Investigations on *Aspergillus fumigatus* double-stranded RNAs and their effects on the fungus

By:

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Dedicated

to

My Parents and Grandparents

Statement of Originality

The material in this thesis has not been previously submitted for a degree in any university, and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

Abstract

The aim of this research was to assess the incidence of dsRNA mycoviruses in the opportunistic human pathogenic fungus *Aspergillus fumigatus*, where previously no dsRNA viruses had been reported and to investigate the effects of any dsRNAs on the growth and pathogenicity of the fungus. Thus far 366 isolates (clinical and environmental) have been screened, 24 of which posses dsRNA elements. Successful efforts were made to completely characterise the two dsRNA segments of the isolate 88, partitivirus to obtain novel sequence information.

Fungal viruses or mycoviruses are widespread and they usually infect their hosts persistently without any detectable phenotypic effects. They have been however linked with both hypovirulence and hypervirulence but are normally cryptic. To obtain information on the effect of the dsRNAs on their respective hosts, efforts were made to 'cure' isolate 88 of its dsRNA infection by cycloheximide treatment. However, following cycloheximide treatment, a sensitive reverse transcription polymerase chain reaction (RT-PCR) amplification assay showed that the dsRNA elements, whilst being reduced in amount, were not eliminated completely and that high levels of cycloheximide also interfered with spore production, pigmentation and overall growth of the isolate. In further experiments attempts were made to mobilise the dsRNAs from 4 isolates *viz.* A-56, A-54, A-78 and isolate 88 into isolate Af-273y, which is hygromycin resistant and yellow in colour, by hyphal tip fusion, protoplast fusion and protoplast transfection with purified virus. Protoplast fusion and viral transfection experiments were successful for some isolates, as assessed by the RT-PCR assay and small scale extractions of nucleic acids. Subsequently comparative growth experiments by radial growth assay and mycelial weight measurements between isolate Af-273y and Af-273y transfected with isolate 88 partitivirus in essentially the same genetic background were performed. These experiments showed that the partitivirus infection resulted in a sectored phenotype and significantly lowered the growth of the fungus. All efforts to initiate the molecular characterisation of uncharacterised dsRNA elements found in isolates A-54, A-78 and isolate-66 have thus far proven unsuccessful but a new approach (cDNA library construction) is proposed for the characterisation of these dsRNAs.

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Abbreviations

Buffers and Media

LB	Luria-Bertani
MM	Minimal media
ACM	Aspergillus complete media
SOC	Super optimal broth
STE buffer	Sodium chloride–Tris EDTA
SSPE buffer	Saline-sodium phosphate-EDTA
SSC buffer	Saline-sodium citrate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
Tris	Tris (hydroxyl methyl) aminomethane
HEPES	(4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)

Chemicals and bio-chemicals

PEG	Polyethylene glycol
DMSO	Dimethyl sulphoxide
APS	Ammonium persulphate
BSA	Bovine serum albumin
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
IPTG	Isopropyl-1-thio-β-D-galactoside
TEMED	N, N, N', N', -tetramethylethylenediamine
SDS	Sodium dodecyl sulphate
Magnesium chloride	$MgCl_2$
Potassium chloride	KCl
Magnesium sulphate	$MgSO_4$
4-aminobenzoic acid	$H_2NC_6H_4CO_2H$
Biotin	$C_{10}H_{16}N_2O_3$
Meso-inositol	$C_6H_{12}O_6$
Nicotinic acid	$C_6H_5NO_2$
DL-pantothenic acid (hemicalcium salt)	$C_{18}H_{32}CaN_2O_{10}$

Pyridoxine monohydrochloride	$C_{19}H_{26}O_3$
Riboflavin	$C_{17}H_{20}N_4O_6$
Choline chloride	$C_5H_4NO.Cl$
Sodium tetraborate	$Na_2B_4O_7.10H_2O$
Copper chloride pentahydrate	$CuCl_2.5H_2O$
Iron chloride	$FeCl_3.H_2O$
Magnesium sulphate tetrahydrate	$MnSO_4.4H_2O$
Sodium molybdate	$Na_2MoO_4.2H_2O$
Zinc sulphate heptahydrate	$ZnSO_4.\ 7H_2O$
Lithium chloride	LiCl
Sodium acetate	$C_2H_3NaO_2$
Potassium dihydrogen orthophosphate	KH_2PO_4
Dipotassium hydrogen orthophosphate	K_2HPO_4
Sodium citrate	$Na_3C_6H_5O_7$
Sodium dihydrogen orthophosphate monohydrate	$NaH_2PO_4 \cdot H_2O$
Disodium hydrogen orthophosphate	Na_2HPO_4
Ammonium sulphate	$(NH_4)_2SO_4$

Others.

Da	Dalton
X *g*	Relative centrifugal field
kcal	Kilo calorie
M	Molar
OD	Optical density
rpm	Revolutions per minute
U	Unit of enzyme activity
RLM RACE	RNA linker-mediated rapid amplification of cDNA ends
vic	Vegetative incompatibility
het	Heterokaryon incompatibility
VCG	Vegetative compatibility group
CP	Coat protein
RdRP	RNA dependent RNA polymerase

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Chapter -1

Introduction

1. Introduction

1.1 Fungal viruses

Viral infections are ubiquitous in the kingdom fungi and are termed mycoviruses (Buck, 1998). They are commonly resident in the cytoplasm and occasionally exist in association with mitochondria (Buck, 1986; Polashock and Hillman, 1994; Varga *et al.*, 2003). The genome of mycoviruses frequently contains segmented double-stranded (ds) RNA and packaged in isometric structures. DsRNA viruses are a significantly large group of pathogens, which have effects on a wide range of species, including terrestrial and non-terrestrial vertebrates, invertebrates, plants, prokaryotes and fungi (Mertens, 2004). However, limited numbers of host species have been studied (Ghabrial and Suzuki, 2009).

Mycoviruses may or may not be associated with morphological changes which range from highly debilitating effects to no effect on its host and can lead to either hypovirulence (attenuation of its host) or may confer hypervirulence and increase fungal virulence (Ghabrial and Suzuki, 2009). The potential effect of these viruses on the levels of toxins and metabolites produced by fungi and also the possible implications of these effects on interferon induction, toxin production, genetics of fungi, and plant diseases, enhance their significance in environmental health research (Bozarth, 1972). The presence of viruses in fungi opened a new horizon in experimental mycology as these viruses may affect the metabolism and genetics of fungal cells (Lemke and Nash, 1974).

1.2 Discovery of dsRNAs in fungi

Mycoviruses were first isolated in 1962 from cultivated mushrooms (Hollings, 1962). In 1967, further evidence of fungal viruses was found in ascomycetes (Dieleman-van Zaayen, 1967). In the late 1960s, studies on interferon induction by factors found in *Penicillium stoloniferum* and *P. funiculosum* revealed that the interferons (antiviral proteins) generated were of viral origin, which were associated with dsRNA elements (Banks *et al.*, 1968). Biochemical characterisation of these and other mycoviruses isolated since that time have led to the conclusion that the genome of most fungal viruses consists of dsRNA (Buck, 1986) but single stranded RNA (ssRNA) containing viruses are also known (Pearson *et al*., 2009).

The discovery of mycoviruses increased the prospects of mycological research since virus infection was thought to be the cause of abnormalities and unusual activities associated with

certain fungi and the viruses with potentially adverse effects on their fungal hosts might be exploited as a means of biological control (Rawlinson *et al*., 1973; Ghabrial, 1980).

Several benign dsRNA species in the genus *Endornavirus* have also been isolated from plants and appear to be non-pathogenic (Fukuhara *et al.,* 1993). New pathogenic, enigmatic mycoviruses are also constantly emerging (Rao *et al.,* 2007). According to most recent reports of the International Committee on Taxonomy of Viruses (ICTV), over 90 fungal viruses have been identified thus far (Pearson *et al.,* 2009). However, the host range of these mycoviruses is not confined to all four major phyla of true fungi *viz.* Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota, but they also infect plant pathogenic oomycetes, like *Phytophthora* spp. (Ghabrial and Suzuki, 2009). It is also predicted that a great number of unrecognised mycoviruses exist in nature (Ghabrial and Suzuki, 2008). There is need for extensive research in the field of mycology and virology to elucidate the role of emerging mycoviruses in different fungi.

1.3 Classification of dsRNA mycoviruses

The ICTV report on virus taxonomy lists 10 mycoviral families (Fauquet *et al.,* 2005). Five of the dsRNA families harbour fungi as their primary host. Important families of dsRNA viruses along with their structures can be seen in Fig. 1.1 Over 20% of these viruses are unassigned to a genus and a similar number to a viral family. Uncertainty in assignment is due to a lack of sufficient sequence data which makes it difficult to confidently assign these viruses to families and genera. Moreover, in fungi many unencapsidated dsRNA species have been reported, which are believed to be viral in nature, but because of lack of sequence data remain uncharacterised.

The largest numbers of these dsRNA viruses are found in the family *Reoviridae*. It has the largest and most diverse host range and includes fungi, protozoa, plants and vertibrates. The particles of this family are spherical and possess icosahedral symmetry. Fungal infecting members of the family *Reoviridae* (genus *Mycoreovirus*) are composed of 11-12 segments of dsRNA ranging from 732 bp-4127 bp in size (Mertens *et al.,* 2005).

The *Totiviridae* are another unique family of dsRNA viruses. Their genomes consist of non-segmented, encapsidated, dsRNA which ranges in size from 4.6-7.0 kbp and infect fungi and protozoa. The genome consists of two open reading frames (ORFs) which usually overlap, encoding the coat protein (CP) and the RNA dependent RNA polymerase (RdRP; Wickner *et*

al., 2005). Two more dsRNA families, the *Partitiviridae* and *Chrysoviridae* are discussed in detail in sections 1.3.1 and 1.3.3.

Moreover a number of ssRNA mycoviruses exist for instance, family *Hypoviridae* and *Endornaviridae* which do not posses CPs and their existence in the host is predominantly in the dsRNA replicative form and are unencapsidated, an important characteristic of a true virions. Although these viruses are classified and grouped with dsRNA viruses their lineage and mode of replication are indicative of ssRNA viruses. *Hypoviruses* have a linear genome 9-13 kbp in size whilst, endornaviruses are comprised of a single large dsRNA 14-17 kbp in size and *Phytophthora* endornavirus (PEV1) is only non-plant virus in the genus *Endornavirus* (Ghabrial and Suzuki, 2009).

Table **1.1** shows the dsRNA virus families, the number of genomic segments, their hosts and morphology of virus particles.

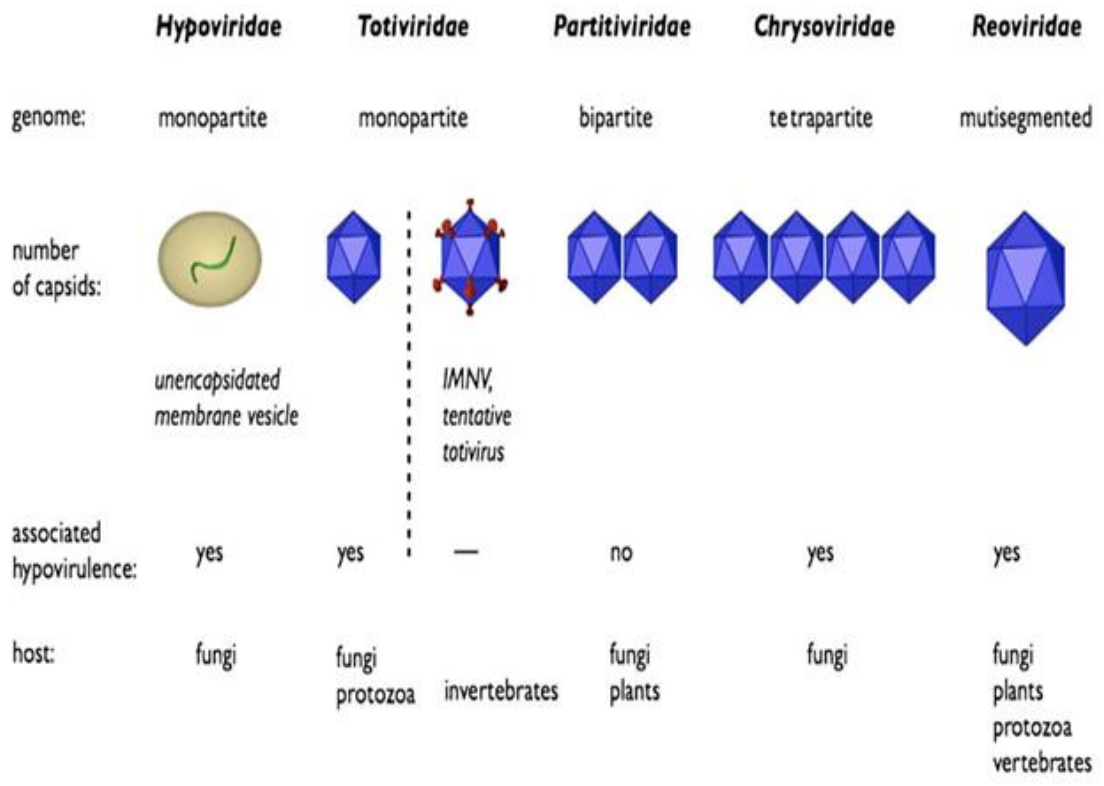


Figure 1.1 Important mycovirus families shown with the genome composition, virus structure, associated hypovirulence and host range of respective viral families (adapted from Van de Sande *et al.*, [2010]).

Table 1.1 DsRNA mycovirus families

dsRNA family	Number of genomic segments	Host	Morphology of virus particles
Reoviridae	10, 11 or 12 (co-packaged)	Fungi	70–90 nm diameter icosahedral (one, two or three layered capsid protein.
Chrysoviridae	4 (packaged separately)	Fungi	30–40 nm diameter, icosahedral capsid protein, multiple components.
Totiviridae	1 (packaged singly)	Fungi	30–40 nm diameter, icosahedral capsid proteins.
Hypoviridae	1 (unpackaged)	Fungi	50–80 nm diameter, pleomorphic vesicles (no capsid).
Partitiviridae	2 (separately packaged)	Fungi, plants	30–40 nm diameter, icosahedral protein capsid.

(Table 1.1 Adapted from Mertens, 2004; Fauquet *et al.*, 2005).

1.3.1 Family *Partitiviridae*

Mycoviruses, obtained from *Penicillium* spp. were among the first to be characterised at the molecular level and were shown to have segmented genomes and those with bipartite genomes are currently classified in the genus *Partitivirus*, in the family *Partitiviridae* (Ghabrial *et al.*, 2008a). The name *Partitiviridae* originates from the Latin word *partitus*, which means 'divided' and that the genome of these viruses is bipartite (separately encapsidated). The family *Partitiviridae* consists of viruses which possess a monocistronic bipartite genome, with the RdRP and CP genes found as ORFs on respectively the larger and smaller individual dsRNA species (Ghabrial, 1998). Each of the bipartite genomic segments of partitiviruses range in size from 1.8-2.2 kbp and the virus possess unenveloped, icosahedral particles *ca.* 30-40 nm in diameter (Crawford *et al.*, 2006). The virion capsid consists of 12 capsomeres and 120 CP

subunits arranged in a T=2 lattice formation, with a molecular mass ranging from 57 to 76 kDa. (Tavantzis, 2008).

Partitiviruses may also possess smaller, satellite or defective dsRNAs which are probably generated as a consequence of deletion or recombination of extant dsRNAs. The family *Partitiviridae* had been divided into four genera namely *Partitivirus*, *Chrysovirus*, *Alphacryptovirus* and *Betacryptovirus*, the first two of which exclusively infect fungi whilst the latter two infect plants (Ghabrial, 2001). The *Chrysovirus* genus has now been placed in a separate family called *Chrysoviridae* and the family *Partitiviridae* now contains only 3 genera (Coutts *et al.*, 2004).

Bearing this in mind the differences that exist concerning host affinity and transmission it would be of interest to investigate further the taxonomy of the family *Partitiviridae* particularly regarding structural homology of the plant-infecting members (Ochoa *et al.*, 2008). The complete genomic sequences of numerous fungal partitiviruses are available in databases and the list is being added to on a regular basis. Below Table 1.2 shows the different genera and species of partitiviruses known to date.

Table 1.2 List of recognised and tentative members in the family *Partitiviridae*, length of their genomes, size of encoded gene products, and GeneBank accession numbers of their genomic sequence.

Sr. No.	**Virus**	**Abbreviation**	**DsRNA segment no. (size in bp, encoded protein, size in kDa)**	**GenBank accession**
	Genus: Partitivirus			
1	*Aspergillus fumigatus* partitivirus*	AfuPV	1 (1779; RdRP, 63)	FN376847
			2 (1623; CP, 48)	FN398100
2	*Aspergillus ochraceous* virus*	AoRV	1 (1754; RdRP, 62)	EU118277
			2 (1555; CP, 47)	EU118278
			3 (1220; unknown, 34)	EU118279
3	*Atkinsonella hypoxylon* virus*	AhV	1 (2180; RdRP, 78)	L39125
			2 (2135; CP, 74)	L39126
			3 (1790; satellite)	L39127
4	*Botryotinia fuckeliana* partitivirus-1*	BfPV	1 (1793; RdRP, 63)	AM491609
			2 (1566; CP, 48)	AM491610

			3 (1383; unknown, 14)	AM491611
5	*Ceratocystis resinifera* virus	CrV	1 (2207; RdRP, 77)	AY603052
			2 (2305; CP, 73)	AY603051
6	*Ceratocystis polonica* virus	CpV	1 (2315; RdRP, 77)	AY260756
			2 (2252; CP, 73)	AY247205
7	*Discula destructiva* virus 1*	DdV1	1 (1787; RdRP, 62)	NC_002797
			2 (1585; CP, 48)	NC_002800
			3 (1181; satellite)	NC_002801
			4 (308; satellite)	NC_002802
8	*Discula destructiva* virus 2*	DdV2	1 (1781; RdRP, 62)	NC_003710
			2 (1611; CP, 50)	NC_003711
9	*Flammulina velutipes browning virus*	FvBV	1(1915; RdRP,66)	AB465308
			2(1730; CP, 60)	AB465309
10	*Flammulina velutipes* isometric virus	FvIV	1 (1919; RdRP, 69)	AB428575
11	*Fusarium poae* virus 1*	FpV-1	1 (2203; RdRP, 78)	NC_003884
			2 (2185; CP, 70)	NC_003883
12	*Fusarium solani* virus 1*	FsV-1	1 (1645; RdRP, 60)	D55668
			2 (1445; CP, 44)	D55669
13	*Gremmeniella abietina*	GaV-MS1	1 (1782; RdRP, 61)	NC_004018
	virus MS1*		2 (1586; CP, 47)	NC_004019
			3 (1186; satellite)	NC_004020
14	*Gremmeniella abietina*	GaV-MS2	1 (1781; RdRP, 62)	NC_006444
	virus MS2*		2 (1586; CP, 47)	NC_006445
			3 (1186; satellite)	NC_006446
15	*Helicobasidium mompa* partitivirus*	HmV-V1-1	1 (2247; RdRP, 83)	AB110979
		-V1-2	1 (1776; RdRP, 63)	AB110980
		-V-70	1 (1928; RdRP, 70)	AB025903
16	*Heterobasidion* RNA virus 3	HetRV3-ec1	1 (1885; RdRP, 69)	FJ816271
			2(1826; CP, 57)	FJ816272
17	*Heterobasidion* RNA virus 2	HetRV2-pa1	1 (2290; RdRP, 85)	HM565953
			2 (2238; CP, 73)	HM565954
18	*Heterobasidion annosum* P-type	HaPV	1 (2325; RdRP, 87)	AF473549
	partitivirus*			
19	*Ophiostoma* partitivirus 1	OPV-1	1 (1744; RdRP, 63)	AM087202
			2 (1567; CP, 46)	AM087203
20	Oyster mushroom isometric virus 2	OMV	1 (2038; RdRP, 70)	AY308801

21	*Penicillium stoloniferum* virus F*	PsV-F	1 (1677; RdRP, 62)	NC_007221
			2 (1500; CP, 47)	NC_007222
			3 (677; unknown)	NC_007223
22	*Penicillium stoloniferum* virus S*	PsV-S	1 (1753; RdRP, 62)	AM040148
			2 (1581; CP, 47)	AM040149
23	*Pleurotus ostreatus* virus	PoV	1 (2296; RdRP, 82)	NC_006961
			2 (2223; CP, 71)	NC_006960
24	*Rhizoctonia solani* virus 717*	RhsV-717	1 (2363; RdRP, 86)	NC_003801
			2 (2206; CP, 76)	NC_003802
25	*Rosellinia necatrix* virus 1-W8	RnV-1-W8	1 (2299; RdRP, 84)	NC_007537
			2 (2279; CP, 77)	NC_007538
26	*Sclerotinia sclerotiorum*	SsPV-S	1 (1874; RdRP, 68)	NC_013014
	partitivirus S			
	Genus: *Alphacryptovirus*			
1	Beet cryptic virus 1*	BCV-1	1 (2008; RdRP, 73)	EU489061
			2 (1783; CP, 53)	EU489062
2	Beet cryptic virus 3*	BCV-3	2 (1607; RdRP, 55)	S63913
3	Carrot cryptic virus*	CaCV	1 (1971; RdRP, 73)	FJ550604
			2 (1776; CP, 54)	FJ550605
4	*Chondrostereum purpureum*	CPCV	1 (1920; RdRP, 68)	AM999771
	cryptic virus 1*		2 (1757; CP; 53)	AM999772
5	*Fig cryptic virus*	FCV	1 (1696; RdRP, 54)	FR687854
			2 (1415; CP, 38)	FR687855
6	*Fragaria chiloensis* cryptic virus*	FCCV	1 (1734; RdRP, 56)	NC_009519
			2 (1479; CPA, 39)	NC_009521
			2 (1465; CPB, 39)	NC_009520
7	*Pyrus pyrifolia* cryptic virus*	PpV	1 (1592; RdRP, 55)	AB012616
8	*Raphanus sativus* cryptic virus 1*	RSCV-1	1 (1866; RdRP, 67)	NC_008191
	(or Radish yellow edge virus*)	(RYEV)	2 (1791; CP, 56)	NC_008190
			3 (1778; CPB, 55)	EU285027
9	*Raphanus sativus* cryptic virus 2*	RSCV-2	1 (1717; RdRP, 55)	DQ218036
			2 (1521; unknown)	DQ218037
			3 (1485; unknown)	DQ218038
10	*Raphanus sativus* cryptic virus 3*	RSCV-3	1 (1609; RdRP, 55)	NC_011705
			2 (1581; unknown)	FJ461350
11	Rose cryptic virus*	RoCV	1 (1749; RdRP, 56)	NC_010346
12	*Rosa multiflora* cryptic virus*	RmCV	1 (1762; RdRP; 56)	EU024675
			2 (1475; unknown)	EU024676
			3 (1384; unknown)	EU024677

13	Vicia cryptic virus*	VCV	1 (2012; RdRP, 73)	NC_007241
			2 (1779; CP, 54)	NC_007242
14	White clover cryptic virus 1*	WCCV-1	1 (1955; RdRP, 73)	NC_006275
			2 (1708; CP, 54)	NC_006276
	Genus: *Betacryptovirus*			
1	*Carrot temperate virus 2*	CTeV-2	No molecular information	
2	*Hop trefoil cryptic virus 2*	HTCV-2	-do-	
3	*Red clover cryptic virus 2*	RCCV-2	-do-	
4	*White clover cryptic virus 2*	(WCCV-2)	-do-	
	Unclassified viruses in the family			
	Partitiviridae			
1	Amasya cherry disease-associated	ACDPV	1 (2002; RdRP, 73)	NC_006441
	partitivirus		2 (1839; CP, 55)	NC_006440
2	Black raspberry cryptic virus	BRCV	1 (1283: RdRP; 44) [b]	ABU55400
3	Cherry chlorotic rusty spot associated partitivirus	CCRSAPV	1 (2021; RdRP, 73)	NC_006442
			2 (1841; CP, 55)	NC_006443
4	*Helicobasidium purpureum*	HpV	1 (486; RdRP, 19) [b]	AY949837
	partitivirus			
5	*Heterobasidion annosum* virus	HaV1	1 (1101; RdRP, 43) [b]	AF348136
6	*Heterobasidion parviporum* partitivirus	HpPV	1 (1025; RdRP, 34) [b]	CBJ23785
7	*Nectria radicicola* virus L1	NrV-L1	1 (1286; RdRP, 50) [b]	AF251278
8	*Ophiostoma quercus* partitivirus	OqV	1 (399; RdRP, 15) [b]	AM111099
9	Pepper cryptic virus	PCV	1 (1069; RdRP, 35) [b]	ABC96789
10	*Picea sitchensis* virus1	PsV	1 (1392; RdRP, 47) [b]	BT122981
11	*Pinus sylvestris* partitivirus	PsPV	1 (1078; RdRp, 41) [b]	AAY 51483
12	*Pittosporum tobira* cryptic virus-1	PCV-1	1 (676; RdRP, 26) [b]	GU595166
13	*Primula malacoides* virus 1	PmV1	1 (2390; RdRP, 84)	EU19536
			2 (2344; CP, 75)	EU195327
14	*Vicia faba* partitivirus 1	VfPV-1	1 (1915; RdRP, 67)	DQ910762

[a] An asterisk next to the virus name indicates it is presently recognised by the ICTV as a member or a tentative member in the family *Partitiviridae*. Family members or tentative members for which no sequence data is available are not included but partial sequences are included [b].

1.3.2 Cryptoviruses (Alpha and Beta cryptoviruses)

Cryptoviruses (*Alphacryptoviruses* and *Betacryptoviruses*) belong to the family *Partitiviridae*, which exclusively infect plants (Ghabrial, 2001). Cryptoviruses have been found in different plants including sugar beet, ryegrass, carnation, alfalfa, radish, spinach, fire trees, green and brown algae, red pepper, carrot, hop trefoil, and broad beans (Milne and Cristina, 1999). *Cryptoviruses* were first reported in the 1960's where apparently healthy plants of 7 beet species *(Beta* spp.) were found to contain isometric virus-like particles (VLPs) *ca.* 29–30 nm in diameter with unusual properties nominated *Beet cryptic virus* (Pullen, 1968; 1969). It is known that the cryptic viruses do not impact the growth of their hosts. However, Nakatsukasa-Akune *et al.,* (2005) reported that TrEnodDR1 encoding the *White clover cryptic virus 1* CP gene expressed in *Lotus japonicus* after *Agrobacterium* spp. transformation resulted in increased concentration of endogenous abscisic acid and suppressed root nodulation. Plant cryptic viruses can also affect the growth and development of host plants in mixed infections (Blawid *et al.* 2008). Limited sequence information is currently available for *Alphacryptovirus* and *Betacryptoviruses*. The ICTV) listed 15 species of plant cryptic viruses (Table 1. 2) and 10 species that are unassigned members in the genus *Alphacryptovirus.*

1.3.3 Family *Chrysoviridae*

Members of the family *Chrysoviridae* are known to infect a wide range of different fungi. The *Chrysovirus* genus was once part of *Partitiviridae* family but now it is part of family *Chrysoviridae* (Coutts *et al.,* 2004). This family is in fact most closely related family to *Partitiviridae*. Some of their salient features are briefly discussed. The genomic organisation shows that chrysoviruses contain four linear dsRNA elements ranging in size from 2.8 to 3.6 kbp with sequences in the 5' and 3' UTRs (untranslated regions) being highly conserved among the four dsRNA elements (Ghabrial *et al.,* 2005; Jamal *et al.,* 2010) The virions are 35-40 nm in diameter and icosahedral in form. The structure of chrysoviruses follows a T=1 lattice organisation and contains 60 CP subunits (Caston *et al.,* 2003).

Penicillium chrysogenum virus (PcV), the prototype species of the family *Chrysoviridae* has been studied in detail at the biochemical, biophysical, and ultrasructural levels which have revealed that the virions possess an icosahedral shape and are non-enveloped. The four dsRNA segments have sizes ranging from 2902-3562 bp. PcV dsRNA-1 is 3562 bp in length and

encodes the RdRP; PcV dsRNA-2 is 3200 bp in size and encodes the CP; PcV dsRNAs-3 and -4 are respectively 2976 bp and 2902 bp in length encoding proteins of unknown function. There is little known about the replication mechanism of chrysoviruses but it appears that they follow a conservative pattern of replication (Ghabrial, 2008). The complete genomic sequences of several chrysoviruses are now available in databases and the list is being added to on a regular basis. Table 1.3 below shows the different species of chrysoviruses known to date.

Table 1.3 List of recognised and tentative members in the family *Chrysoviridae*, their abbreviations, length of their genomes, size of encoded gene products, and GenBank accession numbers of their genomic sequence.

Sr. No.	**Virus**	**Abbreviation**	**DsRNA segment no. (size in bp, encoded protein, size in kDa)**	**GenBank accession**
	Genus: *Chrysovirus*			
1	*Aspergillus fumigatus* chrysovirus	AfuCV	1 (3560, RdRP, 128) 2 (3159, CP, 107) 3 (3006, P3, 99) 4 (2863, P4, 95)	FN178512 FN178513 FN178514 FN178515
2	Amasya cherry disease associated chrysovirus	ACDACV	1 (3399; RdRP, 124) 2 (3128; CP, 112) 3 (2833; P3, 98) 4 (2498; P4, 77)	AJ781166 AJ781165 AJ781164 AJ781163
3	Cherry chlorotic rusty spot associated chrysovirus	CCRSACV	1 (3399; RdRP, 129) 2 (3125; CP, 112) 3 (2833: P3, 98) 4 (2499; P4, 77)	AJ781397 AJ781400 AJ781399 AJ781398
4	*Cryphonectria nitschkei* virus isolates	CnV-1 -bs131 -bs132 -BS122	2 (2718, CP, 99) [a] 1 (2889, RdRP, 110) [a] 1 (2889, RdRP, 110) [a] 2 (2718, CP, 99) [a] 1 (2889, RdRP, 110) [a] 2 (2721, CP, 99) [a] 4 (2960, P4, 81) [a] 3 (2552, P3, 91) [a]	GQ290650 GQ290646 GQ290651 GQ290647 GQ290649 GQ290645 HM013826 HM013825

		-BS321	1 (2889, RdRP, 110) [a]	GQ290652
			2 (2721, CP, 99) [a]	GQ290648
		-OB-5-11	1 (RdRP, 288, 11; 463, 18) [a]	DQ865185
				DQ865186
			2 (588, CP, 17) [a]	DQ865187
			3 (2718, P3, 92) [a]	DQ865189
			4(839, P4, 30) [a]	DQ865188
5	*Fusarium oxysporum* chrysovirus 1	FoCV-1	1 (2574, RdRP, 99) [a]	EF152346
6	*Fusarium oxysporum* f. sp. melonis	FuOMV 1	Four dsRNA segments	no molecular data
7	*Helminthosporium victoriae* *145S* virus	Hv145SV	1 (3612; RdRP, 125	NC_005978
			2 (3134; CP, 100)	NC_005979
			4 (2763; P4, 81)	NC_005981
			3 (2972; P3, 93)	NC_005980
8	*Penicillium brevicompactum* virus	PbV	Four dsRNA segments	no molecular data
9	*Penicillium chrysogenum* virus	PcV	1 (3562; RdRP, 129)	NC_007539
			2 (3200; CP, 109)	NC_007540
			3 (2976; P3, 101)	NC_007541
			4 (2902; P4, 95)	NC_007542
10	*Penicillium cyaneo-fulvum* virus	Pc-fV	Four dsRNA segments	no molecular data
	Tentative members			
1	*Aspergillus niger* virus	AnV-1816	1 (3440, RdRP, 122) [a]	EU289896
	Agaricus bisporus virus 1	AbV-L1	L1 (3396; RdRP, 122)	X94361
			L5 (2455; ?, 82)	X94362
			L3 (2778; ?, 87)	D10830
2	*Magnaporthe oryzae* chrysovirus 1	MoCV1	1 (3554; RdRP,126)	AB560761
			2 (3250; ?, 99)	AB560762
			3 (3074; P3; 84)	AB560763
			4 (3043; P4; 65)	AB560764
3	*Verticillium chrysogenum* virus	VcV	1 (3592; RdRP, 128)	HM004067
			2 (3313; CP, 113)	HM004068
			3 (2979; P3, ?)	HM004069
			4 (2931; P4, 90)	HM004070

[a] Family members with partial sequence available.

1.3.4 Satellite or defective dsRNA viruses

Some mycovirus families (*Hypoviridae, Narnaviridae, Partitiviridae* and *Totiviridae*) often posses additional dsRNA elements known as satellite or defective dsRNAs (Buck, 1988; Palukaitis *et al.,* 2008). Satellite or defective dsRNAs may be generated under specific conditions such as rapid, high multiplicity of infection during passage of the dsRNA through a vulnerable host. They can also be generated as a consequence of internal deletion and/or recombination of existing dsRNA (Hillman *et al.,* 2000). The production of these dsRNA elements *via* recombination and/or deletions are more frequent at specific regions or hot spots rather than randomly through the viral genome (Nagy and Simon, 1997; Cheng, *et al.,* 2003). The occurrence of defective and/or satellite dsRNA elements in mycoviruses is considered as a regular feature. These satellites are reliant on their helper virus to initiate their replication and encapsidation (Ochoa *et al*., 2008). The sequence information of these dsRNA elements contains the minimal sequence required for the stable replication of dsRNA elements (Nuss and Koltin, 1990). However, these elements (satellite/defective dsRNAs) can interfere with the replication of their helper viruses (Buck, 1998) and thus can be utilised as a source of a dsRNA based expression system to impact that host where they exist. In some fungi where dsRNAs encode small killer proteins which are toxic to other sensitive strains of the same or closely related species the satellite dsRNA confers immunity to the host from the toxins. For instance, killer strains of *Ustilago maydis* secrete one of the three different toxins (KP1, KP4, and KP6) that have been identified so far. These designated toxins have killer activity against susceptible cells of the same and closely related species (Koltin and Day, 1975). Each killer strain contains three different size classes of dsRNA segments including heavy (H), medium (M) and light (L) dsRNAs. Genetic studies revealed that the H segments contain information required for virion production and replication, a specific M segment encodes for the protein toxin and the L segment may confer immunity to the host.

1.4 Transmission of dsRNA elements in fungi

Mycovirus transmission in nature takes place by plasmogamy and cytoplasmic exchange between the compatible host isolates with no extracellular transmission mode (Buck, 1998) or plasmid transmission (Ghabrial, 1980). Unlike other viruses, mycoviruses do not lyse their host and are transmitted primarily through the intracellular route which occurs during the

movement of protoplasm toward the growing tips, so that newly formed hyphal segments are congenitally infected (Buck, 1986). Mycoviruses are entirely reliant on their fungal hosts to enable intracellular transmission and transmission which can be either horizontal *via* protoplast fusion, hyphal anastomosis or vertical by sporulation. Vertical transmission *via* fungal spores, whether sexual or asexual is the primary mode of transmission, although variability in transmission rates exists, depending upon the virus/fungus combination and type of spores (sexual and asexual). Horizontal transmission is also a major route for the transmission of dsRNA mycoviruses (Suzuki *et al.,* 2005). However, dsRNA viruses have to overcome a number of biochemical problems to enable them to infect and replicate within their specific host e. g. in some cases they need their own transcription enzymes to synthesise functional mRNA to initiate viral translation for viral protein synthesis (Mertens, 2004).

The natural host range only permits lateral transmission in closely related vegetative compatible groups of closely related fungi (Ghabrial and Suzuki, 2009). Vegetative incompatibility is one of the main obstacles to the transmission of fungal viruses (McCabe *et al.,* 1999). The existence of vegetative compatibility groups (VCGs) provides a natural barrier to horizontal transmission. Incompatible fusion of two hyphae may result in the isolation of fused cells by septal plugging followed by organelle degradation (Pearson *et al.,* 2009) as shown in Fig. 1. 2.

Furthermore, host cells also contain antiviral defence mechanisms e.g. interferons, which are considered to be the primary defence mechanism against viral invasion (Bozarth, 1972) and gene silencing may also be involved. Similarly, induction of apoptosis, modification of host cell translation mechanisms and RNA silencing are also defence mechanisms which are activated by viral infection (Gitlin and Andino, 2003; Goldbach *et al.,* 2003).

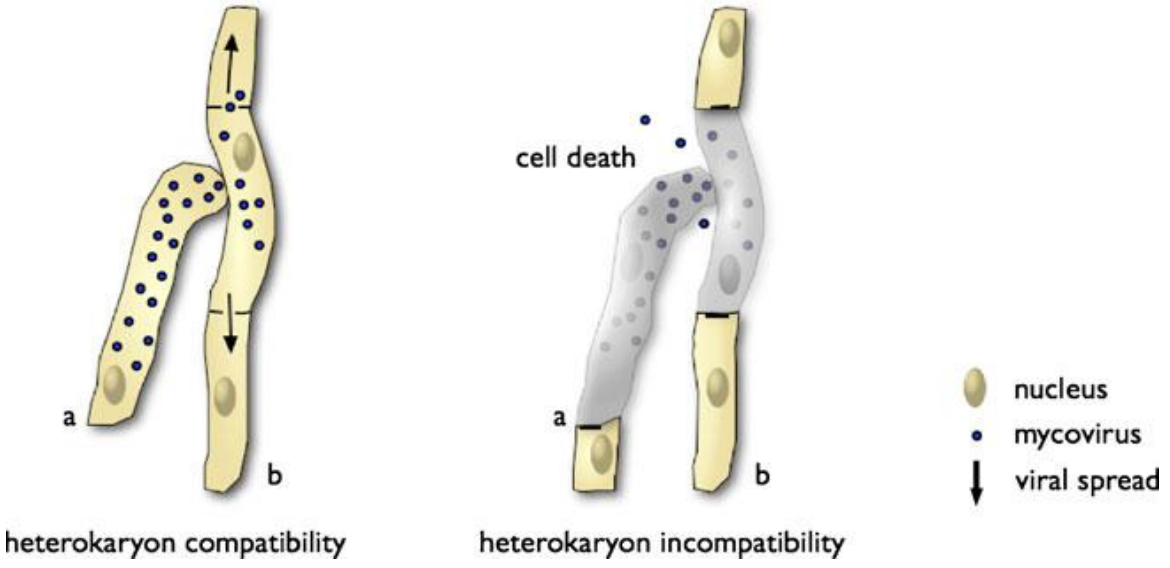


Figure 1.2 Illustration of horizontal transfer of mycoviruses between fungal hyphae (acceptor and donor) occurs successfully in vegetative compatible hyphae.

1.5 Replication of dsRNA viruses

Mycoviruses possess variable replication modes and it is also of interest that they are quite unusual in that they do not kill their host but replicate in parallel with them (Lemke and Nash, 1974). Studies on mycovirus replication were limited in the past mainly due to the cellular complexity of the host. The dsRNA elements of the mycoviruses are considered as a pool of uncoated replicative intermediates that are involved in the synthesis of viruses (Lemke and Nash, 1974). The replication of dsRNA elements in different fungi varies to a great extent. Replication in mycoviruses may be conservative as in *Saccharomyces cerevisiae-L-A virus.* (Nemeroff and Bruenn, 1986; Wickner, 1996) and PcV or they may follow a semi-conservative pattern as in *Aspergillus foetidus virus* (Buck, 1978) and *Penicillium stoloniferum virus* (PsV-S; Ghabrial and Hillman, 1999). A brief overview of replication of dsRNA is given here. Despite genetic and structural complexity, dsRNA viruses share several basic similarities and they produce mRNA transcripts using an endogenous transcription apparatus, which is integral part of the viral architecture.

First, dsRNA is transcribed by RdRP resident within the virion. This results in the generation of (+) strand transcripts which are squeezed out of the virion and can to perform three possible functions. Either it will serve as a messenger (m) RNA which is translated for the synthesis of viral proteins (structural and regulatory). Alternatively (+) strand transcripts can become encapsidated in progeny virions or be used as the template for synthesis of complementary (−) strands (Caston *et al.,* 1997). Both transcription and replication take place inside the nucleocapsid, which is the complete virion for fungal dsRNA viruses or an inner core for higher eukaryotic viruses like those of the *Reoviridae* family e. g. reovirus (Dryden *et al.,* 1993); rotavirus (Labbé *et al.,* 1991) and orbivirus (Huismans *et al.,* 1987). This model also applies to the prokaryotic dsRNA virus, bacteriophage φ6 (Mindich and Bamford, 1988).

Studies have revealed that PsV-S follows a semi-conservative pattern of replication. There is no evidence for the release of newly synthesized dsRNA from particles with two molecules of dsRNA (Ghabrial and Hillman, 1999). As is clear from Fig. 1.3 that PsV-S RdRP acts as a transcriptase and a replicase simultaneously. The transcriptase activity of RdRp accelerates the synthesis of the two complementary strands of dsRNA, within a single virus particle. After the displacement and release of parental, (+) strand RNA of the same polarity, duplexes are

formed. Furthermore, numerous copies can be made through repeated strands and the released parental, (+) strand can be used as a template for protein translation (RdRP and CP) by the host machinery or packaged by RdRP and CP in an assembled progeny virion. However, the replicase activity catalyses the synthesis of (-) strand on the displaced parental strand, to reform a new dsRNA segment (Ghabrial *et al*., 2008a).

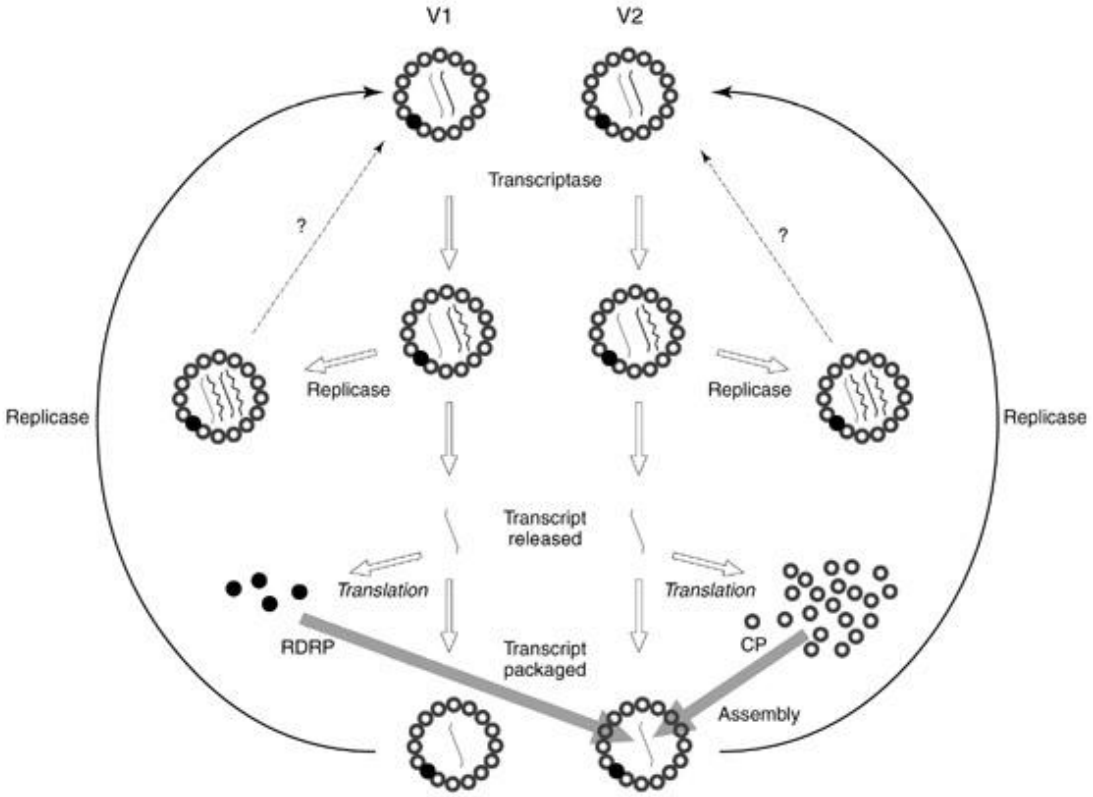


Figure 1.3 Model for replication of PsV-S reproduced from Ghabrial and Hillman (1999).

