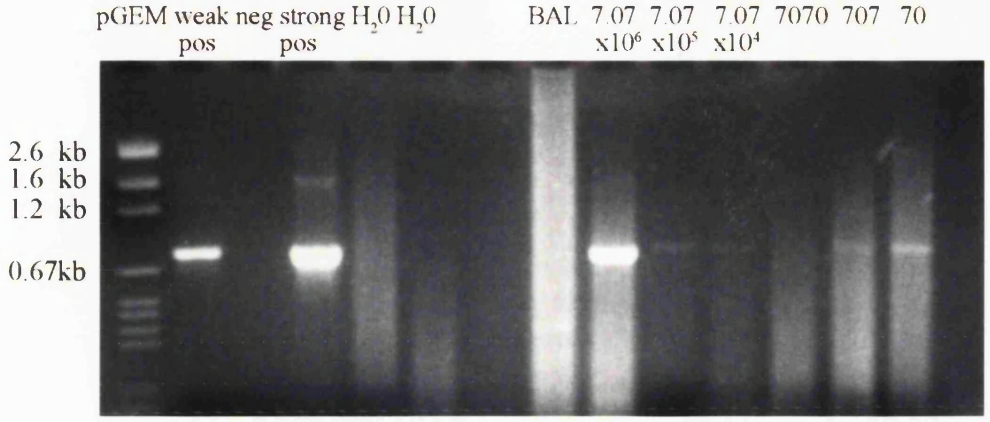


FIGURE 4-11: extraction, then PCR by Method 2 of known numbers of conidia of *A. fumigatus* strain 2109 in water

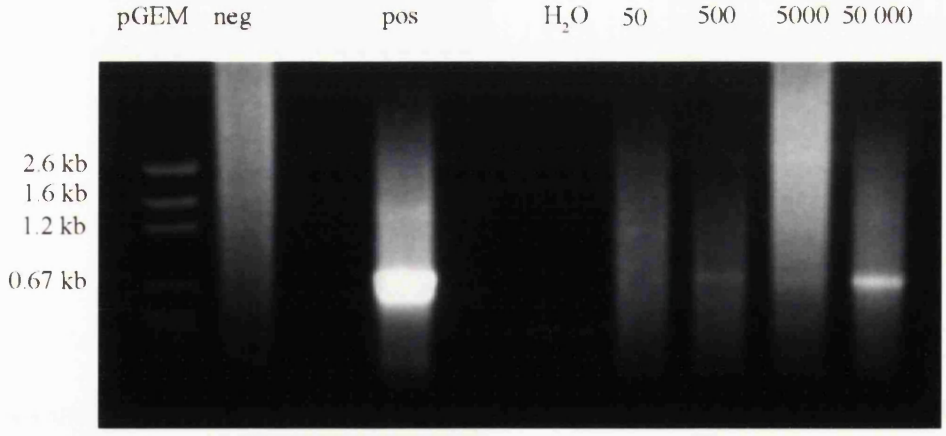


FIGURE 4-12: extraction, then PCR by Method 2 of known numbers of conidia of *A.flavus* strain 30 in water

4.9. CONIDIAL CONCENTRATIONS REQUIRED TO PRODUCE *A. FUMIGATUS* GROWTH ON A SABOURAUD PLATE.

For comparison with PCR results, dilutions of counted conidia of *A. fumigatus* strain 2109 were used to spike 250 µl aliquots of BAL fluid from two of the 13 random *Aspergillus* culture negative patients outside the Haematology unit (samples I and II). The whole spiked sample was used to inoculate a standard Sabouraud plate and incubated at 37°C for up to two weeks. Each dilution of conidia was cultured in duplicate (in one each of two different culture negative BAL fluids), and the unspiked BAL's were also cultured. Inocula of 150 conidia or more produced *A. fumigatus* growth after 2 days in duplicate plates. One 15 conidia inoculum resulted in *A. fumigatus* growth after 2 days, while the other produced no growth at 2 weeks. Unspiked BAL and inocula of conidia produced no *Aspergillus* growth after 2 weeks. This sensitivity level is comparable to that attained by PCR.

4.10.SUMMARY AND CONCLUSIONS

4.10.1. Comparative sensitivity of the two PCR methods

Aspergillus PCR by Method 2, described by Tang *et al* in 1993, is capable of detecting 2 pg by UV visualisation, i.e. 100 times less *A. fumigatus* DNA than Method 1, described by Spreadbury *et al* in the same year. Method 2 has the additional advantages that it is more convenient to use and also suitable for the amplification of *A. flavus* DNA. Furthermore, its performance in the context of the Microbiology Laboratory at the Royal Free Hospital is similar, and

in fact slightly better than in the original report, whereas the performance of Method 1 is considerably worse. The marginally better sensitivity of Method 2 demonstrated in the experiments reported above is probably attributable to the fact that *Aspergillus* DNA dilutions were performed in distilled water here, whereas Tang added human DNA to his assay.

It remains that the excellent reproducibility of Method 2 between laboratories is an additional advantage of this *Aspergillus* PCR technique. The 10 fold lower sensitivity of Method 2 in detecting *A. flavus* DNA rather than *A. fumigatus* DNA is confirmed. In view of its advantages over Method 1, Method 2 was used for all the work on clinical samples presented in Chapter 5.

4.10.2. Possible reasons for poor DNA recovery from BAL fluid

The DNA recovery experiment from BAL following extraction, then PCR shows that 2 ng is the smallest amount of DNA reliably amplified following the combined procedure. This represents up to 1000 fold loss in sensitivity compared to the PCR alone, and could be due to physical loss of DNA during extraction, to properties of BAL which interfere with *Aspergillus* DNA extraction or PCR, or to a combination of both. The recovery of *Aspergillus* DNA from water was not ascertained on a sufficient number of occasions, but the recovery of 20 pg twice, shown in Figure 4-8, suggests that intrinsic properties of BAL may be implicated in the poor recovery of DNA from BAL.

4.10.3. Discussion of results of DNA extraction from conidia

Experiments subjecting known numbers of conidia to the DNA extraction/PCR demonstrate a DNA loss of up to 50 fold from *A. fumigatus* conidia. Taken in conjunction with the purified DNA recovery experiment from BAL discussed above, the data implies that a further sensitivity loss of at least 20 fold within the combined extraction/PCR procedure occurs as a result of intrinsic properties of the BAL. Possible explanations are that viscosity characteristics impair the complete extraction of *Aspergillus* DNA from it, or that substances inhibitory to PCR are present within it.

The ability to obtain a positive PCR result starting from 70 conidia of strain 2109 in 250 µl of water, but the requirement for 2000 conidia of strain 238 or 239 to achieve the same result under the same conditions suggests that the physical properties of separate *A. fumigatus* strains lead to differential susceptibility to DNA extraction by the method used. Such properties could be thickness or chemical composition of the cell wall.

4.10.4. General discussion and implications for future work

The practical implication of these findings is that although the *Aspergillus* PCR by method 2 is capable of detecting 2 pg of *A. fumigatus* DNA or less by UV visualisation, the limitation of the technique appears to be due to a considerable loss of DNA during the extraction procedure, especially when BAL rather than water is used as the diluent for *Aspergillus* DNA. At the same time, the

considerable contamination problems encountered in performing these experiments led to rejection of the idea that BAL or water spiked with conidia should be used as a positive control for DNA extraction in the processing of clinical samples described in Chapter 5.

Finally, the number of organisms detectable by culture and PCR appears to be of a similar order of magnitude with experiments involving conidia of A. *fumigatus* strain 2109. Culture experiments were not performed in the case of strains 238 and 239, but because there is no theoretical reason why their growth characteristics should be different from those of strain 2109, while their DNA appears to be more difficult to extract, it is possible that culture may actually be more sensitive than PCR in such cases.

This situation may be reversed when hyphae, which are notoriously difficult to obtain fungal growth from, are present in the material to be subjected to PCR analysis, as suggested by Melchers data (Melchers *et al* 1994). This hypothesis is difficult to test formally, as hyphae do not lend themselves to straightforward quantification.

On the other hand, respiratory secretions, if containing *Aspergillus*, tend to be a richer source of conidia (also present in colonised but uninvaded airways) than hyphae, which tend to invade unaerated tissues. Because of this, the data presented above predicts that *Aspergillus* PCR technology applied to BAL fluid for diagnostic purposes is unlikely to be significantly superior to culture, particularly in the light of the suboptimal DNA recovery following extraction which was also demonstrated.

CHAPTER 5: APPLICATION OF THE ALKALINE PROTEASE BASED POLYMERASE CHAIN REACTION TO CLINICAL SAMPLES

5.1. RANDOM *ASPERGILLUS* CULTURE NEGATIVE BAL SAMPLES FROM PATIENTS OUTSIDE THE HAEMATOLOGY UNIT

The alkaline protease based PCR (Method 2) was shown in the previous chapter to be preferable to PCR Method 1, and chosen for the processing of clinical samples.

Five of the thirteen *Aspergillus* culture negative BAL samples collected from patients outside the haematology unit were used for validation studies on the DNA extraction and PCR procedures, and some of these experiments have already been described in Chapter 4. The remaining 8 samples were processed as "test BAL's" on several different occasions each, with a view to gaining further familiarity with the technique, and to utilising any sample found to be PCR negative on at least two occasions as an extra negative control (in addition to 2 sterile water controls) with runs of specimens from patients with both haematological and pulmonary disease.

Only samples I and II were available as large volumes (approximately 30 mls). They were used in the experiment described in Chapter 4 which involved spiking 250µl with counted conidia before culture on Sabouraud agar. The rest of both samples was used up in early spiking experiments with conidia prior to extraction/PCR, which resulted in contamination of the sterile water controls run in

parallel. Under these conditions unspiked aliquots of samples I and II were mostly found to be PCR positive and assumed to have become contaminated also.

No further aliquots of sample II were available. One aliquot only of sample I remained. It was subjected to extraction and PCR in a batch with others *Aspergillus* culture negative samples from patients outside the Haematology unit (see Figure 5-1). The other samples on Figure 5-1 and the remaining *Aspergillus* culture negative samples from patients outside the Haematology unit (example shown in Figure 5-2) were all processed at least twice each, and up to 17 times. The results are summarised in Table 5-1.

Sample number	Times PCR positive	Times PCR negative
I	1	not applicable
II	not applicable	not applicable
III	nil	3
IV	nil	4
V	nil	3
VI	nil	4
VII	nil	4
VIII	nil	2
IX	nil	5
X	2	5
XI	nil	5
XII	1	6
XIII	2	15

Table 5-1: Summary of PCR results on random *Aspergillus* culture negative BAL's from 13 patients outside the Haematology Unit.

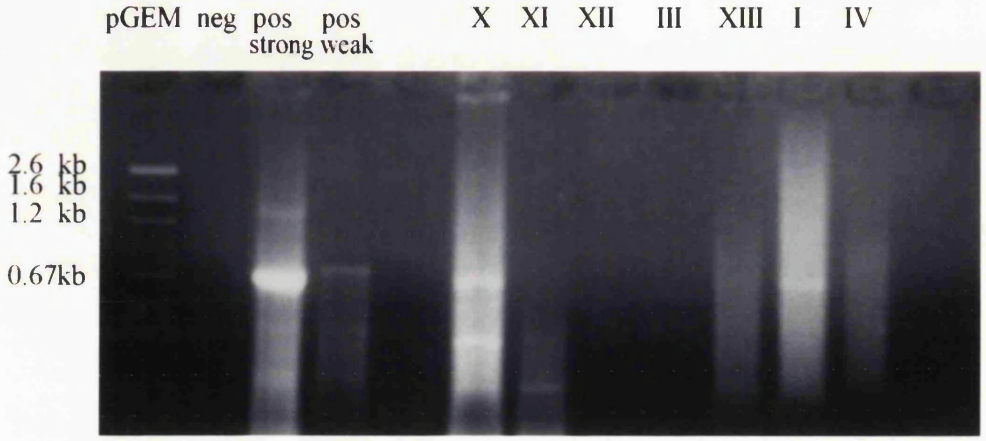


FIGURE 5-1: PCR by Method 2 of *Aspergillus* culture negative BAL's from patients outside the Haematology Unit

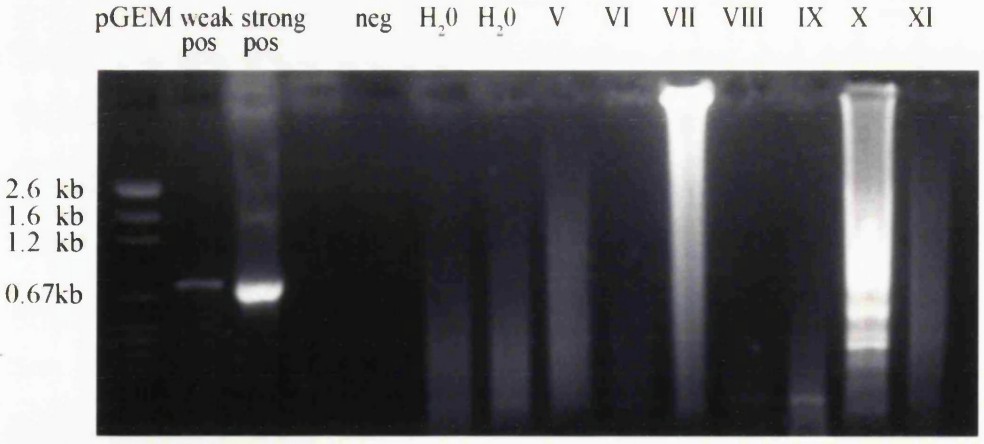


FIGURE 5-2: PCR by Method 2 of *Aspergillus* culture negative BAL's from patients outside the Haematology Unit

Samples III and IV are seen to be PCR negative in Figure 5-1. This result was reproduced two and three more times respectively (see Table 5-1), and the rest of the samples used for the DNA recovery experiment described in Chapter 4.

Eight of 12 BAL samples available for PCR were negative on all 2 to 5 occasions when they were tested.

Sample I was *Aspergillus* PCR positive on the only occasion when it was tested outside the context of spiking experiments. It was obtained from a patient with sickle cell disease (Hb SC) under investigation by the Respiratory Physicians for possible tuberculosis, although the specimen eventually proved sterile. His airway may have been colonised by *Aspergillus*.

Sample X was PCR positive on 2/7 occasions, and was obtained from a patient with pneumonitis following renal transplantation for ANCA positive vasculitis. Her serum was tested for *Aspergillus* antigen using the Pastorex method and found to be strongly positive. These findings were communicated to the physicians in charge of her, but she was already improving in the absence of specific antifungal treatment.

Sample XII was PCR positive on 1/7 occasions, and was obtained from a patient in renal failure with MRSA pneumonia who improved with MRSA specific treatment. The positive result in this case was in fact found when the sample was used as a negative control for another experiment, which did not present any contamination risks. No clinical details are available on patient XIII, whose *Aspergillus*

PCR was positive on 2/17 occasions (negative result shown on Figure 5-1).

It is of interest that the positive PCR results were not reproducible in any of the 3 cases where the assay was repeated several times, even in the case of sample X, where the patient had a history and other laboratory evidence suggestive of aspergillosis.

Sample VII proved unusual in that it was extremely mucoid and difficult to process. Even the DNA obtained at the end of the extraction was sticky and insoluble. The resulting PCR product on Figure 5-2 shows clear evidence of excessive DNA or other substance obscuring the lane. Several repeats with attempts at physical disruption of the mucus by vortexing or vigorous displacement with a pipette were unsuccessful. In order to overcome this problem, the mucus was emulsified with dithiotreitol (Sputasol, Oxoid, Basingstoke, UK) as follows: 1 vial of Sputasol was made up with 10mls of sterile distilled water to produce a 10X concentrated solution, and 25 µl were added to 225 µl of BAL, vortexed, and left at room temperature for 15 minutes before proceeding with extraction as usual. The handling of the specimen improved considerably after this treatment, which was subsequently applied to the occasional mucoid sample, which was received. Sputasol was shown not to be inhibitory to PCR as follows: 20 pg of *A. fumigatus* DNA were diluted in either 10 µl of distilled water, or in 10 µl of a 50:50 water/ Sputasol mixture, i.e. five times the concentration used in clinical samples. Both amplified satisfactorily when used as a template.

5.2. BAL SAMPLES FROM PATIENTS WITH HAEMATOLOGICAL AND PULMONARY DISEASE

Each of the 39 samples from these 32 patients was subjected at least on two occasions to DNA extraction then PCR by Method 2. Every available aliquot from patients on the IPA database was processed (n=3 to 7). The samples were processed in batches as indicated on Table 5-2, with 2 sterile distilled water controls and one random BAL control shown to be PCR negative at least twice. Sputasol was used as described above on samples which appeared mucoid on visual inspection.

Figure 5-3 shows the amount of DNA obtained after extraction of the first batch of BAL's from patients with haematological and pulmonary disease. Each lane represents 10 µl of DNA solution from a total volume of 30 µl. From visual comparison with 1.75 µg of λ *Hind*III DNA contained in 3.5 µl, sample 1A was estimated to have a DNA concentration of 140 ng/µl, with a maximum of 7ng/µl in the negative BAL control (sample XII) and in samples 1B, 1C, 1G and 1H. DNA from samples 1D, 1E, 1F and 1I was barely visible. From Tang's (Tang *et al* 1993) demonstration that 5 pg of *A. fumigatus* DNA is detectable by UV transillumination in the presence of 100 ng of human genomic DNA, it follows that only sample 1A required dilution prior to PCR. On the other hand, samples 1D, 1E , 1F and 1I contained little DNA so that reducing the dilution of the sample was considered to be desirable.

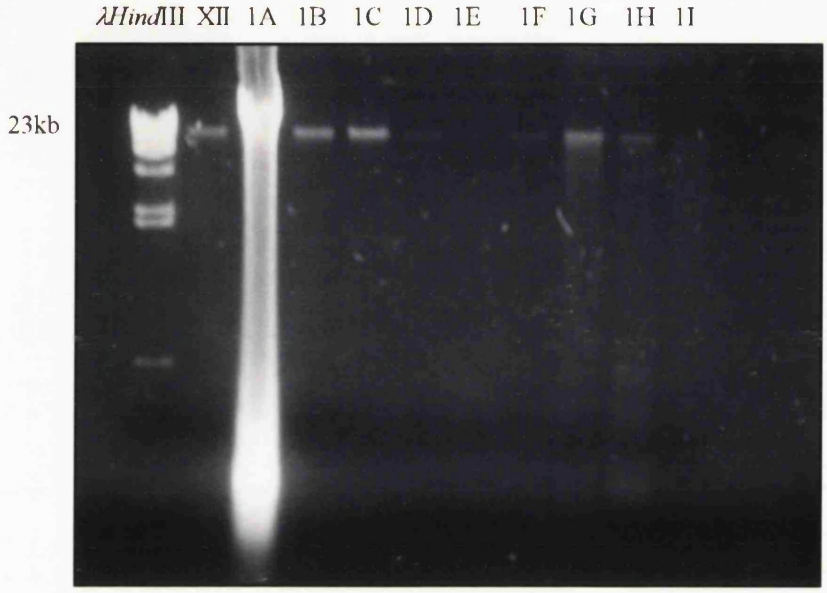


FIGURE 5-3: DNA extracted from batch 1 of *Aspergillus* culture negative BAL's from Haematology patients with pulmonary disease

In order to optimise DNA concentration in all samples, a decision was made to minimise dilution by making up all extractions in 15 μl of water instead of 30, omitting the DNA visualisation step which utilises 10 μl of solution, and amplifying the resulting DNA neat and at a dilution of 1:100 in order to reduce the risk of PCR inhibition by large amounts of human or bacterial DNA, should they be present.

Figure 5-4 shows the PCR's from a different extraction of the samples whose DNA appears in Figure 5-3. All PCR's from the extraction shown in Figure 5-3 were negative. The plain numbers represent amplification of neat DNA solution, and the "prime" numbers amplification of 1:100 solution.

Table 5-2 summarises the PCR results of the 40 samples from patients with both haematological and pulmonary disease. The numbers in brackets in the "other comments" column indicate the batch number of another BAL from the same patient. Experiments when the sterile water controls were contaminated or when the positive control failed are not reported in Table 5.2.

