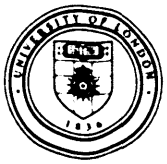

MD Thesis

Evaluation of Diagnostic Methods for Invasive Aspergillosis in Haematological Malignancy

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The work presented in this thesis is my own work.

Signed: .

To my parents, Gerard and Samuel

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ABSTRACT

Invasive aspergillosis (IA) remains a life-threatening infection in immunocompromised patients and is the most important cause of fungal death in cancer patients. In bone marrow transplant recipients the mortality rate is still approaching 90%.

The purpose of this study was to investigate methods for the early diagnosis of invasive aspergillosis in patients with haematological malignancy, and to examine the best strategy for sampling. The study compared clinical, microbiological and histopathological data with newer diagnostic techniques such as antigen detection (Pastorex latex agglutination and sandwich ELISA tests), high resolution computed tomography (CT) scanning and the polymerase chain reaction.

The research involved both a retrospective and a prospective study. The initial retrospective study was performed over 24 months. During this time, 38 bronchoalveolar lavage (BAL) fluid and 178 serum samples were collected from 38 febrile neutropenic and bone marrow transplant (BMT) patients. BAL and serum samples from 12 immunocompetent and 20 asymptomatic HIV+ patients were used as controls.

The results suggested that the sandwich ELISA provides a simple, effective and rapid screening test. The relatively high negative predictive value of all these investigations may allow for better use of empirical antifungal therapy. Regular prospective monitoring of sequential serum samples with BAL and CT scanning in high risk immunocompromised patients allows for the earlier diagnosis of invasive aspergillosis.

Based on the promising findings of the retrospective study, a one year prospective study was performed. However, the prospective study results suggests that

effective prophylaxis with itraconazole may be superior to pre-emptive therapy, even at an early stage of infection.

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ABCD	amphotericin B colloidal dispersion
ABLC	amphotericin B lipid complex
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphocytic leukaemia
AMB	amphotericin B
AML	acute myeloid leukaemia
ANC	absolute neutrophil count
BAL	bronchoalveolar lavage
BD	twice daily
BMT	bone marrow transplant
CDC	Centers for Disease Control
CFR	case fatality rate
CFU	colony forming units
CI	confidence interval
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computed tomography
dCTP	deoxycytidinetriphosphate
DNA	deoxyribonucleic acid
EB-A2	anti-galactomannan monoclonal antibody
ELISA	enzyme linked immunosorbent assay

EORTC	European Organisation for Research and Treatment of Cancer
EORTC-IFICG	Invasive Fungal Infections Co-operative Group of the EORTC
EDTA	ethylenediaminetetraacetic acid
fg	femtograms
FLAG	fludarabine, ara-C, G-CSF
G-CSF	granulocyte-colony stimulation factor
GM	galactomannan
GM-CSF	granulocyte-monocyte-colony stimulation factor
GVHD	graft-versus-host disease
HEPA	high efficiency particulate air
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HSTC	haematopoietic stem cell transplantation
IA	invasive aspergillosis
IFI	invasive fungal infection
IPA	invasive pulmonary aspergillosis
IV	intravenous
kg	kilograms
µl	microlitres
mg	milligrams
MRI	magnetic resonance imaging
MSG	Mycoses Study Group
ng	nanograms

NHL	nonhodgkin lymphoma
NIAID	National Institute of Allergy and Infectious Diseases
NIAID-MSG	Mycoses Study Group of the NIAID
NPV	negative predictive value
OKT3	immunosuppressant brand name
OLB	open lung biopsy
Pastorex LA	Pastorex Latex Agglutination
PCR	polymerase chain reaction
pg	picograms
PPV	positive predictive value
PV	predictive value
QDS	four times daily
rRNA	ribosomal ribonucleic acid
SDD	selective decontamination of the digestive tract
TAE	tris-acetate EDTA
TN	true negative
TP	true positive
UV	ultraviolet
WBC	white blood cells

CHAPTER 1

INTRODUCTION

1.1 *ASPERGILLUS* SPECIES

The aspergilli compose a group of rapidly growing, hyaline moulds that commonly cause opportunistic infections in humans. Of the some 700 *Aspergillus* species described by Raper and Fennell (1965) in their classic text, only 19 species have been cited by Rinaldi (1983) as causing human infections. Of these, only four species are recovered with any frequency from hospitalised patients: *A. fumigatus* (the species causing most allergic pulmonary and invasive diseases), *A. flavus*, *A. niger*, and *A. terreus*.

Aspergilli are common environmental saprophytes, accounting for up to 40% of the fungal flora in hospital and home environments. They are natural inhabitants of soil, water and organic debris and rarely behave as pathogens in a normal host. *Aspergillus* species have a world-wide distribution, and infections in immunocompromised patients have been reported from North America, Europe, Asia and Africa (Degregorio *et al.*, 1982; Bodey *et al.*, 1992; Khoo and Denning, 1994). In many developing countries cases appear to occur less frequently, either because of under diagnosis, as a result of reduced survival of patients with the same underlying disease (Manuel and Kibbler, 1998) or less likelihood of carrying out highly immunosuppressive therapy for haematological malignancies.

1.2 INCIDENCE OF INVASIVE ASPERGILLOSIS

The incidence of invasive aspergillosis (IA) has risen inexorably over the past two decades. A rise of 158% was documented in the USA between 1970 and 1976 (Fraser *et al.*, 1979). Since then, a 14-fold increase has been shown in an autopsy study from Germany which spanned the 12 years up to 1993 (Groll *et al.*, 1996). This almost certainly reflects the more widespread use of aggressive cancer chemotherapy regimens, the expansion of organ transplant programmes and the

advent of the AIDS epidemic.

More recently, the incidence of postengraftment invasive fungal infections, especially invasive aspergillosis, among patients undergoing allogeneic haematopoietic stem cell transplantation (HSCT) increased during the 1990s (Marr *et al.*, 2002). Infections caused by other moulds, such as Zycomycetes and Fusarium species, also increased during the late 1990s (Marr *et al.*, 2002). This increase in invasive mould infections has been attributed to multiple factors, including successful prevention of both candidiasis and cytomegalovirus (CMV) disease early after transplantation and corticosteroid-based treatment of severe graft-versus-host disease (Grow *et al.*, 2002; Marr *et al.*, 2002).

1.3 SOURCES OF INFECTION

1.3.1 Endogenous versus exogenous *Aspergillus* infection

1.3.1.1 Clinical evidence

It is certainly rational to consider that invasive aspergillosis is an endogenous infection in bone-marrow transplant or leukaemic patients in some haematology units. The environment in these settings is strictly controlled and patients are isolated in individual rooms and protected by extensive infection control policies and practices. The potential for cross-infection and exogenous infection is minimised. However, despite this, the incidence of IA varies between 4.5 – 15.1% (Cornet *et al.*, 2002; Grow *et al.*, 2002; Marr *et al.*, 2002; Kojima *et al.*, 2004; Cordonnier *et al.*, 2006; Zaoutis *et al.*, 2006) among allogeneic bone marrow or stem cell transplant recipients. This contrasts with Wingard's study, which showed an incidence of approximately 5% in allogeneic bone marrow transplant recipients (Wingard *et al.*, 1987).

Patients who recover from documented IA and then go on to suffer a relapse during a further course of chemotherapy or bone marrow transplantation clearly have an endogenous infection (Karp *et al.*, 1988), and it is likely that there are other patients who have undiagnosed prior infection. In addition, it seems likely that some patients are admitted into filtered air environments who are already colonised with *Aspergillus* species and develop invasive disease when they are rendered neutropenic. However, the close correlation of clusters of cases with hospital building work indicates that some patients acquire the organism in hospital during their at-risk period (Opal *et al.*, 1986; Perraud *et al.*, 1987; Dewhurst *et al.*, 1990; Goodley *et al.*, 1994).

In the study by McWhinney *et al.* (1993) in BMT patients, it was noted that 60% of the cases were community acquired. This is quite similar to the results obtained from a more recent study in Connecticut (Patterson *et al.*, 1997) during a three year period of epidemiological surveillance for invasive aspergillosis during a period of hospital construction where 70% of their cases were community-acquired. There is a lack of information on the number of community-acquired cases of aspergillosis in other studies (Klimowski *et al.*, 1989; Kramer, 1993). Such information is important whenever nosocomial invasive aspergillosis is suspected, because hospital environmental control measures will not affect community-acquired cases (Patterson *et al.*, 1997). Other control/preventative measures should be considered in these patients, such as home environmental control for at-risk patients and antifungal prophylaxis. Equally, environmental surveillance is likely to be incomplete in even the best studies and it is possible that the infecting strain might have been present in the ward prior to infection in some cases.

1.3.1.2 Evidence from molecular epidemiological studies

Insight into the source of the infecting organism is being gained from studies using a variety of molecular typing methods. A UK study has demonstrated the relatedness of several infecting strains of *Aspergillus fumigatus* to isolates found in the ward environment of patients undergoing bone marrow transplantation. However, all of these strains appeared first in the patients, before being found in the environment, despite prolonged prior sampling of the ward, suggesting that the patient was the source in each case (Radford *et al.*, 1997). A similar longitudinal study from France supports this (Paris *et al.*, 1997). Most strains infecting patients were not found in the environment, and of those that were, all were again present in patients before being isolated from environmental samples. Of interest was the fact that patients involved in an apparent outbreak during this time were all infected with different strains. This has previously been shown in an outbreak investigation using older typing methods. Each patient was found to be infected with a single strain and isolates from very different body locations such as the lung and skin were similar biochemically (Girardin *et al.*, 1994). This again suggests that the organisms were community acquired from different sources and imported into the hospital in the form of colonisation or prior infection. However, we have few data on the incubation period of this disease and on the time taken for clearance of colonised airways in these groups of patients, making it difficult to determine how frequently this occurs.

1.4 SOURCES OF *ASPERGILLUS* SPECIES

Relatively little is know about environmental factors in the determination of risk. Whilst building construction and renovation are recognised risk factors for the development of invasive aspergillosis, the contribution of food, water, flora and fauna are less well studied.

1.4.1 Environment

Aspergillus species are commonly found in soil, decaying vegetable matter, damp cellars, building materials and ventilation systems (Walsh and Dixon, 1989; Denning, 1991). Potted plants, flower arrangements and carpets may also have a high concentration of aspergilli (Nolard *et al.*, 1988).

1.4.2 Food

Dietary exposure to *Aspergillus* species is increasing due to the increased use of processed food. Maize, peanuts, cashews and copra are a source of aspergilli (Pitt *et al.*, 1993). A Thai study showed *Aspergillus flavus* to be the dominant fungus in these commodities (Pitt *et al.*, 1993). *Aspergillus* species are also found in various other food-stuffs including coffee beans, cereals, powdered milk, tea, chocolate, soya sauce and tofu and have been linked with consequent infection in neutropenic patients (Walsh and Dixon, 1989; Marinkovich, 1989; Studer-Rohr *et al.*, 1995). The organism is also used in the production of citric acid, an additive in many foodstuffs (Marinkovich, 1989). Contamination of dietary pepper with *Aspergillus* species has been reported frequently in the literature (De Bock *et al.*, 1989). A study by Eccles and Scott (1992) revealed that the practice of sharing salt and pepper pots between patients in general wards was also being followed in the

haematology unit. They suggested that only autoclaved pepper or pepper in sachets is served to neutropenic patients, which in the study by Vargas *et al.* (1989) showed only a 3% contamination rate. In addition the risk of cross infection associated with redistribution of pepper pots from one patient in protective isolation to another is removed (Eccles and Scott, 1992).

1.4.3 Water

Standing water is readily contaminated with fungi from the air or surrounding environment. Inadequately sterilised nebulisers may also act as a source of aspergilli (Kibbler, 1996). *Aspergillus* species can contaminate ice making machines, and hence ice prepared in this way should not be given to severely immunocompromised patients (Kibbler, 1996).

Shower heads can be a source of aspergillosis in patients in protective isolation. Anaissie (1998) recovered opportunistic fungal pathogens from sinks and showerheads in several hospitals in the USA. *A. terreus* and *A. niger* were cultured from the showerheads, as well as Fusarium species. The clinical significance of these findings remains unclear.

During a three year prospective study, Anaissie *et al.* (2002) identified the water distribution system of a hospital with adequate air filtration as a potential indoor reservoir of *Aspergillus* species, which led to secondary aerosolization of fungal propagules and exposure of patients to the fungus.

1.4.4 Fomites

An outbreak of cutaneous aspergillosis in Manchester caused by *Aspergillus niger* was attributed to fomites within a ward kitchen adjacent to the unit (Loudon *et al.*,

1996). Fomites within the rooms used by patients, the ward and the adjoining kitchen areas (including food items) were sampled. *A. niger* was isolated from two fridges, an ice-making machine, a microwave, a tea caddy, a fire blanket holder and a ceiling airvent within the kitchen. This example highlights the importance of scrupulous hygiene and strict infection control measures in the management of high risk patients.

1.4.5 Occupational exposure

A study by Jensen and co-workers (1993) in a sugar beet refinery was carried out to identify worker exposure to airborne fungi following employee complaints of asthma-like symptoms, and to determine whether engineering controls needed to be revised. It was found that some workers were exposed to an average of 490,000 cfu/m^3 of *Aspergillus* spores during the post production cleanup and maintenance phase in the summer. This was fifty times greater than the average exposure during the production campaign itself in the winter.

The contributing factors for this may be the combination of a higher temperature in June and a longer incubation period for fungal growth because some pellets had been trapped near the bottom and walls of the silo. Reducing exposure to fungal spores may be achieved by improving the design of the silo.

Previously described cases of Farmer's lung and other pulmonary hypersensitivity reactions have been linked to occupational exposure to mouldy corn, mushrooms, grain dust, silage, mouldy hay and mouldy oats (Henderson, 1968; Slavin *et al.*, 1969; Patterson *et al.*, 1973; Patterson *et al.*, 1974; Yocum *et al.*, 1976; Yoshida *et al.*, 1990). The exposure of farmers to spores from mouldy hay and of gardeners to compost has been well documented as the aetiological factor in the pathogenesis

of allergic alveolitis, but does not appear to have been linked with invasive aspergillosis.

1.4.6 Other sources

Contaminated substance abuse material injected intravenously can lead to *Aspergillus* endophthalmitis (Barr *et al.*, 1990). Inhalation of marijuana contaminated with *Aspergillus* species has been linked to the development of invasive pulmonary aspergillosis in patients immunosuppressed for a variety of reasons; one recent case involved a renal transplant recipient (Marks *et al.*, 1996). In this patient, exposure to a large inoculum of *Aspergillus* within 30 days of receiving high dose steroids appeared to be the most important predisposing factor for invasive pulmonary aspergillosis.

1.5 ROUTES OF TRANSMISSION

1.5.1 Airborne route

The concentration of *A. fumigatus* spores in the air undergoes seasonal variation and is generally considered to be higher in the autumn and winter (Noble and Clayton, 1963; Mullins *et al.*, 1984). However, not all studies have shown this seasonal peak (Goodley *et al.*, 1994). A recent study from Connecticut showed a trend for an increased isolation rate in patients and *Aspergillus* cultures during the summer and early fall, although this trend did not reach statistical significance (Patterson *et al.*, 1997). The number of *Aspergillus* hyphal fragments has been shown to correlate with wind speed (Li and Kendrick, 1995).

Most exogenous fungi that cause serious invasive disease are acquired by inhalation. Andersen and colleagues (1996) reported an outbreak of aspergillosis in a paediatric oncology ward, which was attributed to a defective disposal conduit

door as well as the dispersal of a contaminated aerosol from the ward vacuum cleaner which had the highest measured concentrations of *Aspergillus fumigatus* in or around the building (65 cfu/m^3 compared with 0-6 cfu/m^3 elsewhere). Following changes in hygiene practices, no further cases were identified.

As already mentioned, an association between outbreaks of aspergillosis and building works on or near hospital sites has been repeatedly established. The density of *Aspergillus* spores in hospital air is increased considerably during construction, and this supports the view that nosocomial aspergillosis is due to infiltration of conidia into ward air from outside (Arnow *et al.*, 1978; Sarubbi *et al.*, 1982; Rogers and Barnes, 1988; Guillemain *et al.*, 1995; Loo *et al.*, 1996). Hospital ventilation systems can draw in contaminated air from outside either because of malfunction or an inadequate mechanical ventilation and air filtration system (Speller, 1986; Iwen *et al.*, 1993; Loudon *et al.*, 1994). In a 77-month study by Arnow *et al.*, (1991) on the occurrence of aspergillosis in immunocompromised patients, an increased rate coincided with hospital construction or renovation activity and with poor maintenance of air filters. This report is supported by other studies which have shown increases in aspergillosis associated with hospital construction (Arnow *et al.*, 1978; Sarubbi *et al.*, 1982; Opal *et al.*, 1986; Weems *et al.*, 1987). Recognition of the importance of the airborne route in the spread of aspergillosis has lead to the installation of either high efficiency particulate air (HEPA) or laminar air-flow (LAF) systems in most bone marrow transplant and leukaemic units (Rose and Hirsch, 1979; Rhame *et al.*, 1984; Rogers, 1985; Rhame, 1989), with a consequent fall in the incidence of cases.

Contamination can also occur from disturbance of normally closed areas, e.g.

alteration of ceiling spaces (Rhame, 1991). Previous reports have linked cases of aspergillosis to open windows, and gaps in filters and support frames that permit entry of unfiltered outside air (Sarubbi *et al.*, 1982; Weems *et al.*, 1987). Other hospital environmental reservoirs such as bird droppings in air ducts supplying high risk patient areas and contaminated fire-proofing material or damp wood have been associated with aspergillosis in high risk immunosuppressed patients (Aisner *et al.*, 1976). Potted plants near patients are another source of air contamination (Walsh and Dixon, 1989).

1.5.2 Penetration of non-intact skin or mucosa

Conjunctival and corneal trauma and subsequent colonisation of the site with environmental fungi, such as aspergilli, can lead to fungal keratitis, although this is not usually the site of entry of invasive infection. Disruption of the epithelial barrier by eye surgery (Valenton, 1996) or contact lenses can predispose to infection with aspergilli; colonisation of contact lenses with aspergillus species has been demonstrated (Fenelon and Kennedy, 1996).

In line-associated soft tissue infection, *Aspergillus* species gain entry via the catheter puncture site. Wound infections caused by *Aspergillus sp.* in renal (Langlois *et al.*, 1980) and hepatic (Plá *et al.*, 1992) transplant recipients have been previously documented, albeit rarely.

1.5.3 Contact transmission

Contamination of dressings with *Aspergillus* species in burns patients has previously been described (Bruck *et al.*, 1971; Stone *et al.*, 1979). In a cluster of cases in Manchester, contaminated stockinette material was implicated as the

source of infection in one patient (Johnson *et al.*, 1993). To date, there is no evidence to suggest that hand carriage plays a role in the transmission of *Aspergillus* infection.

1.6 THE AT-RISK PATIENT POPULATION

Invasive aspergillosis is an opportunistic fungal infection which primarily affects the respiratory tract. It is the most important cause of death from fungal infection in cancer patients (Denning and Stevens, 1990). Patients at greatest risk for infection are those with inadequate numbers of circulating neutrophils or defective neutrophil function, and patients with mononuclear phagocyte defects.

The at-risk patients include those with haematological and lymphoreticular cancer, particularly acute leukaemia (Young *et al.*, 1970; Meyer *et al.*, 1973; Fisher *et al.*, 1981; Degregorio *et al.*, 1982), organ transplant recipients given long-term immunosuppressive therapy (Saunders and Bieber, 1968; Weiland *et al.*, 1983), diabetics (D'Silva *et al.*, 1982), patients who have undergone major surgery (Gurwith *et al.*, 1971; Williams *et al.*, 1976), and other groups of patients receiving high dose corticosteroids or other immunosuppressive agents such as azathioprine or cyclophosphamide.

The known predisposing factors for *Aspergillus* infection are shown in Table 1-1 (Manuel and Kibbler, 1998).

1.6.1 Neutropenic and Bone Marrow Transplant Patients

The overall incidence of invasive aspergillosis in neutropenic patients is variable and is dependent upon the unit, the underlying disease and the therapy given. The range is given in Table 1-2 (Verweij *et al.*, 1996).

Table 1-1. Factors predisposing to *Aspergillus* infection.

Systemic factors	References
Prolonged granulocytopenia	Gerson 1984
Recent administration of broad-spectrum antibiotics	Fisher 1981, Aisner 1979
Long-term use of corticosteroids	Aisner 1979
Transplantation	Saunders 1968, McWhinney 1993
Use of cytotoxic agents	Degregorio 1982, Young 1970, Fisher 1981
Increased use of immunosuppressive therapy for rejection	McWhinney 1993
Qualitative disorders of granulocyte dysfunction	Cohen 1981
Prolonged use of indwelling catheters and parenteral nutrition	Lowder 1982
Local tissue injury secondary to surgery	Gurwith 1971, Williams 1976
Chronic pulmonary disease or infection	Gurwith 1971, Williams 1976
Cancer	Meyer 1973
Radiation treatment	Gurwith 1971, Williams 1976
Burns	Denning 1991
IV drug abuse	Barr 1990
Chronic alcoholism and cirrhosis	Bodey 1989, Murray 1977
Long-standing diabetes mellitus	D'Silva 1982
Uraemia	Weiland 1983, Zarabi 1984
Tuberculosis	Fisher 1981, Denning 1991
Neonatal period	Denning 1991, Gonzalez-Crussi 1979
Hypoparathyroidism	Kibbler 1996
Tobacco use	Guillemain 1995
Local factors	
Topical steroids	Kibbler 1996
Obesity	Kibbler 1996

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Table 1-2. Incidence of invasive aspergillosis and period of greatest risk in adult and paediatric haematology patients.

Host Group	Incidence of invasive aspergillosis	Time period of highest risk (days)	References
Allogeneic bone marrow or stem cell transplantation	4.5% - 15.1%	41 – 180 days	Cornet *et al.*, 2002; Grow *et al.*, 2002; Kojima *et al.*, 2004; Cordonnier *et al.*, 2006; Zaoutis *et al.*, 2006;
- HLA mismatched or unrelated	10.5%		Marr *et al.*, 2002
- HLA matched or related	7.3%		Marr *et al.*, 2002
Autologous stem cell transplantation	0 – 1.1%	< 30 days	Cornet *et al.*, 2002; Grow *et al.*, 2002; Jantunen *et al.*, 2004; Morgan *et al.*, 2005; Zaoutis *et al.*, 2006
AML	3.7 - 8%	> 21 days	Cornet *et al.*, 2002; Zaoutis *et al.*, 2006
ALL	0.6 - 6.3%	> 21 days	Cornet *et al.*, 2002; Zaoutis *et al.*, 2006

The condition is the second most common opportunistic fungal infection in cancer patients; accounting for 30% of fungal infections in a post-mortem series, and most of those were in patients who were or had been neutropenic (Bodey *et al.*, 1992).

A major risk period is during the profound neutropenia (granulocytes $<0.1 \times 10^9/l$) which follows induction/remission chemotherapy or conditioning for bone marrow transplantation. However, bone marrow transplant (BMT) and allogeneic haematopoietic stem cell transplant (HSCT) recipients who develop graft failure or graft-versus-host disease (and receive corticosteroid therapy, cyclosporin, and other immunosuppressive agents) develop the infection some time after the transplant procedure and in recent series have formed the majority of BMT patients with invasive aspergillosis (McWhinney *et al.*, 1993; Patterson *et al.*, 1997; Maertens *et al.*, 2002). Results of several recent studies also suggest that invasive fungal infections persist in the late time period after nonmyeloablative allogeneic HSCT (Fukuda *et al.*, 2003; Hagen *et al.*, 2003).

An association between CMV disease and fungal infections has been noted not only in solid organ transplant recipients (George *et al.*, 1997; Husni *et al.*, 1998), but also in recipients of HCTs (Marr *et al.*, 2000; Grow *et al.*, 2002). Mechanisms to explain the association between CMV infection (and disease) and fungal infections remain obscure. Neutropenia caused by treatment with ganciclovir was unlikely to be the sole explanation because CMV disease remained an independent risk factor even in models that controlled for neutropenia. Other potential mechanisms includes the immune-modulating effects of CMV itself.

The use of T-cell depleted marrows to reduce the risk of severe graft-versus-host disease is associated with delayed engraftment, more prolonged granulocytopenia

and more severe lymphopenia (Pirsch and Maki, 1986). This was shown by stepwise multivariate regression analysis in the above study to be a predictor of systemic fungal infection ($r = 0.512$, $p < 0.0001$). However, modification of protocols in recent years has almost certainly reduced this effect.

In addition, those undergoing BMT who have had a previous episode of invasive aspergillosis develop recurrence in 50% of cases, unless the patient receives prophylaxis with amphotericin B (Karp *et al.*, 1988), other newer antifungal agents or surgical resection is undertaken (McWhinney *et al.*, 1993).

The mortality is high (60-70%) in neutropenic patients despite the use of amphotericin B and successful outcome is dependent upon early treatment (Aisner *et al.*, 1977) and, to a considerable extent, on bone marrow recovery (Fisher *et al.*, 1981).

1.6.2 Solid Organ Transplantation

The incidence of invasive fungal infection varies according to the organ transplanted, with renal transplant recipients being least at risk. Table 1-3 (Verweij *et al.*, 1996) shows the incidence of invasive aspergillosis in the different patient groups.

Todo *et al.* (1995) reported that there were no cases of IA complicating bowel transplant surgery. However, a recent publication by a French group (Cornet *et al.*, 2002) showed a high risk (10.7%) following small bowel or liver-small bowel transplantation. These results require further confirmation (Jan *et al.*, 1999).

Lung transplantation for cystic fibrosis carries a high risk of invasive aspergillosis. Pre-operative colonisation for some units remains a contraindication to transplantation (Guillemain *et al.*, 1995). In a study by Bertocchi *et al.* (1995) in

Table 1-3. Incidence of invasive aspergillosis in organ transplant recipients

Host Group	Incidence of invasive aspergillosis	Time period of highest risk (days)	References
Kidney transplantation	1.1 – 2.8%	30-90 P	Torre-Cisneros 1993
Liver transplantation	3.8 – 14.7%	14 - 100 P	Collins 1994; Kusne 1988; Wajszczuk 1985
Heart-lung transplantation	3 – 19%	9 – 90 P	Guillemain 1995; Kramer 1993
Heart transplantation	0 – 24%	12 – 45 P	Hofflin 1987
Lung transplantation	9.8%		Bertocchi 1995
Small bowel/liver- small bowel transplantation	4 – 11%		Cornet 2002

P, post-transplant
Data modified after Verweij *et al.*, 1996 and Cornet *et al.*, 2002

lung transplant recipients, 27.5% developed a fungal infection post-operatively, and invasive aspergillosis accounted for 9.8%.

Isolated aspergillus tracheobronchitis is a common finding in lung transplant recipients. Post lung transplant, airway colonization is present in 29% of cases, and subsequent tracheobronchitis occurs frequently, affecting 5%, with the area around the anastomosis being most at risk (Mehrad *et al.*, 2001). The incidence of isolated tracheobronchitis in lung transplant recipients is highest in the first year after transplantation. The presentation of isolated tracheobronchitis has been variously reported as asymptomatic disease identified on surveillance bronchoscopy (Kramer *et al.*, 1993; Shreeniwas *et al.*, 1996), symptomatic infection with fever and cough (Westney *et al.*, 1996), wheezing due to airway compromise (Higgins *et al.*, 1994), and massive hemoptysis (Kessler *et al.*, 1997; Birsan *et al.*, 1998).

There is data to support the association between CMV and fungal infections in solid organ transplant recipients (George *et al.*, 1997; Husni *et al.*, 1998). In the study by Guillemain *et al.* (1995), CMV infection was present in 28.5% of cases of invasive aspergillosis.

The introduction of cytotoxic agents such as azathioprine correlated with the increase in CMV infection seen, and the use of anti-lymphocyte globulin, anti-thymocyte globulin, OKT3 monoclonal antibody and the number of episodes of acute rejection treated are all associated with an increased risk of fungal and viral infections (Wajszczuk *et al.*, 1985; Kusne *et al.*, 1988; Paya, 1993). This emphasises the need for accurate diagnosis and treatment of rejection and stresses the fact that transplant immunosuppressive regimens are still far from optimal (Kibbler, 1995). Studies of tacrolimus (FK506) have shown a reduction in the number of CMV infections (Sakr *et al.*, 1992; European FK506

Multicenter Liver Study Group, 1994) and cases of invasive aspergillosis (Torre-Cisneros *et al.*, 1991) in comparison with patients treated with cyclosporin. Collins *et al.* (1994) studied predictors for invasive fungal infections complicating orthotopic liver transplantation (OLT). These included severe renal insufficiency (creatinine greater than 3.0 mg/dl, primarily due to hepato-renal syndrome), prolonged operative time (greater than or equal to 11 hours), retransplantation, and colonisation within three days of OLT. Patients were stratified into low, moderate or high risk groups dependent on whether no, one, two or more predictors were present. The incidence of invasive fungal infection ranged from 1% in the low risk group to 67% in the high risk group.

The mortality for invasive aspergillosis in organ transplant recipients varies from 50% to 100% and earlier diagnosis probably enhances survival (Weiland *et al.*, 1983; Kirby *et al.*, 1987; Kusne *et al.*, 1988; Paya, 1993; Guillemain *et al.*, 1995; Lin *et al.*, 2001).

1.6.3 HIV Patients

Patients with AIDS are susceptible to tracheobronchitis; many patients exhibit classic risk factors i.e. neutropenia and steroid treatment (Lortholary *et al.*, 1993; Kemper *et al.*, 1993).

Invasive aspergillosis has not been recognised as an AIDS-associated condition until recently, although it might be expected to occur in such immunocompromised patients. Yet a review of the literature shows that it has been well documented in early autopsy series of patients with AIDS (Guardo *et al.*, 1984; Anders *et al.*, 1986; Petito *et al.*, 1986; Gray *et al.*, 1988; Wilkes *et al.*, 1988; Lang *et al.*, 1989; Just-Neubling *et al.*, 1992; Pursell *et al.*, 1992), with a recent incidence of 0.3% (Verweij *et al.*, 1996). The apparently high frequency of

preceding or intercurrent pneumonia due to *Pneumocystis carinii* (*P. jiroveci*) and bacteria (present in 73% of reported cases) in AIDS patients with invasive pulmonary aspergillosis suggests that these infections may be a contributory factor (Khoo and Denning, 1994). However, since pneumonia is a common event in patients with advanced-stage HIV disease, this association remains unproven. It is likely that advanced HIV infection itself constitutes a risk factor for the development of invasive aspergillosis and patients with end stage disease may be neutropenic as a consequence of disease or therapy. In greater than 70% of cases, the lung is the organ most frequently involved; brain involvement was reported in 10% of cases (Khoo and Denning, 1994).

1.7 DIAGNOSTIC METHODS

The diagnosis of IPA is difficult, with the result that, in certain immunocompromised patient groups, such as those with neutropenia, many patients without fungal infection receive empirical antifungal therapy, whilst others with fungal infections are not diagnosed until postmortem.

The diagnostic approaches that are available can be divided into three main areas - clinical, laboratory and imaging techniques. I have further subdivided the laboratory methods into microbiology, histopathology and non-culture based techniques.

1.7.1 Clinical presentation

A common setting for IPA is one of persistent or recurrent fever in a persistently neutropenic patient with pulmonary infiltrates. Development of pulmonary infiltrates may initially be absent, owing to the paucity of the inflammatory response and fever may be the earliest manifestation of pulmonary aspergillosis.

These patients may also have pleuritic pain, non-productive cough, haemoptysis, pleural rub and, occasionally, adventitious breath sounds. *Aspergillus* spp. have a strong propensity for invasion of blood vessels, resulting in vascular thrombosis, infarction and tissue necrosis. This process contributes to many of the clinical and radiographic manifestations of pulmonary aspergillosis; pleuritic pain, pulmonary haemorrhage, haemoptysis and cavitation (Albelda *et al.*, 1984; Panos *et al.*, 1988). Symptoms and signs referable to the lower respiratory tract indicate an urgent need for a chest radiograph in immunocompromised patients.

Fungal rhinosinusitis is occasionally seen in neutropenic and bone marrow transplant recipients but does not occur in solid organ transplant recipients (Denning and Stevens, 1990). It is usually caused by *Aspergillus* species, particularly *A. flavus* (Talbot *et al.*, 1991), but various other fungi have also been implicated (Morrison *et al.*, 1994). Any swelling or pain in the face, nasal blockage or discharge, including epistaxis, is an indication for urgent investigation. Biopsy with histological examination and fungal culture of nasal tissue or maxillary sinus contents will confirm the diagnosis, although cultures for the Mucorales are sometimes negative. Sinus involvement is also suggestive of rhinocerebral aspergillosis or zygomycosis.

1.7.2 Laboratory

1.7.2.1 Microbiology

1.7.2.1.1 Importance of microscopy

Microscopy is an important investigation for several reasons. Firstly, the diagnostic yield is more than that for culture alone. Several studies attest to the substantially higher yield with microscopy than that with culture in

bronchoalveolar fluid (Kahn *et al.*, 1986; Levy *et al.*, 1992; Fischler *et al.*, 1997; Yuen *et al.*, 1997).

The use of staining methods such as lactophenol cotton blue enhances microscopy. Fluorescent whiteners, such as Calcofluor white and Blankophor, bind to cellulose and chitin, and fluoresce when exposed to ultraviolet (uv) light. The advantages of the fluorescent stain are the rapidity with which the stain can be processed and read (<10s), improved sensitivity, and it is inexpensive (Chander *et al.*, 1993; Andreas *et al.*, 2000; Monod *et al.*, 2000). Grocott's methenamine silver (GMS) stain is an essential stain for the detection of fungal elements in tissue sections, and should be used whenever a fungal aetiology is suspected. Periodic acid-Schiff (PAS) may also be useful for the detection of fungal elements in tissue sections (Ellis, 2006).

The second reason microscopy is important is that it is rapid (Yuen *et al.*, 1997). Results should ideally be available within 2-4 hours of the sample being received in the laboratory. Rapid processing is important as delayed diagnosis of a pulmonary invasive mould infection may be fatal for the patient.

Additionally, microscopy may help to distinguish between infection caused by a septate or non-septate mould. The former include *Aspergillus* spp., *Scedosporium apiospermum* and *Fusarium* spp.. The latter include mucorales (zygomycetes) such as *Rhizomucor* spp., *Rhizopus* spp., *Mucor* spp., *Absidia corymbifera* and *Cunninghamella bertholetiae*.

Invasive aspergillosis can be treated with the azoles itraconazole, voriconazole, or posaconazole (when licensed). The mucorales can only thus far be treated with amphotericn B. Pulmonary mucormycosis responds well to surgical resection (11% mortality versus 68% with pharmacotherapy), if clinically possible (Tedder *et al.*, 1994).