1.6 Significance of mycoviruses

Mycoviruses can confer a range of phenotypic effects on their fungal hosts which can range from symptomless to complete destruction of the host and from hypovirulence to hypervirulence (Ghabrial, 1994; MaCabe *et al.,* 1999). Macroscopic symptoms caused by mycoviruses are a result of alterations in complex physiological processes that includes interactions between host and virus factors (Ghabrial and Suzuki, 2009). Some of effects are elucidated below.

1.6.1 Hypovirulence

Virulence is an important factor, which has increased the importance of mycoviruses significantly over the past few years. There are numerous examples where fungal virus infection causes hyper- or hypovirulent effects on fungal growth. The hypovirulent phenotype induced by *Cryphonectria parasitica* hypovirus is an excellent and well documented example which reduces the pathogenicity of the chestnut blight fungus host, *C. parasitica* (Choi and Nuss, 1992; Nuss, 2005) by disturbing fungal development e.g. sexual reproduction and sporulation are affected, attenuating the fungus thus reducing its virulence (Patricia *et al.,* 1999). Furthermore, CHV1-EP713, the prototype member of the family *Hypoviridae*, can replicate and attenuate several members of other fungal genera, for instance, *Endothia gyrosa* and *Valsa ceratosperma*, in addition to its natural host, *C. parasitica* (Ghabrial and Suzuki, 2009). Moreover, the genus *Hypovirus* comprises only mycoviruses which confer hypovirulence (Fauquet *et al*., 2005; Pearson *et al.,* 2009).

Another example of hypovirulence is found in *Ophiostoma novo ulmi,* the fungus that causes Dutch elm disease. The *O. novo ulmi* mycovirus dsRNAs are associated with mitochondria in the fungus and this association results in a reduction of the activity of the mitochondrial cytochrome c oxidase, which in turn causes respiratory deficiency of the fungus. Thus, the debilitated fungus is unable to infect elm trees (Patricia *et al.,* 1999). There are other examples of reduction of virulence caused by fungal viruses e. g. a hypovirulent phenotype can be observed in *Helicobasidium mompa* infected with the totivirus, HmTV1-17 (Sasaki *et al.,* 2005).

Mycoviral infection may cause losses in commercial mushroom production, It has been observed in some commercial mushrooms e. g. the king oyster mushroom, *Pleurotus eryngii* that mycoviruses may affect the mushroom crop by affecting the yield and pigmentation fruiting bodies (Ro *et al.* 2007). Go *et al.,* (1992) and Rickner *et al.,* (1993) has reported yield losses up to 50% due to severe mycoviral infection. Also abnormal fruiting bodies were reported in oyster mushrooms.

Hence, mycoviruses may act as biological control agents of plant pathogenic fungi as confirmed for *C. parasitica* (Nuss, 1992). However, even when mycoviruses clearly have the ability to reduce the virulence of fungal plant pathogens, given that hyphal interaction is the only known mechanism for transmission between fungal colonies, vegetative incompatibility

(as found in many fungal species) is a major hindrance to their adoption as biological control agents.

1.6.2 Hypervirulence

Mycoviruses may be beneficial to their pathogenic hosts. Killer and hypervirulent phenotypes in *S. cerevisiae* and corn smut pathogen, *U. maydis*, represent extremely useful interactions since they secrete proteins which are toxic to sensitive strains of the same and closely related species. For example in *U. maydis* the immunity to the toxin is provided by a satellite dsRNA (Buck, 1988). Hence viruses confer selective advantage to the host by eliminating competitors living in the same ecological niche (Patricia *et al*., 1999).

In some species like *Nectria radicicola*, mycovirus infection regulates fluctuating signal transduction pathways and aids growth. Studies revealed that L1 dsRNA present in *N. radicicola* may alter well known signalling cAMP pathways.While the mechanism remain unknown, dsRNA might alter fungal gene expression by increasing levels of cAMP and subsequently activity of cAMP–dependent protein kinase (PKA). For instance two L1 dsRNA-infected strains (Cy9201 and T-1[3]), had a sixfold increase of cAMP-dependent PKA as compared to that of a cured strain (NcCy9201; Ahn and Lee, 2001).

Another example of hypervirulence is illustrated as a three way symbiosis between a mycovirus, *Curvularia* thermal tolerance virus (CThTV), the endophytic fungus (*Curvularia protuberata*), and panic grass (*Dichanthelium lanuginosum*) in which the fungus and the plant can withstand high temperatures due to dsRNA mycovirus infection. (Marquez *et al*., 2007).

1.6.3 Interferon induction

Interferon induction is another important factor. It has been observed by different research workers, that dsRNA can potentiate interferon induction, which is a primary host defence mechanism against viral invasion. Two fungal products, statolon from *P. stoloniferum* and helenine from *P. funiculosum,* were found to be potent interferon inducers in animal tests. Investigation suggested that the active factors in both of these products were dsRNAs of viral origin (Rytel *et al*., 1966; Bozarth, 1972).

1.6.4 Determination of impact of mycoviruses on the fitness of their hosts

In order to determine the impact of dsRNA mycoviruses on host fitness it is essential to compare infected fungi directly with uninfected types with the same genetic background. This leaves us with two possible solutions, either we can cure the host from the viral infection or transfect the uninfected hosts to attain isogenic lines (infected and uninfected). Numerous methods of curing fungi of dsRNA infection have been illustrated in the literature which include, exposure to high temperature (Golubev *et al.,* 2003), exposure to UV light (Treton *et al.,* 1987), and treatment with a range of different chemicals like emetine (Ahn and Lee, 2001), acridine orange (Cansado *et al*., 1989) and cycloheximide (Dalzoto *et al*., 2006; Elias and Coty, 1996), all of which have been used in this context. Curing and reducing dsRNA levels in infected fungi (completely or partially) by cycloheximide treatment, which blocks translational initiation and elongation during protein synthesis, is the most efficient and successful method used thus far. However cycloheximide treatment is not always completely successful.

Until now, the only method used to assess the efficiency of curing fungi of mycovirus infection was extracting dsRNA from cured and uncured fungal samples and examining the extracts following gel electrophoresis and ethidium bromide staining. However, it is quite possible that the levels of dsRNA present in any one particular sample might be below the level of resolution of the staining procedure and a more sensitive assay such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) amplification using virus-specific primers should be used to check for the presence or absence of viral infection.

1.6.5 Mycoviruses as biological control agents

Most fungal pathogens play a major role in causing plant diseases. Their pathogenicity is controlled mainly by the use of chemicals (fungicides). The extensive use of these fungicides has resulted in the development of fungicide-resistant strains over the years and most importantly, it has increased the risk of potential perilous effects impacting the environment and humans. Various approaches have been used to overcome plant diseases but few options have been found useful for the control of these fungal pathogens and mycoviruses are potential contenders as biocontrol agents. The best and most well documented example of hypovirulent phenotype conferred by a mycovirus is found in chestnut blight fungus (*C. parasitica*). The potential of a dsRNA virus as a biocontrol control agent for chestnut blight was first established

by Choi and Nuss (1992) following construction of a full length complementary DNA (cDNA) copy of hypovirulence-associated viral RNA and transformation into the virulent strain of the fungus which converted the compatible, virulent strain to a hypovirulent strain. The CHV-1 cDNA was successfully used to control chestnut fungus in Europe but failed to control the disease in North America because the fungus showed greater genetic diversity with multiple VCGs limiting the spread of the virus (Nuss, 1992). As hyphal interaction is the only means of transmission of these viruses, it causes limitations for the successful transmission of CHV-1, although it has shown that the dsRNA element greatly reduced virulence.

The development of cDNA infectious clones for transformation (Choi and Nuss, 1992) and other technology has allowed comparative studies of genetically identical, virus infected and uninfected cultures (Xie *et al*., 2006), and experimentation to extend the host range of mycoviruses (Chen *et al.,* 1994), without the obstacles of vegetative incompatibility.

Similarly, in *Helminthosporium victoriae*, the casual agent of Victoria blights of oats, and Dutch elm disease fungus, *O. novo-ulmi,* are also examples of hypovirulence as they attenuate their hosts. However, understanding the molecular basis of the disease in the mycoviral systems would provide excellent opportunities for novel biological control strategies to overcome fungal diseases (Ghabrial and Suzuki, 2009).

1.7 Structure of dsRNA mycoviruses

DsRNA mycoviruses genomic elements are usually segmented and encapsidated. In most cases where the genome is multipartite the dsRNAs are separately encapsidated. There are also some unencapsidated dsRNA genomes which consist of multiple segments (Nuss and Koltin, 1990). These dsRNA viruses have variability in their CP structure. The intricacy of the capsid ranges from a single shell capsid to a multi layered concentric shell capsid (Grimes *et al.,* 1998). A variety of other particle morphologies have also been observed, including rigid rods, flexuous rods, club-shaped particles and enveloped bacilliform particles (Varga *et al.,* 2003).

Most mycoviruses possess an icosahedral virion structure and possess similar structural and biochemical properties. However, different mycoviral proteins have a great deal in common in terms of structure and function possibly because of a similar lineage. This homology is found in the inner capsid layer and the viral enzymes, while the outer capsid region has a greater

diversity in organisation and sequence perhaps as an adaptation to enable transmission to new hosts (Mertens, 2004).

1.8 *Aspergillus* species

Aspergillus is a cosmopolitan filamentous fungus, and is ubiquitous in nature. It was identified by Pietro Antonio Micheli in 1729 and since its discovery, 250 species have been identified (Klich, 2006). It can be isolated from soil, indoor air environments and plant remains. *Aspergillus* spp. are aerobic and they can be seen in all oxygen rich environments but they usually grow as moulds on the surface of substrate (Bennett, 2010). However, in some species like *Aspergillus niger* they can grown in a nutrient depleted environment.

Aspergillus spp. have medical and industrial importance. Different metabolites isolated from *Aspergillus* spp. are used in the preparation of cholesterol lowering drugs e. g. the statins lovastatin and Mevastatin. These drugs lower human cholesterol levels (Bennett, 2010). Likewise, 40 out of 250 species identified have been reported as causative agents of opportunistic infections in man (Klich, 2006). Although *A. fumigatus* is the most common etiologic agent, being responsible for approximately 90% of human infections, it is not the only pathogen in this genus since *A. flavus, A. terreus, A. niger* and *A. nidulans* can also cause human infections (Bodey and Vartivarian, 1989; Kurup and Kumar, 1991).

Regarding commercial importance, some *Aspergillus* spp. e. g. *A. niger* are used in the production of citric acid (Curie, 1917). Citric acid is generally used as food ingredient and widely used in pharmaceutical and cosmetics industries and can also be used to slow down the setting of concrete (Brooke, 1994) Likewise, *A. niger, A. terreus*, and *A. oryzae* are also used in the production of gluconic acid, kojic acid and itonic acid respectively (Ruijter *et al.*, 2002).

1.9 *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus which in nature plays a vital role in recycling environmental carbon and nitrogen. (Latge, 1999; Pitt, 1994) and like most of the other *Aspergillus* spp. produces air borne conidia, green in colour ranging in size from 2.5 to 3 mm, with some isolates that are pigment-free. Though the mechanism of releasing conidia is vague it mainly depends on disturbances in the environment like strong air currents etc. Once the conidia are released small spore size allows the spores to remain suspended in air for extended periods of time. Finally they get deposited on any solid or liquid surfaces; once they come in

contact with them they start to germinate under favourable environmental conditions (Kanaani *et al.*, 2008).

A. fumigatus can grow well at high temperature (up to 55ºC) due to its thermophilic nature and can survive temperatures of 70ºC and above (Haines, 1995; Latge, 1999; Raper and Fennell, 1965). This property of the fungus contributes to its survival. It is a prototypical airborne opportunistic pathogen, affecting a wide range of vulnerable patient groups predominantly those with neutropenia receiving corticosteroids are those with T cell defects such as AIDS patients and the small number with inherited chronic granulomatous disease (Denning, 1998). *Aspergillus* spp. are known to play a role in three different clinical scenarios in man including opportunistic infections, allergic states and toxicoses. Immunosuppression is a major factor enhancing the chances of development of opportunistic infections (Ho and Yuen, 2000). According to a report the mortality rate of untreated patients suffering from invasive aspergillosis is nearly 100% (Dasbach *et al*., 2000). Additionally, *Aspergillus* spp. can cause infections in animals and birds and may induce mycotic abortion in cattle and sheep (St-Germain and Summerbell, 1996; Derouin, 1994).

1.9.1 *Aspergillus fumigatus* as an opportunistic pathogen

A. fumigatus is the dominant species in causing fungal lung diseases in humans and animals (Frisvad *et al*., 2008). According to surveys, all humans inhale several hundred *A. fumigatus* conidia daily (Chazalet *et al.*, 1998; Latge, 1999; Hospenthal *et al.*, 1998). In some cases *A. fumigatus* causes allergic reactions and is known to cause aspergillosis in humans, which is currently rapidly increasing in incidence. However, in immunocompromised patients, it usually causes severe, and often fatal, invasive aspergillosis (Bodey and Vartivarran, 1989; Lin *et al.*, 2001; Anderson *et al.*, 2003) and is associated with severe asthma and sinusitis (Gorman *et al.*, 2009).

In spite of a remarkable increase in incidence, the pathobiology of *A. fumigatus* remains poorly understood. The initial site of infection caused by *A. fumigatus* in invasive aspergillosis (IA) is not clear and two possible sites of infection, the epithelium and alveoli are possibilities.

The role of lung epithelium in IA is still not clearly understood but several observations suggest it may be one of the putative pulmonary sites of infection. Epithelial cells have the ability to engulf conidia and subsequently these conidia survive and live intracellularly (Paris *et al*., 1997; Wasylnka and Moore, 2002; Latge, 2003). While the conidia that are not engulfed come in contact with main lung phagocytic cells known as alveolar macrophage. These conidia are

not exposed to the host immune system to a great extent as this process occurs quickly. The killing of conidia occurs by reactive oxygen intermediates, which initiates 6-8 h after phagocytosis whereas conidial engulfment occurs rapidly (1-2 h).

1.9.2 *Aspergillus fumigatus* as a saprophytic pathogenic fungus

A. fumigatus is indeed one of the most common inhabitants of fungal flora (Chazalet *et al.,* 1998). The default lifestyle of Aspergilli however, is that of a saprophyte, which is characterized by the uptake of nutrients from a decomposing organic matter, and it is likely that the pathogenicity of Aspergilli is based to a great extent on its saprophytic nature **(**Krappmann, 2006).

A. fumigatus potentially grows in soil or organic remains whose most common ecological niche is on the ground (Rementeria *et al.,* 2005**);** its survival depends on its ability to take up all nutrients, including essential micronutrients from the environment such as iron, copper, or zinc, needed to support its growth (Vicentefranqueira *et al.,* 2005). *A. fumigatus*, like most microorganisms, usually lives in environments that are nutrient deficient caused by low initial availability or by depletion caused by the growth of any other microorganisms. *A. fumigatus* has developed different mechanisms to obtain metal nutrients from its substrate and much work has been done regarding the mechanism of iron uptake by pathogenic microorganisms (Howard, 1999). Iron is essential for most organisms like bacteria and yeast in order to enhance their virulence (Ramanan and Wang, 2000; Ratledge and Dover, 2000) suggesting that its acquisition *in vivo* may be required for *A. fumigatus* to cause disease (Haas, 2003; Schrettl *et al.,* 2004).

1.9.3 *Aspergillus* mycoviruses

Mycoviruses were first reported in the genus *Aspergillus* in 1970 (Banks *et al*., 1970) and VLPs were seen to aggregate in the cytoplasm, enveloped by a single membrane (Border *et al*., 1972). Mycoviral infections are found in various *Aspergillus* spp. (Varga *et al*.,, 1998) including *A. foetidus* (Ratti and Buck, 1972; Buck and Ratti 1975), *A. flavus*, (Schmidt *et al*., 1986), *A. niger* (Buck *et al.,* 1973) *A. tubingenesis, A. heteromorphus, A. japonicus, A. carbonarius* (Varga *et al*., 1994), *A. flavus, A. parasiticus, A. nomius* and *A. tamarii* (Elias and Cotty, 1996), *A. ochraceous* (Kim and Bozarth, 1985) and more recently in *A. fumigatus* (Jamal *et al*., 2010). Gel electrophoretic analysis of the dsRNA elements above describes a variety of fragments of

differing size and composition for each *Aspergillus* spp. (Elias and Cotty, 1996; Varga *et al*., 1998; 2001). It is noteworthy that dsRNA elements have been found in both sexual and asexual *Aspergillus* spp., with infection rates of up to 13% and incidence of infection is more prevalent in asexually reproducing *Aspergilli* (Van Diepeningen *et al.*, 2006). In a previous extensive survey no dsRNA elements were discovered in 60 isolates of *A. fumigatus*, which included isolates from type culture collections, clinical and environmental situations (Varga *et al*., 1998). However in a recent survey, dsRNAs were positively identified in some clinical isolates of *A. fumigatus* (Jamal *et al*., 2010; Bhatti *et al.*, 2011a; Bhatti *et al.*, 2011b).

1.10 Aims of the project

The aims of the projects are presented in chapter order.

1.10.1 Chapter 3: Screening of *Aspergillus fumigatus* isolates (clinical and environmental) for dsRNA mycoviruses

The aim of this chapter was to collect information about the incidence of mycoviral infection in *A. fumigatus*. For this purpose a range of clinical and environmental isolates were collected and screened to characterise at least one set of dsRNAs using random cDNA priming and by direct sequencing.

1.10.2 Chapter 4: Developmental technology

The aim of this chapter was to investigate and develop technology and standardise techniques and methodology which will be useful in successfully completing the main project objective (identifying and characterising dsRNAs *Aspergillus fumigatus* mycoviruses).

1.10.3 Chapter 5: Sequencing of the genome of a new partitivirus infecting *Aspergillus fumigatus*

The aim of this chapter was to completely characterise a novel *Partitivirus* (isolate 88) found during the screening of *A. fumigatus* isolates in Chapter 3 in order to obtain novel sequence information. This information was then be used to determine the origin, function and relationship of novel dsRNAs to known dsRNA elements.

1.10.4 Chapter 6: Curing *A. fumigatus* isolate 88 from AfuPV infection and phenotypic screening of AfuPV infected and virus-free *Aspergillus fumigatus* isolates

The aim of this chapter was to check the impact of isolate 88 *Partitivirus* on the fitness of its host. The impact can be checked only in the same genetic background. In order to elucidate the impact of the partitivirus and to create isogenic lines to facilitate a comparison between infected and uninfected isolate 88 not only in in terms of growth and morphology but also pathogenicty in a murine model.

1.10.5 Chapter 7: Transfer *Aspergillus* mycoviruses and dsRNA elements to virus-free isolates of *A. fumigatus*

The main objective of this chapter was to investigate efficient methods to transfer the mycovirus and dsRNA elements to uninfected isolates of *A. fumigatus*. For this purpose different procedures like hyphal fusion and protoplast fusion and protoplast transfection were carried out to transfer mycoviruses in order to elucidate the influence of Aspergillus mycoviruses on growth and morphology of the fungi under the same genetic background.

1.10.6 Chapter 8: Conclusions and future work

Chapter 2

Materials and methods

2. Materials and Methods

2.1 General laboratory practice

All work areas were wiped with 70% ethanol before and after use to ensure sterile working conditions. A protective laboratory coat and disposable gloves were worn at all times in the laboratory, when working with hazardous chemicals, radioactivity, potential carcinogens and toxic materials like ethidium bromide. In addition, a protective face mask was worn when weighing out hazardous/toxic powders. Exposure to UV radiation was minimised by wearing a full-face visor in combination with a laboratory coat and gloves. Work relating to *Aspergillus fumigatus* was carried out in a different laboratory (Flowers building; CMMI) designated only for *Aspergillus* work and prior induction was done before starting work. Elbow visors and a face mask were worn all the times when spores were harvested in an Envair Class II Microbiological safety cabinet. All waste materials were put in disposable autoclave bags, sealed perfectly and placed in a special sealed container for disposal. Safety cabinets were cleaned with Trigene (Medichem), a fungal and bacterial disinfectant and UV was turned on for sterilisation of the cabinet.

2.2 Centrifugation

Smaller volumes (less than >2 ml) were centrifuged in an Eppendorf 5415 C bench top centrifuge using a fixed-angle rotor. The maximum centrifugal force of the bench top microfuge was 16,000 X g. Centrifugation of larger volumes was performed using the fixed-angle rotor JA-14 in a Beckman Coulter Avanti J-26-XP floor standing centrifuge with a maximum centrifugal force of 30,100 X g for the JA-14 rotor. For ultra centrifugation two different types of rotor were used including a fixed angle rotor (Type 45 Ti) and swing bucket rotor (SW41 Ti) employed in a Beckman Optima L-100 XP ultracentrifuge. The maximum centrifugal force of rotor Type 45Ti was 235, 000 X g and 258, 000 X g for SW41 Ti

2.3 Sterilisation

All glassware, plastic and solutions were sterilised by autoclaving at 121^{o}C, 15 p.s.i. for 25 min. All antibiotics and heat sensitive solutions were filter sterilised by passage through a 0.2 µm syringe filter (Fischer Scientific).

2.4 Antibiotics

All antibiotics were filter sterilised through a 0.22 µm filter (Fisher Scientific) before use and aliquots stored at -20^{o}C. Antibiotics are sensitive and easily inactivated by heat. Therefore, all media were cooled to 50^{o}C or below prior to addition of antibiotics.

2.4.1 Ampicillin stock solution

Ampicillin-sodium salt (Sigma-Aldrich)	100 mg
Sterile distilled water	1 ml

Filter through a 0.22 µm filter to sterilise. Aliquots were stored at -20 ^{o}C.

2.4.2 Isopropyl-ß-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- ß -D-galactoside (X-Gal)

The lactose analog (IPTG) and a chromogenic substrate for beta-galactosidase (X-gal) were filter sterilised through a 0.22 µm filter and stored in aliquots at -20^{o}C.

2.4.2.1 X-Gal

X-Gal (Gene)	20 mg
N, N' dimethylformamide (Sigma-Aldrich)	1 ml

The tube was wrapped in aluminium foil to avoid damage by light.

2.4.2.2 IPTG

IPTG (Gene)	2 g
Sterilised distilled water	8 ml

2.5 Common media and solutions

2.5.1 Aspergillus complete medium (ACM)

Aspergillus complete medium (ACM) is a medium specifically designed for optimum growth of *Aspergillus* isolates in solid or liquid culture.

To prepare 1 litre ACM mix the following:-

Final concentrations shown in brackets.

Adenine	0.075g
Glucose 1% ($^{w}/_{v}$)	10 g
Yeast extract 0.1% ($^{w}/_{v}$)	1 g
Peptone 0.2% ($^{w}/_{v}$)	2 g
Casamino acids 0.1% ($^{w}/_{v}$)	1 g
Aspergillus salt solution **(**section 2.5.2)	20 ml
Vitamin solution (section 2.5.4)	10 ml

1% or 2% ($^{w}/_{v}$) Oxoid agar No. 3 (for solid media only)

Finally, make the volume up to 1 litre by adding distilled water and while mixing adjust the pH of the media to 6.5 with 10 M NaOH.

2.5.2 Aspergillus salt solution (1 litre)

KCl	26 g
$MgSO_4$	26 g
KH_2PO_4	76 g
Trace element solution (section 2.5.3**)**	50 ml

Mix and add distilled water to a volume of 1 litre.

2.5.3 Trace element solution (1 litre)

$Na_2B_4O_7.10H_2O$	40 mg
$CuCl_2.5H_2O$	400 mg
$FeCl_3.H_2O$	800 mg
$MnSO_4.4H_2O$	800 mg
$Na_2MoO_4.2H_2O$	800 mg
$ZnSO_4.7H_2O$	8 g

Mix and add distilled water to a volume of 1 litre.

2.5.4 Vitamin solution (1 litre)

4-aminobenzoic acid	400 mg
Aneurin	50 mg
Biotin	1 mg
Meso-inositol	24 g
Nicotinic acid	100 mg
DL-pantothenic acid (hemicalcium salt)	200 mg
Pyridoxine monohydrochloride	250 mg
Riboflavin	100 mg
Choline chloride	1.4 mg

Mix and add distilled water to a volume of 1 litre

2.5.5 Minimal medium

Glucose (1% $^{w}/_{v}$)	10 g
Aspergillus salt solution (section 2.5.2)	20 ml

1.2 % ($^{w}/_{v}$) Oxoid agar number 3 (for solid media only)

Finally, make the volume up to 1 litre by adding distilled water and while mixing adjust the pH of the media to 6.5 with 10 M NaOH. After autoclaving the media, let the media cool down to 50^{o}C. Ammonium tartrate (10 ml; 500 mM) was added to 1 l of the medium prior to pouring of plates.

2.6 Transformation storage solution (TSS)

2 X LB (section 2.7)	5 ml
PEG 8000	1 g
Dimethyl sulphoxide (DMSO)	0.5 ml
$MgCl_2$ (1M)	0.5 ml
Sterilised distilled water	3.0 ml

2.7 LB medium (1 litre)

Tryptone (Oxoid)	10 g
Yeast extract (Oxoid)	5 g
NaCl (BDH)	10 g
Technical agar 3 (Oxoid; for solid media only)	12 g

Sterilised distilled water was added to a volume of 1 litre, stirred to mix all ingredients and autoclaved (section 2.3). Ampicillin was added prior to pouring of making LB plates. Approximately 25 ml of media was poured per Petri dish under aseptic condition. For plates containing antibiotics media was allowed to cool to 50^{o}C and the appropriate amount was added.

2.8 SOC broth

Tryptone (Oxoid)	2.0 g
Yeast extract (Oxoid)	0.5 g
NaCl (BDH)	0.05 g
KCl (250 mM, pH7.0)	1 ml
Sterilised distilled water	95 ml

Final volume (100 ml) was adjusted by addition of water. Aliquots (10 ml) were kept in universal tubes and sterilised by autoclaving. Before use, 100 µl of 2 M $MgCl_2$ stock (filter sterilised) and 200 µl of sterile 2 M glucose solution (filter sterilised) was added in each aliquot under aseptic conditions.

2.9 YG medium (1 litre)

Yeast extract (Oxoid)	5 g
D-glucose (Melford)	20 g
Trace element solution	400 µl
Sterilised distilled water	1000 ml

2.9.1 Selection medium (1 litre)

D-glucose	10 g
Vitamin solution (section 2.5.4)	10 ml
Sucrose	342.3 g
Ammonium tartrate (500 mM)	10 ml
Sterilised distilled water	1000 ml

2.9.2 YES medium (1 litre)

Yeast extract (Oxoid)	5 g
D-glucose	30 g
Vitamin solution	10 ml
Sucrose	40 %
Sterilised distilled water	1000 ml

2.10 Screening of clinical and environmental isolates of *Aspergillus fumigatus* for dsRNA elements

2.10.1 Source of *Aspergillus fumigatus* isolates (clinical and environmental)

Panels of clinical and environmental isolates of *A. fumigatus* were obtained from Dr. Michael Petrou (Faculty of Medicine and Investigative Science, Charing Cross Campus, Du Cane Road, Acton, London W4, United Kingdom) and some environmental isolates were supplied by Dr. Elaine Bignell (Centre of Molecular Microbiology and Infection, South Kensington campus, Imperial College London) in order to screen them for the presence or absence of dsRNA elements. The list of isolates screened to date can be seen in Chapter 3. Screening was carried out in the presence of a positive control (e. g. virus infected isolate-A-78) and a negative control (uninfected isolate) to confirm that the screening procedure was working properly. Isolates were provided in the form of spores in a small volume (5 ml) of water.

2.10.2 Inoculation of isolates on ACM

Individual isolates of *A. fumigatus* were inoculated onto solid ACM plates using an inoculating loop and incubated at 37^{o}C for 5-6 days. When the plates were full of sporulating mycelia the spores were harvested using sterile spreaders and made into a suspension in a small quantity (25 ml) of distilled water. The spore-containing suspension was used to inoculate 500 ml ACM broth in 1 litre Erlenmeyer flasks and incubated at 37^{o}C with shaking at 130 rpm for 5-6 days. Mycelia were harvested through Miracloth, dried with paper towel and stored at -80^{o}C. A schematic pattern of the procedure is described in Fig. 2.1.

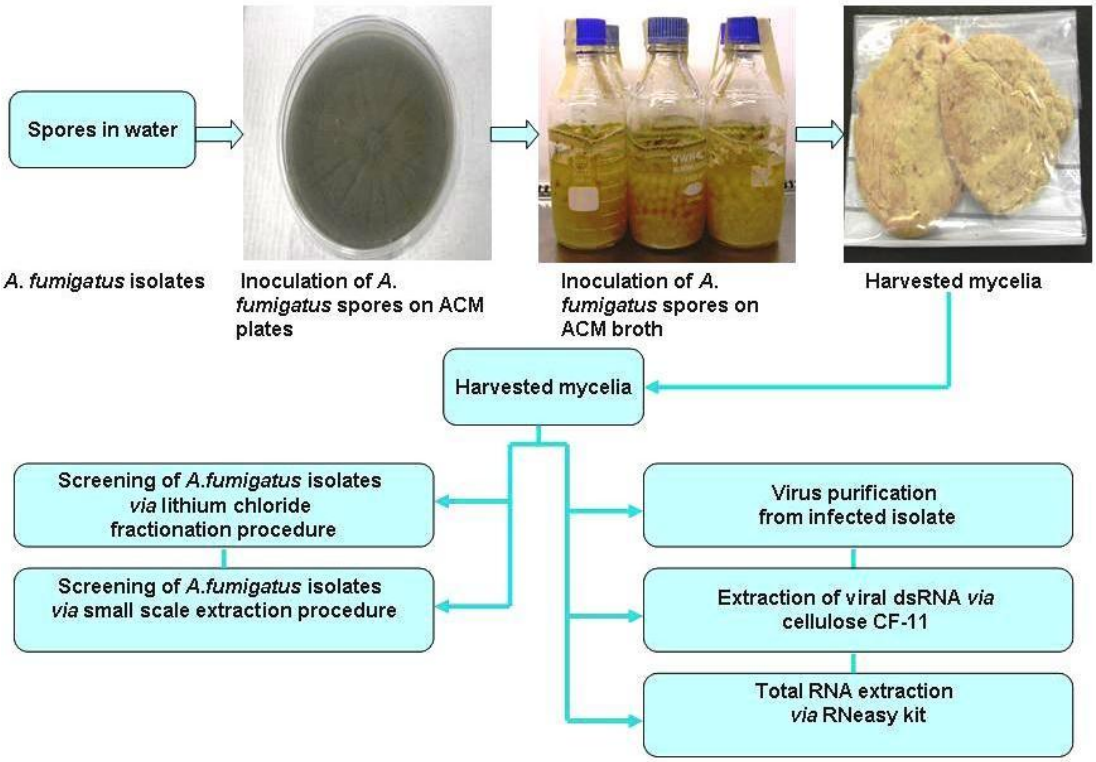


Figure 2.1 Schematic representation of growth stages involved to obtain *Aspergillus fumigatus* mycelia either to determine dsRNA infection *via* screening (either lithium chloride fractionation procedure or small scale extraction) or to obtain dsRNA extracts from fungal tissues using different procedures depending upon the quantity and requirement.

2.10.3 Preparation of glycerol stocks for *A. fumigatus* isolates

Glycerol stocks were prepared for all useful and dsRNA-infected *A. fumigatus* isolates discovered during the screening process with the aim of preservation and convenience.

Spores from the respective plates were preserved in 1 ml of 40 % glycerol (VWR) and kept at -20^{o}C.

2.10.4 Determination of virus infection in *A. fumigatus* isolates

In the work reported here initially dsRNA extraction was performed using relatively large amounts of fungal mycelia and a lithium chloride isolation procedure, which is described in detail (section 2.10.5) and later another procedure was used for the extraction of dsRNA which was faster and more convenient than the large scale extraction using relatively smaller quantities of mycelia (section 2.10.6). The screening was carried out in the presence of appropriate controls.

2.10.5 Isolation of dsRNA from clinical isolates of *Aspergillus fumigatus* using lithium chloride fractionation

Fungal mycelia (25-30gm) were crushed in liquid nitrogen and homogenised to a fine powder using a stainless steel pestle and mortar. Extraction buffer (50 ml/l Tris-HCl buffered phenol, 60 g/l 4-amino salicylate, made up to the desired volume with 50 mM Tris-HCl (pH 8.4) was added to the crushed powder of mycelia and the mixture incubated on ice and stirred for 60 min on a plate stirrer. The mixture was centrifuged at 15,300 X g (Beckman, Model Avanti J-26-XP centrifuge with a JA-14 rotor) for 35 min at 4°C. The aqueous supernatant containing a total nucleic acid extract was transferred into a fresh sterile centrifuge tube and treated once with phenol (15 ml) followed by chloroform (15 ml). The mixture was centrifuged (15,300 X g; 30 min) on each occasion. After phenol and chloroform treatment, lithium chloride fractionation was carried out according to Diaz-Ruiz and Kaper (1978). The supernatant was transferred to a 250 ml sterile tube and an equal amount of 4 M LiCl was added, mixed and then incubated overnight at 4^{o}C. The solution was then centrifuged (15,300 X g; 30 min) to pellet the precipitated ssRNA and dsDNA.

The supernatant was transferred to a 250 ml sterile tube and an equal volume of 8 M LiCl was added, mixed and incubated overnight at 4^{o}C. The solution was centrifuged (15,300 X g; 35 min.) to collect the dsRNA. A 1 ml sample, which served as negative control (eluate), was taken from the supernatant before discarding the remainder, carefully leaving the pellet intact which was dried at room temperature. The dsRNA was then resuspended in water (2 ml) and

small volume (200 µl) of the resuspended pallet was re-precipitated as described in section 2.11.1.

2.10.6 Small scale screening of *Aspergillus fumigatus* isolates

This procedure developed by Coenen *et al*., (1997) is rapid and easy to perform as compared to the large scale extraction procedure. Here 3-5 ml of ACM broth was inoculated with *A. fumigatus* conidiophores in 50 ml Falcon tubes and the samples incubated for 2 days at 30˚C. The mycelia thus obtained were transferred to 2 ml Eppendorf tubes and kept at -80˚C unless processed immediately. Mycelial samples were then crushed and ground to a powder in 350 µl of extraction buffer (section 2.10.6.1) in liquid nitrogen and vortexed to homogenise the material prior to incubation at 70˚C for 1 h. Nucleic acids were then extracted using 350 µl of Tris-HCl saturated phenol (pH 7.5) and an equal volume of Sevag (chloroform and isoamyl alcohol; ratio of 24:1) prior to centrifugation (11,000 X g; 15 min). The supernatant was decanted into a fresh Eppendorf tube, extracted with 500 µl of Sevag and then centrifuged (11,000 X g; 10 min). The supernatant was removed and the nucleic acids were precipitated with 800 µl of absolute ethanol and incubated at -20˚C for 4 h. The precipitated samples were then centrifuged (11,000 X g; 10 min) to pellet the nucleic acid. The pellet was dried under an electric light bulb and resuspended in 15-20 µl of water and kept at -20˚C. The samples were then checked for the presence or absence of dsRNA by 1% agarose gel electrophoresis as described in section 2.11.5.

2.10.6.1 Extraction buffer for small scale screening of dsRNA

Extraction buffer (10 ml; 20 mM EDTA, 20 mM Tris-HCl [pH 7.5], 1% SDS and 1% NaCl) was prepared by mixing the following:- 400 µl 0.5 M EDTA (pH 8); 2) 200 µl 1M Tris-HCl (pH 7.5); 3) 1 ml 10% SDS (sodium dodecyl sulphate); 4) 5 ml 2M sodium chloride; 5) 3.4 ml sterile water to a volume of 10 ml.

2.11 Basic nucleic acid treatments and manipulations

2.11.1 Precipitation of nucleic acid

Nucleic acids were precipitated with a 10% vol of 3 M sodium acetate (pH 5.5) and 2.5 vols of 100% ethanol following storage at -20^{o}C overnight. The precipitated material was centrifuged at 16,000 X g for 25 min. The supernatant was discarded and the pellet washed with 70% ethanol (w/v) before re-centrifugation (16,000 X g; 25 min). The supernatant was discarded and the pellet dried under a lamp to allow any remaining ethanol to evaporate. The pellets were finally resuspended in water (40 µl) and put on ice for 30 min. The resuspended nucleic acid solutions were checked by agarose gel electrophoresis for the presence or absence of dsRNA (section 2.11.5). Preparations of dsRNA were treated with DNase1 and S1 nuclease in succession to remove contaminating DNA and ssRNA.

2.11.2 Purification of dsRNA with DNase1

Contaminating DNA was removed from 20 µl aliquots of purified dsRNA by treatment with 25 µl DNase1 (1 U/µl; Promega) in mixture with 10 µl of DNase1 (10 X) buffer and sterile distilled water (45 µl) and incubation at 37^{o}C for 1 h. DNase-treated dsRNA was then purified and concentrated using phenol-Sevag extraction (See section 2.11.4; Sevag *et al.*, 1938). After this treatment the dsRNA was subjected to S1 treatment in order to remove ssRNA.

2.11.3 Purification of dsRNA through S1 nuclease treatment

Contaminating ssRNA was removed from 20 µl aliquots of DNase treated dsRNA by treatment with 1 µl S1 nuclease (95 U/µl; Promega) in mixture with 5 µl of S1 nuclease (10 X) buffer and sterile distilled water (24 µl) and incubation at 37^{o}C for 1 h. The treated dsRNA was then purified and concentrated using the phenol-Sevag extraction procedure and then analysed by agarose gel electrophoreses to check the purity of the dsRNA.

2.11.4 Phenol-Sevag extraction

Following DNase1 and S1 nuclease treatments samples were adjusted to a volume of 200 μl with sterile distilled water. An equal volume (200 μl) of Tris-HCl saturated phenol (pH 8.0; Sigma) was added and the whole mixed by vortexing in order to deproteinise the extracts. The solution was then micro centrifuged (16,000 X g; 5 min) to separate the two phases. The aqueous layer was transferred to a sterile Eppendorf tube and an equal amount of Sevag (Sevag *et al.*, 1938; chloroform and isoamyl alcohol; ratio of 24:1) added and the whole mixed by vortexing prior to micro centrifugation as above for 5 min to remove excess phenol. The aqueous layer was then transferred to a new sterile Eppendorf tube and nucleic acids precipitated as described (section 2.11.1). The dsRNA solutions were then examined by agarose gel electrophoresis.

2.11.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and visualise nucleic acids. Gels were prepared by melting molecular grade agarose (Bioline) to a final concentration of 1% (w/v) in 1 X TAE buffer (10 X TAE: 400 mM Tris-base [pH 8.0], 11.4 ml/l glacial acetic acid, 10 mM EDTA). Ethidium bromide was added to a final concentration of 50 μg/100 ml when the temperature was 50-55^{o}C. The gel was poured immediately into a plastic casting tray, (100 mm x 65 mm, 1 mm thick) after mixing, and left to set for 45 min. Samples (10 μl) were mixed with 5 X loading buffer (Bioline) containing bromophenol blue before loading. The gel was electrophoresed at 67 V in 1 X TAE for *ca.* 1 h. The bands were observed under a short-wave ultraviolet transilluminator using the Multianalyst software (Bio-Rad), photographed and exported as Tiff or Jpeg files.

2.11.6 Extraction of dsRNA from agarose gels

To extract dsRNA from agarose gels the RNaid kit (BIO-101) was used. Samples of dsRNA were subjected to gel electrophoresis at 67 V for 2 to 3 h so that individual dsRNA species could be well separated from one another and be distinct. Gels were then exposed to UV irradiation for very short time to reduce degradation and dsRNA bands of interest were excised and kept in separate sterile Eppendorf tubes. Three volumes of RNA binding salt

were added to each sample and the solution was incubated at 50-55^{o}C for 10 min with inversion of tube after every 2 min until the agarose was completely melted. Then 13 µl of RNA matrix solution was added so that a homogeneous solution was produced. The solution was incubated for 10 min with occasional manual tapping to encourage solubilisation. The tube was centrifuged (16,000 X g; 1 min) and the supernatant set aside. The pellet was twice resuspended (through pipetting) in 500 µl ethanol washing concentrate and centrifuged (16,000 X g; 1 min). After treatment with wash buffer the supernatant was removed and the pellet was resuspended in 65 µl nuclease free water. The mixture was incubated at 50^{o}C for 5 min and the supernatant collected in a new cold Eppendorf tube after centrifugation (16,000 X g; 1 min). The resulting pellet was again resuspended in 65 µl of water followed by 5 min incubation at 50 ^{o}C and centrifugation (16,000 X g; 1 min) to enhance the recovery of dsRNA. Supernatants were pooled together and dsRNA was precipitated as described (section 2.11.1).

2.11.7 Extraction of DNA from agarose gels

To recover DNA from agarose gels, QIAEX II kit (Qiagen) was used. After electrophoresis, DNA bands were excised from gels and 3 vols of DNA binding salt buffer was added. The solution was incubated at 50^{o}C for 10 min to melt the agarose; 13 µl of DNA binding matrix (QIAEX II) was added. The pellet obtained after micro centrifugation (16,000 X g, 3 min) was air dried for 20 min at room temperature after washing twice with QX1 solution (350 µl) and with PE buffer. The pellet was resuspended in 20 µl of water, vortexed and then incubated for 5 min. The tube was centrifuged (16,000 X g, 1 min) and the clear supernatant was collected in a new cold tube. The remaining contents of tube were resuspended in 20 µl of water, vortexed, incubated for 5 min at room temperature and the supernatant collected after centrifugation (16,000 X g; 1 min). The two aliquots were pooled together and an aliquot electrophoresed on a 1% agarose gel to confirm successful extraction of DNA and the remainder stored at 4^{o}C.

2.11.8 DNA purification using PCR purification kits

For cleaning and concentrating PCR reactions the QIAquick Purification kit (Qiagen) was used. Five volumes of binding buffer (PB) were added to the sample and mixed by inversion.

The pH of the PCR reaction was optimised by addition of Sodium acetate (pH 5.5). The mixture was passed through a QIAquick column and centrifuged at 16,000 X g for 1 min. followed by washing with 700 µl of PE buffer (10 mM Tris- HCl [pH 7.0]). The DNA was eluted in 40 µl of nuclease free water by centrifugation (16,000 X g; 1 min).

2.12 Other methods used to isolate dsRNA from the fungi

2.12.1 Virus purification and extraction of *A. fumigatus* partitivirus dsRNA

This method is adapted from Crawford *et al*., (2006) for the purification of a partitivirus from *Ophiostoma* sp. Here 60 g mycelia was homogenised with 6 g of carborundum (silica carbide-grade 500) using a pestle and mortar and sufficient phosphate buffer (0.03 M PBK buffer; 0.03 M sodium phosphate, 0.3 M potassium chloride, [pH 7.6]) to keep the extract liquidised. The paste formed was diluted with PBK to 1.5 litres, stirred at 4˚C for 1 h. and the extract centrifuged (Beckman rotor JA-14) at a speed of 23,000 X g at 4˚C for 45 min. Polyethylene glycol (PEG)-6000 (10 g) and NaCl (1 g) /100 ml were added to the supernatant and the mixture stirred for 2 h at 4˚C. The solution was centrifuged (Beckman JA-14 rotor) at 15,000 X g for 35 min, and the resultant virus pellet was resuspended in 100 ml of PBK buffer overnight at 4˚C. Following overnight resuspension the virus solution was subjected to low speed centrifugation at 23,000 X g for 30 min to remove unresuspended debris and the supernatant was subjected to ultracentrifugation (Beckman Coulter, Optima L-100 XP ultracentrifuge) at 80,110 X g for 4 h using a Beckman Type 45 Ti rotor. The virus pellet was then resuspended in a small quantity of PBK buffer (1ml). Sucrose density gradients (10-50% w/v), prepared by hand 24 h earlier in 12.5 ml Beckman ultracentrifuge tubes and allowed to form at 4˚C, were loaded with the partially purified virus and ultracentrifuged for 3 h at 140,235 X g in a Beckman rotor SW 41 Ti. After centrifugation the virus, which banded in the gradient as a blue-grey opalescent zone (Fig. 2.2) was removed by puncturing the base of tube and collecting drop fractions in sterile Eppendorf tubes. Gradient fractions of interest were dialysed overnight with stirring, against 500 ml, 10 mM Tris–HCl (pH 7.6) using a dialysis cassette. The dialysate was recovered and aliquots of 100 µl aliquots were stored at -80˚C prior to dsRNA isolation which was performed as described in section 2.11.4

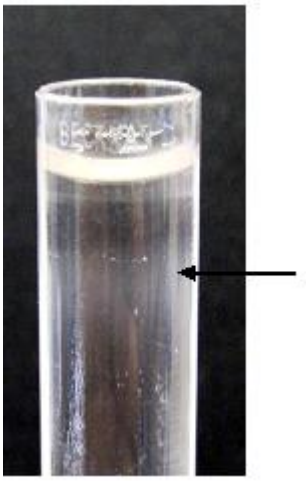


Figure 2.2 Purified *A. fumigatus* isolate 88 virus banded in a 10-20% sucrose density gradient. The opalescent band of the virus is arrowed.