Set	Letter	Number +ive PCR's	Number -ive PCR's	Probability of IPA	Other Comments	IPA Database?
1	A	1	6	probable	*Asp.* 5/7 later	yes
1	B	nil	3	low	Probable PCP (1E)	no
1	C	nil	3	possible	Pneumonitis	no
1	D	1	2	low	Pneumonitis(3D)	no
1	E	nil	3	low	Probable PCP (1B)	no
1	F	nil	3	low	Probable PCP (3G)	no
1	G	nil	3	low	Probable PCP	no
1	H	nil	3	low	Probable PCP	no
1	I	nil	2	possible	Pneumonitis(3H)	no
2	A	nil	3	low	Probable PCP	no
2	B	nil	3	proven	Histo 8/7 before *Asp.*21 10/7 later	yes
2	C	1	1	low	IPA 10/12 later (4A)	yes
2	D	nil	3	low	Probable PCP	no
2	E	nil	4	possible	Patchy pneumonia	no
2	F	nil	3	low	Probable PCP	no
2	K	nil	5	probable	*Asp*10/7 later	yes
3	A	nil	3	possible	Patchy pneumonia	no
3	B	nil	3	low	Probable PCP (5E)	no
3	C	nil	3	possible	Patchy pneumonia	no
3	D	nil	3	low	Pneumonitis(1D)	no
3	E	nil	3	possible	Patchy pneumonia	no
3	F	1	2	possible	Patchy pneumonia	no
3	G	nil	3	low	Probable PCP (1F)	no

Table 5-2: Summary of PCR results of patients with haematological and pulmonary disease

Set	Letter	Number +ive PCR's	Number -ive PCR's	Probability of IPA	Other Comments	IPA Database?
3	H	nil	2	possible	Pneumonitis(3H)	no
3	I	nil	3	low	Proven yeasts	no
4	A	1	4	proven	Histo 12/7 later (2C)	yes
4	B	nil	3	probable	LISA	yes
4	C	2	1	possible	Patchy pneumonia	no
4	D	nil	2	low	Probable PCP (4E)	no
4	E	nil	3	low	Probable PCP (4D)	no
4	F	nil	3	low	Pneumonitis	no
4/5	G	1	4	proven	Histo 7/7 later	yes
4	H	1	2	possible	Patchy pneumonia	no
5	A	nil	2	low	Pneumonitis	no
5	B	nil	2	low	Probable CMV	no
5	C	nil	2	low	Pneumonitis	no
5	D	nil	2	low	Probable PCP (3B)	no
5	E	nil	2	low	Proven yeasts	no
5	F	nil	5	probable	Hyphae in BAL	yes

Table 5-2: Summary of PCR results of patients with haematological and pulmonary disease (Continued)

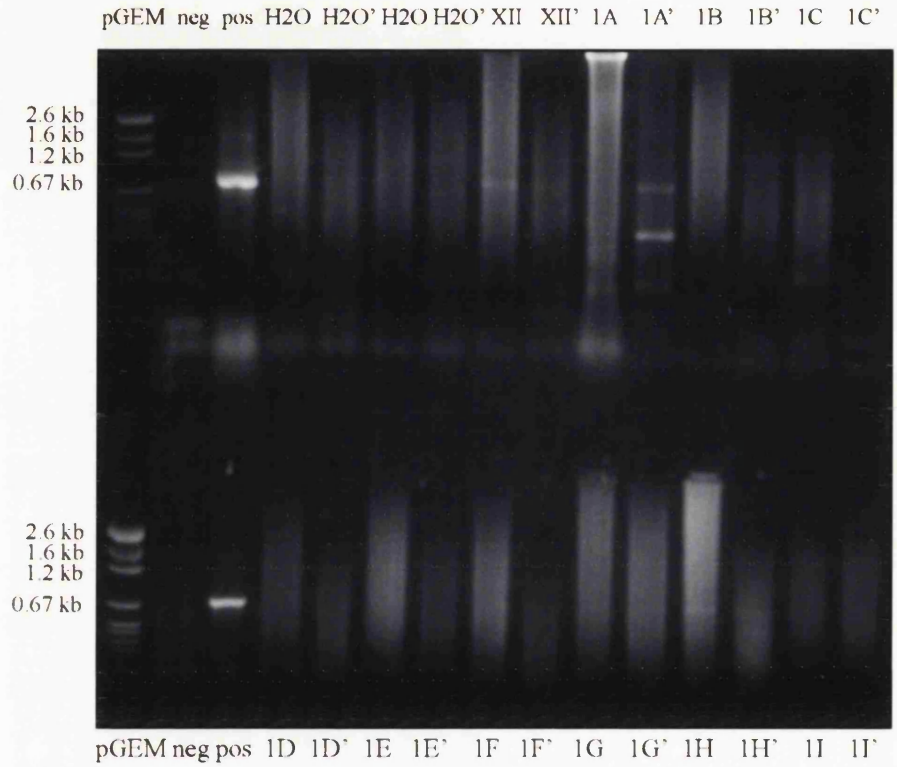


FIGURE 5-4: PCR by Method 2 of batch 1 of *Aspergillus* culture negative BAL's from Haematology patients with pulmonary disease

From Table 5-2, it can be seen that a total of 122 valid extractions/PCR's was performed, and that only 9 were positive. From 39 samples, 7 were positive on one out of 2 to 7 occasions, including 2 BAL's from the same patient 10 months apart. One sample was positive on 2 out of 3 occasions, and the remaining 31 persistently negative. Within the samples with at least one positive result, these results represented 9 out of 31 PCR attempts. Thus reproducibility of positive results appears to be very poor.

Figures 5-5 and 5-6 show batches of PCR's containing at least one positive result. It can be seen that the bands obtained are very faint, and that 6 out of 9 positive results are clustered in 2 out of at least 15 batches tested. This raises the possibility of contamination, although the sterile water controls were negative.

Interestingly, Table 5-2 also shows that the highest rate of positive results on one occasion is within the 7 patients with proven or probable aspergillosis, of whom 4 have such a result (57%). This compares with 1 out of 22 patients with low probability of IPA (4.5%), and 2 out 10 patients with possible IPA, one of who also has 2/3 positive results (30%).

This apparent correlation of percentage of cases with at least one positive PCR and increased probability of IPA is unfortunately of no clinical value, as it is not predictive of aspergillosis in individual cases. On the other hand, a cause of great concern is that 3 patients who were bronchoscoped at a time when they were clearly dying of IPA had PCR negative BAL on the majority or on all of the aliquots that were tested. Their clinical histories were as follows.

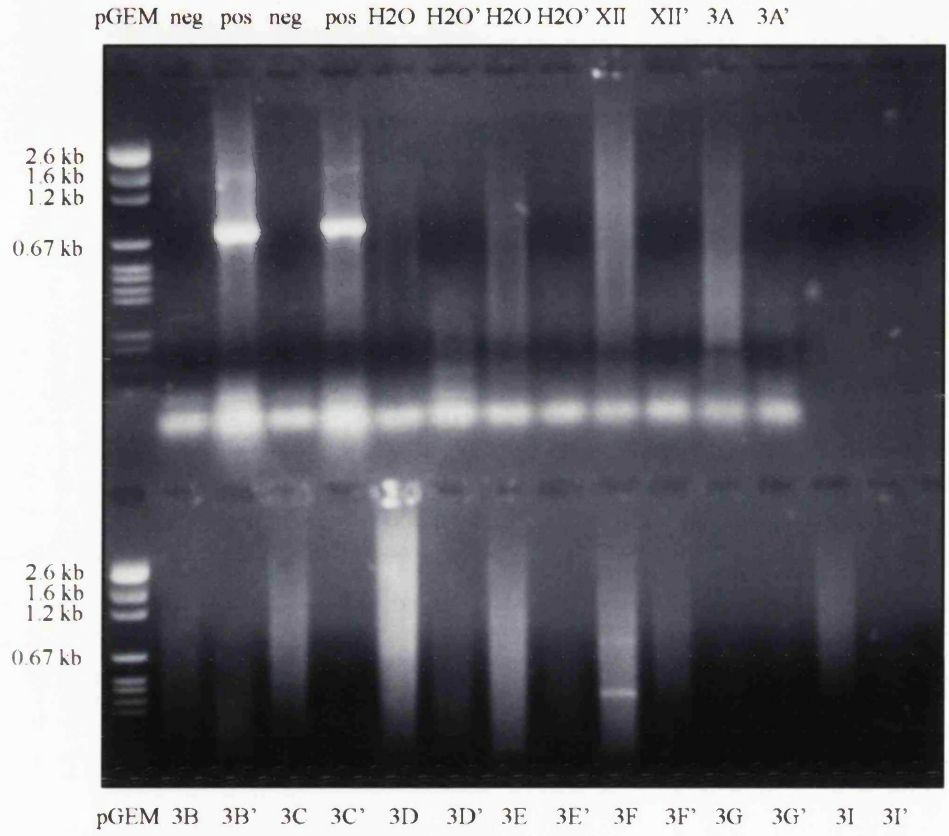


FIGURE 5-5: PCR by Method 2 of batch 3 of *Aspergillus* culture negative BAL's from Haematology patients with pulmonary abnormalities

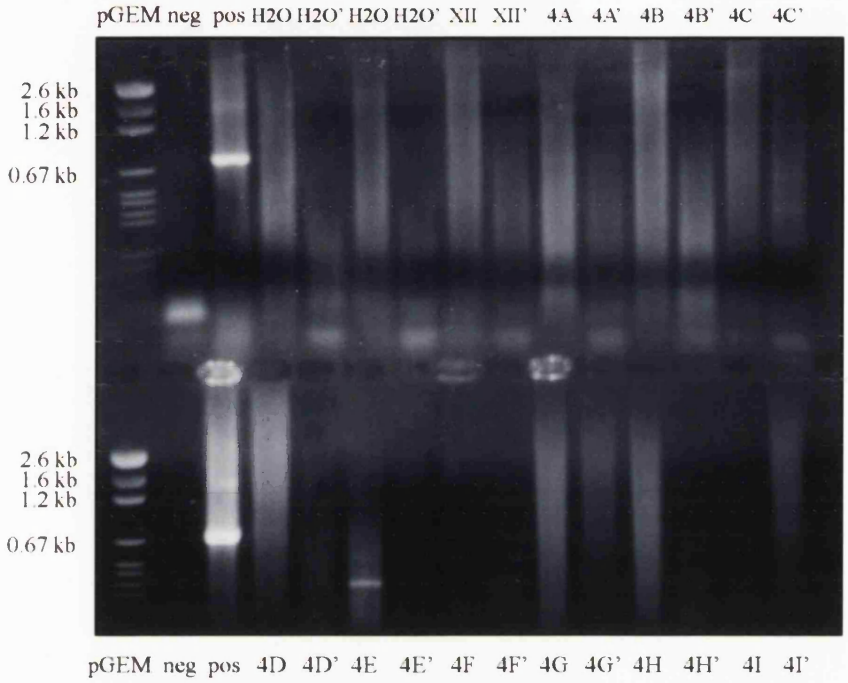


FIGURE 5-6: PCR by Method 2 of batch 4 of *Aspergillus* culture negative BAL's from Haematology patients with pulmonary disease

Patient 1A had diffuse bilateral pulmonary infiltrates and his BAL was PCR negative in 6 out the 7 available aliquots yet 3 different *Aspergillus* species were grown from his sputum 5 days later. He died shortly afterwards. Patient 2B underwent bronchoscopy when she developed diffuse bilateral pulmonary infiltrates 8 days after undergoing a lobectomy for MLS; although the resected tissue was culture negative but histology positive for *Aspergillus*, all 3 available aliquots of BAL were PCR negative. Her condition continued to deteriorate; she developed retinal infiltrates, then required mechanical ventilation. An early retinal aspirate was negative, but *A. fumigatus* strain 21 was cultured from a repeat retinal aspirate 10 days post BAL. She died the following day. Finally patient 4G was bronchoscoped when he developed fever and patchy consolidation in the left mid zone on CXR. Four of 5 aliquots were PCR negative, but an MLS became apparent over the next 5 days, so that a left upper lobectomy with lingula resection was performed on the 7th. He died of respiratory failure of rapid onset the day following operation. *A. flavus* was cultured from the resected lung tissue, which was also histology positive.

Possible reasons for the lack of reproducibility and predictive value of the PCR results in this group of patients with haematological and pulmonary disease are discussed later.

5.3. BAL SAMPLES FROM ASYMPTOMATIC HIV POSITIVE VOLUNTEERS

The 17 culture negative BAL's samples from volunteer asymptomatic HIV positive patients were obtained in the context of

a different research project, and processed for DNA extraction/*Aspergillus* PCR by method 2 on two or three separate occasions each, with two aliquots of sterile distilled water included in each batch. Ten samples were processed 3 times, and seven twice. Only one of the 17 BAL's was PCR positive, on one of three occasions when it was tested, representing 1 of 44 assays (2%). These results confirm both the low numbers of positive PCR results in a population with expected low prevalence of IPA, and the poor reproducibility of the test.

5.4. RESECTED LUNG TISSUE

Two resected lung samples, designated A and B, were available for DNA extraction and PCR by method 2. The extracted DNA was used as a template for the PCR both neat and diluted to 1:100. Sample A was obtained from the patient reported in (Yeghen *et al* 1996) and described in Chapter 3, who developed an MLS and underwent a lobectomy for apparent IPA. The resected lung had compatible histological appearances, but *Chaetomium globosum* rather than *Aspergillus* was grown, and immunocytology confirmed the absence of a common *Aspergillus* species. Sample B was from the only patient included in Chapter 3 who developed IPA on a background of untreated myelodysplasia. No fungus was grown from the resected specimen, but histological appearances were also in keeping with aspergillosis. In addition, sputum produced pre-operatively had grown *A. fumigatus* (strains 23a and 23b) and *A. flavus* (strains 26 and 27). The results are shown in Figure 5-7, which also includes BAL 5F.

Curiously, only sample A tested at a dilution of 1:100 is positive, in spite of its origin from a patient with an MLS shown not to be *Aspergillus*. Conversely, sample B, from a patient with histological IPA but negative *Aspergillus* culture, was PCR negative.

The most likely explanation is that sample B, which was submitted to the microbiology laboratory, was an uninvaded portion of resected lung, while the portion of sample A which underwent PCR could have been an area of dual *Chaetomium/Aspergillus* infection, or have become contaminated with *Aspergillus*. Alternatively, *Chaetomium* may be amplified by PCR method 2 as well as *Aspergillus*, although there was no opportunity to test this hypothesis.

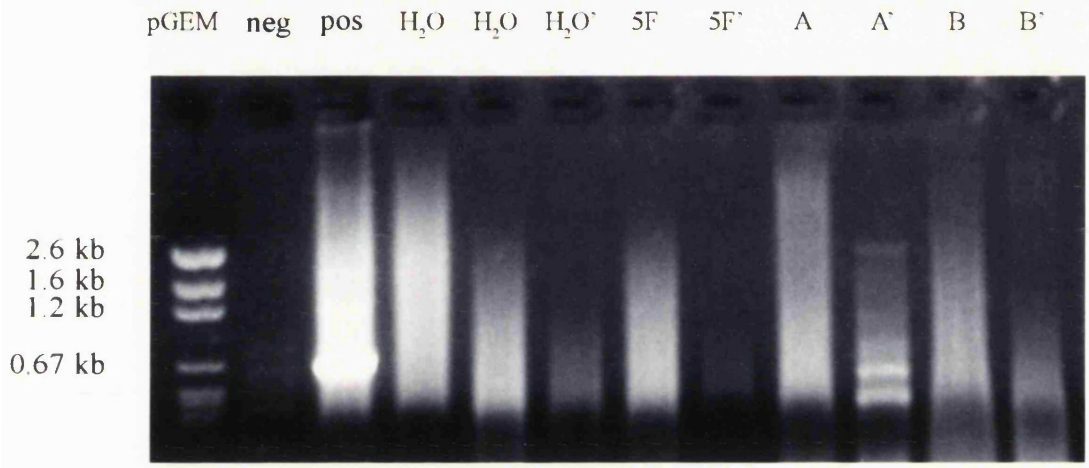


FIGURE 5-7: PCR by Method 2 of two samples of *Aspergillus* culture negative lung resected from haematology patients with histological IPA

5.5. CONCLUSIONS & DISCUSSION

Briefly, the alkaline protease based PCR for *A. flavus* and *A. fumigatus*, when performed on BAL, was no better than culture in predicting IPA in haematology patients with pulmonary disease and culture negative BAL. Occasional positive results were obtained but were not reproducible on different aliquots of the same BAL. Several possible explanations for these findings can be considered.

The first is the contamination hypothesis suggested above in the context of 6 positive results clustering within 2 experiments. It is made less likely by the fact that the water controls were all negative.

The second explanation is that there was a differential distribution of fungal elements in the separate aliquots of BAL samples used, thus accounting for the discrepancies in the PCR results. Only small volumes of unspun BAL were available and aliquoted for these experiments, because the bulk of the specimen was reserved for standard diagnostic tests. A reasonable strategy for future use would be to centrifuge the BAL sample, and perform both culture and extraction/PCR on the deposit, where fungal elements would be more concentrated.

The weakness of the positive bands suggests the third explanation, namely that the amount of *Aspergillus* present in samples found to be intermittently positive is just below the limit of the sensitivity of the combined extraction and PCR assay, so that amplification is detected only occasionally. This is particularly plausible in view of the demonstration in Chapter 4 that much DNA loss occurs both

when a conidial suspension is extracted and when purified DNA is diluted in BAL then assayed.

Related possibilities are that *Aspergillus* DNA is difficult to extract, so that the yield is variable from one extraction of the same sample to the next, or that the assay is dependent on the ratio of human to *Aspergillus* DNA rather than on absolute concentration of either, so that differential extractability of these DNA's leads to discrepancies in the final PCR result.

Southern hybridisation was not considered an appropriate method of circumventing difficulties related to assay sensitivity in view of the serious contamination problems described in Chapter 4, which it would enhance.

It therefore appears that the alkaline protease based *Aspergillus* PCR performed on BAL is subject to the same drawbacks as standard culture methods in that sensitivity is similar, contamination moderately frequent and precision poor. Speed of diagnosis may be an advantage of this PCR in certain cases, although *Aspergillus* growth from as few as 15 conidia was seen after 2 days (see Chapter 4).

Ways in which the assay could be improved should be aimed at enhancing the DNA extraction procedure and minimising contamination, rather than increasing the sensitivity of the PCR itself. The method as it stands cannot be recommended for routine clinical use.

CHAPTER 6: DNA EXTRACTION AND MOLECULAR EPIDEMIOLOGY OF 30 *ASPERGILLUS* ISOLATES

6.1. ENZYMATIC DNA EXTRACTION OF 30 *ASPERGILLUS* STRAINS AND XHOI/SALI RESTRICTION ANALYSIS ON WHOLE GENOMIC DNA

6.1.1. DNA extraction (general)

The enzymatic DNA extraction procedure was applied to all available strains of *Aspergillus fumigatus* and *Aspergillus flavus*, with a view to studying strain variability using an established method based on *Xho*I and *Sal*I polymorphisms (Denning *et al* 1990), then to evaluating PCR/RFLP and PCR/SSCP as possible epidemiological methods for these two species of *Aspergillus*.

The initial experiments were conducted with NCPF strains 2109 and 2140, as well with strains 21 (isolated from retinal fluid of patient on private ward at time when non-irradiated pepper was served) and 22 (from pepper served on private ward). Difficulties in extracting any DNA at all were encountered at first, due mainly to insufficient protoplast isolation. With much experimentation and practise however, DNA was eventually obtained from strains 21 and 22.

DNA concentrations of 130 μg/ml and 150 μg/ml were recorded for strains 21 and 22 respectively, by OD at 260 nm. These concentrations were of the same order of magnitude as those recorded by Denning *et al* at 300 μg/ml. Ratio of OD 260/OD 280

however was 1.52 and 1.58 respectively, indicating poor nucleic acid purity.

Restriction digests of 0.14 ml of these DNA's in the corresponding volumes of appropriate X10 buffer using 40 IU of *Xho*I or *Sal*I were performed. Very sheared DNA and absence of bands were observed, even at high magnifications.

Figure 6-1 shows the DNA used for this experiment against 3.5 µl of 0.5 µg/µl λ*Hind* III. The DNA concentration calculated by comparative electrophoresis is of the order of 1.3 µg/ml.

There was therefore a one hundred fold discrepancy between the DNA concentrations calculated by spectrophotometry and those calculated by comparative electrophoresis. This is almost certainly due to *Aspergillus* pigments and other impurities registering elevated OD's at 260 nm in the absence of adequate amounts of DNA, as is evidenced further by the low OD 260/OD 280 ratios. Spectrophotometry is thus not an appropriate method for measuring DNA concentration when the enzymatic extraction method is used, and the failed restriction digests were due to insufficient amounts of DNA.