In addition to an early indication of the best treatment, pending a positive culture, most cases of pulmonary infection caused by mucorales do not yield positive culture. Mucorales are particularly susceptible to chilling in the refrigerator, and the potential yield may fall with temporary storage of the sample. They can also be damaged by tissue homogenisation and fail to grow. Thus the only means of establishing a causal diagnosis (aside from biopsy or autopsy) is microscopy.

1.7.2.1.2 Cultural characteristics

The genus *Aspergillus* is characterised on culture by hyaline hyphae and conidiophores that bear terminal vesicles, phialides and spores that are termed conidia. *Aspergillus* conidia are spherical, hydrophobic structures measuring 2.5-3.5 μm in diameter, which arise from phialides and are hence known as phialoconidia. Speciation of the genus aspergillus is based upon the morphology of the phialides, conidia and conidiophores.

1.7.2.1.3 The significance of positive cultures of *Aspergillus* spp.

Biopsy and culture of tissue are the most definitive means by which to establish a diagnosis of invasive aspergillosis. However, since many patients at risk of invasive aspergillosis also have haemostatic defects that preclude invasive diagnostic procedures, alternative approaches to establish a presumptive diagnosis are often initially pursued. Additionally, culturing of body fluids has a low diagnostic yield and does not always discriminate between invasive disease, colonisation, and contamination (Horvath and Dummer, 1996).

Early studies conducted by Aisner *et al.* (1979) found that positive nasal surveillance cultures of *A. flavus* in the midst of an outbreak of nosocomial aspergillosis in neutropenic patients correlated significantly with invasive pulmonary aspergillosis.

These findings have not been consistently corroborated in non-outbreak settings. Indeed, the absence of a positive nasal surveillance culture in a persistently febrile neutropenic patient with a pulmonary infiltrate does not exclude a diagnosis of pulmonary aspergillosis. Conversely, isolation of *Aspergillus* spp. from the nares does not consistently predict the development of invasive aspergillosis.

In comparison, isolation of *Aspergillus* spp. from respiratory secretions of febrile neutropenic patients with pulmonary infiltrates is strongly associated with invasive pulmonary aspergillosis. Yu *et al.* (1986), in a prospective study, found that isolation of *Aspergillus* spp. from respiratory secretions of high-risk patients was highly predictive of invasive pulmonary aspergillosis. Among 108 consecutive patients from whom *Aspergillus* spp. were isolated, 17 patients with neutropenia and/or leukaemia had lung tissue examined; all had invasive pulmonary aspergillosis. Invasive aspergillosis was not found in non-immunosuppressed patients or in non-neutropenic patients with solid tumours. Multivariate analysis demonstrated that neutropenia and the absence of smoking were the most significant predictors of invasive aspergillosis in patients with respiratory tract cultures growing *Aspergillus* spp. The findings of Treger *et al.* (1985), in a retrospective study, also underscored the significance of the isolation of *Aspergillus* spp. from respiratory secretions of high-risk populations. *Aspergillus* spp. were rarely contaminants in respiratory secretions. In contrast to the situation in neutropenic patients, Yu *et al.* (1986) found a low predictive value for invasive disease when *Aspergillus* spp. were recovered from respiratory secretions of non-neutropenic smokers with chronic lung disease. Thus, isolation of *Aspergillus* spp. from respiratory tract cultures of febrile neutropenic patients with pulmonary infiltrates should be considered *a priori* evidence of pulmonary aspergillosis.

Mucosal eschars may be observed along the nasal septum by careful otolaryngological

examination of patients with aspergillus sinusitis. Biopsy and culture of these lesions may reveal invasive aspergillosis and prompt the initiation of appropriate antifungal therapy without the need for a more invasive sinus drainage procedure. Similarly, if nasal septal lesions are not observed, a sinus aspirate may preclude the need for bronchoscopy if fungus is demonstrated in the aspirate. Although aspergillus is the most common fungus isolated from the sinuses of immunocompromised patients, other fungi, including zygomycetes, fusarium, *S. apiospermum*, curvularia and alternaria may be recovered.

Several investigators have studied bronchoalveolar lavage (BAL) fluid and found variable results in patients with tissue-proven invasive aspergillosis, with yields of 50-59% (Albelda *et al.*, 1984; Kahn *et al.*, 1986). Some studies to date have indicated that culture only has a sensitivity of between 15-30% (McWhinney *et al.*, 1993).

The presence of *Aspergillus* spp. in BAL fluid in a febrile neutropenic patient with new pulmonary infiltrates is indicative of invasive aspergillosis; however, the absence of hyphal elements or positive culture does not exclude the diagnosis (Saito *et al.*, 1988).

The isolation of *Aspergillus* species from blood cultures is frequently considered to be the result of contamination, although true fungemia with these organisms has been well documented (Martino *et al.*, 1993; Duthie and Denning 1995).

Percutaneous needle biopsy of focal lesions will yield the diagnosis in approximately 50-80% of cases of opportunistic infection in immunosuppressed patients (Yang *et al.*, 1992; Haramati, 1995). The procedure should be performed under radiological guidance and large needles (eg. 18 gauge) should be used. Specimens should be processed histologically as well as for cytology and culture.

If the foregoing methods do not yield a microbiological diagnosis of new infiltrates in the recurrently febrile neutropenic patient, open lung biopsy (OLB) should be considered. For patients with a localised infiltrate, however, OLB will require a major thoracotomy,

using either a lateral or mediastinal approach. It is imperative that the surgeon obtains biopsies of both the peripheral and the central areas of abnormal lung, since the distribution of the organism may vary. McCabe *et al.* (1985) found that many patients undergoing OLB had no change in their therapy, as they were receiving broad-spectrum empirical therapy, including amphotericin B. However, since the completion of this study, newer data now indicates that use of high doses of amphotericin B (1.0-1.5 mg/kg per day), and of lipid formulations, may be more active against pulmonary aspergillosis than standard empirical dosages (0.5-0.6 mg/kg per day) (Karp *et al.*, 1988; Denning and Stevens, 1990). As these higher doses are more nephrotoxic than conventional empiric doses, a microbiological or histopathological diagnosis should preferably be established before implementing high-dose amphotericin B therapy. Therefore, given an improved response of pulmonary aspergillosis in neutropenic patients to higher but more nephrotoxic doses of amphotericin B, an OLB demonstrating *Aspergillus* carries therapeutic implications beyond the empirical dosage of amphotericin B.

Although culture often provides the definitive diagnosis of invasive aspergillosis, it also has some limitations. Chief amongst these is failure to recover the organism.

1.7.2.2 Histopathology

Histopathology remains the gold standard for the diagnosis of invasive aspergillosis. However, the presence of severe thrombocytopenia often precludes the possibility of obtaining a specimen by invasive procedures.

The prevailing policy for surgery at the Royal Free during this study was that surgery was the second line treatment of choice. This was because of the recent introduction of treatment with granulocyte-monocyte-colony stimulating factor (GM-CSF; molgramostim) and liposomal amphotericin B. The recommendation at

the time was that patients should first be treated with a combination of amphotericin B or a lipid-based formulation plus GM-CSF (5 μg/kg/day).

Medical management was continued for patients with stable or improving lesions, unless haemoptysis threatened or occurred, in which case a resection was performed immediately. Surgery (lobectomy or pneumonectomy) was done for those with worsening lesions when technically feasible, or if a lesion with imaging suggestive of aspergillosis was close to a major blood vessel. Surgical resection also allowed patients to proceed with further chemotherapy or bone marrow transplantation. Open lung biopsy was not performed at the Royal Free during this study period.

Autopsies were only performed on a few patients who died of suspected fungal infections. This was because in most cases an antemortem diagnosis had been made, and also because consent for autopsies was difficult to obtain as a significant proportion of patients were from the Middle East and South Asia, and of Muslim faith, and had to be buried by sundown the next day.

All tissues from immunocompromised (including corticosteroid-treated) patients with suspected infection should be stained with fungal stains such as periodic acid-Schiff, silver, or fluorescent stains, in parallel with regular stains. Hyphae and yeasts are commonly invisible on standard sections stained with haematoxylin and eosin or Gram stain alone (Ramos *et al.*, 1995). Hyphae are best visualised by specialised stains for fungus, as recommended in anastomotic biopsy samples in lung transplant recipients (Husain *et al.*, 1996). The inclusion of good positive control sections is mandatory, as some fungi, for example, Mucorales may require longer staining times, whereas other fungi can easily be left for too long.

The pattern of angular, dichotomously branching, septate hyphae may be observed in

invasive tissue infection due to *Aspergillus* spp., *Scedosporium apiospermum*, *Fusarium* spp., and several less common fungi. Consequently, a culture diagnosis is the only way to distinguish these invasive fungi. Since *S. apiospermum* may be resistant to amphotericin B and may be more susceptible to itraconazole and voriconazole (Kontoyiannis *et al.*, 2003), this distinction has therapeutic importance. An outline of the conventional approaches to the diagnosis of invasive aspergillosis in patients with neoplastic disease is given in Table 1-4.

1.7.2.3 Non-culture based diagnostic techniques

Non-culture based methods of rapid identification and diagnosis of invasive aspergillosis may permit the early initiation of effective antifungal therapy and therapeutic monitoring, with an anticipated improvement in survival.

Although these methods were not in routine use at the time this study was performed, in most major haematology or BMT units it is now used as part of the diagnostic armamentarium.

Table 1-4. Outline of conventional aproaches to the diagnosis of invasive aspergillosis in patients with neoplastic disease.

Conventional methods	Diagnosis of invasive aspergillosis
History	Prolonged neutropenia
	Corticosteroid therapy
	Persistent pulmonary infiltrates
	Complaint of sinus congestion or pleuretic chest pain
Physical examination	Pleural or pericardial friction rub
	Rhonchi or consolidation
	Sinus tenderness
	Cutaneous lesions
Radiology	X-Rays of chest and sinuses
Microbiology	Direct microscopy and culture of induced sputum, BAL fluid, OLB or cutaneous lesions
Histopathology	Lung, sinuses and skin

BAL, bronchoalveolar lavage; OLB, open lung biopsy

1.7.2.3.1 Serological methods for diagnosis of invasive aspergillosis

1.7.2.3.1.1 *Antibody response*

Antibody detection assays are of little practical value for diagnosis of invasive aspergillosis in neutropenic patients and bone marrow transplant recipients because many such patients are also incapable of mounting an antibody response. Also, measurement of titres has not been shown to distinguish between colonisation, local infection, and invasive disease. 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.

1.7.2.3.1.2 *Antigen detection*

One of the most promising diagnostic approaches is the detection of *Aspergillus* antigen in the serum, urine or bronchoalveolar lavage (BAL) fluid of a patient (Andrews and Weiner, 1982; Sabetta *et al.*, 1985; Rogers *et al.*, 1990; Warnock *et al.*, 1991; Patterson *et al.*, 1995; Patterson *et al.*, 1997). Galactomannan is a major cell wall component of *Aspergillus* species, and it is a major circulating antigen in IPA (Stynen *et al.*, 1992a).

Early work from the United States (Lehmann and Reiss, 1978; Reiss and Lehmann, 1979) reported the presence of galactomannan antigenaemia by counter-immunoelectrophoresis in experimental disseminated aspergillosis. Dupont *et al.* (1987) measured galactomannan in patients with invasive aspergillosis, and also in experimentally infected rabbits by using a radioimmunoassay and an enzyme-linked immunosorbent assay; they found galactomannan to be present in both serum and urine. This study also found that detection of galactomannan in urine was more sensitive than that in serum.

The detection of *Aspergillus* antigen in serum is increased by testing frequently obtained

samples, and using a sensitive assay technique (Andriole *et al.*, 1993). The need for serial sampling to detect circulating antigen is likely to reflect the low quantity of antigenaemia in some patients (de Repentigny, 1992), particularly those with isolated pulmonary disease (Sabetta *et al.*, 1985), and may be due to the rapid clearance of antigen by immune complexes or by specific galactomannan receptor-bearing reticuloendothelial cells (Bennett *et al.*, 1987).

Aspergillus antigen detection in serum is also increased by dissociating immune complexes (Patterson *et al.*, 1995). Antibody was detected in 40% of patients with antigenaemia, and antigen levels were not significantly different in patients with or without detectable antibody. The success of antigen detection in the above study may be due in part to increased detection of *Aspergillus* antigens using polyclonal antibody made against live *A. fumigatus* cells.

An antigalactomannan monoclonal antibody (EB-A2) is the basis of the commercial Pastorex latex agglutination test (Bio-rad). It recognises the (1→5)-β-D-galactofuranoside side chains of the *Aspergillus* galactomannan (Stynen *et al.*, 1992a). This antigen test has a detection limit of 10-15 ng/ml of circulating *Aspergillus* galactomannan per ml of serum (Verweij *et al.*, 1996). The Pastorex latex agglutination test was the first antigen test to become commercially available, but its use to diagnose invasive aspergillosis at an early stage is controversial. Several investigators (Dupont *et al.*, 1990; Haynes and Rogers, 1994) have reported a high sensitivity of this test, up to 95%, and found that the Pastorex test allowed diagnosis of invasive aspergillosis to be made earlier in 68% of cases when compared to conventional methods (Haynes and Rogers, 1994).

However, other studies have shown that the Pastorex *Aspergillus* test had a low sensitivity (Manso *et al.*, 1994; Hopwood *et al.*, 1995; Verweij *et al.*, 1995a; Rath *et al.*,

1996) and a poor positive predictive value (Ansorg *et al.*, 1994; Haynes and Rogers, 1994) in necropsy proven cases of invasive aspergillosis. A high specificity of this test was noted by Manso *et al.* (1994) compared to that noted by others (Hopwood *et al.*, 1995).

The development of a commercial sandwich ELISA (Platelia *Aspergillus*, Bio-rad) has proved more promising than the Pastorex latex agglutination test (Verweij *et al.*, 1995b; Verweij *et al.*, 1995c; Verweij *et al.*, 1996; Bretagne *et al.*, 1997; Williamson *et al.*, 2000b). The sandwich ELISA employs the same antibody as both a captor and a detector, and thus lowers the detection limit 10-fold. This may allow earlier diagnosis of IPA and increase the sensitivity of the test.

The increase in sensitivity has been associated with false-positive results in up to 8% of the serum samples (Stynen *et al.*, 1995; Verweij *et al.*, 1995b; Sulahian *et al.*, 1996), which may be due to cross-reactivity with unidentified serum components (Sulahian *et al.*, 1996). False-positive reactions were found to occur especially within 30 days following bone marrow transplantation (Sulahian *et al.*, 1996), and within 10 days after the administration of cytotoxic therapy to patients with haematological malignancies (Verweij, personal communication). During this period, patients are often profoundly granulocytopenic and at high risk for both bacterial and fungal infections, including IA. Therefore, false-positive ELISA reactions may be due to the presence in the serum of antigens from pathogens other than *Aspergillus* which cross-react with the EB-A2 monoclonal antibody.

Recently, several prospective studies have assessed the performance characteristics of Platelia *Aspergillus* in patients with haematological malignancy (Maertens *et al.*, 2001; Sulahian *et al.*, 2001; Herbrecht *et al.*, 2002a; Maertens *et al.*, 2002; Becker *et al.*, 2003; Pinel *et al.*, 2003). The findings from some of these studies (Herbrecht *et al.*, 2002a;

Pinel *et al.*, 2003) is that although this test contributes to improving IA diagnosis, the sensitivity was disappointing in proven and probable IA cases and was lower than previously described (Maertens *et al.*, 1999).

1.7.2.3.2 Biochemical methods

In recent years techniques to improve timely diagnosis have focused on the detection of surrogate markers including fungal cell wall components such as (1→3)-β-D glucan (*Reiss et al.*, 2000).

Another diagnostic method that has been studied in this patient group is the detection of fungal metabolites such as D-mannitol in invasive aspergillosis (Wong *et al* 1989; Megson *et al.*, 1994).

1.7.2.3.3 Polymerase chain reaction

The polymerase chain reaction was first described in 1985; the detection of fungal DNA is another surrogate marker that has been extensively studied in recent years (Tang and Cohen, 1992; Hopfer *et al.*, 1993; Einsele *et al.*, 1997; Ruhnke and Maschmeyer, 2002). Laborious extraction of fungal DNA and detection of PCR-amplified fungal DNA may be speeded up by using commercially available DNA extraction kits and amplicon detection with a PCR-ELISA system (Jones *et al.*, 1998; Loffler *et al.*, 1998).

A multicentre study from Germany (Hebart *et al.*, 2000b) in febrile neutropenic patients without prior history of invasive fungal infections found that the PCR assay had a sensitivity of 100% and a specificity of 73% for the development of proven or probable invasive fungal infections. The detection of IA after allo-HSCT by the same group (Hebart *et al.*, 2000a) revealed a sensitivity of 100% and a specificity of 65%. However, serial samples were needed to achieve sufficient specificity. The positive predictive value

and the specificity of the assay were improved without a loss of sensitivity if calculations were based on two positive PCR tests (positive predictive value, 27.8% [95 CI, 10%-54%]; specificity, 84% [95% CI, 74-91%]).

Analysis of BAL fluid from neutropenic patients may help in the diagnosis of IPA. Initial studies in four patients with invasive aspergillosis demonstrated the utility of identifying *A. fumigatus* and *A. flavus* by PCR from BAL in four patients with proven or probable aspergillosis, while 6 (13%) out of 46 BAL specimens from control patients had a positive PCR signal (Tang *et al.*, 1993).

Another study to detect *Aspergillus* DNA in 197 BALs (including samples from 141 neutropenic patients) had a sensitivity of 93.9%, specificity of 94.4%, positive predictive value of 83.8% and negative predictive value of 98.1% (Buchheidt *et al.*, 2002). Other authors have highlighted that it may be difficult to distinguish between *Aspergillus* infection and colonisation using a nested PCR assay (Hayette *et al.*, 2001).

Some studies have shown that PCR-based methods are more sensitive (with detection limits of ≤10 fg of *Aspergillus* DNA) than antigen detection methods, particularly for patients with IPA (Kawamura *et al.*, 1999; Buchheidt *et al.*, 2001). The nested PCR assay developed by Kawamura *et al.* (1999) was successfully used in serum samples from 44 patients with pulmonary aspergillosis, although only four had invasive disease. Unlike the sandwich ELISA however, conventional PCR methods cannot be used to monitor the fungal load during antifungal treatment. This limitation has been overcome by the introduction of real-time PCR assays (Kami *et al.*, 2001; Costa *et al.*, 2002). Real-time PCR can be more helpful than conventional PCR because it provides quantitative information on the fungal burden that can be used to distinguish between infection and simple colonisation. A study by Rantokokko-Jalava (2003) found that semi-quantitative detection of *A. fumigatus* DNA could not discriminate between colonisation and

invasion very well. The use of molecular diagnostics appears promising, but a standardised approach has yet to be developed.

Specimen type (serum, whole blood or bronchoalveolar lavage) may affect the sensitivity of the PCR assay (White *et al.*, 2005; Loeffler *et al.*, 2000b; Buchheidt *et al.*, 2001). Serum has been reported to be an appropriate sample for the diagnosis of IA (Yamakami *et al.*, 1996; Costa *et al.*, 2002; Challier *et al.*, 2004). Serum samples were tested using the DNA extraction method described by Yamakami *et al.* (1996), as this specimen was readily available (used for GM ELISA). However, only free-circulating Aspergillus DNA would be targeted, because hyphae or phagocytosed intracellular fungal fragments would have been removed by clot formation and centrifugation. In retrospect, whole blood may have given better results, as subsequent studies have shown (White *et al.*, 2006).

1.7.2.3.4 Microarrays

A technique capable of overcoming the limitations of PCR and increasing the diagnostic output of PCR assays is hybridisation on a DNA microarray. Due to its unrestricted capacity to accommodate hundreds to thousands of individual gene probes, an array allows the simultaneous detection of potentially any amplifiable pathogen present in a specimen. As a result, this technique is ideal for the extensive parallel identification and differentiation of various pathogens and their strains.

The technique is rapidly evolving from a novel research technology (Freeman *et al.*, 2000) to a practical tool for the identification of bacterial species (Chizhikov *et al.*, 2001; Hamels *et al.*, 2001) and the genotyping of viruses (Li *et al.*, 2001). However, the microarray approach has not yet been extended to the clinical

diagnosis of invasive fungal infections.

1.7.3 Imaging

1.7.3.1 High resolution computed tomography scanning

The current British Society of Medical Mycology guidelines (1997) states that the major advances in the diagnosis of invasive fungal infection in patients with haematologic malignancy or solid organ transplantation have been in the use of imaging techniques, rather than in the development of new mycological methods in the routine laboratory.

1.7.3.1.1 Pulmonary infection

The radiographic manifestations of invasive pulmonary aspergillosis include bronchopneumonia, lobar consolidation, segmental pneumonia, multiple nodular lesions resembling septic emboli and cavitary lesions (Orr *et al.*, 1978; Kuhlman *et al.*, 1987). The chest X-ray may show progressive pulmonary infiltrates, leading to complete opacification of entire lobes and corresponding with clinical deterioration.

Computed tomography (CT) scanning of the chest has made a major impact in the management of this patient group. With greater awareness of the effectivenes of CT scanning in detecting lung infections, several authors have reported its early use and consequent benefits (Graham *et al.*, 1991; Caillot *et al.*, 1997). It is more sensitive than chest radiography and is particularly valuable when the chest radiograph is negative or shows only subtle changes (Denning *et al.*, 1997).

In a study by Graham *et al.* (1991), CT scanning detected intrathoracic complications of bone marrow transplantation in 57% of patients in whom chest x-ray (CXR) was

negative. CT scanning can often differentiate between IPA in neutropenic patients and bacterial or viral infections (Graham *et al.*, 1991).

Caillot *et al.* (1997) performed CT scans of the lungs in febrile neutropenic patients once pulmonary infiltrates appeared on chest radiography and reduced the mean time of diagnosis of IPA from 7 to 1.9 days. This was associated with a reduction in mortality directly attributable to IPA from 50% to 17%. A further study by the same author (Caillot *et al.*, 2001a) showed that the extent of lung infiltrates in patients with IPA almost inevitably quadrupled during the first 2 weeks of effective antifungal therapy, before a regression of the inflitrates could be noted. The findings of this study support the warning against designating patients as refractory to treatment with an established antifungal agent after only one week.

Computed tomography scanning is also useful in defining whether bronchoscopy is the best modality for confirming diagnosis, or in guiding further invasive diagnostic procedures, such as the best location for needle biopsy or open lung biopsy (Graham *et al.*, 1991; Plunkett *et al.*, 1992). A diffuse picture on CT scan correlates with the best yield from BAL, whereas solitary, and particularly peripheral, nodules are unlikely to be amenable to microbiological diagnosis by bronchoscopy (Janzen *et al.*, 1993; McWhinney *et al.*, 1993). This diagnostic imaging information has modified patient management and more clearly established the extent of pulmonary disease.

1.7.3.1.2 Sinus and nasal disease

Aspergillus sinusitis may develop before or concomitantly with pulmonary aspergillosis (Berkow *et al.*, 1983; Swerdlow and Deresinski, 1984; Viollier *et al.*, 1986). X-rays of the paranasal sinuses will reveal sinus opacification, and CT

scanning of the infected sinuses may reveal bony destruction. CT scanning is initially preferable to MRI scanning because bone architecture is better visualised by CT. Extension into the orbit, cavernous sinus, carotid artery, and brain is frequently demonstrated.

1.7.3.1.3 Central nervous system infection

For central nervous system infections, evidence of cerebral infarction provides strong support for invasive aspergillosis in this patient population. These radiological changes may progress to the appearance of cerebral abscesses. Hypodense lesions on CT scans are subtle and easy to miss but are highly suggestive of cerebral aspergillosis.

1.7.3.2 ^{99m}Tc-Infecton (INFECTON®)

The diagnosis of deep seated infection can be a challenging problem. Radiolabelled leucocyte imaging (WBC imaging) is currently the main method used for imaging infection. Neutrophils migrate to the site of infection through diapedesis and chemotaxis (Datz, 1993).

A group at St Bartholomew's Hospital, London (Hall *et al.*, 1998) have developed a novel radiopharmaceutical, ^{99m}Tc-Infecton (INFECTON®), based on the 4-fluoroquinolone antibiotic ciprofloxacin. They have demonstrated that INFECTON® is specific for detecting sites of bacterial infection in the body. The high positive predictive value displayed by the technique is clinically valuable because a positive image strongly supports a diagnosis of bacterial infection. A negative result does not rule out an infection, and may be the result of previous or current antibiotic treatment and/or infection with organisms that do not take up

INFECTON®.

This agent is currently unavailable in the UK as DRAXIS Health Inc. (USA) now own the patent for INFECTON® and has received FDA approval to initiate a Phase II clinical study on patients with signs and symptoms of bacterial osteomyelitis. Two other trials with INFECTON® are currently being conducted in Canada, one involving diabetic patients suffering from bacterial infections of the foot and the other in patients with either known soft tissue infections or chronic inflammatory conditions.

INFECTON® would have been ineffective in diagnosing *Aspergillus* spp. infections. However, the group at St Bartholomew's Hospital is currently working on radiolabelled AmBisome and caspofungin (Das *et al.*, personal communication). As the labelling needs refinement, this agent may be up to 5 years away from clinical trials. It will be interesting to observe future developments.

1.8 THERAPEUTIC STRATEGIES

Invasive aspergillosis has emerged as the leading cause of death among fungal infections (Groll *et al.*, 1996). An expanding armamentarium of antifungal compounds is improving the range of therapeutic options against this infection.

Figure 1-1 shows the sites of action of antifungal agents. A comparison of the price of various antifungal agents is given in Figure 1-2.

1.8.1 Amphotericin B deoxycholate

Amphotericin B deoxycholate (AMB) has been the cornerstone of antifungal therapy in immunosuppressed patients for decades (Gallis *et al.*, 1990). However,

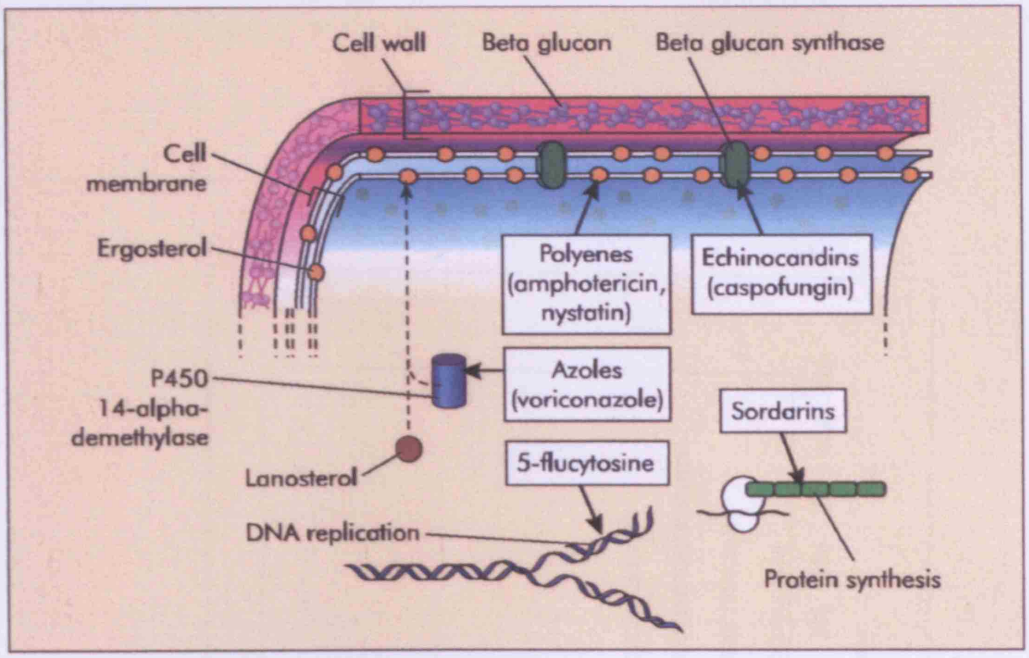
because of significant dose-limiting nephrotoxicity and infusion-related toxicity, lipid formulations of the drug have been developed with the aim of reducing toxicity and improving efficacy. A recently published paper by Eriksson *et al.* (2001) indicated that infusion-related side effects and nephrotoxicity of AMB may be reduced significantly by the prolongation of AMB application to 24 hours.

1.8.2 Lipid formulations of amphotericin B

Three lipid formulations of AMB are commercially available: liposomal AMB (L-AMB), AMB lipid complex (ABLC), and AMB colloidal dispersion (ABCD). The consensus opinion from various studies is that lipid formulations of AMB appear to be at least as effective as AMB in patients having fever and neutropenia, and result in less nephrotoxicity and infusion-related toxicity (Oppenheim *et al.*, 1995; Prentice *et al.*, 1997; Walsh *et al.*, 1998; Walsh *et al.*, 1999; Dix and Andriole, 2000; Johansen and Gotzsche, 2000; Wingard *et al.*, 2000). However, this was not the finding in the study by Bowden *et al.*, (2002) where the rate of acute infusion-related toxicity was higher in patients who received ABCD than in those who received conventional amphotericin B for both chills and fever. At the licensed doses of 3-5 mg/kg/day for L-AMB, 5 mg/kg/day for ABLC, and 3-6 mg/kg/day for ABCD, these agents appear to be equally potent, although determining their optimal dose for serious infections requires further study.

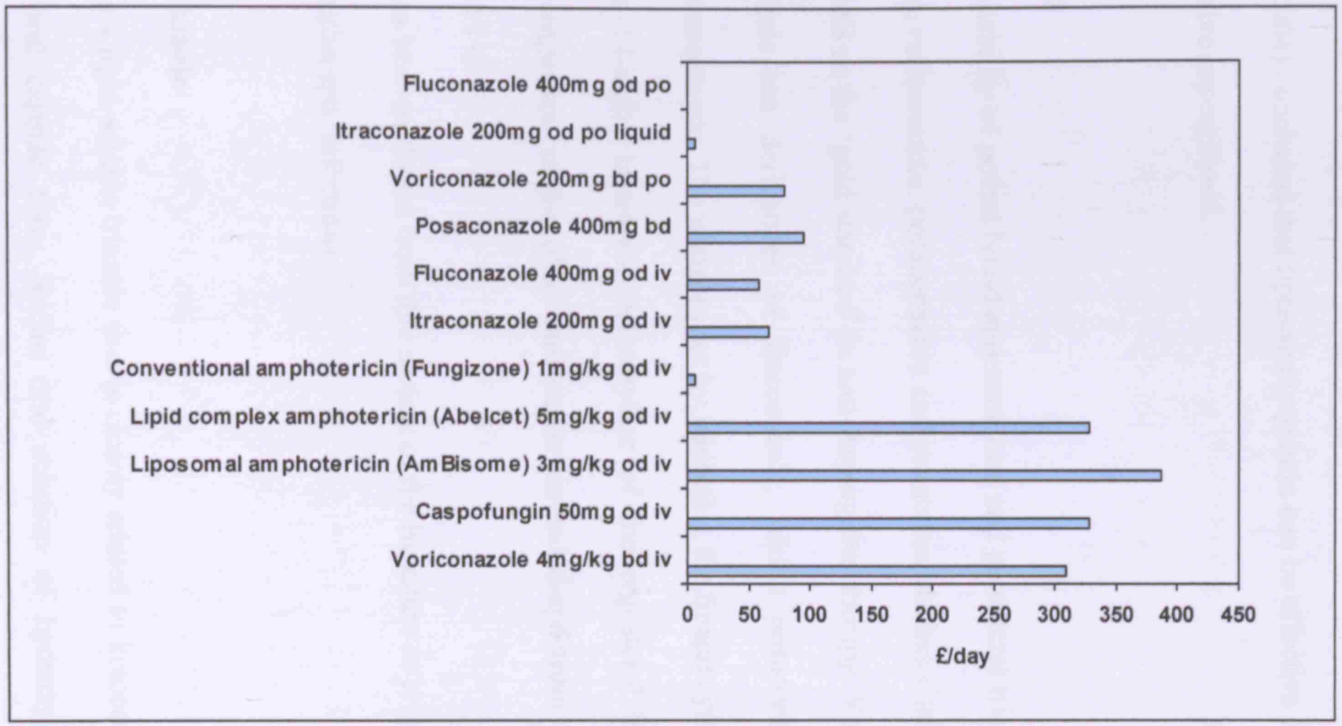
Although these lipid products represent attractive alternatives to delivery of AMB from a toxicity standpoint, their use should be limited to patients who cannot tolerate, or whose infection does not respond, to AMB, because they are all substantially more expensive than the parent drug, with L-AMB being the most expensive.

Figure 1-1. Sites of action of antifungal agents.



Reproduced with permission from Hospital Medicine (Shetty and Barnes, 2004).

Figure 1-2. Comparison of acquisition costs. From the British Medical Association and the Royal Pharmaceutical Society of Great Britain (2007).



1.8.3 Liposomal nystatin

Liposomal nystatin, a broad spectrum antifungal agent, is nystatin incorporated into liposomes containing dimyristoyl phosphatidylcholine and dimyristoyl phostatidylglycerol. Studies have shown that it is active against Aspergillus in neutropenic mice (Wallace *et al.*, 1997). A recently published EORTC study (Offner *et al.*, 2004) concluded that liposomal nystatin can be effective for salvage therapy of invasive aspergillosis.

1.8.4 Triazoles

The recent availability of potent broad-spectrum oral and parenteral triazoles such as itraconazole, voriconazole, posaconazole and ravuconazole has challenged the standing of AMB as the 'gold standard' in anti-Aspergillus therapy. Voriconazole and ravuconazole are derivatives of fluconazole, whilst posaconazole is a derivative of itraconazole. The triazoles act by inhibiting the fungal cytochrome P-450-dependent 14-alpha lanosterol demethylase of the ergosterol biosynthetic pathway. A comparison of the older and newer extended-spectrum triazoles is shown in Table 1-5.

Fluconazole has been excluded from this review of the literature as it is ineffective against *Aspergillus* spp. infections.

1.8.4.1 Itraconazole

Itraconazole is a lipid-soluble triazole that is closely related to ketoconazole. It is available in oral capsule form, as an oral solution of hydroxypropyl-beta-cyclodextrin, and an intravenous formulation (Prentice *et al.*, 1999).

Recent encouraging data from an open-label multicentre European study of 31

cases of invasive aspergillosis showed that administration of intravenous itraconazole followed by oral itraconazole is safe, reliable, and effective (Caillot *et al.*, 2001b).

The results of a large randomised, controlled, multicentre trial of intravenous and oral itraconazole versus amphotericin B as empirical antifungal therapy for persistent fever in neutropenic patients was published recently (Boogaerts *et al.*, 2001). This study involved 384 neutropenic patients with cancer and persistent fever that did not respond to antibiotic therapy. The response rate was 47% with itraconazole, and 38% with amphotericin B. There were significantly fewer drug-related adverse events and withdrawals due to toxicity in the itraconazole arm.