2.12.2 Extraction of viral dsRNA using cellulose CF-11

The method was adapted from Coffin and Coutts (1992) for the extraction of dsRNA from plant tissues. Freshly harvested mycelia (100 g) was homogenised in liquid nitrogen and added into a mixture containing 150 ml GPS (GPS buffer 5 X: 87.66 g NaCl, 37.54 g glycine, 35.49 g Na_2HPO4, [pH 9.5]), 150 ml GPS saturated phenol, 150 ml Sevag, 15 ml 10 % SDS and 1.5 ml β-mercaptoethanol and was left to stir at 4^{o}C for 1 h. The material was strained through a double layered muslin cloth to remove solid material and subjected to centrifugation (13,000 X g; 10 min) at 4^{o}C. The aqueous phase was removed and ethanol was added to a final concentration of 17% together with CF-11 cellulose (3 g/l) and the mixture stirred for 1 h to allow binding of dsRNA and then centrifuged (13,000 X g; 10 min). The resulting pellet was resuspended in *ca*. 45 ml of STE buffer (STE buffer 10 X: 29.22 g NaCl, 30.29 g Tris-HCl, 1.86 g EDTA, 500 ml H2O, [pH 7.5]) with 17% ethanol and shaken for 5 min and the cellulose was repelleted by centrifugation (2000 X g; 2 min). This step was repeated four times. The dsRNA was eluted using 20 ml of STE buffer (without ethanol) in four steps and centrifuged as before, with the final elution being left for 1 h on a rotating wheel to maximise yield. The combined supernatants were collected and centrifuged (23,700 X g; 30 min) to remove any residual cellulose. Nucleic acids were then precipitated from the supernatant as described in section 2.11.1. Following overnight precipitation dsRNA was pelleted, dried and resuspended in 1 ml aliquots and frozen at -80^{o}C prior to be being treated with DNase (section 2.11.2) and S1 nuclease (section 2.11.3) and agarose gel electrophoresis analysis (section 2.11.5).

2.12.3 RNeasy extraction of total RNA from fungal mycelia

Total RNA was extracted from mycelium using the RNeasy plant minikit (Qiagen) by following the instructions to extract total RNA from filamentous fungi. Small quantities of mycelia (100 mg) were ground in Eppendorf tubes in liquid nitrogen using a plastic pestle and mortar ensuring that the tissue did not thaw during the procedure. The tissue was thawed into 450 µl of lysis buffer RLC, vortex mixed vigorously for 30 sec and incubated at 56˚C for 3 min, to enhance disruption of tissue**.** Cell debris was removed from the homogenised lysate by application of the mixture to a QIAshredder spin column and micro-centrifugation for 2 min at 16,000 X g followed by transfer of the supernatant to a fresh Eppendorf without disturbing the cell-debris pellet in the collection tube. The volume of the supernatant was estimated and half that volume of absolute ethanol was added and the whole mixed well by pipetting to clear the lysate. The mixture was then applied onto an RNeasy mini spin column sitting in a 2 ml collection tube and centrifuged for 15 sec at 16,000 X g. The flow through was discarded and 700 µl of wash buffer RW1 was pipetted onto the RNeasy column, and centrifuged for a further 15 sec at 16,000 X g to wash the nucleic acid. The flow through and collection tube were both discarded and the RNeasy column placed into a new collection tube. Then 500 µl of wash buffer RPE was added to the RNeasy column and centrifuged at 16,000 X g for 15 sec. A further 500 µl RPE was added and the column centrifuged for 2 min at 16,000 X g. To avoid carryover of RPE buffer the empty column was re-centrifuged (16,000 X g; 15 sec) prior to transfer to new Eppendorf tube. The RNA was eluted in 30 µl RNase free water by centrifugation at 16,000 X g for 1 min.

2.13 Genome walking technique for characterisation of mycoviruses

To characterise and sequence the mycoviruses of *A. fumigatus*, a genome walking procedure was adapted in which primers were designed on the basis of known sequence and RT-PCR was performed.

2.13.1 Design of oligonucleotide primers for RT-PCR amplification

A series of oligonucleotide primers were designed for reverse transcription polymerase chain reaction (RT-PCR). Primers designed were 18-22 bases in length and in most cases had a melting temperature of between 60^{o}C-75^{o}C. Care was taken during primer design to avoid or

minimise primer/dimer and secondary structures and that the G/C content of any particular primer was at least 45-60%. These features were checked on the Sigma-Genosys website: http://www.sigma-genosys.com/calc/DNACalc.asp from where all primers were purchased.

2.13.2 Reverse transcription polymerase chain reaction (RT-PCR) amplification

RT-PCR amplification of dsRNAs was performed using a standard procedure. DsRNA (8 µl) extracts from various sources were denatured by addition of 2 µl methyl mercuric hydroxide (CH_3HgOH; 100 mM, SERVA) and 1 µl of an oligonucleotide primer (100 µM) of known sequence designed to amplify mycoviral dsRNA sequences. The mixture was incubated at room temperature for 20 min, and then chilled on ice for 1 min. For first strand cDNA synthesis, denatured dsRNA was added to the first strand cDNA synthesis reaction mixture that had been pre-heated at 45°C for 1 min. The entire mixture was incubated at 45°C for 1 min followed by addition of 1 µl (200 U/µl) of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) prior to further incubation at 45°C for 1 h.

First strand cDNA synthesis reaction mix:

Sterile distilled water	26.55 µl
M-MLV Reverse Transcriptase reaction buffer (5x; Promega)	10 µl
dNTP mix (20 mM; Promega)	1.20 µl
RNasin™ RNase inhibitor (40U/µl; Promega)	1.25 µl

The solution was size-fractionated and concentrated using a Nanosep column (30 K, Millipore). The volume of the first strand cDNA solution was increased to 500 µl with water and centrifuged in the column (5,000 X g; 5 min) resulting in the recovery of *ca*. 150 µl of solution. PCR reactions were then performed using undiluted cDNA and a 1:10 dilution of the cDNA.

PCR Mixture:

H_2O	26.3 µl/71.3 µl
First strand cDNA	50 µl /5 µl
PCR reaction buffer (Go-Taq buffer, 5 X)	20 µl

Specific primer (e.g. MF-1; 100 μM)	1 μl
Random hexamer (100 μM) or other oligonucleotide	1 μl
Oligonucleotide primer	1 μl
dNTPs mix (20 mM; Promega)	1.2 μl
Go-Taq polymerase (5 U/μl; Promega)	0.5 μl

The reaction was run in a PCR thermocycler (Peltier) using the different thermocycling conditions.

2.13.2.1 Thermocycling conditions used in PCR thermocycler

The reaction was run in a PCR thermocycler (Peltier) using the following cycling regimes.
Thermocycling condition 1:-

96^{o}C for 5 min	1 cycle
96^{o}C for 30 sec/50^{o}C for 30sec/72^{o}C for 2 min	32 cycles
72^{o}C for 10 min	1 cycle
4°C	forever

Thermocycling condition 2 :-

96^{o}C for 5 min	1 cycle
96^{o}C for 30/55^{o}C for 45 sec/72^{o}C for 1.5 min	30 cycles
72^{o}C for 10 min	1 cycle
4°C	forever

One tenth of each reaction mixture was analysed on a 1% agarose gel (section 2.11.5) along with a molecular weight size marker in order to check the size of any amplicons produced. Amplicons generated were ligated into a suitable vector to generate recombinant DNA for transformation.

2.13.3 RNA linker mediated random amplification of cDNA ends (RLM-RACE)

This protocol was taken from Coutts and Livieratos (2003). This procedure permits fast and reliable modification of 3' termini using viral dsRNA as template. In this procedure a primer

(LIG-Rev [5'-kinated-PO_4; 3'-OH-blocked]; 5'-GATCCAACTAGTTCTAGAGCGG-3') is ligated to the 3'-ends of dsRNA using T4 RNA ligase (New England Biolabs [NEB]). To achieve this, 7 µl aliquot of dsRNA was added to 1 µl of LIG-Modified REV primer, and the mixture heated to 90°C for 2 min followed by snap cooling on ice. The ligation mixture was prepared as described below:

	Final concentrations in the ligation mixture
1 µl 2 M MgCl2	20 mM
1 µl ATP	1 mM
10 µl DMSO	10%
2.5 µl RNase inhibitor	1 U/µl
2.5 µl 2 M HEPES (4-[2-hydroxyethyl] -1-piperazineethanesulfonic acid)	50 mM
5 µl DTT (100 mM)	5 mM
5 µl T4 RNA ligase (NEB)	100 U
Sterile distilled water	to 100 µl

The RNA ligase was added last and the mixture microfuged briefly to bring contents at the bottom, prior to incubation at 17^{o}C for 15 h. Whilst the buffer supplied by the manufacturer with the T4 RNA ligase was used in the ligation reaction, only DMSO, RNase inhibitor and RNA ligase were used for setting up the reaction. Here 10 µl of buffer was added to 64.5 µl of water and incubated at 37^{o}C for 10 min after that DMSO and RNase inhibitor were added followed by addition of RNA ligase and mixture was incubated at 17^{o}C for 15 h. After incubation, sterile water was added to increase the reaction volume to 500 µl and the solution was then size-fractioned and concentrated using a Nanosep 30K column (VWR) by centrifugation (5,000 X g; 5 min), resulting in the recovery of *ca*. 150 µl. The ligated nucleic acid was precipitated as before (section 2.11.1) and following pelletting and drying was resuspended in 7 µl of sterile water and put on ice for 20 min. The ligated dsRNA was then denatured by adding 2 µl of CH_3HgOH (100 mM, SERVA) together with 1 µl (100 µM) of (LIG-For primer; 5'-CCGCTCTAGAACTAGTTGGATC-3'). The mixture was incubated at room temperature for 20 min, and then chilled on ice for 1 min. For first strand cDNA synthesis, denatured dsRNA was added to the first strand cDNA synthesis reaction mixture that had been pre-heated at 45^{o}C for 1 min. The entire mixture was incubated at 45^{o}C for 1

min followed by addition of 1 μl (200 U/μl) of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) prior to further incubation at 45^{o}C for 1 h.

First strand cDNA synthesis reaction mix:

Sterile distilled water	26.55 μl
M-MLV Reverse Transcriptase reaction buffer (5x; Promega)	10 μl
dNTP mix (20mM; Promega)	1.2 μl
RNasin^{TM} RNase inhibitor (40U/μl; Promega)	1.25 μl

The solution was then size-fractioned and concentrated using a Nanosep 30K column as before (section 2.13.2). The volume of the first strand cDNA solution was increased to 500 μl with H_2O and centrifuged in the column (5000 X g for 10 min), resulting in the recovery of *ca*. 150 μl of solution. A PCR amplification reaction was then set up as described below:

PCR Mixture:

First strand cDNA	50 μl
Go*Taq* buffer (5 X, Promega)	20 μl
LIG-FOR primer (100 μM)	1 μl
Sequence specific primer (100 μM)	1 μl
dNTP mix (20 mM, Promega)	1.2 μl
Sterile distilled water	26.3 μl
GoTaq polymerase (5 U/μl, Promega)	0.5 μl

The reaction was run in a PCR thermocycler (Peltier) using thermocycling program 1 (see section 2.13.2.1). One tenth of each reaction mixture was electrophoresed in a 1% agarose gel (section 2.11.5) in order to check the size of any amplicons produced.

2.14 Complementary DNA (cDNA) library construction

This method of cDNA library construction is a useful tool in the production of cDNA clones from dsRNA (Coffin and Coutts, 1992) and is described below:-

A small aliquot of dsRNA (7 µl) was denatured using methyl mercuric hydroxide (2 µl; 100 mM CH_3HgOH, SERVA) in the presence of random hexameric primers (2 µl; 0.5µg/µl Promega) following incubation at room temperature for 20 min, followed by 1 min incubation on ice. Denatured dsRNA was added to first strand cDNA synthesis reaction mixture (10 µl; 5 X reverse transcription buffer, 4 µl; 100 mM DTT, 1.25 µl; RNasin™ ribonuclease inhibitor, 1 µl; 20 mM dNTPs and 23.75 µl sterile distilled water) pre-heated at 50-55 ˚C. The mixture was incubated at 25˚C to ensure efficient binding of random primers before the initiation of first strand synthesis and then at 55°C for 1 min followed by the addition of 1 µl of Superscript III Reverse Transcriptase (Invitrogen) and further incubation at 55°C for 1 h.

The first strand synthesis reaction (50 µl) was added to second strand reaction mixture and the mixture incubated at 16°C for 3h. Then T4 DNA polymerase (2 µl; 5U/µl; Invitrogen) was added and the mixture incubated for a further 5 min. and the reaction was arrested by heating at 65°C for 10 min. Products obtained were purified using the QIA quick PCR purification kit (section 2.11.8) collecting the product into a small volume of sterile distilled water.

Second strand cDNA synthesis reaction mixture:

Second strand cDNA synthesis reaction mixture consists of the following ingredients:-

5x second strand buffer (Invitrogen)	30 µl
10mM dNTP mix	3 µl
10U/µl *E.coli* DNA ligase (Invitrogen)	1 µl
10U/µl *E.coli* DNA polymerase (Invitrogen)	4 µl
2U/µl *E.coli* RNAse H (Promega)	1 µl
H_2O	61 µl

The purified product (6 µl) is then phosphorylated. Following addition of 1 µl of (10 X) polynucleotide kinase (PNK) buffer, and 1 µl of ATP (20 mM) and heating at 70°C for 5 min. the mixture is placed on ice followed by the addition of 2 µl of T4 PNK (5-10U/µl; Promega) prior to 30 min incubation at 37°C. The reaction is terminated by heating at 68°C for 10 min and cleaned using the QIAquick PCR purification kit eluting the product into 30 µl sterile distilled water. Following phosphorylation an adenine residue is added to the product to

facilitate ligation into the pGEM-T Easy vector (Promega). Here 30 µl of phosphorylated dsDNA is added to the A tailing reaction mixture (4 µl; 10 X *Taq* polymerase buffer, 2 µl; 10 mM dATP, 1 µl; 50 mM $MgCl_2$, 2.8 µl; sterile distilled water and 0.2 µl; 5 U/µl *Taq* DNA polymerase) prior to 20 min incubation at 72^{o}C. Finally, the product is purified using the QIAquick PCR purification kit and eluted into 30 µl sterile distilled water and half of the reaction volume ligated into the pGEM-T Easy vector.

2.15 Ligation of PCR amplicons

Amplicons and randomly primed cDNAs were ligated into a suitable vector to generate recombinant DNA for transformation. PCR products were ligated into pGEM-T Easy vector (Promega). After gel recovery or PCR product cleanup through a Qiagen column, 250 ng (*ca.* 3 µl) of PCR product was added to 5 µl 1x T4 ligation buffer, 1 µl of pGEM-T Easy plasmid and 2 U T4 ligase (1 µl) was added. Ligation reactions were incubated at 4^{o}C for a minimum of 16 h.

2.16 Preparation of *E. coli* JM109 competent cells

2.16.1 The $CaCl_2$ method for preparation of *E.coli* competent cells

A small aliquot of frozen *E.coli* JM109 cells (Promega) were inoculated into sterile LB media (5 ml) and allowed to grow in a shaking incubator at 37 ^{o}C. Then 1 ml of the overnight culture was added to sterile LB broth (100 ml) in a 250 ml flask and allowed to grow at 37^{o}C until the OD (at 600 nm) reached 0.4-0.5. Cells were then placed on ice for 5 min and total culture (100 ml) was aliquoted into two (50 ml) universal tubes and centrifuged at 3000 X g; 10 min at 4^{o}C. Supernatants were decanted carefully without disturbing the pellets into bacterial disinfectant. Cold 25 ml $CaCl_2$ (100 mM) was added to each pellet and mixed with care on ice for 20 min. Centrifugation as above was repeated and the supernatant was removed prior to finally resuspending the pellets very carefully in 1 ml storage buffer (300 µl of 100% glycerol (VWR) plus 700 µl of 100 mM $CaCl_2$). Aliquots (100 µl) of the prepared cells were flash-frozen with liquid nitrogen and stored at -80^{o}C for future use.

2.16.2 Transformation storage solution (TSS) method for preparation of *E.coli* competent cells

This method was adapted from Chung *et al.,* (1989) for the preparation of competent cells. The cells were grown overnight in 5 ml LB broth at 37^{o}C. The overnight culture was diluted 1:1000 into 50 ml LB broth and incubated at 37^{o}C until an OD 0.40-0.50 at 600 nm was reached. The cells were chilled on ice for 5 min in pre-cooled 50 ml centrifuge tubes before centrifugation (3000 X g; 10 min) at 4^{o}C. They were then resuspended in 5 ml chilled TSS (section 2.6) and aliquoted (100 µl) into Eppendorf tubes, flash-frozen with liquid nitrogen and stored at -80^{o}C for future use.

2.17 Transformation of competent *Escherichia coli* cells

Frozen JM 109 *E. coli* competent cells (section 2.16) were left on ice to thaw and an aliquot of 40 µl was carefully transferred and mixed with 5 µl of ligation reaction mixture in a chilled Eppendorf tube. Tubes were rapidly flicked manually to mix, and then kept on ice for 20 min. The cells were then heat shocked for 45 sec at 42^{o}C, snap cooled on ice for 2 min and 900 µl of chilled SOC solution (section 2.8) ; including 200 µl 2 M glucose and 50 µl 2 M $MgCl_2$) added and the mixture incubated with shaking (150 rpm) for 60 min at 37^{o}C. The cells were pelleted (16,000 X g; 10 sec), resuspended and spread on Luria-Bertani (LB; section 2.7) solid medium in Petri dishes supplemented with 100 µg/ml ampicillin (section 2.4.1), 0.1 mM IPTG; (section 2.4.2.2) and 50 µg/ml X-Gal (section 2.4.2.1) for blue/white selection. Plates were incubated at 37^{o}C for 16 h. Colonies representing recombinant plasmids were identified.

2.18 Colony picking

The colonies which were transformed were white and untransformed cells remained blue. Recombinant colonies were picked aseptically with sterile toothpicks and inoculated into 5 ml LB broth containing 7 µl of ampicillin (50 mg/ml; section 2.4.1) and the cultures incubated at 37^{o}C overnight with shaking at 150-200 rpm.

2.19 Isolation and purification of plasmid DNA using the QIAprep spin miniprep kit

For isolation and purification of plasmid DNA, the QIAprep Spin Miniprep kit was used (Qiagen). Overnight cultures produced as in section 2.18 were centrifuged (3000 X g; 10 min) and the pelleted bacterial cells resuspended in 250 µl of P1 buffer (resuspension buffer) with mild vortexing of the pellet until there were no cellular clumps visible. The liquid was carefully transferred to a fresh Eppendorf tube. The cells were lysed by the addition of 250 µl P2 buffer (lysis buffer) and inversion of the mixture. When the suspension was clear, 350 µl of N3 (neutralisation buffer) were added and the mixture inverted gently. After centrifugation (16,000 X g; 10 min), the pellet (containing cellular debris) was discarded and the supernatant was carefully transferred to a QIA prep spin column and centrifuged (16,000 X g; 1 min). The column was washed once with 500 µl of PB buffer. Then column was washed with 750 µl PE buffer (ethanol added) and re-centrifuged (16,000 X g; 1 min) until the surface was dry and free of PE buffer. The DNA was eluted in 50 µl of nuclease free water by centrifugation for (16,000 X g; 1 min).

2.20 Restriction digestion of recombinant clones

Restriction digestion of recombinant clone DNA (6 µl) was carried out in digestion buffer (2 µl; Buffer H; 10 X, Promega) with 5 U (2 µl) *Eco*RI restriction enzyme plus 10 µl of water. Reactions were incubated for 1 h at 37^{o}C and the products examined by electrophoresis on agarose gels to confirm the presence of a DNA insert in any putative recombinant clones.

2.21 Sequencing

Recombinant plasmids containing inserts of interest were sequenced using either of the oligonucleotide primers below which primed within the pGEM-T Easy vector.
The reverse oligonucleotide primer was used when inserts were larger than 600 bp.

M13 Forward (Universal) 5’-GTAAAACGACGGCCAGT-3’
M13 Reverse (16 mer) 5’-AACAGCTATGACCATG-3’

For each sequencing reaction 8.50 µl of the purified miniprep DNA sample was mixed with 1.50 µl of oligonucleotide primer making a final volume of 10 µl. The sequence of each

recombinant plasmid DNA was determined by the Medical Research Council facility at Hammersmith Hospital. Sequence data received was then analysed, aligned and compared for similarity with database accessed sequences using the NCBI, BLAST program (Altschul *et al.*, 1990). Sequence alignments were performed using the LALIGN http://www.ch.embnet.org/software/LALIGN_form.html and MulTALIN alignment programs http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html.

2.22 Quantitation of nucleic acid

For the quantitation of nucleic acids a spectrophotometer and a nanodrop were used.

2.22.1 Spectrophotometry

For quantitation of nucleic acids and for the measurement of the optical density (OD) of bacterial cultures, a spectrophotometer was used. Samples were placed in plastic or quartz cuvettes with a 1 cm path length and absorbance of light at an appropriate wavelength was compared with that of appropriate blank samples and concentrations can be calculated as under

$1\ A_{260} = 50\ \mu g/ml$ dsRNA or DNA

$1\ A_{260} = 40\ \mu g/ml$ ssRNA

The following equation can be used to quantify the dsRNA concentration.

$A_{260} \times$ dilution factor $\times 50 = \mu g/ml$ dsRNA.

2.22.2 Nanodrop spectrophotometer

For the quantitation of small amounts of nucleic acids, the Nanodrop 2000C (Thermo Fischer) was used according to the manufacturer's instructions. After cleaning the lower and upper optical surfaces of the microspectrophotometer and opening the nucleic acid module, 2 µl of deionised water was loaded on the lower optical surface and a blank measurement was taken. Nucleic acid sample was measured by loading 2 µl of sample. The software automatically calculates the nucleic acid concentration if the appropriate constant has been chosen for the measurement (e. g. 50 for dsDNA or RNA and 40 for ssRNA).

2.23 Hybridisation of nucleic acid separated on non-denaturing agarose gels

2.23.1 Nucleic acid blotting

Agarose gel electrophoresis (section 2.11.5) of dsRNAs was performed until the bromophenol blue in the loading buffer reached the end of the gel. The gel was photographed and nucleic acids nicked by immersion in 0.25 N HCl for 20 min, followed by nucleic acid denaturation by soaking in 100 mM NaOH for 30 min. The nucleic acid were then neutralised twice in (100 mM Tris HCL [pH 8.0]) for 20 min on each occasion. The gel was reversed and placed on top of four sheets of 3 mm Whatman paper, which served as a bridge with the blotting buffer (20 x SSC: 3 M NaCl, 0.3 M $C_6H_5Na_3O_7$ [pH 7]). Pre-wetted Hybond-N membrane (Amersham) in 2 X SSC, exactly the gel size was placed on the top of the gel followed by four pieces of 3mm Whatman paper and paper towels 5 cm thick. The blot was covered with Saran wrap (Sigma) in order to prevent evaporation and a weight (~500gm) was placed on the top and the assembly left overnight so that nucleic acids were capillary transferred onto the membrane. After dismantling the assembly, the membrane was briefly rinsed in 2 X SSC and left to dry. Nucleic acids were fixed on the membrane by exposure to UV radiation for 60 sec using a Stratalinker 1800 (Stratagene) at 120 mg/cm^2. Membranes were prehybridised in 25 ml of a pre-hybridisation buffer (2.23.1.1) in a hybridisation oven in soda glass tubes at 65^{o}C.

2.23.1.1 Pre-hybridisation solution

20X SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA [pH 7.4])	6.25 ml
50 X Denhardt's solution (for 0.51: 5 g Ficoll Type 400, 5 g PVP, 5 g BSA)	2.5 ml
Sheared denatured salmon sperm DNA (20mg/ml)	25 μl
0.5% SDS	0.625 ml
Sterile distilled water	to 25 ml

2.23.2 Radioactive labelling of DNA

Radiolabelled probes for the dsRNA components of the *A. fumigatus* partitivirus **(**AfuPV**)** genome were produced using representative recombinant clones for each genomic

component. Clones were labelled with (α^{32}P)-dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Promega) using the oligolabelling method of Feinberg and Vogelstein (1984). Individual recombinant plasmids (5 µl; 20 µg/µl) were heat denatured at 95-100^{0}C for 3', snap-cooled on ice for 2 min and each added to a separate mixture below to achieve a final volume of 50 µl for each probe.

Nuclease-free water	24 µl
Oligolabeling buffer (5 X)	10 µl
Nuclease-free bovine serum albumin (10 µg/µl)	2 µl
Mixture of dGTP dATP AND dTTP (1.5 mM)	2 µl each
[α^{32}] dCTP (50 µCi: 3000 Ci/mmol; GE., Healthcare)	2 µl
Klenow fragment of DNA polymerase 1 (Promega)	1 µl

The mixtures were incubated at room temperature for 60 min, denatured at 95-100^{0}C for 3 min, snap cooled on ice for 2 min and briefly microfuged to bring evaporated contents in the bottom of tube. Each probe was added to their respective pre-hybridised blots behind the protective screen and the blots hybridised overnight at 65^{0}C in a Rotisserie.

2.23.3 Filter washing of the membranes and autoradiography

After hybridisation, the membranes were washed in Rotisserie washing solutions in the following order with increased stringency of SDS and reduced ionic strength.

2 × SSPE (section 2.23.1.1), 0.1% (w/v) SDS	(Twice at 65^{o}C)
1 × SSPE, 0.1% SDS (w/v)	(Once at 65^{o}C)
0.1 × SSPE, 0.1% SDS (w/v)	(Once at 65^{o}C)

The membranes were wrapped in Saran wrap and exposed overnight or for longer periods of time to X-ray film (Kodak) at -80^{o}C, using a cassette with an intensifying screen.

2.24 Methods used to investigate the curing *A. fumigatus* isolate 88 from AfuPV infection, hyphal fusion and protoplast transfection of virus-free isolates of *A. fumigatus*

2.24.1 Curing of *A. fumigatus* isolate 88 from AfuPV infection

Attempts were made to cure *A. fumigatus* isolate 88 of AfuPV infection and eradicate the viral dsRNA by treatment with cycloheximide (protein synthesis inhibitor). Cycloheximide, which blocks translational initiation and elongation during protein synthesis, has been used before to successively eradicate mycoviral infection from several different fungi (Elias and Coty, 1996; Robinson and Deacon, 2002; Dalzoto *et al*., 2006). The procedure used to 'cure' the isolate of dsRNA infection was adapted from Van Diepeningen *et al.,* (2006). Initially, sequential hyphal tip isolation was performed and fast growing hyphal tips were isolated and grown over a period of three weeks on ACM media to 'cure' or to reduce the virus titre. Secondly, ACM media (section 2.5.1) treated with a range of concentrations of range of cycloheximide concentrations (0.01 mM to 150 mM) to impact the fungi ACM containing Petri plates which were inoculated with *A. fumigatus* isolate 88, once the cycloheximide had been added to the medium at ~50^{o}C and the agar allowed to solidify. All inoculated plates were incubated at 37^{o}C until confluent mycelial growth was obtained and the phenotypic characteristics of the fungal growth patterns scored. In order to screen for any effect of cycloheximide treatment on the titre of individual genomic dsRNA components of AfuPV, small amounts of mycelia were harvested and total RNA extracted using the either small scale isolation procedure (2.10.6) or RNeasy kit (section 2.12.3) for RT-PCR (section 2.13.2). The oligonucleotide primers used in these assays were designed to generate amplicons not only representing fragments or complete coding regions predicted from the sequences of two of the three AfuPV genomic components, encoding the RdRp and CP.

2.24.2 Intraspecific virus transfer *via* hyphal tip fusion

Fungal anastomosis, which is an easy and useful tool to transfect virus to recipient hosts and has been used successfully for several fungi, was attempted to transfer dsRNAs from infected to uninfected isolates. Isolates A-54 A-56 and A-78 were selected as donor isolates for virus transfer to a yellow coloured, hygromycin resistant recipient isolate (Af-237y).

All isolates of *A. fumigatus* were grown on ACM, which in the case of Af-237y contained hygromycin (30 µl/25 ml ACM). Spores were harvested after 3 days and enumerated using a

haemocytometer. Glycerol stocks (section 2.10.3) for each isolate were stored at -20^{o}C. Spores of each donor isolates were co-cultured with Af-237y in 200 ml of ACM incubated at 37^{o}C with shaking at 130 rpm for 5-6 days. Then a small quantity of mycelia was used as inoculum to inoculate ACM plates, with and without hygromycin (30 µl/25 ml ACM). Selection was made on the basis of yellow pigmentation and hygromycin resistance. Colonies obtained, were inoculated on ACM plates and left for 5 days to sporulate. Spores were harvested and a suspension was used to inoculate 200 ml ACM broth in 500 ml Erlenmeyer flasks, which were incubated at 37^{o}C with shaking at 130 rpm for 5-6 days. The mycelia obtained were then subjected to RNeasy procedure to extract total RNA (section 2.12.3) and diagnostic RT-PCR assay to assess transfection.

2.24.3 Protoplast transfection-using PEG

This procedure was adapted from Szewczyk *et al*., (2007). Spores of *A. fumigatus* isolate Af-237y were quantified (1 X 10^{8}) using a haemocytometer (section 2.25.1.1) and inoculated into 20 ml of YG medium (section 2.9) in an Erlenmeyer flask (50 ml) and incubated with shaking (120-150 rpm) at 30^{o}C overnight. Mycelium was harvested, washed with water, filtered through sterile Miracloth (VWR), and resuspended in 8 ml ACM (section 2.5.1) in a sterile 50 ml Erlenmeyer flask, loosely covered with aluminium foil and mixed after adding, 8 ml of freshly prepared 2 X filter sterilised (0.22 µm) protoplasting solution (10 ml KCl [1.1 M] citric acid [100 mM] solution, add 1.28 g Vinoflow FCE [Novo Nordisk]). The mixture was incubated with gentle shaking at 30^{o}C for 2 h and then small quantity is analysed under the microscope to check the state of protoplasts; if not enough protoplasts were released from mycelia the incubation was prolonged for 30 min. Then protoplast containing mixture was layered on a 1.2 M sucrose cushion in a sterile, universal tube and centrifuged (2000 X g; 10 min) at 4^{o}C to remove any residual, undigested hyphal material. The protoplasts were collected gently from the top of the sucrose solution, placed in a sterile centrifuge tube and an equal volume of 0.6 M KCl was added and the solution was centrifuged again as above. The supernatant was discarded without disturbing the protoplast pellet which was resuspended in 2 ml of 0.6 M KCl. The solution was aliquoted equally into two sterile 1.5 ml Eppendorf tubes and protoplasts were pelleted by centrifugation (13,000 X g; 3 min) in a bench top microfuge with a fixed angle rotor. The supernatant was removed and the protoplasts were resuspended in 1 ml of 0.6 M KCl. This step was repeated twice and after discarding the supernatant the protoplasts were finally resuspended in 0.5 ml of a 0.6 M KCl, 50 mM $CaCl_2$

solution. The contents of both Eppendorf tubes were combined in one tube and re-centrifuged (13,000 X g; 3 min) to pellet the protoplasts. The protoplasts were resuspended completely in 100 µl of 0.6 M KCl, 50 mM $CaCl_2$ solution by pipetting and 10 µl (0.05 µg/ml) of purified, filter sterilised virus added and the mixture vortexed briefly 6-8 times (1 sec on and 1 sec off). Then freshly filtered 50 µl PEG (3350) solution (0.6 M KCl, 50 mM $CaCl_2$ and 75 mM PEG 3350) was added to the protoplasts and the mixture vortexed 4-5 times as above followed by addition of 1 ml of PEG 3350 solution before incubation in an ice water bath for 25 min. A control experiment was performed simultaneously which omitted PEG 3350 from the transfection procedure but included the purified virus. Both the control and test transfection mixtures were inoculated onto agar plates which contained a selection medium (section 2.9.1) and incubated at 37^{o}C for 36-48 h and then glycerol stocks of mycelia were made from both plates Subsequently spores of both plates were inoculated into ACM broth and mycelia harvested 4 days after incubation and checked for transfer and replication of the virus following isolation of total RNA (section 2.12.3) and RT-PCR as described in section 2.13.2. The glycerol cultures were passaged sequentially and tested as above to assess any retention of virus on the surface of fungal mycelia during the inoculation process.

2.24.4 Transfection of macerated mycelia

This transfection procedure was modified from an original protocol as described by Schmidt *et al.* (1986). *A. fumigatus* isolate Afv-237y was inoculated from a glycerol stock (section 2.10.3) onto an ACM plate and incubated at 37^{o}C for 3 days. Spores were harvested on Miracloth and inoculated into 10 ml liquid YES medium (section 2.9.2) and grown at 37^{o}C for 4 days with shaking at 100 rpm. Following incubation the mycelia were macerated using a hand held homogeniser by blending for 1 min. Aliquots of the macerated and unmacerated (control) mycelium (500 µl) were mixed with 100 µl of AfuPV particles (0.07 µg/ml) and incubated in screw-capped tubes with shaking in a horizontal orientation at 37^{o}C, for 4 days. Mycelia were harvested and checked for transfer and replication of the virus following isolation of total RNA and RT-PCR as described in sections 2.12.3 and 2.13.2 respectively.

2.25 Effects of dsRNA on growth of *Aspergillus fumigatus*

2.25.1 Radial growth analysis

In order to determine the effects of AfuPV infection on *A. fumigatus* the virus-free strain (Af-237y; yellow isolate) and isolate 88 AfuPV transfected *A. fumigatus* were analysed for mycelial growth rate on solid media and in liquid media.

An average of 100 spores in a 10 µl volume (section 2.25.1.1) for each isolate were spotted in the centre of ACM (section 2.5.1) and MM (section 2.5.5) plates in triplicate. Radial growth measurement for each isolate was recorded on a daily basis and the results averaged and graphs for radial growth were plotted against number of days of incubation.

For growth rate determination, equal numbers of spores (150) of both isogenic lines were counted (centrally inoculated onto MM (section 2.5.5) and ACM (section 2.5.1) plates and incubated at 37^{o}C. Colony diameters were measured every 24 h over a period of 5 days.

2.25.1.1 Spore counting *via* haemocytometer

Spores were enumerated using a haemocytometer. To facilitate counting of concentrated spore suspensions they were diluted 1:10 or 1:100 in water. Spores were counted after the surface of the counting chamber was cleaned with absolute ethanol and 10 µl of the spore suspension was introduced into the V-shaped wells. A clean glass cover slip was placed over the droplet in order to spread the droplet. The suspension was introduced into one of the V-shaped wells with a Gilson pipette and the area under the cover slip was filled by capillary action. The counting chamber was then placed on the microscope stage and the counting grid was brought into focus at lower magnification (25 X). Five readings were taken for counting (4 from the side and 1 in the centre) and these were averaged.

2.25.2 Comparisons of mycelial dry weight of viral infected isolates of *Aspergillus fumigatus*

In order to assess the impact of virus on the fitness of its host, growth rate comparisons were made between AfuPV infected and virus free by dry mycelial weight assessment. Individual plates for each isolate were grown and conidia were harvested in a small quantity of water (25 ml) and enumerated (as described in section 2.25.1.1). An average of 3 X 10^{8} spores was

used as inoculum for 60 ml cultures of ACM broth from which an identical number of spores for each isolate was used to inoculate 30 Schott bottles containing 60 ml of ACM broth. Mycelial weight was measured by harvesting the mycelia for each individual isolate from 3 replicate bottles by filtration through pre-weighed sterile Miracloth. Samples were taken every 12 h over a period of 5 days. Mycelial mats were dried with paper towels and kept at 37 ^{o}C for 2-3 days to ensure complete dryness prior to weighing. Growth curves were plotted for each isolate using mycelial weight as ordinate and time interval as abscissa. Student's t test was used to analyse the data for differences in growth rate and mycelial biomass production.

2.26 Gel electrophoresis of proteins

2.26.1 SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

Proteins were separated under denaturing conditions using SDS-PAGE, as described by Laemmli (1970). The Mini-protean II electrophoresis system (Bio-Rad) was used and slab plates were cast between two glass plates (12x 10x 0.2 cm) separated by a 1.5 mm spacer. The gel consists of two main parts (resolving and stacking). The resolving gel was cast first and the components of which are shown in section (2.26.1.1). Isopropanol on the top surface of the gel to level the surface the system was left to set for 1 h. After polymerisation the isopropanol was removed, the surface of gel was washed with water and the stacking gel (section 2.26.1.2) was poured using a pipette. Comb was adjusted and gel was allowed to set for 1 h before electrophoresis. Protein samples were solublised in 2 X Laemmli buffer (Sigma) and were electrophoresed through the gel at 100 V.

2.26.1.1 Resolving gel (10%; 5ml)

1.5 M Tris-HCL (pH 8.8)	1.3ml
10% SDS	0.05 ml
30% acrylamide-bisacrylamide (30:0.8)	1.7 ml
TEMED	0.002 ml
10% (w/v) APS	10% (w/v) APS
H_2O	1.9 ml

2.26.1.2 Stacking gel (5%; 2ml)

1.0 M Tris-HCl (pH 6.8)	0.25 ml
10% SDS	0.02 ml
30% acrylamide-bisacrylamide (30:0.8)	0.33 ml
TEMED	0.002 ml
10% (w/v) APS	0.02 ml
H_2O	1.4 ml

2.26.2 Staining of protein gels

Protein gels are usually stained by Coomassie blue stain as it is a simple and sensitive staining procedure for visualising electrophoresed proteins. The gel was stained at room temperature in staining solution (0.1 % Coomassie brilliant blue R250 in methanol: water: glacial acetic acid [5:5:1 by volume]) for at least 20 min on an orbital shaker followed by overnight destaining of the protein gel in a destaining solution (methanol: water: glacial acetic acid [5:5:1 by volume]) with gentle shaking or with several changes of destain solutions over 2-3 h.

Chapter 3

Screening of *Aspergillus fumigatus* isolates (clinical and environmental) for dsRNA mycoviruses

3. Screening of *Aspergillus fumigatus* isolates (clinical and environmental) for dsRNA mycoviruses

To investigate the incidence of double stranded (ds) RNA mycoviruses in *A. fumigatus,* a panel of clinical isolates and environmental isolates (Table 3.1; see Appendix) were obtained from Dr. Michael Petrou (Faculty of Medicine and Investigative Science, Charing Cross Campus, Du Cane Road, Acton, London W4, United Kingdom) and Dr. Elaine Bignell (Centre of Molecular Microbiology and Infection, South Kensington campus, Imperial College London) in order to screen them for the presence or absence of dsRNA elements. *Aspergillus fumigatus* isolates were cultured as described in section (2.10.2) and harvested mycelia was used to determine dsRNA infection *via* screening (either LiCl fractionation procedure or small scale extraction; sections 2.10.5 and 2.10.6).

3.1 Results and discussion for screening *A. fumigatus* isolates

Previous investigations concerning the incidence of dsRNA mycoviruses revealed that the dsRNA infection rate of *Aspergillus* strains ranged from 0% to 13% and that the dsRNA electrophoretic profiles of mycoviruses in *Aspergilli* varied greatly in dsRNA fragment number and type even in one species (Varga *et al.,* 1998; Liu *et al*., 2008)**.** In my investigations 366 isolates of *A. fumigatus* were screened for the presence of dsRNAs, 24 of which were found to contain dsRNA elements (Table 3.2) with an incidence of *ca.* 6.5 %, which is lower than the incidence in other *Aspergillus* spp. including the black Aspergilli where 10% of the 668 isolates sampled contained dsRNAs (Van Diepeningen *et al.,* 2006).

Screening was carried out simultaneously with isolating dsRNA from *A. fumigatus* isolates known to contain dsRNAs *viz.* isolates A-56, A-54, isolate 80 and A-78 as positive controls and negative control isolates, which do not contain dsRNAs. Screening for dsRNA-containing isolates was initially carried out using a large scale extraction procedure (LiCl extraction; section 2.10.5) which was lengthy and time consuming. Later this procedure was superseded by more rapid and facile small scale extraction procedure (section 2.10.6). Results of extractions were analysed following agarose gel electrophoreses (section 2.11.5). As can be seen clearly in Fig. 3.1., which analysed the dsRNAs isolated from two *A. fumigatus* isolates (3 and A-54) using the LiCl procedure, isolate A-54 (lane 4) contained dsRNAs, while the negative controls contained no dsRNAs (lanes 3 and 5) and isolate 3 was also dsRNA-free (lane 2). Furthermore, Fig. 3.2 Illustrates the dsRNAs isolated from a range of *A. fumigatus*

isolates, using the small scale extraction procedure; *A. fumigatus* isolate 80 (Fig. 3.2; lane 3) contained a mixed infection of a chrysovirus with an uncharacterised dsRNA virus (positive control), while the negative control (isolate 3; lane 2) was dsRNA-free. Among the range of isolates shown in the Fig 3.2, only isolate 12 were found to contain dsRNA mycoviruses.

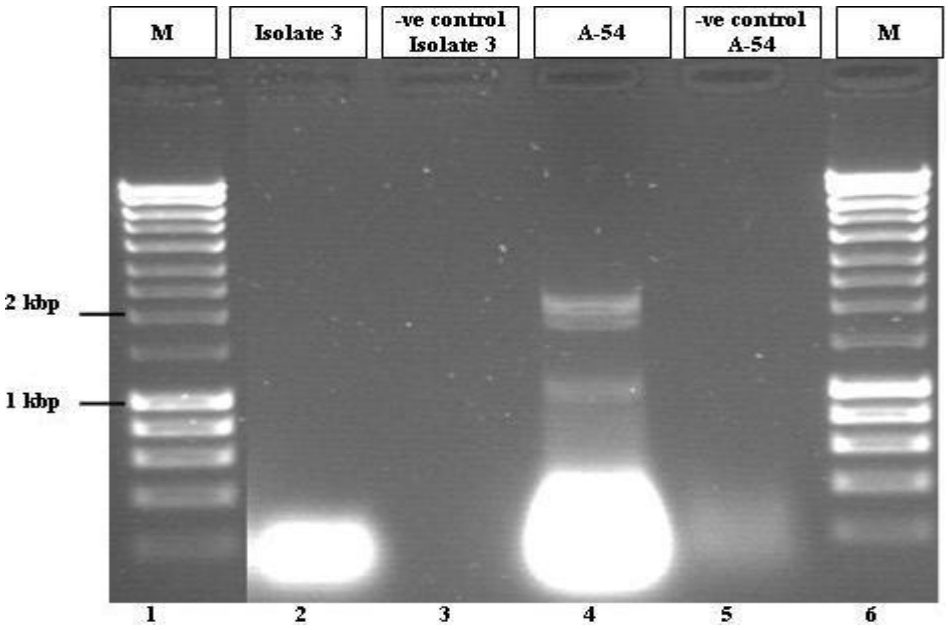


Figure 3.1 Agarose gel electrophoresis of *A. fumigatus* isolate 3 (accession no. 40907) dsRNAs were isolated using the LiCl fractionation procedure together with a positive control A-54 dsRNA. Lane 2 contains dsRNA extracts from isolate 3 and lane 3 shows a negative control of the same isolate (eluates). Similarly lane 4 contains dsRNA extracts from A-54 (positive control) and lane 5 contains eluates (negative control for A-54). Lane M contains the Bioline molecular weight marker DNAs.