After several more unsuccessful attempts (mainly involving increases in the amount of processed fungal mass) to extract adequate amounts of DNA, help was sought from Dr Denning, who kindly arranged a site visit to his laboratory at the University of Manchester. Insufficient DNA concentration was confirmed to be the cause of the failed restriction digests in this laboratory, and the final enzymatic DNA extraction method described in Chapter 2 was

recommended. This includes some new refinements as compared to the original published method, particularly the drying of the *Aspergillus* strain using a vacuum prior to weighing, and the heating of the DNA extraction buffer prior to adding to ensure the inactivation of DNA ases. Advice to avoid DNA shearing by using wide mouthed pipettes and avoiding shaking was also given.

Marginal improvement only in the quality and concentration of the extracted DNA were observed. In addition, some DNA loss was found to occur in the course of dialysis, although this particular problem was easily circumvented by additional precautions in securing the ends of the dialysis tubing. Another visit to Dr Denning's laboratory was requested, approximately 10 weeks after the first, and the opportunity was created to observe and to participate in a complete enzymatic DNA extraction in the laboratory where the method originated. An *Aspergillus* DNA sample produced in Manchester (referred to as "Man") was kindly donated to test restriction enzyme methodology and to serve as a standard for the concentration of future DNA extractions.

Much improvement was noted in DNA extraction from this point, although even the best DNA samples obtained failed to reach the high concentration of the "Man" DNA, as shown in Figures 6-2 and 6-3. The main reason for failure to achieve the same very high DNA quality standard as in Dr Denning's laboratory in the time allocated to this project is likely to have been insufficient expertise with a difficult technique, which other laboratories did not always succeed in reproducing either (Mike Birch, personal communication).

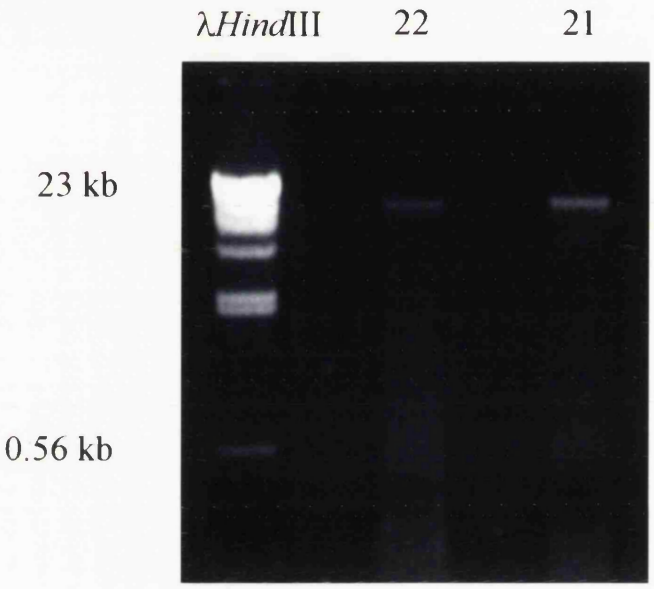


FIGURE 6-1:calculation by comparative electrophoresis of DNA concentration from *A. fumigatus* strains 21 and 22

6.1.2. Strain variability in ease of DNA extraction

DNA was obtained without further difficulties from *A. fumigatus* strains 2109, 2140, 7, 8, 9, 17b, 20, 21, 22, 23a, 31, 210, 233, 234, 235, 237, 238 and 239 and *A. flavus* strains 10, 12, 29 and 30. DNA extraction was much more difficult, with protracted fungal wall digestion times, for *A. fumigatus* strains 6, 16, and 17a. The process had to be repeated at least twice before any DNA could be visualised.

Despite repeated efforts, *A. fumigatus* strain 23b and *A. flavus* strains 5, 15, 26 and 27 yielded no visible DNA whatsoever. Attempts to amplify the material obtained from these strains by PCR (Method 2) were also unsuccessful, confirming absence of any significant amounts of DNA.

The fact that *Aspergillus* strains lend themselves differently to DNA extraction is suggestive of strain variability in cell wall structure or thickness. These findings are in keeping with those in Chapter 4, where conidia of *A. fumigatus* strain 2109 was detected in much smaller numbers than those of strains 238 and 239. Proportionately more *A. flavus* than *A. fumigatus* strains failed to yield DNA, and even in those where extraction was successful, weaker DNA solutions were generated.

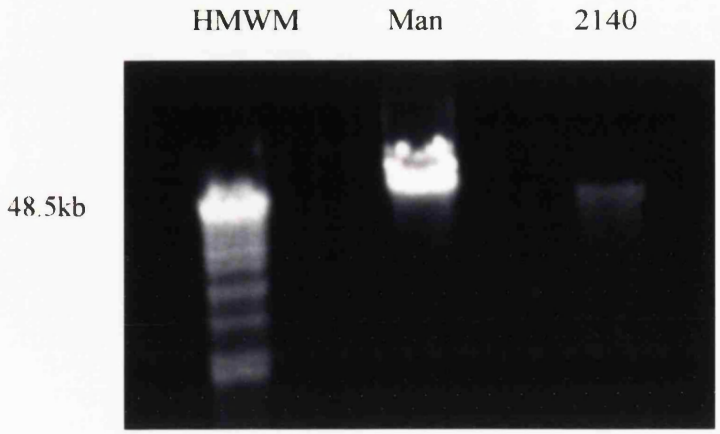


FIGURE 6-2: comparative quality of DNA extracted in Manchester and at RFH

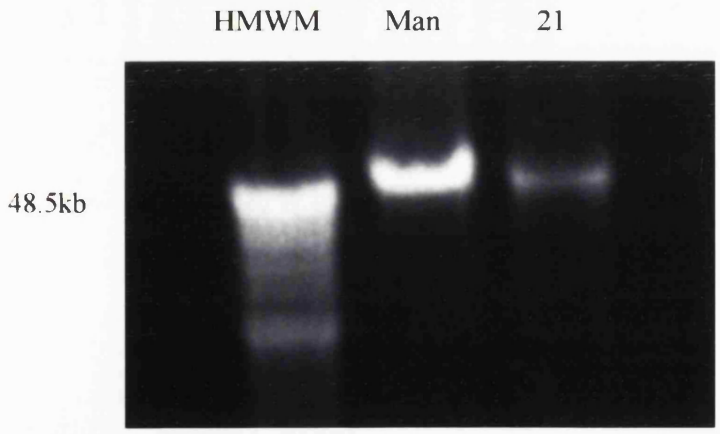


FIGURE 6-3: comparative quality of DNA extracted in Manchester and at RFH

It is of interest that, although strains 23a and 23b were cultured from the same patient, no DNA was obtainable from strain 23b, whereas DNA extraction from strain 23a presented no problem. This suggests that the patient was infected by two different strains. Similarly, strains 17a and 17b, both from another patient, behaved very differently in the way they lent themselves to DNA extraction, again suggesting dual infection. The same applies to *A. fumigatus* isolate pair 6 and 7. Although valid observations, these findings are too subjective and methodology dependent to be of epidemiological use.

6.1.3. *SalI* and *XhoI* restriction digests

Attempts at performing *Sal*I and *Xho*I restriction digests on the improved DNA samples continued to be disappointing and essentially uninterpretable, even if the whole of the available DNA (rather than 0.14 ml) was used in order to optimise the amount of material for digestion. Figure 6-4 demonstrates the most successful attempt at differentiating between strains 21 and 22 using *Sal*I digests on a whole extraction. Although no definite DNA type can be attributed to the 2 strains according to the published scoring system (Denning *et al* 1990), strain 22 has only one band above 23 kb, whereas strain 21 appears to have several. They are thus different, and this suggests that the outbreak of IPA which occurred on the private patients ward following the inadvertent use of non-irradiated pepper may in fact not have been related to the contaminated pepper.

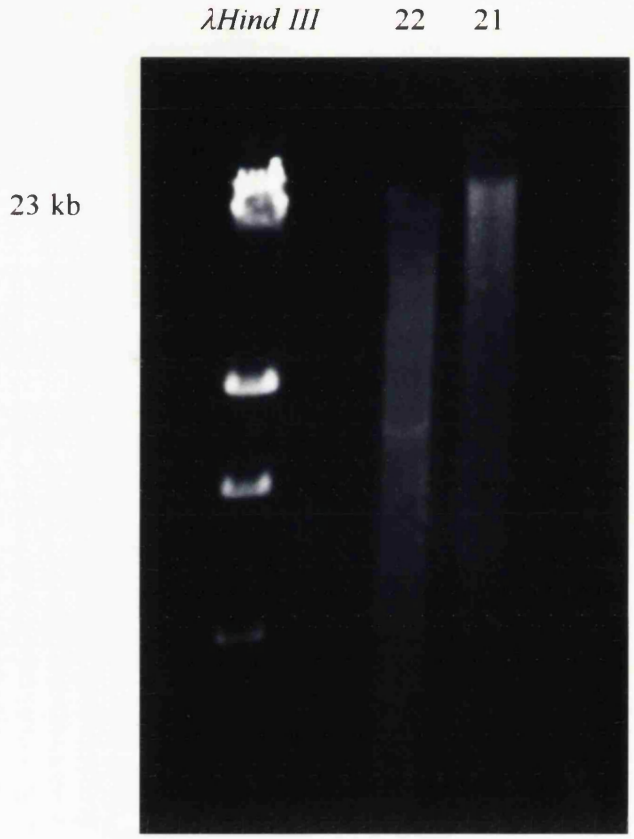


FIGURE 6-4: *Sal I* restriction digests of DNA from *A. fumigatus* strains 21 and 22

Figure 6-5 shows "Man" DNA digested with *Xho*I. The strain can clearly be allocated to DNA type (1, 6) on this basis. This confirms that DNA quality, rather than any problem with restriction digests or optimisation of their visualisation or imaging is the reason for the poor results of these experiments. Further attempts at epidemiological studies by RFLP's of whole genomic DNA were abandoned.

6.2. PCR OF EXTRACTED *ASPERGILLUS* STRAINS BY METHODS 1 AND 2

All strains whose DNA had been successfully extracted were processed by PCR Method 2. These were all *A. fumigatus* strains except 23b and *A. flavus* strains 10, 12, 29 and 30. All yielded a product of appropriate size except *A. flavus* strain 29, which failed to amplify on at least 4 different occasions. This suggests that strain 29 lacks the alkaline protease gene, in contrast to the rest. The patient who harboured it had a particularly fulminant course and died the day after a lobectomy of widespread pneumonia, so that strain 29 clearly did not have reduced virulence in spite of lacking the alkaline protease gene or part of it.

All *A. fumigatus* strains except 23b, for which no DNA was available, were processed by PCR Method 1 and all yielded a product of appropriate size.

All PCR products were purified using the Promega Wizard™ PCR Preps DNA purification System for Rapid Purification of DNA Fragments, in preparation for restriction digests and/or SSCP.

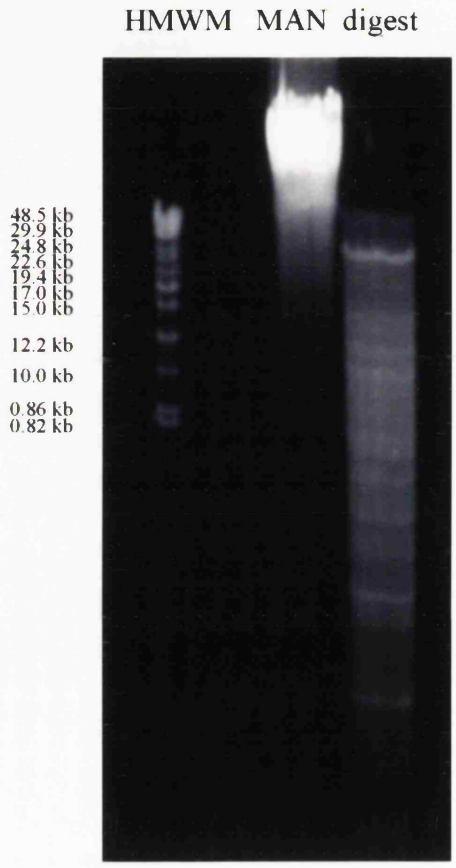


FIGURE 6-5: *XHOI* digest of Manchester DNA

6.3. DDEI AND STYI RESTRICTION ANALYSIS OF PRODUCTS BY PCR METHOD 2

A GeneBank search was undertaken to determine the restriction sites available on the 747 bp product obtained by PCR Method 2 when applied to *A. fumigatus*. *DdeI* and *StyI* were found to yield two fragments of similar size. Both were tried on *A. fumigatus* DNA, and Figure 6-6 shows more complete digestion with *DdeI*, which was selected for future RFLP and RFLP/SSCP experiments.

Figure 6-7 shows *DdeI* restriction digests of PCR products by Method 2 on all 21 strains of *A. fumigatus* whose DNA was available, including "Man", and 2 strains of *A. flavus*, 10 and 12. Both *A. flavus* isolates exhibit the same three bands pattern, in contrast with most *A. fumigatus* isolates, which have a 2 bands pattern. The notable exception is isolate 8, which exhibits the same restriction pattern as *A. flavus*. *A. flavus* strain 30 was restricted with *Dde*I on a separate occasion and shown to exhibit the same pattern as the other two *A. flavus* isolates. These findings are in keeping with the presence of an additional *DdeI* restriction site common to *A. flavus* and to *A. fumigatus* strain 8.

Strain 8 was cultured again and confirmed morphologically to be *A.fumigatus* once more. It had been isolated from the resected lung tissue of a microbiology laboratory scientist from Oman, who developed a MLS 14 days post allogeneic

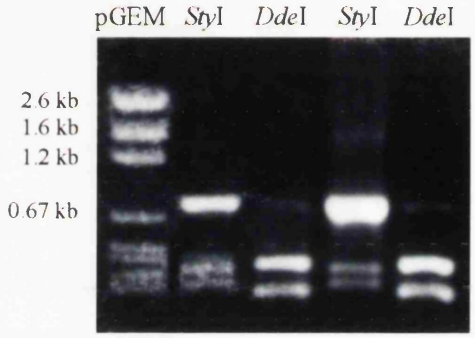


FIGURE 6-6: restriction digests of *A.fumigatus* DNA with *Dde*I and *Sty*I

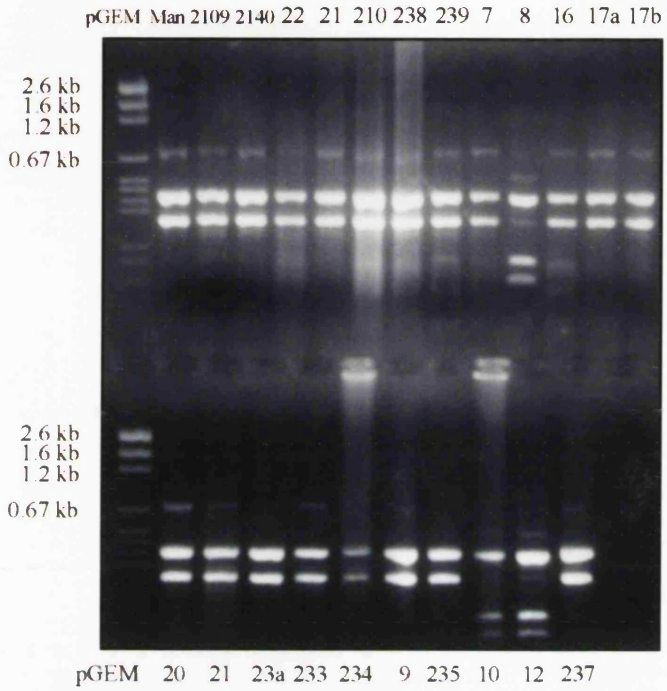


FIGURE 6-7: *Dde*I digests of PCR products by Method 2 of different strains of *A. fumigatus* and *A. flavus*

bone marrow transplantation for chronic granulocytic leukaemia in chronic phase, and who had not undergone intensive chemotherapy episodes prior to this. The unusual nature of the strain is likely to be related to the geographical origin and occupation of the patient; the short period between his arrival in the UK and his IPA would be consistent with a pre-existing colonising organism causing the aspergillosis.

6.4. SSCP ANALYSIS OF RESTRICTED AND UNRESTRICTED PCR PRODUCTS

SSCP was carried out with unrestricted PCR products by Method 1 and Method 2, and also with the *Dde*I restricted Method 2 PCR products described above.

Figure 6-8 shows the area of interest in an SSCP analysis carried out on whole PCR products from 18 *A. fumigatus* strains by Method 1. There are subtle differences between strains in the banding patterns within zone A. In particular strains 21 and 22 of local epidemiological interest appear to be slightly different. However, the small differences that are present are in the relative intensities of the small bands within zone A, rather than their number or overall position. This does not represent an objective or robust method for differentiating between *A. fumigatus* strains, particularly when the starting material is a glycerol stabilised PCR product. Further experiments using this method were not undertaken.

Figure 6-9 shows the area of interest in an SSCP analysis carried out on whole PCR products from 8 *A. fumigatus* and 2 *A. flavus* strains

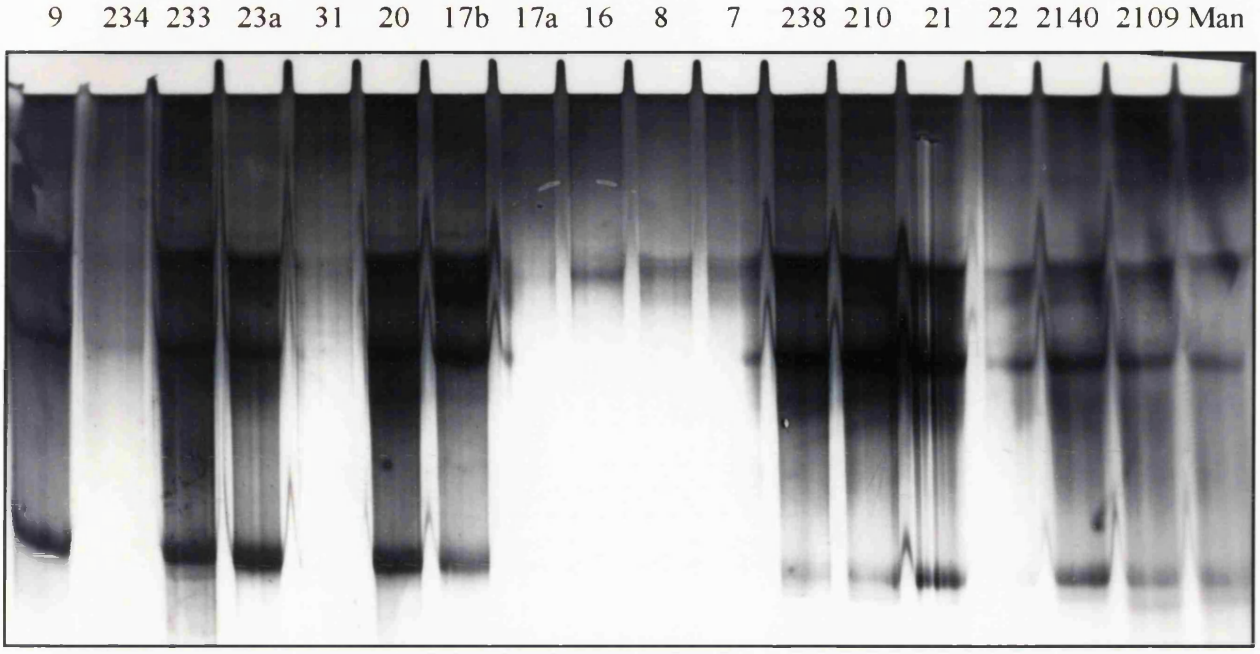


FIGURE 6-8: SSCP of PCR products by Method 1 of various *A. fumigatus* strains

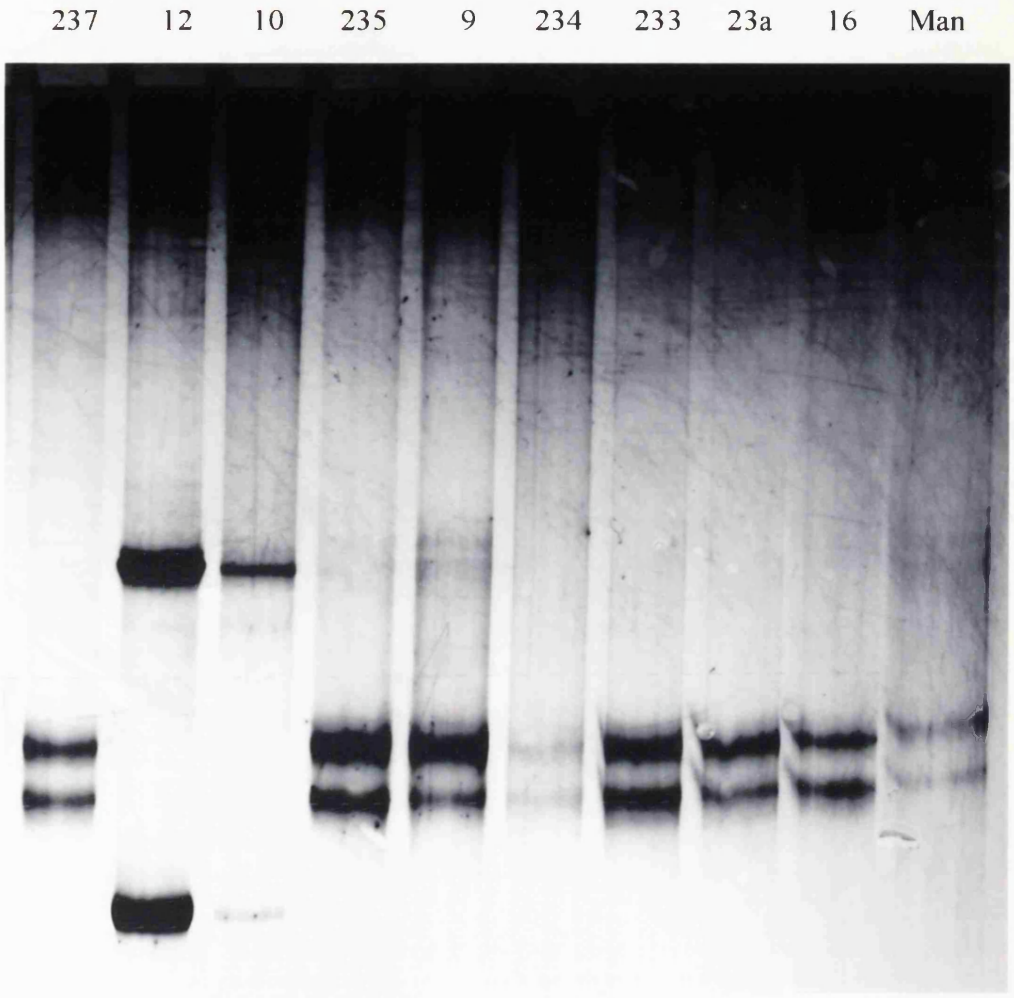


FIGURE 6-9: SSCP of PCR products by Method 2 of various *A. fumigatus* and *A. flavus* strains

by Method 2. Although there are no real differences within the *A. fumigatus* strains or between the two *A. flavus* strains, *A. flavus* and *A. fumigatus* display dramatically different patterns on SSCP, in contrast to the subtle difference in the size of the PCR product between the 2 species.