The mortality and incidence of breakthrough fungal infections were similar in both arms.

1.8.4.2 Voriconazole

Voriconazole, a broad spectrum triazole which is available in both intravenous and oral preparation, has been licensed by the FDA for primary therapy of invasive aspergillosis. The Herbrecht study (N Eng J Med 2002) shows the superiority of voriconazole over amphotericin B as initial therapy for invasive aspergillosis, in terms of response rate, survival rate, and safety. The efficacy of voriconazole in invasive aspergillosis shown in this trial is consistent with the results of the recently published comparison of voriconazole with liposomal amphotericin B for empirical antifungal therapy in persistently febrile patients with neutropenia (Walsh *et al.*, 2002). Remarkably, voriconazole is the first antifungal showing relevant response and survival rates in patients treated for aspergillosis with cerebral involvement.

Table 1-5. Pharmacologic differences between the triazoles*

	Fluconazole	Itraconazole	Voriconazole	Posaconazole
Spectrum	Narrow, limited mostly to *C. albicans*, *C. tropicalis*, *C. parapsilosis* +/- C. glabrata (at higher doses). No activity against *Aspergillus* spp.	Broader than fluconazole-similar *Candida* coverage as fluconazole plus *Aspergillus* spp.	Broad, includes *Candida*, *Aspergillus*, and *Fusarium* spp., hyalohyphomycetes.	Broadest-spectrum triazole, potent activity against *Candida* and *Aspergillus* spp., hyalohyphomycetes, *Zygomycetes* spp.
Oral absorption	Tablet - excellent	Capsule - irregular, stomach pH-dependent; cyclodextrin solution - improved absorption	Tablet - excellent	Suspension - improved with multiple daily dosing and high-fat meal
IV Formulation	Available, no solubilizer	Available, hydroxy-propyl-ß-cyclodextrin	Available, sulphobutyl ether-ß-cyclodextrin	Currently undergoing phase III clinical trials
Clearance	80% renal	Hepatic	Hepatic	Excreted in faeces (90%)
Serum half-life (h)	24	24-35	6-24	8-24
Cerebrospinal fluid penetration	Excellent	Poor	Excellent	Poor-moderate
Vulnerability to hepatic enzyme induction	Moderate	Significant	Significant	Moderate
Adverse effects - rash	+	++	++	++
Adverse effects - other	Occasional nausea and vomiting, increase in hepatic transaminase level	Nausea and vomiting, diarrhoea with solution formulation, increase in hepatic transaminase level, congestive heart failure with prolonged oral therapy	Nausea and vomiting, increase in hepatic transaminase level, transient visual disturbances with IV infusion	Nausea and vomiting, fever
Contraindicated when CrCl <30 mL/min	No, but dosage should be adjusted	Oral - no IV - yes	Oral - no IV - yes	Oral - no

* Adapted from Groll *et al.*, (1998)

1.8.4.3 Ravuconazole

Ravuconazole is structurally similar to fluconazole and voriconazole, and is currently undergoing Phase III clinical trials. Activity against resistant fungi, high bioavailability, and long half-life are the key properties of this new triazole (Groll *et al.*, 1998).

1.8.4.4 Posaconazole

Posaconazole is a new generation extended spectrum triazole with in-vitro activity against a wide spectrum of medically important fungi, including species of candida, aspergillus, Zygomycetes, and fusarium. (Groll and Walsh, 2005; Sabatelli *et al.*, 2006) Studies of animals and humans have shown clinical activity of posaconazole in the treatment of invasive infection with moulds and yeasts. (Petraitiene *et al.*, 2001; Raad *et al.*, 2006; Ullmann *et al.*, 2006; van Burik *et al.*, 2006). This drug has recently been licensed for use in the refractory setting as well as for prophylaxis by the Food and Drug Administration in the USA, and the European Medicines Evaluation Agency.

An open-label externally controlled multicentre trial using posaconazole as salvage therapy for invasive aspergillosis in patients who were refractory or intolerant of conventional therapy found that posaconazole was a suitable alternative. The overall success rate and survival rate for posaconazole treated patients was greater than that for the external control group (Walsh *et al.*, 2007).

A recently published paper in the New England Journal of Medicine (Cornely *et al.*, 2007) comparing prophylaxis with posaconazole versus fluconazole or itraconazole in neutropenic patients found that posaconazole prevented invasive fungal infection more effectively than either comparator drug, and improved

overall survival in patients undergoing chemotherapy for acute myelogenous leukaemia or the myelodysplastic syndrome. There were however more serious adverse events likely related to treatment with posaconazole.

A limitation of this drug is that it is currently only available in oral formulation. The prophylactic posaconazole study was limited in its ability to provide data on the usefulness of azole prophylaxis in patients who had severe mucusitis and were unable to eat or take oral medication. An intravenous formulation has now been developed, and phase III clinical studies have just started worldwide (Angus Campbell, Schering-Plough; personal communication).

1.8.5 Echinocandins

Echinocandins are a new class of antifungal drugs derived from several fungal species. The first of the class to be licensed was caspofungin, for refractory invasive aspergillosis (about 40% response rate) and the second was micafungin. These drugs are cell-wall active agents that are inhibitors of (1,3)-β-D-glucan synthesis, an action that damages fungal cell walls (Georgopapadakou, 2001). No drug target is present in mammalian cells.

The echinocandins are rapidly fungicidal against most *Candida* spp., and fungistatic against *Aspergillus* spp. (Groll and Walsh, 2001). They are not active at clinically relevant concentrations against *Fusarium* spp., *Zygomycetes* spp., or *Cryptococcus neoformans* (Table 1-6).

Micafungin is undergoing Phase III clinical trials in BMT recipients, as primary prophylaxis in patients having fever during protracted neutropenia.

All the echinocandins that are currently available are administered parenterally. Dosing is once daily, drug interactions are few, and there are substantially fewer

Table 1-6. Spectrum of the echinocandins

Covered	Not covered
Candida spp. Fungicidal, including azole-resistant species	*C. neoformans*
Aspergillus spp. Inhibition of apical tips and branching	*Fusarium* spp.
P. carinii Not effective alone in disseminated infection	*Zygomycetes* spp.
Histoplasma spp. Other endemic fungi?	Dematiaceous moulds

toxic effects. One potential use of the echinocandins is in combination with other antifungal drugs (AMB or triazoles). Animal data suggest that there is a synergistic effect when an echinocandin is combined with AMB or a triazole for the treatment of aspergillosis (Kohno *et al.*, 2000).

1.8.6 Surgical resection

Some haematology units have a policy of resecting focal lesions that show features characteristic of invasive pulmonary fungal infection on CT scanning. Resection is performed for therapeutic purposes; to allow cure, to prevent the risk of massive hemoptysis and to reduce the risk of relapse following subsequent neutropenic episodes or bone marrow transplantation (McWhinney *et al.*, 1993; Wong *et al.*, 1992).

1.8.7 Cytokines

In neutropenia, the most important factor that determines the resolution of IA is neutrophil recovery (Bodey and Vartivarian, 1989). The degree and duration of neutropenia is reduced by administration of cytokines such as G-CSF and GM-CSF (Vadhan-Raj *et al.*, 1987; Moore *et al.*, 1998). The stimulation of neutrophil production in myelocompromised patients by the use of these agents has also been shown to diminish the frequency of infective complications (Yoshida *et al.*, 1990; Bodey *et al.*, 1993; Bodey *et al.*, 1994). Hence they may play a significant role in the management of fungal infections when used in conjunction with amphotericin B and amBisome (Catalano *et al.*, 1997). Animal experiments also suggest that interferon(IFN)-gamma has a protective role in IA, but further studies are required before any firm conclusions can be drawn (Nagai *et al.*, 1995).

1.8.8 Granulocyte transfusions

Some investigators believe that granulocyte transfusions may be useful in cases of severe uncontrollable fungal infections (Bhatia *et al.*, 1994; Catalano *et al.*, 1997; Dignani *et al.*, 1997). Transfusions of high numbers of granulocytes to the donor, obtained after administration of G-CSF, with or without dexamethasone, is done by some clinicians; to date, there is no convincing evidence of its efficacy. Significant toxicities in recipients include transmission of cytomegalovirus, alloimmunization associated with fever, graft-versus-host reactions if granulocytes are not irradiated, progressive platelet refractoriness and possibly, respiratory insufficiency associated with concomitant administration of amphotericin B (Hughes *et al.*, 2002).

1.9 PREVENTION OF INVASIVE ASPERGILLOSIS

The case fatality rate (CFR) from invasive aspergillosis varies considerably depending on the patient population studied; a recent systematic review of the literature gave an overall CFR of 58% (Lin *et al.*, 2001). Rates as high as 86-90% have been reported in bone marrow transplant recipients (Denning, 1996; Lin *et al.*, 2001). In contrast, patients with leukaemia or lymphoma have a lower but still high mortality rate of 49.3%. Patients with central nervous system or disseminated aspergillosis have the highest mortality rate (88.1%; Lin *et al.*, 2001).

Prevention of severe fungal infections should be a high priority in the management of all at-risk patients such as neutropenic, bone marrow transplant or solid organ transplant recipients; outbreaks of invasive aspergillosis reinforce the importance of maintaining an environment as free of *Aspergillus* spp. spores as possible for these patients.

Risk factors for invasive aspergillosis need to be identified in each patient group and patients at risk should be monitored especially carefully, with a high index of suspicion for infection. Several strategies can be adopted to reduce the morbidity and mortality associated with invasive aspergillosis; the most obvious one is to reduce exposure of immunocompromised patients to *Aspergillus* conidia by using environmental control.

1.9.1 Environmental strategies

The Centers for Disease Control and Prevention (CDC) in the United States (Tablan *et al.*, 1994; Tablan *et al.*, 2004) have set out the following recommendations for the prevention of nosocomial pulmonary aspergillosis: when planning specialised-care units for high risk patients, the following environmental strategies should be implemented to minimise fungal spore counts.

1. Air filtration.

High efficiency particulate air (HEPA) filters are 99.97% efficient in filtering 0.3μ-sized particles and should be placed in the unit air supply either centrally or at the point of use, i.e. at patient room-air inflow ducts.

2. Directed room air flow.

Air-intake and exhaust ports should be placed such that room air comes in from one side of the room, flows across the patient's bed and exits on the opposite side of the room.

3. Room-air pressure.

Room-air pressure should be maintained continuously above that of the corridor unless there are clinical-care or infection-control contraindications for doing so. To maintain positive pressure ventilation, room air must be supplied at a rate that

is 10-20% more than the rate of exhausting air from the room.

4. Well sealed room.

Windows, doors and intake and exhaust ports should be constructed to achieve complete sealing of the room against air leaks. In addition, facilities should be designed without false ceilings.

5. Number of air changes.

A high (≥12) number of air changes per hour is recommended.

The integrity of the air filtration system needs to be closely monitored with regular planned preventative maintenance, particulate counting, pressure monitoring, and air flow changes being performed. In addition, the guidelines recommend that hospital policies to minimise exposure of high risk patients to potential sources of *Aspergillus* spp., such as hospital construction and renovation, cleaning activities, carpets, food, potted plants, and flower arrangements should be in place.

In existing facilities with no cases of aspergillosis, dust accumulation should be prevented by daily damp-dusting horizontal surfaces and regularly cleaning ceiling and air duct grates when the rooms are not occupied by patients. The Bristol study (Humphreys *et al.*, 1991) emphasises the importance of thorough and regular cleaning of all surfaces in clinical areas to prevent the accumulation of dust. Systematic review and coordination of infection control strategies with hospital personnel in charge of engineering, maintenance and catering should be undertaken. When hospital construction and renovation activities are being planned, a strategy should be implemented to prevent patients at high risk of aspergillosis from exposure to high ambient air spore levels. During construction and renovation, barriers which should be impermeable to *Aspergillus* spp. (plastic, drywall) must be constructed between patient-care and construction areas to

prevent dust from entering patient-care areas. Maintenance of a negative pressure in these areas relative to adjacent patient-care areas is essential unless there are contraindications for such pressure differential. Direction of pedestrian traffic away from construction areas prevents dust dispersion, entry of contaminated air or tracking of dust into patient areas. Air and environmental monitoring for fungal spores may be indicated when building works are taking place adjacent to a high dependency unit.

When a case of nosocomial aspergillosis occurs, the CDC recommend a prospective search for additional cases in hospitalized patients and an intensified retrospective review of the hospital's microbiologic, histopathologic, and post-mortem records should be carried out. If evidence of continuing *Aspergillus* spp. infection exists, an environmental investigation should be conducted to determine and eliminate the source. If an environmental source is identified, corrective measures must be performed to eliminate the source from the high-risk patient's environment.

1.9.2 General strategies

Education of healthcare workers regarding invasive aspergillosis and its associated risks in immunosuppressed patients, and implementation of infection control policies to decrease its occurrence should be a priority. Other risk factors should also be reduced, such as ensuring that the patient is in protective isolation when the absolute neutrophil count is less than $0.5 \times 10^9/l$, restricting the use of broad-spectrum antimicrobials, or minimising the duration of granulocytopenia by administering haemopoietic growth factors. Transplant recipients should be specifically proscribed from marijuana use during periods of high steroid

administration (Marks *et al.*, 1996).

1.9.3 Prophylactic antifungal strategies

The use of anti-fungal prophylaxis has become widespread although convincing data regarding efficacy is lacking (Working Party of the British Society for Antimicrobial Chemotherapy, 1993). There have been no large randomised studies which have adequately evaluated the optimal prophylaxis against invasive aspergillosis in immunosuppressed patients or in those undergoing bone marrow or solid organ transplantation.

Non-absorbable polyene antibiotic prophylaxis has been used for several decades in the neutropenic host, although the benefits of this have been marginal.

A multicentre prospective randomised study comparing itraconazole solution (which has improved absorption over previous formulations) with fluconazole in neutropenic patients with haematological malignancy suggests this formulation may be effective (Morgenstern *et al.*, 1999). There were no cases of invasive aspergillosis in the itraconazole treated patients compared with four in the fluconazole arm. Previous preparations of itraconazole have not prevented cases of invasive aspergillosis as 14% of all cases collected by the EORTC during a prospective survey had received itraconazole prophylaxis (Denning *et al.*, 1996b).

Itraconazole has largely replaced fluconazole as the most frequently administered azole for prophylaxis of invasive fungal infections in blood and BMT recipients, especially in centres with a high incidence of aspergillosis (Glasmacher *et al.*, 1996; Bohme *et al.*, 2000; Boyle and McCann, 2000). There is currently considerable speculation regarding the potential use of voriconazole for antifungal prophylaxis. To date, there is conflicting evidence for the efficacy of

inhaled/nebulised amphotericin B (Conneally *et al.*, 1990; Jeffery *et al.*, 1991; Beyer *et al.*, 1994; Richenspurner *et al.*, 1997).

A meta-analysis by Gotzsche and Johansen (1997) showed no significant survival benefit gained from the use of prophylactic or empirical antifungal agents in neutropenic patients with cancer. The authors concluded that the use of antifungal agents should be restricted to neutropenic patients with proven fungal infections. Although this paper has been criticised (Kibbler *et al.*, 1997) for considering prophylaxis along with early and late empirical therapy, it is reasonable to conclude that there is no overwhelming evidence for the efficacy of any antifungal agent in preventing invasive aspergillosis (Prentice *et al.*, 2000)..

Patients with previously documented fungal infection (proven or probable invasive aspergillosis or other mould infections, or fungaemia) should receive secondary prophylaxis during subsequent neutropenia and graft-versus-host disease. This may be oral itraconazole (unless the previous infection broke through itraconazole prophylaxis or was unresponsive to itraconazole). Amphotericin B 0.75 mg/kg/day or amBisome 1mg/kg/day may be given as an alternative. In cases where patients required voriconazole for treatment of the initial infection, this would also be a suitable secondary prophylactic agent.

Patients who develop CMV infection or start corticosteroid therapy for GVHD should be monitored closely for subsequent development of invasive mould infections. It is possible that one of the mould-active azole antifungals or echonocandins may be used to prevent infections in patients with recognized high-risks (Morgenstern *et al.*, 1999; Denning *et al.*, 2002; Herbrecht *et al.*, 2002b; Pacetti and Gelone, 2003).

Some liver transplant centres have used selective decontamination of the digestive

tract (SDD) with gentamicin, polymyxin E and nystatin, and demonstrated a reduction in the number of bacterial and fungal infections in those in whom SDD effectively eliminated aerobic faecal flora (Wiesner *et al.*, 1988; Rosman *et al.*, 1990; Van Zeijl *et al.*, 1990; Kibbler 1995). However, SDD has not been shown to reduce the incidence of IA.

PURPOSE AND SCOPE OF THIS THESIS

Earlier diagnosis of invasive aspergillosis may lead to improved survival. The purpose of this thesis was to investigate methods that lead to earlier diagnosis of invasive aspergillosis and consequently improve the likelihood of a successful outcome.

I performed both a retrospective and a prospective study looking at various diagnostic methods that were available. As the primary site of entry of *Aspergillus* conidia is the respiratory tract and most patients (>90%) present with pulmonary infection, the purpose of the retrospective study was:

1. to compare two different antigen detection assays (Pastorex latex agglutination and Platelia sandwich ELISA tests), with a PCR assay and high resolution CT scanning
2. to compare bronchoalveolar lavage fluid samples with serum samples

in the early diagnosis of invasive pulmonary aspergillosis in febrile neutropenic and BMT patients with respiratory symptoms and signs.

A one year prospective study was then performed to evaluate further the promising findings of the retrospective study.

CHAPTER 2

CLINICAL AND LABORATORY METHODS

2.1 INTRODUCTION

Invasive aspergillosis has become a leading cause of death due to infection in allogeneic haematopoietic stem-cell-transplant (HSCT) recepients (Fukuda *et al.*, 2003). It remains an important cause of morbidity and mortality among those treated for haematological malignancy (Martino and Subira, 2002) and those receiving a solid organ transplant, especially of the lung (Singh and Husain, 2003).

2.2 STUDY DESIGN

2.2.1 Retrospective study

2.2.1.1 Patient recruitment

Registers of prospective patients with suspected, proven or probable invasive aspergillosis were kept by the haematology, microbiology and radiology departments of the Royal Free Hospital. The fact that comprehensive databases were already available made accurate case finding easier. We identified all patients with haematological malignancies who developed invasive aspergillosis between July 1995 to July 1997 and studied their medical records. All consecutive patients who fulfilled the entry criteria were entered into the study.

Patients were enrolled if they fulfilled the following criteria: febrile neutropenia or BMT, with pulmonary infiltrates on high resolution thoracic computed tomography (CT) scan and who had a BAL performed.

2.2.1.2 Sample processing

Once patients had been enrolled, any stored sera obtained during the period from six weeks before until three weeks after the BAL were retrieved for galactomannan testing. The BAL fluid and serum samples were then stored at -70°C until antigen

testing and PCR was performed. For the serological analysis, each sample was tested in triplicate, and the consensus (majority) result taken. Microscopy and culture for bacteria and fungi, and virus isolation studies were also performed on all BAL fluid samples in the routine diagnostic laboratory.

The clinical notes including the microbiological and histopathological records of all patients were reviewed for evidence of IPA or other respiratory disease, and compared with the non-culture based diagnostic techniques above. BAL and serum samples from 12 immunocompetent and 20 asymptomatic HIV positive patients were used as controls.

2.2.2 Prospective study

All patients were hospitalised in single reverse-isolation rooms in a unit equipped with high-efficiency particulate air filters until recovery of the neutrophils above 500 cells per μl. Patients were also on a "clean" diet.

In our unit, we routinely administer antifungal prophylaxis to all high risk neutropenic and BMT patients. Until January 1998, oral fluconazole (100 mg/day) and amphotericin B (500 mg qds) were the prophylactic agents that were used.

However, six months after the onset of the prospective study, itraconazole solution was licensed for use as a prophylactic antifungal agent. This drug has been shown to have excellent serum and tissue levels, both in animal studies and in-vivo studies.

Because of the relatively high incidence and attributable mortality associated with invasive aspergillosis, the decision was made to change the prophylactic agent to itraconazole solution 200 mg bd (from mid-January 1998).

A patient's ability to swallow is rarely compromised immediately after induction

chemotherapy, but oral intake may decrease owing to mucositis later in the course of treatment. Few patients in this study were unable to tolerate oral medications at entry or during the course of treatment.

For the first month after we started using itraconazole prophylaxis, serum itraconazole levels (Mycology Reference Laboratory, Bristol) were measured on all samples. As all trough levels were > 0.5 mg/ml (satisfactory level), no further levels were performed.

Subsequent to this study, more recent studies have shown the benefit of measuring itraconazole levels (Marr *et al.*, 2004; Maertens and Boogaerts, 2005; Potter, 2005).

2.2.2.1 Patient recruitment

Serum and BAL fluid samples were collected prospectively over a one year period from all at-risk patients in the haematology unit. Serum samples were collected thrice weekly from all at-risk patients. Specimens from sterile or deep respiratory sites were obtained whenever the clinical condition deemed it necessary; this is in contrast to the retrospective study in which both CT scans and BALs were obtained from all patients. Microbiological and histological data were collected on a prospective basis.

High resolution CT scanning of the thorax, sinuses and head was performed based on an individual patients clinical symptoms and signs. The CT scans were as previously independently and anonymously reviewed by a radiologist (LAB) for evidence of IPA.

2.2.2.2 Sample processing

Microscpy and culture for bacteria, fungi and viruses were performed on all BAL fluid

samples. Galactomannan analysis by the sandwich ELISA (Platelia *Aspergillus*, Bio-Rad) technique was performed on all samples. PCR analysis was performed only on serum samples.

PCR analysis was only performed on serum samples in the prospective study because analysis of data from the retrospective study showed that false positive PCR results were more likely to occur with BAL specimens [15.8%]. There were no false positive results with the serum samples in the retrospective study.

The aim of the prospective study was to examine the best strategy for sampling, and to determine the best methodology for the earlier diagnosis of IA.

2.3 PATIENT CATEGORISATION

During the past several decades, there has been a steady increase in the frequency of opportunistic invasive fungal infections (IFIs) in immunocompromised patients. However, there is substantial controversy concerning optimal diagnostic criteria for these IFIs.

The definitions used here are based on the NIAID Mycoses Study Group (MSG) guidelines (Denning *et al.*, 1994). Proven IPA was defined as histologically proven disease with or without positive respiratory tract (specimens include sputum, nasopharyngeal aspirates, BAL fluid and endotracheal secretions) or tissue cultures for *Aspergillus*. Probable IPA was defined as clinical evidence of infection plus positive culture for *Aspergillus* spp. (minimum of 2 cultures from sputum, endotracheal secretions or nasopharyngeal secretions, or one from BAL fluid), or positive cytology on BAL, but without histological confirmation. Clinical evidence of IPA included compatible radiological evidence. Possible cases were defined as those with clinical and radiological evidence of pulmonary infection but without culture evidence for any

infection. Patients in the negative group were defined as those with proven clinical infection due to another aetiologic agent, or where the pulmonary infiltrates were attributed to another cause.

Recently, a consensus committee composed of members from the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer (EORTC-IFICG), and the Mycoses Study Group of the National Institute of Allergy and Infectious Diseases (NIAID-MSG) has taken a lead in developing standardized definitions of invasive fungal infection (IFI) in cancer patients and stem cell transplant recipients for use in clinical research (Ascioglu *et al.*, 2002). Three levels of probability are proposed: "proven", "probable", and "possible". The definitions are intended for use in the context of clinical and/or epidemiological research, not for clinical decision making.

A proven deep tissue infection is defined as histopathologic or cytopathologic examination showing hyphae from needle aspiration or biopsy specimen with evidence of associated tissue damage (either microscopically or unequivocally by imaging); or positive culture result for a sample obtained by sterile procedure from normally sterile and clinically or radiologically abnormal site consistent with infection, excluding urine and mucous membranes.

A probable IFI has at least one host factor criterion, one microbiological criterion and one major (or 2 minor) clinical criteria from abnormal site consistent with infection (refer Table 2-1).

Possible IFIs should have at least one host factor criterion, one microbiological criterion or one major (or two minor) clinical criteria from abnormal site consistent with infection. This category is not recommended for use in clinical trials of antifungal agents but might be considered for studies of empirical treatment, epidemiological studies, and studies of

health economics.

The EORTC/MSG Consensus group is in the process of revising the consensus definitions (draft VI, Dec 18 2005; Dr Fungus website). The definitions still exclude PCR results. It is the opinion of the consensus group that until a PCR system is developed that has been externally validated, a positive PCR result for blood, tissue or BAL fluid for the specific fungus studied will not be considered microbiological evidence of invasive fungal disease.

Table 2-1. Host factor, microbiological, and clinical criteria for invasive fungal infections in patients with cancer and recipients of hematopoietic stem cell transplants.

Type of criteria	Criteria
Host factors	Neutropenia (<500 neutrophils/mm^3 for >10 days). Persistent fever for >96 h refractory to appropriate broad-spectrum antibacterial treatment in high-risk patients. Body temperature either >38°C or <36°C and any of the following predisposing conditions: prolonged neutropenia (>10 days) in previous 60 days, recent or current use of significant immunosuppressive agents in previous 30 days, proven or probable invasive fungal infection during previous episode of neutropenia, or coexistence of symptomatic AIDS. Signs and symptoms indicating graft-versus-host disease, particularly severe (grade ≥2) or chronic extensive disease. Prolonged (>3 weeks) use of corticosteroids in previous 60 days.
Microbiological	Positive result of culture of mould (including *Aspergillus, Fusarium,* or *Scedosporium* species or Zygomycetes) or *Cryptococcus neoformans* or an epidemic fungal pathogen[a] from sputum or bronchoalveolar lavage fluid samples. Positive result of culture of findings of cytologic/direct microscopic evaluation for mould from sinus aspirate specimen. Positive findings of cytologic/direct microscopic evaluation for mould or *Cryptococcus* species from sputum or bronchoalveolar lavage fluid samples. Positive result for *Aspergillus* antigen in specimens of bronchoalveolar lavage fluid, CSF, or ≥ 2 blood samples. Positive findings of cytologic or direct microscopic examination for fungal elements in sterile body fluid samples (e.g., *Cryptococcus* species in CSF).
Clinical	Must be related to site of microbiological criteria and temporally related to current episode.
Lower respiratory tract infection	
Major	Any of the following new infiltrates on CT imaging: halo sign, air-crescent sign, or cavity within area of consolidation[b].
Minor	Symptoms of lower respiratory tract infection (cough, chest pain, hemoptysis, dyspnea); physical finding of pleural rub; any new infiltrate not fulfilling major criterion; pleural effusion.
Sinonasal infection	
Major	Suggestive radiological evidence of invasive infection in sinuses (i.e., erosion of sinus walls or extension of infection to neighboring structures, extensive skull base destruction).
Minor	Upper respiratory symptoms (e.g., nasal discharge, stuffiness); nose ulceration or eschar of nasal mucosa or epistaxis; periorbital swelling; maxillary tenderness; black necrotic lesions or perforation of hard palate.
CNS infection	
Major	Radiological evidence suggesting CNS infection (e.g., mastoiditis or other parameningeal foci, extradural empyema, intraparenchymal brain or spinal cord mass lesion).
Minor	Focal neurological symptoms and signs (including focal seizures, hemiparesis, and cranial nerve palsies); mental changes; meningeal irritation findings; abnormalities in CSF biochemistry and cell count (provided that CSF is negative for other pathogens by culture or microscopy and negative for malignant cells).
Disseminated fungal infection	Papular or nodular skin lesions without any other explanation; intraocular findings suggestive of hematogenous fungal chorioretinitis or endophthalmitis.

[a] *H capsulatum* variant *capsulatum, Blastomyces dermatitidis, Coccidioides immitis,* or *Paracoccidioides brasiliensis*

[b] In absence of infection by organisms that may lead to similar radiological findings including cavitation, such as *Mycobacterium, Legionella* and *Nocardia* species

Data modified after Ascioglu *et al.*, 2002

2.4 METHODOLOGY

2.4.1 Galactomannan detection methods

2.4.1.1 Pastorex latex agglutination test

The *Pastorex Aspergillus* (Bio-Rad Laboratories, Marnes-La-Coquette, France) test is a qualitative and semiquantitative test which uses a simple agglutination technique to detect the Aspergillus polysaccharide galactomannan in serum. The principles of this test, based on the user manual are outlined below.

This test uses latex particles coated with monoclonal antibody to galactomannan. The particles react with the *Aspergillus* polysaccharide antigen, resulting in agglutination visible to the naked eye. The sensitivity limit is 15 ng/ml. Prior treatment of the sera (heat treatment at 100°C followed by centrifugation) is necessary in order to dissociate the circulating immune complexes and eliminate nonspecific reactions.

2.4.1.1.1 Collection, storage and processing of serum and BAL fluid samples

The serum and BAL fluid samples that were collected from all patients who met the criteria for the study, and from the control patients, were stored at –70°C until tested.

Sera were processed according to the manufacturer's instructions. Bronchoalveolar lavage fluid samples were also processed the same way although the manufacturer has not licensed the test for this purpose.

2.4.1.1.2 Treatment of sera and BAL fluid

Three hundred microlitres of test serum or BAL fluid were transferred into a 1.5 ml Eppendorf tube. One hundred microlitres of treatment reagent (edetic acid) was added. The mixture was vigorously homogenized and heated to 100°C for 3 minutes, then centrifuged at 10 000g for 10 minutes. The supernatant was then tested. The same treated

samples were used to perform the sandwich ELISA.

2.4.1.1.3 Agglutination reaction

Forty microlitres of supernatant was mixed with 10 µl of sensitised *Aspergillus* latex on an agglutination card. This was mixed with a stick, and the agglutination card was then placed on an agitator for 5 minutes (160 rpm), at room temperature. The result was read by eye. A positive reaction is manifested as agglutination of the latex particles.

2.4.1.1.4 Quality control

Two controls supplied by the manufacturer were used when testing specimens. The negative control involved checking for the absence of latex agglutination with the glycine buffer provided, and the positive control was *Aspergillus fumigatus* galactomannan antigen, 75 ng/ml.

2.4.1.2 Platelia sandwich ELISA test

Platelia *Aspergillus* is a 1-stage immunoenzymatic sandwich microplate technique, allowing the detection of galactomannan in human serum. It uses the rat monoclonal antibody EB-A2, directed against *Aspergillus* galactomannan and characterised during previous studies (Stynen *et al.*, 1991; Stynen *et al.*, 1992a; Latge *et al.*, 1994). The monoclonal antibody is used to sensitise the wells of the microplate and to bind the antigen, as well as functioning as the detector (peroxidase-linked mAb).

The test has a detection limit of 1ng of galactomannan per ml of test serum. The principles of this test, based on the manufacturer's instructions are outlined below.

Sera were processed according to the manufacturer's instructions. Bronchoalveolar lavage (BAL) fluid samples were also processed in the same way although the test is not

validated for BAL specimens.

2.4.1.2.1 Treatment of sera and BAL fluid

The same treated samples as used in the Pastorex LA test were also used to perform the ELISA.

2.4.1.2.2 Reconstitution of reagents

2.4.1.2.2.1 *10-fold concentrated washing solution*

Tris NaCl pH 7.4 buffer containing 1% Tween 20 and 0.01% sodium merthiolate. The solution was diluted 10-fold in distilled water.

2.4.1.2.2.2 *Negative/threshold/positive control sera*

The content of one bottle was reconstituted with 1000 µl of sterile distilled water. This was mixed thoroughly after allowing 2-3 minutes for rehydration of the serum. The mixture was distributed into three aliquots of 300 µl into Eppendorf tubes. The two tubes that were not used the same day were frozen at –20°C until required.

The control sera had to be prepared just before the ELISA was performed, and required the same treatment as all the other clinical samples (300 µl of serum + 100 µl of treatment solution, etc.).

2.4.1.2.3 Procedure for the ELISA

The diluted washing solution was prepared. 50 µl of conjugate and 50 µl of treated serum or BAL fluid supernatant were successively dispensed into the wells. One positive, two weakly positive and a negative control serum were also tested with each batch of test samples. The microplate was covered with adhesive film which was pressed tightly over the entire surface to ensure watertightness, and then incubated in a waterbath for 90

minutes at 37°C.

Following incubation, the adhesive film was removed. The plates were washed five times by filling the wells with 370 µl of washing solution. The strips were dried by inverting them over a sheet of absorbent paper.

Working away from bright light, 200 µl of substrate-chromogen reaction solution was rapidly dispensed into each well. The reaction was allowed to develop in darkness for 30 ± 5 minutes at room temperature (18-25°C). Adhesive film was not used during this incubation.

The enzymatic reaction was stopped by adding 100 µl of stopping solution (1.5 N sulphuric acid) to each well, by using the same sequence of distribution as for the substrate solution.

The bottom of each plate was wiped thoroughly. The optical density was read at 450 nm by using a plate reader within 30 minutes of stopping the reaction.

2.4.1.2.4 Calculation and interpretation of results

2.4.1.2.4.1 *Calculation of the cut-off value*

The cut-off value corresponds to the mean optical density of the wells containing the threshold serum.

2.4.1.2.4.2 *Calculation of an index (I) for each test serum*

The following ratio is calculated for each test serum:

$$I = \frac{\text{Optical density of the sample}}{\text{Mean optical density of the threshold serum}}$$

This calculation limits the inter-test optical density variations due to the different ELISA test conditions (room temperature, washing method, etc.).

In this study the results were interpreted as follows:

Initial Method	*Revised Method*
≥ 1.5 : positive	≥ 0.5 : positive
≥ 1 < 1.5 : grey zone	
< 1 : negative	< 0.5 : negative

2.4.1.2.4.3 *Validation of the test*

Under normal test conditions, the control sera give the following results:

The optical density of the threshold serum : ≥ 0.3 ≤ 0.8

The index of the positive control serum: > 2.0

$$\text{i.e. } I = \frac{\text{OD positive control}}{\text{OD of threshold serum}} > 2.0$$

Initial Method	*Revised Method*
The index of the negative control serum: < 0.5	The index of the negative control serum: < 0.4
$\text{i.e. } I = \frac{\text{OD negative control}}{\text{OD of threshold serum}} < 0.5$	$\text{i.e. } I = \frac{\text{OD negative control}}{\text{OD of threshold serum}} < 0.4$

2.4.1.2.4.4 *Interpretation of the results*

2.4.1.2.4.4.1 *Initial methodology*

The cut-off value (index of 1), corresponding to 1 ng of galactomannan per ml of serum, was determined from the results of internal and external evaluations (Stynen *et al.*, 1995; Verweij *et al.*, 1995b; Sulahian *et al.*, 1996).

Whenever a sample had an index >1, positivity was confirmed by re-testing the same sample (the treatment process was again carried out) and by testing another sample

obtained from the patient. This confirmation was necessary in order to eliminate any false-positive results due to contamination of the sample after collection.