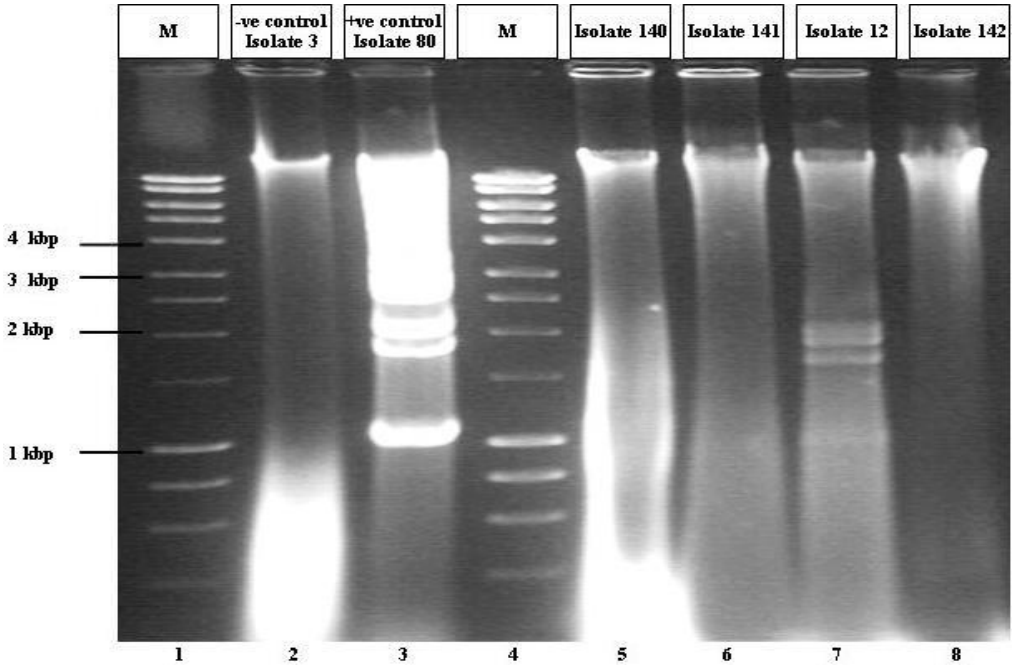


Figure 3.2 Agarose gel electrophoresis of dsRNAs isolated from *A. fumigatus* isolates 140, 141, 12 and 142 (lanes 5-8 respectively) together with negative control (isolate 3; lane 2) and positive control, (isolate 80; lane 3) using the small scale extraction procedure. Lane M contains the Bioline molecular weight marker DNAs.

Table 3.2 Clinical and environmental isolates of *A. fumigatus* isolates containing dsRNA elements

Isolate	**Source**	**Hospital**[a]	**Patient age and sex** [b]	**Date of isolation**
	Uncharacterised mycoviruses			
54	40047-Respiratory sample-Respiratory	WM	-	12/01/2006
78	40350-Respiratory sample-General Practitioner (GP)	HH	-	05/05/2006
26	41092-Respiratory sample-Haematology	HH	76M	15/12/2006
66	41144-Respiratory sample-Renal	HH	51F	28/09/2007
80	41218-Respiratory sample-Respiratory	XCH	48F	25/10/2007 [c]

103	420 BAL-Renal	HH	63F	13/06/2008
123	612-Sputum-Renal	HH	70M	25/08/2008
674	H987406-Sputum sample-Renal	HH	-	14/09/2008
729	H1034308-Sputum sample-Haematology	HH	-	10/10/2008
8	40051-Ear-GP	HH	-	16/01/2009
11	40630-Sputum sample-Respiratory	XCH	-	09/07/2009
12A	40695-BAL-ID	CW	-	30/07/2009
20	40283-Sputum sample-UNK	STM	-	30/03/2009
12	40081-Sputum sample-Respiratory	HH	-	26/01/2009
	Partitiviruses			
88	41249-Respiratory sample-Respiratory	HH	72M	13/11/2007
49	40049-H1208044-Sputum sample-GICU	HH	-	12/01/2009
6.2	Exeter-Environmental-Soil/compost	-	-	2009
10.2	Exeter-Environmental-Soil/compost	-	-	2009
11.7	Exeter-Environmental-Soil/compost	-	-	2009 [c]
23.1	Exeter-Environmental-Soil/compost	-	-	2009
25	40400-Knee aspirate-Surgery	CW	-	14/05/2009
	Chrysoviruses			
56	40093-Ear-ENT	HH	-	19/01/2006
9	40924-Oesophageal sample-Respiratory	HH	41M	06/112006

80	41218-Respiratory sample-Respiratory	XCH	48F	25/10/2007 [c]
11.7	Exeter-Environmental-Soil/compost	-	-	2009 [c]
24	40581-Sputum sample-A&E	HH	-	02/07/2009

[a] Hospital of isolation: WM=West Middlesex; HH=Hammersmith; XCH=Charing Cross; CW=Chelsea and Westminster; STM=St Marys. [b] Patient age and sex-when known: (M = male; F = female). [c] Mixed mycovirus infection.

3.1.1 Profiles of different *Aspergillus fumigatus* mycoviruses found during screening

Previous investigations revealed that the gel electrophoretic profiles of dsRNA elements in aspergilli differ widely within a species both in number and composition (Elias and Cotty, 1996; Varga *et al.*, 2001; Hammond *et al.*, 2008). These studies are considered to underestimate the diversity of mycoviruses genomes, which occur in *Aspergillus* spp. which are also thought to contain as yet uncharacterised satellite and defective dsRNAs (Varga *et al.*, 2001). The size of the dsRNA elements detected following electrophoresis of extracts ranged from *ca.* 1-4 kbp. Among infected isolates dsRNA element ranged in number from 2–5 segments. Representative different mycoviral profiles can be seen in Figs. 3.3 and 3.4. All mycoviral infections in *A. fumigatus* are divided into four different groups i.e. *A. fumigatus* chrysoviruses, partitiviruses, uncharacterised viruses and mixed mycoviral infections.

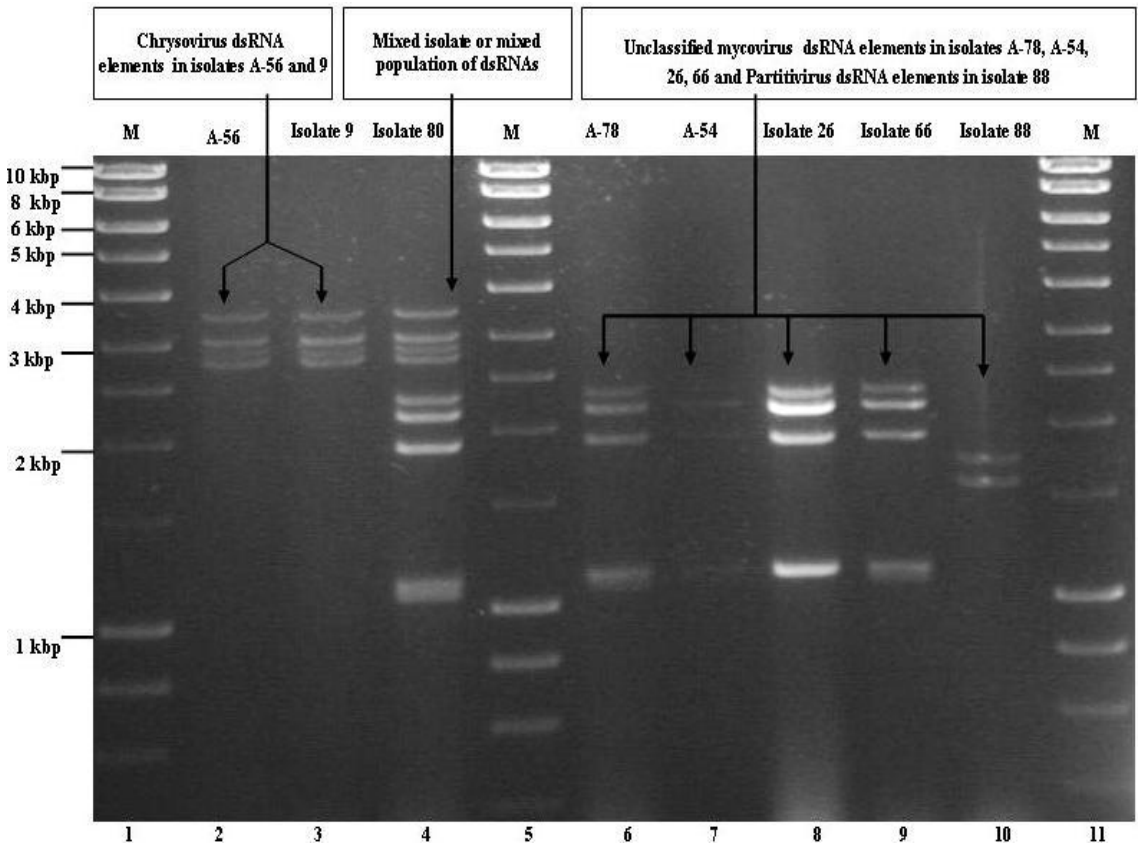


Figure 3.3 Overnight agarose gel electrophoresis of DNase and S1 nuclease treated nucleic acid extracts of those *A. fumigatus* isolates which contain dsRNAs,. Lanes 2-4 (A-56, 9, and 80); lanes 6-10 (A-78, A-54, 26, 66 and 88) Lanes 1, 5 and 11 contains the Bioline molecular weight marker (M) DNAs.

3.1.1.1 *A. fumigatus* chrysovirus (AfuCV)

A. fumigatus isolate A-56 was the first isolate of the fungus found to be infected with a mycovirus with a dsRNA profile apparently comprising three segments ranging in size from 2.5-3.5 kbp (Fig. 3.3; lane 2). The sizes and profile of these dsRNAs are reminiscent of members of the family *Chrysoviridae* apart from the fact that all the members of this family have a genome comprised of four segments. Confirmation that the A-56 isolate was infected with a chrysovirus was obtained following cloning and sequencing the dsRNA elements associated with the fungus (Jamal *et al.*, 2010). The banding profile of the dsRNAs found in *A. fumigatus* isolate A-56, isolate 9 and 24 are similar to the chrysovirus found in isolate PCV the type species of the family *Chrysoviridae* (Jiang and Ghabrial, 2004).

3.1.1.2 *A. fumigatus* partitivirus (AfuPV)

Partitiviruses were also found during this screening process. Isolate 88 partitivirus (Fig. 3.3; lane 10) was the first partitivirus found during the screening which was then cloned and sequenced (Bhatti *et al.,* 2011a). The dsRNA elements associated with the family *Partitiviridae*, consist of 2-3 segments, ranging in sizes from *c.a.* 1.0- 1.8 kbp (Fig. 3.3; lane 10). The banding profiles of the dsRNAs discovered in the *A. fumigatus* isolates 88, 49, 6.2, 10.2, 11.7, 23.1 and 88 are similar to those found for isolates of the putative partitivirus found in *Botryotinia fuckeliana* (De Guido *et al.,* 2007) and *A. ochraceus* (Kim *et al.,* 2006).

3.1.1.3 *A. fumigatus* uncharacterised viruses

During the screening of *A. fumigatus* isolates for the presence of dsRNAs another group of uncharacterised mycoviruses was found which contain 4-5 elements ranging in size from 1.0-2.2 kbp (Fig. 3.3 lanes 6-9). This type of dsRNA profile has not been described previously for any mycovirus associated with *Aspergillus* spp. Although they have been found in different Aspergilli like *A. niger* (Van Diepeningen *et al.,* 2006) and the elements were not investigated further. These uncharacterised viruses were the most frequently found dsRNA mycoviruses in *A. fumigatus*.

3.1.1.4 *A. fumigatus* mixed infections

Mixed mycoviral infection was also observed in two isolates, isolate 80 and 11.7 respectively. Mycoviral infection in isolate 11.7 (Fig. 3.4; lane 8) is a mixture of chrysovirus and a partitivirus. Such infections were also seen during investigations of mycoviruses in black aspergillus populations (Van Dipeningen *et al.,* 2006). Whilst, isolate 80 contains a mixture of a chrysovirus and the uncharacterised mycovirus (Fig. 3.3; lane 4).

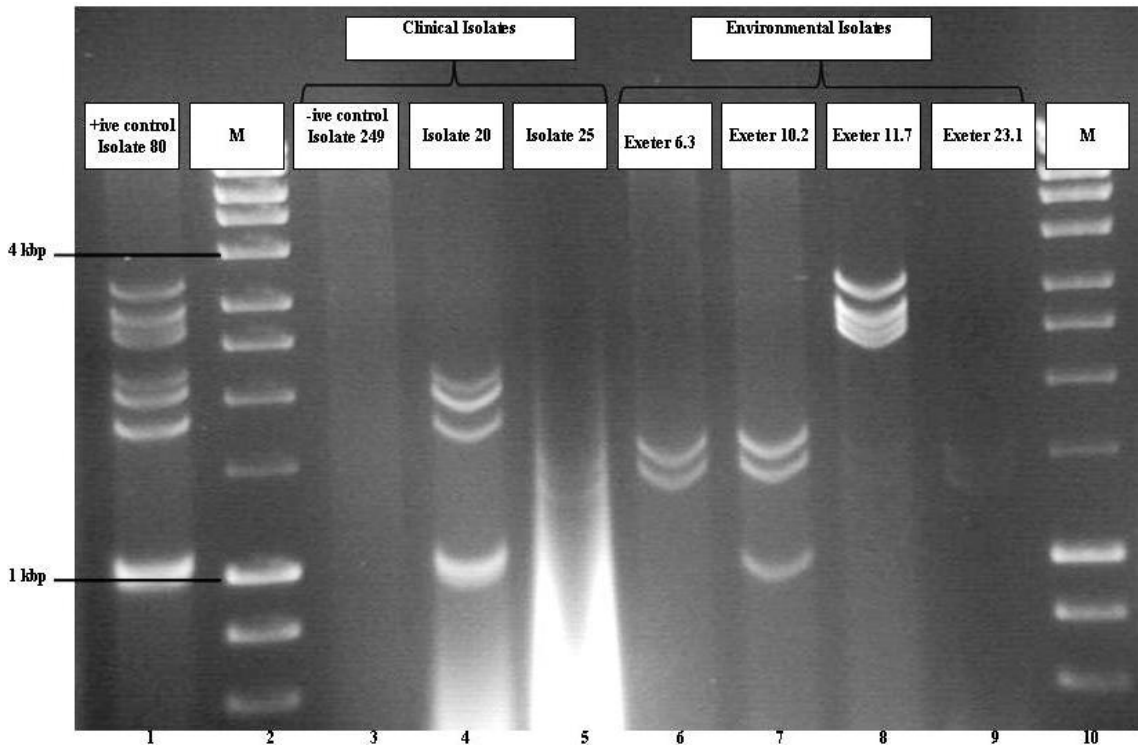


Figure 3.4 Overnight agarose gel electrophoresis for representative clinical and environmental isolates of those *A. fumigatus* isolates which contain dsRNAs, along with a negative (isolate 249; lane 3) and a positive control (isolate 80; lane 1).

3.1.2 Morphology of different *Aspergillus fumigatus* isolates infected with mycoviruses found during screening

Mycoviruses are widespread in fungi and are usually associated with symptomless infections but beneficial and detrimental effects are known (Hillman and Suzuki, 2004). However, correlation of fungal phenotype with a particular mycovirus is often troubled by the lack of an infectivity assay to connect the two conclusively (McCabe *et al*., 1999; Pearson *et al*, 2009). This problem is intensified by the frequent occurrence of mixed infections of multiple mycoviruses, for example in *Agaricus bisporus* (Romaine and Schlagnhauffer, 1995) and *Botryotinia fuckeliana* (Howitt *et al.*, 2006). In various fungi detrimental impact can be seen in terms of reduced sporulation and presence of debilitating sectors when they are grown on solid medium. Unlike animal and plant viruses, which are commonly associated with disease, many of the known mycoviruses cause no obvious symptoms. Only a few fungal viruses are known to affect their host, causing hypovirulence or hypervirulence (Herrero *et al.*, 2009).

Amongst the infected *A. fumigatus* isolates, on solid media some possessed a sectoring phenotype, a feature often characteristic of fungi containing mycoviruses. It is believed that the dsRNA mycovirus titre in these sectors are higher than in the surrounding mycelium (Van Diepeningen *et al.*, 2006) but this is yet to be investigated with the *A. fumigatus* isolates investigated here. However, most virus infections did not result in any obvious phenotypic alterations. The morphology of some of the *A. fumigatus* isolates infected with AfuCV are shown in Fig. 3.5 together with two, control uninfected isolates (clinical isolate 147and an environmental isolate Exeter 7.3) and an isolate originally infected with AfuCV cured of infection following treatment with cycloheximide, kindly supplied by Dr A. Jamal. The morphology of some *A. fumigatus* isolates infected with AfuPV are shown in Fig. 3.6 together with the same uninfected isolates shown in Fig. 3.5 and isogenic lines of wild-type, uninfected isolate Af-237y and the same isolate transfected with AfuPV (see section 2.23.3). The morphology of some *A. fumigatus* isolates infected with the unclassified dsRNA mycovirus are shown in Fig. 3.7 along with the same negative controls as before.

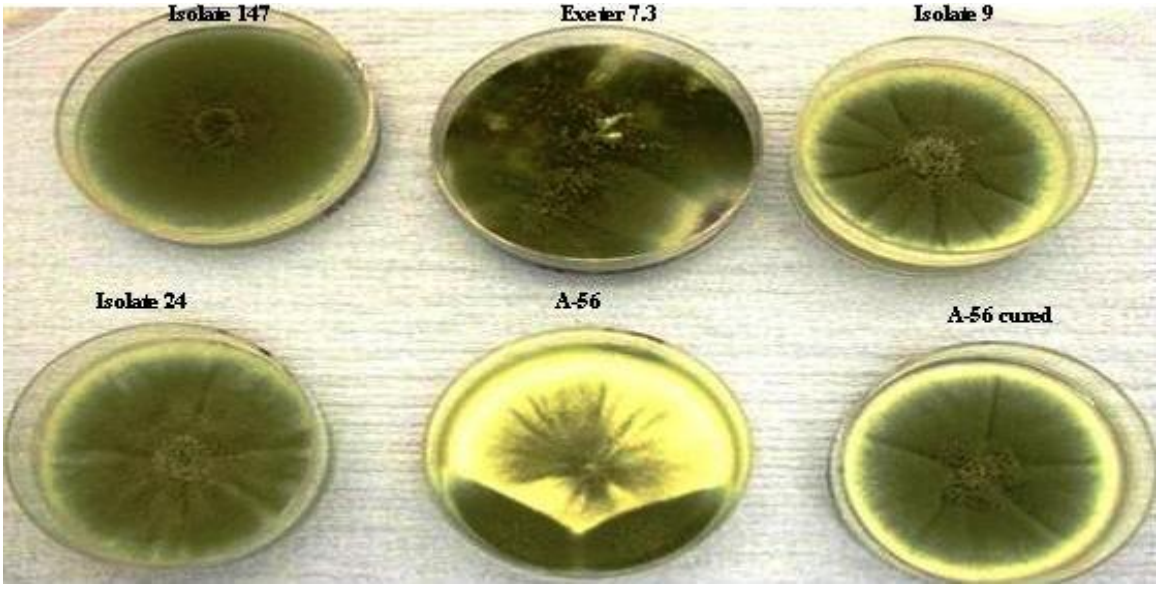


Figure 3.5 Colony morphology of *A. fumigatus* isolates (9, 24 and A-56) infected with AfuCV. The sectored morphology of A-56 (AfuCV infected) as compared to virus-free (A-56 cured) and control uninfected isolates (clinical and environmental; isolate 147 and Exeter 7.3 respectively).

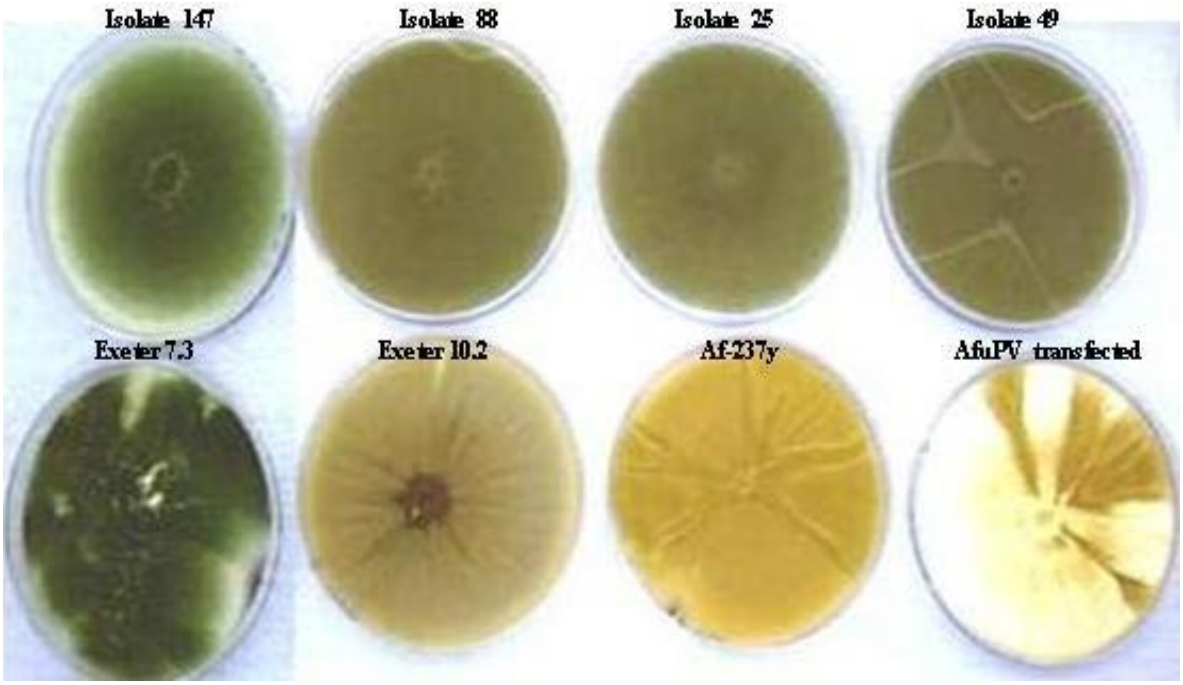


Figure 3.6 Colony morphology of AfuPV containing clinical (isolates 88, 25, 49) and environmental isolates (Exeter 10.2) along with uninfected isolates (clinical and environmental; isolate 147 and Exeter 7.3 respectively). The lower panel illustrates that transfection of the virus free isolate (Af-237y) with AfuPV results in a sectored morphology and less sporulation similar to that found in isolate Exeter 10.2.

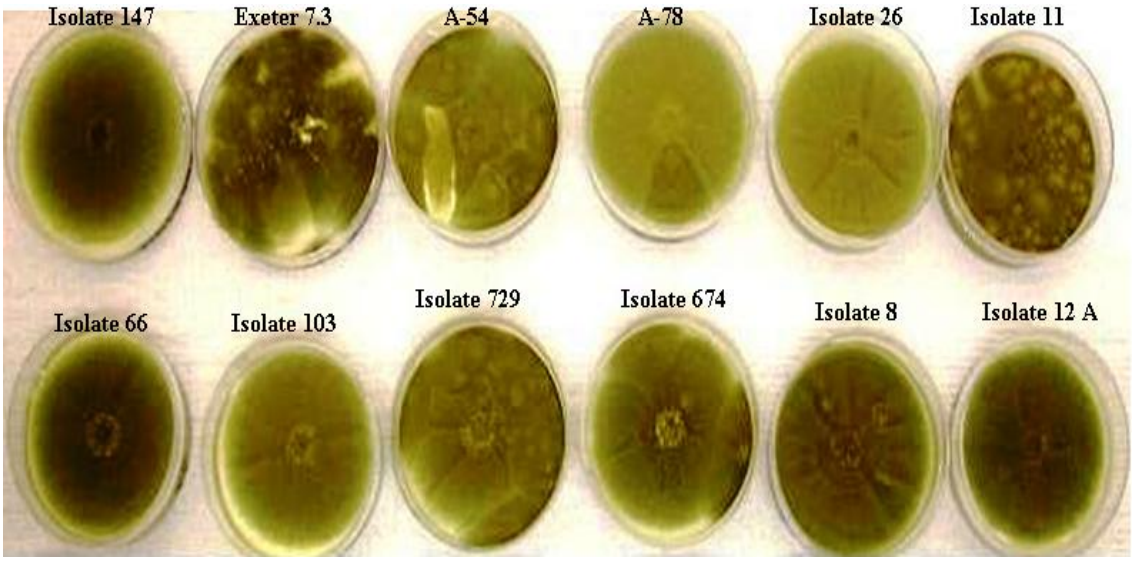


Figure 3.7 Colony morphology of mycovirus infected isolates infected with the same uncharacterised dsRNAs (isolates A-54, A-78, 26, 11, 66, 103, 729, 674, 8 and 12A) along with

controls (uninfected clinical and environmental isolates; isolate 147 and Exeter 7.3 respectively)

3.2 Conclusions

A total of 366 wild-type *A. fumigatus* isolates screened for the presence of dsRNA segments, 24 were found to contain dsRNAs (6.5%) and these are shown in Table 3.2. The mycovirus infection frequency in another study, which sampled over twice as many isolates, was similar with *ca.* 10% recorded for all black Aspergilli (van Diepeningen *et al*., 2006). The profiles of the dsRNAs found in a selection of virus-infected isolates are shown in Figs. 3.3 following extended agarose gel electrophoresis to separate the nucleic acids. Fourteen isolates of *A. fumigatus* contained an as yet uncharacterised mycovirus, whose genome apparently consists of four dsRNAs 1-2.5 kbp in size (e. g. Fig 3.3, lanes 4, 6-9). Seven isolates contained a mycovirus with a genome consisting of two dsRNAs, 1.5-2.0 kbp in size characteristic of members of the family *Partitiviridae* (e. g. Fig 3.3, lane 10, isolate 88; Ghabrial *et al*., 2008) which has been completely sequenced (Bhatti *et al*., 2011a). Five isolates contained four dsRNAs of a size and profile characteristic of members of the family *Chrysoviridae* (e. g. Fig 3.3, lanes 2-4; Ghabrial, 2008). The genome of one isolate A-56 (Fig 3.3, lane 2) has also been completely sequenced (Jamal *et al*., 2010). Occasionally some *A. fumigatus* isolates were infected with more than one mycovirus and contained multiple dsRNA segments (e. g. Fig 3.3, lane 4, isolate 80; see Table 3.2). Mixed mycovirus infections in fungi are relatively common (Ghabrial and Suzuki, 2009) and have also been recorded in the Aspergilli including *A. foetidus* (Ratti and Buck, 1972) and the black Aspergilli (van Diepeningen *et al*., 2008). In this investigation isolate 88, infected with a partitivirus and isolate A-56, infected with a chrysovirus were selected for further investigation. Interestingly dsRNAs with a profile almost identical to that of the uncharacterised mycovirus (e. g. Fig 3.3, lanes 4, 6-9) have been noted previously in total nucleic acid extracts of the prototype *A. fumigatus* isolate AF293 whose genome has been completely sequenced (Warn *et al*., 2006) but these were not investigated further. The population dynamics of the *A. fumigatus* mycoviruses appeared to be independent of the source of the fungus or its location.

Chapter 4

Developmental technology

4. Developmental technology

The aim of this chapter was to investigate and develop technology and standardise techniques and methodology which will be useful in successfully completing the main project objective (identifying and characterising dsRNAs *Aspergillus fumigatus* mycoviruses). Therefore projects were undertaken initially to optimise techniques.

4.1.1 *Aspergillus foetidus* virus

Aspergillus foetidus virus (AfV) was the first ever report of mycoviral infection in the genus *Aspergillus* (Banks *et al.*, 1970; Ratti and Buck, 1972). *Aspergillus foetidus* (isolate IBI 41871) contains a mixture of two distinct isometric viruses and possess 8 dsRNA elements (see Fig. 4.1) which confirms the presence of multiple infection and supports the idea that the more than one virus can coexist in fungi. On the basis of the electrophoretic mobility on agarose gels the *Aspergillus foetidus* viruses were designated *A. foetidus* virus slow (AfV-S) and *A. foetidus* virus fast (AfV-F; Ratti and Buck, I972). AfV-S contains four RNA species nominated S1 and S2 as the major components with respective molecular weights of 2.24×10^6 and 2.76×10^6 Da together with two minor, smaller components nominated S3 and S4 (Fig.3.1; Buck and Ratti, 1975). AfV-F was separated into four components nominated F1, F2, F3 and F4 with molecular weights of 1.44×10^6, 1.70×10^6, 1.87×10^6 and 2.31×10^6 Da respectively. The virus particles isolated were isometric in form, *ca.* 40 nm in diameter. The RNA isolated from the virus particles was shown to be dsRNA and particles were separated into discrete bands following isopynic centrifugation in caesium chloride density gradient (Banks *et al*., 1970). Electrophoretic analysis of the dsRNA obtained from different virus fractions revealed that the multiple RNA components are not fragments released from a single virus particle, but are separately encapsidated in different particles (Ratti and Buck, 1972).

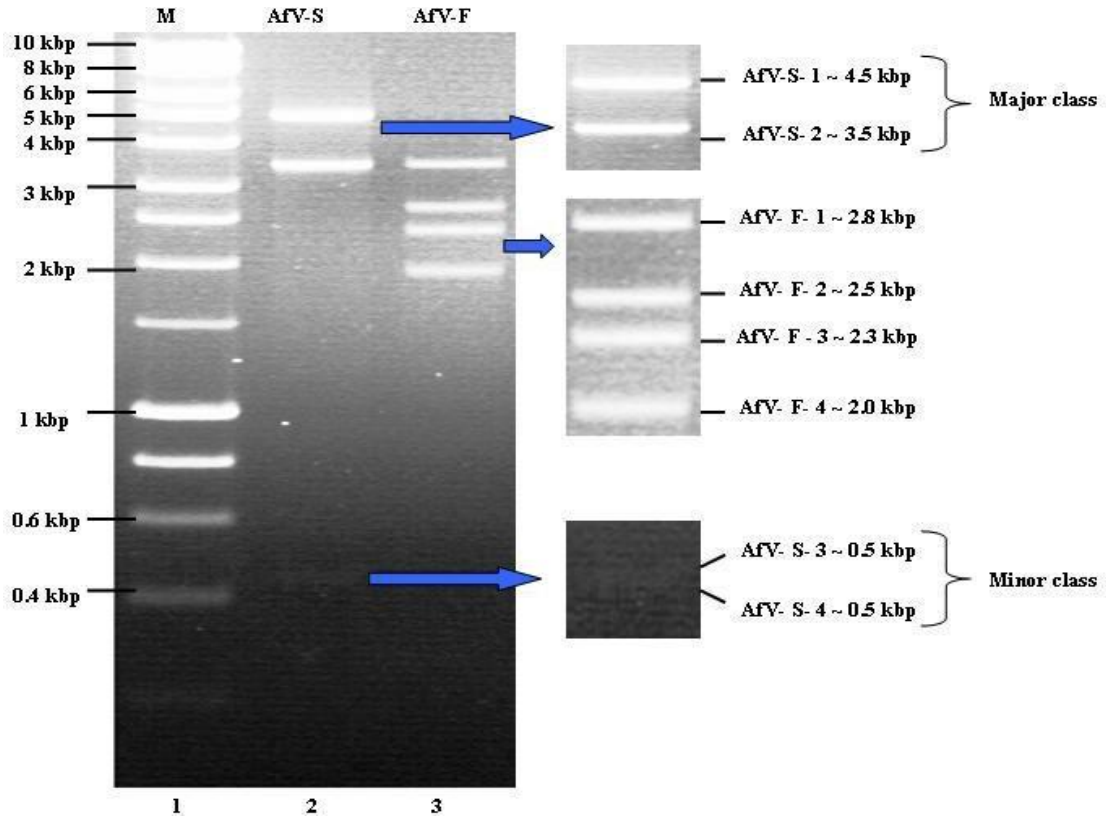


Figure 4.1 Agarose gel electrophoresis of *A. foetidus* virus RNAs showing RNA profile for slow (S) and fast (F) components. Hyperladder 1 (M; 10 kbp; Bioline) was used as a molecular weight marker.

4.1.2 Characterisation of *Aspergillus foetidus* virus

Partial characterisation of the sequences of AfV-S had been initiated in the laboratory in order to sequence these viruses. As large amounts of glycerol stocks of these viruses were available the aim was to complete the molecular characterisation of these viruses and develop cloning and sequencing technologies which would be applicable to investigations in the main project.

4.1.3 Modified RLM RACE procedure

This procedure was adapted from Coutts and Livieratos (2003) with some modifications that filled in the gaps of the dsRNA before first strand cDNA synthesis was performed. In order to sequence the 3'-terminal sequence of the AfV-S2 dsRNA a modified RLM RACE technique was used. In this procedure an oligonucleotide primer (LIG-REV [5'-kinated-PO_4; 3'-OH-

blocked]; 5'-GATCCAACTAGTTCTAGAGCGG-3') is ligated to the 3'-ends of dsRNA using T4 RNA ligase (New England Biolabs [NEB]).

To achieve this, 7 µl of dsRNA was added to 1 µl of LIG- REV primer, and the mixture heated to 90°C for 2 min followed by snap cooling on ice. The ligation mixture was prepared as described below:

Reaction buffer (T4 RNA ligase) supplied by the manufacturer (10 X; 10 µl; NEB) was added to 63.5 µl of water. This mixture was preheated at 37°C (10 min) followed by cooling at room temperature (2 min) prior to setting up the ligation reaction. This step is crucial for dissolving adenosine triphosphate in the reaction buffer which is an essential energy component for the reaction. The following components were then added (DMSO (10 µl; 10 %), RNase inhibitor (2.5 µl; 1 U/µl), T4 DNA ligase (1 µl; 3U; Promega) and T4 RNA ligase (5 µl; 100 U; NEB)) prior to setting up the reaction at 17^{o}C for 15 h. After incubation, sterile water was added to increase the reaction volume to 500 µl and the solution was then size-fractioned and concentrated using a Nanosep 30K column (VWR) by centrifugation (5,000 X g; 5 min), resulting in the recovery of *ca*. 100 µl. The ligated RNA mixture was treated with Go*Taq* DNA polymerase (Promega) to fill in gaps. This reaction was prepared as described below:-

Ligated RNA mixture	100 µl
Go*Taq* buffer (5 X, Promega)	40 µl
dNTP mix (20 mM, Promega)	2.4 µl
Sterile distilled water	57.1µl
Go*Taq* polymerase (5 U/µl, Promega)	0.5 µl

This total reaction (200 µl) was incubated at 68 ^{o}C for 3 h. followed by overnight precipitation of nucleic acids as before (section 2.11.1) This was followed by pelletting, drying and resuspension of nucleic acids in 7 µl of sterile water which were then placed on ice for 20 min. The ligated dsRNA was then denatured by adding 2 µl of CH_3HgOH (100 mM, SERVA) together with 1 µl (100 µM) of (LIG-FOR primer; 5'-CCGCTCTAGAACTAGTTGGATC-3'). The mixture was incubated at room temperature for 20 min, and then chilled on ice for 1 min prior to adding it to the first strand cDNA synthesis reaction mixture that had been pre-heated at 45^{o}C for 1 min. The entire mixture was incubated at 45^{o}C for 1 min followed by the addition

of 1 μl (200 U/μl) of M-MLV reverse transcriptase, RNase H minus, point mutant (Promega) prior to further incubation at 45°C for 1 h.

First strand cDNA synthesis reaction mix:

Sterile distilled water	26.55 μl
M-MLV Reverse Transcriptase reaction buffer (5x; Promega)	10 μl
dNTP mix (20mM; Promega)	1.2 μl
RNasin™ RNase inhibitor (40U/μl; Promega)	1.25 μl

The solution was then size-fractioned and concentrated using a Nanosep 30K column as before (section 2.13.2). The volume of the first strand cDNA solution was increased to 500 μl with H_2O and centrifuged in the column (5000 X g for 10 min), resulting in the recovery of *ca*. 150 μl of solution. A PCR amplification reaction was then set up as described below:

PCR Mixture:

First strand cDNA	50 μl
Go*Taq* buffer (5 X, Promega)	20 μl
LIG-FOR primer (100 μM)	1 μl
Sequence specific primer (100 μM)	1 μl
dNTP mix (20 mM, Promega)	1.2 μl
Sterile distilled water	26.3 μl
GoTaq polymerase (5 U/μl, Promega)	0.5 μl

The reaction was run in a PCR thermocycler (Peltier) using thermocycling program 1 (see section 2.13.2). One tenth of each reaction mixture was electrophoresed in a 1% agarose gel (section 2.11.5) in order to check the size of any amplicons produced.

4.1.4 RLM RACE results for AfV-S

AfV-S2 dsRNA was extracted from agarose gel fragments using the RNaid kit (BIO-101; Qiagen) as previously (section 2.11.6). The extracted dsRNA was treated with DNase (section 2.11.2) and S1 nuclease (section 2.11.3) and used in RLM-RACE procedures

(section 2.13.3) comparing the efficiency of the original with the modified procedure In both experiments AfV-S2 cDNA was amplified with oligonucleotide LIG-FOR (5'-CCGCTCTAGAACTAGTTGGATC-3') in combination with an oligonucleotide primer designed from the known sequence of AfV-S2, nominated AfV-S2-3 (5'-CCGTCACTTCCTTAGGGAAT-3'). Attempts were also made to obtain full length amplicons using LIG-FOR only in the PCR amplification procedure. Both procedures generated amplicons, however the modified method produced a greater quantity of DNA (Fig. 4.2).

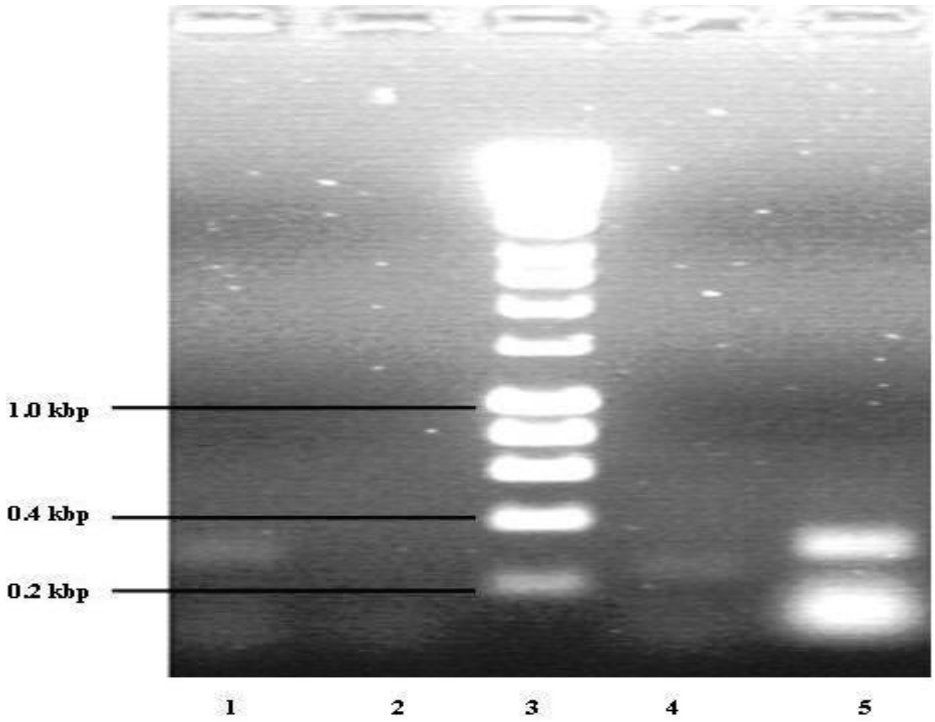


Figure 4.2 Agarose gel electrophoresis comparison of the amplicons derived from AfV-S2 dsRNA following the original and modified RLM-RACE procedures. Lanes 1 and 2 contain amplicons produced from AfV-S2 dsRNA using respectively oligonucleotide primer LIG-FOR alone or a combination of oligonucleotide primers (AfV-S2-3 and LIG-FOR) using the original procedure; Lane 3 contains a DNA molecular weight marker (Bioline Hyperladder 1); lanes 4 and 5 contain amplicons produced with the same templates and primers as those

used to generate amplicons shown respectively in lanes 1 and 2 using the modified procedure.

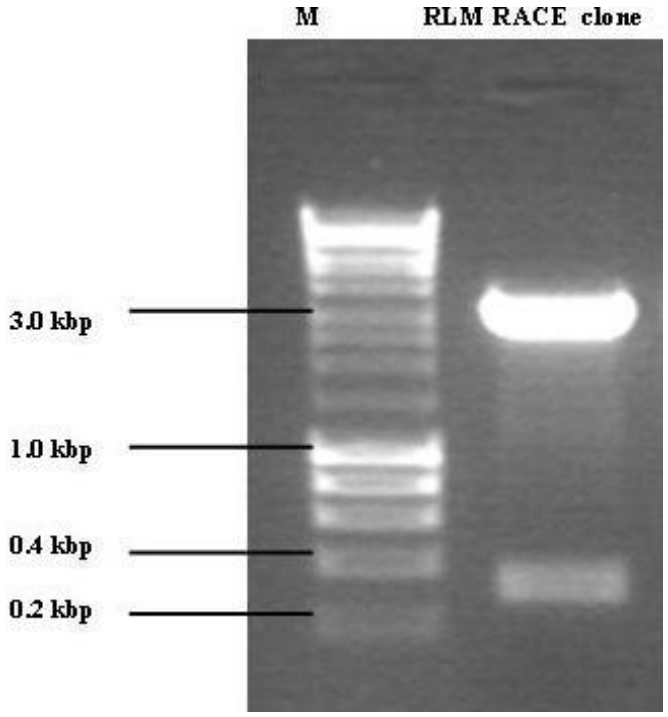


Figure 4.3 Agarose gel electrophoresis showing *EcoR*I digestion of a AfV-S2 cDNA clone containing a 254 bp insert. Hyperladder 1 was used as a marker (M; 10 kbp; Bioline)

Amplicons were cloned and sequenced as described before (sections 2.15- 2.20). Analysis of one of the clones produced from the amplicon shown in Fig 4.2 (lane 5) by restriction analysis and subsequent sequencing revealed that it was a 3'-prime specific AfV-S2 cDNA clone (Fig 4.3) the 254 bp sequence of which had been obtained previously, confirming previous data.

Sequence of a 3'-prime specific AfV-S2 cDNA:-

ACGATACTATTATCTTAGTCTACTGGTTGCTGGACCCGTCTGACAAACCTGGTTCG
TTACCAATTATGCATGTATCTCCATGACCATGGATAAGTGTATAAGGAAGTAATTC
CCCAGATATGATAATTGGCTATTATCATATAAATAGGACTACCACTAGAAATGTCT
ACACATTCCGACAGACATTGACAGTAGAGGTAGAAGCCTCTCCTAGGAATCCGCC
TAGGAGGCTAAAACCCCAGCCAAGAAAAAAA.

The sequence obtained represented the 3' terminus of AfV-S2 which contains a small poly-A tail (highlighted in green). A BLASTP search of this sequence plus further upstream sequence (not shown) identified conserved region belonging to the RdRP ORF of the ssRNA containing *Broad bean wilt virus* (BBWV; Fig. 4.4) which belongs to genus *Fabavirus*, which confer

serious threats to plants of agricultural importance. BBWV causes diseases like wilting and necrosis in broad bean, streak of peas, blight of spinach and ring spot of petunia (Lee *et al*., 2000). Recently, BBWV was discovered in plants of medicinal importance like *Bupleurum chinense* (Sui *et al.*, 2009). Further analysis of the AfV-S is required to understand its phylogeny.