Figure 6-10 shows that *A. fumigatus* strain 8, which demonstrated a *Dde*I restriction pattern akin to those obtained with *A. flavus*, is clearly distinct both from the other *A. fumigatus* strains. Unfortunately no SSCP of unrestricted PCR products showing strain 8 in the presence of both *A. fumigatus* and *A. flavus* is available.

When SSCP was performed on *Dde*I restricted Method 2 PCR products, the patterns seen in Figure 6-11 (4 *A. fumigatus* isolates including strain 8, and all 3 available *A. flavus* isolates) and Figure 6-12 (17 *A. fumigatus* isolates including strain 8) are observed.

Again, strain 8 is clearly different from all other *A. fumigatus* isolates, and from *A. flavus* isolates, while *A. fumigatus* isolates are also distinct from *A. flavus* isolates. In addition, strains 17a and 20, isolated from patients on separate wards several months apart, both appear to lack the band transecting zone A', which is present in the other strains. Again, no differences were observed between strains 21 and 22 of local epidemiological interest.

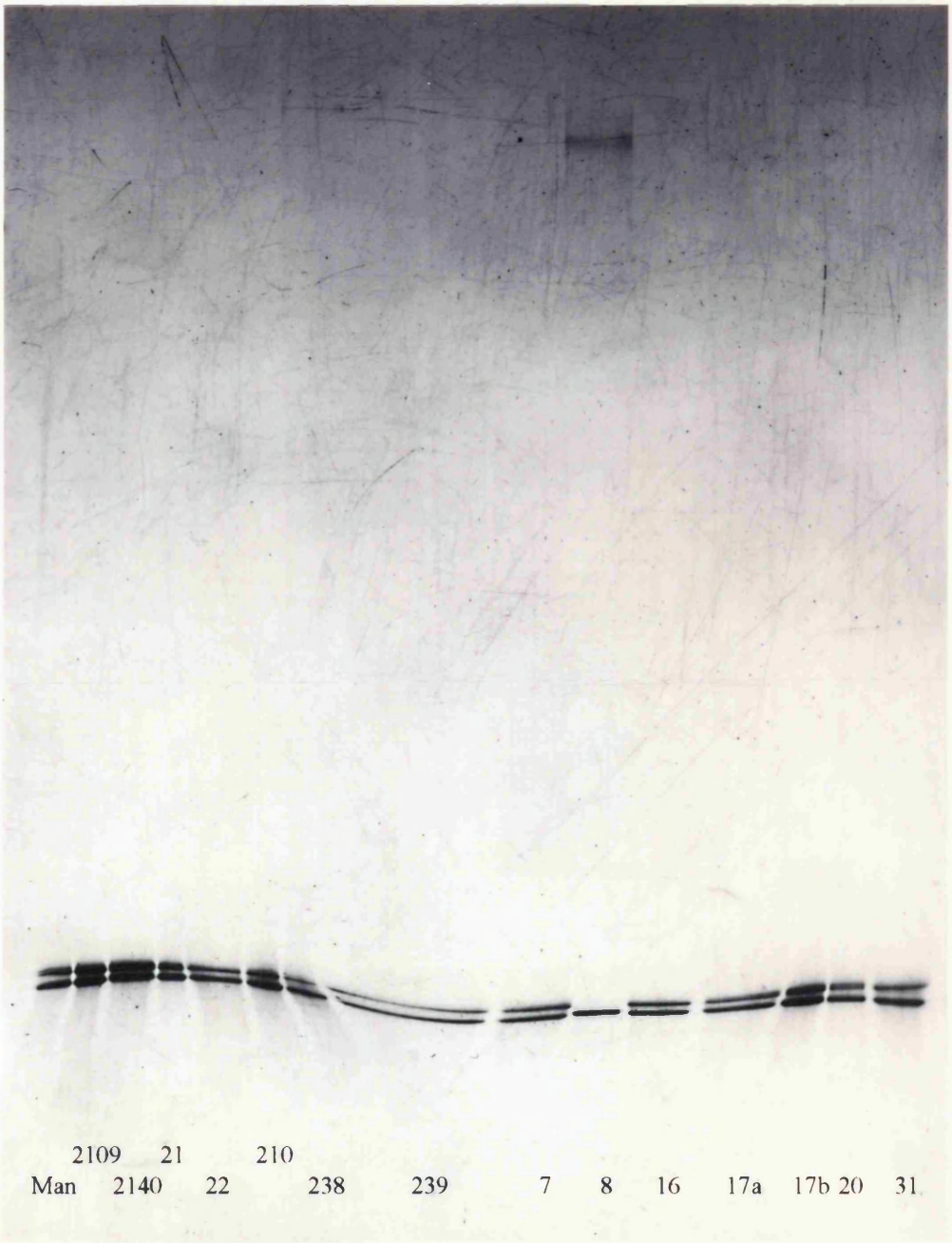


FIGURE 6-10: SSCP of PCR products by Method 2 of 15 *A. fumigatus* strains

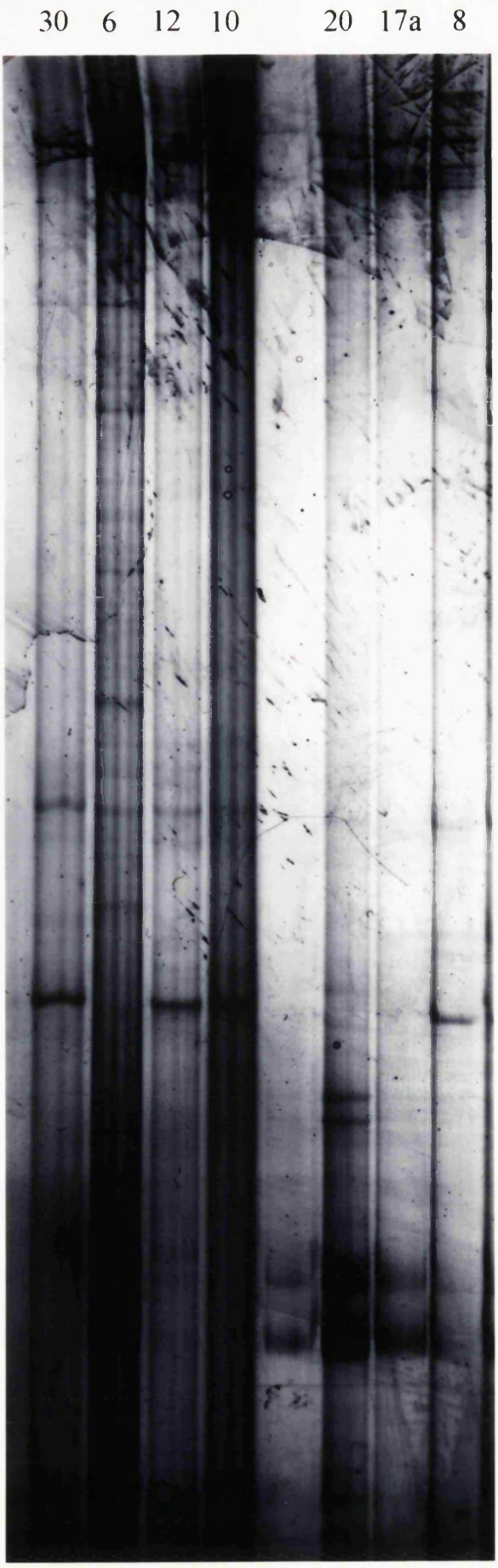


FIGURE 6-11: SSCP of *Dde*I restrictions of PCR products by Method 2 from various *A. fumigatus* and *A. flavus* strains

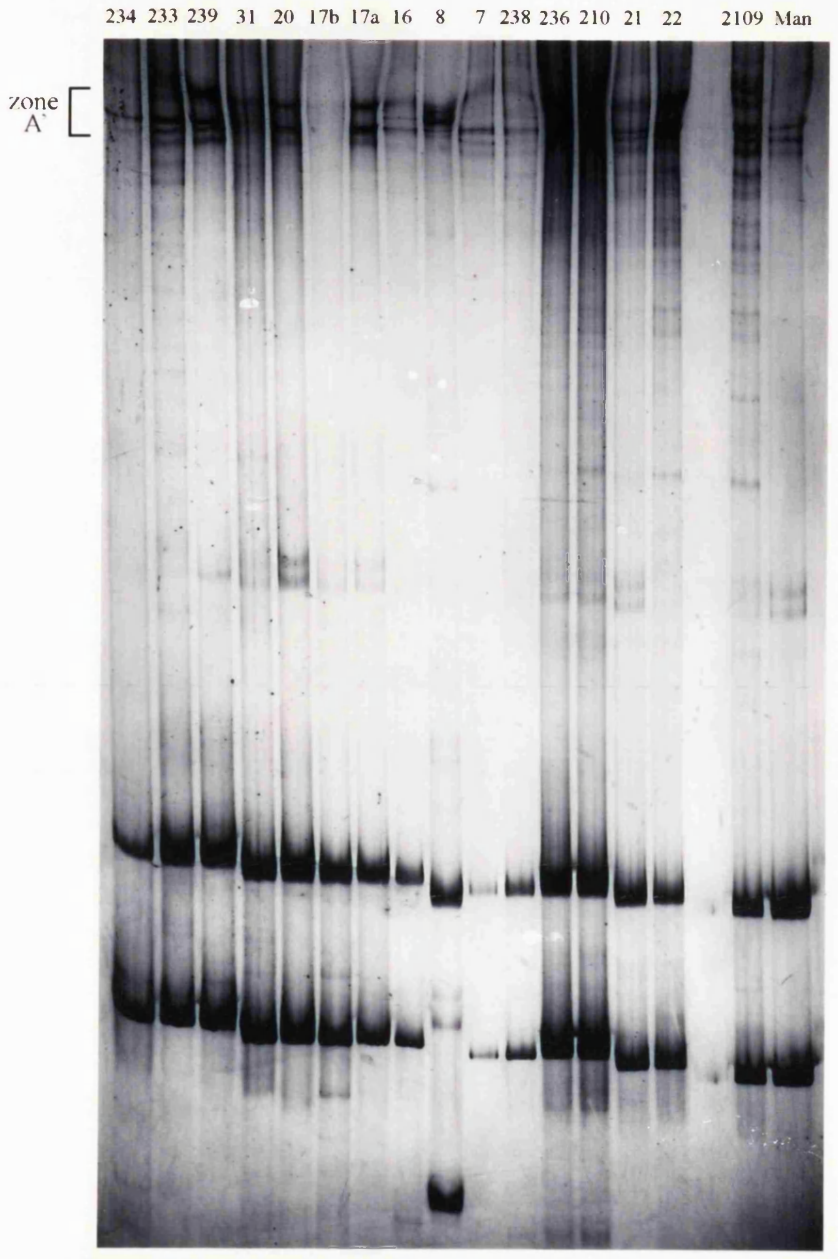


FIGURE 6-12: SSCP of *Dde*I restrictions of PCR products by Method 2 of 17 *A. fumigatus* strains

6.5. DISCUSSION OF EPIDEMIOLOGICAL STUDIES & CONCLUSIONS

The *Aspergillus fumigatus* typing method based on *Xho*I and *Sal*I restriction of genomic DNA proved too difficult to apply in the setting of the experiments described above, because of inability to extract DNA of sufficient quality. Possible reasons for this have been discussed. As a result of these difficulties, no control epidemiological method was available for the evaluation of PCR/RFLP and PCR/SSCP based techniques as typing methods. Such methods, if found to be suitable, would have the great advantage that
they do not require an isolate for their performance and that they can be applied to amplified material direct.

DNA was unobtainable from 1 *A. fumigatus* and 4 *A. flavus* strains. PCR of the 21 remaining *A.fumigatus* strains by Method 1 and Method 2 demonstrated appropriate amplification by both assays for all strains. One of 4 *A. flavus* strains for which DNA was available did not amplify with the alkaline protease gene based PCR Method 2, suggesting an alkaline protease deficient strain.

*Dde*I restriction digests of Method 2 PCR products demonstrated a variant pattern in 1 of 21 *A. fumigatus* isolates (strain 8), presumably attributable to an additional restriction site.

SSCP of the unrestricted Method 1 PCR products of 21 *A.fumigatus* strains demonstrated no definite strain variability, while SSCP of

unrestricted Method 2 products again showed two patterns in that strain 8 was unique and the others similar to each other.

SSCP on *Dde*I restriction digests of Method 2 PCR products, in addition to confirming an idiosyncratic pattern in strain 8, revealed 2 different patterns amongst the other strains. This method thus differentiates 3 patterns between 18 *A. fumigatus* isolates.

Although no control typing method was available for comparison, most successful *Aspergillus* typing techniques that have been described in the literature (as summarised in Chapter 1) tend to differentiate at least one type for every two isolates. Also, unlike the PCR based techniques tested in this chapter, *Sal*I digestion of genomic DNA demonstrated a difference between strains 21 and 22 on the only occasion when it was performed successfully. By implication, the PCR based techniques evaluated here are insufficiently discriminatory for use as typing methods as they are.

However, this category of method presents such definite attractions in *Aspergillus* typing that more work based on PCR with RFLP and /or SSCP would be worthwhile, using the same or more recently described PCR assays. The ultimate technique might then be based on the combined result of PCR with RFLP and/or SSCP, using several different PCR assays and restriction enzymes in order to achieve maximum discriminatory power with minimal requirement for DNA whilst avoiding techniques lacking precision or requiring intensive labour.

CHAPTER 7: CONCLUSIONS

A database of 87 immunosupressed haematology patients with invasive pulmonary aspergillosis was reviewed. From this group, 57 patients had lesions with imaging suggestive of aspergillosis (LISA) in the form of mycotic lung sequestrum or halo sign lesion. Forty were operable, and 37 actually underwent surgical resection of the affected lobe(s) in addition to systemic antifungal treatment. The survival for the operated group was 89% at one month, 45% at 1 year and 36% at two years. These findings, taken in the context of data reporting a partial response to antifungal treatment in 75% of cases as highly satisfactory, suggest that surgery may convey a therapeutic advantage as well as being feasible and safe.

This hypothesis was tested by assessing, within the database, the impact on death from all causes of factors including radiological appearances and whether surgical treatment was undertaken. This analysis suggests that the apparent favourable prognosis of patients undergoing resection is due to their underlying form of aspergillosis rather than to surgery itself, although the two variables were strongly correlated and difficult to disentangle. Relapsed haematological disease, as expected, was an independent predictor of death. Thus patients with LISA have a better prognosis than those with other forms of aspergillosis. The question of whether surgery improves the prognosis of such patients can now only be answered in the context of a randomised controlled trial.

The detection of LISA was found to have a positive predictive value of 90% for IPA in 39 cases verified histologically following surgical resection. Because all but 3 operable patients within the group actually underwent surgery, this favourable positive predictive value within operated patients is likely to be a true reflection of the diagnostic value of radiology in an entire population of immunosupressed haematology patients. The presence of LISA could reasonably be recognised as a sufficient diagnostic criterion for IPA on this basis and would be particularly helpful in epidemiological studies and for the enrolment of patients to trials of antifungal treatments.

Aspergillus was isolated from 54% of resected lung tissue of patients with histological aspergillosis, where the organism had survived despite a mean of 13.7 days of amphotericin B treatment. The duration of pre-operative amphotericin is not significantly different in those whose resected lung with histological aspergillosis was sterile. One such sample was found to be *Aspergillus* PCR (alkaline protease method) negative as well as culture negative, in spite of originating from a patient whose sputum was *Aspergillus* positive on several occasions. This raises the question of whether sampling errors account, at least partly, for these surprising results.

The alkaline protease based PCR (Tang *et al* 1993) and the ribosomal DNA/intergenic spacer region PCR (Spreadbury *et al* 1993) were evaluated comparatively for *A. fumigatus* detection. The alkaline protease based PCR was found to have a minimal detection level of 2 pg of *A.fumigatus* DNA (approximately 40 organisms) by ethidium bromide UV visualisation, compared with 200 pg for the

ribosomal DNA based PCR, and was chosen for subsequent experiments in view of its greater sensitivity and convenience.

Experiments designed to assess the overall performance of the extraction/PCR procedure combined showed that up to 2000 conidia of *A. fumigatus* (100 pg) needed to be processed to register a positive PCR result, implying 50 fold DNA loss during extraction or incomplete extraction of conidia. Both strain variability in conidial "extractability" and poor recovery of purified DNA from BAL were evident, suggesting that both phenomena do occur. Because the recovery of purified DNA from BAL was 20 fold worse than that from unextracted conidia in water, intrinsic properties of the BAL, such as its viscosity, were considered to also interfere with the DNA extraction procedure. While DNA loss was a problem, it was also difficult to avoid contamination of the assay whenever a strongly positive sample was present in a batch, in spite of very rigorous precautions.

For comparison, *A.fumigatus* growth on culture plates was reliably obtained within 2 days from 150 conidia, a detection rate intermediate between that of some actual extraction/PCR experiments and that of a PCR result assuming no DNA loss during extraction.

With these issues in mind, 39 *Aspergillus* culture negative BAL samples from Haematology patients with pulmonary disease were processed in a total of 122 separate PCR assays, ranging from 2 to 7 per BAL. Positive results were registered only on 9 occasions. They were only weakly positive and the findings were not reproducible,

even in patients with pulmonary aspergillosis documented by other means. In one such patient, the BAL was even persistently negative.

A variety of technical reasons can be used to explain these disappointing results, including sampling errors and the fact that the performance of the DNA extraction/PCR procedure combined is at odds with the sensitivity of the PCR in isolation. More important, however, is the striking resemblance between the drawbacks of IPA diagnosis by BAL fluid PCR and those of IPA diagnosis by BAL fluid or sputum culture.

Both methods lack reproducibility, both are susceptible to false positivity due to airway colonisation or atmospheric contamination of samples/equipment, and both appear to lack sufficient sensitivity. It is also known that individuals with localised forms of IPA (MLS or halo sign lesion) are less likely to have culture positive respiratory secretions than those with widespread disease; equivalent findings for PCR seem likely although such data is not available as yet.