2.4.1.2.4.4.2 *Revised methodology*

The presence or absence of galactomannan antigen in the test sample is determined by calculation of an index for each patient specimen. Sera with an index < 0.5 are considered to be negative for galactomannan antigen. Sera with an index ≥ 0.5 are considered to be positive for galactomannan antigen. Positive results obtained should be considered in conjunction with other diagnostic procedures such as microbiological culture, histological examination of biopsy samples and radiographic evidence. For all positive patients, it is recommended that a new aliquot of the same sample be repeated as well as collection of a new sample from the patient for follow-up testing.

2.4.2 Polymerase chain reaction

Molecular techniques have revolutionised the diagnosis and understanding of microbial infections. Numerous in-house PCR assays have been developed to diagnose Aspergillus infections in the past decade (Tang *et al.*, 1993; Verweij *et al.*, 1995c; Einsele *et al.*, 1997; Skladny *et al.*, 1999; van Burik *et al.*, 1998). One such method was evaluated in this study (Einsele *et al.*, 1997); this assay was performed on blood specimens and involves the amplification of a highly conserved sequence of the multicopy 18s rRNA gene.

2.4.2.1 DNA extraction

The method described by Tokimatsu *et al.* (1995) was used to extract the fungal DNA. Briefly, 100 µl of test serum sample was mixed with 100 µl lysis buffer [100 mM KCl, 20 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.2 mg of gelatin per ml, and 0.9% polysorbate

20 (Tween 20) solution]. Proteinase K was added to a final concentration of 60 μg/ml. The mixture was then incubated for one hour in a waterbath at 55°C, and the proteinase K was inactivated by heating the mixture to 95°C for 10 minutes. Following centrifugation at 12 000 × *g* for 10 min at 4°C, the supernatant was used for PCR amplification.

Controls were incorporated into each run of DNA extraction; 100 μl of serum spiked with 500fg of Aspergillus DNA was the positive control, and 100 μl of serum from a healthy volunteer was the negative control.

2.4.2.2 Polymerase chain reaction

2.4.2.2.1 Amplification

Amplication reactions were performed in a 100 μl volume [containing 10 mM Tris (pH 9.6)], 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mmol of dNTPs per liter,100 pmol of forward and reverse primers, 1.5U of Taq polymerase (Bioline, UK) and template DNA. Using a Biomed thermocycler, 35 cycles of repeated denaturation, primer annealing and enzymatic chain extension was carried out on the reaction mixture.

Positive and negative controls were tested in each run. The positive control for the PCR assay was 100fg of genomic DNA (Aspergillus *fumigatus* ATCC 7100) and the negative control was molecular grade water.

2.4.2.2.2 Validation of assay

To validate and determine the sensitivity of the PCR assay developed by Einsele *et al.*, (1997), various concentrations (10fg, 100fg, 1000fg[1pg], 10pg, 100pg) of genomic DNA of *Aspergillus fumigatus* (ATCC 7100) were amplified using the panfungal primers (refer Appendix 7-2).

The lowest concentration of genomic DNA reliably amplified by the PCR was found to be 100fg. The Southern blot increased the sensitivity 100-fold and therefore lowered the detection limit to 1fg. The probe used in the study was the probe hybridising sequences of *Aspergillus fumigatus*, *A. flavus* and *A. versicolor* (Appendix 7-2).

2.4.2.2.3 Detection of amplified products

The PCR products were electrophoretically separated in a 1.8% agarose gel (containing ethidium bromide) in 1 x TAE buffer (pH 8.0; 40mM Tris-acetate [pH 7.5], 2mM EDTA). The amplicons were transferred onto a nylon membrane by southern blotting.

2.4.2.2.4 Transfer of DNA

The gel was trimmed and the bottom left hand corner snipped off before being placed in a clean bioassay dish. Depurination with 100ml of 0.2 N HCl was performed for 10 minutes. The gel was rinsed three times with sterile distilled water. Denaturation with 1.5 M NaCl, and 0.5 N NaOH was followed by neutralisation with 1 M Tris (pH 7.4), and 1.5 M NaCl, each for 45 minutes.

The gel was then inverted onto a clean sheet of 3MM (prewet with 10 x SSC) in a tank was filled with transfer buffer (10 x SSC). A Hybond N nylon membrane, the same size as the gel, was placed on the gel. Sheets of 3MM (wet with 2 x SSC) and blotting paper (Quickdraw) were placed on top, followed by a 500g weight. The DNA was left to transfer for 2 hours.

After 2 hours the sheets were removed and the membrane (blot) separated from the gel. The blot was then rinsed in 6 x SSC (30 ml of 20 x SSC and 70 ml of water), and left to air dry in a 3MM envelope. DNA was fixed to the membrane by uv cross linking (DNA

side down) for 2.5 to 3 minutes.

2.4.2.2.5 Radioactive DNA probe labelling

To 15.5 µl of sterile water, 1 µl of DNA (ie. 20 - 40 ng of probe) was added, mixed well and spun when necessary. The mixture was boiled for 7 minutes to separate the dsDNA, pulse centrifuged, and held in ice for 5 minutes to prevent re-annealing. Five µl of labelling mix (contains dinucleotides except for dCTP and oligoprimers) was then added, followed by 1 µl of enzyme (Klenow). The labelling mix was thawed at 37°C for 5 minutes. In the hot room, 2.5 µl of hot [^{32}P]dCTP was added (should be stored in –50°C fridge, and ideally be less than a week old) and incubated for 2 hours at room temperature. Following incubation, 75 µl of water was added to a final volume of 100 µl. Finally, unincorporated nucleotides were removed by spinning in a sephadex G 50 column for 2 minutes at 1800. The probe was collected in an eppendorf tube.

2.4.2.2.6 Hybridisation

The dried filter paper was transferred into a dry plastic bag. Fifteen millilitres of prehybridisation solution was introduced (this solution contains salmon DNA which binds to the filter and prevent non-specific binding of the probe). The bag was heat sealed and incubated in a shaking water bath for 1-6 hours at 65°C. To the labelled probe 400 µl of water, 50 µl of carrier DNA, and 50 µl of 1 M Tris was added, and the mixture boiled for 10 minutes. The probe was then cooled in ice for 5 minutes.

After cooling, the probe was added to 20 ml of hybridisation mixture in a 50 ml Falcon tube. One corner of the bag was cut off and the prehybridisation solution poured off. The hybridisation solution containing the boiled probe was added to the bag. The bag was then re-sealed, and the contents mixed. Overnight incubation of the bag in a shaking

water bath at 65°C was performed.

2.4.2.2.7 Washing

Five hundred microlitre of washing solution (0.2 x SCC and 0.1% SDS) that had been prewarmed to 65°C was added to a sandwich box. After gentle agitation the washing solution was poured off and refilled with fresh prewarmed wash. The filter was then placed in a shaking water bath at 65°C for 45 minutes.

2.4.2.2.8 Autoradiography

The filter was dried on 3MM Whatman paper and wrapped in clingfilm. The filter was placed in a cassette with X-ray film (at -70°C) for 24 hours.

2.4.3 High resolution CT scanning

In order to grade infections accurately, high resolution CT scanning with thin slices is necessary (Graham *et al.*, 1991). The 'halo' sign, an area of low attenuation around a nodule or area of consolidation, although seen with other infections, is virtually pathognomonic of IPA in the clinical setting of an immunocompromised patient undergoing chemotherapy or following BMT (Herbert and Bayer, 1980; Kuhlman *et al.*, 1985; Caillot *et al.*, 1997). This characteristic sign was present in patient 3 (Figure 3-2), who is one of the case reports discussed in Chapter 4, to illustrate the combined use of techniques.

Cavitation, characteristically as an air crescent (Figure 3-3), though less frequent is also diagnostic (Orr *et al.*, 1978; Curtis *et al.*, 1979; Herbert and Bayer, 1980; Kuhlman *et al.*, 1985). Cavitary lesions (Figure 3-4) appear to be a later stage of development, representing necrosis within the lesion. It is usually seen when the bone marrow is

recovering, although it may also be seen in solid organ transplant recipients and lymphoma patients with this disease.

Other features that are suggestive of IPA are the presence of single or multiple pulmonary lesions which are wedge-shaped and pleurally-based (Figure 3-5), with or without cavitation, or nodular areas of consolidation, often related to blood vessels (Kuhlman *et al.*, 1985; Graham *et al.*, 1991; Denning *et al.*, 1997).

Early recognition of these lesions contributes to more prompt initiation of antifungal therapy appropriate for pulmonary aspergillosis (Aisner *et al.*, 1977; Kuhlman *et al.*, 1987). However, other infective causes include other fungi, such as the Mucorales, as well as atypical mycobacteria, *Nocardia* spp., and *Staphylococcus aureus*.

In about 30% of patients, the pulmonary infiltrates are solely peribronchial or peribronchiolar, and indicate an air-borne infection, without evidence of blood vessel involvement. Initially the features, though suggestive of IPA may not be sufficiently clear-cut to make the diagnosis. In such cases a follow-up CT scan is essential and will often be diagnostic (Berger, 1998).

CT scanning has been used at the Royal Free Hospital since 1988 to aid in the diagnosis of IPA, and the use of high resolution CT scanning was first introduced in 1995. Both thick slices (10mm) and additional 1mm thin slices through any suspected fungal lesions are routinely performed (Berger, 1998).

An experienced radiologist (LAB) independently and annonymously reviewed all the thoracic CT scans for evidence of IPA. CT scans of the paranasal sinuses, head, and/or abdomen were also reviewed where relevant.

2.5 ITRACONAZOLE IN-VITRO STUDY

Prevention of severe fungal infections should be a high priority in the management of all at risk patients such as neutropenic, BMT or solid organ transplant recipients. In several prospective studies that assessed the performance of antigen detection, patients received antifungal prophylaxis with itraconazole (Maertens *et al.*, 2001; Becker *et al.*, 2003). This prophylaxis may have a significant effect on the sensitivity of the assay and requires careful attention in evaluating the ELISA test.

Since mid-January 1998, we have used itraconazole solution 200 mg bd as antifungal prophylaxis in neutropenic patients with haematological malignancies and in BMT recipients. Hydroxy-itraconazole is the major metabolite and is present in greater concentration than itraconazole in the steady state.

Since using itraconazole, the vast majority of serum samples from these patients have been negative for galactomannan by sandwich ELISA. Because of this, we decided to perform an in-vitro study to establish the effect, if any, of itraconazole and hydroxy-itraconazole on serum galactomannan levels when assayed using the Platelia *Aspergillus* kit (Bio-Rad).

2.5.1 Methodology

Aliquots of serum with 5.9 ng/ml of GM were inoculated with the following concentrations of itraconazole: 500ng, 200ng, 100ng and 50ng. The 5.9 ng/ml concentration of GM was used as it was available in freeze dried pellets from the Platelia Aspergillus kit.

All tubes were incubated at 37°C aerobically. At 0, 24 and 48 hours, 150 µl of each test solution was aspirated, and stored at 4°C until tested. The sandwich

ELISA (Platelia *Aspergillus*) was used to detect the GM in the samples.

Briefly, 150 µl of a test sample was mixed with 50 µl of treatment solution and boiled for 3 minutes. After centrifugation, 50 µl of the test supernatant was added to 50 µl of conjugate in a well of the microtitre plate coated with antigalactomannan immunoglobulin (EB-A2). One positive, 2 weakly positives and a negative control were also tested with each batch of test samples.

The plates were washed thoroughly after 90 minutes incubation in a water bath at 37°C, and 200 µl of substrate buffer containing ortho-phenylenediamine hydrochloride were added to each well. The reaction was allowed to develop at room temperature in darkness for 30 minutes. The reaction mixtures were stopped with 100 µl of 1.5 N sulphuric acid.

The optical densities were measured at 450 nm using a plate reader, and the index of each sample was calculated. The ratio between the optical density of the weakly positive samples and each test sample was calculated, and an index of > 1.5 was considered positive as recommended by the manufacturer.

This is not the only way to assess effect. Further discussion of different methodologies can be found in chapter 5.

2.6 STATISTICAL ANALYSIS

The objective of this study was to assess the diagnostic potential of galactomannan, high resolution CT scanning and PCR in a cohort of adult and paediatric haematology patients at high risk for IPA. But, because the histopathologic confirmation of fungal tissue invasion is still considered the reference diagnostic test, the true status of disease of many study patients remains unknown in the absence of tissue specimens (Kaufman *et al.*, 1997). In this setting, the sensitivity and specificity of any noninvasive diagnostic test

remain ill-defined and may dramatically be influenced by the proposed case definitions (Ilstrup, 1990). Therefore, several different estimates were used to calculate sensitivity, specificity and predictive values from 2 x 2 tables (Horvath and Dummer, 1996).

For example, PPVs were calculated using different estimates for the number of true-positive results (numerator) and number of true- plus false-positive results (denominator). Method A assumes that all positive results from proven and probable cases are true-positive results, while those from possible and uninfected cases are false-positive results. Method B calculates the PPVs using only those results that are known with certainty to be true-positive results (proven cases) or false-positive results (uninfected cases). Methods C and D provide extreme estimates of PPVs, assuming that all probable and possible cases have either true-positive results (method C) or false-positive results (method D). It is likely that the actual performance of the tests almost certainly lies between method B and method C, and that these estimates are the most accurate for clinical practice.

CHAPTER 3

RESULTS

3.1 RETROSPECTIVE STUDY

3.1.1 Patient recruitment and categorisation

CT thorax was routinely performed on all febrile neutropenic or BMT patients 72 hours after onset of fever that was unresponsive to first or second line antimicrobial therapy, at the time when AmBisome 1mg/kg/day would have been started. CT thorax was performed irrespective of whether or not the patient had pulmonary symptoms or signs.

As mentioned previously, all patients in the study had had a BAL performed as this was a criterion for inclusion into the study. The criterion was included because we wanted to assess the usefulness of BAL in the early diagnosis of IPA, taking into consideration that many patients are pancytopenic and prone to bleeding diastheses.

Thirty eight BAL and 178 serum samples were collected from 38 consecutive febrile neutropenic and BMT patients receiving oral fluconazole (100 mg/day) and amphotericin B (500 mg qds) as antifungal prophylaxis. In 37 patients with respiratory signs or symptoms, a bronchoscopy was performed because pulmonary infiltrates were found on CT scan and fever (temperature >38°C) persisted for greater than 72 hours despite broad-spectrum antimicrobial treatment. However, in one patient a bronchoscopy was performed because of progressively worsening dyspnoea and hypoxia despite a normal thoracic CT scan.

The characteristics of the 38 patients with haematological malignancies are shown in Table 3-1.

Table 3-1. Characteristics of 38 patients with haematological malignancies and results of analysis of bronchoalveolar lavage (BAL) fluid and serum samples.

	Proven and probable IPA	Possible IPA	No fungal infection
Number of patients	7	13	18
Sex M/F	4/3	6/7	9/9
Mean age, years (range)	34 (16 - 63)	33 (13 - 76)	30 (16 - 52)
Underlying diseases			
ALL	4	1	4
AML	1	7	8
Other	2	5	6
Allogeneic bone marrow transplant	3	8	9
Autologous bone marrow transplant	1	1	4
Galactomannan positive patients			
ELISA (BAL/Serum)	5/6	4/4	0/0
LA (BAL/Serum)	2/2	2/2	0/0
PCR positive patients (BAL/serum)	7/7	6/4	4/0
CT scan features of IPA	7	4	0
Fungal culture	Aspergillus fumigatus: 2 Aspergillus flavus: 3		

IPA	Invasive pulmonary aspergillosis
ELISA	Enzyme linked immunosorbent assay
LA	Pastorex latex agglutination test
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia

3.1.2 Galactomannan detection methods

3.1.2.1 Pastorex latex agglutination test

In the 5 patients with histologically proven aspergillosis, only two of the five BALs were positive with the LA test. Sera from both patients were also positive. Serial serum samples remained consistently negative in the other three patients. The BAL from both patients with probable IPA were negative, as were serial serum samples.

Of the 13 patients with possible IPA, 2 patients tested positive by LA in both BAL and serum samples. No false positive results occurred in BAL fluid or serum samples from patients in the negative or control group using this assay.

3.1.2.2 Platelia sandwich ELISA test

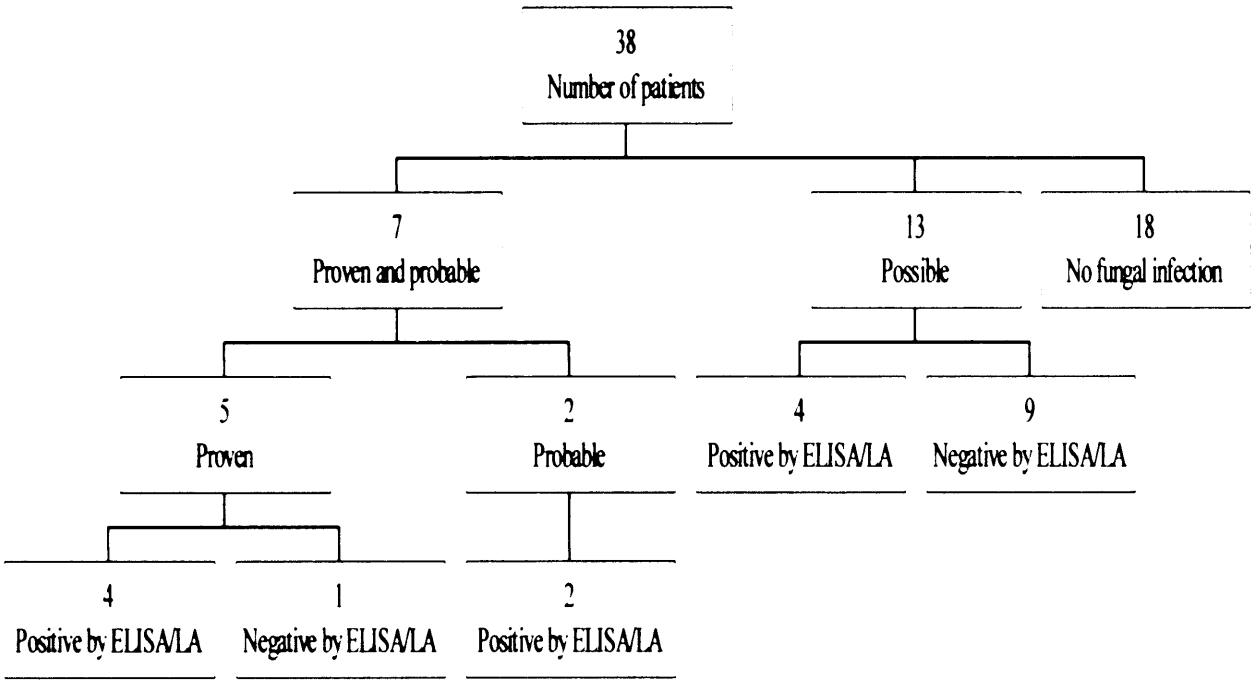
In the five patients with histologically proven aspergillosis, four BAL samples tested positive for galactomannan by ELISA. Sera from the same four patients were also positive. In patient 5 however, galactomannan titres in both BAL and serial serum samples remained persistently negative.

The BAL from only one of the two patients with probable IPA tested positive with the ELISA. However, serial serum samples gave positive results for both patients.

Of the 13 patients with possible IPA, four BAL samples tested positive by ELISA. Serum samples from these four patients were also positive by ELISA. There were no false positive results amongst patients in the negative or control group.

Figure 3-1 is a tree diagram showing the categorisation of the 38 patients with haematological malignancies and overview of results of the Pastorex LA and sandwich ELISA tests in sera, BAL fluid or both.

Figure 3-1. Tree diagram showing the categorisation of the 38 patients with haematological malignancies and overview of results of the Pastorex LA and sandwich ELISA tests in either sera, BAL fluid or both.



3.1.3 Polymerase chain reaction

All 5 patients with histologically proven IPA were PCR positive in both BAL and serial serum samples. Likewise, Aspergillus DNA was detected in both BAL and serial serum samples in the two patients with probable IPA.

Four of the 13 patients with possible IPA had serial serum and BAL samples that tested positive by PCR. These were the same 4 patients with positive ELISA antigenaemia results. A further 2 patients were PCR positive in BAL fluid, but serial serum samples remained negative.

Amongst patients in the negative group, four had a positive PCR result in BAL fluid, but as with the 2 patients in the possible group, serial serum samples remained negative.

3.1.4 High resolution CT scanning

All patients with histologically proven IPA had thoracic CT scan evidence of IPA. Only one of the 2 patients with probable IPA had a positive thoracic CT scan at initial presentation. This case will be discussed further later in this chapter. Four of the thirteen patients in the possible group had positive thoracic CT scan features suggestive of IPA. These were the same 4 patients with positive antigenaemia and PCR results. There were no positive CT scan results amongst patients in the negative group.

Table 3-2 illustrates the thoracic CT scan features of IPA present prior to BAL in patients with proven, probable or possible disease. Figures 3-2, 3-3, 3-4 and 3-5 illustrate thoracic CT signs that were present in patients 3, 8, 2 and 11 respectively.

3.1.5 Comparative analysis of results

The results of analysis of BAL fluid and serum samples are shown in Table 3-1. Among the 70 patients evaluated (which included the negative controls), 17 cases (5/5 proven, 2/2 probable, 6/13 possible, 4/50 negative) gave positive results, either by thoracic CT, sandwich ELISA, or PCR, or a combination of methods (Table 3-3a and 3-3b). In the 5 patients with histologically proven aspergillosis, 4 BAL samples tested positive for GM by ELISA but only 2 of 5 with the LA. Sera from these 4 patients were positive by ELISA, and from 2 of these by LA (same 2 who were LA positive in BAL fluid). Thoracic CT scans of patient 5 demonstrated both the halo and air crescent signs, features which are highly characteristic of IPA. Galactomannan titres however remained persistently negative in both BAL and serial serum samples. All 5 patients were PCR positive in both BAL and serial serum samples.

Table 3-2. Thoracic CT scan features of IPA present prior to BAL in patients with proven, probable or possible* disease.

Patient number	Halo sign	Air crescent sign or cavitation	Wedge shaped pleurally based infiltrates
1	+	-	-
2	-	+	-
3	+	-	-
4	+	-	-
5	+	+	-
6	-	-	-
7	+	+	-
8	-	+	+
9	+	-	-
10	+	+	+
11	-	-	+
12	-	-	-
13	-	-	-

*Patients with possible IPA were only included if they were positive using one or more of the diagnostic techniques under evaluation

Figure 3-2. The characteristic CT 'halo' sign of IPA in the left lower lobe of patient 3. This patient will be discussed later in Chapter 4.

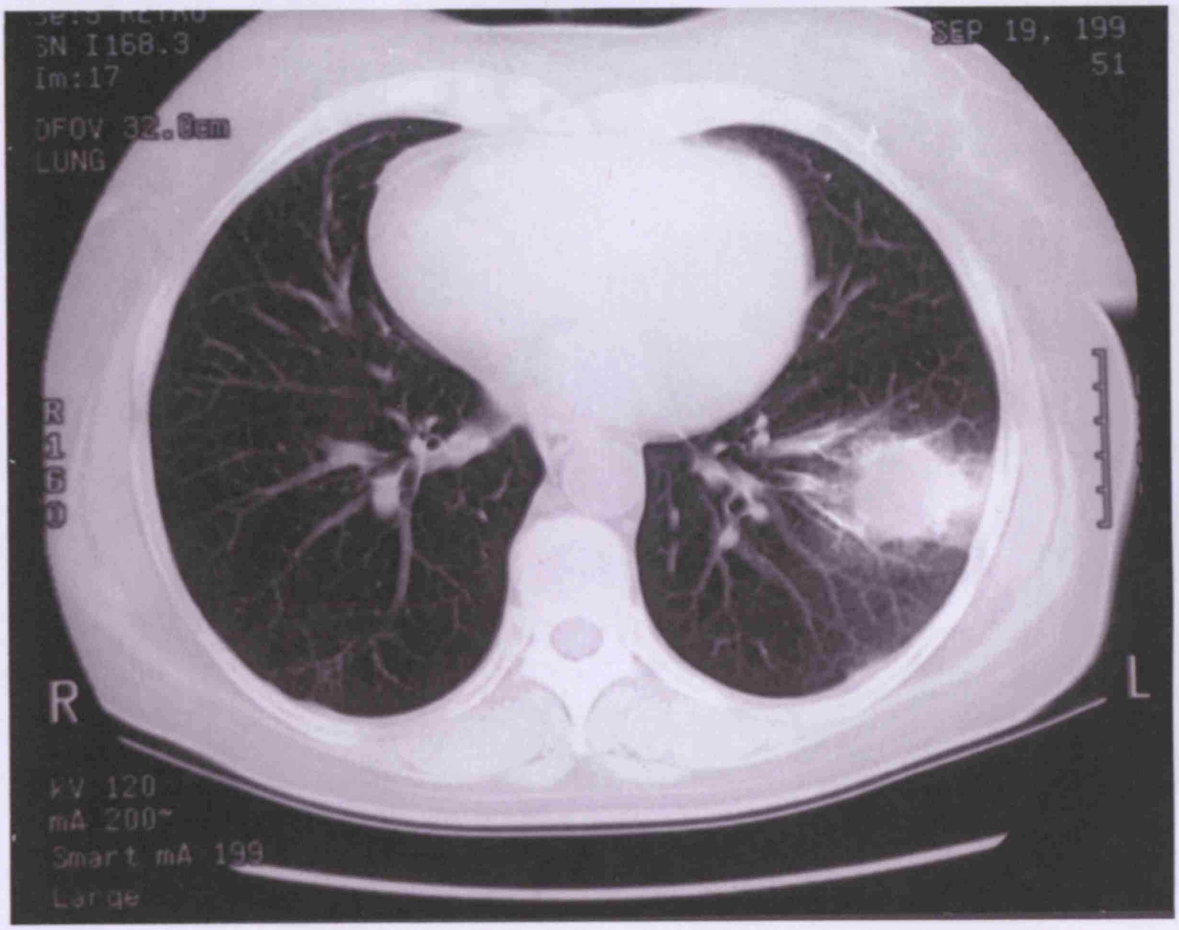


Figure 3-3. Cavitating lesion on CT thorax with the characteristic air-crescent sign present in patient 8.

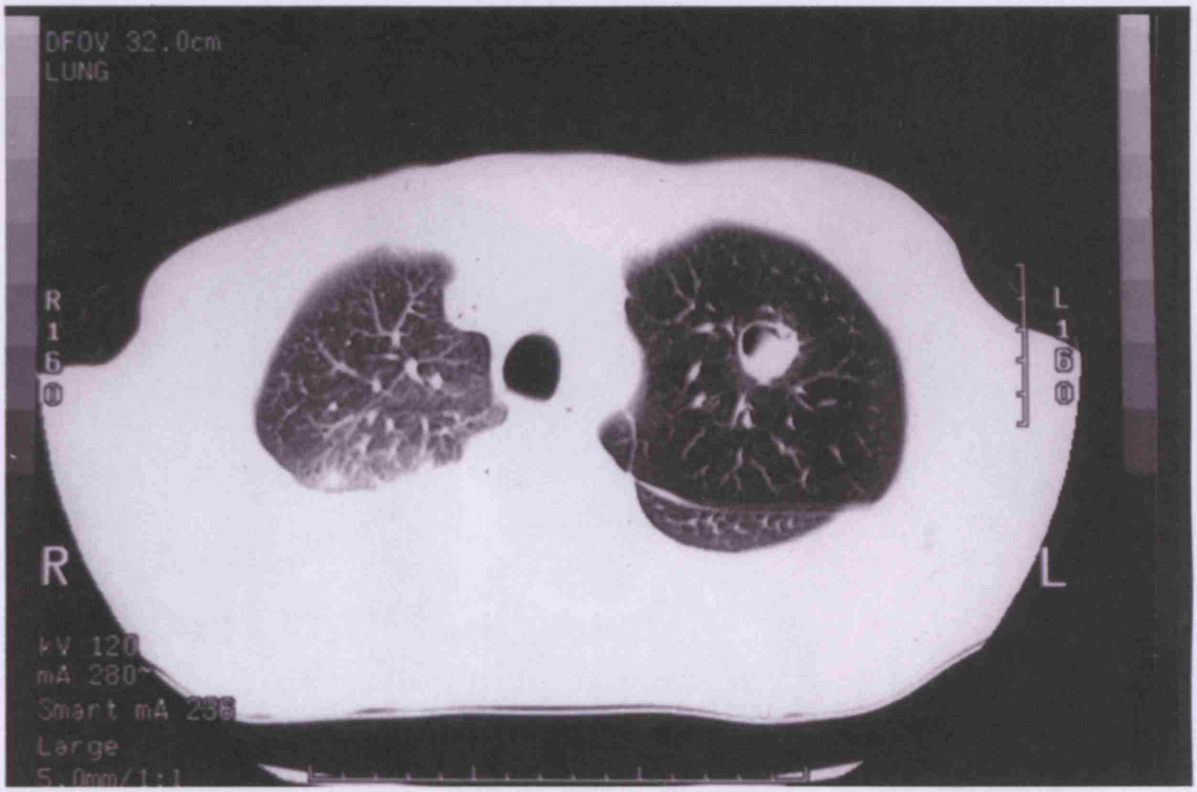


Figure 3-4. A cavitating lesion at a later stage of development that was present in patient 2.

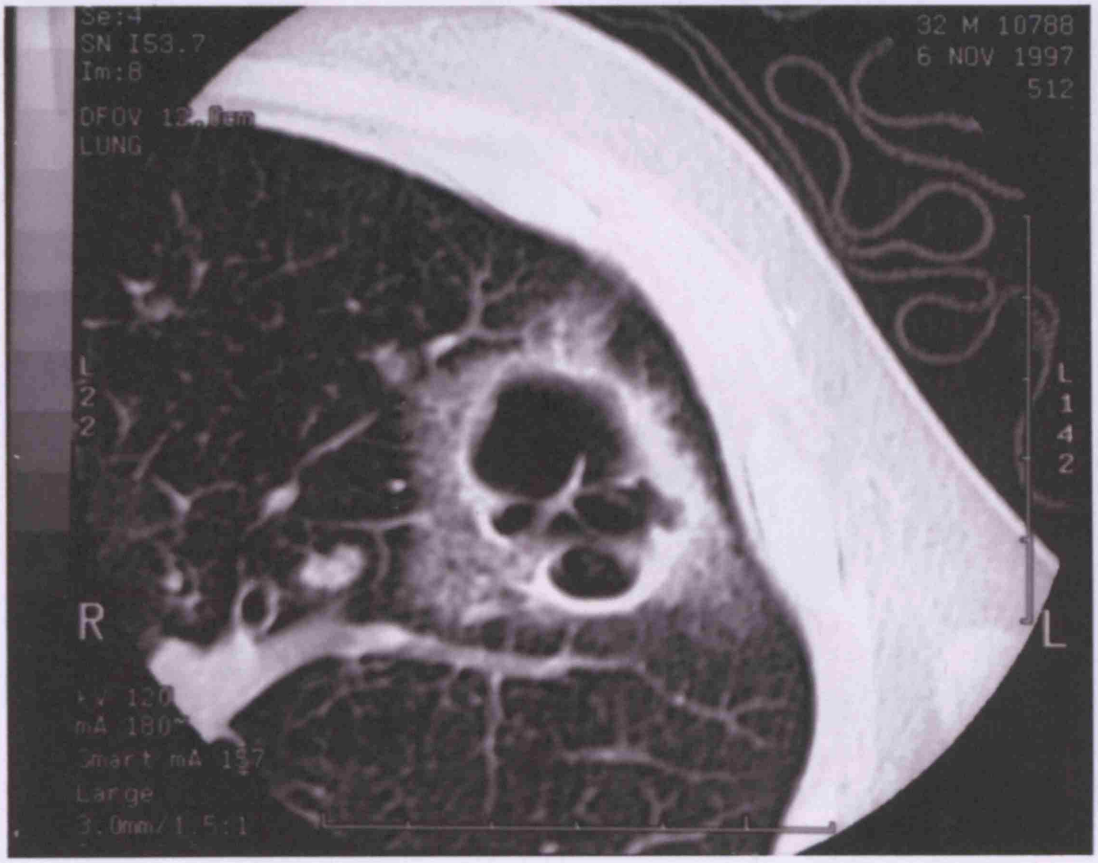


Figure 3-5. CT thorax demonstrating wedge-shaped pleurally-based infiltrates in patient 11.