Figure 4.4 Alignment of the amino acid sequence of AfV-S dsRNA-2 compared to the amino acid sequence of *Broad bean wilt virus* (BBWV) using MAFFT alignment software http://mafft.cbrc.jp/alignment/software/. Asterisks show identical residues; colons indicate highly conserved amino acid residues, whilst single dots indicate less conserved, but related residues.

4.2 Optimisation of dsRNA denaturation

Heat denaturation is the most frequently used procedure to denature nucleic acids. However, for those nucleic acids with significant amounts of secondary structure such as dsRNAs, chemical denaturation e. g. using methyl mercuric oxide (Gruenwedel and Davidson, 1966) is preferred. In order to select a best procedure for denaturation of dsRNA templates and use in future experimentation two different dsRNA mycoviruses *viz.* the uncharacterised virus of *Aspergillus fumigatus* (isolate 66; see Chapter 3 for further details) and AfV-S virus were compared using two different denaturation (heat and chemical) procedures prior to the analysis on agarose gel electrophoresis (section 2.11.5).

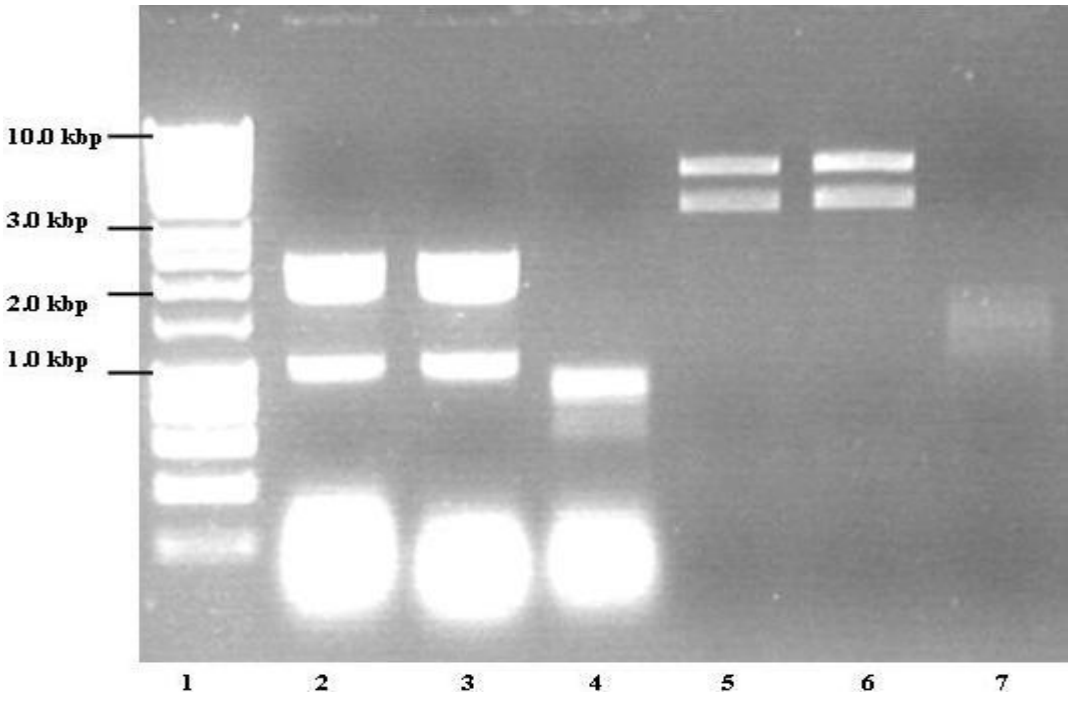


Figure 4.5 Agarose gel analysis of denatured dsRNAs. Lane 1, Hyperladder 1; lane 2, untreated uncharacterised virus of isolate 66 of *A. fumigatus* ; lane 3, heat denatured dsRNA of isolate 66; lane 4, isolate 66 dsRNA denatured with methyl mercuric oxide; lane 5, untreated AfV-S; lane 6, heat denatured AfV-S dsRNA; lane 7, methyl mercuric oxide treated AfV-S.

The experiment was performed simultaneously together with untreated virus, to check the effect of the treatments. Agarose gel analysis showed that the use of methyl mercuric oxide for

denaturing dsRNA is more effective then heat treatment (Fig. 4.5) as the bands move faster through the gel and represent true denatured ssRNAs.

4.3 Conclusions

On the basis of results obtained it is concluded that modification to the RLM-RACE protocol, where an extra step was added after the ligation step to fill in any gaps in the sequence, was a useful modification which will be useful in future experimentation to produce larger quantities of amplicons.

Chapter 5

Sequencing of the genome of a new partitivirus infecting *Aspergillus fumigatus*

5. Sequencing of the genome of a new partitivirus infecting *Aspergillus fumigatus*

The aim of this chapter was to completely sequence a partitivirus discovered during screening of a collection of both clinical and environmental isolates of *A. fumigatus* for dsRNA elements.

5.1 Results and discussion

A collection of *A. fumigatus* isolates were screened for the presence of dsRNA elements following their isolation by a lithium chloride fractionation procedure (section 2.10.5) and/or a small scale extraction method (section 2.10.6). Out of 366 isolates 24 (6.5%) were found to contain dsRNAs. Seven of the infected isolates contained dsRNA elements, the profile of which was reminiscent of members of the family *Partitiviridae* (Ghabrial *et al*., 2008a) which contain two genomic dsRNAs, nominated 1 and 2 (Fig. 5.1). I selected one putative partitivirus containing isolate, 88 for further investigation. As compared to uninfected isolates, infection of *A. fumigatus* isolate 88 with a mycovirus, nominated *A. fumigatus* partitivirus (AfuPV) did not cause any alterations to colony morphology, sectoring or pigmentation which are rare, but known, effects found in other mycovirus-infected *Aspergillus* species (Van Diepeningen *et al*., 2008; Jamal *et al*., 2010).

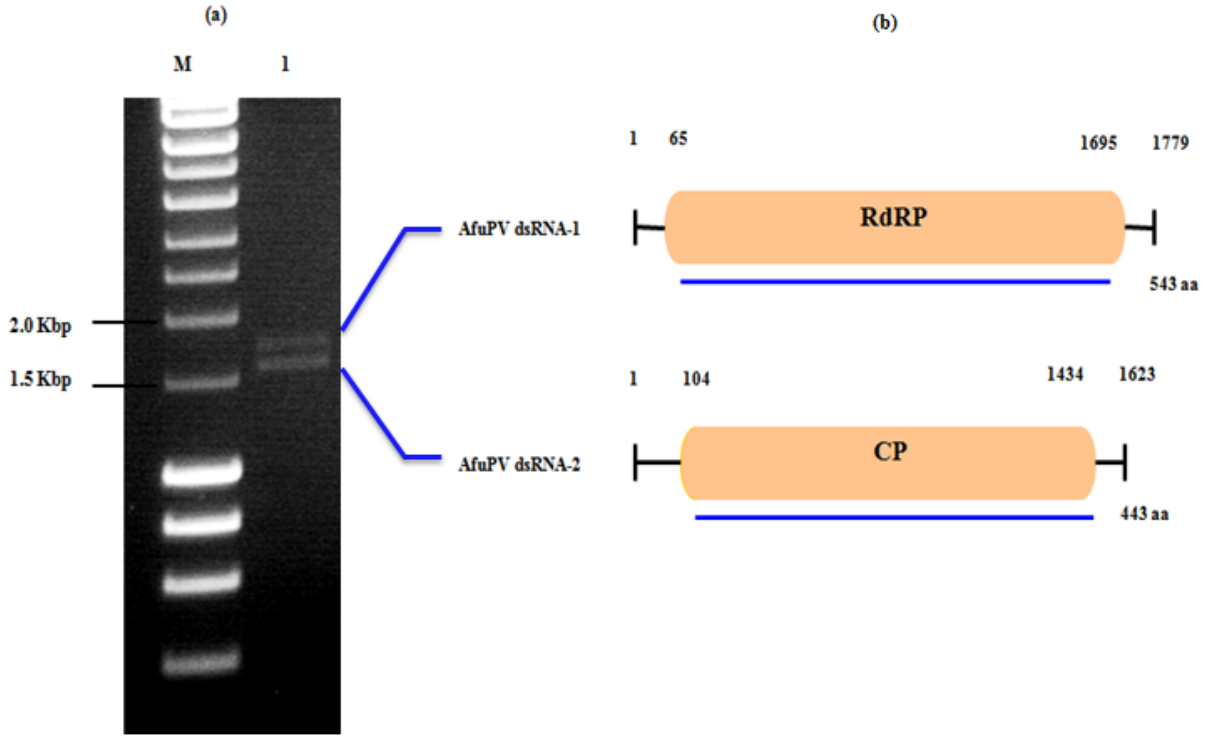


Figure 5.1 (a). Occurrence of partitivirus dsRNAs in *Aspergillus fumigatus*. Agarose gel (1%) electrophoresis of dsRNAs purified from *A. fumigatus* isolate 88: Lane 1; *Aspergillus*

fumigatus partitivirus (AfuPV) dsRNAs; Lane M, DNA marker showing 1.5 and 2 kbp bands; (b) Schematic representation of the genomic organisation of AfuPV dsRNA1 and dsRNA2. The genome consists of two dsRNA segments; each is monocistronic. The RNA-dependent RNA polymerase (RdRP), open reading frame (ORF; nucleotide [nt] positions 66–1694 on dsRNA1) and the coat protein (CP) ORF (nt positions 105–1433 on dsRNA2) are shown.

5.2 Sequencing strategy for AfuPV

The dsRNAs isolated from AfuPV virions, isolated and purified from isolate 88 as described (section 2.12.1) were resolved into two bands by agarose gel electrophoresis (section 2.11.5) and their sizes ranged from ~1.6- 1.8 kbp. The dsRNA obtained was contaminated with host nucleic acids. So, the partially purified dsRNA was treated with DNase (section 2.11.2) and S1 nuclease (section 2.11.3) in order to remove host contaminating DNA and ssRNA respectively. The complete nucleotide sequence of the two dsRNA segments was determined from overlapping cDNA clones obtained using random hexamers and/or degenerate, sequence-specific primers (Table 5.1) and RLM-RACE. Initially the primers were designed on the basis of conserved regions found in the family *Partitiviridae.* To locate areas of conservation, at least 10 family members were aligned using BLAST (Altschul *et al*., 1997) however the sequence of the primers were either degenerate primers or they were exclusively derived from the conserved region of the *Aspergillus ochraceous* partitivirus sequence (Table 5.1). However, in the later stages of the project sequence-specific primers were designed from the known sequence of AfuPV dsRNA-1 and RNA-2. The amplicons generated were purified using the PCR purification kit (section 2.11.8) ligated into the pGEM-T Easy vector and transformed into *E.coli* JM-109 competent cells.

The recombinant clones were sequenced and their sequences analysed using the BLASTX program (Altschul *et al.*, 1997) to identify homology with previously characterised protein sequences in the database. Once sequencing data for both dsRNA elements was available, primers were designed upstream (5’-) and downstream (3’-) of the extant sequences for genome walking and an RLM-RACE PCR procedure was used to determine the 5'- and 3'-terminal sequences of the dsRNAs (Fig 5.2.; Coutts and Livieratos, 2003). All clones were sequenced in triplicate as described before (sections 2.15- 2.20).

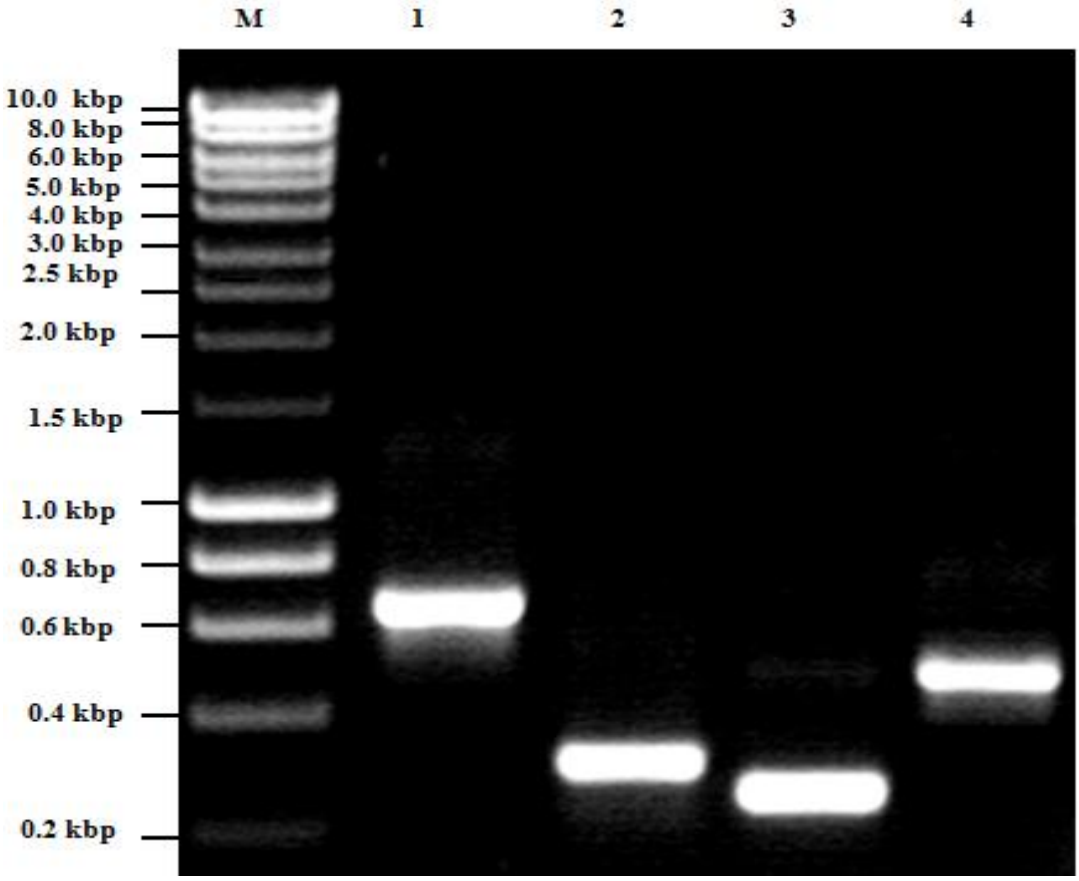


Figure 5.2 Agarose gel electrophoresis showing amplicons produced following the RLM-RACE procedure to determine the 5'- and 3'- ends of the AfuPV dsRNAs. Respectively shown in lanes 1-4 are amplicons corresponding to 1; the 3' end of dsRNA-1(RdRP). 2; the 5' end of dsRNA-1. 3; the 3' end of dsRNA-2 (CP) and 4; the 5' end of dsRNA-2. Lane M; Hyperladder-1 DNA markers.

Table 5.1 Sequence of oligonucleotide primers used to complete the sequence of AfuPV dsRNA(s).

Primers name	**Primer sequences**	**Position in genome (bp)**
RNA-1 primers		
MF-1	5'- CGCTGGTGTACCTTCTGGCAT- 3'	1125
MF-2	5'- GTTCGCTCAACTCTTGTATCC -3'	849
MF-3	5'- GAGCCAACTCGTGACCACGAATTGC -3'	1302

MF-5	5’- GTCGTGAAGACGTCGCTTGCTTGTCT- 3’	1516
MF-8*	5’- CGATCCAGAGACCGATTAABC -3’.	1480
MF-11	5’- GTGGACGCAGATGATCTATTC -3’	1145
MF-12	5’-CCTTCGCAACGTCCAGATCG-3’	1274
MF-13	5’- CCCAAGGATCAGTGTTGGATG -3’	262
MF-21	5’- GAGGTATGGTGGCTACTCTAC- 3’	230
MF-22	5’- GGTCCGATACCATCTTGCTTC -3’	632
MF-28	5’-ATGGAAGATTATACTCAAGATC -3’	66
MF-29	5’- GCCATAGGCGTAGAAGATTGAT -3’	1670
RNA-2 primers		
MF-4	5’- GTGTTGGAGGATGCTGTTCTCTC -3’	867
MF-6*	5’ -CCAATTNAGNTGDCCNGC -3’	1455
MF-7*	5’ –GAACTCVGTHNCNCNTTG- 3’	1255
MF-14	5’- GCCTCCTTCATCTTGAGACC -3’	85
MF 15	5’- GTCGTTTTTCAGACGGGTGC -3’	315
MF-16	5’-CTCATGTGAACATGGGCCTAC -3’	532
MF 17	5’-CCTGGTTCCTAGAGCAGGAAC -3’	828
MF 18	5’- CAAGAGAGAATTCTGGCGCAG -3’	1294
MF-19	5’- GAGATCCCACAGCGGACCTG -3’	209
MF 20	5’- CTGATCTGGGCTGCTGACCA -3’	699
MF-31	5’- GTGTCCTGGGACCTGTCTTAT- 3’	376
MF-32	5’ GTCCAAGGCAAGCCTAGCTCG 3’	1055

RNA-2 FS	5’CCCCCAAGCTTGTCGACTAATACGACTCACT ATAGGGCGCAAAAGGACCCTTTTGAC- 3’	1
RNA-2 RS	5’CCCCCCGGATCCGGTACCTAATACGACTCAC TATAGGGTGGATTTACAGTGAACACGTA -3’	1603

* An asterisk indicates degenerate primers (Where B= C/G/T; D= A/G/T; H= A/C/T/; N= A/G/C/T).

5.3 Confirmation of size and number of AfuPV dsRNAs

Nucleotide sequence analysis revealed the presence of two distinct dsRNA segments. The complete nucleotide sequence analysis revealed the dsRNA-1 of AfuPV is 1779 base pairs (bp) long and dsRNA-2 is 1623 bp long (Fig. 5.1). The dsRNA1-2 sequences have been deposited in the GenBank database under accession numbers FN376847 and FN398100 respectively. Sequence analysis of full-length AfuPV dsRNA-1 cDNA indicated that it contains one major ORF from nt position 66 to 1694 which potentially encodes a protein with a molecular mass of 63 kDa as calculated from the 542 amino acid (aa) residues encoded by the ORF (Table 1.2; Fig. 5.1). The major ORF of AfuPV dsRNA2, from nt position 105 to 1433, corresponding to 442 aa, putatively encodes a protein with a estimated molecular mass of 48 kDa. Computer analysis predicted a number of minor ORFs on both strands of both dsRNAs but presently no information is available as to whether the short polypeptides corresponding to these ORFs are synthesised.

The 5′-untranslated regions (UTRs) flanking the major ORFs of the plus strands of dsRNA1 and dsRNA2 are respectively 65 and 104 nt long (Fig. 5.1.). The 5′-UTRs show a sequence identity of ~60%, the difference mainly arising from the size difference in their 3′-proximal region and the identical stretches include the terminal motif 5′-CGCAAAAG-3’ (Fig. 5.2.). Interestingly the AfuPV 5’-UTRs were closely related to the similarly sized 5’-UTRs of *Botryotinia fuckeliana* partitivirus-1 (BfPV-1) dsRNA1 (61 nt and 61% identical) and dsRNA2 (101 nt and 51% identical). As distinct from most partitiviruses the AfuPV 5′-terminal regions were devoid of CAA repeats which are characteristic for members of the family *Partitiviridae* (Ghabrial *et al.*, 2008) and the regions were not significantly A/U rich. The 3′-UTRs of the plus strands of AfuPV dsRNA1 and dsRNA2 differ in length even more than the 5’-UTRs (85 and 190 nt, respectively) and there was little sequence conservation between them apart from the terminal motif 5’-AUCCA-3 (Fig. 5.3).

The deduced amino acid sequence of the major ORF of AfuPV dsRNA1 contains characteristic sequence motifs (motifs III–VIII; see Fig. 5.4) found in genes of putative RdRPs of RNA viruses (Breunn, 1993) and we therefore conclude that dsRNA1 encodes the AfuPV RdRP. A comparison of the amino acid sequence of the putative AfuPV RdRP with sequences in the NCBI database using the BLASTP algorithm revealed highly significant similarities to the RdRPs of viruses in the *Partitiviridae* family. The RdRP sequences of four fungal partitiviruses, namely those of BfPV, *Discula destructiva virus 2* (DdV2), *Discula destructiva virus 2* (DdV1) and *Ophiostoma* partitivirus 1 showed 70–80% aa sequence identity to AfuPV without any gaps (Fig. 5.4; Bruenn, [1993]).

Figure 5.3 Alignment of the 5'-UTRs (Fig. 5.3a) and 3'-UTRs (Fig. 5.3b) of AfuPV dsRNAs 1 and 2, both of which show similarities, indicated by asterisks, especially at the termini of the dsRNAs.


AfuPV-RdRP
BfPV-1-RdRP
DdV2-RdRP
OPV-1-RdRP
PsV-S-RdRP
III
IV
V
VI
VII

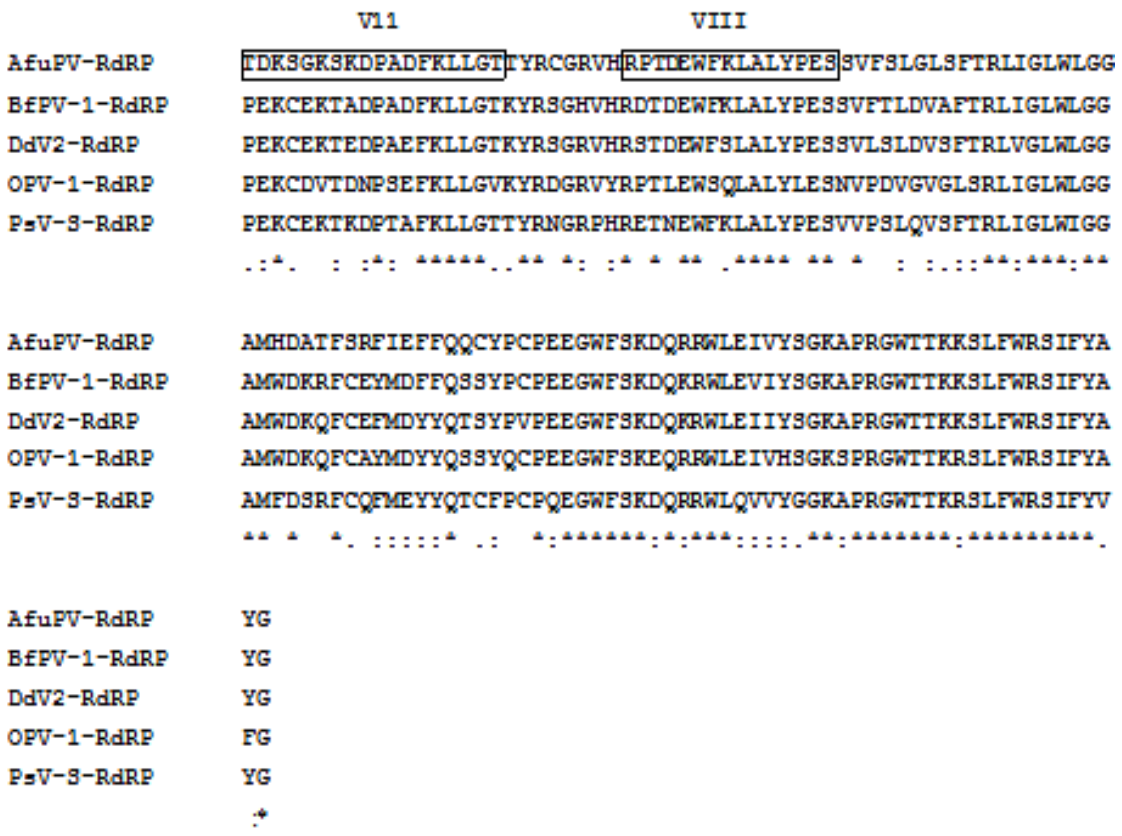
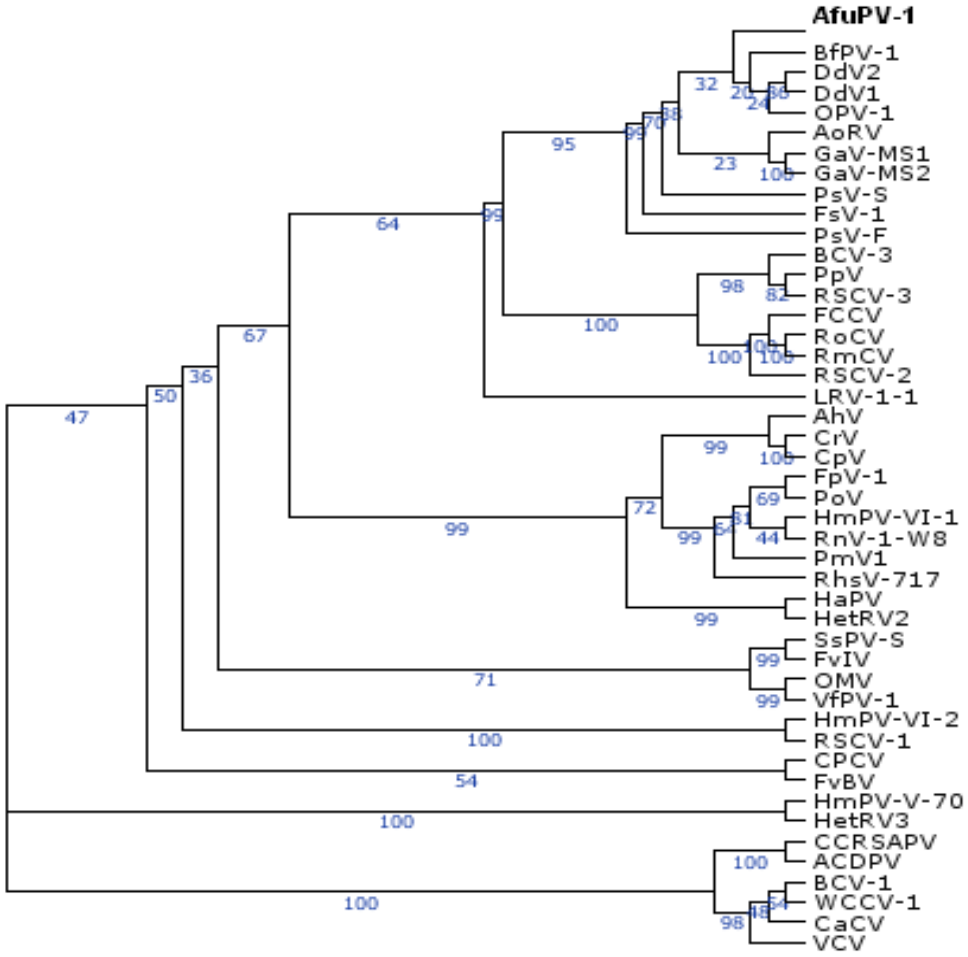


Figure 5.4 Amino acid sequence alignment of the putative RNA-dependent RNA polymerases of AfuPV, BfPV-1, DdV2, OPV-1 and PsV-S, for full names of the viruses see Table 1.2 (Chapter 1). The positions of the conserved RdRP motifs (I-V111; Bruenn, 1993) are indicated above the sequences. The amino acid positions corresponding to conserved motifs I and II for the RdRPs of viruses in the family *Partitiviridae*, which are not well defined, are not presented. Asterisks signify identical residues; colons signify highly conserved amino acid residues; single dots less conserved, but related residues. The sequence identity between the four proteins is around 70 %.

Based on the complete RdRP aa sequences of different members of the family *Partitiviridae* (Table 1.2) a phylogenetic tree was generated by the NJ method (Saitou and Nei, 1987; Fig. 5.5.). This phylogram gives further support for the strong relatedness of the viruses mentioned above. The tree has three major branches, one contains cryptoviruses and several fungal partitiviruses the second branch contains fungal partitiviruses and the third branch contains AfuPV and partitiviruses which infect ascomycetous fungal hosts (Fig. 5.4), essentially coincidental with the current taxonomy of the genus (Ghabrial *et al.*, 2008).

(a)


AfuPV-1
BfPV-1
DdV2
DdV1
OPV-1
AoRV
GaV-MS1
GaV-MS2
PsV-S
FsV-1
PsV-F
BCV-3
PpV
RSCV-3
FCCV
RoCV
RmCV
RSCV-2
LRV-1-1
AhV
CrV
CpV
FpV-1
PoV
HmPV-VI-1
RnV-1-W8
PmV1
RhsV-717
HaPV
HetRV2
SsPV-S
FvIV
OMV
VfPV-1
HmPV-VI-2
RSCV-1
CPCV
FvBV
HmPV-V-70
HetRV3
CCRSAPV
ACDPV
BCV-1
WCCV-1
CaCV
VCV

(b)

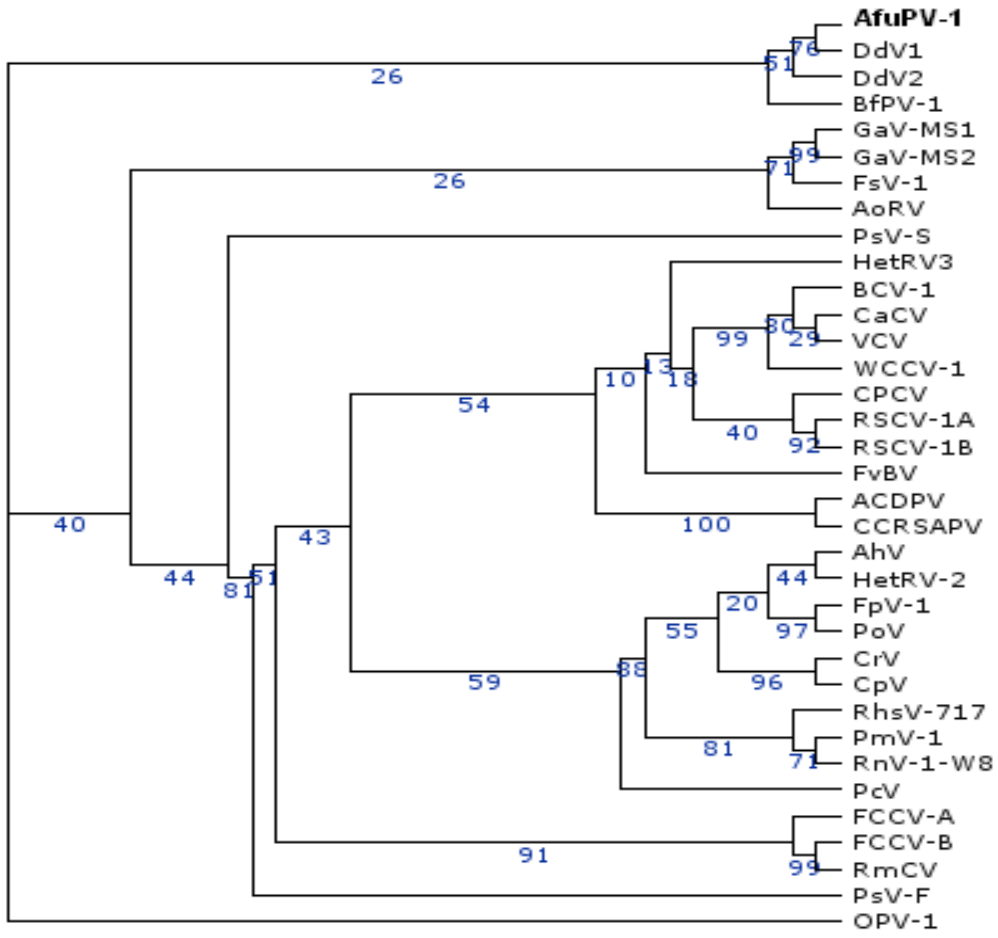


Figure 5.5 Phylogenetic trees of the putative RNA-dependent RNA-polymerase (RdRP) and coat protein (CP) amino acid sequences of members of the *Partitiviridae* family and AfuPV dsRNA1 and dsRNA2 (embolded). Both trees of the RdRP and CP sequences, shown in (a) and (b) respectively, were generated by the Neighbor-Joining method (Saitou and Nei, 1987). The numbers shown at branch points indicate bootstrap values. The data sets were subjected to 1,000 bootstrap replicates. Only complete RdRP and CP sequences taken from the GenBank were analysed and these are shown in detail in Table 1.2 which also includes the abbreviations for the individual viruses. The RdRP and CP trees were respectively rooted with the RdRP of *Leishmania RNA* virus *1-1* (LRV-1-1; M92355), the type species of the genus *Leishmaniavirus* in the family *Totiviridae* and the CP of *Penicillium chrysogenum* virus (PcV; NC_007540) the type species of the genus *Chrysovirus* in the family *Chrysoviridae.*

The deduced amino acid sequence of the major ORF of AfuPV dsRNA2 encodes a putative 442 aa protein with a molecular mass of 48 kDa. This predicted size is similar to that estimated by SDS-PAGE for the AfuPV CP subunit derived from purified virions (Fig. 5.6.) and is within the range (39-73 kDa) described for the CPs of members of the genus *Partitivirus* (Ghabrial *et al.*, 2008). However, the CP can be further characterised by mass spectrophotometry to obtain sequence data for the characterisation of genomic proteins (Thomas *et al.*, 2000). The variability in size of the CPs among members of the genus is shown in Table 1.2 (Chapter 1). Multiple protein sequence alignment of the AfuPV CP aa sequence showed a similar trend to that found for the AfuPV RdRP sequences and the CP sequences of DdV1, DdV2 and BfPV showed ~60% aa sequence identity. A phylogenetic tree, indicating the relationships among representative, sequenced partitiviruses based on the full-length sequences of the CPs, (Table 1.2; Fig. 5.5 b) supported the relationships observed by phylogenetic analysis of the RdRP sequences (Fig. 5.5a). The high bootstrap values associated with these relationships support classification of AfuPV as a new member of the genus *Partitivirus*, family *Partitiviridae*. Some isolates of *A. fumigatus* containing AfuPV dsRNAs 1 and 2 also appeared to contain a smaller dsRNA element similar in size to those described for DdV1, DdV2 and BfPV but these are considered to be satellite-like dsRNAs and not partitivirus genomic components (Ghabrial *et al.*, 2008) and were not investigated further.

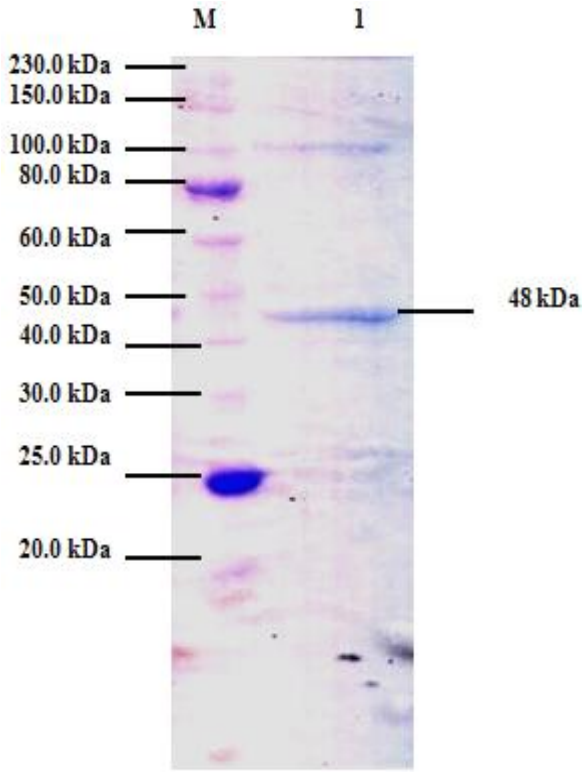


Figure 5.6 SDS PAGE in a 10% acrylamide gel of denatured, purified AfuPV virions (lane 1; 48 kDa) compared to proteins of known molecular weights, (lane M; Prestained protein ladder, NEB). Coomassie blue staining after SDS-PAGE analysis showed a major protein with an apparent molecular mass of 48 kDa.

5.4 Secondary structures for AfuPV UTRs

The UTRs are mostly involved in initiation of RNA synthesis, transcript recognition and virion packaging or may contain signals to assist these processes. The 5' and 3' UTR sequence of the sense strands of two dsRNAs segments of AfuPV were investigated regarding their potential secondary structures. Secondary structures were constructed using the Mfold program (Zuker, 2003). The 5' UTRs of both dsRNA molecules contain stable stem loop structures (Fig. 5.7.), while the 3' UTR region of the dsRNA molecules both appear to possess a terminal hairpin loop (Fig. 5.8.). At the nucleotide sequence level, the 5' UTR regions are strongly conserved as compared to the 3' UTR regions, although both sets of ends contain distinct areas with nucleotide sequence identity (Fig. 5.2). The similarity in all the sequences shown after alignment is reflected in the similarity of the predicted secondary structures (Figs. 5.7. and 5.8.).

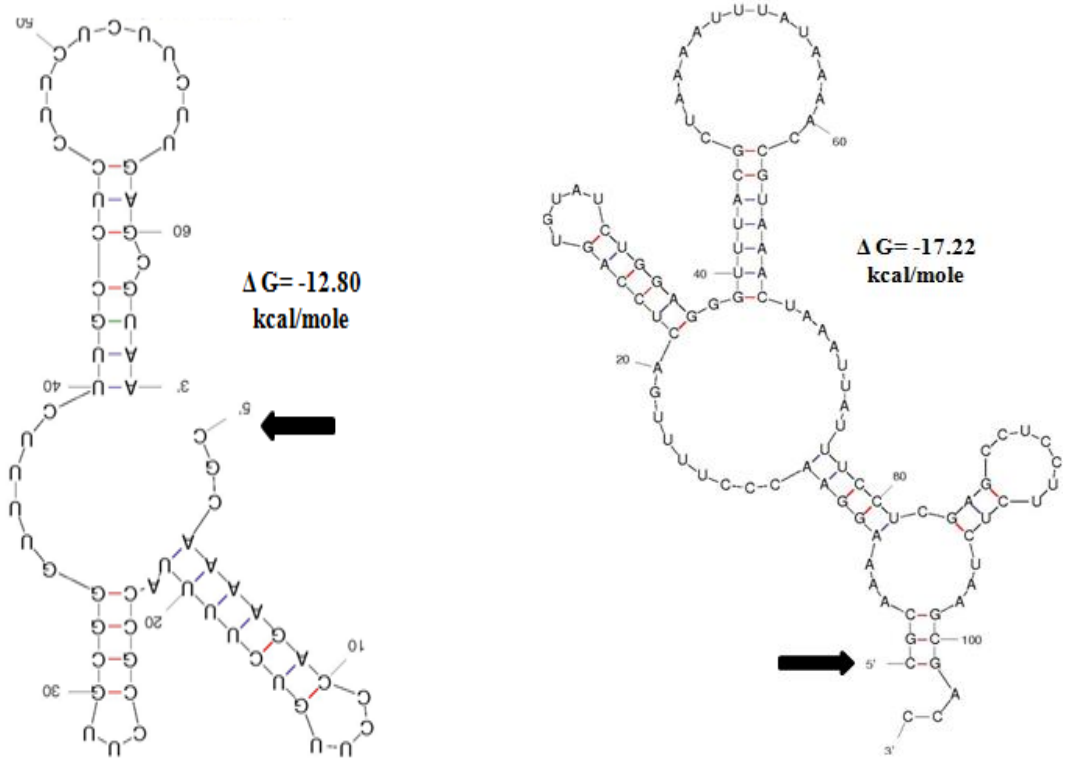


Figure 5.7 Predicted folding of the 5'-UTR regions of AfuPV dsRNA 1 and dsRNA 2. The ΔG values were calculated using the Mfold program. The horizontal arrows point to the start of the sequences.

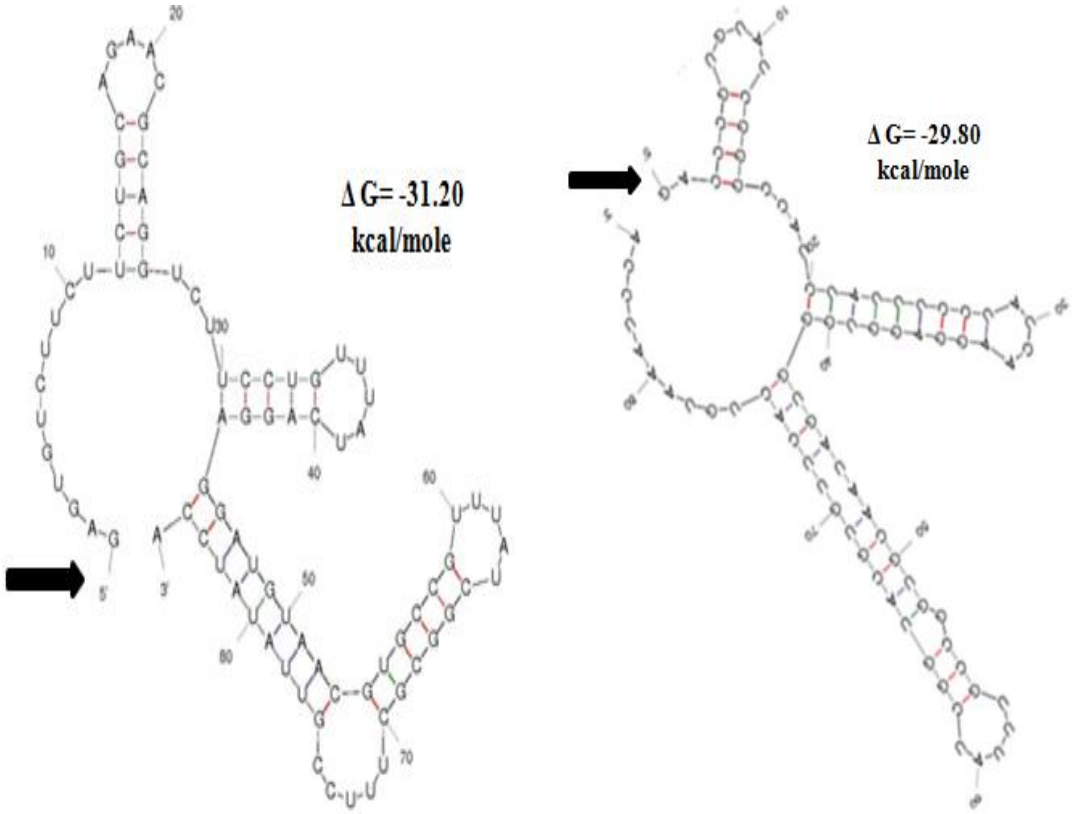


Figure 5.8 Predicted folding of the 3'-UTR regions of AfuPV dsRNA 1 and dsRNA 2. The ΔG values were calculated using the Mfold program. The horizontal arrows point to the start of the sequences.

5.5 Conclusions

The molecular features of the two dsRNAs found in *A. fumigatus* isolate 88 parallel those of the genus *Partitivirus*. The larger segment, dsRNA-1, encodes for an RdRP, whereas, the smaller segment, dsRNA-2, encodes for a CP. On the basis of size, sequence and structural features of their 5'-UTRs it is proposed that they are the genomic components of a new species in this genus, for which the complete genomic sequence has been described (GenBank accessions FN376847 and FN398100). After the successful characterisation, it was also revealed that the AfuPV genome, like other members of *Partitiviridae* family, possesses conserved motifs (III-VIII; Fig 5.4) whilst the location of first two conserved motif of RdRP were not well defined (Bruenn, 1993). The phylogenetic analysis illustrates that, within the

clusters the RdRP gene of AfuPV is closely related to *Botryotinia fuckeliana* partitivirus-1 (BfPV-1; Fig. 5.5a). However, the CP is closely related to *Discula destructiva* virus 1 (DdV-1; Fig. 5.5b; Rong *et al.*, 2002). The 5'- UTRs of both dsRNA were found to contain stem loop structures as found in most of the viruses of this genus (Tavantzis, 2008; Fig. 5.7) and 3'-UTRs contains terminal hairpin loops (Fig. 5.8).