The excellent preliminary results for IPA diagnosis of rapid diagnostic methods based on the detection in blood rather than in BAL of *Aspergillus* nucleic acids (Einsele *et al* 1997)or antigen suggests that the choice of body fluid for diagnosis may be at least as important as the detection technique itself or its basic sensitivity. The choice of blood rather than BAL as a diagnostic specimen is certainly logical for those forms of IPA which spread by angioinvasion rather than along the bronchial tree, and which tend to be the more frequently encountered. As well as ease of sampling, diagnostic techniques using blood have the additional advantage that

they are less susceptible to false positives due to airway colonisation. If validated, they are likely to gain widespread clinical use in future.

A technique using SSCP of *Dde*I restricted PCR products by the alkaline protease method was evaluated as a typing method and found to differentiate 3 patterns in 18 strains of *A. fumigatus*. This method lacks sufficient discrimination, but because *Aspergillus* isolation can be elusive, the prospect of a PCR based epidemiological method not requiring an isolate at all is sufficiently attractive to prompt more research in this field.

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APPENDICES

APPENDIX I: ACCESS IPA DATABASE

ID1	AGE	SEX	DIAGNOSIS	ONSET IPA	RADIOLOGY	*ASPERGILLUS* IN SECRETIONS?	ISOLATE	IPA DIAGNOSIS	BMT PRE	SAMPLE	?OPERABLE	OUTCOME	DATE LAST FU	BMT POST	Type BMT	DIED IPA	LEUKAEMIC RELAPSE
1	58	M	AML	4/6/92	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	RIP	21/7/92	NO	Nil	NO	YES
2	55	M	ALL	1/10/92	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	NO	NO	DIFFUSE	RIP	15/10/92	NO	Nil	YES	NO
3	31	M	AML	23/7/92	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	NO	NO	DIFFUSE	RIP	7/9/92	NO	Nil	YES	YES
4	30	M	CGL-ACC	10/3/92	LISA	NO	FUMIGATUS LUNG	HISTOLOGICAL	YES	ISOLATE 8	OPERATED	RIP	1/4/92	NO	ALLO	YES	NO
5	29	M	ALL	8/1/92	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	20/6/92	NO	Nil	WITH	YES
6	36	M	AML	8/9/92	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	3/11/92	NO	Nil	NO	YES
7	15	M	AML	10/3/92	OTHER	YES	*FUMIGATUS*	MICROBIOLOGY	NO	NO	NO	RIP	11/5/92	NO	Nil	NO	YES
8	17	M	ALL	6/6/92	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	25/9/92	NO	Nil	NO	YES
9	29	M	ALL	27/11/92	LISA	YES	*FLAVUS*	MICROBIOLOGY	NO	NO	NO	RIP	16/12/92	NO	Nil	YES	NO
10	25	F	AML	12/11/92	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	A+W	22/9/96	NO	Nil	N/A	NO
11	59	M	AML	25/12/92	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	LFU	30/1/93	NO	Nil	N/A	NO
12	48	F	AML	13/1/93	OTHER	NO	NIL	CYTOLOGY	YES	NO	YES	A+W	22/9/96	NO	ALLO	N/A	NO
13	49	F	MDS	10/3/93	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	NO	NO	OPERATED	RIP	21/3/93	NO	Nil	YES	NO
14	16	F	AML	2/3/93	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	YES	NO	OPERATED	RIP	13/5/93	NO	MUD	YES	NO
15	64	F	AML	2/4/93	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	NO	NO	DIFFUSE	RIP	14/4/93	NO	Nil	WITH	NO
16	56	F	AML	28/4/93	LISA	NO	NIL	RADIOLOGICAL	YES	NO	NO	A+W	22/9/96	NO	AUTO	N/A	NO
17	57	F	AML	13/7/93	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	RIP	14/9/93	NO	Nil	WITH	YES
18	37	F	CGL	15/11/93	DIFFUSE	YES	*FLAVUS*	MICROBIOLOGY	YES	NO	DIFFUSE	RIP	28/11/93	NO	ALLO	YES	NO
19	27	M	ALL	25/11/93	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	YES	BAL 1K	DIFFUSE	RIP	27/12/93	NO	MUD	YES	YES
20	29	F	AML	14/12/93	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	LFU	11/2/94	YES	ALLO	N/A	NO
21	37	F	CGL-CP	6/1/94	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	YES	ISOLATE 21 BAL 1B	OPERATED	RIP	10/2/94	NO	MUD	YES	NO
22	14	F	ALL	29/12/93	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	LFU	8/2/94	NO	Nil	N/A	YES
23	68	F	CLL	17/1/94	OTHER	NO	NIL	CYTOLOGY	NO	NO	NO	RIP	7/2/94	NO	Nil	YES	YES
24	56	M	MDS	11/3/94	LISA	YES	*FLAVUS* *FUMIGATUS*	HISTOLOGICAL	NO	ISOLATES 23A, 23B, 26, 27 + LUNG	OPERATED	RIP	16/4/94	NO	Nil	YES	YES
25	30	F	AML	23/4/94	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	LFU	9/7/95	YES	AUTO	N/A	YES
26	50	M	AML	25/5/94	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	YES	NO	DIFFUSE	RIP	29/6/94	NO	AUTO	YES	NO
27	26	M	CGL-CP	31/7/94	DIFFUSE	YES	*FLAVUS* + *FUMIG* + *TERREUS*	MICROBIOLOGY	YES	BAL 2A	DIFFUSE	RIP	4/8/94	NO	ALLO	YES	NO
28	17	F	ALL	11/11/88	LISA	YES	*FLAVUS*	HISTOLOGICAL	NO	NO	OPERATED	RIP	13/3/90	YES	Auto	NO	YES
29	32	M	AML	16/9/91	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	NO	ISOLATES 6+7	OPERATED	RIP	20/10/91	NO	Nil	WITH	YES
30	16	M	ALL	2/1/91	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	YES	NO	OPERATED	A+W	22/9/96	NO	ALLO, AUTO RESCUE	N/A	NO
31	23	M	AML	25/8/89	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	NO	NO	OPERATED	LFU	1/11/92	YES	ALLO	N/A	NO

ID1	AGE	SEX	DIAGNOSIS	ONSET IPA	RADIOLOGY	*ASPERGILLUS* IN SECRETIONS?	ISOLATE	IPA DIAGNOSIS	BMT PRE	SAMPLE	?OPERABLE	OUTCOME	DATE LAST FU	BMT POST	Type BMT	DIED IPA	LEUKAEMIC RELAPSE
32	27	M	AML	17/4/91	LISA	YES	*FUMIGATUS*	MICROBIOLOGY	NO	NO	NO	RIP	17/5/91	NO	Nil	WITH	YES
33	35	M	AML	31/8/91	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	LFU	1/11/92	YES	ALLO	N/A	NO
34	29	M	AML	18/8/91	OTHER	YES	*FLAVUS*	MICROBIOLOGY	NO	ISOLATE 5	NO	RIP	30/8/91	NO	Nil	YES	YES
35	23	F	ALL	1/3/89	OTHER	YES	*FUMIGATUS*	MICROBIOLOGY	YES	NO	NO	RIP	10/3/89	NO	ALLO	YES	NO
36	27	F	ALL	5/10/90	LISA	YES	*FUMIGATUS*	HISTOLOGICAL	NO	NO	OPERATED	A+W	22/9/96	YES	ALLO	N/A	NO
37	15	M	ALL	26/12/91	DIFFUSE	NO	NIL	PM	NO	NO	DIFFUSE	RIP	30/12/91	NO	Nil	YES	NO
38	19	M	ALL	16/12/87	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	YES	NO	OPERATED	RIP	27/1/88	NO	ALLO	YES	NO
39	76	M	AML	22/1/92	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	16/2/92	NO	Nil	YES	YES
40	40	M	MDS	12/6/91	OTHER	NO	*FUMIGATUS* LUNG	PM	NO	NO	NO	RIP	3/7/91	NO	Nil	YES	YES
41	62	M	AML	13/5/92	LISA	NO	NIL	CYTOLOGY	NO	NO	NO	LFU	20/7/92	NO	Nil	N/A	YES
42	18	M	ALL	26/3/85	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	LFU	10/5/85	NO	Nil	N/A	NO
43	27	M	ALL	9/2/91	LISA	NO	*FLAVUS LUNG*	HISTOLOGICAL	NO	NO	OPERATED	LFU	22/11/92	NO	Nil	N/A	NO
44	38	F	AML	31/8/88	LISA	NO	*ASPERGILLUS* Sp. LUNG	BIOPSY	NO	NO	NO	LFU	8/9/92	NO	Nil	N/A	NO
45	29	F	NHL	29/8/90	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	NO	NO	OPERATED	LFU	19/10/90	NO	Nil	N/A	NO
46	28	M	AML	24/12/89	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	25/3/90	NO	Nil	NO	YES
47	24	F	AML	1/6/91	LISA	NO	*FUMIGATUS* LUNG	HISTOLOGICAL	NO	NO	OPERATED	RIP	6/7/91	NO	Nil	YES	YES
48	47	F	ALL	22/2/89	LISA	YES	*FUMIGATUS*	HISTOLOGICAL	NO	NO	OPERATED	RIP	31/12/89	NO	Nil	NO	YES
49	22	M	AML	15/1/91	OTHER	YES	*FUMIGATUS*	MICROBIOLOGY	YES	NO	NO	RIP	17/1/91	NO	ALLO	YES	NO
50	57	M	AML	28/9/84	LISA	NO	*FUMIGATUS LUNG*	HISTOLOGICAL	NO	NO	OPERATED	RIP	31/1/86	NO	Nil	NO	YES
51	17	F	AML	13/9/89	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	LFU	26/3/90	NO	Nil	N/A	NO
52	56	M	CGL-BLAST	8/8/88	LISA	NO	*FLAVUS* LUNG	HISTOLOGICAL	NO	NO	OPERATED	LFU	30/11/88	NO	Nil	N/A	NO
53	33	M	AML	20/11/88	LISA	NO	NIL	BIOPSY	YES	NO	NO	RIP	13/4/89	NO	ALLO	WITH	YES
54	54	M	CGL-CP	6/5/92	LISA	NO	NIL	HISTOLOGICAL	YES	NO	OPERATED	RIP	7/6/92	NO	ALLO	YES	NO
55	17	M	ALL	5/1/86	OTHER	YES	*ASPERGILLUS* Sp.	BIOPSY	YES	NO	NO	RIP	8/1/86	NO	ALLO	YES	NO
56	50	F	SEZARY	27/9/90	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	NO	NO	DIFFUSE	RIP	10/10/90	NO	Nil	YES	NO
57	34	F	CGL-CP	7/6/92	DIFFUSE	YES	*NIGER*	MICROBIOLOGY	YES	NO	DIFFUSE	RIP	6/7/92	NO	ALLO	YES	NO
58	19	M	ALL	1/11/82	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	14/4/84	YES	ALLO	NO	YES
59	18	F	AML	1/8/85	OTHER	YES	*FUMIGATUS*	CYTOLOGY	NO	NO	NO	RIP	25/7/86	YES	ALLO	WITH	NO
60	13	F	AML	15/1/87	LISA	YES	*FUMIGATUS*	HISTOLOGICAL	NO	NO	OPERATED	A+W	22/9/96	YES	ALLO	N/A	NO

ID1	AGE	SEX	DIAGNOSIS	ONSET IPA	RADIOLOGY	*ASPERGILLUS* IN SECRETIONS?	ISOLATE	IPA DIAGNOSIS	BMT PRE	SAMPLE	?OPERABLE	OUTCOME	DATE LAST FU	BMT POST	Type BMT	DIED IPA	LEUKAEMIC RELAPSE
61	24	M	ALL	1/5/85	OTHER	NO	NIL	PM	YES	NO	NO	RIP	30/5/85	NO	ALLO	YES	NO
62	18	M	CGL-BLAST	1/7/85	OTHER	YES	*FLAVUS*	MICROBIOLOGY	YES	NO	NO	RIP	12/7/85	NO	ALLO	YES	NO
63	40	M	AML	1/8/86	DIFFUSE	YES	*FLAVUS*	MICROBIOLOGY	YES	NO	DIFFUSE	RIP	25/11/86	NO	ALLO	YES	NO
64	48	M	AML	1/8/86	OTHER	YES	*FLAVUS*	PM	YES	NO	NO	RIP	12/10/86	NO	ALLO	YES	NO
65	23	F	ALL	9/2/87	OTHER	YES	*FUMIGATUS*	PM	YES	NO	NO	RIP	25/2/87	NO	ALLO	YES	NO
66	30	F	ALL	27/12/85	OTHER	YES	*ASPERGILLUS* Sp.	MICROBIOLOGY	YES	NO	NO	RIP	8/1/86	NO	ALLO	YES	NO
67	44	M	AML	3/5/82	LISA	NO	NIL	PM	YES	NO	NO	RIP	9/6/82	NO	ALLO	YES	YES
68	37	M	AML	26/1/84	OTHER	NO	*ASPERGILLUS* Sp. LUNG	PM	YES	NO	NO	RIP	6/2/84	NO	ALLO	YES	YES
69	25	M	AML	25/7/85	OTHER	YES	*FUMIGATUS*	PM	YES	NO	NO	RIP	8/8/85	NO	ALLO	YES	NO
70	28	M	ALL	22/1/95	LISA	NO	*FLAVUS* LUNG	HISTOLOGICAL	YES	ISOLATE 29 BAL 4/5G	OPERATED	RIP	31/1/95	NO	MUD	YES	YES
71	47	M	MDS	26/1/95	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	RIP	20/4/95	YES	MUD	YES	YES
72	18	M	AML	23/1/95	LISA	NO	NIL	RADIOLOGICAL	NO	BAL 4B	NO	RIP	22/2/95	NO	Nil	YES	YES
73	22	M	SAA	17/3/95	OTHER	YES	*FLAVUS*	MICROBIOLOGY	NO	ISOLATE 30	NO	RIP	17/3/95	NO	Nil	YES	N/A (SAA)
74	44	F	MDS	9/2/95	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	RIP	27/4/95	NO	Nil	WITH	YES
75	50	M	AML	6/5/95	DIFFUSE	YES	*FUMIGATUS*	MICROBIOLOGY	NO	ISOLATE 31	DIFFUSE	RIP	12/5/95	NO	Nil	YES	YES
76	13	F	AML	10/4/95	LISA	NO	NIL	RADIOLOGICAL	NO	NO	NO	RIP	10/8/95	NO	Nil	YES	YES
77	47	F	AML	21/6/95	LISA	NO	NIL	RADIOLOGICAL	YES	NO	YES	LFU	17/6/96	NO	ALLO	N/A	NO
78	62	M	MDS	18/10/94	LISA	NO	NIL	PM	YES	NO	NO	RIP	16/11/94	NO	ALLO	NO	NO
79	43	F	AML	27/5/92	LISA	NO	NIL	NIL AT SURGERY	YES	NO	OPERATED	RIP	30/7/92	NO	ALLO	NO	YES
80	49	F	AML	19/9/95	LISA	NO	NIL	HISTOLOGICAL	YES	BAL 1C + BAL 4A	OPERATED	A+W	22/9/96	NO	AUTO	N/A	YES
81	27	M	ALL	30/10/95	LISA	NO	NIL	HISTOLOGICAL	YES	NO	OPERATED	A+W	22/9/96	NO	AUTO	NO	YES
82	28	M	CGL-ACC	5/10/95	LISA	NO	NIL	RADIOLOGICAL	YES	NO	NO	RIP	4/1/96	NO	AUTO 10 years pre	WITH	YES
83	41	M	ALL	10/11/95	LISA	YES	*FLAVUS*	NIL AT SURGERY	YES	NO	OPERATED	RIP	20/1/96	NO	ALLO	NO	YES
84	46	F	AML	20/10/95	LISA	NO	NIL	HISTOLOGICAL	NO	NO	OPERATED	RIP	13/5/96	NO	Nil	NO	YES
85	62	M	HCL	27/11/95	OTHER	NO	NIL	CYTOLOGY	NO	BAL 5F	NO	RIP	20/12/95	NO	Nil	YES	YES
86	29	F	CGL-CP	14/1/94	LISA	YES	*FUMIGATUS*	MICROBIOLOGY	YES	ISOLATE 20	YES	A+W	22/9/96	NO	MUD	N/A	NO
87	37	F	CGL-BLAST	23/1/92	LISA	NO	NIL	RADIOLOGICAL	YES	NO	NO	RIP	25/3/92	NO	ALLO	NO	YES

APPENDIX II: BUFFERS, ETC

Sabouraud Medium

62g of commercial Sabouraud medium (MAST Diagnostics DM 200) was made up to 1l with water. 400mg of chloramphenicol was added. The solution was sterilised and poured into Petri dishes.

Sterilisation of buffers and equipment

All buffers were either autoclaved in a portable bench top autoclave (Dixons Surgical Ltd) for 25 minutes at 10-15 lb/in^2 or filter sterilised prior to use. Those buffers suitable for filter sterilisation only, using a 0.3 μm filter, are marked **FSO**.

Phosphate Buffered Saline (PBS)

8g NaCl, 0.2g KCl, 1.11g Na2HPO4 and 0.24g KH2PO4 were dissolved in 800ml of distilled water. The pH was adjusted to 7.4 with HCl and the volume made up to a litre with distilled water.

Vogel's minimal nutrient solution x50

Vogel's trace element solution

Citric acid	5g
$Zn\ SO_4.7H_2O$	5g
$Fe(NH_4)2(SO_4)2.6H_2O)$	1g
$CuSO_4.5H_2O$	0.25g
$MnSO_4.4H_2O$	0.05g
H_3BO_3	0.05g
$Na_2MoO_4.2H2O$	0.05g

were made up in 100 mls distilled water and autoclaved.

Vogel's stock solution(x50)

Na_3 citrate	125g
KH_2PO_4 anhydrous	250g
NH_4NO_3 anhydrous	100g
$MgSO_4.7H2O$	10g
$CaCl_2.2H2O$	5g
Trace element solution	5mls

To make x50 Vogel's minimal medium

Each component was dissolved sequentially to prevent precipitation, in a total volume of 1000mls distilled water and the solution autoclaved. When cool, 2.5 mls of filter sterilised biotin solution, 0.1mg/ml was added.

To make up 50mls of Vogel's minimal medium, 1 ml of solution was added to 49 mls of distilled water containing 0.5 g of glucose, and filter sterilised.

Novozyme buffer

59.16g $MgSO_4$. 7 H_2O was dissolved in 100mls of deionised water. The pH was adjusted to 5.8 with 0.54 ml KH_2PO_4 0.5M and 3.46 ml of 0.5 M K_2HPO_4. The volume was made up to 200mls. **FSO**.

Protoplast separation buffer A

Sorbitol 600mM , Tris 100mM pH 7.0. **FSO** and stored at 4°C.

Protoplast separation buffer B

Sorbitol 1.2M, Tris 10 mM, Na_2EDTA 50mM pH 7.5. **FSO** and stored at 4°C.

TE (Tris EDTA) buffer (pH 7.5)

Tris 0.01M, sodium EDTA 0.001M, pH 7.5

1.21 g Tris base was weighed out and dissolved in 700 ml distilled water. 2ml sodium EDTA 0.5M pH 8.0 was added. The pH was altered to 7.5 using HCl, the volume made up to 1000ml and the solution autoclaved.

Preparation of dialysis tubing (Sigma D0405)according to manufacturers' instructions

The glycerine, included as a humectant, was removed by washing for 3-4 hours under a running tap. The sulphur contents was removed by treating the tubing with a 0.3% solution of sodium sulfide at 80°C for 1 minute. The tubing was washed with hot water for 2 minutes at 60°C followed by acidification with 0.2% v/v solution of sulphuric acid then rinsed with hot water to remove the acid.

GYEP broth

2% glucose, 0.3% yeast extract, 1% peptone.

Promega x10 buffer D (suitable for use with *DdeI*, *SalI* and *XhoI* restriction endonucleases)

pH at 37°C: 7.9, Tris-HCl 60mM, $MgCl_2$ 60mM, NaCl 1500 mM, DTT 10mM.

Promega x10 buffer F (suitable for use with *StyII* restriction endonuclease)

pH at 37°C: 8.5, Tris-HCl 100mM, $MgCl_2$ 1000mM, NaCl 1000 mM, DTT 10mM

TBE buffer (Tris Borate EDTA) x5

54g Tris base, 27.5g boric acid, 20 ml 0.5M EDTA (pH 8.0) and made up to 1000 ml.

TPE (Tris Phosphate EDTA) buffer x10

Tris -phosphate 80mM, Sodium EDTA 2 mM.

108g Tris base was weighed out and dissolved in 900ml distilled water. 15 ml 85% phosphoric acid and 40 ml 0.5M Na_2EDTA (pH 8.0) were added. The solution was made up to 1000 ml.