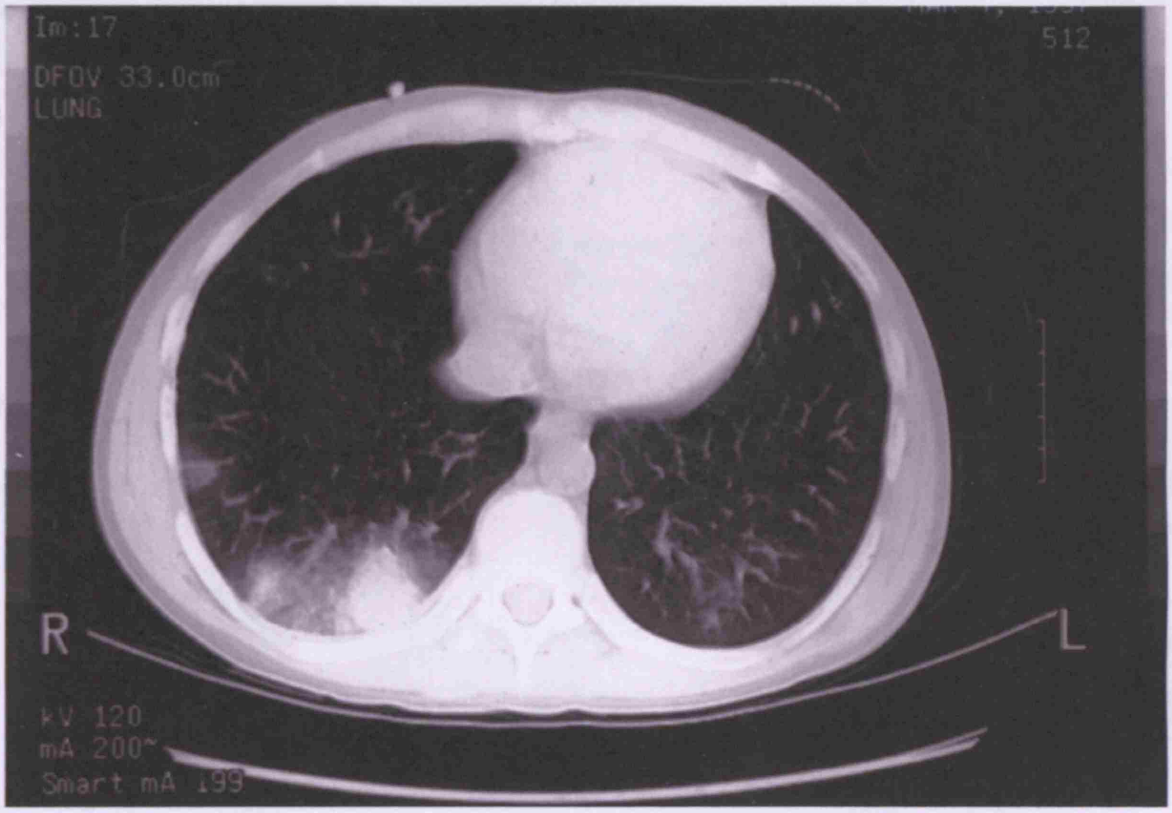


Table 3-3a. Clinical characteristics, radiological findings, mycological data, bronchoalveolar lavage (BAL) and serum analysis of patients with proven, probable or possible# invasive pulmonary aspergillosis (IPA)

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13
Sex/Age	F, 51	M, 28	F, 18	M, 33	F, 16	M, 28	M, 63	M, 26	F, 76	M, 32	M, 33	M, 51	F, 18
Underlying diagnosis	AML	ALL	ALL	MDS	ALL	ALL	Hairy cell leukaemia	AML	AML	ALL	AML	AML	MDS
Bone marrow transplant	Autologous	Allogeneic	No	Allogeneic	No	Allogeneic	No	Allogeneic	No	No	Allogeneic	Autologous	Autologous
Aspergillus infection	Proven	Proven	Proven	Proven	Proven	Probable¥	Probable	Possible	Possible	Possible	Possible	Possible	Possible
Thoracic CT scan features of IPA*													
CT halo sign	+	-	+	+	+	-Ψ	+	-	+	+	-	-	-
CT air-crescent sign or cavitation	-	+	-	-	+	-	+	+	-	+	-	-	-
Wedge-shaped pleurally based infiltrates	-	-	-	-	-	-	-	+	-	+	+	-	-
ANC when CT scan performed	0.2	0.1	0.0	0.0	0.1	0.1	0.8	0.1	0.3	0.0	0.0	0.3	0.0
Respiratory tract cultures	-	-	*A. flavus*	*A. flavus*	*A. fumigatus*	*A. fumigatus*	*A. flavus*	-	-	-	-	-	-
BAL fluid analysis													
ELISA	+	+	+	+	+(+)	-	+	+	+	+	+	-	-
Latex agglutination	+	-	+	-	-	-	-	-	-	+	+	-	-
PCR	+	+	+	+	+	+	+	+	+	-	+	+	+
Number of serum samples tested	5	4	27	66	20	8	3	4	6	3	4	8	5
Number positive by ELISA	3	2	16	9	0 (4)	5	2	3	4	2	3	0	0
Number positive by LA	1	0	8	0	0	0	0	1	0	0	1	0	0
Number positive by PCR	3	4	20	38	4	2	1	4	4	3	4	0	0
Time (days) between positive BAL & first positive serum by ELISA	-2	+8	-42	+3	NA (+2)	NA	-5	0	+4	-1	-12	NA	NA
Time (days) between positive BAL & first positive serum by PCR	-2	+6	-36	+2	+3	+8	-7	0	+5	-1	-14	NA	NA
Time (days) between antigen positivity and first positive CT scan	-1	-1	+37	-13	NA (-1)	+6	-1	-2	-3	-2	+8	NA	NA
Time (days) between PCR positivity and first positive CT scan	-1	-3	+31	-14	-4	+14	-3	-2	-2	-2	+6	NA	NA
Recurrence of IPA	No	No	Yes¥	Yes¥	No	No	No	No	No	No	No	No	No
Outcome(1 year)	Survived	Deceased	Deceased	Deceased	Deceased	Deceased	Deceased	Deceased	Survived	Deceased	Deceased	Deceased	Deceased

Patients in the possible group were only included if they had a positive result using one or more of the diagnostic techniques under evaluation. * Features present prior to BAL. Ψ When BAL was performed thoracic CT was normal. Twenty days later respiratory tract cultures were positive. ¥ These 2 patients are discussed in the results section. ANC Absolute neutrophil count. NA Not Applicable

( ) result obtained using revised index factor ≥ 0.5

Table 3-3b. Clinical characteristics, radiological findings, mycological data, bronchoalveolar lavage (BAL) and serum analysis of patients with no evidence of invasive pulmonary aspergillosis[#] (IPA).

Patient	14	15	16	17
Sex Age	M, 38	M, 28	F, 20	M, 26
Underlying diagnosis	CML	NHL	AML	CML
Bone marrow transplant	Allogeneic	Autologous	No	Allogeneic
Aspergillus infection	No	No	No	No
Thoracic CT scan features of IPA*				
CT halo sign	-	-	-	-
CT air-crescent sign or cavitation	-	-	-	-
Wedge-shaped pleurally based infiltrates	-	-	-	-
ANC when CT scan performed	4 5	0 4	0 0	0 1
Respiratory tract cultures	-	-	-	-
BAL fluid analysis				
ELISA	-	-	-	-
Latex agglutination	-	-	-	-
PCR	-	-	-	-
Number of serum samples tested	4	3	6	4
Number positive by ELISA	0	0	0	0
Number positive by LA	0	0	0	0
Number positive by PCR	0	0	0	0
Time (days) between positive BAL & first positive serum by ELISA	NA	NA	NA	NA
Time (days) between positive BAL & first positive serum by PCR	NA	NA	NA	NA
Time (days) between antigen positivity and first positive CT scan	NA	NA	NA	NA
Time (days) between PCR positivity and first positive CT scan	NA	NA	NA	NA
Recurrence of IPA	No	No	No	No
Outcome(1 year)	Deceased	Deceased	Deceased	Deceased

Patients with no evidence of IPA were included only if they had a positive result using one or more of the diagnostic techniques under evaluation.

* Features present prior to BAL

ANC Absolute neutrophil count

NA Not Applicable

The BAL from one of the 2 patients with probable IPA tested positive with the ELISA, but both were negative by LA. However, serial serum samples gave positive results for both patients by ELISA but not by LA. In patient no.6, in whom a bronchoscopy was performed because of clinical deterioration despite a normal thoracic CT, galactomannan antigen was not detected in the BAL fluid. Twenty days after bronchoscopy and BAL, when repeat thoracic CT showed the presence of pulmonary infiltrates which were typical of IPA, *Aspergillus fumigatus* was isolated from sputum and endotracheal secretions. Serial serum samples during the intervening time were positive for galactomannan by ELISA. Aspergillus DNA was detected in
both the BAL fluid and serial serum samples.

Of the 13 patients with possible IPA, 4 BAL samples tested positive by ELISA and 2 of the same 4 by LA. Serum samples from these 4 patients were also positive by ELISA and from 2 of these by LA. Serial serum and BAL samples in the same 4 patients with positive ELISA results were PCR positive. These same four patients had positive thoracic CT scan features suggestive of IPA. A further 2 patients were PCR positive in BAL fluid, but serial serum samples remained negative.

Apart from patients 5 and 6, the other nine patients (4 proven, 1 probable, 4 possible) were all galactomannan positive in both BAL and serial serum samples and had CT scans which were highly suggestive of IPA.

As mentioned previously, amongst patients in the negative group, four had a positive PCR result in BAL fluid, but as with the 2 patients in the possible group, serial serum samples remained negative. No false positive results occurred in BAL fluid or serum samples from patients in the negative or control groups using the antigen detection assays. There were no positive CT scan results amongst patients in the negative group.

The clinical characteristics, mycological data, BAL and serum analysis of thirteen

patients with proven, probable or possible IPA who were positive by one or more of the tests under investigation are detailed in Table 3-3a. All but three patients (patients 5, 12 and 13) had detectable galactomannan levels within 72 hours following the CT scan performed at the onset of illness.

The results of CT scanning, and galactomannan and PCR analysis of BAL fluid and serum samples are shown in Figure 3-6. Galactomannan was detected in BAL fluid from 9 patients using the sandwich ELISA, but only from 4 patients using the Pastorex LA test. In four of the 9 ELISA positive BAL samples, the BAL ELISA was positive at or before the serum ELISA. In the PCR positive cases, Aspergillus DNA was detected in BAL fluid a mean of 4 days prior to detection in serum.

In three patients, serum GM results were positive prior to thoracic CT scanning or initiation of empirical antifungal therapy (Table 3-3a). Likewise, serum PCR results were positive for the same three patients prior to initiation of antifungal therapy. For these three patients, analysis of serum GM and PCR results could have been used to initiate pre-emptive therapy.

3.1.6 Statistical analysis

Table 3-4 demonstrates the statistical analysis performed by comparing thoracic CT, sandwich ELISA and PCR tests, using different definitions of true positive and true negative results (Horvath and Dummer, 1996).

3.1.6.1 Use of NIAID definitions (Denning *et al.*, 1994), with a galactomannan index ≥ 1.5

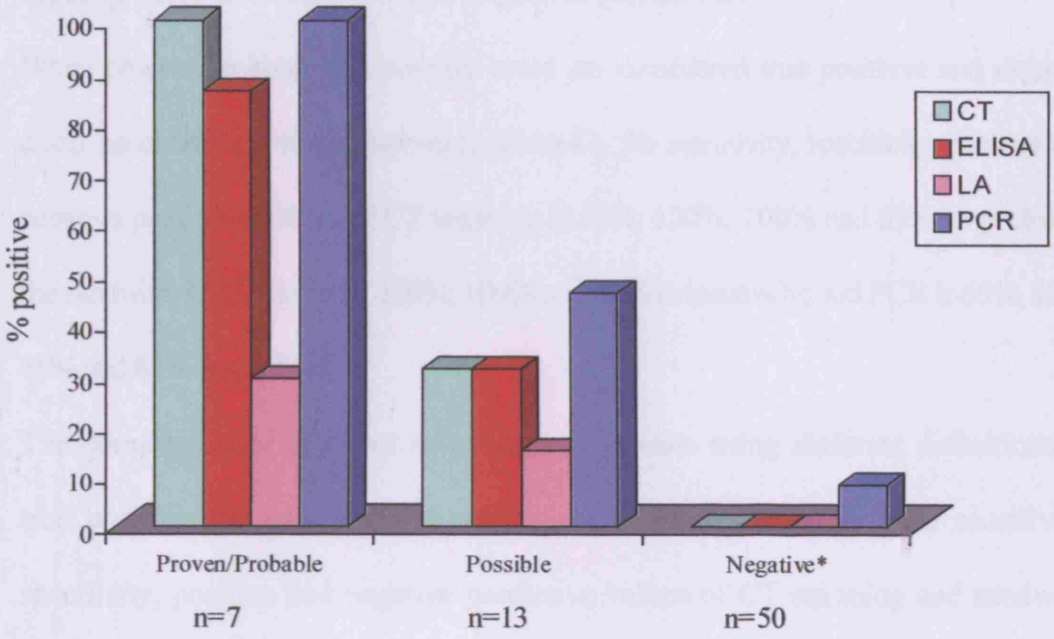
The sensitivity, specificity, positive and negative predictive values of CT scanning is 100%, 100%, 100% and 100% respectively; the sandwich ELISA is 80%,

Table 3-4. Statistical analysis 1. Retrospective study. Comparison of thoracic CT, sandwich ELISA and PCR tests using different definitions of true positive and true negative results.

Statistical analysis	A				B				C				D			
	CT	GM		PCR	CT	GM		PCR	CT	GM		PCR	CT	GM		PCR
		I ≥1.5	I ≥0.5*			I ≥1.5	I ≥ 0.5			I ≥1.5	I ≥0.5			I ≥1.5	I ≥0.5	
Sensitivity	86	86	100	100	100	80	100	100	50	50	55	65	100	80	100	100
Specificity	87	94	100	84	100	100	100	92	100	100	100	92	85	91	91	82
PV Pos	60	60	100	41	100	100	100	56	100	100	100	76	50	40	45	30
PV Neg	96	98	100	100	100	98	100	100	64	83	85	88	100	98	100	100

A. TP = Proven + probable groups (n = 7); TN = Possible + negative groups (n = 63).
B. TP = Proven group only (n = 5); TN = Negative group only (n = 50).
C. TP = Proven + probable + possible groups (n = 20); TN = Negative group only (n = 50).
D. TP = Proven group only (n = 5); TN = Probable + possible + negative groups (n = 65).
TP: True positive
TN: True negative
* Includes EORTC-MSG consensus definitions: A. TP = Proven + probable groups (n = 11); TN = Possible + negative groups (n = 59).

Figure 3-6. Results of CT, galactomannan and PCR analysis.



* Including 32 immunocompetent and asymptomatic HIV positive controls

100%, 100% and 98% respectively; and PCR is 100%, 92%, 56% and 100% respectively, when only proven cases are considered true positives and only negative cases are considered true negatives (column B).

When proven, probable and possible cases are considered true positives and negative cases are considered true negatives (column C), the sensitivity, specificity, positive and negative predictive values of CT scanning is 50%, 100%, 100% and 83% respectively; the sandwich ELISA is 50%, 100%, 100% and 83% respectively; and PCR is 65%, 92%, 76% and 88% respectively.

The comparison of different combinations of tests using different definitions of true positive and true negative results is shown in Table 3-5. The sensitivity, specificity, positive and negative predictive values of CT scanning and sandwich ELISA combined is 100%, 100%, 100% and 100% respectively; and CT scanning, sandwich ELISA and PCR combined is 100%, 92%, 56% and 100% respectively, when only proven cases are considered true positives and only negative cases are considered true negatives (column B).

When proven, probable and possible cases are considered true positives and negative cases are considered true negatives (column C), the sensitivity, specificity, positive and negative predictive values of CT scanning and sandwich ELISA combined is 55%, 100%, 100% and 85% respectively; and CT scanning, sandwich ELISA and PCR combined is 65%, 92%, 76% and 88% respectively.

3.1.6.2 Use of EORTC-MSG definitions (Ascioglu *et al.*, 2002), with a galactomannan index ≥ 0.5

Four patients from the possible group are now in the probable group. The sensitivity, specificity, positive and negative predictive values of the sandwich

Table 3-5. Statistical analysis 2. Retrospective study. Comparison of different combinations of tests using different definitions of true positive and true negative results

Statistical analysis	A		B		C		D	
	CT ± GM	CT ± GM ± PCR	CT ± GM	CT ± GM ± PCR	CT ± GM	CT ± GM ± PCR	CT ± GM	CT ± GM ± PCR
Sensitivity	100	100	100	100	55	65	100	100
Specificity	94	84	100	92	100	92	91	82
PV Pos	64	41	100	56	100	76	45	29
PV Neg	100	100	100	100	85	88	100	100

A. TP = Proven + probable groups (n = 7); TN = Possible + negative groups (n = 63).
B. TP = Proven group only (n = 5); TN = Negative group only (n = 50).
C. TP = Proven + probable + possible groups (n = 20); TN = Negative group only (n = 50).
D. TP = Proven group only (n = 5); TN = Probable + possible + negative groups (n = 65).
TP: True positive
TN: True negative

ELISA, using an index cut-off of ≥ 0.5, are increased or unchanged for all the different patient groups.

3.2 PROSPECTIVE STUDY

3.2.1 Patient recruitment and categorisation

During the year, 1066 samples were collected from 75 patients. This accounted for 143 episodes of neutropenia or bone marrow transplantation (BMT). The characteristics of the 75 patients are detailed in Table 3-6.

Twenty-nine specimens were from sterile or deep respiratory sites; 24 bronchoalveolar lavages, four pleural fluids and one nasopharyngeal aspirate.

3.2.2 Comparative analysis of results

Among the 75 patients evaluated, 17 cases (3/3 proven, 3/3 probable, 2/8 possible, 9/60 negative) gave positive results, either by thoracic CT, sandwich ELISA, or PCR, or a combination of methods. One of the 3 patients with histologically proven disease had persistently negative galactomannan results. Thoracic CT scans however showed the halo sign with evidence of cavitation, and serial serum samples were PCR positive. Of the 3 patients diagnosed with probable IPA, all had characteristic CT findings, and serial serum samples were positive by PCR. Two of the three patients had a BAL performed; both were galactomannan positive by sandwich ELISA. All 3 patients were galactomannan positive in serial serum samples.

Eight patients had a diagnosis of possible IPA. According to the new Mycoses Study Group guidelines (Ascioglu *et al.*, 2002), one of the eight patients in the possible group would be placed in the probable group based on galactomannan

Table 3-6. Characteristics of 75 patients with haematological malignancies and results of analysis of serum and respiratory samples.

	Proven and probable IPA	Possible IPA	No fungal infection
Number of patients	6	8	61
Sex M/F	3/3	7/1	39/21
Mean age, years (range)	24 (8 - 43)	35 (17 - 74)	32 (18 - 83)
Underlying diseases			
ALL	1	2	21
AML	2	3	17
Other	3	3	22
Allogeneic bone marrow transplant	3	2	10
Autologous bone marrow transplant	0	1	6
ELISA positive patients	5	1	0
PCR positive patients	6	2	9
CT scan features of IPA	6	1	0
Fungal culture	Aspergillus fumigatus: 3 Aspergillus flavus: 3		

IPA	Invasive pulmonary aspergillosis
ELISA	Enzyme linked immunosorbent assay
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia

positivity and the presence of the 'halo' sign on CT thorax. The other seven patients in the group were all galactomannan negative, and thoracic CT scans were inconclusive for IPA. However, one of the seven patients had a positive serum PCR result; this result was reproduced in three subsequent serial serum samples. The other six patients in the possible group remained PCR negative.

Amongst patients in the negative group, 9 patients had positive PCR results. Ten of the positive PCR results (1 possible, 9 negative) were thought to be false positives.

Statistical analysis was performed using the method described previously ([Horvath and Dummer, 1996] [Table 3-7]). The sensitivity, specificity, positive and negative predictive values of CT scanning is 100%, 97%, 75% and 100% respectively; the sandwich ELISA (GM index $\geq$ 1.5) is 83%, 97%, 71% and 99% respectively; and PCR is 100%, 84%, 35% and 100% respectively, when proven and probable cases are considered true positives, and possible and negative cases are considered true negatives (column A).

The sensitivity, specificity, positive and negative predictive values of CT scanning is 100%, 100%, 100% and 100% respectively; the sandwich ELISA (GM index $\geq$ 1.5) is 67%, 100%, 100% and 98% respectively; and PCR is 100%, 85%, 25% and 100% respectively, when only proven cases are considered true positives and only negative cases are considered true negatives (column B).

When proven, probable and possible cases are considered true positives and negative cases are considered true negatives (column C), the sensitivity, specificity, positive and negative predictive values of CT scanning is 57%, 100%, 100% and 91% respectively; the sandwich ELISA (GM index $\geq$ 1.5) is 50%, 100%, 100% and 90% respectively; and PCR is 57%, 85%, 47% and 90%

respectively.

Re-analysis of the GM data was performed using the revised index cut-off ≥ 0.5. This is discussed in section 5.2.

As discussed previously, six months after the onset of the prospective study, we changed our prophylactic antifungal to itraconazole solution 200 mg bd. The number of proven or probable cases of invasive aspergillosis appeared to fall following the use of itraconazole prophylaxis, as illustrated in Figure 3-7. Figures 3-8a and 3-8b compares the results of galactomannan, CT scanning and PCR analysis pre and post itraconazole.

Figure 3-9 shows the time to positivity of the newer diagnostic tests, which on average became positive 3 days before clinically apparent disease. Galactomannan was positive up to 12 days before clinically apparent disease using the revised GM index cut-off of ≥ 0.5.

The scatter plot in Figure 3-10 depicts galactomannan assay results for the 1066 serum samples obtained from the 75 patients in the prospective study. In order to illustrate the correlation of the investigations with clinical events, two case reports are presented in Chapter 4.

3.3 ITRACONAZOLE IN-VITRO STUDY

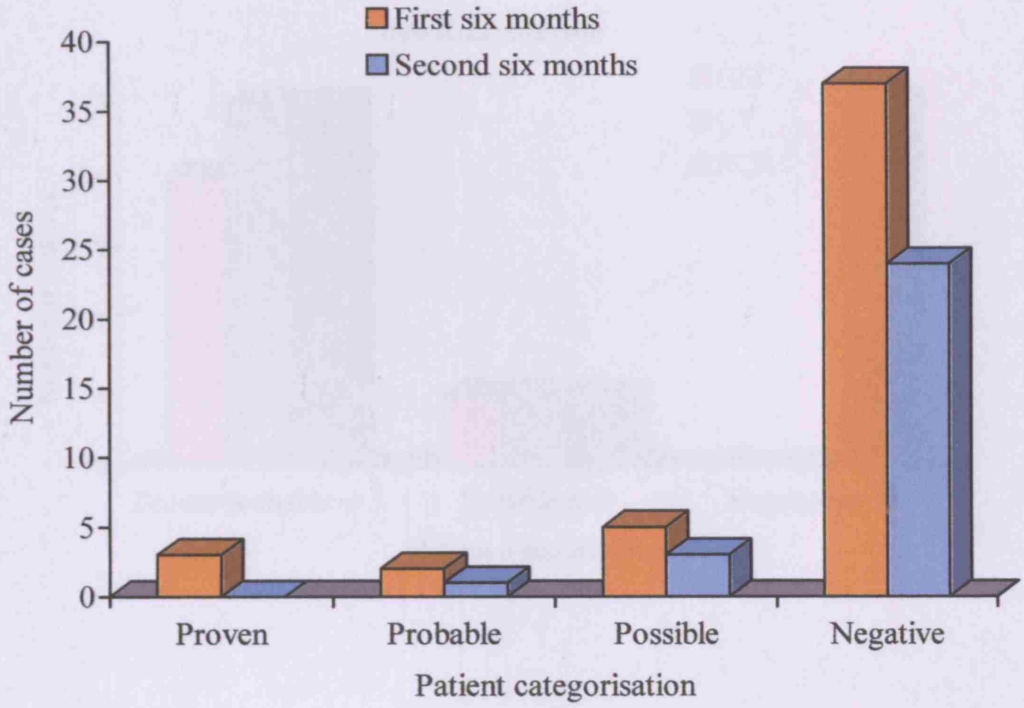
The results showed no significant or consistent change in the concentration of galactomannan using the different concentrations of itraconazole, as demonstrated in figures 3-11 and 3-12.

Table 3-7 Statistical analysis. Prospective study. Comparison of thoracic CT, sandwich ELISA and PCR tests using different definitions of true positive and true negative results.

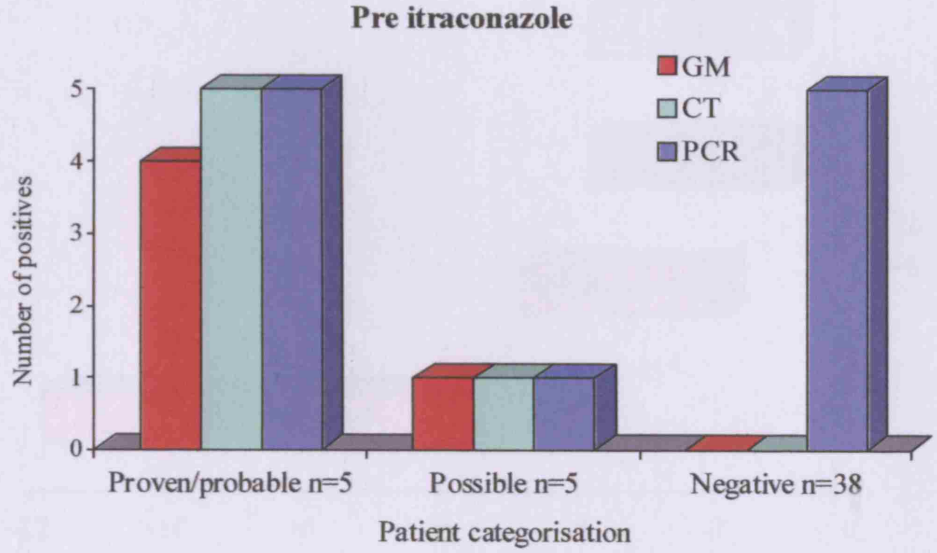
Statistical analysis	A				B				C				D			
	CT	GM		PCR	CT	GM		PCR	CT	GM		PCR	CT	GM		PCR
		I ≥ 1.5	I ≥ 0.5*			I≥1.5	I ≥ 0.5			I ≥ 1.5	I ≥ 0.5			I ≥ 1.5	I ≥ 0.5	
Sensitivity	100	83	100	100	100	67	100	100	57	50	57	57	100	67	100	100
Specificity	97	97	100	84	100	100	100	85	100	100	100	85	93	93	93	80
PV Pos	75	71	100	35	100	100	100	25	100	100	100	47	38	29	38	18
PV Neg	100	99	100	100	100	98	100	100	91	90	91	90	100	99	100	100

A. TP = Proven + probable groups (n = 6); TN = Possible + negative groups (n = 69).
B. TP = Proven group only (n = 3); TN = Negative group only (n = 61).
C. TP = Proven + probable + possible groups (n = 14); TN = Negative group only (n = 61).
D. TP = Proven group only (n = 3); TN = Probable + possible + negative groups (n = 72).
TP: True positive
TN: True negative
* Includes EORTC-MSG consensus definitions: A. TP = Proven + probable groups (n = 8); TN = Possible + negative groups (n = 67).

Figure 3-7. The number of cases investigated for possible/suspected invasive aspergillosis before and after itraconazole prophylaxis.



Figures 3-8a and 3-8b. Comparison of galactomannan, CT scanning and PCR analysis pre and post itraconazole.



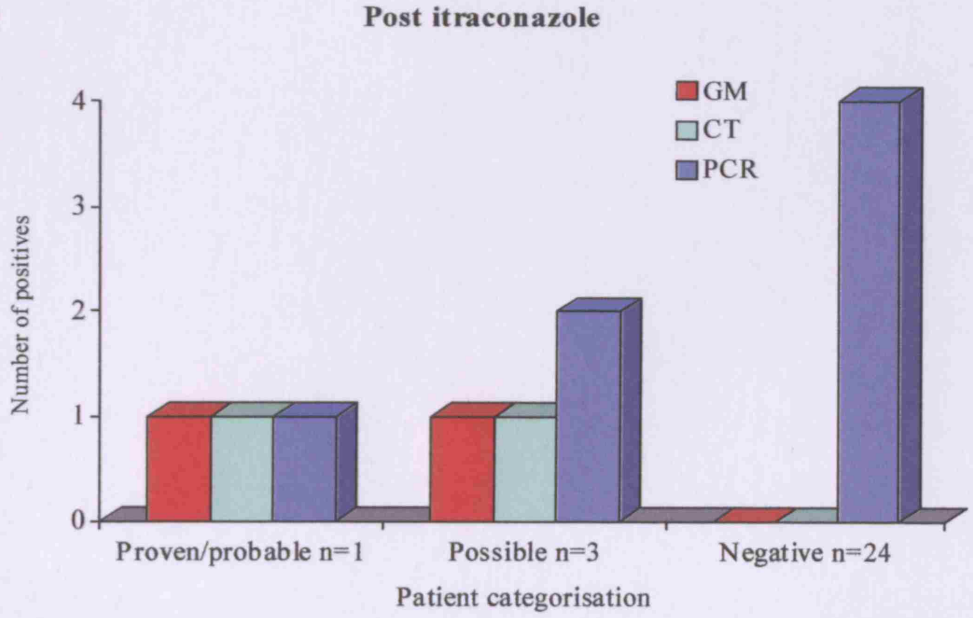


Figure 3-9. Time to positivity (days) of the sandwich ELISA, CT and PCR.

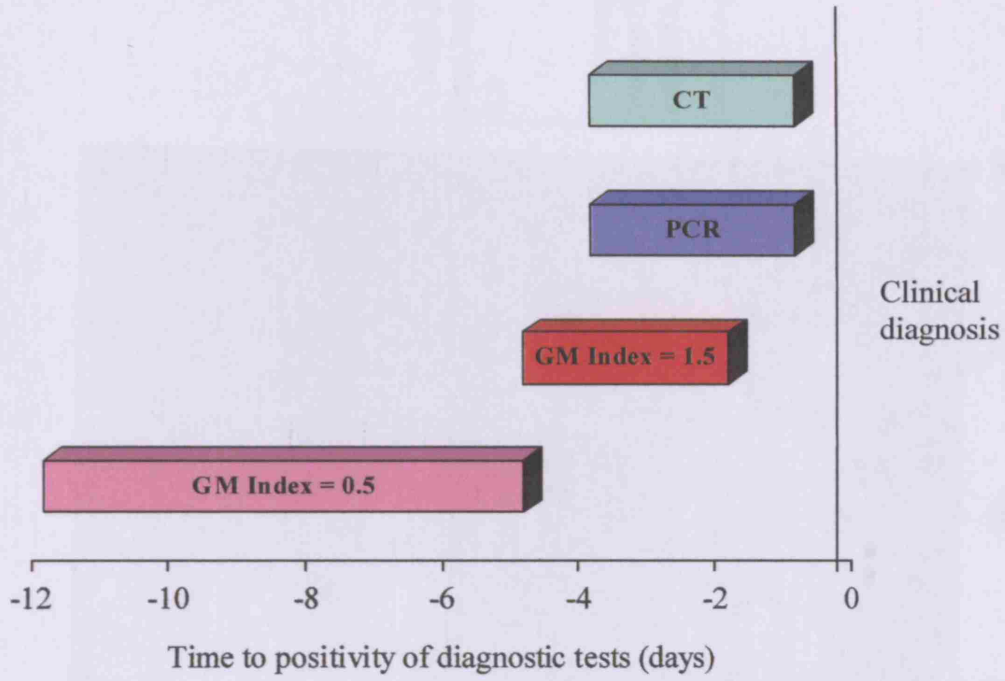


Figure 3-10. Distribution of serum index value from all 75 patients in the prospective study, n=1066.

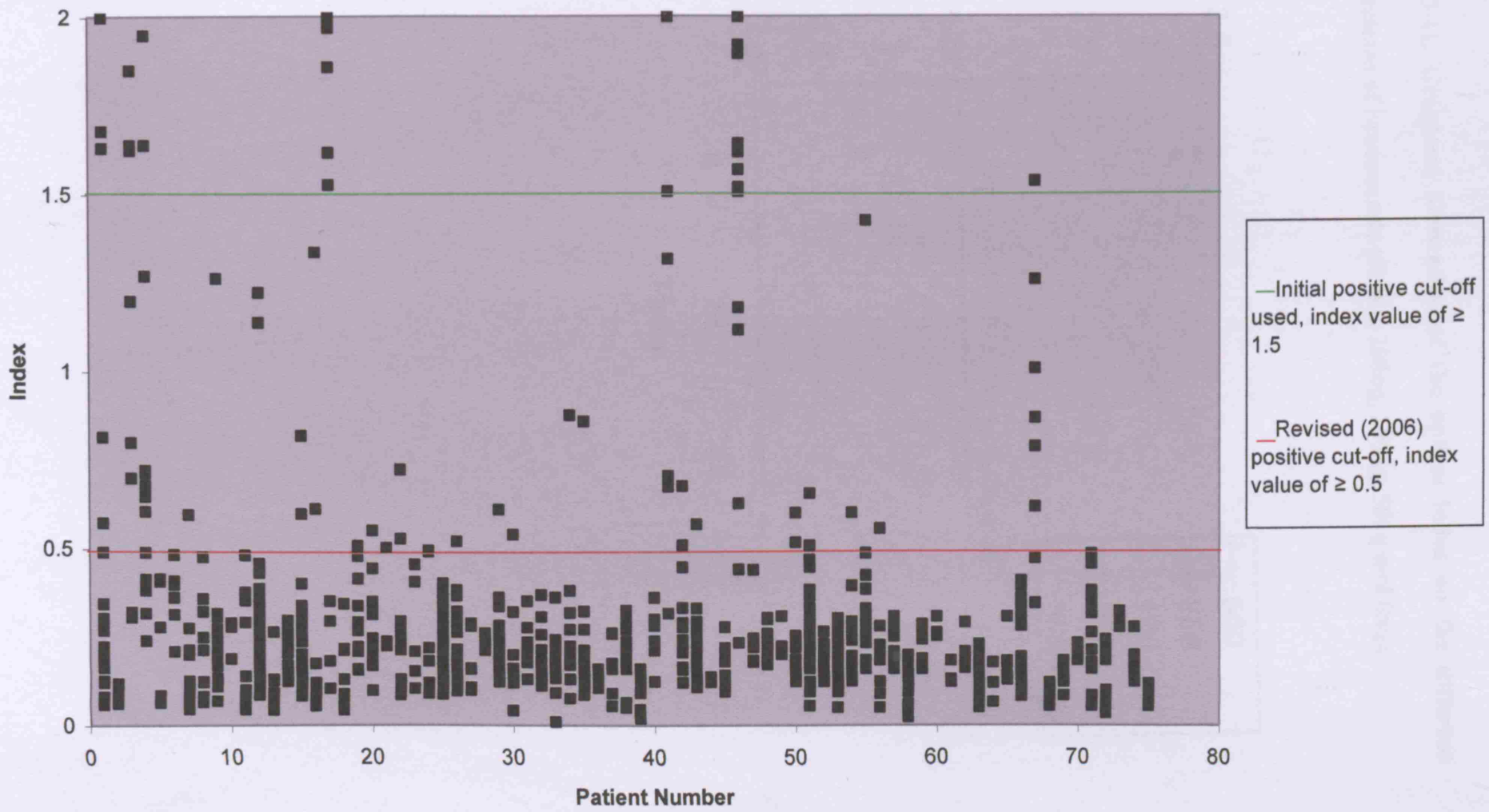


Figure 3-11. Graphical illustration of the optical index on the different concentrations of itraconazole (500ng, 200ng, 100ng, 50ng and 0ng).