The UTRs (3'- and 5'-) of both dsRNAs (RNA-1 and 2) significantly vary in length. Despite these variations the UTRs contain conserved regions at both termini, which suggest that the sequence is correct and ends have been reached. This also illustrates that, for multi component dsRNA viruses terminal sequences are important as recognition sites for viral RdRP (Crawford *et al.*, 2006; Buck, 1996) and this high conservation at 5'- and 3'- termini in of both AfuPV dsRNAs (Fig. 5.3) suggests that they may be replicated by the same RdRP, as previously observed for other paritiviruses.

The infection of *A. fumigatus* isolate 88 with AfuPV results in no obvious morphological changes on agar, we do not know how it impacts the growth or pathogenicity in susceptible hosts (including mammals). In order to understand the role of AfuPV on the fitness of the fungus further experimentation was performed (See Chapters 6 and 7).

Chapter 6

Curing *A. fumigatus* isolate 88 from AfuPV infection and phenotypic screening of AfuPV infected and virus-free *Aspergillus fumigatus* isolates

6. Curing *A. fumigatus* isolate 88 from AfuPV infection and phenotypic screening of AfuPV infected and virus-free *Aspergillus fumigatus* isolates

6.1 Curing of *A. fumigatus* isolate 88 from mycoviral infection

In order to elucidate the impact of AfuPV on *A. fumigatus* several attempts were made to cure *A. fumigatus* isolate 88 from infection with AfuPV dsRNA by cycloheximide treatment. To achieve this ACM media was amended with a range of concentrations of cycloheximide (0.1 mM-15 mM) and inoculated with the fungus. Following 7 days incubation at 37°C, (Fig. 6.1) spores were harvested from all plates, inoculated into 5 ml ACM broth and grown for 3 days prior to dsRNA purification using the small scale isolation procedure (section 2.10.6) followed by agarose gel electrophoretic analysis. Examination of the plates revealed no significant alterations to the morphology or pigmentation of the fungal colonies. Comparisons between the electrophoretic profiles of dsRNA isolated from virus-infected *A. fumigatus* isolate isolate 88 (positive control) and the treated samples indicated that curing was unsuccessful following treatment with increasing concentrations of cycloheximide (Fig.6.2) as dsRNA elements were detected in all of the amended cultures however, the virus titres were reduced in amount.

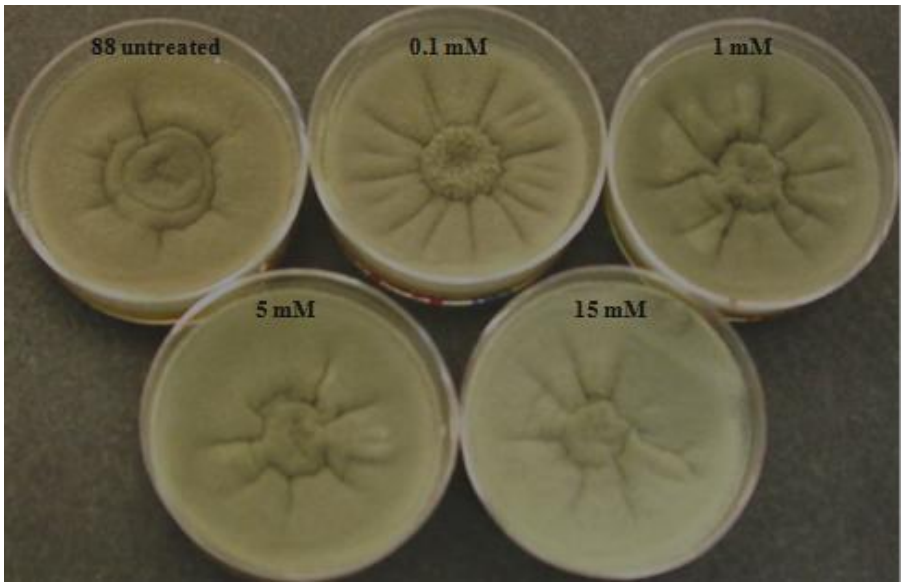


Figure 6.1 Colony morphology of *Aspergillus fumigatus* isolate 88 grown on ACM amended with different concentrations of cycloheximide (0.1, 1, 5 and 15 mM) along with an untreated isolate as a positive control.

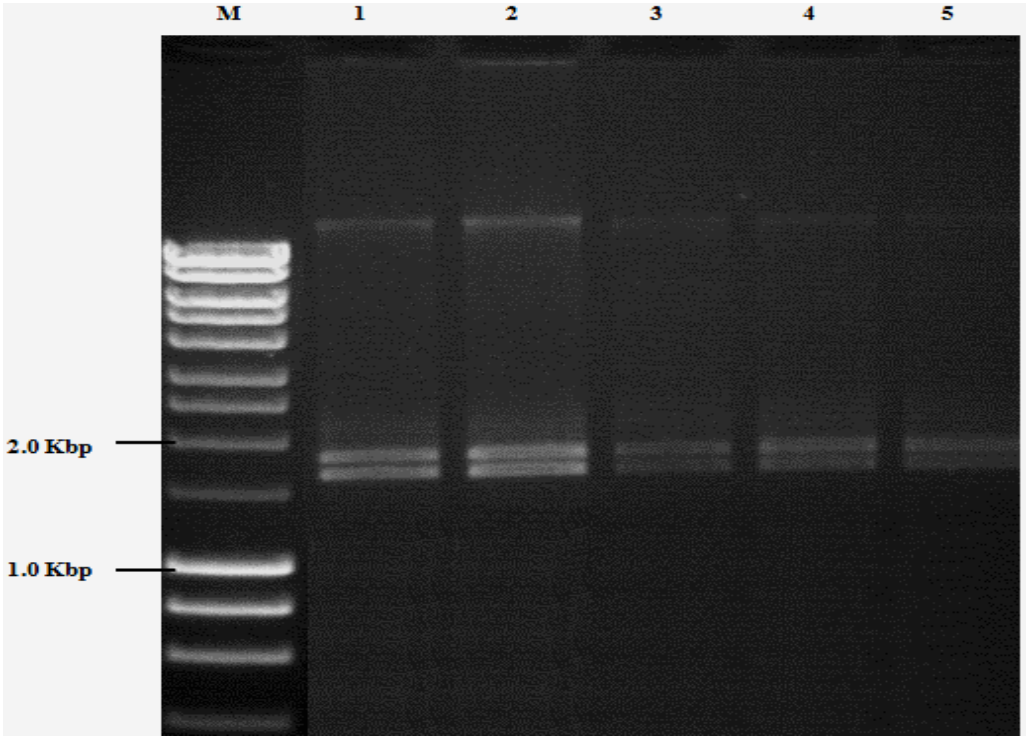


Figure 6.2 Agarose gel electrophoresis of total nucleic acids extracted from *Aspergillus fumigatus* isolate 88 amended with increasing concentrations of cycloheximide (0.1, 1, 5 and 15 mM) in lanes 2 to 5 respectively along with the untreated control isolate (lane 1). Hyperladder 1 (lane M; 10 kbp; Bioline) was used as marker.

Following the failure to eradicate AfuPV from *A. fumigatus* isolate 88 in the initial experiments which reduced the virus titres, investigations on the effect of increasing the concentration of cycloheximide were initiated. Inoculations and incubation were carried out as above (section 6.1) and the amended media contained cycloheximide at concentrations of 25 mM, 50 mM and 75 mM. Following incubation at 37°C for 7 days, exposure to all 3 concentrations of cycloheximide resulted in significant alterations to fungal growth (Fig. 6.3). Mycelia from the margins of cultures amended with 50 mM and 75 mM were inoculated onto ACM plates and incubated at 37°C for 7 days prior to harvesting spores in an attempt to select individual fungal colonies the RNA of which was isolated using the small scale isolation procedure (section 2.10.6).

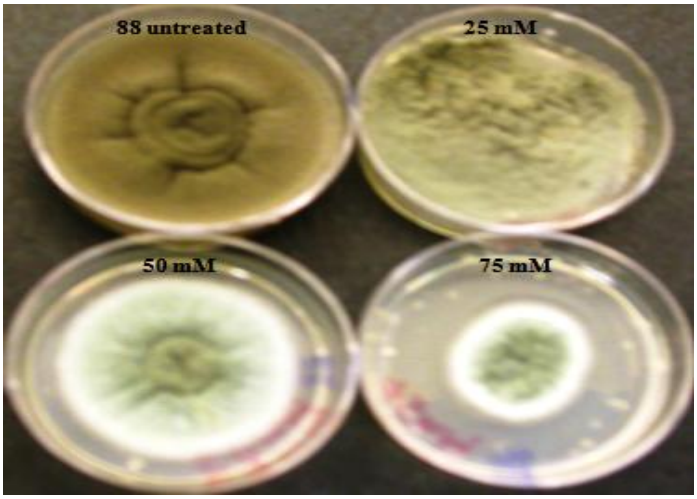


Figure 6.3 Colony morphology of *Aspergillus fumigatus* isolate 88 grown on ACM amended with cycloheximide (25 mM, 50 mM and 75 mM concentration) 7 days after inoculation.

Comparisons between the electrophoretic profiles of dsRNAs isolated from the partitivirus *A. fumigatus* isolate 88 (positive control), and from mycelia from single colonies selected from cultures treated with 25 mM, 50 mM and 75 mM cycloheximide indicated that all three were apparently free of infection with AfuPV (Fig. 6.4) and that cycloheximide impacts dsRNA accumulation significantly. Therefore single colonies of both virus-infected and apparently virus-free isolates were checked for infection following RT-PCR using dsRNA and compared with a positive control. A CP specific amplicon of 700 bp was amplified in all cases (Fig. 6.5) indicating that curing was not successful and had only reduced the dsRNA titres in the fungus below the limits of resolution of staining dsRNA with ethidium bromide in agarose gels.

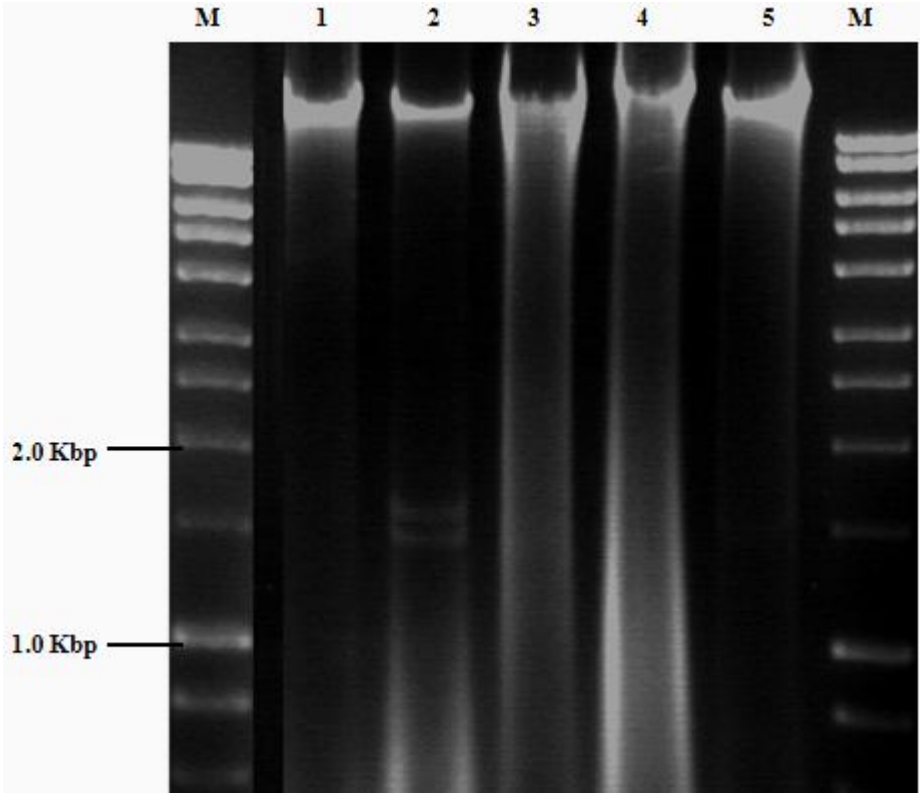


Figure 6.4 Small scale isolation of RNA from representative single colonies (sub-isolates of isolate 88 AfuPV) following treatment with cycloheximide at 25 mM, 50 mM and 75 mM concentrations (lanes 3, 4 and 5 respectively). Lane M contains Bioline molecular weight marker DNAs. Lanes 1 and 2; Nucleic acid extracts of negative control (uninfected, isolate 75; Chapter 3) and AfuPV- infected isolate 88 (positive control) extracts respectively.

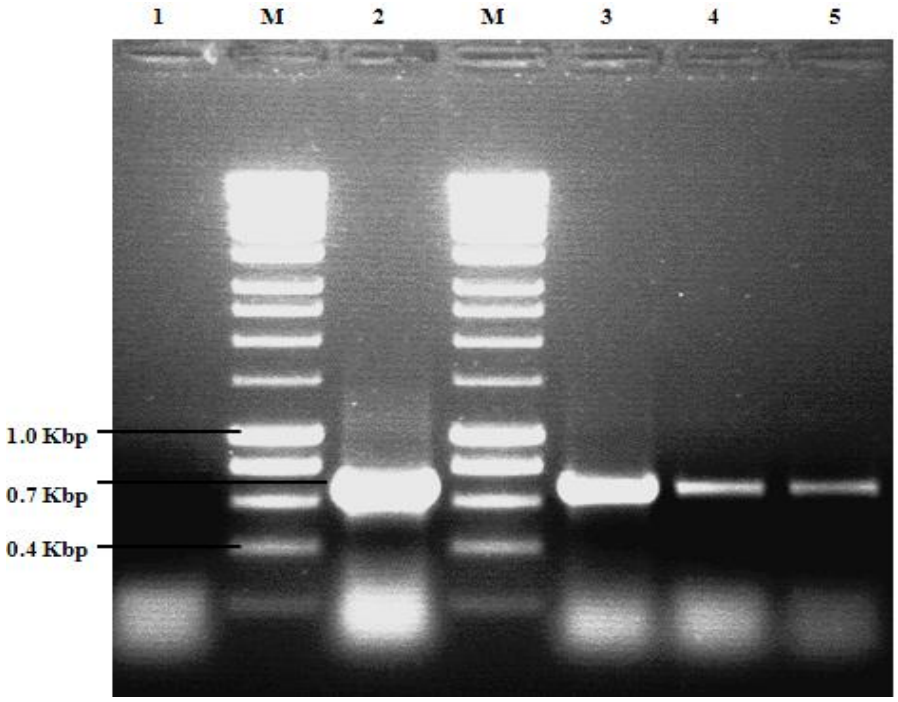


Figure 6.5 Agarose gel electrophoresis of amplicons (~700 bp) generated by RT-PCR using CP-specific primers to determine the effect of increasing concentrations of cycloheximide (25 mM, 50 mM and 75 mM; Lanes 3, 4 and 5 respectively) in curing *A. fumigatus* isolate 88 of infection with AfuPV. Lanes 1 and 2 are respectively negative and positive controls for RT-PCR. Lane M; Bioline molecular weight marker DNAs.

Further experiments investigated even higher concentrations of cycloheximide between 85 mM-150 mM in an attempt to eradicate AfuPV dsRNAs from isolate 88. These levels of the drug severely impaired the growth of the fungus and almost completely hindered its growth (Fig. 6.6). Nevertheless six colonies from these cultures were processed and examined for the presence of AfuPV dsRNA which was isolated using the small scale extraction procedure (Fig 6.7; section 2.10.6) which once more suggested that curing had been successful. To check if these cultures were indeed free of AfuPV, two colonies were selected randomly (F4 and F5; Fig. 6.8) and their mycelia grown in bulk for dsRNA analysis isolation (Fig 6.9). From these results it would appear that both isolates were indeed virus-free and these were subjected to RT-PCR to confirm this status. RT-PCR amplification of the entire CP-ORF and a fragment of the RdRP gene (section 2.13.2) revealed that isolate F5 was not cured of virus infection (Fig 6.10) while isolate F4 appeared to have lost the CP-encoding dsRNA component of the AfuPV genome (Fig 6.10) but retained the RdRP encoding dsRNA or a

fragment of it (Fig. 6.11). Later, RT- PCR amplification of the entire ORF of dsRNA-1(RdRP) also confirmed the results shown in Fig. 6.11. (data not shown).

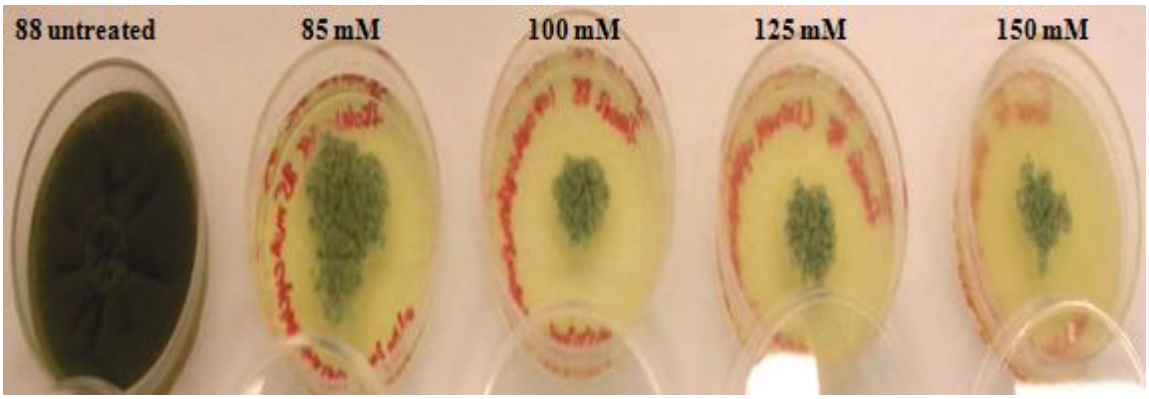


Figure 6.6 Colony morphologies of *A. fumigatus* isolate 88 grown on ACM amended with cycloheximide (85 mM-150 mM), 7 days after inoculation. Cultures amended with cycloheximide exhibit slow growth and altered colony pigmentation.

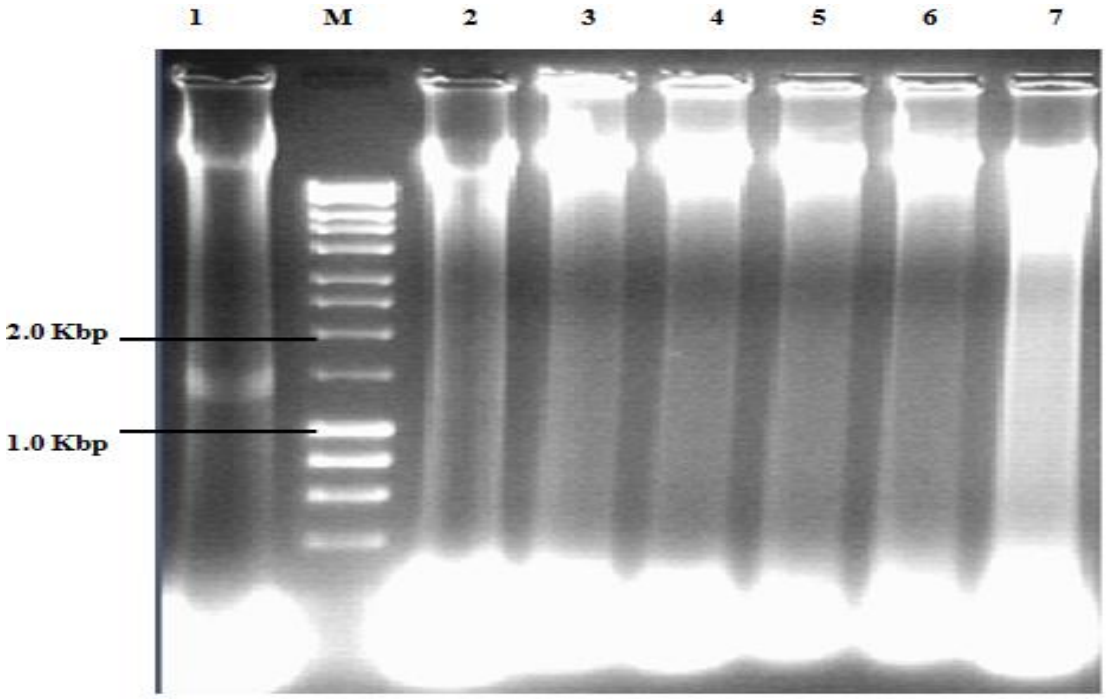


Figure 6.7 Agarose gel electrophoresis of 6 representative single colonies (sub-isolates of isolate 88 AfuPV) following treatment with 150 mM cycloheximide (F-1- F6; lanes 2-7 respectively). Lane M contains Bioline molecular weight marker DNAs. Lane 1; Nucleic acid extract of AfuPV- infected isolate 88-postive control.

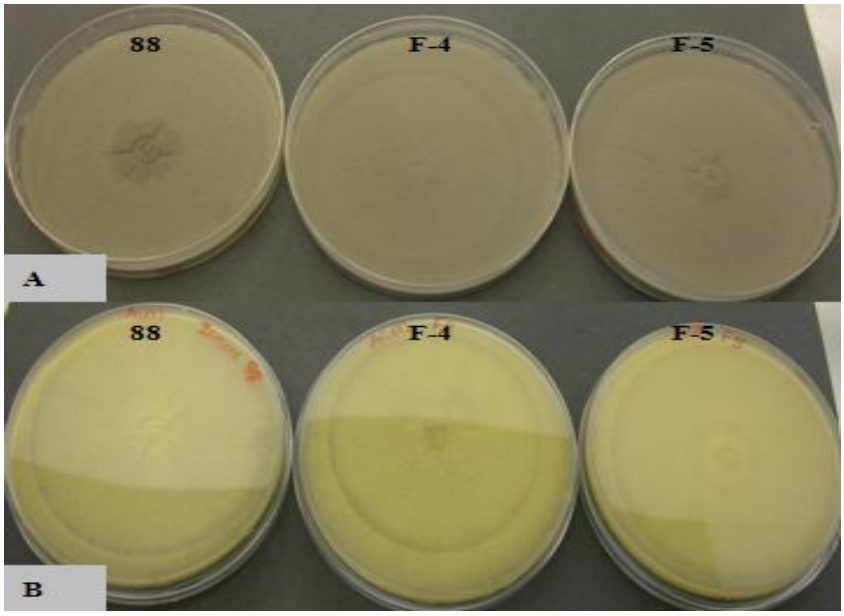


Figure 6.8 Colony morphologies of *Aspergillus fumigatus* isolate 88 (AfuPV infected); isolate F-4 (partially cured) and isolate F-5 (AfuPV-infected) grown on ACM 5 days after inoculation. Photographs were taken from the front (A) and from the back (B) of the plates to emphasise similarities and differences.

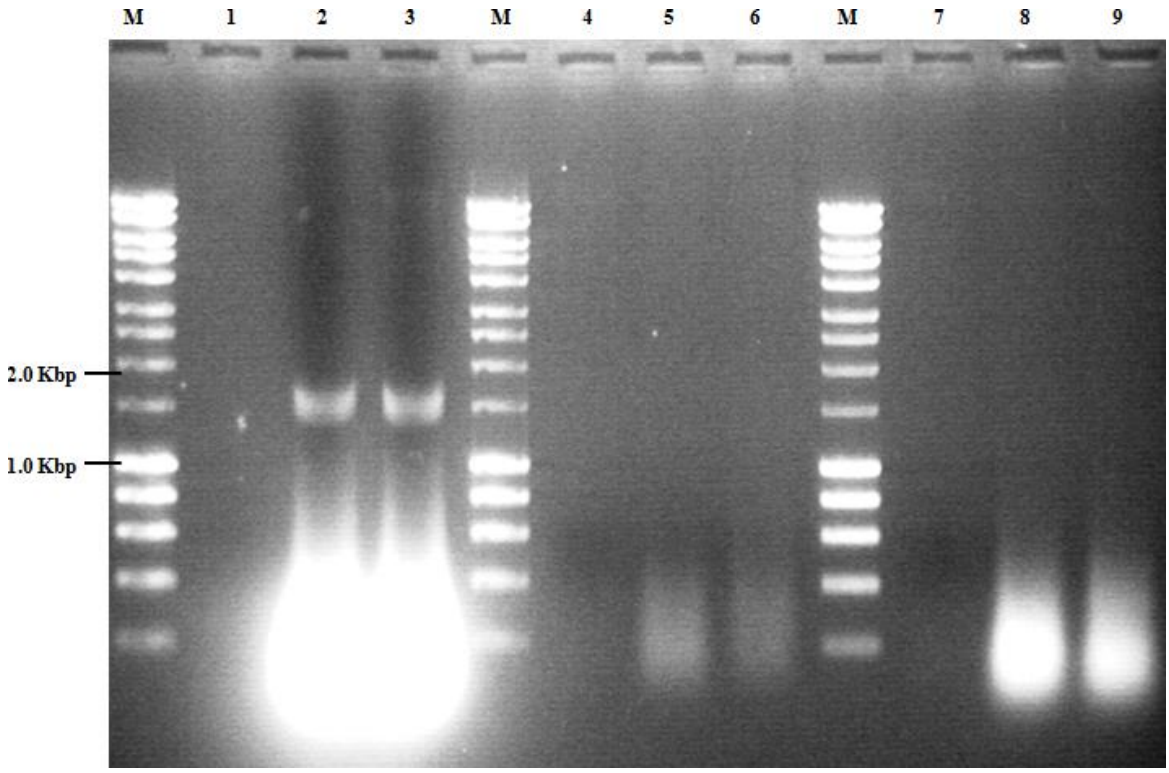


Figure 6.9 Agarose gel electrophoresis of dsRNAs isolated from LiCl purified virions processed from potentially cured isolates (F-4 and F-5) plus a positive control (isolate 88).

Lane M; Hyperladder1 (Bioline); lane 1, eluates of isolate 88 (negative control); lanes 2 and 3, dsRNA extracts obtained from isolate 88 (positive control); lane 4, eluate (negative control) of potentially cured isolate (F-4); lanes 5 and 6 dsRNA extracts obtained from virions isolated from isolate F-4; lane 7, eluates (negative control) of potentially cured isolate (F-5); lanes 8 and 9, dsRNA extracts obtained from virions isolated from isolate F-5.

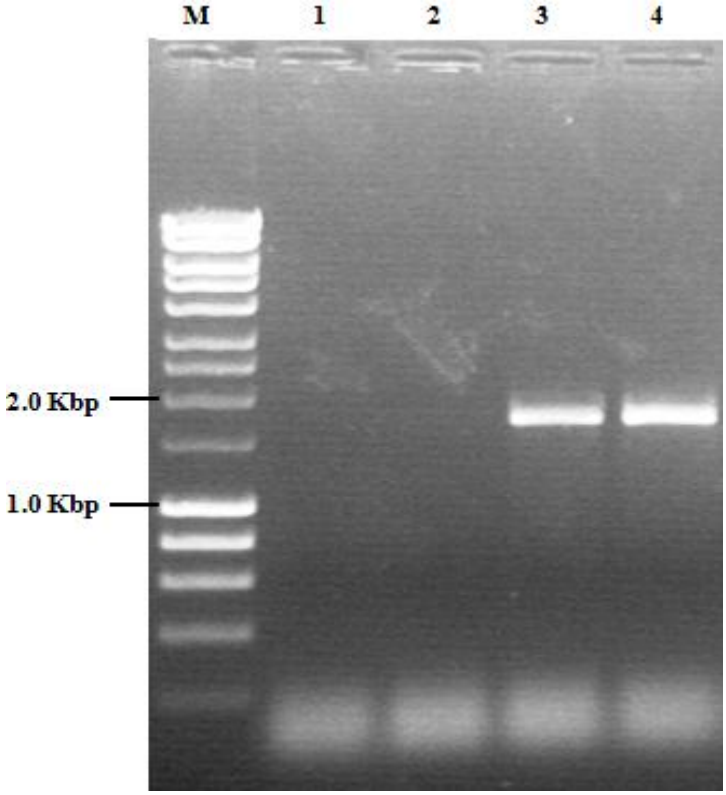


Figure 6.10 Amplicons generated by RT-PCR using oligonucleotide primers designed to produce a full length AfuPV CP amplicon. Lanes 1 and 4; negative and positive controls, respectively, for the complete CP gene; lanes 2 and 3; RNA extracts of potentially cured colonies F-4 and F-5 respectively, amplified using CP specific primers (RNA-2-FS and RNA-2 RS; Table 5.1).

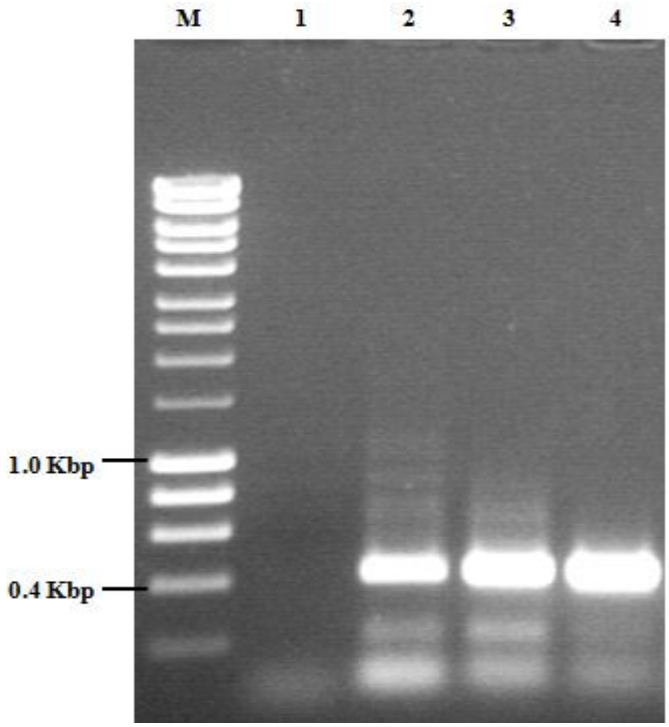


Figure 6.11 Amplicons generated by RT-PCR using oligonucleotide primers designed to produce a fragment of the AfuPV RdRP gene (~420 bp). Lanes 1 and 4; negative and positive controls respectively for the amplicon. Lanes 2 and 3; RNA extracts of potentially cured isolates F-4 and F-5 respectively, amplified using RdRp specific primers (MF-21 and MF-22; Table 5.1).

6.2 Sub-culturing of partially cured isolate F4

From the data described above it was anticipated that further treatment of the partially cured isolate F4 with cycloheximide at the elevated concentration of 150 mM may free it of any remaining dsRNA and cure *A. fumigatus* isolate 88 completely of AfuPV infection. In an attempt to achieve this isolate 4 was passaged a further 4 times on amended ACM and spores were harvested, serially diluted to 10^{-5} , grown on drug-free medium and 12 random individual colonies were selected 2 days post inoculation for further analysis. These colonies were point inoculated on ACM and grown at 37°C for 5 days. A change in morphology was observed among the selected colonies. The individual colony isolates were then passaged on ACM medium 5 times to confirm that the alterations in morphology persisted. Two different morphologies were observed during the selection process, a light or a wild type morphology and a dark green morphology (Fig 6.13). When the RNA from those isolates exhibiting the darker morphology was subjected to RT-PCR analysis both components of the AfuPV

genome were found to be present (Figs. 6.14 and 6.16). However when the RNA from those isolates exhibiting the lighter morphology were examined a fragment of the RdRP gene was amplified once more (Fig. 6.16) suggesting only partial curing in these isolates and that elimination of the AfuPV infection by cycloheximide treatment was not possible.

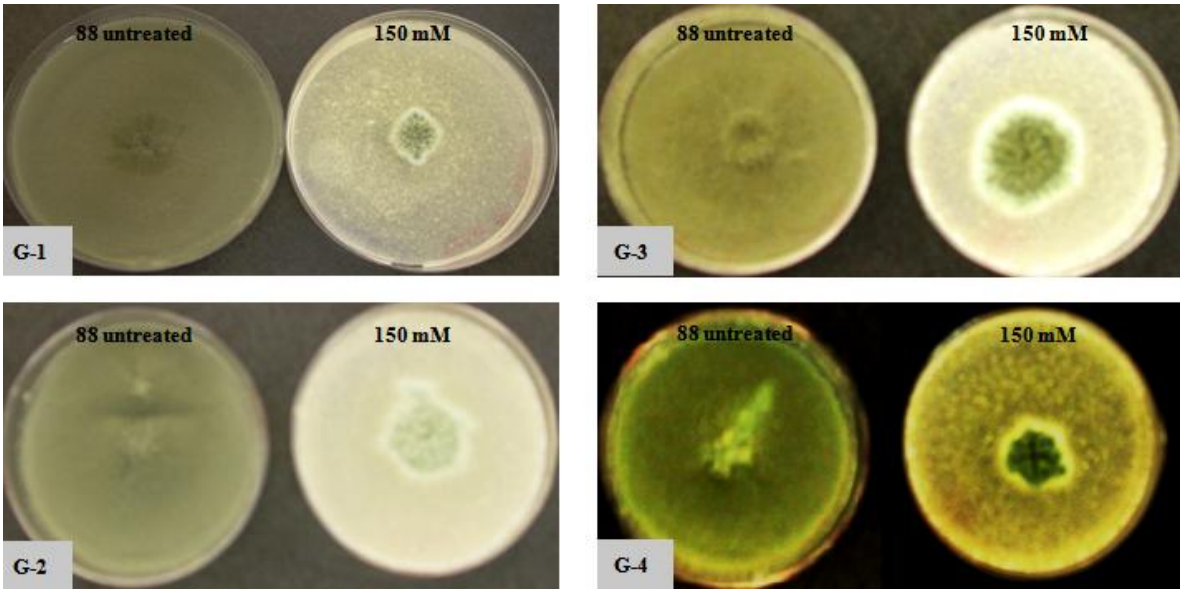


Figure 6.12 Four generations of partially cured isolate F-4 grown on ACM containing 150 mM cycloheximide serially diluted to 10^{-4} and incubated at 37°C for 7 days.

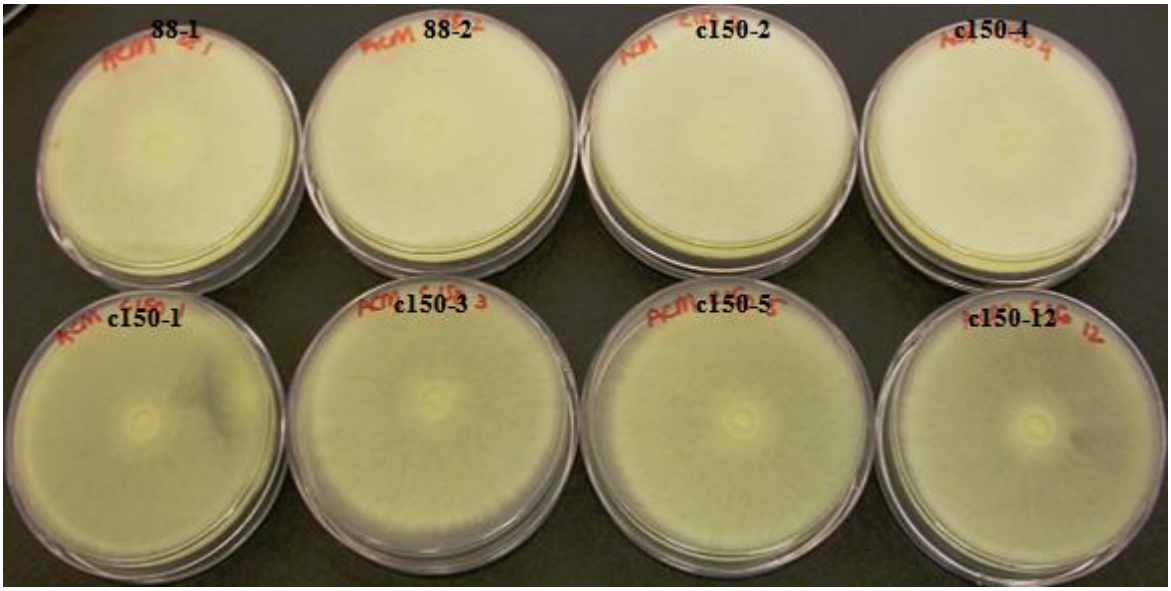


Figure 6.13 Colony characteristics of representative cured isolates (c-150) with two different morphologies (light and darker) obtained after serial passage of isolate 88 (isolate F-4) for 4

generations on ACM containing 150 mM cycloheximide, followed by passage on ACM without cycloheximide.

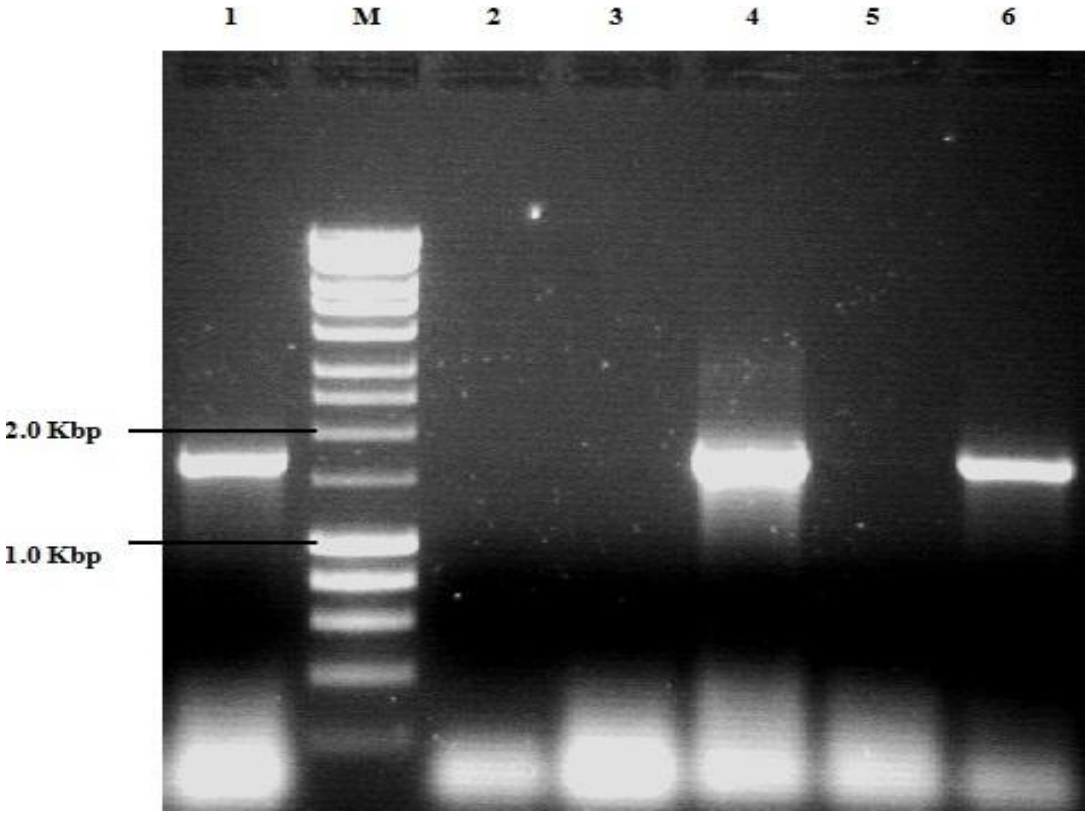


Figure 6.14 Agarose gel electrophoresis of amplicons generated by RT-PCR using oligonucleotide primers designed to produce a full length AfuPV CP amplicon. Lanes 1 and 2 are positive and negative controls respectively for the complete CP gene. Lanes 3-6 are the RT-PCR amplicons generated using RNA extracts from 4 representative colonies (c150-2, c150-3, c150-4 and c150-5) cultured as described in the text and amplified using CP specific primers (RNA-2-FS and RNA-2 RS; Table 5.1).

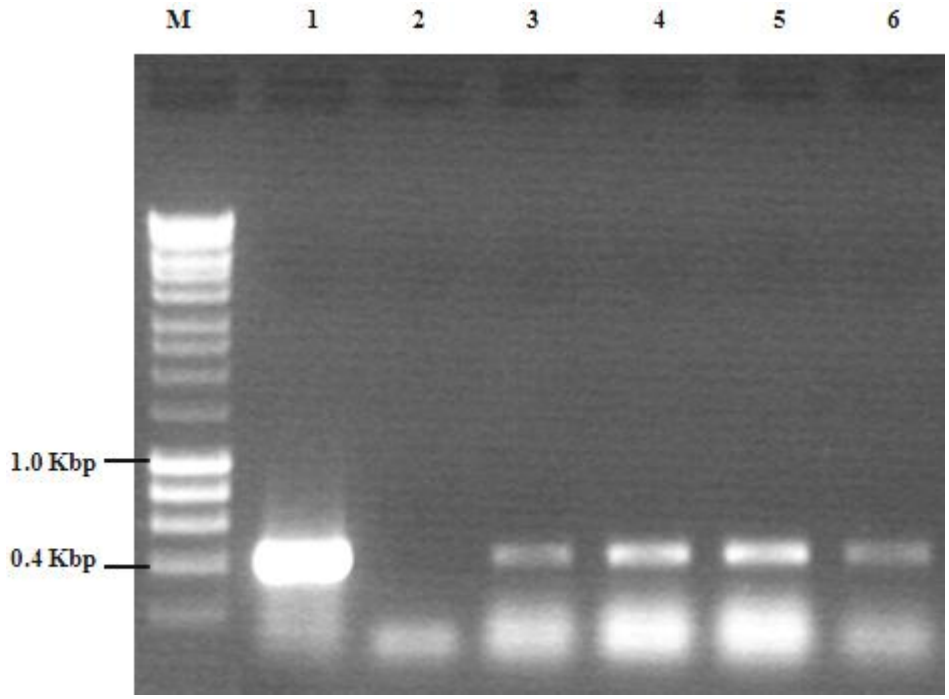


Figure 6.15 Agarose gel electrophoresis of amplicons generated by RT-PCR using oligonucleotide primers designed to produce a fragment of the AfuPV RdRP gene. Lanes 1 and 2 are positive and negative controls respectively for the fragment of the AfuPV RdRP gene. Lanes 3-6 are the RT-PCR amplicons generated using RNA extracts from 4 representative colonies (c150-2, c150-3, c150-4 and c150-5) cultured as described in the text and amplified using RdRP specific primers (Table 5.1).

6.3 Discussion

In order to determine the impact of dsRNA viruses on fungal hosts it is important to compare infected fungi directly with uninfected types in the same genetic background. Until recently, the only method used to assess the efficiency of curing fungi of mycovirus infection was extracting dsRNA from infected and cured fungal samples and examining the extracts following gel electrophoresis. However, the levels of dsRNA present in any one particular sample might be below the levels of visual detection in stained gels and a more effective and sensitive assay such as RT-PCR amplification using virus-specific primers to check for the presence and absence of virus should be used (see Chapter 5 for details). The sensitivity of such assays has been illustrated by Park *et al.,* (2006), who studied the efficiency of curing *C. elegans* from infection with *Chalara elegans* mitovirus (CeMV). Attempts were made to eradicate CeMV infection following sequential mycelial transfer and heat curing by

incubation at 35-37^{o}C. The treatment was apparently successful in eliminating the CeMV 2.8 kbp mitovirus dsRNA element as assessed by dsRNA extraction, gel electrophoresis and staining and northern blot hybridisation. However following sequencing of the products generated by RT-PCR using mitovirus-specific oligonucleotide primers the presence of mitovirus was confirmed in both uncured and apparently cured mycelia invalidating the original conclusions.

Several procedures are available to cure fungi from mycovirus infection and these include exposure to ultra violet (UV) light (Treton *et al.*, 1987), elevated temperature (Golubev *et al.*, 2003) and treatment with a range of different chemicals including acridine orange (Cansado *et al*., 1989), emetine (Ahn and Lee, 2001) and cycloheximide (Dalzoto *et al*., 2006; Elias and Coty, 1996; Robinson and Deacon, 2002; Yamada *et al*., 1991) all of which have been used in this context. The most reliable and frequently used method to cure and reduce dsRNA levels in mycovirus infections (completely or partially) involves treating fungi with cycloheximide. Changes in the phenotype, sporulation and pigmentation of virus-infected fungi following treatment with cycloheximide were first observed by Fulbright (1984) where eradication of dsRNAs from *E. parasitica* strain GHU4pc10 restored the normal morphology of the fungus.