Bind Silane mixture

3µl Bind Silane, 5µl glacial acetic acid, 1ml distilled water.

MDE (Mutation Detection Enhancement)gel

MDE (Flowgen)	25 ml
Distilled water	63 ml
5x TBE	12 ml
Fresh 10% APS	0.4 ml
TEMED (Sigma)	0.04 ml

Preparation of solutions using Promega silver staining kit

Stop/fix solution: 1800 ml distilled water and 200mls glacial acetic acid.

Staining solution: 2l distilled water, 2g Ag NO_3, 3mls formaldehyde (1 vial).

Developing solution: 2l distilled water, 60g Na_2 CO_3 were stirred to dissolve, then placed in -20°C freezer until required. Just before use, 3ml formaldehyde (1 vial) and 400 µl sodium thiosulphate (1 vial) were added.

APPENDIX III: METHOD FOR PROMEGA WIZARD™ PCR PREPS DNA PURIFICATION SYSTEM

- For each completed PCR reaction, the aqueous phase transferred into a fresh Eppendorf tube, to avoid contamination with mineral oil.
- 100 µl of Direct Purification Buffer were aliquoted into a large Eppendorf tube. 300 µl of the PCR reaction were added and the preparation vortexed briefly to mix.
- 1 ml of Resin was added, and the preparation vortexed briefly 3 times over a one minute period.
- For each PCR product, one Wizard Minicolumn was prepared by removing and setting aside the plunger from a 3ml Luer-Lok syringe. The syringe barrel was attached to the Luer-Lok extension of each Minicolumn. A container was made available to collect waste.
- The resin/PCR product mix was pipetted into the syringe barrel. The syringe plunger was inserted slowly, and the slurry pushed gently into the Minicolumn.
- The syringe was detached from Minicolumn, and the plunger removed. The syringe was reattached to the Minicolumn, and 2 ml of 80% isopropanol pipetted into the barrel. The plunger was re-inserted, and the isopropanol pushed gently through the column to wash.
- The syringe was removed, and the Minicolumn transferred to 1.5 ml Eppendorf tube, then spun for 20 seconds at 9000g to dry the Resin.
- The Minicolumn was transferred to a fresh Eppendorf tube and 50 µl of water applied to the Minicolumn. After 1 minute,

centrifugation at 9000g for 20 seconds was performed to elute the bound DNA fragment.

APPENDIX IV: PUBLICATIONS ARISING FROM THIS THESIS

Yeghen T., Fenelon L., Campbell C. K., Warnock D. W., Hoffbrand A. V., Prentice H. G., Kibbler C. C. (1996): *Chaetomium* Pneumonia in Patient with Acute Myeloid Leukaemia. *J. Clin. Pathology* 49, 184-186.

Yeghen T., Kibbler C. C. , Prentice H. G., Berger L. A., Wallesby R. K., McWhinney P. H. M., Lampe F. C., Gillespie S.(2000): Management of Invasive Pulmonary Aspergillosis in Haematology Patients: A Review of 87 Consecutive Cases in a Single Institution. *Clinical Infectious Diseases* 31, 859-868.

Reprints of the above publications are inserted in the sleeve situated within the binding of this manuscript.

Giant cell hepatitis associated with systemic lupus erythematosus

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Abstract

Giant hepatocytes are commonly found in several neonatal and infantile liver diseases, but are rarely found in adult liver disease. A 42 year old white woman presented with a five month history of paraesthesia and numbness of both the upper and lower limbs and with vague abdominal pain. Abnormal liver function was noted on routine screening. Ultrasound scan of the abdomen showed gallstones; barium enema, ERCP and computed tomography scan were all normal. IgG antibodies to double stranded DNA were present at a titre of 40 units. Anti-cardiolipin antibodies, anti-mitochondrial antibodies and rheumatoid factor were not detected. Serology for hepatitis A, B, C, and paramyxoviruses was negative, as was the Paul Bunnell test. A clinical diagnosis of systemic lupus erythematosus (SLE) with an axonal sensory polyneuropathy was made, the latter confirmed on biopsy of the sural nerve. Giant cells were noted on liver biopsy. The patient was treated with corticosteroids; liver function had improved after two years of follow up. When extensive giant cell transformation is noted on liver biopsy, particularly when neuropathy is also a feature, the possibility of an association with SLE should be considered.

(*J Clin Pathol* 1996;**49**:183–184)

Keywords: giant cells, hepatitis, systemic lupus erythematosus.

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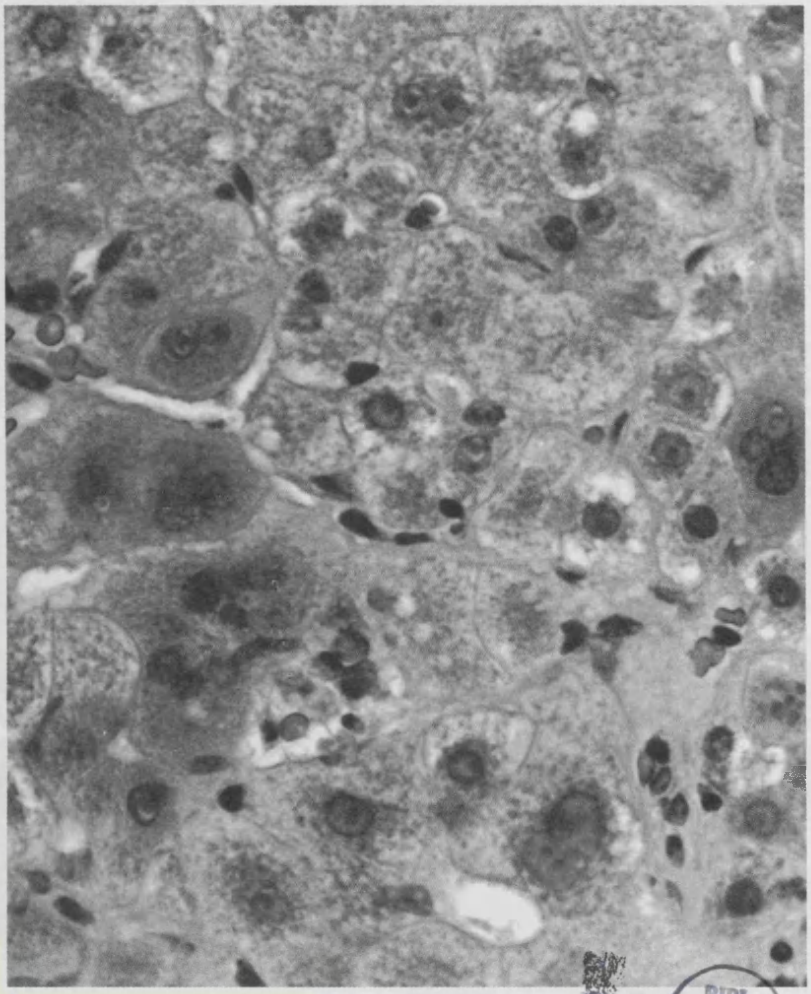
Case report

A 42 year old white woman presented with a five month history of paraesthesia and numbness of both the upper and lower limbs and with vague abdominal pain. Routine screening revealed raised liver function tests (aspartate aminotransferase (AST), 147 IU/l; alanine aminotransferase (ALT), 149 IU/l; alkaline phosphatase, 395 IU/l; bilirubin, 37 μmol/l; γ-glutamyltransferase (γGT), 217 IU/l). Ultrasound scan of the abdomen showed gallstones; barium enema, endoscopic retrograde cholangiopancreatography and computed tomography scan were all normal. IgG antibodies to double stranded DNA were present at a titre of 40 units. Anti-cardiolipin antibodies, anti-mitochondrial antibodies and rheumatoid factor were not detected. Serology for hepatitis A, B, C, and paramyxoviruses was negative, as was the Paul Bunnell test. A clinical diagnosis of systemic lupus erythematosus (SLE) with an axonal sensory polyneuropathy was made, the latter confirmed on biopsy of the sural nerve.

Examination of a liver biopsy specimen revealed nodularity with portocentral septum formation, not amounting to cirrhosis. The most striking feature was the extensive giant cell transformation of hepatocytes, occurring predominantly in periportal/periseptal regions (figure). Portal tracts were expanded by a predominantly lymphocytic infiltrate and there was extensive irregular periportal and periseptal piecemeal necrosis with marked ductular proliferation. Hydropic swelling and a noticeable accumulation of copper associated protein within hepatocytes, especially the multinucleated variety, were noted. No viral nucleocapsids could be identified on electron microscopy.

The patient was treated with corticosteroids. Follow up at two years revealed an improvement in liver function (AST, 50 IU/l; ALT, 58 IU/l; alkaline phosphatase, 334 IU/l;



Periportal region with hepatocytes demonstrating giant cell transformation.

bilirubin, 10 μmol/l; γGT, 270 IU/l). The polyneuropathy was relatively quiescent. A second liver biopsy specimen demonstrated a reduction in giant cell transformation, but inflammation and fibrosis, not amounting to cirrhosis, were still present.

Discussion

Giant cells are present frequently in neonatal liver disease, but are rarely found after infancy. Post-infantile giant cell hepatitis refers to hepatitis associated with extensive giant cell change in the adult liver. It is a purely descriptive term, encompassing a heterologous group of disorders in terms of clinical, serological and histological features.[1]

Aetiological factors implicated include drugs (methotrexate, 6-mercaptopurine and clonmetacin), viruses (hepatitis A, B and C, Epstein–Barr virus and paramyxoviruses) and autoimmune chronic hepatitis.[1] Devaney *et al*[2] found evidence of autoimmune disease (positive antinuclear antibody or direct Coombs reaction and anaemia) in 40% of patients.

About 8–23% of patients with SLE develop liver disease, which is usually of modest clinical relevance and occurs in patients with a higher frequency of positive DNA antibody of double stranded type.[3] Liver biopsy diagnoses have included granulomatous hepatitis, cirrhosis, chronic active hepatitis, chronic persistent hepatitis, and steatosis.[4]

Giant cell hepatitis has been reported in association with neonatal SLE[5] and, more recently, with SLE in an adult.[6] The first two cases of giant cell hepatitis in association with neurological features have also been published recently.[1] The current case shares similarities in that the patient presented with neurological features and abnormal liver function tests were detected only on routine screening. This suggests that when extensive giant cell transformation is noted on liver biopsy, particularly when neuropathy is also a feature, the possibility of an association with SLE should be considered.

We thank Drs RM Bernstein, JM Braganza and W Schady for permission to report this case.

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J Clin Pathol 1996;**49**:184–186

Chaetomium pneumonia in patient with acute myeloid leukaemia

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Abstract

A patient with relapsed refractory acute myeloid leukaemia developed typical fungal lung lesions despite intravenous amphotericin B prophylaxis. *Chaetomium globosum* was cultured from the resected right lower lobe. Histology showed branching hyphae negative for common *Aspergillus* species by immunohistochemical staining. Previous reports of invasive disease caused by *Chaetomium* and some applications of immunohistochemical staining for *Aspergillus* are discussed.

(*J Clin Pathol* 1996;**49**:184–186)

Keywords: *Chaetomium globosum*, pneumonia, immunohistochemical staining, *Aspergillus*.

Patients with haematological malignancies are susceptible to invasive fungal infections. Although the great majority of such infections are caused by *Aspergillus* or *Candida* species, various fungi of low pathogenicity have occasionally been reported to cause invasive disease in these immunosuppressed patients.

Because invasive fungal infections are common and devastating complications of cytotoxic therapy in this group of patients, treatment with intravenous amphotericin B is usually introduced within 96 hours of onset of a fever resistant to broad spectrum antibiotics and for which no cause has been determined, or as soon as lesions raising the suspicion of invasive fungal infection (IFI) appear. For those patients undergoing surgical resections as part of the treatment of mycotic lung sequestra (MLS), systemic amphotericin B may already have been given for several days before the operation and, in our institution, fungi are frequently not isolated from the operative sample, so that the only evidence of IFI is the presence of fungal elements on tissue sections.

These hyphae tend to be identified as those of *Aspergillus* species by default even when not entirely typical. Under such circumstances there is no routine method for proving that the

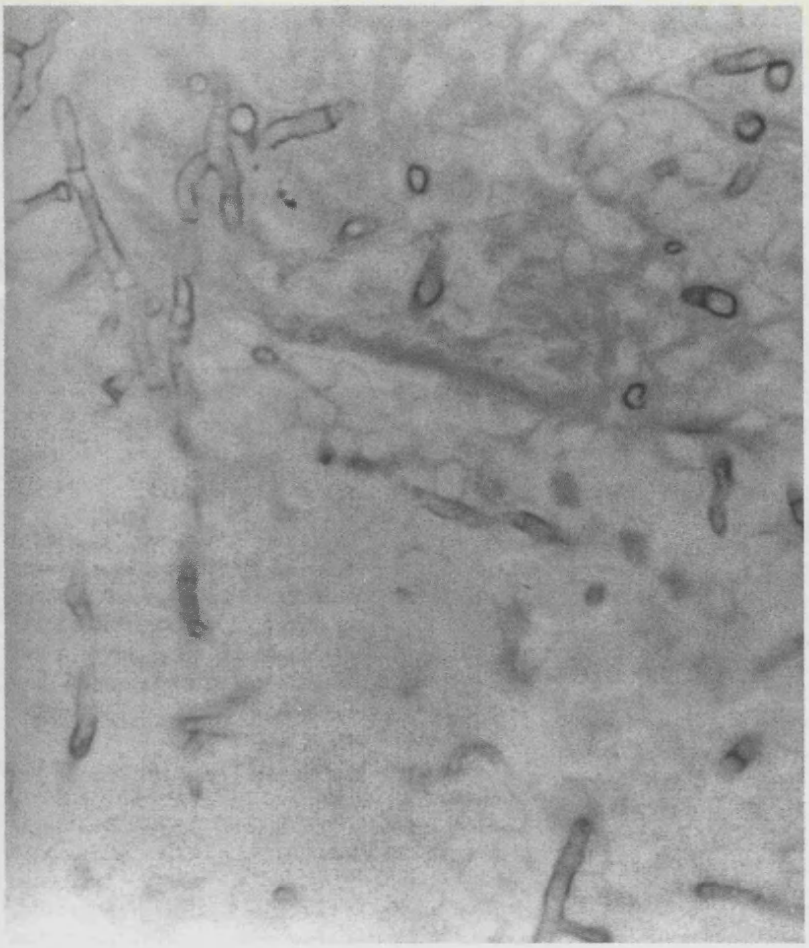

Figure 1 Histological section of lung biopsy specimen, showing branched septate hyphae. (Grocott's silver stain, × 1200).

hyphae really are *Aspergillus*. Recently, a specific monoclonal antibody directed against the common species of *Aspergillus* has been developed.[1] The sensitivity of the antibody in the published study is 88·8%, and its specificity 100%. This antibody may be helpful in clarifying the epidemiology of culture negative, histology positive IFI.

Figure 2 Culture of the isolated strain of Chaetomium globosum, *showing ruptured ascocarp wall and ascospores (× 1200).*

Chaetomium species are rarely implicated in human pathology, but are a well recognised, albeit uncommon, cause of onychomycosis in people with normal immunity. *Chaetomium* has been reported as a confirmed or suspected pathogen in the immunocompromised host on four occasions only.[2–5] Empyema was described once[4] in association with *Chaetomium*. In another case *Chaetomium* was grown from the bronchoalveolar lavage specimen of a patient whose pulmonary radiological changes probably preceded her admission to hospital, and who was nursed in a room where three patients colonised with the organism had been housed before. The air filter system was subsequently found to be contaminated with *Chaetomium* and it is likely that the isolate in this patient represented colonisation rather than infection.[3] We report what we believe is the first case of *Chaetomium* pneumonia in a patient with acute myeloblastic leukaemia (AML) and highlight potential difficulty in identifying the causal organism in IFI.

Case report

A 19 year old man from Israel was diagnosed with AML FAB type M4 Eo in March 1993. Cytogenetic studies showed inversion 16 and 7q-. He received two courses of chemotherapy in his own country and achieved remission. The second course was apparently complicated by "pulmonary infiltrates suggestive of invasive aspergillosis". He responded to intravenous amphotericin B although no fungi were isolated from his sputum and no bronchoalveolar lavage was performed. He received one further course of consolidation chemotherapy but suffered a leukaemic relapse one month later. Re-induction was not successful, and he was transferred to our institution for further treatment.

On admission, a computed tomography (CT) scan of his chest was normal and a CT scan of his sinuses showed no evidence of aspergillosis. His bone marrow was heavily infiltrated with leukaemic cells and he had no circulating neutrophils. He received amphotericin B 1 mg/kg on alternate days as secondary antifungal prophylaxis and a course of modified timed sequential chemotherapy[6 7] was given.

After nine days on amphotericin B, the patient's renal function deteriorated and prophylaxis was changed to liposomal amphotericin B 1 mg/kg daily.

On day 24 of re-induction chemotherapy, the patient developed right sided pleuritic chest pain and fever. He remained totally neutropenic. Chest *x* ray and CT scan showed cavitating lesions in the right lower lobe. The dose of liposomal amphotericin B was increased to 5 mg/kg daily and a right lower lobectomy was performed. Histology revealed branching hyphae invading blood vessels (fig 1). The hyphae were fragmented and were not present in sufficient numbers to permit further characterisation. A filamentous fungus, off-white in

colour, was grown on one of three Sabouraud plates (the 37°C plate) from the lobectomy specimen pool. Septate hyphae were seen, but no other characteristics were noted.

The fungus was submitted to the Mycology Reference Laboratory where it was subcultured onto slopes of Sabouraud's glucose agar, potato sucrose agar and Borelli's lactrimel agar, and incubated at 30°C in the dark. After one week, the off-white mould had developed a central dark pigment, especially on the lactrimel agar, but no sporing structures were produced after one month and the cultures were allowed to continue to grow at room temperature in natural light. After growth in these conditions for a further six weeks, the culture on the lactrimel plate contained mature perithecia of *C globosum* (fig 2). An attempt to repeat subcultures from the submitted culture which had been stored at 4°C for nearly three months resulted in no growth. However, a subculture stored at the Royal Free Hospital and submitted to the Mycology Reference Laboratory was found to be *C globosum*.

We were concerned that the *C globosum* might be a contaminant and that an *Aspergillus* species, which had not been successfully isolated, was the real cause of the infection. In order to investigate this, immunohistochemical staining of sections of the affected lung was performed, using a monoclonal antibody directed against the commonest species of *Aspergillus* found in human disease: *A fumigatus*, *A flavus*, and *A niger*.[1] *Aspergillus* hyphae were not seen on examination of these lung sections, suggesting that *C globosum* was indeed the cause of the infection.

The patient did not achieve remission and salvage chemotherapy was attempted with high dose melphalan with a view to proceeding to a volunteer unrelated donor transplant.

He developed new cavitating lesions in the right lung seven weeks after the initial operation in spite of continuous treatment with high doses of liposomal amphotericin B (5 mg/kg). He had remained profoundly neutropenic throughout. A right pneumonectomy was performed in mid-February 1994. Two weeks following surgery, after a good initial recovery, he suffered a respiratory arrest and died in spite of respiratory support. No fungi were isolated from the pneumonectomy sample, but branching hyphae were again seen in tissue sections. A necropsy was not performed.

The patient had died before the *Chaetomium* was characterised, and the results of the immunostaining became available.

The culture of *C globosum* was added to the UK National Collection of Pathogenic Fungi as NCPF 7115.

Discussion

Speciation of fungi involved in invasive infection in neutropenic patients is not possible when such an infection is diagnosed by histological examination with no confirmatory culture results. Although *Aspergillus* is said to have characteristic appearances in tissue sections (dichotomous branching at 45°), a definite diagnosis is not possible if few hyphae are present, or the invaded part of the tissue small. Fusariosis may give similar appearances, although this infection is extremely rare in our institution.

A precise diagnosis is not always considered necessary for therapeutic purposes, as amphotericin B is the drug of choice for most infections with filamentous fungi. *Fusarium* species and *Pseudallescheria boydii* respond poorly to this drug, however, and confirmed infection with these organisms warrants a change of treatment to an azole antifungal. Exclusion of an *Aspergillus* infection may thus be useful for therapeutic purposes, given the different treatment required for these alternative pathogens.

In our case, the fungus that was isolated is a rare pathogen and a frequent contaminant. It might have been dismissed as such without the immunopathological study. Although proving that a common species of *Aspergillus* was not responsible for the patient's infection does not prove that the *Chaetomium* was the pathogen, it does make it more likely.