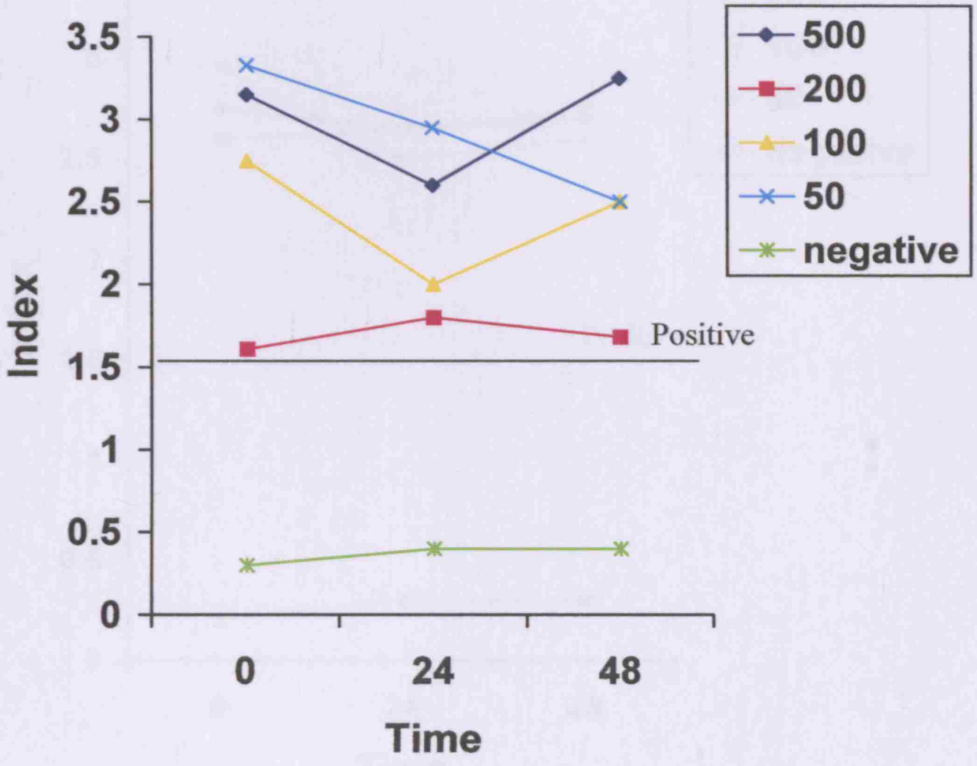
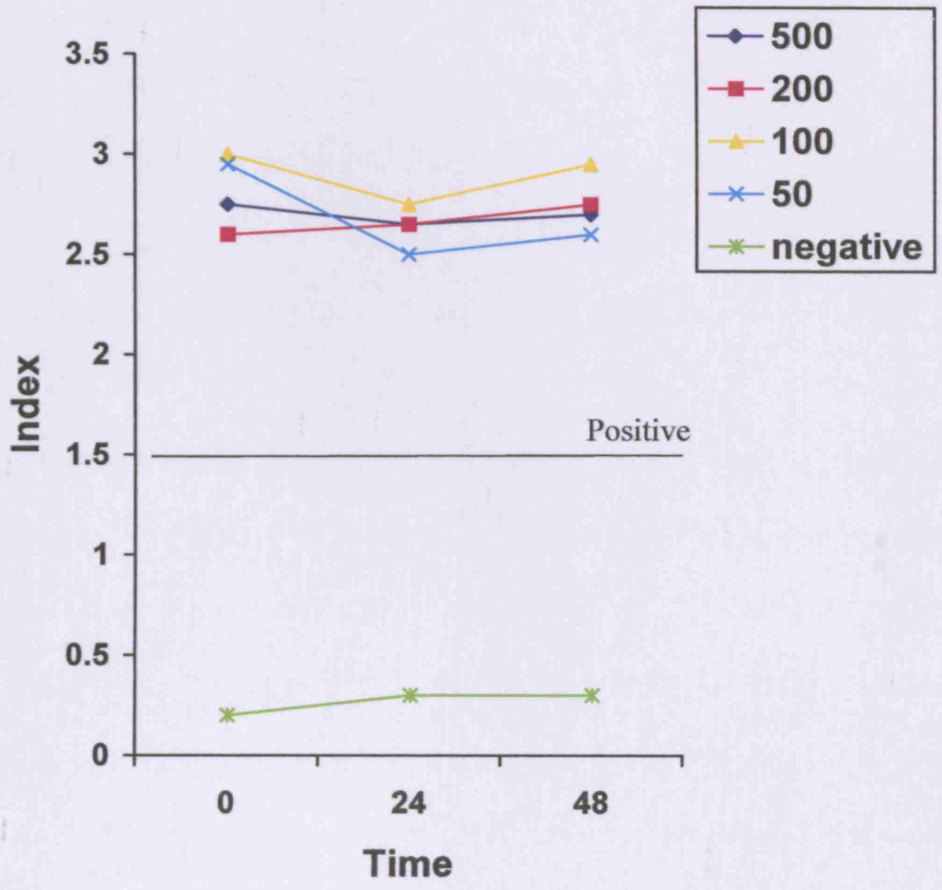


Figure 3-12. Graphical illustration of the optical index on the different concentrations of hydroxy-itraconazole (500ng, 200ng, 100ng, 50ng and 0ng).



CHAPTER 4

CASE REPORTS

4.1 INTRODUCTION

Invasive pulmonary aspergillosis is the most important cause of infective death in children with acute myeloid leukaemia or allogeneic bone marrow transplant recipients (Vogeser *et al.*, 1999). The optimal management of this opportunistic infection is a major challenge for clinicians.

Cultures for *Aspergillus* species are characterised by low sensitivity (Denning *et al.*, 1997; Denning, 1998). Histopathology remains the gold standard for the diagnosis of IPA. However, the presence of severe thrombocytopenia often precludes the possibility of obtaining a specimen by invasive procedures.

Promising alternatives to culture or biopsy include the commercial sandwich ELISA ([Platelia *Aspergillus;* Bio-Rad] [Verweij *et al.*, 1995b]) for the detection of galactomannan antigen in serum or bronchoalveolar fluid, the commercial β-D glucan test (Glucatell; Associates of Cape Cod), high resolution computed tomography scanning (Caillot *et al.*, 1997; Denning *et al.*, 1997) and PCR-based methods for the detection of *Aspergillus* specific DNA (Einsele *et al.*, 1997).

The two case histories presented here illustrate the clinical relevance of these investigations.

4.2 CASE HISTORY 1

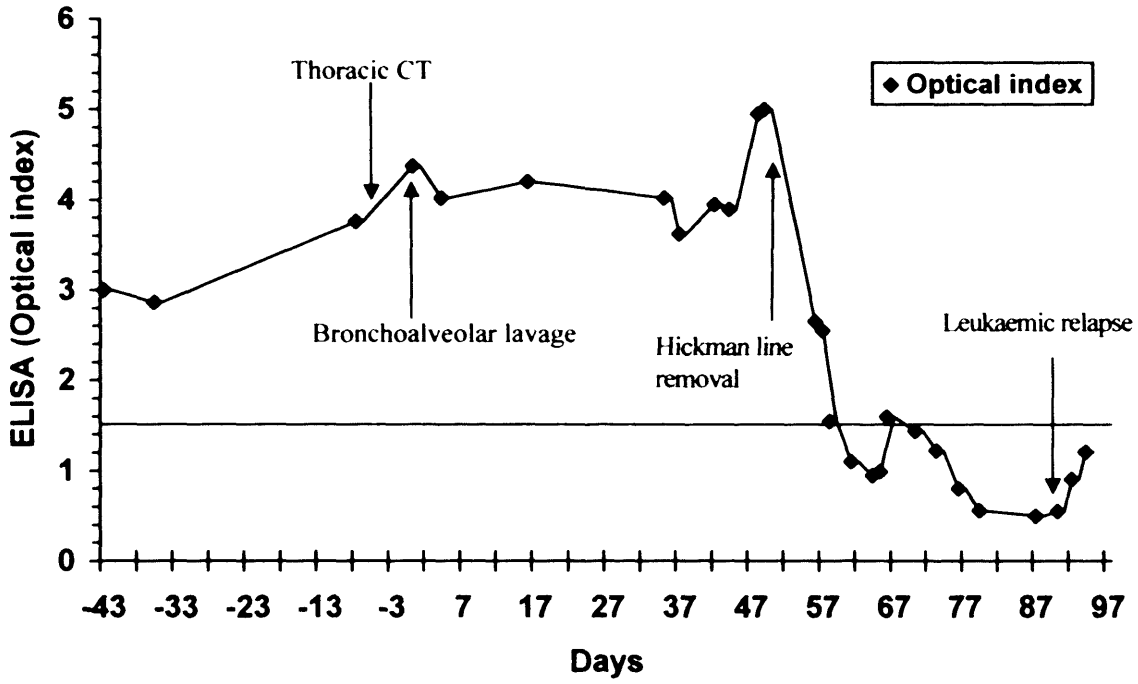
Patient 3 was an 18 year old girl followed for 18 months for the treatment of acute lymphoblastic leukaemia (ALL). Following her block of "late intensification" therapy while still neutropenic, she was readmitted with a fever >39°C associated with lethargy and malaise. Physical examination was otherwise unremarkable. Thoracic CT scanning however, showed a pulmonary lesion in the left lower lobe with the characteristic halo sign of IPA, and she was commenced on liposomal amphotericin B 3 mg/kg daily and

GM-CSF 5 μg/kg/day. The patient then developed erythematous skin lesions and repeat thoracic CT scan two days later showed an increase in the number and size of the pulmonary lesions, which were now present in both lung fields. A skin biopsy and bronchoscopy was performed the same day; galactomannan antigen and *Aspergillus* DNA were detected in BAL fluid but culture remained negative. Histology of the skin biopsy showed acute branching septate hyphae compatible with invasive aspergillosis. Multiple blood cultures taken during this period yielded *Aspergillus flavus*. Retrospective analysis of stored blood samples showed that galactomannan antigenaemia was present 42 days prior to BAL. *Aspergillus* DNA was detected 36 days prior to BAL.

Oral itraconazole solution 200 mg bd, gamma-interferon and white cell transfusions were added, and neutrophil recovery occurred 5 days after bronchoscopy. The Hickman catheter was removed as this was thought to be a possible source of infection, and culture of the tip grew *A. flavus*. Repeat thoracic CT scans showed continuing resolution of the pulmonary lesions. Galactomannan titres showed little change until the Hickman catheter removal when they fell rapidly (Figure 4-1). *Aspergillus* PCR of serial serum samples however gave varying results throughout the course of her illness.

Unfortunately, relapse of IPA following recurrence of her ALL occurred three months after the initial diagnosis of disseminated aspergillosis and she returned home to receive palliative care.

Figure 4-1. Evolution of galactomannan antigen concentrations evaluated by the sandwich ELISA in serum of patient 3. Galactomannan antigenemia was present 37 days prior to the first positive thoracic CT scan.



4.3 CASE HISTORY 2

Patient 4 was a 33 year old man diagnosed as having myelodysplastic syndrome. Sixteen months after the initial diagnosis, he was referred for consideration of an allogeneic bone marrow transplant from his HLA-identical brother. He became febrile 21 days after pre transplantation consolidation with FLAG (Fludarabine 45mg daily for 5 days, Cytarabine 3.1 g daily for 5 days and G-CSF 300 μg daily) chemotherapy, whilst neutropenic. A thoracic CT scan three days later showed an infiltrate in the right perihilar region, which was typical of an early fungal lesion. Conventional amphotericin B 1 mg/kg/day was therefore added. A repeat thoracic CT scan five days later showed that the lesion had progressed and G-CSF was substituted by GM-CSF 5 μg/kg/day. A bronchoalveolar lavage was performed and this was culture negative, but galactomannan antigen and *Aspergillus* DNA were detected in BAL fluid. Serum was negative for galactomannan and DNA. Due to the rapid progression of the lung lesions with a perceived associated risk of haemorrhage (Kibbler *et al.*, 1988), a right upper lobectomy was performed 6 days later, under platelet cover. The histological appearance of the resected lung was consistent with invasive pulmonary aspergillosis but culture was negative. The patient made a good post-operative recovery and was discharged home three weeks later on oral itraconazole solution 200 mg bd. Thoracic CT scan at this stage showed no evidence of fungal infection.

Six weeks later he was re-admitted for a sibling allograft. The oral itraconazole therapy was changed to AmBisome 1 mg/kg daily prior to the conditioning regimen. Nineteen days post transplant the patient developed acute graft-versus-host disease of the skin and liver for which he required long term high-dose steroids (≥40 mg/day). His post transplant course was also complicated by an episode of cytomegalovirus (CMV) pneumonitis and recurrent viremia, as demonstrated by PCR.

Surveillance cultures from his nose grew *Aspergillus flavus* one hundred and five days post BMT. A nasopharyngeal aspirate performed 3 days later also grew *A. flavus* and the dose of AmBisome was increased to 3 mg/kg daily. At this stage galactomannan antigen and *Aspergillus* DNA was not detected in serum and he was clinically well. However, six days later antigenemia developed and a subsequent thoracic CT scan showed the development of a cavity in the left mid-zone. Five days after detection of antigenemia, *Aspergillus* DNA was detected in serum. The patient then developed focal neurological signs and a CT brain scan showed a lesion in the left frontoparietal region, suggestive of cerebral aspergillosis (Figure 4-2). The patient's respiratory and neurological status deteriorated rapidly and he died 128 days post transplant, 14 days following redevelopment of antigenemia (Figure 4-3) and 9 days following recurrence of PCR positivity.

Figure 4-2. CT brain scan showing a ring enhancing mass in the left fronto-parietal region of patient 4, suggestive of disseminated aspergillosis.

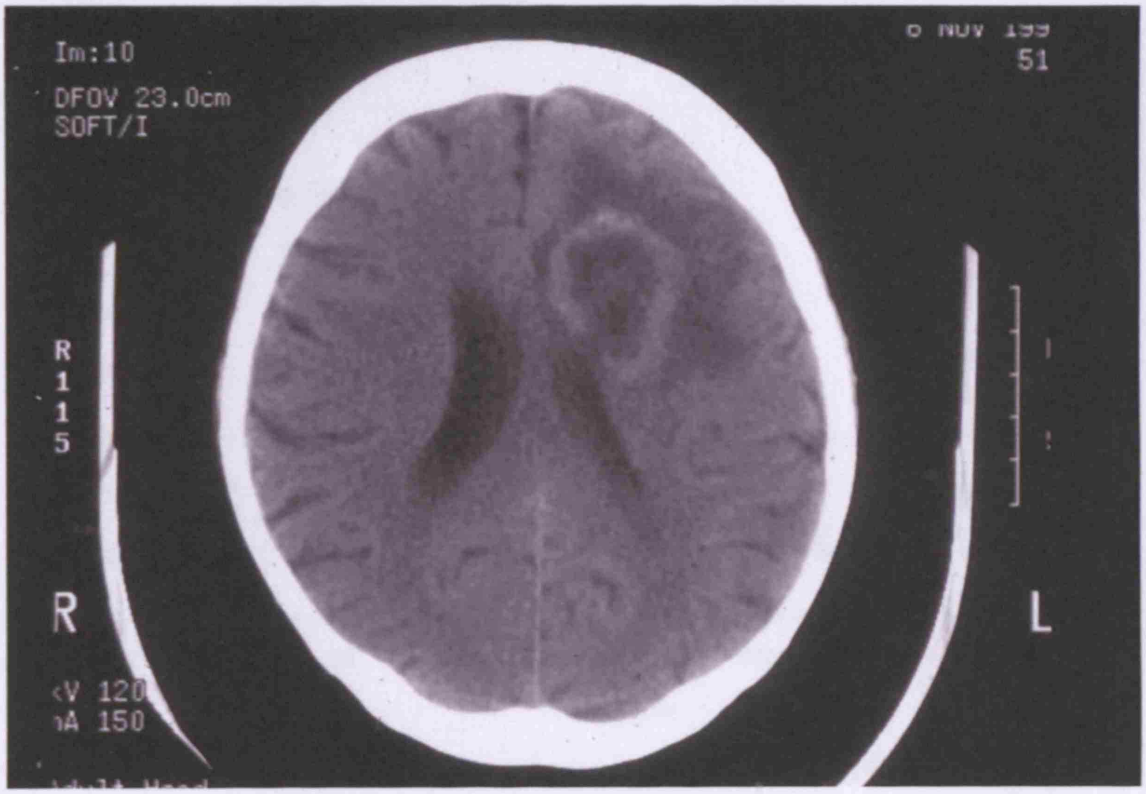
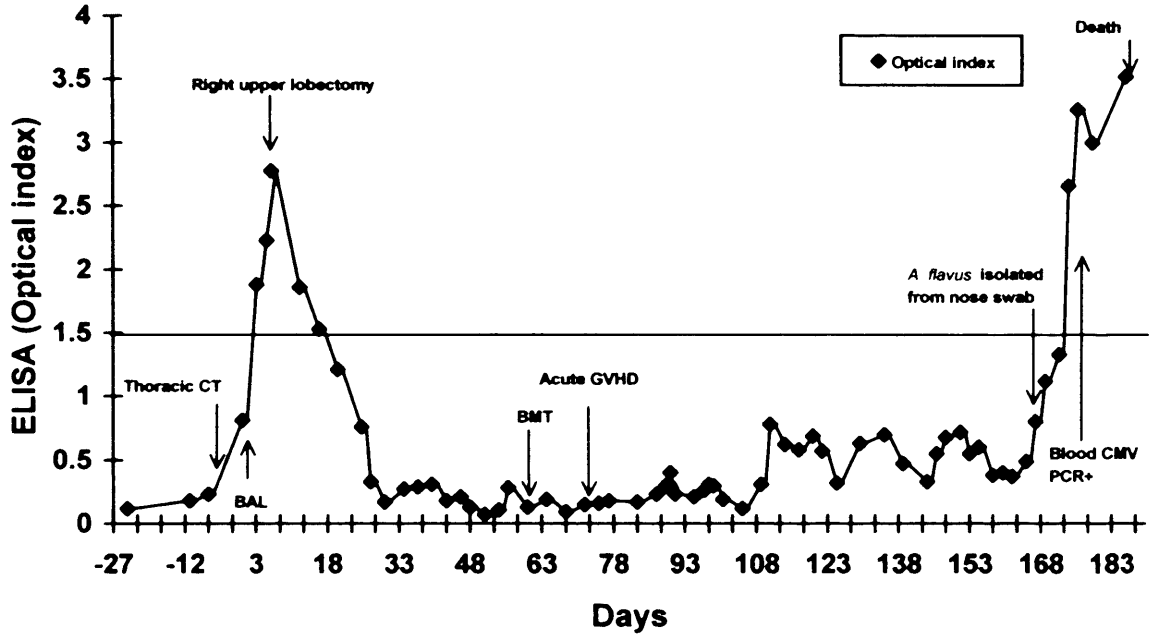


Figure 4-3. Evolution of galactomannan antigen concentrations evaluated by the sandwich ELISA in serum of patient 4. Bronchoalveolar lavage fluid galactomannan was positive whilst serum galactomannan levels were still negative.



CHAPTER 5

DISCUSSION

5.1 RETROSPECTIVE STUDY

This study has shown that the detection of the *Aspergillus* antigen galactomannan in BAL fluid and serum samples correlates strongly with the development of IPA, and that galactomannan antigen may be detectable in BAL fluid before it is present in serum. In the present study, the BAL ELISA was positive at or before the serum ELISA in four of the nine ELISA positive BAL samples. A limited number of studies have compared *Aspergillus* antigen detection in both BAL and serum of patients at high risk of IPA (Andrews and Weiner, 1982; Lortholary *et al.*, 1993; Verweij *et al.*, 1995c; Rath *et al.*, 1996; Caillot *et al.*, 1997). A positive correlation was found between detection of galactomannan in BAL fluid and antigenaemia (Lortholary *et al.*, 1993; Verweij *et al.*, 1995c; Caillot *et al.*, 1997). PCR of BAL fluid was positive at or before serum positivity in 6 of 11 patients. However, in 6 other patients (2 possible, 4 negative) BAL PCR was positive whilst serial serum samples remained persistently PCR negative.

The performance data of the sandwich ELISA, PCR and CT scanning were analysed in different ways (Table 3-4) to demonstrate the range of performance of the tests given the relatively large size of the possible group which almost certainly includes true positives and true negatives. In group C, patients with proven, probable and possible IPA are classified as true positives. Statistical analysis of this group yields a sensitivity of 50% for CT and galactomannan (55% combined), and 65% for PCR. However, CT and galactomannan have a high specificity and positive predictive value (100%), compared with PCR (92% and 76% respectively). Analysing the data by this "worst case" method demonstrates the value of a positive result and even the negative predictive values (87% combined) are approaching levels which can be used for guiding empirical antifungal therapy.

If one considers only proven cases as true positives and negative cases as true negatives (column B), the performance of both CT and galactomannan ELISA is excellent (sensitivity, specificity, PPV and NPV of both combined: 100%). However, if analysis of CT, galactomannan ELISA and PCR is combined (Table 3-5), the specificity and PPV drop to 92% and 56% respectively, whilst sensitivity and NPV remain 100%. The actual performance of these investigations almost certainly lies somewhere between this "best-case" analysis and that of column C.

To combine the variables into a scoring system, logistic regression analysis was considered. However, it is generally accepted that you must have no more predictor variables than 10% of the number of events observed. For these data, only seven events were observed suggesting that logistic regression was inappropriate.

The low sensitivity of the latex agglutination test (Table 3-4) makes this assay unsuitable for the early diagnosis of IPA in immunocompromised patients, as supported by earlier studies (Lortholary *et al.*, 1993; Saugier-Veber *et al.*, 1993; Kappe *et al.*, 1996; Rath *et al.*, 1996). Even in the LA positive patients, this test only detected the presence of the antigen a few days before the death of the patient (Latgé 1995; Stynen *et al.*, 1995). Conflicting results have been described in studies using the same assay where sensitivities of up to 95% were found (Dupont *et al.*, 1990; Haynes and Rogers, 1994). However, other studies have found this test to lack both sensitivity and specificity (Warnock *et al.*, 1991; Ansorg *et al.*, 1994; Hopwood *et al.*, 1995).

Increased sensitivity of the latex agglutination test may be achieved by application of a non-cavitating ultrasonic standing wave field (Ellis and Sobanski, 2000). A study by Grundy *et al.* (1995) demonstrated a × 500 sensitivity enhancement of the Pastorex latex agglutination assay using this method.

The ELISA on average provided positive results 21 days earlier than the LA test. The

galactomannan detection rate was more than twice as high using the ELISA than the LA test (Table 3-1). My findings are similar to those of Maertens and colleagues (1999), who showed that, with the ELISA test, the diagnosis could be made a median of six days before other diagnostic clues appeared. Monitoring of galactomannan concentrations is valuable in the assessment of therapeutic response, as illustrated by Figures 4-1 and 4-3. My study differs from that of Maertens because I investigated biopsy proven rather than postmortem proven cases. It therefore gives more information on patients who survive, and is also less selected for severe (i.e. fatal) disease. As the study was performed retrospectively, the use of the ELISA had no impact on therapy.

When using the Platelia sandwich ELISA test, it is important to remember that galactomannan is rapidly cleared from the blood in this group of patients, and tests for their detection may be helpful in management only if they are performed on a regular basis, at least twice weekly in high-risk patients. The case reports presented in Chapter 4 emphasise the importance of continuous surveillance for IPA in high-risk patients by regular monitoring of galactomannan and CT scanning (Figures 4-1 and 4-3). Patient 6 had a BAL performed when he was clinically unwell despite a normal thoracic CT scan. A repeat CT scan 14 days after bronchoscopy revealed the presence of pulmonary infiltrates and *Aspergillus fumigatus* was isolated from sputum and endotracheal secretions six days later. The patient was therefore placed in the "probable" group based on the above findings. Retrospective analysis of serum samples showed positive galactomannan antigenemia eight days post BAL, once again highlighting the benefits of regular monitoring of galactomannan and weekly CT scanning in the early diagnosis of IPA.

False negative results may be attributed to limited angioinvasion, the rapid clearance of circulating antigen from body fluids, the variable release of galactomannan from the

primary focus of infection or the initiation of antifungal therapy (Verweij *et al.*, 1995b). The use of consensus testing allowed us to demonstrate a high reproducibility of the serological tests, and to give confidence in the galactomannan results obtained. Other investigators have defined a positive result as positive samples collected on two different days and having optical density ratios of more than 1.0 (Maertens *et al.*, 1999).

Dupont *et al.* (1990), in the first paper on the *Aspergillus* latex test reported that samples from patients with invasive aspergillosis may turn negative during storage in a freezer. Given the usually low amounts of circulating galactomannan in patients, this instability during storage may account for true-positive samples turning negative. Studies by Warnock *et al.* (1991) and Knight and Mackenzie (1992) also report poor reproducibility of the Pastorex *Aspergillus* test, making the interpretation of positive agglutination results difficult. However, these reactions may have been false positives as the manufacturer's instructions were not followed (Stynen *et al.*, 1992b).

A study conducted by Verweij *et al.* (1995a) to determine the impact of the duration of storage and repeated freezing and thawing on the antigen titre showed that serum samples which had been stored at –20°C for longer than six months tended to lose reactivity. Also, the titre found at retesting tended to be lower as the period of storage became longer. The authors recommended that to maintain a good reproducibility, serum samples should be stored at -70°C when the period of storage exceeds six months.

In this study, unlike in some others (Stynen *et al.*, 1995; Verweij *et al.*, 1995b; Verweij *et al.*, 1995c; Sulahian *et al.*, 1996), the increased sensitivity of the sandwich ELISA was not associated with false positive results. Other workers have found ELISA reactivity to occur in serum samples from patients without evidence of invasive aspergillosis (Swanink *et al.*, 1997; Denning 2000). However, this has not been our experience. All of our patients with positive ELISA tests had CT scan evidence compatible with IPA.

False-positive antigenaemia may be induced by the agents used for immunosuppressive therapy. Indeed, false-positive reactions by the Pastorex *Aspergillus* latex agglutination test, which employs the same monoclonal antibody used by the sandwich ELISA, have been reported with the urine of rats treated with cyclophosphamide (Hashiguchi *et al.*, 1994). Severe mucositis, which is present in both bone marrow transplant recipients and patients receiving cytotoxic chemotherapy, may play a role by enhancing the resorption of galactomannan from food, or cross-reacting factors from the intestine (Swanink *et al.*, 1997). Therefore, these may be true positive results, but they do not indicate invasive aspergillosis.

The membrane-associated lipoteichoic acid of *Bifidobacterium* spp. is recognised by EB-A2, and may cause ELISA reactivity after gastrointestinal translocation. The high load of *Bifidobacterium* spp. in the gut of newborn babies corresponds with the high number that show false-positive reactivity with serum (Mennink-Kersten *et al.*, 2004). Recently, intravenous administration of piperacillin-tazobactam has been shown to be associated with serum ELISA reactivity in patients without evidence of invasive aspergillosis (Sulahian *et al.*, 2003).

Clinicians and laboratories employing the EB-A2 monoclonal antibody in the Pastorex assay must beware of cross-reactivity. A study by Kappe and Schulze-Berge (1993) found that the antigalactomannan monoclonal antibody used in the Pastorex latex agglutination assay cross-reacted with the potential laboratory contaminants *Penicillium chrysogenum, Cladosporium herbarum, Acremonium* spp., and *Alternaria alternata*, as well as known fungal pathogens such as *Fusarium oxysporum, Wangiella dermatitidis* and *Rhodotorula rubra*. *Penicillium marneffei*, the dimorphic, endemic fungal pathogen of South-East Asia, and *Penicillium digitatum*, have also been found to cross-react with the EB-A2 monoclonal antibody (Stynen *et al.*, 1992a).

Swanink *et al.* (1997) tested the specificity of the Platelia sandwich ELISA with exoantigens of 29 fungi cultured from clinical specimens. Cross-reactivity was observed with *Penicillium chrysogenum, Penicillium digitatum* and *Paecilomyces variotii.* In contrast to the findings of Kappe and Schulze-Berge (1993), they were unable to find cross-reactivity with antigens from *Fusarium oxysporum, Rhodotorula rubra* and *Cladosporium* species, which may be due to differences in the preparation of the exoantigens. Their study suggests that the exoantigens of the tested fungi and bacteria are not responsible for the false-positive reactions by the sandwich ELISA, and therefore other factors may be of importance.

As mentioned previously, all patients in the study had had a BAL performed as this was a criterion for inclusion into the study. The criterion was included because we wanted to assess the usefulness of BAL in the early diagnosis of IPA, taking into consideration that many patients are pancytopenic and prone to bleeding diastheses. However, one negative consequence of the strict inclusion criterion is that patients who did not have a BAL performed, but who had a proven, probable or possible diagnosis of IPA were excluded from the study. The number of cases described here is therefore not a true reflection of the number of cases of IPA seen during the 24-month study period.

Although culture of BAL fluid in patients with focal pulmonary radiological shadowing is known to have a very low yield (McWhinney *et al.*, 1993), confirmed again in this study, these results suggest that galactomannan analysis of BAL fluid is a useful adjunct in the investigation of pulmonary infiltrates in these patients. We suggest that bronchoscopy and bronchoalveolar lavage be performed on all patients with clinical symptoms and signs suggestive of IPA, in whom a definitive diagnosis has not been made, as galactomannan may be detectable in the BAL fluid before it is detected in serum. The ELISA is more sensitive than the LA test and allows earlier detection of

antigen.

Although the ELISA test is not validated for BAL and CSF, several studies have shown high levels of galactomannan in these specimens of infected patients. Antigen detection appears to be superior to culture and PCR.

When the ELISA kit was launched in Europe a decade ago, a cut-off serum ratio of 1.5 was recommended by the manufacturers. Although it is generally accepted that the course of antigenaemia is more important than the actual cut-off, several studies found that 1.5 was too high. In recent years, many investigators have used 0.7-1.0 as the cut-off. Recently, the ELISA test was approved for use in cancer patients by the US Food and Drug Administration with a cut-off ratio of 0.5 (Mennink-Kersten *et al.*, 2004).

In the light of new information on improved sensitivity of the galactomannan-ELISA by using a lower cut-off for positivity of 0.5, a presentation of the original galactomannan data from this study, presented by diagnostic group, was performed (Table 3-4). The sensitivity, specificity, positive and negative predictive values all increased for the different groups of patients ([A, B, C, D] [Table 3-7]). Interestingly, for proven disease, and proven or probable IA, the sensitivity, specificity, positive and negative predictive values were 100%.

Analysis of the data suggests that the new index cut-off ≥ 0.5 could improve sensitivity without loss of specificity in this patient group. A rising ratio above 0.5 strongly indicates the presence of IA and should lead to an additional diagnostic work-up (Maertens *et al.*, 2006) .

As mentioned previously, there were no false positive PCR results amongst the serum samples. This is reassuring, but I would like to point out that the samples were processed in a class 2 safety cabinet. In the UK, most routine microbiology laboratories will not have access to a class 2 safety cabinet, and therefore the risk of contamination with

environmental aspergilli increases. The six false positive BAL PCR results is worrying, and suggests that perhaps the PCR result should ideally be interpreted in conjunction with other indices suggestive of IPA. However, other studies have shown that PCR is highly sensitive (Williamson *et al.*, 2000a; Raad *et al.*, 2002) and predictive for IPA (Raad *et al.*, 2002).

PCR on tissue specimens may aid in the early diagnosis and confirmation of invasive aspergillosis as culture can often be negative. A recent study by Rantakokko-Jalava and colleagues (2003) found that PCR is well suited for the verification of the presence of *A. fumigatus* in tissue biopsy specimens.

Paterson *et al.* (2003) from the Royal Free Hospital performed a study comparing two methods for extracting fungal DNA from paraffin wax embedded tissue sections, based on the QIAmp® DNA mini kit and the TaKaRa DEXPAT™ kit. They found that a method based on the TaKaRa DEXPAT kit, with the addition of lyticase and ethanol precipitation of extracted DNA was more sensitive than one based on the QIAmp DNA mini kit. It was possible to detect less than 10 conidia per sample using spiked samples, and a positive result was obtained with 100% of clinical samples known to be culture positive for *A. fumigatus*.

Many different DNA extraction methods and PCR techniques have been used to detect fungal DNA since this study was performed. DNA extraction methods that have been compared include a freeze-thaw method, a freeze-boil method, enzyme extraction and a bead-beating method (Loeffler *et al.*, 2002; Fredricks *et al.*, 2005; Griffiths *et al.*, 2006).

PCR based molecular diagnostic tests for IA are not commercially available and remain largely unstandardised. Moreover, despite good published performance, interlaboratory reproduction of these assays is poor and no consensus has been

reached for an optimal method. In view of these variables, in 2001, a United Kingdom-Ireland consensus group was set up to evaluate the various DNA extraction techniques and PCR assays available. Real-time PCR was the assay evaluated using different platforms, e.g. Rotagene, Taqman and LightCycler (Sanguinetti *et al.*, 2003; Challier *et al.*, 2004; Halliday *et al.*, 2005). It was subsequently decided to evaluate amplification methods to begin with and therefore, DNA extracts, rather than suspensions of viable fungi, were distributed. Subsequently many discussions were based on whether the optimal specimen to test was serum or whole blood. At present, consensus opinion is that the optimal specimen is whole blood (White *et al.*, 2006). A recent publication by White and Barnes (2006) discussed the benefits and limitations that occurred throughout the process of molecular testing.

As mentioned previously, the value of PCR for diagnosing invasive fungal infections has yet to be determined, and PCR results are not included in the current EORTC-MSG criteria for defining invasive fungal infection. This highlights the difficulties in determining a proven/probable case of IA. No patients in this study received a diagnosis of other filamentous fungal infections; however, the number of cases of non-Aspergillus filamentous fungal infections is rising, and other assays are needed to avoid false-negative results of non-Aspergillus infections or to withhold therapy.

Whenever a CT scan feature that is strongly suggestive of IPA and/or galactomannan seropositivity are present, antifungal therapy should be commenced. Adopting this strategy would allow the selective targeting of patients with a high probability of IPA. Our results support the new EORTC/ Mycoses Study Group consensus on the definitions of invasive fungal infections, which proposes that the combination of aspergillus

antigenaemia (on two occasions) and a halo sign in a patient with a high-risk haematological disorder is evidence that is almost equivalent to a biopsy sample showing hyphae, with or without a positive culture of *Aspergillus* (Ascioglu *et al.*, 2002).

However, I suggest that if both CT and galactomannan ELISA are negative, PCR of blood should be performed on all high-risk patients. If PCR of blood is also negative, the investigations should be repeated on a regular basis and consideration given to an alternative diagnosis. It would seem reasonable to withold anti-Aspergillus therapy in these cases, whilst keeping the patient under close review.

In conclusion, the sandwich ELISA could function as a simple and rapid screening test to detect the presence of *Aspergillus* in bronchial washings and serum samples. The relatively high negative predictive value of all these investigations may allow for better use of empirical antifungal therapy. Regular monitoring of sequential serum samples in conjunction with bronchoalveolar lavage and CT scanning in high risk immunocompromised patients allows earlier diagnosis of IPA.

The combination of a positive BAL or serum ELISA, with a thoracic CT that fulfills this study's criteria for IPA should be considered definitive evidence of IPA. This justifies commencement of pre-emptive antifungal therapy (Table 5-1). The opposite is true for a negative PCR result. By using this strategy, we hope to improve the outlook for patients at risk of IPA, both in terms of morbidity and mortality.

Table 5-1. Proposals for Initiation of Antifungal Therapy.

Features of IPA on CT scan	BAL fluid and/or serum galactomannan	PCR of BAL fluid and/or serum	Diagnostic definitions for IPA	Initiation of antifungal therapy
Positive	Positive	Positive or Negative	Probable	Yes
Positive	Negative	Positive or Negative	Possible	Yes
Negative	Positive	Positive or Negative	Possible	Yes
Negative	Negative	Negative	Negative	No

IPA Invasive Pulmonary Aspergillosis
BAL Bronchoalveolar lavage

5.2 PROSPECTIVE STUDY

The literature suggests that studies such as this are biased towards positivity in patients with invasive fungal infections, because sampling occurs frequently during prolonged hospital stays. This is true of patients with suspected, proven or probable invasive aspergillosis who are included in this study. However, a significant proportion of samples were sent from high-risk patients with no evidence of fungal infection.