In the experiments reported here all attempts to completely eradicate the dsRNA elements associated with AfuPV infection of *A. fumigatus* isolate 88 by treatment with cycloheximide at elevated concentrations (<150 mM) failed (sections 6.1 and 6.2) even though alterations to the phenotype were recorded. Whilst elimination of the AfuPV dsRNA2 element encoding the CP gene was achieved following cycloheximide treatment complete eradication of the dsRNA1 element encoding the RdRP was not. Once the antibiotic stress was removed, the concentration of AfuPV dsRNA1 increased and the fungus remained infected albeit apparently only with one component of the genome.

Since it has not been possible to produce a virus-free, isogenic line of *A. fumigatus* isolate 88 it is not possible to state with any certainty that the presence of a mycovirus in this isolate alters the fungus phenotype. Indeed few mycovirus infections of Aspergilli cause alterations to phenotype, growth or mycotoxin production and most infections appear to be cryptic (Van Diepeningen *et al.*, 2006).

There are several reports of successful eradication of mycoviral eradication following cycloheximide treatment. For instance, successful curing of *Aspergillus fumigatus chrysovirus* (AfuCV) present in isolate A-56 (Bhatti *et al.*, 2011b), *Endothia parasitica* strain GHU4pc10 (Fullbright, 1984) and *Beauveria bassiana* strain CG25 (Dalzoto *et al.*, 2006). However, there are also reports of unsuccessful eradication of dsRNA elements from some fungal isolates e. g. *M. anisopliae* (Martins *et al.*, 1999), *Fusarium oxysporum* (Sharzei *et al*., 2007) and *Aspergillus* section *flavi* isolate 91-182A infected with a chrysovirus (Elias and Cotty, 1996). These failures might be due to using low titres of cycloheximide during treatment. The presence of high titres of virus and also the inability to cure mycovirus infection might be a reflection of the general stability of dsRNA. Fink and Styles (1972) reported that since cycloheximide inhibits protein synthesis, cytoplasmic ribosomal protein synthesis must be necessary for the replication of the genetic determinants. Occasionally treatment with cycloheximide was accompanied by hyphal tip isolation to achieve success e.g. with virus-infected *Alternaria alternata* (Aoki *et al*., 2009).

The results presented here indicate partial curing of a partitivirus infection in a fungus and similar results were obtained when cured and partially cured isolates of virus-infected *C. elegans* were compared (Punja, 1995), and also when the pigmentation of virus-infected *M. pinodes* pycnidia changed from dark brown to light pink following curing, coincident with a restoration of the normal phenotype (Yamada *et al*., 1991). Similarly Yamada *et al.*, (1991) were unable to detect one of the three dsRNA elements present in the plant pathogenic fungus *Mycospharella pinodes* following treatment with cycloheximide. However, cycloheximide treatment is not always completely successful and Robinson and Deacon, (2002) were unable to completely cure *Rhizoctonia solani* AG-3 of mycovirus infection comprising several dsRNA elements 1.0 to 12.5 kbp in size. Whilst the smaller segments were cured after cycloheximide treatment, the larger segments 9.0 to12.5 kbp were always retained. This may be an indication of the fact that partial eradication of dsRNA elements might not affect the replication of other dsRNAs and they continue to replicate. Our findings has also revealed that after sucessful eradication of CP genome with the cycloheximide treatment, the RdRP genome was still present which means replication of RdRP genome is not affected by the CP eradication.

6.4 Conclusions

In order to elucidate the effects of the partitivirus, attempts were made to completely cure the AfuPV infection. For this purpose a range of cycloheximide concentrations (0.1 mM- 150 mM) were used. These levels of the drug severely impaired the growth of the fungus and almost completely hindered its growth. As assessed by RT-PCR amplification assays, *A. fumigatus* isolate 88 was not cured of AfuPV infection by cycloheximide treatment at a higher concentration (150 mM) of drug. However, this treatment with cycloheximide resulted in partially cured isolate (F-4) in which the smaller component of the AfuPV (CP; dsRNA2) was eradicated. However, presence of larger component (RdRP; dsRNA1) confirms the AfuPV infection was only partially cured.

Chapter 7:

Transfer of *Aspergillus* mycoviruses and dsRNA elements to uninfected isolates of *A. fumigatus*

7. Transfer of *Aspergillus* mycoviruses to uninfected isolates of *A. fumigatus*

The aim of these experiments was to transfer *A. fumigatus* virus and dsRNA elements to virus-free isolates of *A. fumigatus*. Three methods of transferring dsRNAs were investigated including co-cultivation (anastomosis), protoplast fusion and transfection with purified virions. Before the discovery of AfuPV, pilot experiments were carried out on two other groups of Aspergillus mycoviruses i. e. *A. fumigatus* chrysovirus and *A. fumigatus* uncharacterised virus (see Chapter 3 for further details).

7.1.1 Transfer of Aspergillus mycovirus *via* co-cultivation (hyphal anastomosis)

The success of hyphal tip fusion and protoplast fusion in the transfer of the chrysovirus dsRNAs from *A. fumigatus* isolate A-56 to the yellow pigmented and hygromycin resistant isolate Af-237y was monitored using the diagnostic RT-PCR assay described earlier. The potential transfer of the uncharacterised *A. fumigatus* mycovirus was assessed by small scale extraction procedure as there was no sequence information available to design primers for a diagnostic RT-PCR. Initially chrysovirus containing isolate (A-56; AfuCV) was transferred to the yellow isolate of *A. fumigatus* Af-237y *via* co-cultivation (hyphal anastomosis). Putative positive fusion events were recorded as those yellow pigmented mycelia which grew on selective solid media containing hygromycin (section 2.24.2) and RNA was isolated from it using the RNeasy kit (section 2.12.3). Successful dsRNA transfer was assessed by RT-PCR which sought to amplify a fragment of the *A. fumigatus* isolate A-56 AfuCV CP gene using oligonucleotide primers AJ-9A (5'-ACTACGCTGGTTAGGGAATGT-3') and AJ-10A (5'-GTCCTCTTCCGAAGCTTGTT-3'), (kindly provided by Dr Atif Jamal). The results of these experiments showed that only one colony isolated was suspected to contain AfuCV specific amplicon (Fig. 7.2; lane 4). To confirm that amplicon was not artifactual, the restriction digestion of that AfuCV CP amplicon was carried out. The restriction digestion of that amplicon with *Ava1* resulted into 2 anticipated and equally sized fragments, confirming the successful transfer of AfuCV (Fig. 7.3).

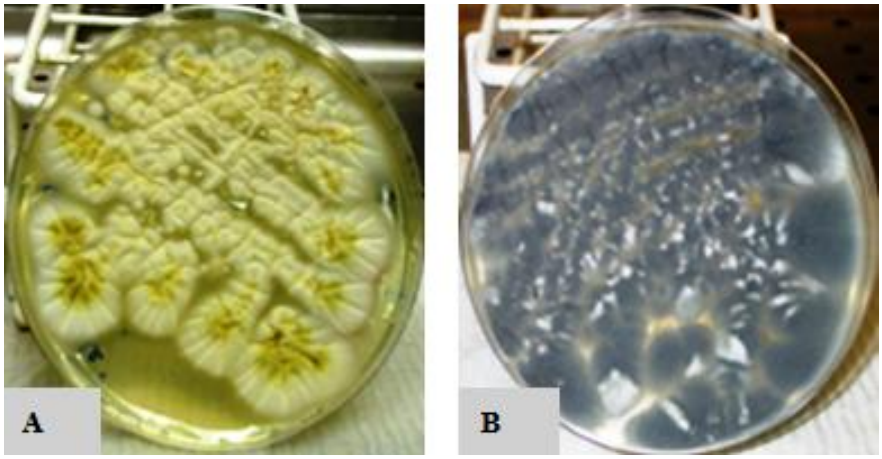


Figure 7.1 ACM plates containing mixed conidia of A-56 and Af-237y with (A) and without hygromycin (B).

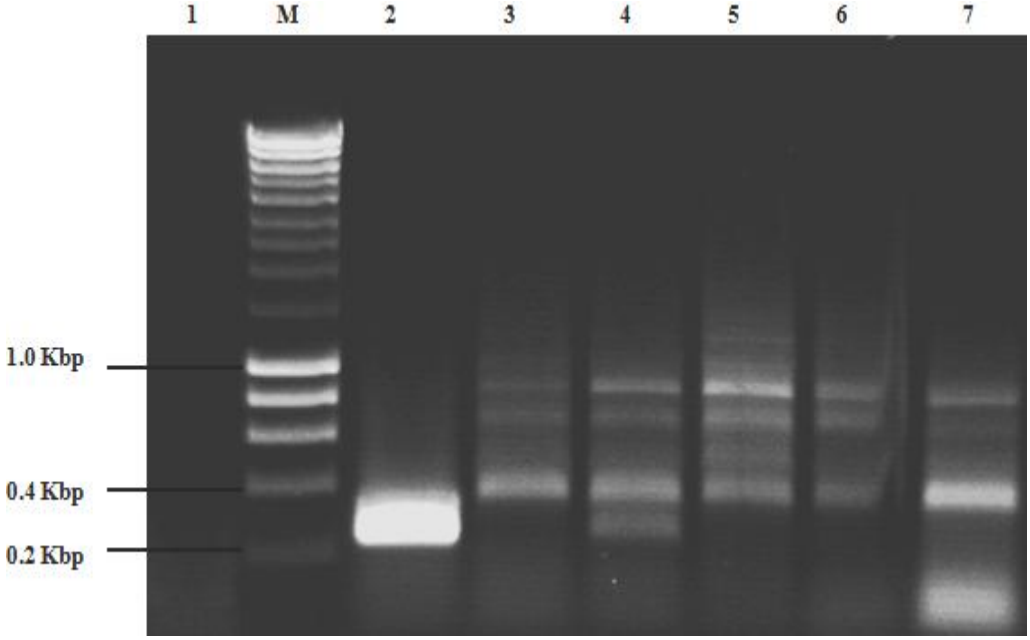


Figure 7.2 Determination of transfection of Af-237y with AfuCV by RT-PCR following RNA isolation with the RNeasy kit. RNA extracts were used for RT-PCR in an attempt to amplify a fragment of the AfuCV CP gene using primers AJ-9A and AJ-10A. Lane M, Hyperladder1 (Bioline); lanes 1 and 2; negative and positive controls; lanes 3-7; random Af-237y colonies (1-5 respectively).

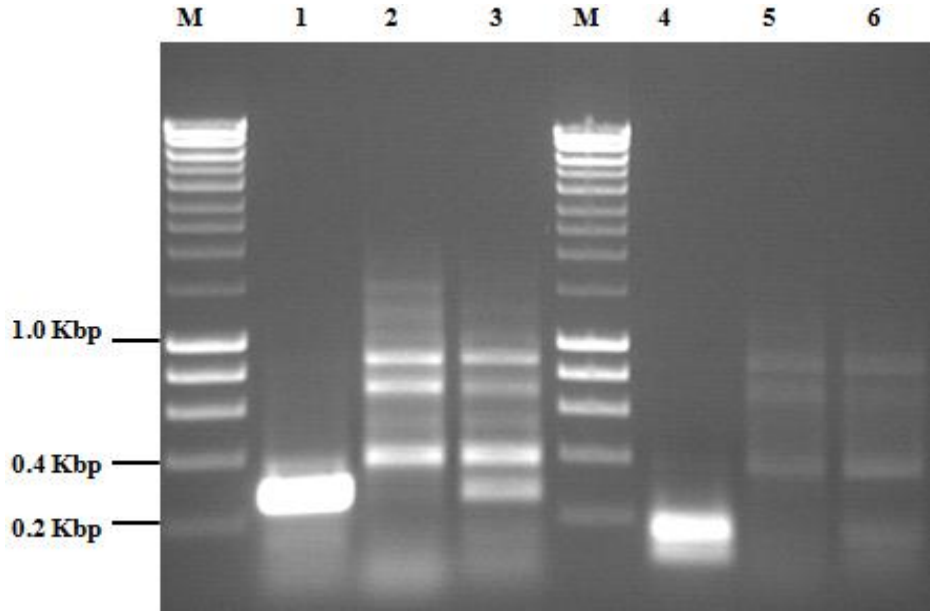


Figure 7.3 Similar gel to Figure 7.2 to determine transfection of Af-237y *via* hyphal anastomosis with AfuCV; lane M, Hyperladder1 (Bioline); lanes 1 and 4 respectively, CP amplicon generated with A-56 chrysovirus using specific primers (AJ-9A and AJ-10A) and the same amplicon digested with *Ava*1 (positive control); lanes 2 and 5; Negative control; lanes 3 and 6; amplicons generated from dsRNA isolated from putatively infected Af-237y colony 2 (Fig. 7.2; lane 4) and the same amplicon digested with *Ava*1.

The lack of sequence information for isolates A-78 and A-54, both infected with the uncharacterised dsRNAs, precluded development of a diagnostic RT-PCR assay for these elements and attempts to check for any transfer of dsRNA elements to strain Af-237y were made using the small scale extraction procedure only. These experiments showed no transfer of dsRNAs by anastomosis (Fig.7.4; lanes 1-5 for isolate A-78 and lanes 6-10 for isolate A-54). Confirmation that the RNA extraction procedure was working was obtained following successful extraction of dsRNAs from cultures of isolate A-78 and A-54 (Fig. 7.4; lanes 11 and 12 respectively).

These results indicate that transfer of the A-78 and A-54 dsRNAs did not occur following hyphal tip fusion with isolate Af-237y. However the amounts of dsRNA present in the fused mycelia might be below the levels required to be detectable by ethidium bromide staining as suggested before.

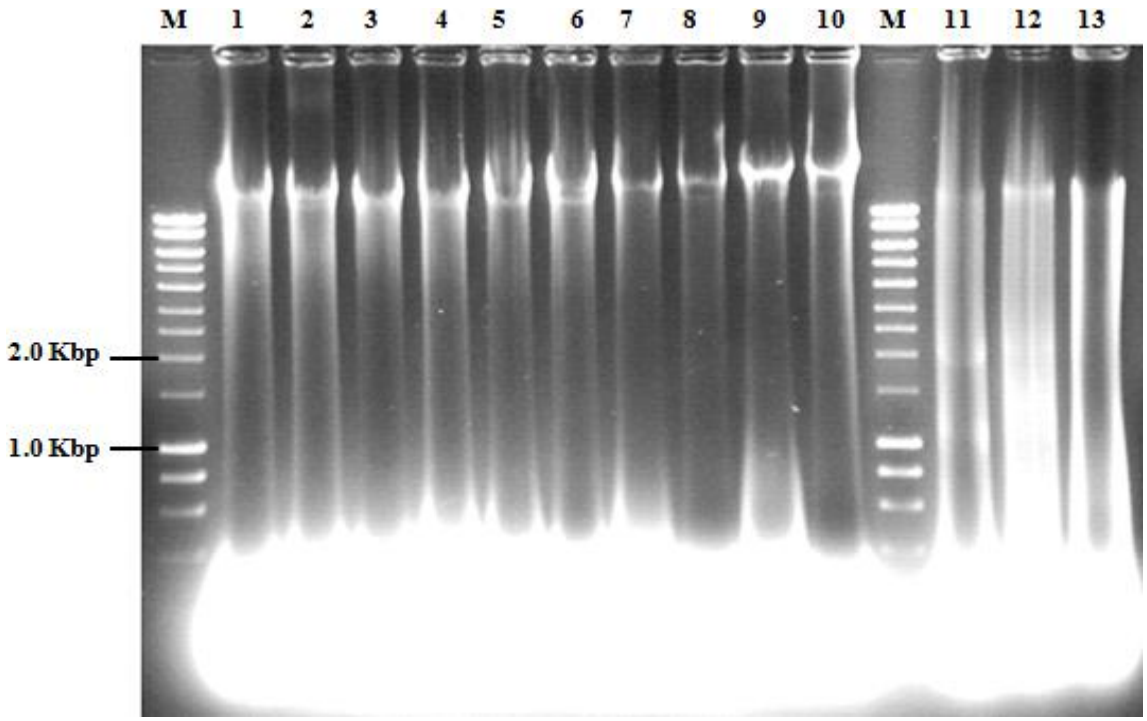


Figure 7.4 Analysis of the dsRNA extracts of mycelia isolated by the small scale isolation procedure following attempted transfer of uncharacterised dsRNAs from *A. fumigatus* isolates A-78 and A-54 to isolate Af-237y by hyphal anastomosis. Lane M Hypperladder1 (Bioline); Lanes 1-5; five randomly picked Af-237y colonies fused with isolate A-78; lanes 6-10; five randomly picked Af-237y colonies fused with isolate A-54; lanes 11 and 12; dsRNA isolated from respectively isolates A-78 and A-54 respectively as positive control; lane 13; dsRNA isolated from respectively isolate Af-237y as a negative control.

7.1.2 Transfer of *Aspergillus fumigatus* mycoviruses *via* protoplast fusion and transfection

7.1.2.1 Transfer of *A. fumigatus* chrysovirus (AfuCV) and uncharacterised virus dsRNAs *via* protoplast fusion

Protoplast fusion and transfection were two other approaches used to transfer mycoviruses in to uninfected isolates of *A.fumigatus*. The purpose of investigating these methodologies were to utilise these techniques for not only generating the isogenic lines of different mycoviruses but also to establish a system through which *A. fumigatus* mycoviruses can be transmitted to a broad range of host fungi so to use these mycoviruses as a potential biological control agent. As natural host ranges of fungal viruses are limited to single species or to very closely related

species and only proven route of mycovirus horizontal transmission in filamentous fungi is via hyphal fusion (Kanematsu, *et al.*, 2010). The transfer of mycoviruses through hyphal fusion is often hindered by vegetative incompatibility. To over come this phenomenon protoplast fusion and transfection are the two important approaches which have broadened the range of mycoviral infection to other fungi.

Attempts to transfer dsRNAs between some of the same combinations of *A. fumigatus* isolates as described earlier (section 7.1.1) using protoplast fusion and direct transfection with virions were apparently successful. Small scale RNA extracts from mycelia grown on replica hygromycin-plates following fusion of protoplasts from isolate Af-237y with isolates A-78, A-54 and A-56 with positive controls for each respective dsRNA were analysed. Similarly RNA extracted from mycelia grown on replica plates following transfection of protoplasts from isolate Af-237y with purified AfuCV virions was examined.

The results obtained suggest successful transfer of the uncharacterised dsRNAs from both isolates A-78 and A-54 to isolate Af-237y following protoplast fusion (Fig. 7.5; lanes 2 and 3; 5 and 6 respectively) and a failure to transfer the AfuCV dsRNAs, either by protoplast fusion (Fig. 7.5; lanes 8 and 9) or by transfection with virions (Fig. 7.5; lanes 10 and 11). However further anlaysis of RNA extracts of mycelia generated following fusion of protoplasts from isolates A-56 and Af-237y and protoplasts transfected with AfuCV virions by RT-PCR as described earlier (section 2.13.2) revealed that these experiments were apparently successful too, since amplicons of the expected size were generated in both cases. Confirmation that these amplicons were not artefactual was made following digestion with *Ava*1, which is known to restrict the CP amplicon into two fragments of known size (Fig.7.6). Overall these observations indicate that the levels of AfuCV dsRNA present in the mycelia resulting from protoplast fusion and transfection are below that required to be visualised by the small scale extraction procedure and ethidium bromide gel staining but easily detectable by RT-PCR. However the efficiency of transfection in both cases was low.

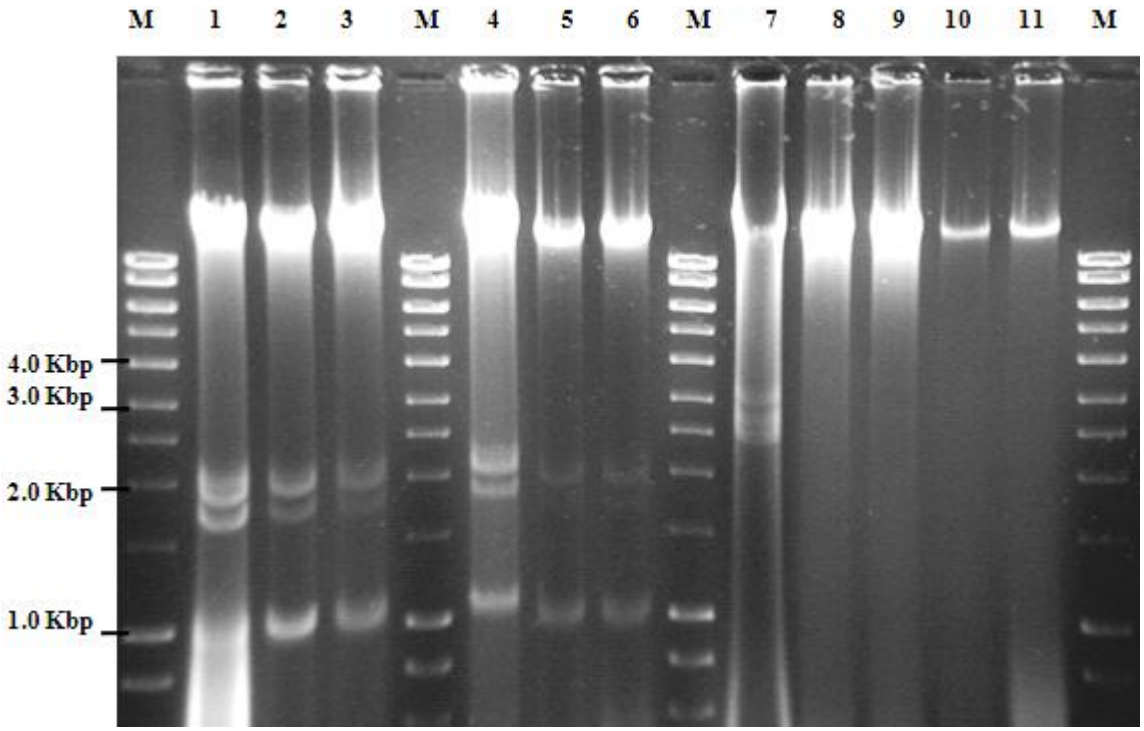


Figure 7.5 Analysis of the dsRNA extracts of mycelia isolated by the small scale isolation procedure following attempted transfer of uncharacterised dsRNAs from *A. fumigatus* isolates A-78 and A-54 to isolate Af-237y by protoplast fusion (lanes 2 and 3-A-78; lanes 5 and 6-A-54) and AfuCV to isolate Af-237y by protoplast fusion (lanes 8 and 9) and viral transfection with purified A-56 chrysovirus (lanes 10 and 11). Lane M, Hyperladder1 (Bioline); lanes 1, 4 and 7 positive controls of dsRNA isolated from isolates A-78, A-54 and A-56 respectively.

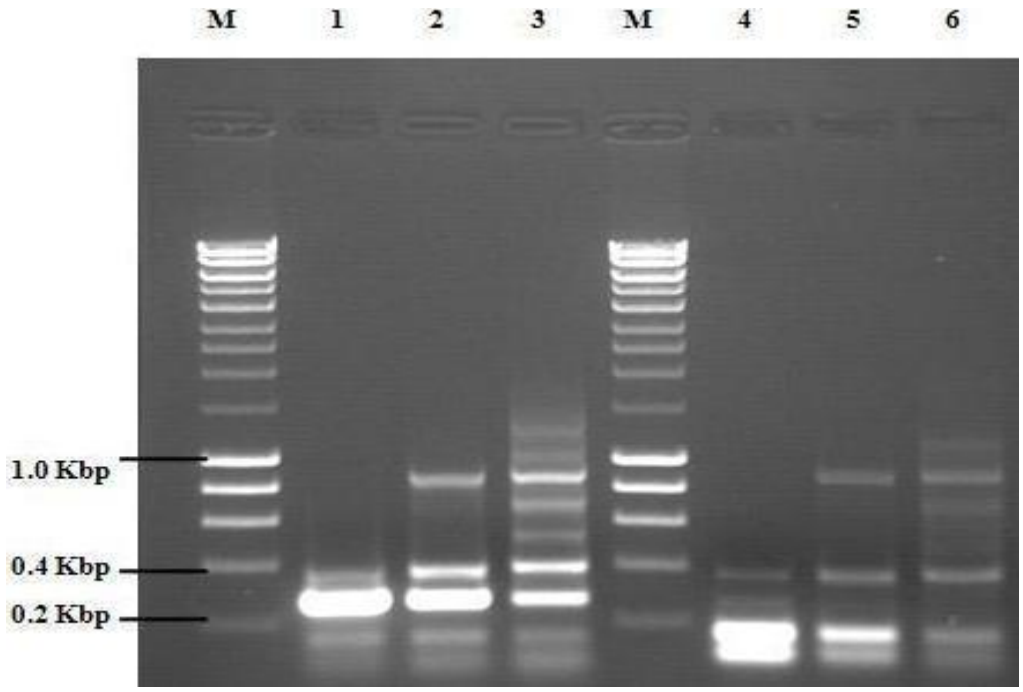


Figure 7.6 Determination of transfection of Af-237y with AfuCV by RT-PCR following small scale isolation procedure. Total RNA preparations were used for RT-PCR in an attempt to amplify a fragment of the AfuCV CP gene using primers AJ-9A and AJ-10A. Lane M, Hyperladder1 (Bioline); lane 1, positive control (CP fragment generated by AJ-9A and AJ-10A with purified AfuCV); lanes 2 and 3, amplicons generated from 2 random Af-237y colonies; lanes 4 to 6 *Ava*1 digested amplicons from products displayed in lanes 1-3.

7.1.2.2 Transfection of the *A. fumigatus* yellow isolate (Af-237y) with purified AfuPV

Following the successful transfer of AfuCV and uncharacterised virus, attempts were made to launch an infection of AfuPV in the virus-free *A. fumigatus* yellow isolate (Af-237y) to create isogenic lines of AfuPV. To achieve this, isolated protoplasts of Af-237y (Fig. 7.7) were inoculated with purified AfuPV using PEG (section 2.24.3). Control preparations of protoplasts were inoculated with water. Following transfection all protoplasts both control and virus-transfected were washed, and cultured on selective media (section 2.9.1) at 37^{o}C. The resultant mycelia were harvested and extracts serially diluted and spread on solid ACM for the selection of individual colonies (Fig.7.8). All plates were incubated at 37^{o}C for 3 days and 15 individual colonies were then selected and re-inoculated onto fresh media and incubated further. Observations of the resulting colonies revealed that, as compared to

control colonies, virus-transfected colonies had four different phenotypes including different levels of pigmentation and sectoring.

A representative isolate of each of the four phenotypes was randomly selected (Fig.7.9; colonies 5, 8, 11 and 14) and grown in ACM broth to harvest mycelia in bulk. Virus was then purified from the mycelia of the four isolates (section 2.12.1) and the RNA isolated from each (section 2.11.4) was analysed on 1% agarose gels. Confirmation of successful *in vitro* infection of the virus-free, *A. fumigatus* strain (Af-237y) with AfuPV was obtained following RT-PCR (section 2.13.2) amplification using oligonucleotide primers (MF-28 and MF-29; Table 5.1) producing an amplicon corresponding to the complete AfuPV dsRNA-1 RdRP open reading frame. Gel electrophoresis of the amplicons together with a positive control amplicon produced using the same procedure with RNA from *A. fumigatus* isolate 88 revealed the presence of an identical band (Fig. 7.11), confirming the presence of AfuPV dsRNA1 and successful introduction and replication of AfuPV in *A. fumigatus* stain Af-237y.

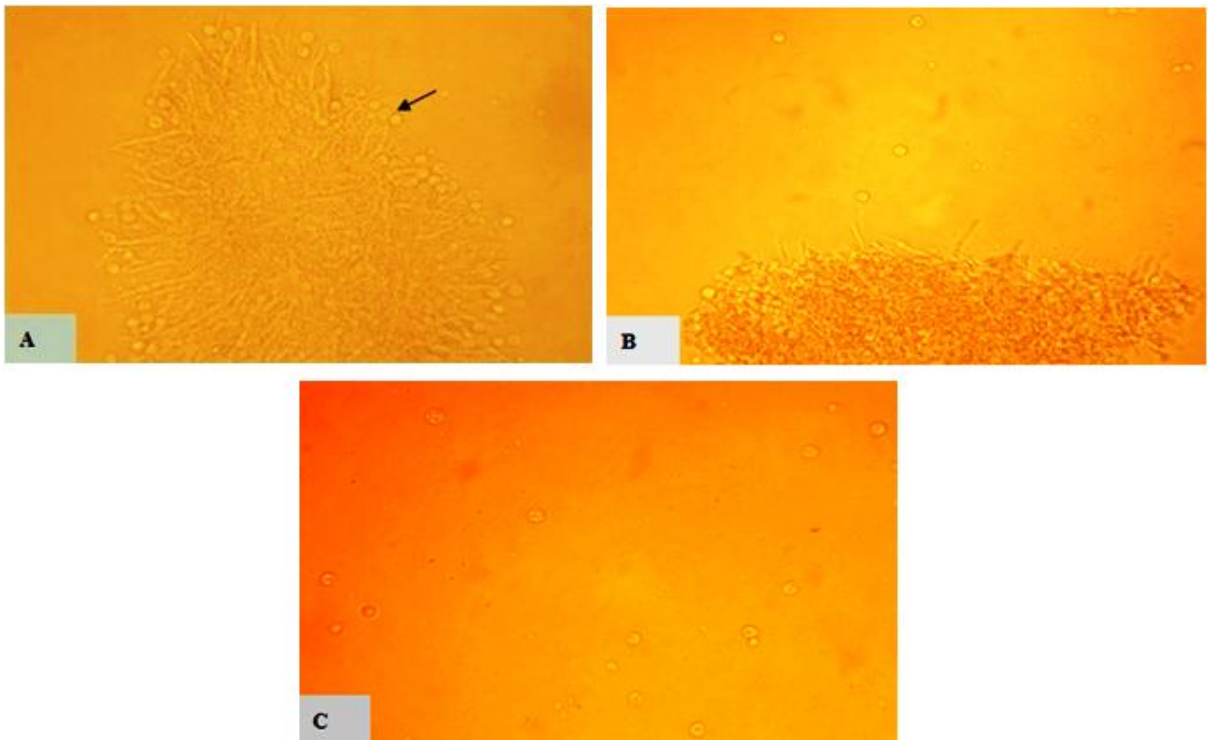


Figure 7.7 Freshly isolated protoplasts from *A.fumigatus* (isolate Af-237y). The hyphal tips bulge and protoplasts form at the end of hyphae (arrowed). Protoplasts were monitored 2 h (A) and 2.5 h (B) after incubation in the protoplasting solution, following purification on a sucrose cushion (C).

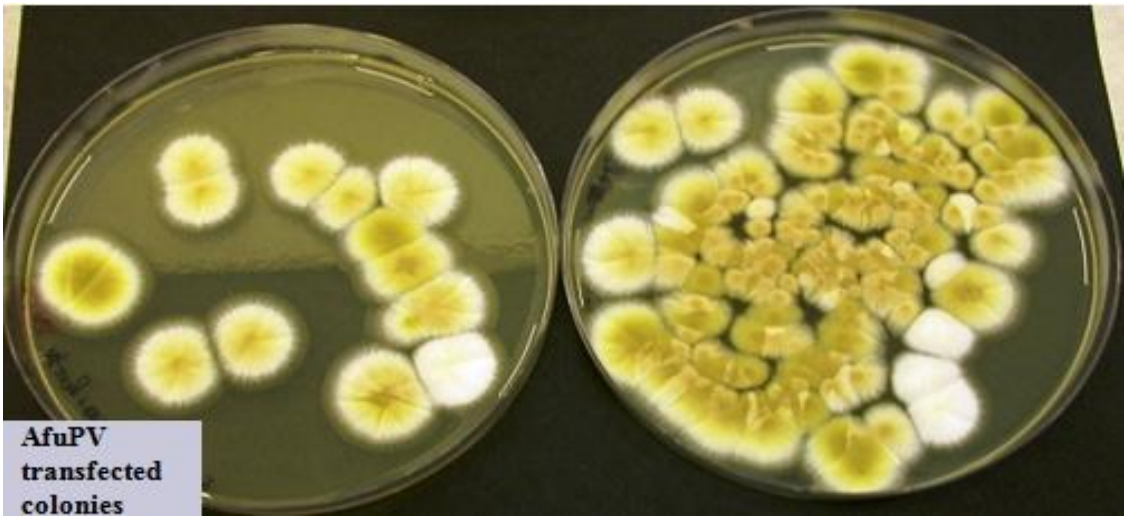


Figure 7.8 AfuPV transfected individual colonies on solid ACM.

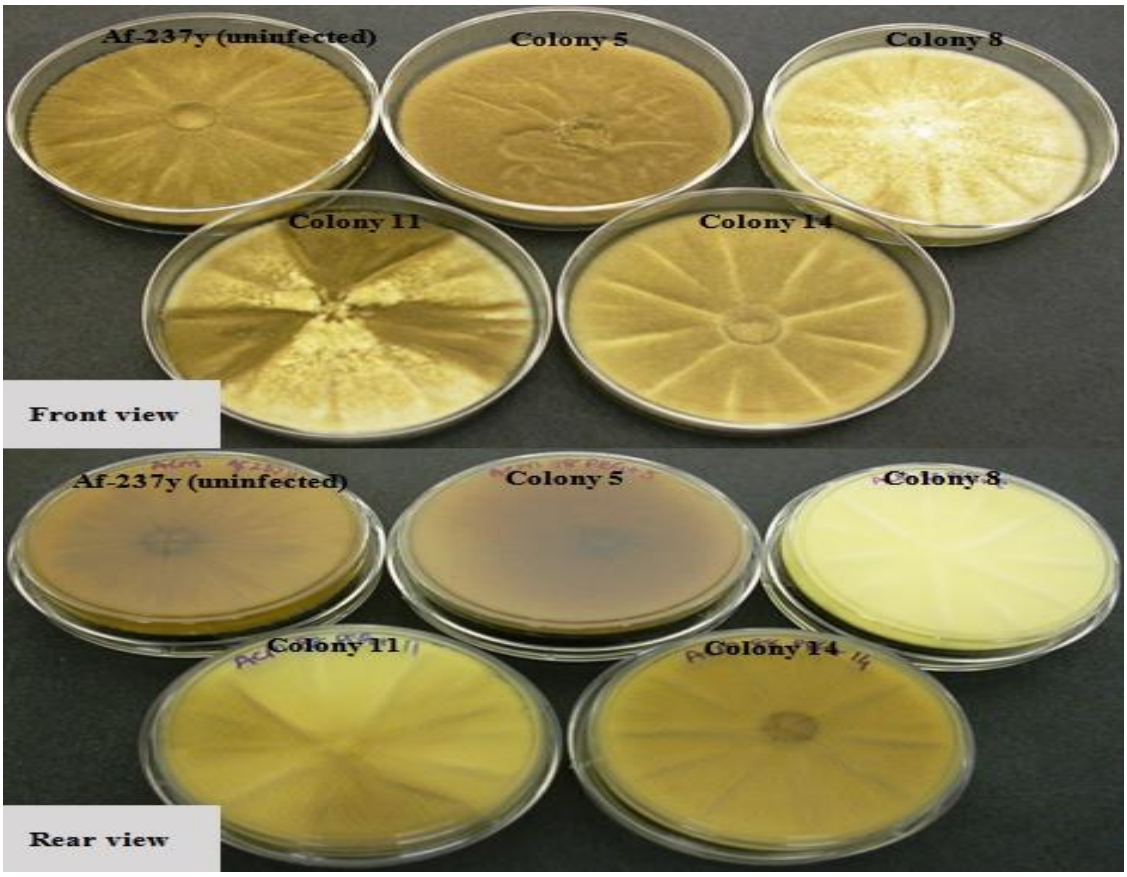


Figure 7.9 Colony morphology of virus-free *A. fumigatus* strain Af-237y and AfuPV transfected colonies (colonies 5, 8, 11 and 14). Sectoring and differences in morphology can be seen can be seen from the front (upper panel) and back side of the plate (lower panel) as compared to the uniform morphology of the control, uninfected isolate.

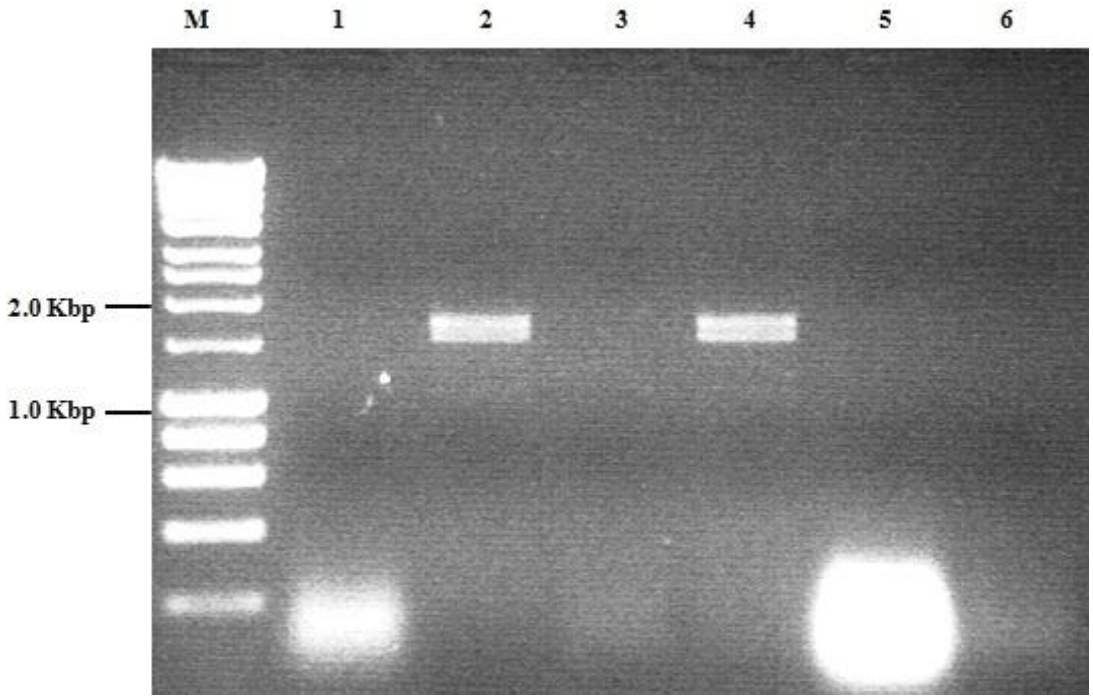


Figure 7.10 Agarose gel electrophoretic analysis of RNA isolated from purified AfuPV virions in transfected colonies of *A. fumigatus* isolate Af-237y. Lane M; Hyperladder 1 marker DNAs (Bioline); lane 1; RNA extract obtained from virus-free, *A. fumigatus* isolate Af-237y (negative control); lane 2; RNA isolated from purified virus derived from *A. fumigatus* isolate 88 (positive control); lanes 3, 4, 5 and 6; RNA derived from purified virus isolated from four potential Af-237y transfectants (colonies 5, 8, 11 and 14 respectively).

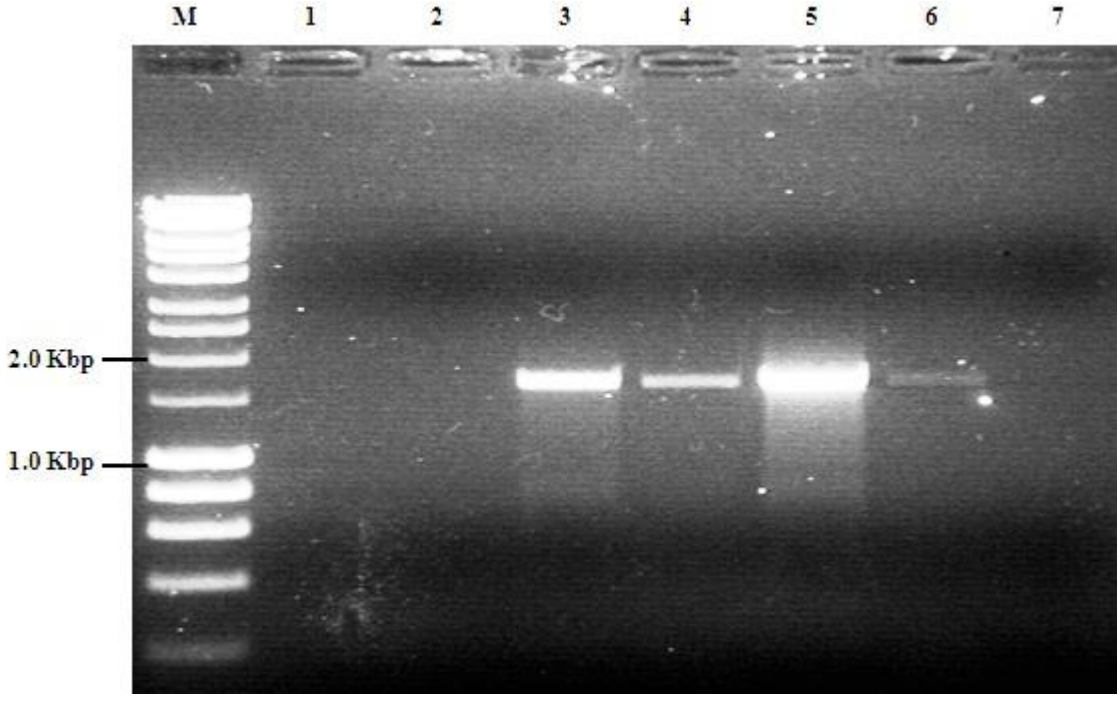


Figure 7.11 Transfection of *A. fumigatus* isolate Af-237y with AfuPV. Agarose gel electrophoretic analysis of amplicons generated by RT-PCR using oligonucleotide primers designed to produce an amplicon corresponding to the complete AfuPV dsRNA1 RdRP gene. Lane M; Hyperladder 1 marker DNAs (Bioline); lane 1; complete RT-PCR reaction with no RNA template (negative control); lane 2; RNA extract obtained from virus-free, *A. fumigatus* isolate Af-237y (negative control); lane 3; RNA isolated from purified virus derived from *A. fumigatus* isolate 88 (positive control); lanes 4, 5, 6 and 7; total RNA derived from four potential Af-237y transfectants (colonies 5, 8, 11 and 14 respectively).

7.1.2.3 Transfection of the *A. fumigatus* yellow isolate (Af-237y) with purified AfuPV dsRNA

Following the successful transfection of protoplasts isolated from *A. fumigatus* yellow isolate (Af-237y) with virions attempts were made to launch infection with AfuPV dsRNA using protoplasts in a similar procedure. These experiments were carried out with a simultaneous positive control reaction with purified AfuPV and a negative control with no virus or virus dsRNA. After infection protoplasts were washed and cultured on selective media (section 7.1.2.2; Fig. 7.12) at 37^{o}C, harvested and spores inoculated onto solid ACM plates and the phenotype of the resulting colonies observed and recorded (Fig. 7.13). As compared to virus-free examples, cultures with unusual phenotypes were found for all AfuPV inoculated colonies and in one out of ten colonies inoculated with AfuPV dsRNA following individual colony selection (Fig. 7.14; +dsRNA-C8).