There were no treatment implications in this particular instance, both because a fulminant clinical course preceded full characterisation of the organism, and because invasive *Chaetomium* infection is extremely rare, so that treatment guidelines based on previous experience are unavailable. Of the documented cases, one was not treated with systemic antifungals,[2] one was treated with intravenous amphotericin B with initial success,[4] and the third—the only case where in vitro sensitivity tests were carried out—was resistant to amphotericin B but sensitive to ketoconazole, which was given to the patient with good clinical, but poor mycological response[5]; isolation of *Chaetomium* ceased only after removal of the peritoneal dialysis catheter.

In conclusion, immunostaining was helpful on this occasion in excluding *Aspergillus* infection and ascribing pathogenicity to *C globosum* in a case of fungal pneumonia. The development of immunostaining techniques capable of identifying a variety of different fungi would be a considerable aid in the investigation of invasive fungal infections.

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Management of Invasive Pulmonary Aspergillosis in Hematology Patients: A Review of 87 Consecutive Cases at a Single Institution

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Eighty-seven patients with hematologic malignancies and invasive pulmonary aspergillosis (IPA) were identified between 1982 and 1995. Of these, 39 underwent lung resection on the basis of radiological detection of at least 1 lesion with imaging suggestive of aspergillosis (LISA). IPA was confirmed histologically in 35. The presence of LISA had 90% positive predictive value for IPA. The actuarial survival at 2 years was 36% for 37 patients treated surgically, 20% for 12 patients with unresected LISA but no cultures of *Aspergillus* species, and 5% for 21 patients diagnosed only by isolation of *Aspergillus* from respiratory secretions. Analysis by proportional hazard models showed a significant independent negative association between the radiological appearance of LISA and death from all causes. Relapsed hematologic disease was independently significantly associated with death. Age, sex, surgery, previous bone marrow transplantation, or *Aspergillus* isolation were not independent predictors of death. IPA presenting as LISA carries a relatively good prognosis, possibly explaining the better survival of patients undergoing surgery for such lesions.

Invasive pulmonary aspergillosis (IPA) is a frequently lethal complication of hematologic malignancies and occurs following both conventional chemotherapy and bone marrow transplantation (BMT), as well as in untreated severely neutropenic patients. The incidence of invasive aspergillosis is increasing [1].

IPA remains a diagnostic and therapeutic challenge. Diagnostic difficulties arise from the protean clinical and radiological manifestations of the disease as well as from the high incidence of sterile cultures from respiratory secretions, even from patients exhibiting postmortem evidence of the disease subsequently [2]. In particular, sputum or bronchoalveolar lavage (BAL) specimens are nearly always negative in focal forms of IPA [3]. Furthermore, *Aspergillus* is difficult to culture from lungs showing histological evidence of aspergillosis, with *Aspergillus* culture–negative lungs found at postmortem in up to 30% of untreated patients with IPA [4].

The opposite problem, environmental contamination by *Aspergillus* of clinical specimens (usually sputum) taken from a nonsterile site, also occurs. Because of this, cultures positive for *Aspergillus* found on one occasion only are generally classified as being due to colonization or environmental contamination. The diagnostic status of patients with such cultures can be controversial, and only cases satisfying a set of rigorous but variable criteria [5] tend to be reported in the literature.

For example, criteria for "definite aspergillosis" according to current published definitions by the Mycoses Study Group [6] include histological diagnosis with or without positive culture, or a positive culture from tissue obtained by an invasive procedure such as transbronchial biopsy or percutaneous needle aspiration. "Probable aspergillosis," according to the same definitions, is applicable only to pulmonary disease and includes the radiological appearance of new nodules or new cavities in a host predisposed to IPA, who must also have a minimum of 2 cultures from sputum or one from BAL fluids or positive cytology on BAL. Several of these criteria require the use of invasive procedures often avoided in clinical practice.

The incidence of IPA is therefore almost certainly underestimated, and this results in difficulty in assessing prognosis and therapeutic intervention. On the other hand, IPA is often treated in clinical practice on the basis of typical radiological appearances that, when found in patients susceptible to aspergillosis, strongly suggest the diagnosis even in the absence of any microbiological or histological evidence. Such appearances are well described and include the mycotic lung sequestrum [4] on plain radiograph or CT scan and the "halo sign" lesion on CT scan [7–9], as well as cavitating lesions.

For convenience, herein we have grouped mycotic lung sequestrum, cavitation, and halo sign lesions together and will refer to them as lesions with imaging suggestive of aspergillosis (LISA). These lesions by definition are focal and therefore amenable to surgery. If not resected, they can lead to life-threatening hemoptysis due to pulmonary vascular infiltration.

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Recent guidelines for the investigation of invasive fungal infections [10] state that resection of isolated pulmonary LISA is the best way to confirm the diagnosis. The therapeutic benefit of such surgery, on the other hand, is by no means established [3, 4, 11–18], and encouraging results, such as those previously reported by our institution [12], must be treated with caution in view of the lack of randomized trials comparing medical and surgical treatment of IPA.

Because of the risk of hemoptysis associated with LISA, and the prospect that excision of diseased lung tissue would cure the condition, most patients with LISA in our program who could sustain an operation were referred for resection until the recent introduction of treatment with granulocyte-macrophage–colony stimulating factor (GM-CSF; molgramostim) and liposomal amphotericin B. Since then surgery has become the second-line treatment of choice. Patients with typical radiological findings or other evidence of IPA are currently given a "trial of therapy" with amphotericin B or a lipid-based amphotericin B formulation plus GM-CSF before surgical intervention is contemplated. The choice of amphotericin B preparation is made according to a local standard protocol. This is based mainly on factors relating to toxicity, particularly nephrotoxicity, and the concurrent use of other necessary nephrotoxic drugs, although treatment with high-dose lipid-based amphotericin B is considered for patients with worsening inoperable lesions.

To optimize the detection of such typical radiological lesions, CT scans of the thorax (which were previously requested sporadically) have been performed routinely since 1990 for all neutropenic patients with a normal chest radiograph who fail to respond to broad-spectrum antibiotics after 4 days, the time at which amphotericin B is introduced in our neutropenic fever protocol. Resections were mostly done in the absence of supportive evidence from culture or examination of a biopsy specimen.

Data from these cases have been systematically gathered, as have data from other cases of aspergillosis in patients not undergoing surgery. Three publications have already considered various aspects of some of the surgically treated cases [3, 4, 12].

This study has examined all of these data with the aim of establishing the positive predictive value of the radiological diagnosis of LISA, based on cases with subsequent histological confirmation after surgery; the operative details and complication rates of those whose IPA was treated surgically; a comparison of outcomes of patients with IPA according to demography, diagnostic criteria, and treatment modalities, with a particular view to assessing the influence of surgery on prognosis; and the relationship between isolation of *Aspergillus* from resected lung tissue and the duration of preoperative treatment with amphotericin B.

Patients and Methods

Prospective registers of patients with likely or proven systemic mycoses are kept by the hematology, microbiology, and radiology departments. We identified all patients with hematologic malignancies who developed IPA (according to our definitions detailed below), with or without aspergillosis at other sites, during the period from 1 March 1982 to the end of 1995 and studied their medical records.

The following definitions for IPA were used. Patients were classified as having a "histological diagnosis after surgery," a "biopsy diagnosis," or a "postmortem diagnosis" according to whether lobectomy, biopsy, or postmortem, respectively, yielded lung tissue that showed evidence of septate hyphal with dichotomous branching at 45°, whether or not *Aspergillus* was isolated from any source at any stage.

Patients were classified as having a "radiological diagnosis" if they were at risk of IPA and ≥ 1 LISA was detected in the chest by radiological examination and they had fever that did not respond to antibiotics.

Patients were classified as having a "microbiological diagnosis" if they had a clinical course and radiological appearances in keeping with IPA and *Aspergillus* cultured from at least 1 sputum or BAL sample (routinely incubated on Sabouraud medium at 37°C for a minimum of 10 days) but no subsequent histological confirmation. (In otherwise convincing pulmonary cases, throat or nasal swabs positive for *Aspergillus* were also taken as supporting evidence for IPA).

Patients were classified as having a "cytology diagnosis" if they had a clinical course and radiological appearances compatible with IPA and positive cytology for *Aspergillus* at a relevant site but no subsequent histological confirmation.

Three of these diagnostic categories are clearly based on different clinical presentations of IPA. The category "histological diagnosis after surgery" includes all patients with LISA (therefore likely to be *Aspergillus* culture–negative) who underwent surgery, "radiological diagnosis" includes all patients with LISA who were culture-negative and did not undergo surgery, and "microbiological diagnosis" includes patients unlikely to have LISA or sustain an operation. We determined the actuarial survival rates at various times for these diagnostic categories and for the biopsy and cytology categories grouped together, as well as the number of deaths from all causes, the number of deaths from IPA, and follow-up data. The IPA survival time was taken as the date of onset of symptoms or signs leading to the diagnosis of IPA until the date of death, loss to follow-up, or September 1996, whichever was soonest.

The impact of the following factors on death from all causes was assessed by use of Cox's proportional hazards survival analysis models: age, sex, radiological appearances, isolation of *Aspergillus* from respiratory secretions, previous hematologic treatment (BMT or not), status of hematologic disease (relapsed or not), and whether IPA was treated or treatable surgically. These models were also used to adjust for several factors simultaneously to determine which factors were independently predictive of death. Results are presented as relative risks with 95% confidence intervals. Statistical significance was assessed by use of the likelihood ratio statistic. Survival curves comparing different levels of each individual factor were constructed by use of the Kaplan-Meier technique.

Results

Demography and previous hematologic treatment. A total of 87 patients (35 female, 52 male; age range, 13–76 years) fulfilled

Table 1. Demography and previous hematologic treatment of patients with invasive pulmonary aspergillosis (IPA) according to diagnostic category and whether the patients underwent surgery, compared with Mycoses Study Group criteria for IPA.

Diagnostic category	*n*, (female/male)	Age in y, median (range)	Prior BMT		Chemotherapy alone or no treatment	No. with LISA	Mycoses Study Group criteria		
			Allogeneic	Autologous			Definite IPA	Probable IPA	Possible IPA
Histological diagnosis after resection	35 (14/21)	31 (13–76)	7	2	26	35	35	0	0
Radiological diagnosis only	12 (8/4)	37 (13–58)	3	1	8	12	0	0	12
LISA, resection, no diagnosis	2 (1/1)	42 (41–43)	2	0	0	2	0	0	2
Microbiological diagnosis	21 (7/14)	29 (15–55)	10	1	10	3	0	11	10
Cytological diagnosis	5 (3/2)	62 (18–68)	2	0	3	1	0	5	0
Biopsy diagnosis	3 (1/2)	33 (17–38)	1	0	2	2	3	0	0
Postmortem diagnosis	9 (1/8)	37 (15–62)	7	0	2	2	9	0	0
Total	87 (35/52)	36 (13–76)	32	4	51	57	47	16	24

NOTE. For patients with histological diagnosis following surgery and postmortem diagnosis, final Mycoses Study Group status is given. For definitions of the diagnostic categories, see Methods. BMT, bone marrow transplantation; LISA, lesions with imaging suggestive of aspergillosis.

our criteria for pulmonary aspergillosis during the study period. Thirty-two (11 female, 21 male; age range, 16–62 years) had undergone allogeneic BMT before developing IPA. Five of the allogeneic BMT patients had received transplants from volunteer unrelated donors. The grafts of 4 patients had failed before they developed IPA. A further 5 allografted patients had relapse of their original disease. They were being treated again at the time they developed IPA. Four patients (2 men, 2 women; ages, 56, 27, 49, and 50 years) had undergone autologous BMT before they developed IPA. Two of these were being treated for relapsed leukemia when they developed IPA. The remaining 51 patients (21 female, 30 male; ages, 13–76 years) had received chemotherapy alone ($n = 47$) or no treatment ($n = 4$) when they developed IPA. Ten of these patients underwent BMT after their aspergillosis was treated (1 volunteer unrelated donor, 2 autologous BMT, 7 sibling allogeneic BMT). These data are summarized in table 1.

Table 1 also shows the classification of patients according to our diagnostic criteria and to Mycoses Study Group criteria [6]. In cases in which a diagnosis of IPA became definite after surgical resection or postmortem examination, the final Mycoses Study Group status of the patient is shown.

Positive predictive values of radiological diagnosis of IPA on the basis of histological and microbiological findings in resected lung tissue. In all surgical cases, the presumptive diagnosis based on radiology was IPA, so that histological and microbiological analysis of resected samples has enabled us to establish the predictive values of our radiological diagnosis. The data are summarized in table 2.

Of 59 patients with LISA, 39 underwent resection, after which aspergillosis was histologically confirmed in 35. Alternative infectious causes for IPA were found for 2 of the remaining patients (*Chaetomium* species in 1 and *Mycobacterium avium intracellulare* in the second). Although no pathology was identified for a third patient, and only adhesions with loculated fluid were found in a fourth, IPA remained the likely explanation for their lung disease; therefore 57 patients had LISA due to proven or probable IPA.

Of note, *Aspergillus* was cultured from respiratory tract samples or nasal swabs of only 8 (14%) of these 57 patients, including preoperatively in 5 (14%) of the 35 cases subsequently confirmed histologically. The positive throat swab was taken from the patient with LISA found to have a sterile loculated pleural effusion at thoracotomy.

The positive predictive value of LISA detection for IPA is 90%, as 2 of 39 cases were "misdiagnosed" and 2 not confirmed.

Table 2. Histology and microbiology results for 59 patients with invasive pulmonary aspergillosis (IPA) and lesions with imaging suggestive of aspergillosis (LISA) according to whether they underwent surgery.

Surgical treatment	No. with LISA	Supportive findings from culture or biopsy at detection of LISA (no. of patients)	Findings on examination of resected tissue or at PM	
			IPA confirmed, no. of patients	Other diagnosis confirmed (no. of patients)
Resection	39	Positive cultures of sputum (3); positive nasal swab (1); positive throat swab (1); positive lung biopsy sample with positive culture of sputum (1); fungal elements seen on bronchoalveolar lavage, no growth (1)	35	*Chaetomium* species (1); *Mycobacterium avium intracellulare* (1); loculated adhesions, decorticated, nonfocal (1); loculated adhesions, decorticated, no focal lesion (1); no pathology found (1)
No resection	20	Positive biopsy of facial tissue (1); positive subcarinal biopsy (1); positive cultures of sputum (2); positive nose swab (1); positive pleural cytology (1)	2 (PM)	Negative lung biopsy (1)
Total	59	14	37	5

NOTE. Patients from whom *Chaetomium* species and *M. avium intracellulare* were isolated are not included in table 1 or in the general IPA data but are shown here to demonstrate the number of false-positive results (thus the number of patients with LISA is 57 in table 1 but 59 in table 2). Two patients with LISA who underwent surgery and were not found to have focal IPA at thoracotomy possibly both had IPA and are included in all tables. PM, postmortem.

In practical terms, however, the patient with the *Chaetomium* infection [19] had the same treatment requirements as they would if they had a case of IPA, and the patient with the *M. avium intracellulare* infection also benefited from lung resection.

The decortication performed for the patient with a loculated pleural effusion had diagnostic and therapeutic intent, but 1 patient did undergo an unnecessary thoracotomy (no lesion was identified and no tissue was resected). This was 12 days after the initial diagnosis by CT scan. She had received amphotericin for the 24 days before surgery. A repeat CT scan 11 days after surgery confirmed resolution of the lesion, a result in keeping with late success of the amphotericin B treatment.

The negative predictive value of LISA is more difficult to establish, but we assume it to approach 100% with CT scanning, since we have had no postmortem reports of such focal forms of IPA that did not have LISAs detected in life.

Background of patients with histologically documented IPA after resection. The demographic details and prior hematologic treatment of the 35 patients who had IPA confirmed histologically after surgery (with or without isolation of *Aspergillus*) are shown in table 1. Nine of these patients had developed IPA after BMT, of which only 2 were uncomplicated sibling allogeneic BMTs. Twenty-five patients developed IPA after receiving chemotherapy alone, and 1 patient with newly diagnosed myelodysplasia had received no cytotoxics. Eight subsequently underwent BMT (1 autologous, 7 allogeneic) without relapse of their aspergillosis. These patients had also received additional prophylaxis with amphotericin B to cover the transplant procedure.

Patients underwent the following surgical procedures: partial lobectomies, 4 patients (including 1 who had a partial pleurectomy simultaneously); multiple lobectomies/segmentectomies, 11; right upper lobectomies, 12; right lower lobectomies, 2; left upper lobectomies, 2 (including a patient whose nasopharyngeal aspergillosis was debrided at the same time); left lower lobectomies, 2; and pneumonectomies, 2.

Complications and recurrence of IPA following surgery. Eighteen patients had an uncomplicated postoperative course, including the 1 patient who had emergency surgery for massive hemoptysis. Three patients had postoperative hemorrhage, and 1 required a repeat thoracotomy. One wound and 1 nonfungal chest infection were also seen after surgery. In another case, *Aspergillus*-infected tissue was accidentally spilled in the thoracic cavity during surgery, with no ill effects.

Two patients underwent revision pneumonectomies after initial lobectomies for progressive *Aspergillus* infection. One of them (with relapsed refractory acute myelogenous leukemia) died soon afterwards, and the other is a long-term survivor. Two patients died of microbiologically proven *Aspergillus* pneumonia 3 and 8 days after surgery. Another died of disseminated aspergillosis (including *Aspergillus fumigatus* grown from a biopsy specimen of the vitreous humor) 18 days after surgery. Three further patients died of respiratory failure 1–10 days after surgery, although no microbiological cause was found or postmortem examination carried out. Another died of apparent septic shock (culture yielded no organism) 4 days after surgery. All 4 of these early postoperative deaths were presumed due to progression of IPA.

Finally, 2 patients died of unclassified interstitial pneumonitis after allogeneic BMT, including matched unrelated donors (MUD), 1 at 27 days after surgery and 1 at 52 days. Although radiologically not typical, recurrent IPA cannot be excluded. These postoperative complications occurred in 9 (35%) of the 26 who had not undergone BMT before developing IPA, whereas all but 1 of the BMT patients (89%) had postoperative complications.

Reasons for not operating on patients with radiological and microbiological diagnosis. We examined the reasons for not operating on the patients with radiological diagnosis and also on those with a microbiological diagnosis who did not have diffuse lung disease. Ten patients with microbiological diagnosis but without diffuse lung disease did not undergo a surgical procedure. The reasons were as follows: 1 patient (the only patient with single LISA) improved and survived, 2 had disseminated aspergillosis, 3 had refractory hematologic disease, and 4 deteriorated very rapidly, including 1 patient who had initially improved.

The 12 patients with radiological diagnosis did not undergo surgery for the following reasons: 1 had multiorgan failure, 1 improved, 6 had untreatable hematologic malignancies (of whom 2 had stable aspergillosis until their death from other causes), and 4 had multiple lesions in sites that precluded operation, all but 1 of whom made complete recoveries. Thus, only 3 patients could be operated on but did not undergo surgery.

Survival. Table 3 shows the survival data for patients according to their diagnostic category; some of the data are represented in the form of a survival curve in figure 1. Both the table and the figure show that for patients with microbiological diagnosis, the survival rate was low at all points, and there was a high proportion of early deaths. The survival rate at 2 years was only 5%, which represents the single patient with 1 LISA and positive *Aspergillus* culture from the nose (rather than from lower respiratory secretions). Of the deaths in this group, 85% were due to IPA. The cytology or biopsy group had a 2-year survival rate of 14%, and 80% of deaths were due to IPA.

Table 3 and figure 1 also show the higher survival rate for the 2 groups constituted exclusively of patients with LISA, namely the postoperative group, with 36% actuarial survival rate at 2 years, and the radiological diagnosis group, with 20%. The percentages of deaths due to IPA were very similar in these groups, at 54% and 50% of all deaths, respectively.

Table 4 and figure 2 show Cox's proportional hazards analysis of the impact of various individual factors on death from all causes and corresponding survival curves. The adjustments for several factors simultaneously are shown in table 5.

Table 3. Survival rates of patients with invasive pulmonary aspergillosis (IPA), by diagnostic category.

Diagnostic category	*n*	Patients who died: Total no.	Patients who died: No. from IPA (% of total)	No. lost to follow-up	Days of follow-up, median (range)	% of patients surviving at: 2 w	1 mo	3 mo	12 mo	2 y
Radiological diagnosis only	12	8	4 (50)	3	77 (30–1243)	100	91	31	20	20
Microbiological diagnosis	21	20	17 (85)	0	13 (0–982)	45	29	9	5	5
Cytological or biopsy diagnosis	8	5	4 (80)	2	106 (3–1469)	87	58	44	29	14
Histological diagnosis after resection[a]	37	22	12 (54)	10	81.5 (9–3538)	94	89	56	45	36
All patients[b]	87	64	44 (69)	15	46 (0–3538)	79	67	41	26	26

NOTE. For definitions of the diagnostic categories, see Methods.