As discussed previously, PCR was not performed on BAL specimens in the prospective study because analysis of the retrospective study data had shown that false positive PCR reactions were more likely to occur in BAL as compared to serum samples, however subsequent analysis of the prospective study data has shown that false positive results also occur in serum samples (70/1066 [6.6%]).

A study by Buchheidt *et al.* (2002) reported that BAL samples gave higher sensitivities and specificities than blood samples. Although PCR sensitivity may be improved by testing BAL samples, improved specificity must be questioned. Indeed, 25% of BAL samples from healthy donors are PCR positive through inhalation of airborne Aspergillus spores (Bart-Delabesse *et al.*, 1997).

The findings from several prospective studies suggest that monitoring of patients during a high-risk period will help to identify those that require a diagnostic work-up when galactomannan is detected in serum or plasma, thus enabling prompt provision of pre-emptive antifungal therapy (Verweij *et al.*, 1996; Severens *et al.*, 1997).

Statistical analysis was performed using the method described previously (Horvath and Dummer, 1996). In analysing the GM data, I used different indices, i.e. positivity was defined as an index ≥ 1.5 (initial positive cut-off) and now ≥ 0.5

(revised positive cut-off; Figure 3-10). The NIAID definitions (Denning *et al.*, 1994) were compared with the EORTC-MSG consensus definitions ([Ascioglu *et al.*, 2002] [Table 3-7]).

Using the revised index cut-off of ≥ 0.5, the sensitivity, specificity, positive and negative predictive values all increased for the different groups of patients ([A, B, C, D] [Table 3-7]). As discussed previously, the sensitivity, specificity, positive and negative predictive values for patients with proven and probable IA were 100%. Therefore, lowering the GM index to ≥ 0.5 would have allowed for the earlier diagnosis of IA in this group of patients and may have improved outcome. The number of probable cases of IA was not affected by a reduction of the index factor to 0.5. Galactomannan was positive up to 12 days before clinically apparent disease using the revised GM index cut-off of ≥ 0.5.

The use of a lower positive cut-off also gave unique false positive results (false positives were defined as cases where an initial sample had an index factor ≥ 0.5 but subsequent samples gave results < 0.5). This had no impact on the sensitivity or specificity of the assay.

The first six months of my study showed that despite the use of methods aimed at securing an earlier diagnosis of IA, the incidence and attributable mortality of the disease continued to increase. For these reasons, when itraconazole was licensed as a prophylactic antifungal agent halfway through the study period, the decision was made to change our routine antifungal prophylaxis to itraconazole solution (200 mg bd). By using this strategy, we hoped to reduce the incidence and prevalence of IA in high risk patients.

The strategy appeared to have worked because the number of proven or probable cases of invasive aspergillosis appeared to fall following the use of itraconazole

prophylaxis, as illustrated in Figure 3-7 . There were no further cases of proven IPA during the second half of the study period, and only one case of probable IPA. Using the EORTC-MSG consensus definitions (Ascioglu *et al.*, 2002), we would have had two cases of probable IPA. This may represent an artificial fall, but it does correlate very strongly with the change in antifungal prophylaxis. Although this data is a few years old, there is a continued apparent benefit in terms of proven cases at the Royal Free Hospital. This is especially significant as we had a 100% mortality rate associated with proven or probable disease. However, it is important to stress that the six patients with proven or probable IPA had poor risk disease, with a high incidence of relapse or persistent neutropenia. We can therefore argue that it is still better at this stage to give itraconazole prophylaxis.

During this period, the attributable mortality rate was lower compared to our historical data (Yeghen, 2000). This trend continued over the next 2 years (Paterson *et al.*, 2001). Recent audit data from our centre however suggests that itraconazole prophylaxis does not appear to have altered the incidence of invasive aspergillosis (Paterson *et al.*, 2001). I would have expected the incidence to increase with the use of more immunosupressive chemotherapy and transplantation regimens. The stable rate may be due to a combination of factors including earlier diagnosis, itraconazole prophylaxis and therapy with GM-CSF. Randomised comparative multicentre trials with sufficient patients in high-risk groups need to be performed before any definitive conclusions can be made.

5.3 ITRACONAZOLE IN-VITRO STUDY

The study that was performed was a preliminary study investigating the effect of antifungals by adding itraconazole to galactomannan from the ELISA kit.

The results of the study suggest that itraconazole prophylaxis should have no effect on the detection of circulating GM levels in patients with breakthrough invasive aspergillosis. In retrospect, it would also have been useful to have examined GM levels when itraconazole was added to suspensions of *Aspergillus*. The fungus could be incubated over a period of time, with aliquots of suspension tested periodically for galactomannan concentrations.

In animal models, serum GM index values correlate with fungal burden (Francis *et al.*, 1994; Becker *et al.*, 2003; Petraitis *et al.*, 2003; Petraitiene *et al.*, 2001). One study based on a rabbit model of invasive pulmonary aspergillosis demonstrated that antifungal prophylaxis with posaconazole decreases circulating GM indices (Petraitiene *et al.*, 2001). Two studies that evaluated the utility of the GM-ELISA applied to bronchoalveolar-lavage fluids noted that the assay sensitivity was reduced in patients who were already receiving antifungal therapy (Becker *et al.*, 2003; Musher *et al.*, 2004). This is supported by a recent study by Marr *et al.*, (2005) which demonstrated that the sensitivity of the GM-ELISA is impaired by administration of mould-active antifungal therapy.

The result of the itraconazole in-vitro study, together with the CT scan and PCR data in the prospective study, supports the likelihood that the decrease in galactomannan positivity is the consequence of fewer cases because of improved prophylaxis. This is supported by the study by Marr *et al.* (2005) which suggests that overall fungal burden may be decreased by use of antifungal prophylaxis.

Itraconazole concentrations (trough > 0.5 mg/ml) should be measured to ensure

therapeutic drug levels are achieved. In an early study (Boogaerts *et al.*, 1989), breakthrough infection occurred on oral itraconazole tablets; the development of fatal fungal infection was due largely to failure to reach adequate plasma levels. The value derived from the Boogaerts study was 250 ng/ml.

5.4 OVERALL SUMMARY AND CONCLUSION

Prevention of severe fungal infections should be the aim of all clinicians managing at-risk patients. The use of itraconazole solution as antifungal prophylaxis in high-risk patients is the preferred option. However, in cases where prophylaxis is either not effective, or not administered, it is important to make an early diagnosis so that pre-emptive treatment can be started. The use of an index cut-off ≥ 0.5 increases sensitivity and allows for earlier detection, and may lead to an improved outcome.

In this study, the role of PCR remains controversial. However, a negative PCR result is extremely helpful as it indicates that the patient does not have IA. This reduces unnecessary use of empirical antifungal therapy, with its associated side effects and costs.

There is now an enormous number of susceptible hosts immunocompromised by cancer chemotherapy, bone marrow and organ transplantation, and the HIV epidemic. These patient numbers will continue to increase, as will the number of opportunistic fungal infections. The optimal management of these opportunistic complications remains a major challenge for clinicians.

"...when a condition is foreseen, it is easy to remedy.

If you wait for the disease to manifest itself, it may well be too late."

Machiavelli 1513

CHAPTER 6

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CHAPTER 7

APPENDIX

7.1 EXCEL SPREADSHEET FOR PROSPECTIVE STUDY GALACTOMANNAN DATA

Patient Name	Date	Sample	Index	ELISA I ≥ 1.5	ELISA I ≥ 0.5	PCR	CT Scan
Patient 1	06.08.97	serum	0.061	neg	neg	neg	
Episode 1	08.08.97	serum	0.081	neg	neg	neg	
Proven	11.08.97	serum	0.083	neg	neg	pos	
	13.08.97	serum	0.124	neg	neg	neg	
	13.08.97	BAL	0.124	neg	neg		
	15.08.97	serum	0.182	neg	neg	neg	
	18.08.97	serum	0.18	neg	neg	neg	
	20.08.97	serum	0.187	neg	neg	neg	
	22.08.97	serum	0.182	neg	neg	neg	
	25.08.97	serum	0.166	neg	neg	neg	
	27.08.97	serum	0.127	neg	neg	neg	
Patient 1	24.09.97	serum	0.271	neg	neg	neg	
Episode 2	26.09.97	serum	0.211	neg	neg	neg	
	01.10.97	serum	0.191	neg	neg	neg	
	03.10.97	serum	0.346	neg	neg	pos	
	06.10.97	serum	1.68	pos	pos	pos	
	08.10.97	serum	0.275	neg	neg	neg	
	10.10.97	serum	0.172	neg	neg	neg	
	13.10.97	serum	0.191	neg	neg	neg	
	15.10.97	serum	0.209	neg	neg	pos	
	17.10.97	serum	0.225	neg	neg	neg	
	20.10.97	serum	0.306	neg	neg	neg	
	22.10.97	serum	0.491	neg	neg	neg	
	23.10.97	Nose swab grew *Aspergillus flavus*					
	24.10.97	serum	0.574	neg	pos	pos	
	27.10.97	serum	0.817	neg	pos	pos	Brain pos
	29.10.97	serum	1.631	pos	pos	pos	
	31.10.97	serum	2	pos	pos	pos	
	03.11.97	serum	2	pos	pos	pos	
	10.11.97	serum	2	pos	pos	pos	
	11.11.97	RIP					
Patient 2	16.07.97	serum	0.064	neg	neg	neg	
	18.07.97	serum	0.121	neg	neg	neg	
	20.07.97	serum	0.099	neg	neg	neg	
	23.07.97	serum	0.082	neg	neg	neg	
	25.07.97	serum	0.084	neg	neg	neg	
	27.07.97	serum	0.099	neg	neg	neg	
Patient 3	14.08.97	serum	0.324	neg	neg	neg	
Probable	14.08.97	BM	0.309	neg	neg	neg	
	15.08.97	serum	0.702	neg	pos	neg	Chest?asp
	20.08.97	BAL	1.626	pos	pos		Sinus pos
	20.08.97	serum	0.802	neg	pos	pos	
	21.08.97	serum	1.2	doubt	pos	pos	
	23.08.97	serum	1.64	pos	pos	pos	
	24.08.97	serum	1.85	pos	pos	pos	
Patient 4	15.07.97	serum	0.242	neg	neg	neg	

Proven	23.07.97	serum	0.65	neg	pos	pos	
	24.07.97	serum	0.723	neg	pos	pos	
	25.07.97	serum	0.716	neg	pos	pos	Chest pos
	29.07.97	serum	0.416	neg	neg	pos	
	01.08.97	serum	0.608	neg	pos	pos	
	04.08.97	serum	0.492	neg	neg	neg	
	07.08.97	serum	0.386	neg	neg	neg	
	12.08.97	serum	0.32	neg	neg	neg	
	15.08.97	serum	0.682	neg	pos	pos	
	17.08.97	serum	0.67	neg	pos	neg	
	19.08.97	serum	1.27	doubt	pos	pos	
	20.08.97	serum	1.64	pos	pos	pos	
	22.08.97	serum	1.945	pos	pos	pos	
Patient 5	01.08.97	serum	0.087	neg	neg	neg	
	03.08.97	serum	0.067	neg	neg	pos	
	05.08.97	serum	0.282	neg	neg	pos	
	07.08.97	serum	0.411	neg	neg	pos	
	10.08.97	serum	0.412	neg	neg	pos	
	11.08.97	FNAC	0.418	neg	neg	pos	
	12.08.97	serum	0.083	neg	neg	pos	
	Scedosporium apiospermum isolated from sputum and histology positive						
	RIP						
Patient 6	12.08.97	serum	0.486	neg	neg	neg	
Episode 1	13.08.97	serum	0.402	neg	neg	neg	
	15.08.97	serum	0.365	neg	neg	neg	
Patient 6	27.08.97	BAL	0.212	neg	neg		
Episode 2	27.08.97	serum	0.318	neg	neg	neg	
	29.08.97	serum	0.412	neg	neg	neg	
Patient 7	24.09.97	serum	0.051	neg	neg	neg	
	26.09.97	serum	0.096	neg	neg	neg	
	29.09.97	serum	0.048	neg	neg	neg	
	03.10.97	serum	0.599	neg	pos	neg	
	06.10.97	serum	0.127	neg	neg	neg	
	09.10.97	serum	0.068	neg	neg	pos	
	10.10.97	serum	0.063	neg	neg	neg	
	14.10.97	serum	0.126	neg	neg	neg	
	15.10.97	serum	0.203	neg	neg	neg	
	17.10.97	serum	0.216	neg	neg	neg	
	20.10.97	serum	0.279	neg	neg	neg	
	RIP						
Patient 8	24.09.97	serum	0.084	neg	neg	neg	
Episode 1	26.09.97	serum	0.068	neg	neg	neg	
	29.09.97	serum	0.327	neg	neg	neg	
	03.10.97	serum	0.48	neg	neg	neg	
	04.10.97	serum	0.362	neg	neg	neg	
	06.10.97	serum	0.216	neg	neg	neg	
Patient 8	15.11.97	serum	0.252	neg	neg	neg	
Episode 2	16.11.97	serum	0.126	neg	neg	neg	
	17.11.97	serum	0.125	neg	neg	neg	
Patient 9	29.09.97	serum	0.136	neg	neg	neg	

Episode 1	01.10.97	serum	0.134	neg	neg	neg
	03.10.97	serum	0.171	neg	neg	neg
	06.10.97	serum	0.174	neg	neg	neg
	08.10.97	serum	0.071	neg	neg	neg
	10.10.97	serum	0.183	neg	neg	neg
	13.10.97	serum	0.171	neg	neg	neg
	15.10.97	serum	0.113	neg	neg	neg
	17.10.97	serum	0.268	neg	neg	neg
	20.10.97	serum	0.277	neg	neg	neg
	22.10.97	serum	0.184	neg	neg	neg
Patient 9	26.11.97	serum	1.265	doubt	pos	neg
Episode 2	28.11.97	serum	0.216	neg	neg	neg
	30.11.97	serum	0.317	neg	neg	neg
	01.12.97	serum	0.32	neg	neg	neg
	03.12.97	serum	0.312	neg	neg	neg
	05.12.97	serum	0.227	neg	neg	neg
	11.12.97	serum	0.26	neg	neg	neg
	RIP					
Patient 10	26.08.97	serum	0.192	neg	neg	neg
	28.08.97	BAL	0.292	neg	neg	
	28.08.97	serum	0.283	neg	neg	neg
	29.08.97	serum	0.294	neg	neg	neg
Patient 11	24.09.97	serum	0.486	neg	neg	neg
	26.09.97	serum	0.382	neg	neg	neg
	29.09.97	serum	0.072	neg	neg	neg
	01.10.97	serum	0.046	neg	neg	neg
	03.10.97	serum	0.358	neg	neg	neg
	06.10.97	serum	0.142	neg	neg	neg
	08.10.97	serum	0.075	neg	neg	neg
	10.10.97	serum	0.296	neg	neg	neg
	13.10.97	serum	0.103	neg	neg	neg
Patient 12	29.10.97	serum	0.184	neg	neg	neg
Episode 1	30.10.97	serum	0.192	neg	neg	neg
	31.10.97	serum	0.247	neg	neg	neg
Patient 12	10.11.97	serum	0.33	neg	neg	neg
Episode 2	12.11.97	serum	1.141	doubt	pos	neg
Possible	14.11.97	serum	0.199	neg	neg	neg
	17.11.97	serum	0.098	neg	neg	neg
	19.11.97	serum	0.094	neg	neg	neg
	21.11.97	serum	0.088	neg	neg	neg
	24.11.97	serum	0.139	neg	neg	neg
	26.11.97	serum	0.303	neg	neg	neg
	28.11.97	serum	1.225	doubt	pos	neg
	28.11.97	p.fluid	0.462	neg	neg	
	01.12.97	serum	0.219	neg	neg	neg
	03.12.97	serum	0.458	neg	neg	neg
	05.12.97	serum	0.288	neg	neg	neg
	08.12.97	serum	0.224	neg	neg	neg
	11.12.97	serum	0.257	neg	neg	neg
	17.12.97	serum	0.215	neg	neg	neg
	19.12.97	serum	0.258	neg	neg	neg
	22.12.97	serum	0.215	neg	neg	neg

	24.12.97	serum	0.232	neg	neg	neg
Patient 12,	02.01.98	serum	0.241	neg	neg	neg
Episode 3	05.01.98	serum	0.211	neg	neg	neg
	07.01.98	serum	0.144	neg	neg	neg
	09.01.98	serum	0.216	neg	neg	neg
	12.01.98	serum	0.181	neg	neg	neg
	14.01.98	serum	0.138	neg	neg	pos
	16.01.98	serum	0.135	neg	neg	neg
	19.01.98	serum	0.14	neg	neg	neg
Patient 12	09.02.98	serum	0.139	neg	neg	neg
Episode 4	11.02.98	serum	0.142	neg	neg	neg
	13.02.98	serum	0.172	neg	neg	neg
	16.02.98	serum	0.22	neg	neg	neg
	18.02.98	serum	0.288	neg	neg	neg
	19.02.98	p.fluid	0.161	neg	neg	
	20.02.98	serum	0.128	neg	neg	neg
	23.02.98	serum	0.362	neg	neg	neg
	25.02.98	serum	0.285	neg	neg	neg
	27.02.98	serum	0.329	neg	neg	neg
	01.03.98	serum	0.378	neg	neg	neg
	02.03.98	serum	0.392	neg	neg	neg
	04.03.98	serum	0.437	neg	neg	neg
Patient 12	01.04.98	serum	0.387	neg	neg	neg
Episode 5	03.04.98	serum	0.286	neg	neg	neg
Patient 12	22.04.98	serum	0.286	neg	neg	neg
Episode 6	24.04.98	serum	0.345	neg	neg	neg
	27.04.98	serum	0.356	neg	neg	neg
	29.04.98	serum	0.187	neg	neg	neg
	01.05.98	serum	0.384	neg	neg	neg
	03.05.98	serum	0.392	neg	neg	neg
	06.05.98	serum	0.365	neg	neg	neg
	08.05.98	serum	0.216	neg	neg	neg
	11.05.98	serum	0.252	neg	neg	neg
	13.05.98	serum	0.278	neg	neg	neg
	15.05.98	serum	0.345	neg	neg	neg
	18.05.98	serum	0.276	neg	neg	neg
	20.05.98	serum	0.36	neg	neg	neg
	22.05.98	serum	0.287	neg	neg	neg
	25.05.98	serum	0.328	neg	neg	neg
	27.05.98	serum	0.45	neg	neg	neg
	29.05.98	serum	0.521	neg	pos	neg
Patient 12	17.06.98	serum	0.362	neg	neg	neg
Episode 7	19.06.98	serum	0.345	neg	neg	neg
	22.06.98	serum	0.268	neg	neg	neg
	24.06.98	serum	0.216	neg	neg	neg
	26.06.98	serum	0.239	neg	neg	neg
	30.06.98	serum	0.214	neg	neg	neg
	01.07.98	serum	0.389	neg	neg	neg
	03.07.98	serum	0.412	neg	neg	neg
	06.07.98	serum	0.452	neg	neg	neg
	08.07.98	serum	0.387	neg	neg	neg
Patient 13	24.09.97	serum	0.09	neg	neg	neg
	26.09.97	serum	0.045	neg	neg	neg
	01.10.97	serum	0.057	neg	neg	neg

	03.10.97	serum	0.082	neg	neg	neg
	06.10.97	serum	0.268	neg	neg	neg
	09.10.97	serum	0.134	neg	neg	neg
	10.10.97	serum	0.129	neg	neg	neg
	12.10.97	serum	0.094	neg	neg	neg
	13.10.97	serum	0.068	neg	neg	neg
Patient 14	03.09.97	serum	0.142	neg	neg	neg
Epsiode 1	04.09.97	serum	0.127	neg	neg	neg
Patient 14	16.12.97	serum	0.129	neg	neg	neg
Episode 2	17.12.97	serum	0.207	neg	neg	neg
	19.12.97	serum	0.252	neg	neg	neg
	22.12.97	serum	0.209	neg	neg	neg
	24.12.97	serum	0.222	neg	neg	neg
Patient 14	02.01.98	serum	0.213	neg	neg	neg
Episode 3	05.01.98	serum	0.218	neg	neg	neg
	07.01.98	serum	0.268	neg	neg	neg
	09.01.98	serum	0.172	neg	neg	neg
	12.01.98	serum	0.254	neg	neg	neg
	14.01.98	serum	0.302	neg	neg	neg
Patient 15	29.09.97	serum	0.241	neg	neg	neg
Episode 1	01.10.97	serum	0.302	neg	neg	neg
Possible	04.10.97	serum	0.344	neg	neg	neg
	06.10.97	serum	0.256	neg	neg	neg
	09.10.97	serum	0.1	neg	neg	neg
	10.10.97	serum	0.129	neg	neg	neg
	13.10.97	serum	0.17	neg	neg	neg
	15.10.97	serum	0.193	neg	neg	neg
	17.10.97	serum	0.176	neg	neg	neg
	20.10.97	serum	0.321	neg	neg	neg
Patient 15	03.11.97	serum	0.295	neg	neg	neg
Episode 2	06.11.97	serum	0.164	neg	neg	neg
	10.11.97	serum	0.204	neg	neg	neg
	12.11.97	serum	0.824	neg	pos	neg
	13.11.97	serum	0.275	neg	neg	neg
	14.11.97	serum	0.085	neg	neg	neg
	17.11.97	serum	0.099	neg	neg	neg
	19.11.97	serum	0.13	neg	neg	neg
	24.11.97	serum	0.089	neg	neg	neg
	01.12.97	serum	0.406	neg	neg	neg
	08.12.97	serum	0.604	neg	pos	neg
	10.12.97	serum	0.193	neg	neg	neg
	12.12.97	BAL	0.111	neg	neg	
	15.12.97	serum	0.168	neg	neg	neg
Patient 15	30.12.97	BAL	0.162	neg	neg	
Episode 3	30.12.97	serum	0.154	neg	neg	neg
	07.01.98	serum	0.197	neg	neg	neg
	09.01.98	serum	0.127	neg	neg	neg
	12.01.98	serum	0.184	neg	neg	neg
	RIP					
Patient 16	17.09.97	serum	0.056	neg	neg	neg
Episode 1	19.09.97	serum	0.067	neg	neg	neg
	20.09.97	serum	0.121	neg	neg	neg

	29.09.97	serum	0.077	neg	neg	neg	
Patient 16	02.10.97	serum	0.069	neg	neg	neg	
Episode 2	03.10.97	serum	1.34	doubt	pos	neg	
Patient 16	17.11.97	serum	0.097	neg	neg	neg	
Episode 3	18.11.97	BAL	0.178	neg	neg		
	19.11.97	serum	0.09	neg	neg	neg	
	21.11.97	serum	0.123	neg	neg	neg	
	24.11.97	serum	0.105	neg	neg	neg	
	26.11.97	serum	0.618	neg	pos	neg	
Patient 17	03.10.97	BAL	1.86	pos	pos		
Probable	03.10.97	serum	1.53	pos	pos	pos	Chest pos
	06.10.97	serum	0.3	neg	neg	pos	
	07.10.97	serum	1.97	pos	pos	pos	
	08.10.97	*Aspergillus fumigatus* isolated from BAL					
	09.10.97	serum	0.357	neg	neg	pos	
	13.10.97	serum	0.108	neg	neg	pos	
	15.10.97	serum	1.62	pos	pos	pos	
	17.10.97	serum	0.186	neg	neg	neg	
	20.10.97	serum	2	pos	pos	neg	
	31.10.97	serum	2	pos	pos	neg	
	04.11.97	serum	2	pos	pos	neg	
	14.11.97	serum	2	pos	pos	neg	
	RIP on 15.11.97						
Patient 18	10.10.97	serum	0.074	neg	neg	neg	
Episode 1	12.10.97	serum	0.086	neg	neg	neg	
	13.10.97	serum	0.133	neg	neg	neg	
	15.10.97	serum	0.085	neg	neg	neg	
	17.10.97	serum	0.086	neg	neg	neg	
Patient 18	02.11.97	serum	0.092	neg	neg	neg	
Episode 2	03.11.97	serum	0.221	neg	neg	neg	
	05.11.97	serum	0.216	neg	neg	neg	
	07.11.97	serum	0.134	neg	neg	neg	
	09.11.97	serum	0.045	neg	neg	neg	
	10.11.97	serum	0.349	neg	neg	neg	
Patient 19	15.10.97	serum	0.159	neg	neg	neg	
	16.10.97	serum	0.162	neg	neg	neg	
	17.10.97	serum	0.226	neg	neg	neg	
	20.10.97	serum	0.272	neg	neg	neg	
	22.10.97	serum	0.223	neg	neg	neg	
	24.10.97	serum	0.421	neg	neg	neg	
	27.10.97	serum	0.297	neg	neg	neg	
	29.10.97	serum	0.213	neg	neg	neg	
	31.10.97	serum	0.291	neg	neg	neg	
	03.11.97	serum	0.513	neg	pos	neg	
	10.11.97	serum	0.344	neg	neg	neg	
	12.11.97	serum	0.484	neg	neg	neg	
	RIP on 13.11.97						
Patient 20	24.10.97	serum	0.172	neg	neg	neg	
Episode 1	27.10.97	serum	0.221	neg	neg	neg	
Patient 20	21.11.97	serum	0.102	neg	neg	neg	
Episode 2	26.11.97	serum	0.348	neg	neg	neg	

	28.11.97	serum	0.251	neg	neg	neg
	01.12.97	serum	0.557	neg	pos	neg
	03.12.97	serum	0.356	neg	neg	neg
	05.12.97	serum	0.335	neg	neg	neg
	08.12.97	serum	0.192	neg	neg	neg
	10.12.97	serum	0.319	neg	neg	neg
	12.12.97	serum	0.222	neg	neg	neg
	17.12.97	serum	0.449	neg	neg	neg
	19.12.97	serum	0.212	neg	neg	neg
Patient 21	29.10.97	serum	0.229	neg	neg	neg
	31.10.97	BAL	0.507	neg	pos	
	31.10.97	serum	0.242	neg	neg	neg
Patient 22	03.11.97	serum	0.274	neg	neg	neg
	10.11.97	serum	0.534	neg	pos	neg
	12.11.97	serum	0.253	neg	neg	neg
	14.11.97	serum	0.133	neg	neg	neg
	17.11.97	serum	0.089	neg	neg	neg
	19.11.97	serum	0.134	neg	neg	neg
	21.11.97	serum	0.09	neg	neg	neg
	24.11.97	serum	0.102	neg	neg	neg
	26.11.97	serum	0.3	neg	neg	neg
	28.11.97	serum	0.729	neg	pos	neg
	01.12.97	serum	0.297	neg	neg	neg
	03.12.97	serum	0.231	neg	neg	neg
	05.12.97	serum	0.234	neg	neg	neg
	08.12.97	serum	0.242	neg	neg	neg
	11.12.97	serum	0.218	neg	neg	neg
Patient 23	24.10.97	BAL	0.461	neg	neg	
Episode 1	24.10.97	serum	0.213	neg	neg	neg
Patient 23	10.11.97	serum	0.412	neg	neg	neg
Episode 2	12.11.97	serum	0.157	neg	neg	neg
	14.11.97	serum	0.108	neg	neg	neg
Patient 24	10.11.97	serum	0.5	neg	pos	neg
	12.11.97	serum	0.224	neg	neg	neg
	14.11.97	serum	0.096	neg	neg	neg
	17.11.97	serum	0.122	neg	neg	neg
	19.11.97	serum	0.093	neg	neg	neg
	21.11.97	serum	0.124	neg	neg	neg
	24.11.97	serum	0.185	neg	neg	pos
	26.11.97	serum	0.497	neg	neg	neg
	28.11.97	serum	0.123	neg	neg	pos
Patient 25	10.11.97	serum	0.334	neg	neg	neg
Episode 1	12.11.97	serum	0.128	neg	neg	neg
Possible	14.11.97	serum	0.116	neg	neg	neg
	17.11.97	serum	0.132	neg	neg	neg
	19.11.97	serum	0.09	neg	neg	neg
	21.11.97	serum	0.129	neg	neg	neg
	24.11.97	serum	0.096	neg	neg	neg
	28.11.97	serum	0.408	neg	neg	neg
	01.12.97	serum	0.182	neg	neg	neg

	03.12.97	serum	0.197	neg	neg	neg
	05.12.97	serum	0.22	neg	neg	neg
	08.12.97	serum	0.204	neg	neg	neg
	12.12.97	serum	0.323	neg	neg	neg
	17.12.97	serum	0.267	neg	neg	neg
	19.12.97	serum	0.179	neg	neg	neg
	22.12.97	serum	0.259	neg	neg	pos
	24.12.97	serum	0.189	neg	neg	neg
Patient 25	02.01.98	serum	0.226	neg	neg	neg
Episode 2	04.01.98	serum	0.271	neg	neg	neg
	06.01.98	serum	0.162	neg	neg	neg
	09.01.98	serum	0.216	neg	neg	neg
	11.01.98	serum	0.287	neg	neg	neg
Patient 25	09.02.98	serum	0.245	neg	neg	neg
Episode 3	11.02.98	serum	0.161	neg	neg	neg
	13.02.98	serum	0.184	neg	neg	neg
	16.02.98	serum	0.175	neg	neg	neg
	18.02.98	serum	0.358	neg	neg	pos
	20.02.98	serum	0.186	neg	neg	neg
	23.02.98	serum	0.212	neg	neg	neg
	25.02.98	serum	0.392	neg	neg	neg
	27.02.98	serum	0.181	neg	neg	neg
	02.03.98	serum	0.169	neg	neg	neg
	04.03.98	serum	0.189	neg	neg	neg
	09.03.98	serum	0.155	neg	neg	neg
	11.03.98	serum	0.295	neg	neg	neg
Patient 25	26.03.98	serum	0.167	neg	neg	pos
Episode 4	30.03.98	serum	0.235	neg	neg	neg
	01.04.98	serum	0.165	neg	neg	neg
	03.04.98	serum	0.312	neg	neg	neg
	06.04.98	serum	0.268	neg	neg	neg
	12.04.98	serum	0.367	neg	neg	neg
	15.04.98	serum	0.145	neg	neg	neg
	17.04.98	serum	0.187	neg	neg	neg
	24.04.98	serum	0.292	neg	neg	neg
	27.04.98	serum	0.367	neg	neg	neg
	01.05.98	BAL	0.163	neg	neg	
Patient 26	06.11.97	BAL	0.188	neg	neg	
MDR TB	06.11.97	serum	0.373	neg	neg	neg
	08.11.97	serum	0.124	neg	neg	neg
	02.01.98	BAL	0.186	neg	neg	
	05.01.98	serum	0.11	neg	neg	neg
	09.01.98	serum	0.1	neg	neg	neg
	20.01.98	serum	0.104	neg	neg	neg
	21.01.98	serum	0.147	neg	neg	neg
	22.01.98	serum	0.158	neg	neg	neg
	23.01.98	serum	0.114	neg	neg	neg
	26.01.98	serum	0.116	neg	neg	neg
	28.01.98	serum	0.197	neg	neg	neg
	30.01.98	serum	0.208	neg	neg	pos
	02.02.98	serum	0.328	neg	neg	neg
	04.02.98	serum	0.527	neg	pos	neg
	06.02.98	serum	0.285	neg	neg	neg
	09.02.98	serum	0.219	neg	neg	neg

	11.02.98	serum	0.312	neg	neg	neg
	12.02.98	serum	0.379	neg	neg	neg
	13.02.98	serum	0.268	neg	neg	pos
	16.02.98	serum	0.315	neg	neg	neg
	20.02.98	serum	0.389	neg	neg	neg
Patient 27,	20.10.97	serum	0.294	neg	neg	neg
Episode 1	17.11.97	serum	0.101	neg	neg	neg
	19.11.97	serum	0.163	neg	neg	neg
Patient 27,Ep. 2	15.12.97	serum	0.165	neg	neg	neg
Patient 27	05.01.98	serum	0.286	neg	neg	neg
Episode 3	12.01.98	serum	0.106	neg	neg	neg
	26.01.98	serum	0.11	neg	neg	neg
Patient 28	22.11.97	serum	0.246	neg	neg	neg
Episode 1	24.11.97	serum	0.265	neg	neg	neg
Patient 28	15.12.97	serum	0.233	neg	neg	neg
Episode 2	18.12.97	serum	0.222	neg	neg	neg
	23.12.97	NPA	0.216	neg	neg	
	RIP on 23/12/97					
Patient 29	05.12.97	serum	0.181	neg	neg	pos
Episode 1	08.12.97	serum	0.217	neg	neg	neg
	10.12.97	serum	0.616	neg	pos	neg
	17.12.97	serum	0.204	neg	neg	neg
	19.12.97	serum	0.23	neg	neg	neg
	22.12.97	serum	0.21	neg	neg	neg
Patient 29	02.01.98	serum	0.158	neg	neg	neg
Episode 2	05.01.98	serum	0.127	neg	neg	neg
	07.01.98	serum	0.126	neg	neg	neg
	09.01.98	serum	0.168	neg	neg	neg
	12.01.98	serum	0.252	neg	neg	neg
	14.01.98	serum	0.134	neg	neg	neg
	16.01.98	serum	0.367	neg	neg	neg
Patient 29	11.02.98	serum	0.342	neg	neg	neg
Episode 3	13.02.98	serum	0.359	neg	neg	neg
	16.02.98	serum	0.256	neg	neg	neg
	20.02.98	BAL	0.287	neg	neg	
	23.02.98	serum	0.216	neg	neg	neg
	25.02.98	serum	0.238	neg	neg	neg
	27.02.98	serum	0.185	neg	neg	neg
	02.03.98	serum	0.18	neg	neg	neg
	04.03.98	serum	0.179	neg	neg	neg
Patient 30	12.12.97	serum	0.203	neg	neg	neg
Possible	15.12.97	serum	0.126	neg	neg	neg
	15.12.97	BAL	0.546	neg	pos	
	19.12.97	serum	0.326	neg	neg	neg
	22.12.97	serum	0.136	neg	neg	neg
	24.12.97	serum	0.043	neg	neg	neg
	06.01.98	BAL	0.124	neg	neg	
	09.01.98	serum	0.145	neg	neg	neg
	12.01.98	serum	0.142	neg	neg	neg
	14.01.98	serum	0.131	neg	neg	neg
	16.01.98	serum	0.123	neg	neg	neg