[a] Excluding 1 patient from whom *Chaetomium* was isolated and 1 patient from whom *Mycobacterium avium intracellulare* was isolated, but including 1 patient with a normal thoracotomy and 1 patient with sterile loculated effusion (thus, 35 of 37 patients fit the diagnostic category "histological diagnosis after resection").

[b] Includes postmortem diagnosis.

Analysis by individual risk factor reveals a nonsignificant trend toward increased risk of death with older age, with hematologic relapse, and with BMT as a previous hematologic treatment. The actuarial curves also show that although survival rate was similar at 2 years between the BMT and non-BMT groups, most deaths occurred very early in the BMT group.

On the other hand, compared with radiological presentation as LISA, other radiological changes were significantly associated with increased risk of death, particularly if diffuse. Other factors that decreased the risk of death considerably were having undergone pulmonary resection for IPA and being able to sustain an operation (although only 3 patients who could be operated on were not in fact operated on).

The isolation of *Aspergillus* species from sputum and male sex were associated with a 2-fold increased risk of death, but these relative risks were attenuated and no longer significant when several factors were adjusted for simultaneously (table 5). In this adjusted model, the only factors that had a significant independent effect on risk of death were radiological presentation and hematologic relapse. Hematologic relapse more than doubled the risk of death, but its effect was not apparent in unadjusted analysis because of the higher proportion of relapsed patients who had LISA rather than other radiographic presentation.

Because radiological findings and pulmonary resection were very strongly correlated, it is not surprising that these 2 variables were not independent predictors of mortality. In the adjusted model, radiological findings remained strongly predictive of death: findings other than LISA increase the risk 4-fold, whereas the RR associated with not undergoing resection was greatly reduced. This seems to suggest that the apparent favorable prognosis for patients undergoing resection is due to their underlying form of IPA rather than to the surgery itself. However, it is difficult to disentangle the effects of these 2 factors in this study because nearly all patients who could be operated on actually underwent resection.

The outcome in a group of 8 patients whose IPA was treated with lung resection before BMT (allogeneic in 7, autologous in 1) is worth noting. Their actuarial survival rate was high, 71% at 2 years. Both deaths were from leukemic relapse and none of these 8 patients suffered a relapse of their aspergillosis.

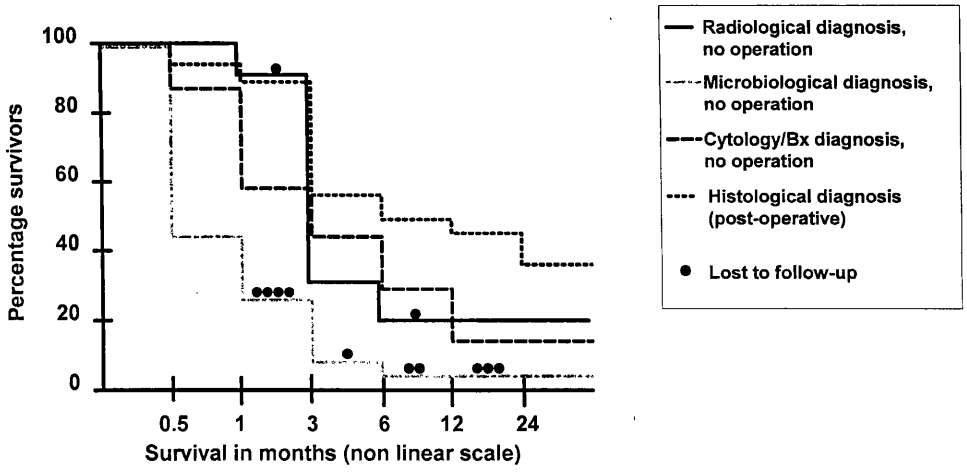

Figure 1. Survival rate of patients with invasive pulmonary aspergillosis, according to method of diagnosis and whether patients received surgical treatment or not.

Table 4. Impact of individual patient characteristics on deaths from all causes in patients with invasive pulmonary aspergillosis (IPA), expressed as unadjusted relative hazards (RH).

Characteristic	*n*	Unadjusted RH (95% CI)	Likelihood ratio statistic χ^2 (df)	*P*
Age, years			1.6 (2)	.45
<30	41	1		
30–49	28	1.1 (0.6–1.9)		
≥50	18	1.5 (0.8–2.9)		
Sex			6.8 (1)	.009
Female	35	1		
Male	52	2 (1.2–3.4)		
Isolate grown from sputum sample			9.7 (1)	.002
No	55	1		
Yes	32	2.2 (1.4–3.7)		
BMT before infection			2.2 (1)	.14
No	51	1		
Yes	36	1.5 (0.9–2.4)		
Radiological findings			29.2 (2)	<.001
LISA	57	1		
No (diffuse)	12	5.2 (2.6–10.3)		
No (other)	18	4.0 (2.2–7.1)		
Could be operated on			25.6 (2)	<.001
Yes	40	1		
No (bulky IPA)	12	6.3 (3.0–13.3)		
No (other)	35	2.9 (1.6–5.0)		
Operated on			11.1 (1)	<.001
Yes	37	1		
No	50	2.4 (1.4–4.0)		
Relapsed hematologic disease[a]			2.0 (1)	.16
No	45	1		
Yes	41	1.4 (0.9–2.4)		

NOTE. BMT, bone marrow transplantation; LISA, lesions with imaging suggestive of aspergillosis.

[a] One patient did not have hematologic malignancy (aplastic anemia).

Aspergillus isolates from resected lung and correlation with duration of amphotericin B before resection. *Aspergillus* isolates were obtained from lung tissue obtained during surgery from 14 (54%) of 26 patients with a histological diagnosis after resection and no positive *Aspergillus* culture before surgery whose tissue was submitted to the microbiology laboratory. The operative lung samples were not submitted for microbiological study for 4 patients, and cultures from the remaining 12 were sterile (46%).

In addition, 5 patients with histological diagnosis after resection had *Aspergillus* isolated from a sputum sample or nose swab before surgery. The resected lung was culture-positive for *Aspergillus* for 4 of these patients and culture-negative for 1.

Five patients had *Aspergillus* isolated before surgery; of these isolates, 3 (60%) were *A. fumigatus,* 1 (20%) was *Aspergillus flavus,* and 1 (20%) was a combination of *A. fumigatus* and *A. flavus.* Fourteen patients had no *Aspergillus* isolated before surgery, but cultures from resected lung tissue were positive for *Aspergillus. A. fumigatus* was cultured from tissue samples of 11 (79%) of these patients; and *A. flavus* from samples of 3 (21%).

The 14 patients with no *Aspergillus* isolate before surgery but *Aspergillus* growth from resected lung specimens received amphotericin B for 0–29 days (median, 15 days; mean, 13.7 days) before surgery. The 12 patients without *Aspergillus* isolates from resected lung specimens received amphotericin B for 0–45 days (median, 12.5 days; mean, 16 days). There was no statistical difference in the duration of amphotericin B treatment between these 2 groups ($P > .5$, Student's *t* test).

Discussion

We have demonstrated that radiological detection of LISA has a positive predictive value of 90% for IPA in cases in which histological verification took place. This compares with a positive predictive value of 81.5% reported by another group [18] and suggests that cases of IPA diagnosed in this manner could usefully be included in epidemiological studies and in trials of antifungal agents. It should be noted that a joint European Organization for Research on Treatment of Cancer/Mycoses Study Group proposal to modify the current Mycoses Study Group criteria has been published [20]. This takes into account CT scanning evidence and antigenemia, as well as the patient's underlying disease, and will have the effect of assigning a more definite initial diagnosis of invasive aspergillosis to many of these cases.

We have confirmed that *Aspergillus* is only occasionally isolated from respiratory secretions of patients with LISA (14%).

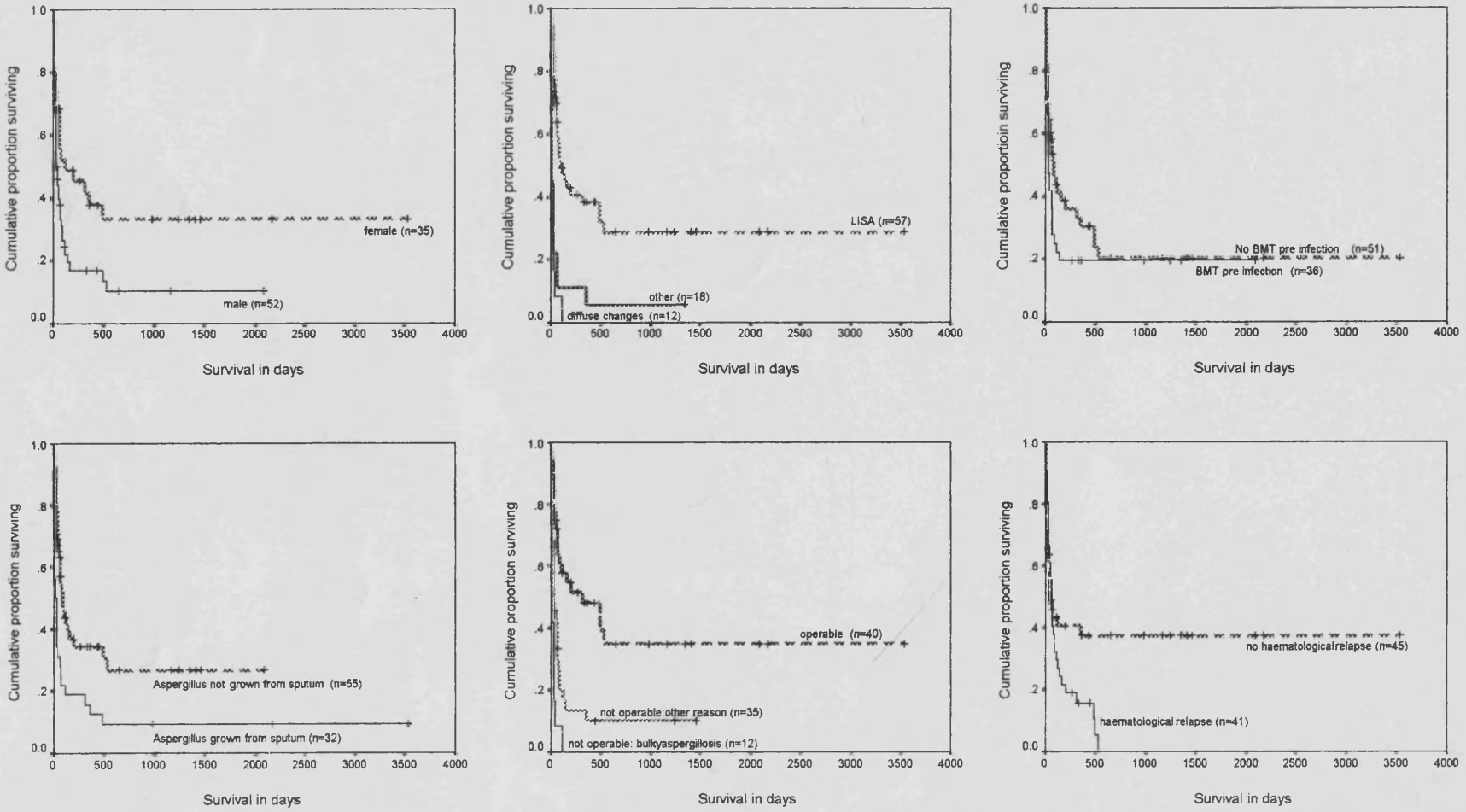

Figure 2. Kaplan-Meier curves showing the impact of various individual factors on death from all causes of patients with invasive pulmonary aspergillosis. LISA, lesion with imaging suggestive of aspergillosis; BMT, bone marrow transplantation.

Table 5. Effect of several patient characteristics simultaneously on death from all causes in patients with invasive pulmonary aspergillosis, expressed as adjusted relative hazards (RH).

Characteristic	Adjusted RH (95% CI)	Likelihood ratio statistic χ^2 (df)	*P*
Age, years		3.0 (2)	.22
<30	1		
30–49	0.8 (0.5–1.6)		
≥50	1.6 (0.8–3.3)		
Sex		1.7 (1)	.19
Female	1		
Male	1.5 (0.8–2.7)		
Isolate grown from sputum sample		0.7 (1)	.38
No	1		
Yes	1.4 (0.7–2.7)		
BMT before infection		2.0 (1)	.15
No	1		
Yes	1.5 (0.8–2.8)		
Radiological findings		11.7 (2)	.003
LISA	1		
Diffuse	4.1 (1.4–12)		
Other	4.2 (1.8–9.7)		
Relapsed hematologic results		7.4 (1)	.007
No	1		
Yes	2.3 (1.3–4.3)		
Operated on		0.1 (1)	.75
Yes	1		
No	1.1 (0.5–2.3)		

NOTE. BMT, bone marrow transplantation; df, degrees of freedom; LISA, lesions with imaging suggestive of aspergillosis.

This compares well with the postmortem series of Young et al. [2], in which only 13% of patients with IPA had a single sputum or BAL specimen culture-positive for *Aspergillus* while alive, and only 7.5% had ≥2 such positive cultures.

We have also shown that isolation of *Aspergillus* from sputum is significantly associated with death. This significance is lost when analysis is adjusted for other factors, and radiological appearances different from LISA become most significantly associated with death. The isolation of *Aspergillus* from secretions thus appears to be only a poor prognostic indicator because of its association with cases that do not present as LISA.

We have recorded *Aspergillus* isolates from lung tissue specimens of 54% of patients with histological aspergillosis; in these cases the organism had survived despite a mean of 13.7 days of amphotericin B treatment. The duration of preoperative treatment with amphotericin B is not significantly different for those whose resected lung with histological aspergillosis was sterile.

We have shown that surgical resection of LISA is both feasible and safe in a population of patients with hematologic malignancies. This is in contrast to a series of 36 patients treated surgically (wedge biopsy in 13, resection in 23) and subsequently shown to have fungal infections (IPA in 23 cases), in which 39% early postoperative mortality was reported [13]. The single most important predictor of death following surgery was angioinvasive fungal disease, which was associated with severe neutropenia ($< 0.1 \times 10^9$/L).

On the other hand, a French group [14] undertook pulmonary resections in 16 patients with LISA and subsequently proven IPA, at least 6 of whom were stated to have residual granulocytopenia. The death rate was only 6.25%. Twenty further patients were treated conservatively. There was a 72% improvement or cure rate from IPA in the group of 36 surgical and nonsurgical patients, although no comparison of the prognosis was made on the basis of whether surgery took place or not. Half of the operated patients underwent surgery for prophylaxis of hemoptysis on the basis of the novel criterion of CT evidence of a lesion adjacent to the pulmonary artery or its main branches.

The same authors subsequently reviewed the indications for surgery in a series of 19 patients with IPA suspected on the basis of radiological findings, among whom there was only 1 death from IPA [15, 16]. Another group [17] reports 69% clearance of IPA in 13 immunocompromised patients treated surgically, with 31% of deaths from IPA.

In our study, surgical resection, in conjunction with prophylactic conventional or liposomal amphotericin B, prevented relapse of IPA in 100% of cases (all but 1 reported in [3]) who underwent resection of lesions before BMT and had an actuarial survival rate of 71% at 2 years. Another group, however, published a similar success in 7 patients treated with amphotericin B, of whom only 3 underwent surgery [11].

All but 3 of our patients eligible for surgery underwent pulmonary resections. The actuarial survival rate of patients who had surgery for LISA was 89% at 1 month and 36% at 2 years (compared with 23% at 21 months in [17]), whereas the actuarial survival rate of patients with radiological diagnosis—that is, with LISA but not able to sustain an operation—was 91% at 1 month and 20% at 2 years. Although the patients treated surgically appear to have a somewhat better prognosis, analysis of the data with adjustment for several factors simultaneously demonstrates that it is likely that presentation of IPA as LISA is the most influential factor.

No direct comparison between medical and surgical treatment of IPA has been published. A review of the literature [5] reports a response rate of 51% among patients with IPA (some with diffuse and some with focal forms) who were treated with systemic antifungals. However, this cannot be usefully compared with outcomes of patients treated surgically because only focal forms of IPA are amenable to resection and because diffuse and focal forms of IPA may respond differently to amphotericin B.

We conclude that IPA presenting as LISA has in itself the best prognosis, and that this, rather than any benefit conveyed by the operation itself, may be the reason surgical resection of such lesions appears to improve the survival rate. A randomized trial would be necessary to determine the respective advantages of conservative treatment versus surgical plus systemic antifungal treatment in operable cases of IPA.

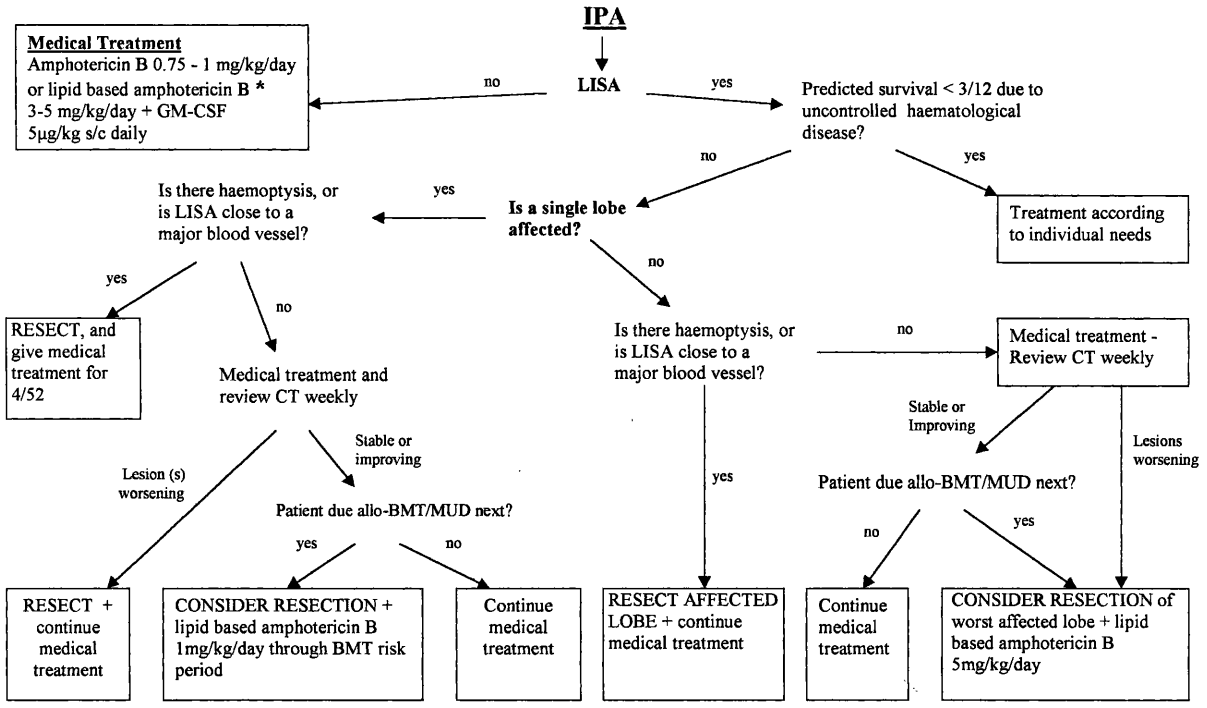

Figure 3. Suggested algorithm for the management of a lesion with imaging suggestive of aspergillosis (LISA) in patients with invasive pulmonary aspergillosis (IPA). Allo, allogeneic; BMT, bone marrow transplantation; GM-CSF, granulocyte-macrophage–colony stimulating factor; 4/52, 4 weeks; 3/12, 3 months. *Amphotericin B should be replaced by lipid-based amphotericin B if it is poorly tolerated or if other nephrotoxic drugs are added to the treatment regimen.

Our current recommendation is that patients with LISA should first be treated with a combination of amphotericin B or a lipid-based formulation plus GM-CSF (5 μg/kg/day). The choice of amphotericin B preparation should be made according to a local standard protocol. This should be based on factors relating to toxicity, especially nephrotoxicity, and on the concurrent use of other necessary nephrotoxic drugs. A CT scan should be done weekly and medical management should be continued for those with stable or improved lesions, unless hemoptysis threatens or occurs, in which case a resection should be performed immediately. Surgery should be done for those with worsening lesions when technically feasible and should also be considered before allogeneic/MUD BMT. The main circumstances that preclude surgery are the following: predicted death from hematologic disease within 3 months, inability of the patient to tolerate a surgical procedure, and/or a lesion that is technically inoperable. A suggested algorithm for the management of LISA is presented in figure 3.

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