	19.01.98	serum	0.145	neg	neg	neg
	26.01.98	serum	0.131	neg	neg	neg
	02.02.98	serum	0.164	neg	neg	neg
	09.02.98	serum	0.134	neg	neg	neg
Patient 31	12.12.97	serum	0.206	neg	neg	neg
Episode 1	19.12.97	serum	0.357	neg	neg	neg
	22.12.97	serum	0.218	neg	neg	neg
	24.12.97	serum	0.24	neg	neg	neg
Patient 31	05.01.98	serum	0.24	neg	neg	neg
Episode 2	07.01.98	serum	0.281	neg	neg	neg
	09.01.98	serum	0.133	neg	neg	neg
	12.01.98	serum	0.182	neg	neg	neg
Patient 32	12.12.97	serum	0.374	neg	neg	neg
Episode 1	15.12.97	serum	0.261	neg	neg	neg
	19.12.97	serum	0.184	neg	neg	neg
	22.12.97	serum	0.167	neg	neg	neg
	24.12.97	serum	0.158	neg	neg	neg
	29.12.97	serum	0.218	neg	neg	neg
	31.12.97	serum	0.126	neg	neg	neg
Patient 32,	16.01.98	serum	0.134	neg	neg	neg
Episode 2	19.01.98	serum	0.117	neg	neg	neg
	23.01.98	serum	0.119	neg	neg	neg
	26.01.98	serum	0.114	neg	neg	neg
	28.01.98	serum	0.127	neg	neg	neg
	30.01.98	serum	0.312	neg	neg	neg
	02.02.98	serum	0.124	neg	neg	neg
Patient 33	19.12.97	serum	0.247	neg	neg	neg
	22.12.97	serum	0.233	neg	neg	neg
	24.12.97	serum	0.206	neg	neg	neg
	31.12.97	serum	0.012	neg	neg	neg
	06.01.98	serum	0.367	neg	neg	neg
	07.01.98	serum	0.18	neg	neg	pos
	09.01.98	serum	0.145	neg	neg	neg
	12.01.98	serum	0.163	neg	neg	neg
	14.01.98	serum	0.185	neg	neg	neg
	16.01.98	serum	0.137	neg	neg	pos
	19.01.98	serum	0.094	neg	neg	neg
	23.01.98	serum	0.136	neg	neg	neg
	26.01.98	serum	0.145	neg	neg	neg
Patient 34	19.12.97	serum	0.191	neg	neg	pos
Episode 1	22.12.97	serum	0.174	neg	neg	neg
	24.12.97	serum	0.196	neg	neg	pos
	29.12.97	serum	0.212	neg	neg	neg
	31.12.97	serum	0.196	neg	neg	neg
Patient 34	14.01.98	serum	0.134	neg	neg	neg
Episode 2	16.01.98	serum	0.078	neg	neg	neg
	18.01.98	serum	0.882	neg	pos	neg
Patient 34	30.01.98	serum	0.192	neg	neg	neg
Episode 3	02.02.98	serum	0.186	neg	neg	neg
	04.02.98	serum	0.214	neg	neg	neg
	06.02.98	serum	0.228	neg	neg	neg

	09.02.98	serum	0.217	neg	neg	neg
	11.02.98	serum	0.214	neg	neg	neg
	11.02.98	BAL	0.275	neg	neg	
	14.02.98	serum	0.324	neg	neg	neg
	16.02.98	serum	0.386	neg	neg	neg
Patient 35	13.12.97	serum	0.216	neg	neg	neg
Episode 1	15.12.97	serum	0.275	neg	neg	neg
Patient 35 Ep. 2	12.01.98	serum	0.328	neg	neg	neg
Patient 35	26.01.98	serum	0.114	neg	neg	neg
Episode 3	28.01.98	serum	0.141	neg	neg	neg
	31.01.98	serum	0.185	neg	neg	neg
Patient 35	01.04.98	serum	0.089	neg	neg	neg
Episode 4	02.04.98	serum	0.864	neg	pos	neg
	03.04.98	serum	0.167	neg	neg	neg
	04.04.98	serum	0.145	neg	neg	neg
	RIP on 4/4/98 of multiorgan failure					
Patient 36	07.01.98	serum	0.149	neg	neg	pos
	09.01.98	serum	0.128	neg	neg	neg
	12.01.98	serum	0.162	neg	neg	neg
	16.01.98	serum	0.116	neg	neg	neg
	19.01.98	serum	0.109	neg	neg	neg
	23.01.98	serum	0.142	neg	neg	pos
	26.01.98	serum	0.115	neg	neg	neg
Patient 37	07.01.98	serum	0.162	neg	neg	neg
	09.01.98	serum	0.178	neg	neg	neg
	12.01.98	serum	0.057	neg	neg	pos
	19.01.98	serum	0.093	neg	neg	neg
	23.01.98	serum	0.265	neg	neg	neg
	28.01.98	serum	0.262	neg	neg	neg
Patient 38	09.01.98	serum	0.329	neg	neg	neg
Episode 1	09.01.98	BAL	0.216	neg	neg	
	12.01.98	serum	0.207	neg	neg	neg
Patient 38	02.03.98	serum	0.241	neg	neg	neg
Episode 2	03.03.98	BAL	0.285	neg	neg	
	05.03.98	serum	0.214	neg	neg	neg
	06.03.98	serum	0.223	neg	neg	neg
Patient 38	18.03.98	serum	0.211	neg	neg	neg
Episode 3	20.03.98	serum	0.213	neg	neg	neg
	23.03.98	serum	0.275	neg	neg	neg
	25.03.98	serum	0.067	neg	neg	neg
	27.03.98	serum	0.163	neg	neg	neg
	30.03.98	serum	0.325	neg	neg	neg
	06.04.98	serum	0.318	neg	neg	neg
Patient 38	22.04.98	serum	0.064	neg	neg	neg
Episode 4	24.04.98	serum	0.052	neg	neg	neg
	27.04.98	serum	0.187	neg	neg	neg
Patient 39	19.12.97	BAL	0.134	neg	neg	
Episode 1	19.12.97	serum	0.045	neg	neg	neg
	22.12.97	serum	0.022	neg	neg	neg
	24.12.97	serum	0.094	neg	neg	neg

Patient 39	09.01.98	serum	0.126	neg	neg	neg
Episode 2	10.01.98	serum	0.097	neg	neg	neg
	12.01.98	serum	0.162	neg	neg	neg
Patient 39	01.02.98	serum	0.124	neg	neg	neg
Episode 3	02.02.98	serum	0.047	neg	neg	neg
Patient 40	10.12.98	serum	0.214	neg	neg	neg
Episode 1	12.12.98	serum	0.242	neg	neg	neg
	15.12.98	serum	0.126	neg	neg	neg
	17.12.98	serum	0.297	neg	neg	pos
	19.12.98	serum	0.286	neg	neg	neg
	22.12.98	serum	0.304	neg	neg	neg
Patient 40,	12.01.98	serum	0.217	neg	neg	pos
Episode 2	14.01.98	serum	0.289	neg	neg	pos
	16.01.98	serum	0.366	neg	neg	neg
	Disseminated *C. krusei* infection					
Patient 41	10.01.98	serum	0.68	neg	pos	neg
Possible/Probable	12.01.98	serum	0.71	neg	pos	neg
	14.01.98	serum	1.32	doubt	pos	pos
	16.01.98	serum	1.51	pos	pos	pos
	19.01.98	serum	2	pos	pos	pos
	21.01.98	serum	2	pos	pos	pos
	23.01.98	serum	0.321	neg	neg	pos
	26.01.98	serum	2	pos	pos	pos
	28.01.98	serum	2	pos	pos	pos
	30.01.98	serum	2	pos	pos	pos
	02.02.98	serum	2	pos	pos	pos
Patient 42	13.01.98	BAL	0.209	neg	neg	
Proven	14.01.98	serum	0.243	neg	neg	pos
	17.01.98	serum	0.245	neg	neg	pos
	19.01.98	serum	0.159	neg	neg	pos
	21.01.98	serum	0.167	neg	neg	pos
	23.01.98	serum	0.123	neg	neg	pos
	26.01.98	serum	0.234	neg	neg	pos
	28.01.98	serum	0.295	neg	neg	pos
	30.01.98	serum	0.337	neg	neg	pos
	02.02.98	serum	0.287	neg	neg	pos
	04.02.98	serum	0.243	neg	neg	pos
	06.02.98	serum	0.514	neg	pos	pos
	09.02.98	serum	0.682	neg	pos	pos
	11.02.98	serum	0.58	neg	pos	pos
	13.02.98	serum	0.569	neg	pos	pos
	Aspergillus fumigatus grown from lung tissue, histology positive					
	RIP on 13.02.98					
Patient 43	14.01.98	serum	0.132	neg	neg	pos
Episode 1	16.01.98	serum	0.146	neg	neg	neg
	19.01.98	serum	0.131	neg	neg	neg
	21.01.98	serum	0.164	neg	neg	neg
	23.01.98	serum	0.11	neg	neg	pos
	26.01.98	serum	0.197	neg	neg	neg
	28.01.98	serum	0.202	neg	neg	neg
	30.01.98	serum	0.24	neg	neg	neg

	02.02.98	serum	0.211	neg	neg	pos
	04.02.98	serum	0.335	neg	neg	neg
	06.02.98	serum	0.267	neg	neg	pos
	09.02.98	serum	0.262	neg	neg	neg
	11.02.98	serum	0.201	neg	neg	neg
Patient 43	25.02.98	serum	0.177	neg	neg	neg
Episode 2	27.02.98	serum	0.171	neg	neg	neg
	01.03.98	serum	0.182	neg	neg	neg
	03.03.98	serum	0.189	neg	neg	pos
	06.03.98	serum	0.198	neg	neg	neg
	09.03.98	serum	0.161	neg	neg	neg
	11.03.98	serum	0.2	neg	neg	neg
	13.03.98	serum	0.573	neg	pos	neg
	16.03.98	serum	0.273	neg	neg	neg
	18.03.98	serum	0.25	neg	neg	pos
	20.03.98	serum	0.277	neg	neg	neg
	23.03.98	serum	0.207	neg	neg	neg
	25.03.98	serum	0.298	neg	neg	neg
	30.03.98	serum	0.245	neg	neg	neg
Post itraconazole prohpylaxis						
Patient 44	19.01.98	serum	0.125	neg	neg	neg
	23.01.98	serum	0.139	neg	neg	neg
	26.01.98	serum	0.134	neg	neg	neg
	30.01.98	serum	0.135	neg	neg	neg
	02.02.98	serum	0.13	neg	neg	neg
Patient 45	19.01.98	serum	0.143	neg	neg	neg
Episode 1	26.01.98	serum	0.131	neg	neg	neg
	28.01.98	serum	0.136	neg	neg	neg
Patient 45	08.04.98	serum	0.183	neg	neg	neg
Episode 2	10.04.98	serum	0.203	neg	neg	pos
Patient 45	18.05.98	serum	0.222	neg	neg	neg
Episode 3	20.05.98	serum	0.097	neg	neg	neg
	27.05.98	serum	0.196	neg	neg	neg
	29.05.98	serum	0.281	neg	neg	neg
Patient 46	07.02.98	serum	0.444	neg	neg	neg
Probable	10.02.98	serum	0.631	neg	pos	neg
	12.02.98	serum	1.57	pos	pos	pos
	17.02.98	serum	1.643	pos	pos	pos
	19.02.98	serum	1.896	pos	pos	pos
	20.02.98	serum	1.92	pos	pos	pos
	05.03.98	BAL	2	pos	pos	
	07.03.98	serum	2	pos	pos	pos
	09.03.98	serum	1.62	pos	pos	pos
	12.03.98	serum	1.51	pos	pos	pos
	14.03.98	serum	1.121	doubt	pos	pos
	16.03.98	serum	1.184	doubt	pos	pos
	17.03.98	serum	1.522	pos	pos	pos
	19.03.98	serum	0.236	neg	neg	pos
	BMT. RIP. *Aspergillus fumigatus* grown from ETS.					
Patient 47	06.02.98	serum	0.442	neg	neg	neg
	09.02.98	serum	0.224	neg	neg	pos

	11.02.98	BAL	0.186	neg	neg	
	13.02.98	serum	0.249	neg	neg	neg
	16.02.98	serum	0.182	neg	neg	pos
Patient 48	09.02.98	serum	0.196	neg	neg	pos
	11.02.98	serum	0.187	neg	neg	neg
	13.02.98	serum	0.193	neg	neg	neg
	16.02.98	serum	0.303	neg	neg	pos
	18.02.98	serum	0.234	neg	neg	pos
	20.02.98	serum	0.308	neg	neg	neg
	23.02.98	serum	0.241	neg	neg	neg
	25.02.98	serum	0.206	neg	neg	neg
	27.02.98	serum	0.235	neg	neg	neg
	02.03.98	serum	0.174	neg	neg	neg
	04.03.98	serum	0.262	neg	neg	neg
Patient 49	09.02.98	serum	0.226	neg	neg	pos
	13.02.98	serum	0.312	neg	neg	neg
	16.02.98	serum	0.203	neg	neg	pos
Patient 50	11.02.98	serum	0.188	neg	neg	neg
Episode 1	13.02.98	serum	0.186	neg	neg	neg
	16.02.98	serum	0.223	neg	neg	pos
	18.02.98	serum	0.519	neg	neg	pos
	25.02.98	serum	0.604	neg	neg	neg
	27.02.98	serum	0.171	neg	neg	neg
Patient 50	11.03.98	serum	0.247	neg	neg	neg
Episode 2	13.03.98	serum	0.256	neg	neg	neg
	16.03.98	serum	0.188	neg	neg	neg
Patient 50	03.04.98	serum	0.126	neg	neg	neg
Episode 3	05.04.98	serum	0.168	neg	neg	neg
	08.04.98	serum	0.171	neg	neg	pos
	15.04.98	serum	0.176	neg	neg	neg
	17.04.98	serum	0.152	neg	neg	neg
	20.04.98	serum	0.189	neg	neg	neg
	22.04.98	serum	0.176	neg	neg	neg
	24.04.98	serum	0.162	neg	neg	pos
Patient 51	13.02.98	serum	0.45	neg	neg	neg
Episode 1	16.02.98	serum	0.47	neg	neg	pos
Possible	18.02.98	serum	0.288	neg	neg	neg
	20.02.98	serum	0.289	neg	neg	neg
	23.02.98	serum	0.659	neg	pos	neg
	25.02.98	serum	0.206	neg	neg	neg
	27.02.98	serum	0.258	neg	neg	neg
	02.03.98	serum	0.242	neg	neg	pos
	04.03.98	serum	0.22	neg	neg	neg
	05.03.98	serum	0.204	neg	neg	neg
	11.03.98	serum	0.251	neg	neg	pos
	13.03.98	serum	0.177	neg	neg	neg
	16.03.98	serum	0.212	neg	neg	neg
	18.03.98	serum	0.214	neg	neg	neg
	20.03.98	serum	0.183	neg	neg	neg
	23.03.98	serum	0.168	neg	neg	neg
	25.03.98	serum	0.162	neg	neg	pos

	30.03.98	serum	0.197	neg	neg	neg
	01.04.98	serum	0.216	neg	neg	neg
	03.04.98	serum	0.212	neg	neg	neg
	06.04.98	serum	0.386	neg	neg	neg
	08.04.98	serum	0.324	neg	neg	neg
	13.04.98	serum	0.102	neg	neg	neg
	15.04.98	serum	0.097	neg	neg	neg
	17.04.98	serum	0.056	neg	neg	neg
	22.04.98	serum	0.186	neg	neg	pos
	24.04.98	serum	0.171	neg	neg	neg
	27.04.98	serum	0.125	neg	neg	neg
	29.04.98	serum	0.326	neg	neg	neg
	01.05.98	serum	0.181	neg	neg	neg
	06.05.98	serum	0.056	neg	neg	neg
Patient 51	18.05.98	serum	0.121	neg	neg	neg
Episode 2	20.05.98	serum	0.124	neg	neg	neg
	22.05.98	serum	0.167	neg	neg	neg
	27.05.98	serum	0.204	neg	neg	neg
	29.05.98	serum	0.226	neg	neg	neg
Patient 51	15.06.98	serum	0.204	neg	neg	neg
Episode 3	17.06.98	serum	0.212	neg	neg	pos
	19.06.98	serum	0.3	neg	neg	neg
	22.06.98	serum	0.321	neg	neg	neg
	26.06.98	serum	0.368	neg	neg	neg
	30.06.98	serum	0.372	neg	neg	neg
	01.07.98	serum	0.382	neg	neg	neg
	03.07.98	serum	0.511	neg	pos	neg
	06.07.98	serum	0.322	neg	neg	pos
	08.07.98	serum	0.335	neg	neg	neg
	10.07.98	serum	0.386	neg	neg	neg
	15.07.98	serum	0.218	neg	neg	neg
	17.07.98	serum	0.283	neg	neg	neg
	20.07.98	serum	0.256	neg	neg	neg
	22.07.98	serum	0.169	neg	neg	neg
	24.07.98	serum	0.206	neg	neg	neg
	27.07.98	serum	0.212	neg	neg	neg
	28.07.98	BAL	0.189	neg	neg	
	29.07.98	serum	0.167	neg	neg	neg
	31.07.98	serum	0.286	neg	neg	neg
Patient 52	25.03.98	serum	0.126	neg	neg	neg
Possible	30.03.98	serum	0.212	neg	neg	neg
	06.04.98	serum	0.187	neg	neg	neg
	08.04.98	serum	0.162	neg	neg	neg
	01.05.98	serum	0.191	neg	neg	neg
	03.05.98	serum	0.157	neg	neg	neg
	05.05.98	serum	0.182	neg	neg	neg
	08.05.98	serum	0.179	neg	neg	neg
	30.06.98	serum	0.172	neg	neg	pos
	01.07.98	serum	0.206	neg	neg	neg
	03.07.98	serum	0.212	neg	neg	neg
	06.07.98	serum	0.234	neg	neg	pos
	08.07.98	serum	0.2	neg	neg	neg
	10.07.98	serum	0.19	neg	neg	neg
	15.07.98	serum	0.224	neg	neg	neg

	17.07.98	serum	0.214	neg	neg	neg
	20.07.98	serum	0.197	neg	neg	neg
	22.07.98	serum	0.186	neg	neg	neg
	24.07.98	serum	0.132	neg	neg	neg
	27.07.98	serum	0.187	neg	neg	neg
	29.07.98	serum	0.268	neg	neg	neg
	31.07.98	serum	0.212	neg	neg	neg
Patient 53	16.02.98	serum	0.213	neg	neg	neg
Episode 1	18.02.98	serum	0.306	neg	neg	pos
	20.02.98	serum	0.275	neg	neg	neg
	23.02.98	serum	0.218	neg	neg	pos
	25.02.98	serum	0.187	neg	neg	neg
	27.02.98	serum	0.29	neg	neg	neg
	02.03.98	serum	0.265	neg	neg	neg
	04.03.98	serum	0.201	neg	neg	neg
Patient 53	16.03.98	serum	0.177	neg	neg	neg
Episode 2	18.03.98	serum	0.241	neg	neg	neg
	20.03.98	serum	0.176	neg	neg	neg
	23.03.98	serum	0.152	neg	neg	neg
	25.03.98	serum	0.145	neg	neg	neg
	30.03.98	serum	0.097	neg	neg	neg
	01.04.98	serum	0.126	neg	neg	pos
	03.04.98	serum	0.187	neg	neg	neg
	06.04.98	serum	0.216	neg	neg	pos
	08.04.98	serum	0.202	neg	neg	neg
	15.04.98	serum	0.052	neg	neg	neg
	17.04.98	serum	0.122	neg	neg	neg
Patient 54	11.03.98	serum	0.281	neg	neg	neg
Episode 1	13.03.98	serum	0.397	neg	neg	neg
	16.03.98	serum	0.179	neg	neg	neg
	22.03.98	serum	0.186	neg	neg	neg
	25.03.98	serum	0.163	neg	neg	neg
	30.03.98	serum	0.128	neg	neg	neg
	01.04.98	serum	0.199	neg	neg	pos
	03.04.98	serum	0.243	neg	neg	neg
	06.04.98	serum	0.297	neg	neg	pos
	08.04.98	serum	0.252	neg	neg	neg
Patient 54	15.04.98	serum	0.603	neg	pos	neg
Episode 2	17.04.98	serum	0.192	neg	neg	neg
Patient 54	15.07.98	serum	0.168	neg	neg	neg
Episode 3	20.07.98	serum	0.129	neg	neg	neg
	24.07.98	serum	0.176	neg	neg	neg
	27.07.98	serum	0.171	neg	neg	neg
	29.07.98	serum	0.129	neg	neg	neg
	31.07.98	serum	0.142	neg	neg	neg
Patient 55	02.03.98	serum	0.489	neg	neg	neg
Episode 1	06.03.98	serum	0.242	neg	neg	neg
	13.03.98	serum	0.236	neg	neg	neg
	16.03.98	serum	0.28	neg	neg	neg
	20.03.98	serum	1.428	doubt	pos	neg
	23.03.98	serum	0.187	neg	neg	neg
	27.03.98	serum	0.163	neg	neg	neg

	30.03.98	serum	0.287	neg	neg	neg
Patient 55	01.05.98	serum	0.296	neg	neg	neg
Episode 2	03.05.98	serum	0.312	neg	neg	neg
Patient 55	26.06.98	serum	0.286	neg	neg	neg
Episode 3	30.06.98	serum	0.278	neg	neg	neg
	01.07.98	serum	0.333	neg	neg	neg
	03.07.98	serum	0.389	neg	neg	neg
	06.07.98	serum	0.312	neg	neg	neg
	08.07.98	serum	0.426	neg	neg	pos
	10.07.98	serum	0.387	neg	neg	neg
	15.07.98	serum	0.324	neg	neg	neg
	17.07.98	serum	0.187	neg	neg	neg
	20.07.98	serum	0.287	neg	neg	neg
	22.07.98	serum	0.302	neg	neg	neg
	24.07.98	serum	0.256	neg	neg	neg
Patient 56	04.03.98	serum	0.284	neg	neg	neg
Episode 1	06.03.98	serum	0.22	neg	neg	neg
	11.03.98	serum	0.559	neg	pos	neg
	13.03.98	serum	0.23	neg	neg	neg
	16.03.98	serum	0.215	neg	neg	neg
	18.03.98	serum	0.193	neg	neg	neg
Patient 56	24.04.98	serum	0.198	neg	neg	neg
Episode 2	27.04.98	serum	0.052	neg	neg	neg
	01.05.98	serum	0.187	neg	neg	neg
	03.05.98	p.fluid	0.092	neg	neg	neg
	06.05.98	serum	0.126	neg	neg	neg
Patient 57	16.03.98	serum	0.175	neg	neg	neg
	18.03.98	serum	0.258	neg	neg	neg
	20.03.98	serum	0.204	neg	neg	neg
	23.03.98	serum	0.212	neg	neg	neg
	25.03.98	serum	0.206	neg	neg	neg
	30.03.98	serum	0.312	neg	neg	pos
	01.04.98	serum	0.268	neg	neg	neg
	03.04.98	serum	0.255	neg	neg	neg
	06.04.98	serum	0.257	neg	neg	neg
	08.04.98	serum	0.281	neg	neg	neg
	15.04.98	serum	0.162	neg	neg	pos
	17.04.98	serum	0.184	neg	neg	neg
	22.04.98	serum	0.167	neg	neg	neg
Patient 58	21.03.98	serum	0.097	neg	neg	neg
Episode 1	23.03.98	serum	0.054	neg	neg	neg
	25.03.98	serum	0.102	neg	neg	neg
	27.03.98	serum	0.191	neg	neg	neg
	30.03.98	serum	0.168	neg	neg	neg
Patient 58	22.04.98	serum	0.062	neg	neg	neg
Episode 2	24.04.98	serum	0.024	neg	neg	neg
	27.04.98	serum	0.122	neg	neg	neg
	01.05.98	serum	0.137	neg	neg	neg
	06.05.98	serum	0.168	neg	neg	neg
	15.05.98	serum	0.127	neg	neg	neg
	18.05.98	serum	0.199	neg	neg	neg
	20.05.98	serum	0.164	neg	neg	neg

	22.05.98	serum	0.1	neg	neg	pos
	27.05.98	serum	0.12	neg	neg	neg
Patient 58	10.06.98	serum	0.163	neg	neg	neg
Episode 3	10.06.98	p.fluid	0.202	neg	neg	neg
	12.06.98	serum	0.187	neg	neg	neg
	15.06.98	serum	0.143	neg	neg	neg
	19.06.98	serum	0.123	neg	neg	neg
	22.06.98	serum	0.167	neg	neg	pos
	26.06.98	serum	0.152	neg	neg	neg
	30.06.98	serum	0.193	neg	neg	neg
	01.07.98	serum	0.197	neg	neg	neg
	03.07.98	serum	0.137	neg	neg	neg
Patient 59	25.03.98	serum	0.165	neg	neg	neg
Episode 1	27.03.98	serum	0.192	neg	neg	neg
	30.03.98	serum	0.163	neg	neg	pos
	03.04.98	serum	0.289	neg	neg	neg
Patient 59	20.04.98	c.blood	0.246	neg	neg	neg
Episode 2	20.04.98	serum	0.29	neg	neg	neg
	22.04.98	serum	0.278	neg	neg	neg
Patient 60	17.04.98	serum	0.312	neg	neg	neg
	19.04.98	serum	0.256	neg	neg	neg
	22.04.98	serum	0.267	neg	neg	neg
Patient 61	20.04.98	serum	0.126	neg	neg	neg
	22.04.98	serum	0.134	neg	neg	neg
	24.04.98	serum	0.187	neg	neg	neg
Patient 62	06.05.98	serum	0.296	neg	neg	neg
	15.05.98	serum	0.187	neg	neg	neg
	18.05.98	serum	0.192	neg	neg	neg
	20.05.98	serum	0.203	neg	neg	neg
	22.05.98	serum	0.212	neg	neg	neg
	27.05.98	serum	0.197	neg	neg	neg
	29.05.98	serum	0.165	neg	neg	neg
Patient 63	15.06.98	serum	0.192	neg	neg	neg
	17.06.98	serum	0.203	neg	neg	neg
	19.06.98	serum	0.156	neg	neg	neg
	22.06.98	serum	0.187	neg	neg	pos
	26.06.98	serum	0.134	neg	neg	neg
	30.06.98	BAL	0.083	neg	neg	
	01.07.98	serum	0.189	neg	neg	neg
	03.07.98	serum	0.067	neg	neg	neg
	06.07.98	serum	0.052	neg	neg	neg
	08.07.98	serum	0.162	neg	neg	neg
	10.07.98	serum	0.187	neg	neg	neg
	15.07.98	serum	0.156	neg	neg	neg
	17.07.98	serum	0.219	neg	neg	neg
	20.07.98	serum	0.234	neg	neg	neg
	22.07.98	serum	0.192	neg	neg	neg
	24.07.98	serum	0.12	neg	neg	neg
	27.07.98	serum	0.076	neg	neg	neg
	29.07.98	serum	0.111	neg	neg	neg

	31.07.98	serum	0.097	neg	neg	neg
Patient 64	20.04.98	serum	0.182	neg	neg	neg
	22.04.98	serum	0.068	neg	neg	neg
	27.04.98	serum	0.123	neg	neg	neg
Patient 65	20.04.98	serum	0.186	neg	neg	neg
	22.04.98	serum	0.178	neg	neg	neg
	27.07.98	serum	0.312	neg	neg	neg
	29.07.98	serum	0.145	neg	neg	neg
	31.07.98	serum	0.132	neg	neg	neg
Patient 66	15.5.98	serum	0.197	neg	neg	neg
Episode 1	18.5.98	serum	0.126	neg	neg	neg
	20.5.98	serum	0.086	neg	neg	neg
	22.5.98	serum	0.134	neg	neg	neg
Patient 66	27.05.98	serum	0.312	neg	neg	neg
Episode 2	29.05.98	serum	0.298	neg	neg	neg
	26.06.98	serum	0.345	neg	neg	neg
	28.06.98	serum	0.412	neg	neg	neg
	30.06.98	serum	0.296	neg	neg	neg
	01.07.98	serum	0.181	neg	neg	neg
	03.07.98	serum	0.167	neg	neg	pos
	06.07.98	serum	0.109	neg	neg	neg
	08.07.98	serum	0.397	neg	neg	neg
	10.07.98	serum	0.364	neg	neg	neg
	15.07.98	serum	0.352	neg	neg	neg
	17.07.98	serum	0.286	neg	neg	neg
	20.07.98	serum	0.197	neg	neg	neg
	22.07.98	serum	0.184	neg	neg	neg
	24.07.98	serum	0.087	neg	neg	pos
	27.07.98	serum	0.153	neg	neg	neg
Patient 67	27.02.98	serum	0.475	neg	neg	neg
Possible/probable	28.02.98	serum	0.349	neg	neg	neg
	02.03.98	serum	0.791	neg	pos	pos
	03.03.98	serum	0.621	neg	pos	pos
	05.03.98	serum	1.26	doubt	pos	pos
	07.03.98	serum	1.54	pos	pos	neg
	10.03.98	serum	1.01	doubt	pos	neg
	14.03.98	serum	0.87	neg	pos	neg
Patient 68	04.04.98	serum	0.056	neg	neg	neg
Episode 1	09.04.98	serum	0.124	neg	neg	neg
Patient 68	03.06.98	serum	0.097	neg	neg	neg
Episode 2	05.06.98	serum	0.085	neg	neg	neg
Patient 69	03.04.98	serum	0.126	neg	neg	neg
Episode 1	04.04.98	serum	0.172	neg	neg	neg
	06.04.98	serum	0.145	neg	neg	pos
Patient 69	20.04.98	serum	0.087	neg	neg	neg
Episode 2	22.04.98	serum	0.186	neg	neg	neg
Patient 70	15.06.98	serum	0.216	neg	neg	neg
Episode 1	17.06.98	serum	0.203	neg	neg	neg

	19.06.98	serum	0.187	neg	neg	neg
Patient 70	22.06.98	serum	0.192	neg	neg	neg
Episode 2	26.06.98	serum	0.236	neg	neg	neg
	30.06.98	serum	0.216	neg	neg	neg
	03.07.98	serum	0.21	neg	neg	neg
Patient 71	17.06.98	serum	0.384	neg	neg	neg
Episode 1	19.06.98	serum	0.361	neg	neg	neg
	22.06.98	serum	0.181	neg	neg	neg
	26.06.98	serum	0.267	neg	neg	neg
	30.06.98	serum	0.328	neg	neg	neg
	01.07.98	serum	0.162	neg	neg	neg
	03.07.98	serum	0.087	neg	neg	neg
Patient 71	10.07.98	serum	0.326	neg	neg	pos
Episode 2	15.07.98	serum	0.339	neg	neg	neg
	17.07.98	serum	0.46	neg	neg	neg
	20.07.98	serum	0.32	neg	neg	neg
	22.07.98	serum	0.487	neg	neg	neg
	24.07.98	serum	0.365	neg	neg	pos
	27.07.98	serum	0.342	neg	neg	neg
	29.07.98	serum	0.216	neg	neg	neg
	31.07.98	serum	0.056	neg	neg	neg
Patient 72	17.06.98	serum	0.036	neg	neg	neg
	19.06.98	serum	0.057	neg	neg	neg
	22.06.98	serum	0.102	neg	neg	neg
	26.06.98	serum	0.092	neg	neg	neg
	30.06.98	serum	0.067	neg	neg	pos
	01.07.98	serum	0.187	neg	neg	neg
	03.07.98	serum	0.222	neg	neg	neg
	06.07.98	serum	0.246	neg	neg	pos
	08.07.98	serum	0.2	neg	neg	neg
	10.07.98	serum	0.216	neg	neg	neg
Patient 73	17.06.98	serum	0.306	neg	neg	neg
	19.06.98	serum	0.322	neg	neg	neg
	22.06.98	serum	0.281	neg	neg	neg
	26.06.98	serum	0.326	neg	neg	neg
Patient 74	26.06.98	serum	0.186	neg	neg	neg
	28.06.98	serum	0.172	neg	neg	neg
	30.06.98	serum	0.174	neg	neg	pos
	01.07.98	serum	0.151	neg	neg	neg
	03.07.98	serum	0.126	neg	neg	neg
	06.07.98	serum	0.281	neg	neg	pos
	08.07.98	serum	0.157	neg	neg	neg
	10.07.98	serum	0.202	neg	neg	neg
	12.07.98	serum	0.126	neg	neg	neg
	15.07.98	serum	0.187	neg	neg	neg
	17.07.98	serum	0.195	neg	neg	neg
	20.07.98	serum	0.163	neg	neg	neg
	22.07.98	serum	0.145	neg	neg	neg
Patient 75	15.07.98	serum	0.103	neg	neg	pos
	17.07.98	serum	0.121	neg	neg	neg

20.07.98	serum	0.098	neg	neg	neg
22.07.98	serum	0.057	neg	neg	neg
24.07.98	serum	0.063	neg	neg	neg
27.07.98	serum	0.089	neg	neg	neg
29.07.98	serum	0.097	neg	neg	neg
31.07.98	serum	0.102	neg	neg	neg

7.2 DETAILS OF PRIMER AND PROBE SEQUENCES

Primers 5'-ATTGGAGGGCAAGTCTGGTG and
5'-CCGATCCCTAGTCGGCATAG

DNA probe specifically hybridising with *A. fumigatus*, *A. flavus*, and *A. versicolor*
(TGGGGAACCTCATGGCCTTCACTGGCTGTG)

were used in this study.

7.3 SUGGESTED PRECAUTIONS AGAINST AMPLICON CONTAMINATION OF PCR (Kwok and Higuchi, 1989)

1. All reagents are stored in small aliquots to avoid frequent opening of tubes.
2. Separate sets of pipettes are used for preparing cells and reagents for PCR, and for manipulations of the PCR products.
3. All pipette tips and reaction tubes are disposable single use items.
4. Designated rooms are used for evaluation and manipulation of PCR products.
5. No plastics or reagents used in the PCR reactions are permitted to enter the above designated rooms.
6. Water for use in the PCR reactions is prepared at a remote location and received in sealed bottles. The water is stored in 1ml aliquots, and any residue of an opened aliquot is discarded after single use.
7. The PCR buffer is prepared at a remote location and delivered in small aliquots in screw capped tubes.
8. Cells are prepared for the PCR reaction in an exhaust ventilated safety cabinet until heat inactivated, and all other manipulations of cells as well as preparation of the PCR reaction mix are performed in a vertical laminar flow cabinet.
9. The laminar flow cabinet is positioned in a locked room in a remote part of the building and is not used for any purpose other than preparation of PCR reactions.
10. The laboratory coat is changed on entering and leaving the room housing the laminar flow cabinet.
11. Disposable gloves are worn at all times, and are always changed on entry to the room housing the laminar flow cabinet.

12. To determine if contamination of the reagents or plastics sufficient to cause a false positive result has occurred, negative controls containing all reagents except for the template DNA are included in every set of PCR reactions.

7.4 PUBLICATIONS, ABSTRACTS AND PRESENTATIONS RELATING TO THIS WORK

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