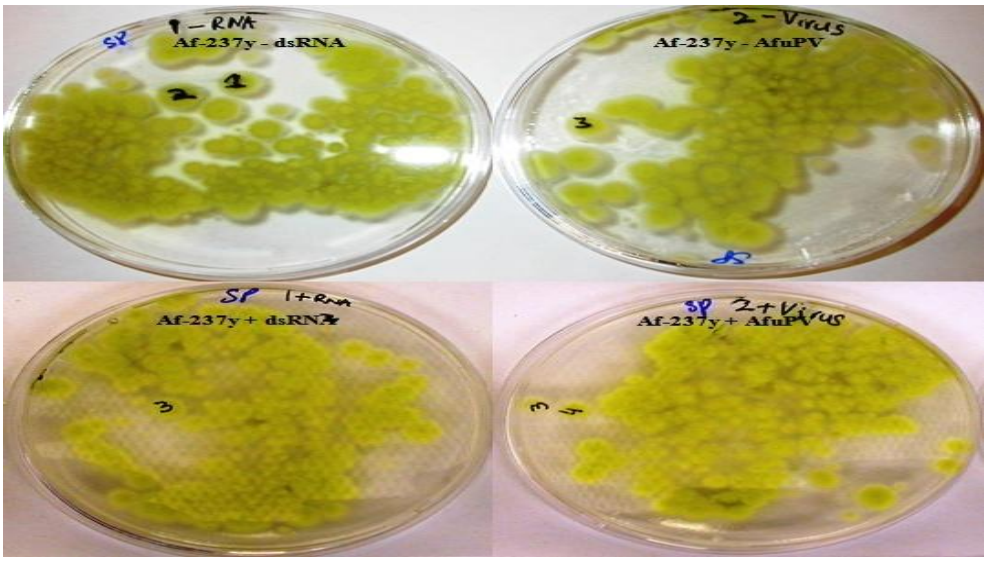


Figure 7.12 AfuPV and AfuPV dsRNA transfected colonies of Af-237y and a negative control (without AfuPV and dsRNA).

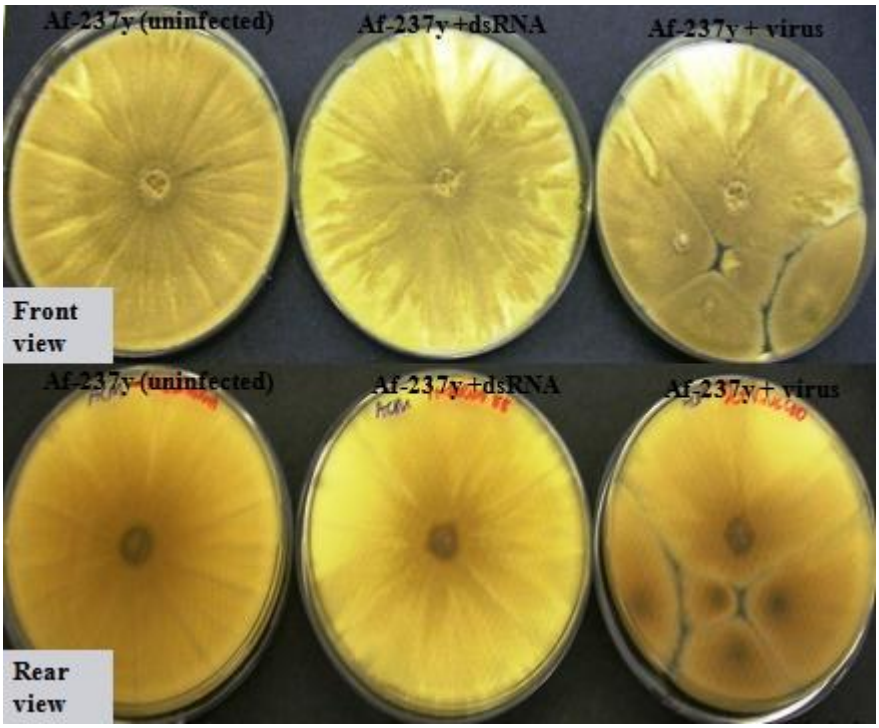


Figure 7.13 AfuPV and AfuPV dsRNA transfected colonies and negative uninfected Af-237y control.

Figure 7.14 Colony phenotypes of 10 selected, individual *A. fumigatus* isolate Af-237y colonies following transfection with AfuPV dsRNA. Control uninfected *A. fumigatus* isolate Af-237y colonies are on the left hand side in the top tier of plates.

7.1.2.4 Assessment of dsRNA transfection of *A.fumigatus* isolate Af-237y

Following virus purification from mycelia produced from +dsRNA-C8 (Fig. 7.14; section 2.12.1) and mycelia regenerated from protoplasts infected with AfuPV or no infectious agent, the dsRNA present in these extracts was examined by agarose gel electrophoresis and used as a template for RT-PCR with oligonucleotide primers (MF-28 and MF-29; Table 5.1) designed to produce an amplicon corresponding to the complete AfuPV RdRP ORF. These analyses revealed that transfection with purified virions of AfuPV is always successful but that transfection with AfuPV dsRNA is not possible. See results in Figs 7.15 and 7.16.

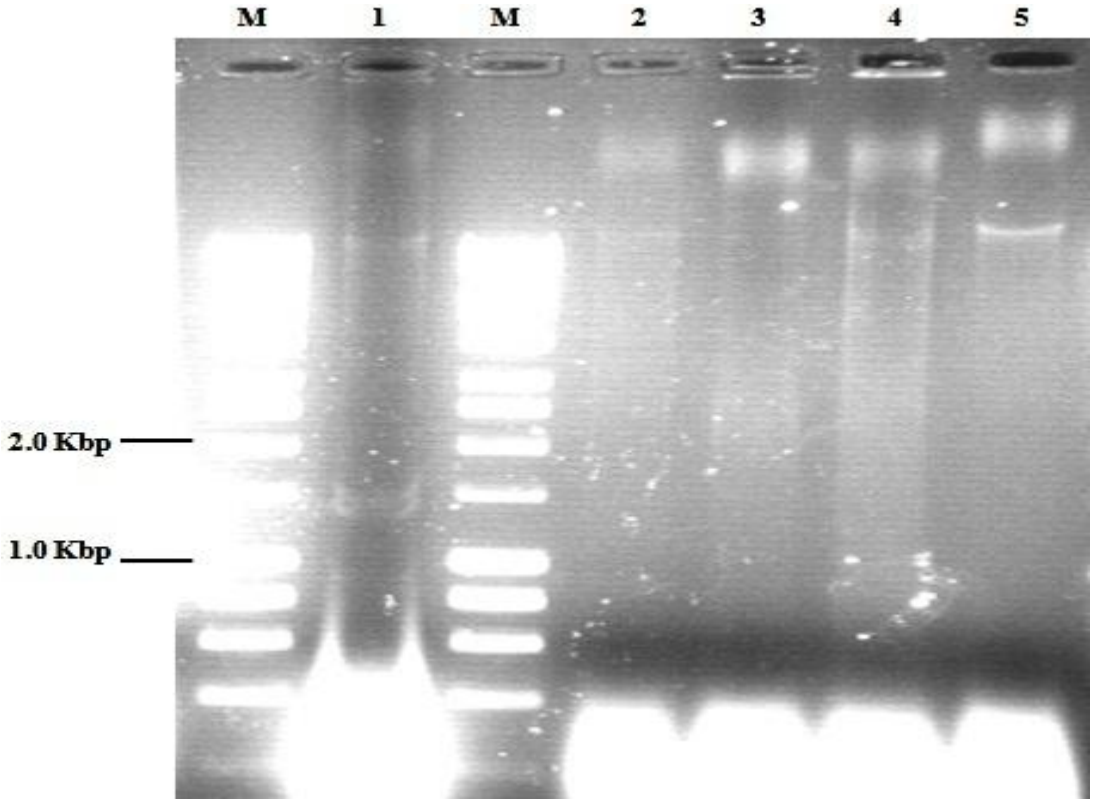


Figure 7.15 Agarose gel electrophoretic analysis of RNA isolated from purified AfuPV virions in transfected colonies of *A. fumigatus* isolate Af-237y. Lane M; Hyperladder 1 marker DNAs (Bioline); lane 1; RNA isolated from purified virus derived from *A. fumigatus* isolate 88 (positive control); lane 2; RNA isolated from virus-free, *A. fumigatus* isolate Af-237y (negative control); lane 3; RNA isolated from *A. fumigatus* isolate Af-237y transfected with AfuPV (positive control for transfection), lane 4; RNA isolated from AfuPV dsRNA transfected multicondial population (Af-237y+dsRNA; Fig 7.13), lane 5; RNA isolated from mycelia derived from single colony with sectored phenotype (+dsRNA-C8; Fig.7.14) following transfection with AfuPV dsRNA.

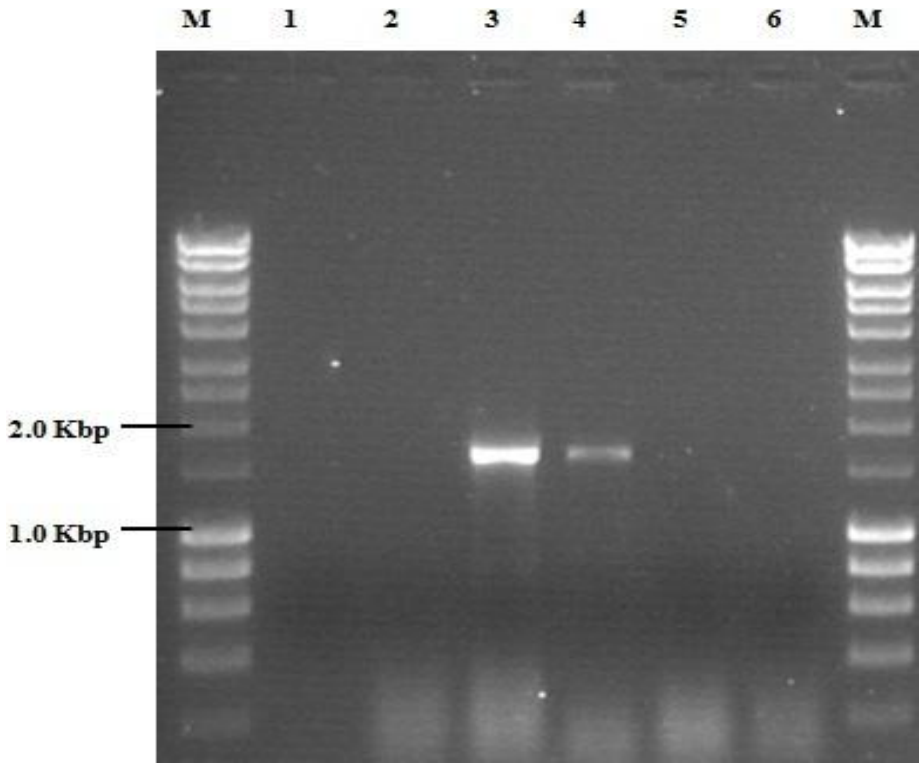


Figure 7.16 Transfection of *A. fumigatus* isolate Af-237y with AfuPV dsRNA. Agarose gel electrophoretic analysis of amplicons generated by RT-PCR using oligonucleotide primers designed to produce an amplicon corresponding to the complete AfuPV dsRNA1 RdRP gene. Lane M; Hyperladder 1 marker DNAs (Bioline); lane 1; complete RT-PCR reaction with no RNA template (negative control); lane 2; RNA extract obtained from virus-free, *A. fumigatus* isolate Af-237y (negative control); lane 3; RNA isolated from purified virus derived from *A. fumigatus* isolate 88 (positive control); lane 4; RNA extract from *A. fumigatus* isolate Af-237y mycelia transfected with AfuPV (positive control for transfection); lanes 5; RNA isolated from the multiconidial population of spores following transfection of *A. fumigatus* isolate Af-237y with AfuPV dsRNA(Af-237y+dsRNA; Fig 7.13); lane 6; RNA isolated from mycelia derived from the single colony with a sectored phenotype (+dsRNA-C8; Fig. 7.14).

7.2 Effects of partitivirus infection on mycelial growth, colony morphology and biomass of *Aspergillus fumigatus*

7.2.1 Mycelial growth rate and morphology of AfuPV-transfected and virus-free isogenic lines of *A. fumigatus* isolate Af-237y

There were significant differences in the colony morphology of both the starting strain, isolate Af-237y (virus-free strain) and a transfected strain (Af-237y-88=colony 8-see earlier; Fig 7.9) on both MM (Fig. 7.17) and ACM (Fig. 7.17) following 5 days incubation at 37^{o}C. Uniform yellow pigmentation was seen in the case of the virus-free strain on both MM and ACM without any sectoring. However, in Af-237y-88 a clear sectoring and reduced pigmentation was observed on both MM and ACM, which is evident from examining both sides of the plates and Colony expansion was faster for both strains on ACM as compared to MM because of the enriched nature of the former media.

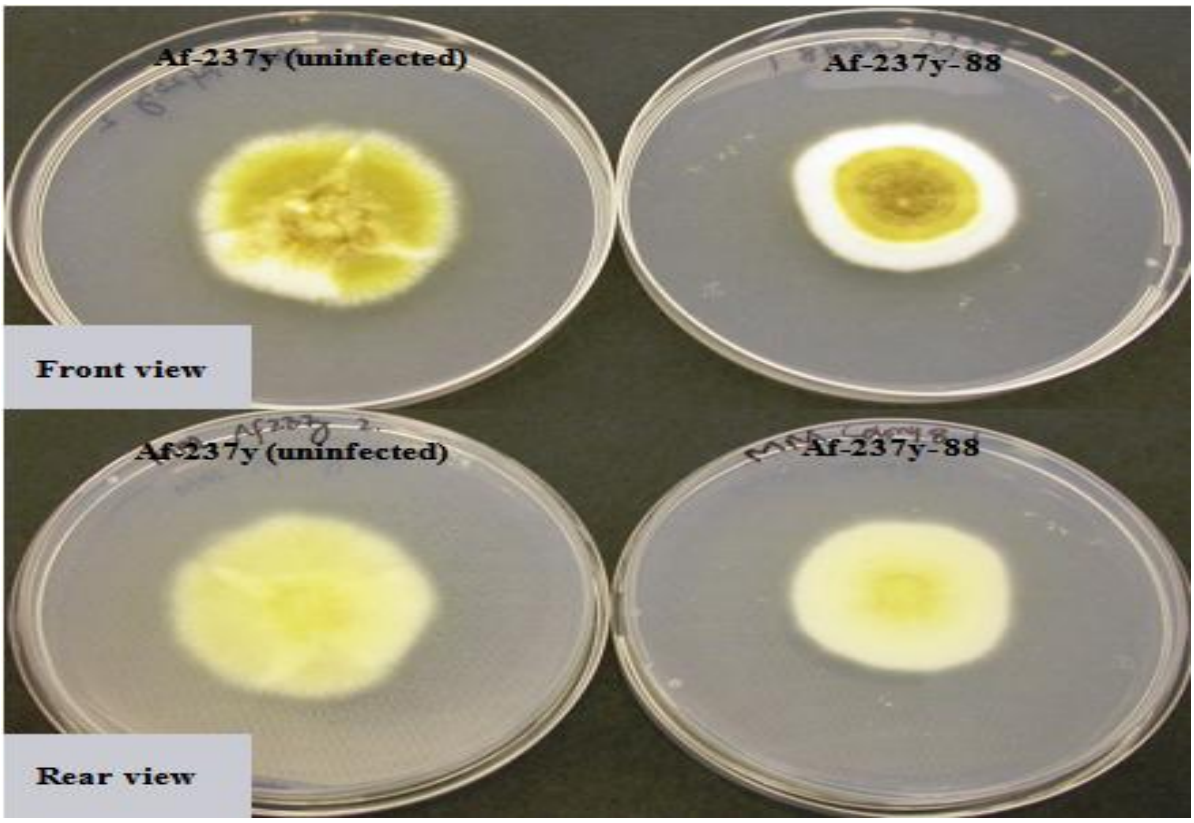


Figure 7.17 Colony morphologies of the virus-free, *A. fumigatus* Af-237y isolate and strain Af-237y-88 on MM plates following incubation at 37^{o}C for 5 days.

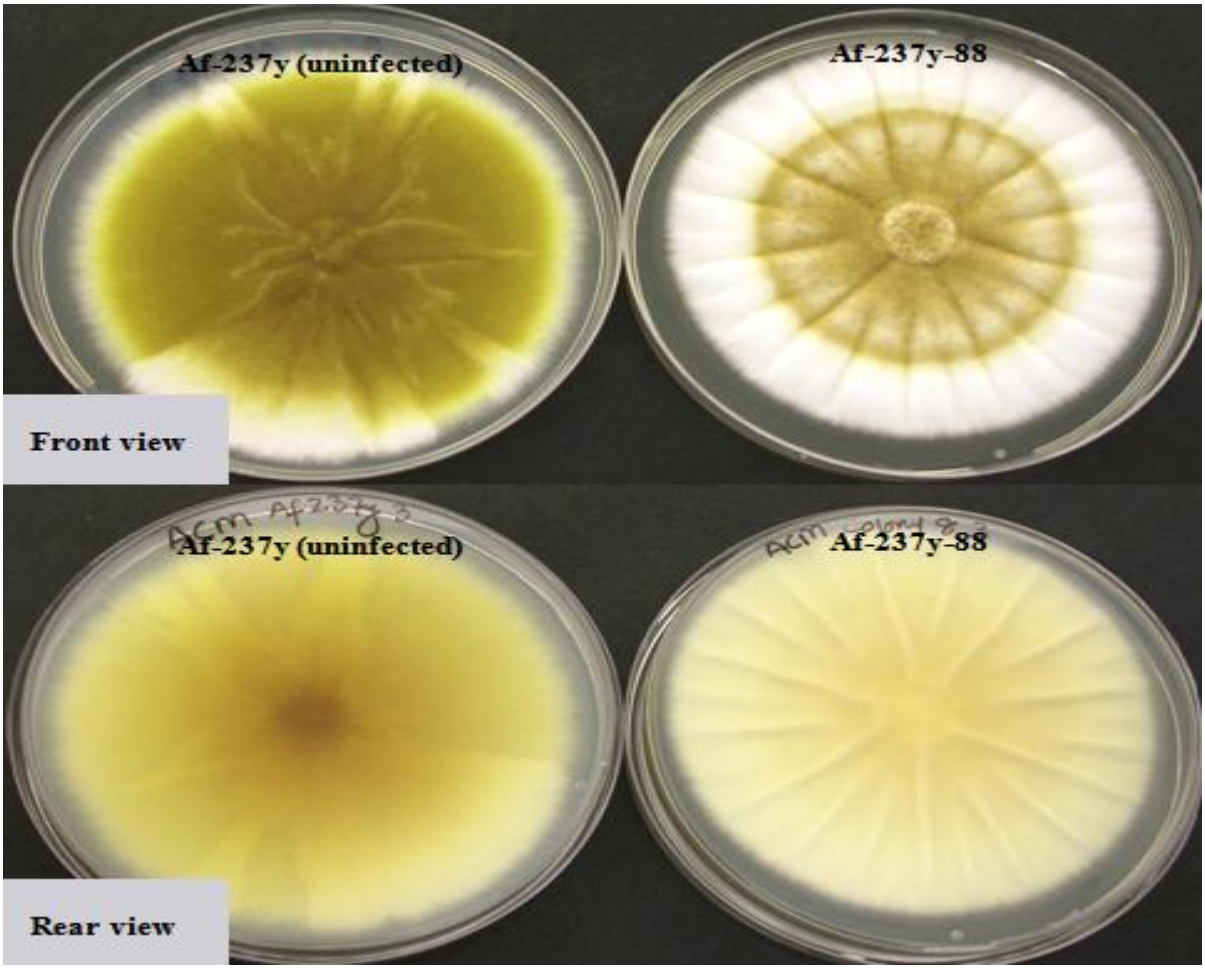


Figure 7.18 Colony morphologies of the virus-free, *A. fumigatus* Af-237y isolate and Af-237y-88 on ACM plates following incubation at 37^{o}C for 5 days. Sectoring and reduced sporulation can be seen from the front (upper panel) and back side of the plate of strain Af-237y-88 as compared to the uniform morphology of the control, virus-free, *A. fumigatus* Af-237y isolate.

7.2.1.1 Mycelial growth rate

The mycelia of the virus-free, *A. fumigatus* Af-237y isolate grew significantly faster and denser than strain Af-237y-88 on solid MM. This trend was observed from the first reading taken after 24 h and continued over the 5 day incubation period (Figs. 7.19 and 7.20). In neither case was mycelial growth confluent. Growth rate, as defined by increasing colony diameter, was measured over a 5 day incubation period for 3 replicate plates (Tables 7.1 and 7.2) the results which when presented as histograms (Figs. 7.21 and 7.22) which show significantly reduced growth of strain Af-237y-88. Similar results were obtained when the

experiments were carried out on ACM except that confluent growth was achieved by both strains after 4 days incubation.

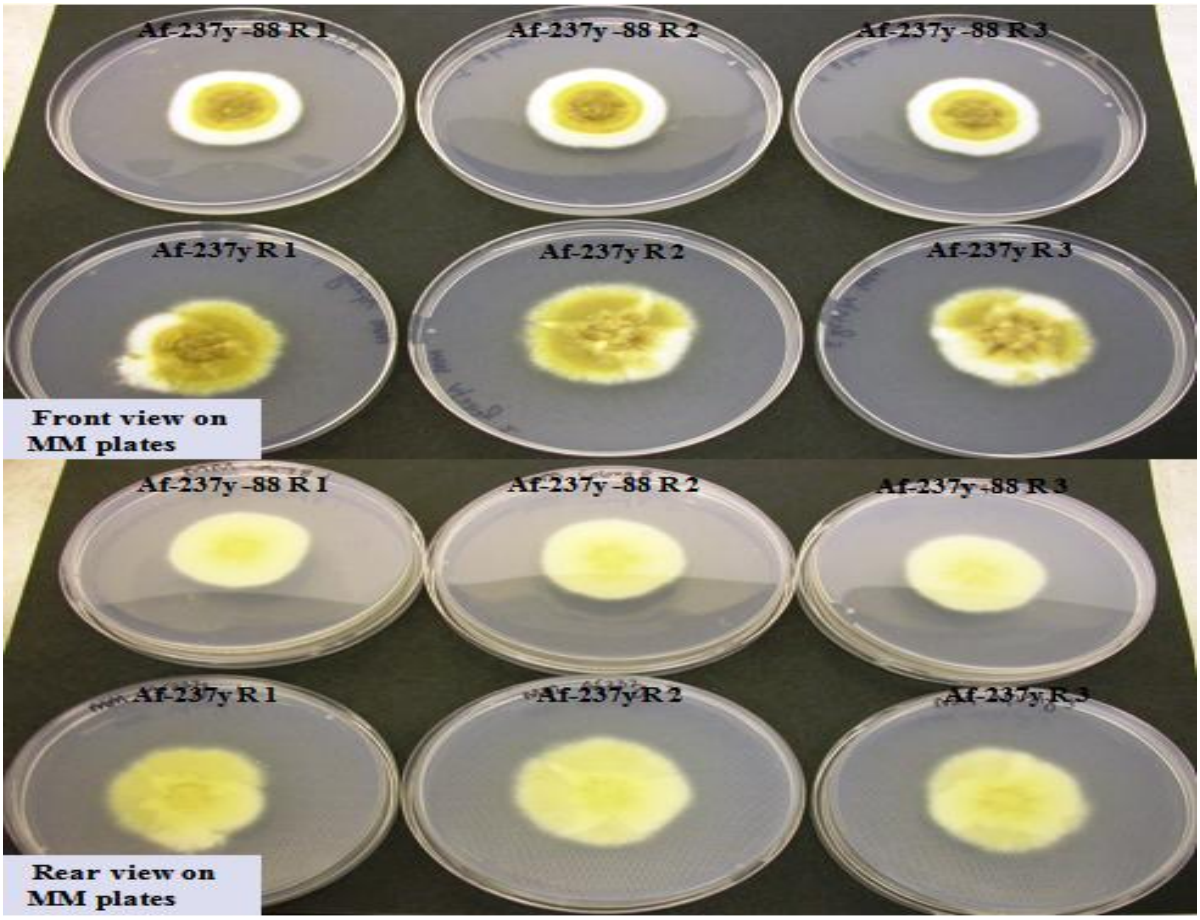


Figure 7.19 Comparison of radial growth of isogenic lines of the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 on MM plates following incubation at 37^{o}C for 5 days.

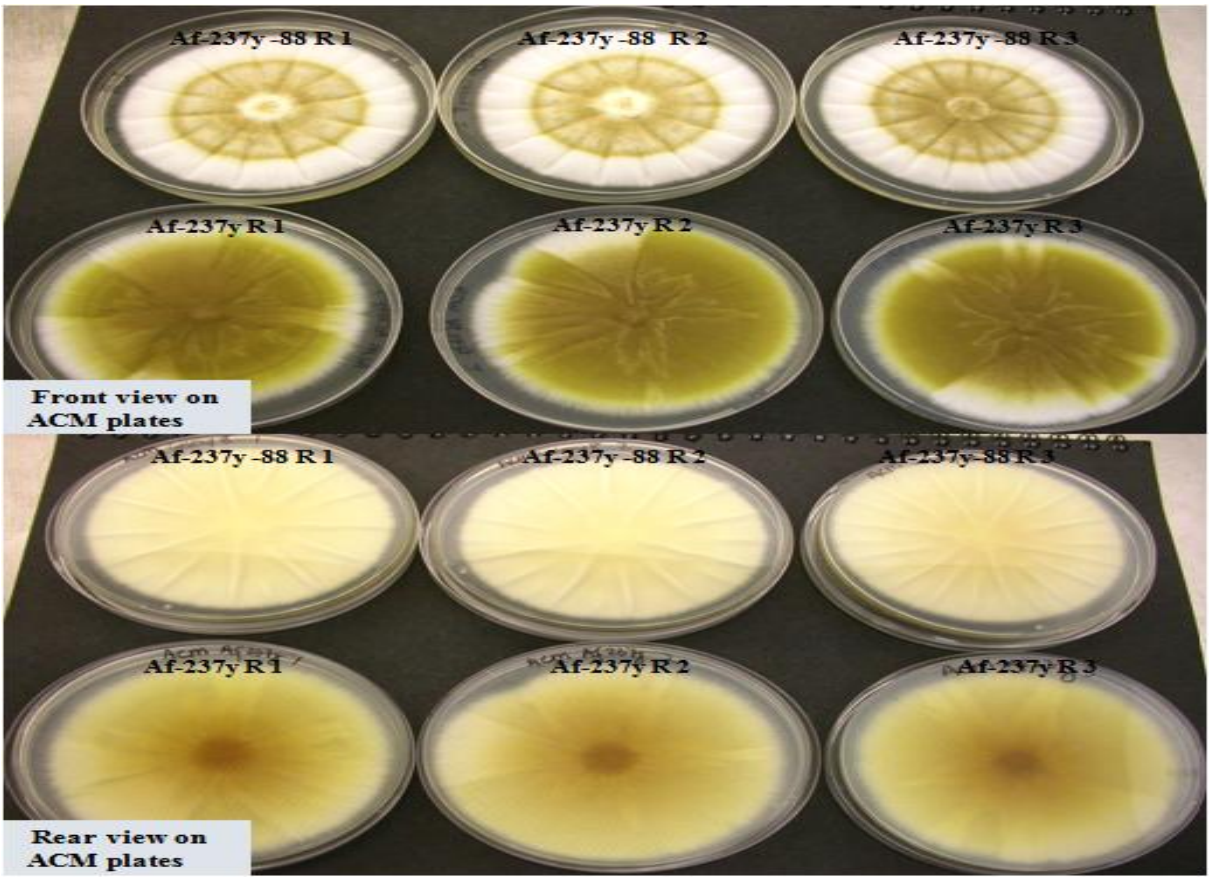


Figure 7.20 Comparison of radial growth of isogenic lines of the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 on ACM plates following incubation at 37^{o}C for 5 days.

Table 7.1 Colony diameters of the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 on MM plates measured over a 5 day incubation period. Measurements were analysed using the Student's t test and P values less than 0.01 indicate significant differences in growth between the two strains.

Colony diameter (cm) on solid MM						
	R	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Af237y	1	0.35	0.75	1.50	2.1	2.80
	2	0.35	0.80	1.60	2.2	2.90
	3	0.40	0.80	1.60	2.2	2.90
Mean± SE		0.366 ± 0.017	0.783 ± 0.017	1.566± 0.017	2.166 ± 0.033	2.866 ± 0.033
AfuPV transfected (Af-237y-88)	1	0.30	0.60	1.30	1.80	2.30
	2	0.25	0.55	1.25	1.75	2.20
	3	0.25	0.55	1.25	1.75	2.20
Mean ± SE		0.266± 0.017	0.566± 0.017	1.266± 0.017	1.766± 0.017	2.233± 0.033
t4		6.396	-	-	-	-
P		0.000	-	-	-	-

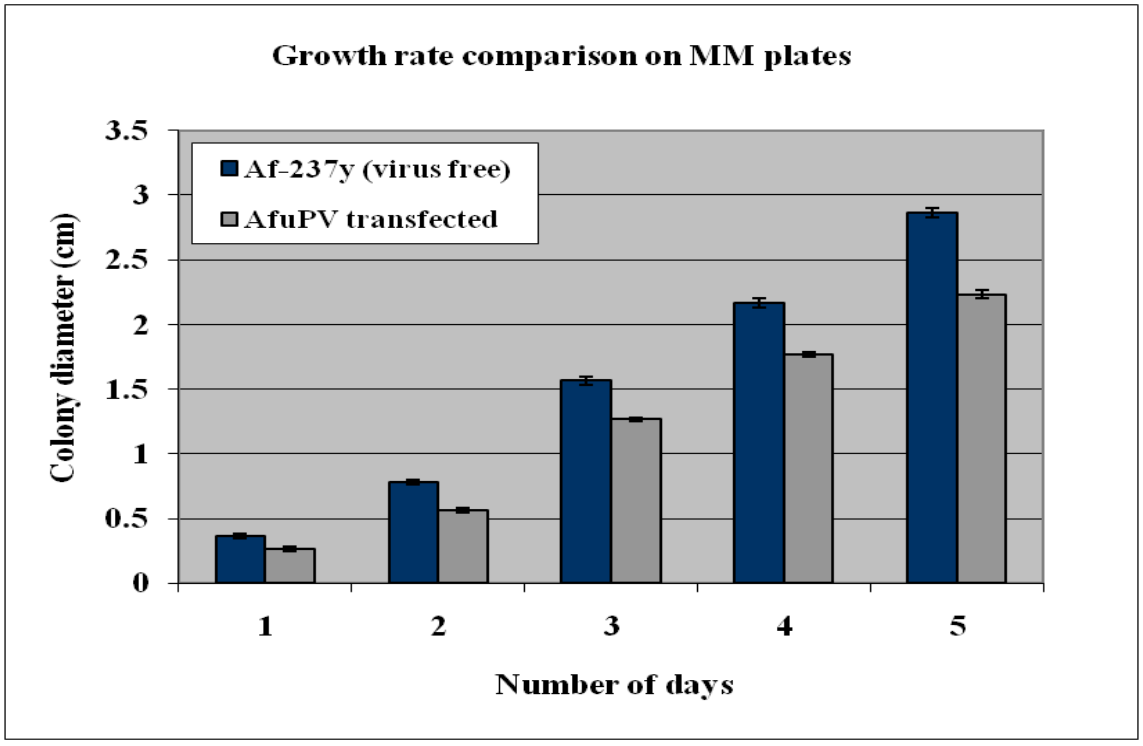


Figure 7.21 Histogram representation of the data presented in Table 7.1 showing a comparison of the growth rates of the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 on MM plates. Vertical bars represent standard deviation of the mean.

Table 7.2 Colony diameters of the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 on ACM plates. Measurements were analysed using the Student's t test and P values less than 0.01 indicate significant differences in growth between the two strains. No results were taken 5 days after incubation as confluent growth had been achieved by both strains.

Colony diameter (cm) on solid ACM						
	R	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Af237y (Virus free)	1	0.50	1.75	2.95	4.1	4.5
	2	0.50	1.75	2.90	4.0	4.5
	3	0.50	1.75	2.95	4.2	4.5
Mean± SE		0.50 ± 0	1.750 ± 0	2.933 ± 0.017	4.10± 0.058	4.50 ± 0
AfuPV transfected (Af237y-88)	1	0.50	1.60	2.75	3.8	4.5
	2	0.50	1.55	2.65	3.7	4.5
	3	0.50	1.50	2.70	3.75	4.5
Mean ± SE		0.50± 0	1.55± 0.029	2.70± 0.029	3.75± 0.290	4.50± 0
t4		4.115	-	-	-	-
P		0.001	-	-	-	-

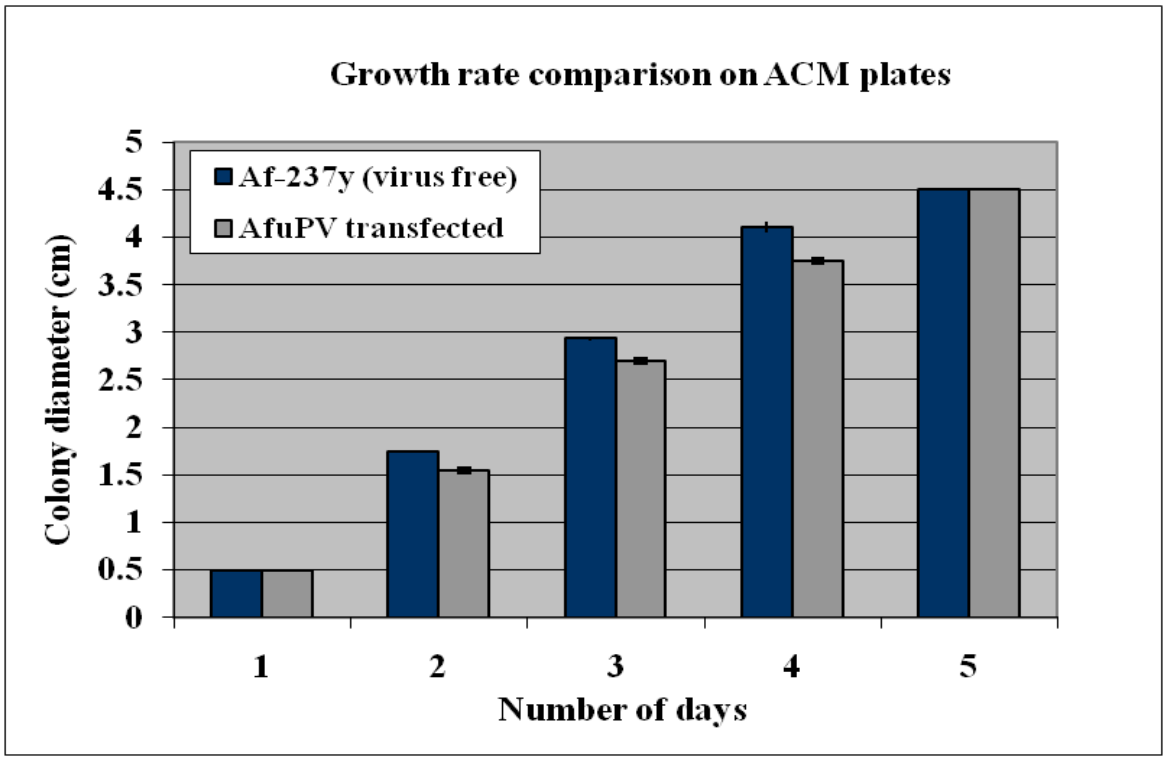


Figure 7.22 Histogram representation of the data presented in Table 7.2 showing a comparison of the growth rates of the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 on ACM plates. Vertical bars represent standard deviation of the mean.

7.2.2 Biomass production

The mycelial biomass produced by the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88 were measured following growth in both ACM and MM broth. In both cases growth was significantly impaired in the virus-infected isolate Af-237y-88 as compared to the virus-free, starting strain Af-237y (Figs. 7.23 and 7.24 and Tables 7.3 and 7.4).

Table 7.3 Biomass production over a 5 day incubation period in MM broth as assessed by dry weight of mycelia produced by the virus-free, *A. fumigatus* Af-237y isolate and Af-237y-88. The weights were analysed using the Student's t test and P values less than 0.01 indicate significant differences in biomass production between the two strains.

Mycelial weight (g) in MM broth						
	R	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Af237y (Virus free)	1	0.060	0.123	0.164	0.198	0.230
	2	0.047	0.134	0.170	0.201	0.222
	3	0.074	0.130	0.166	0.203	0.223
Mean ± SE		0.060± 0.008	0.129± 0.003	0.167± 0.002	0.200± 0.001	0.225± 0.002
AfuPV transfected (Af-237y-88)	1	0.030	0.080	0.138	0.170	0.191
	2	0.033	0.102	0.15	0.165	0.189
	3	0.040	0.110	0.144	0.168	0.193
Mean ± SE		0.034± 0.003	0.097± 0.009	0.144± 0.003	0.168± 0.001	0.191±0.001
t4		14.429	-	-	-	-
P		0.000	-	-	-	-

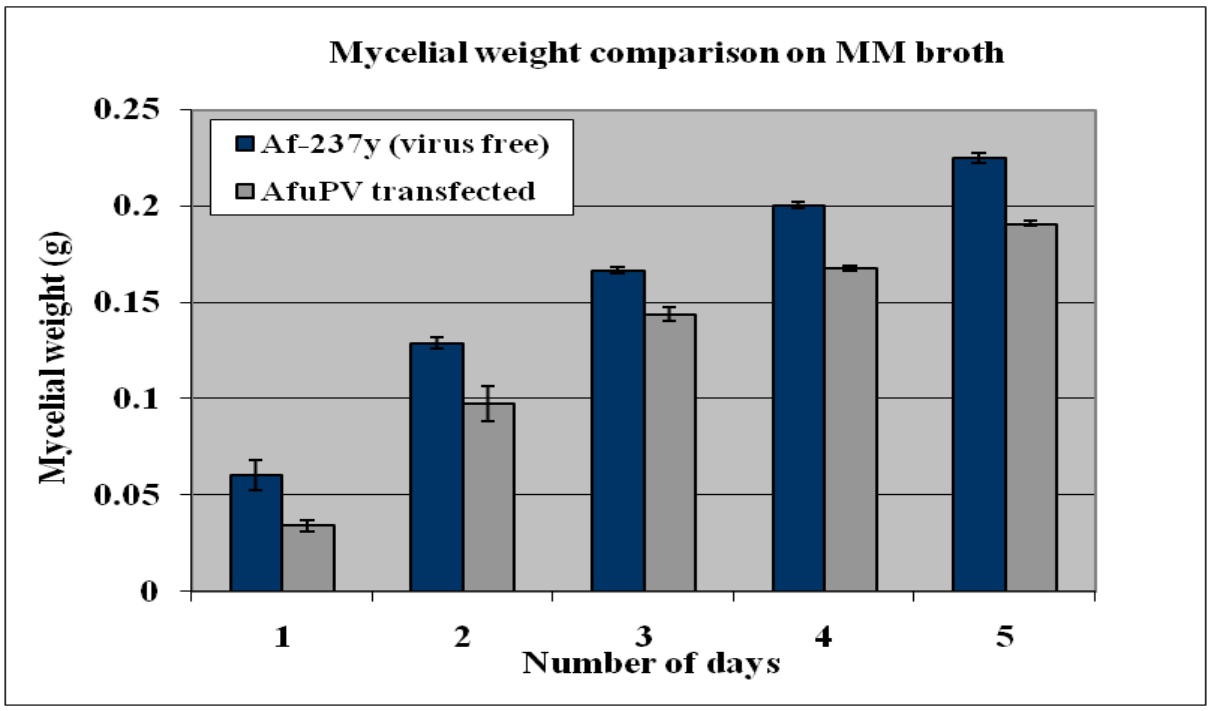


Figure 7.23 Histogram representation of the data presented in Table 7.3 showing a comparison of the biomass production in MM broth by the virus-free, *A. fumigatus* Af-237y isolate and the isolate Af-237y-88. Vertical bars represent standard errors of the mean.

Table 7.4 Biomass production over a 5 day incubation period in MM broth as assessed by dry weight of mycelia produced by the virus-free, *A. fumigatus* Af-237y isolate and isolate Af-237y-88. The weights were analysed using the Student's t test and P values less than 0.01 indicate significant differences in biomass production between the two strains.

Mycelial weight (g) in ACM broth						
	R	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Af237y (Virus free)	1	0.051	0.156	0.225	0.276	0.305
	2	0.051	0.180	0.236	0.253	0.310
	3	0.051	0.160	0.249	0.294	0.314
Mean ± SE		0.051± 0	0.165± 0.007	0.237± 0.007	0.274± 0.012	0.309± 0.003
AfuPV transfected (Af-237y -88)	1	0.041	0.085	0.143	0.214	0.266
	2	0.039	0.098	0.158	0.202	0.255
	3	0.048	0.090	0.190	0.230	0.260
Mean ± SE		0.043± 0.003	0.091± 0.004	0.167± 0.014	0.215± 0.008	0.260± 0.003
t4		7.895	-	-	-	-
P		0.000	-	-	-	-

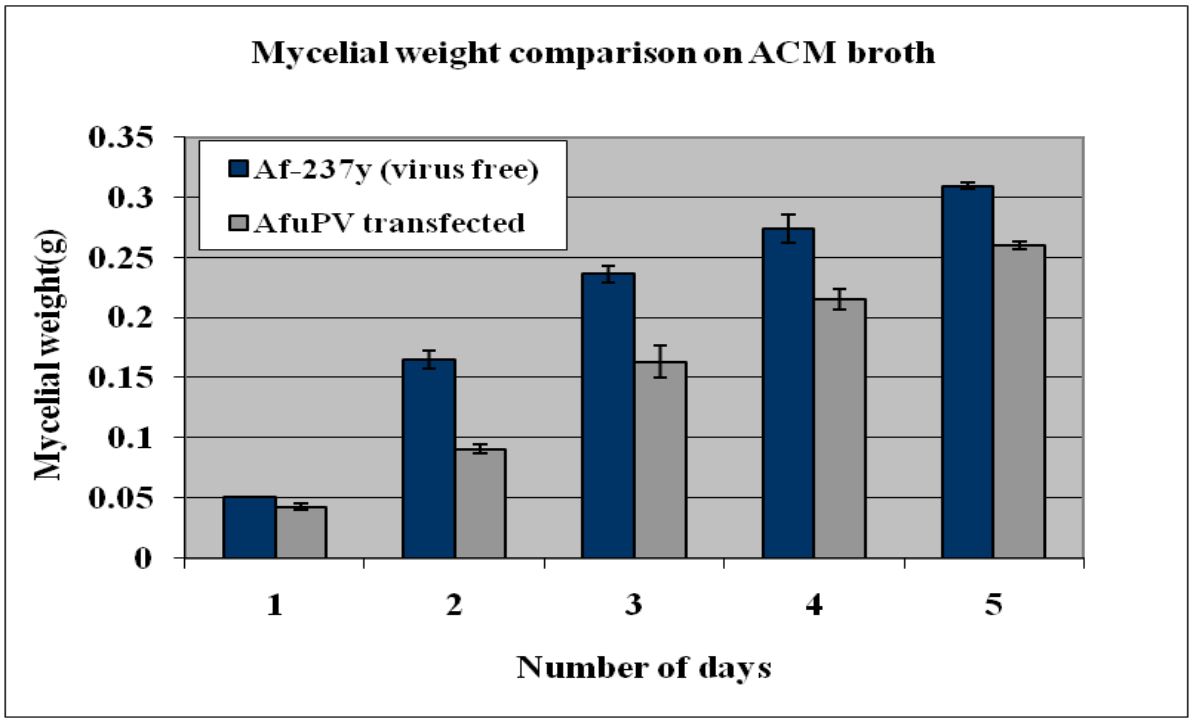


Figure 7.24 Histogram representations of the data presented in Table 7.4 showing a comparison of the biomass production in ACM broth of the virus-free, *A. fumigatus* Af-237y isolate and the isolate, Af-237y-88. Vertical bars represent standard errors of the mean.

7.3 Discussion

Transmission of mycoviruses occurs either horizontally by cell fusion between genetically vegetative compatible strains or vertically through cell division and conidia production (Buck 1998). Mycovirus transmission through asexual spores occurs most frequently however transmission rates vary greatly depending upon the virus and host strains (Ghabrial and Suzuki, 2008). Horizontal mycovirus transmission between strains of the same species is often hampered by vegetative incompatibility. Therefore, the development of inoculation methods is important to elucidate cause-effect relationships and potential utilisation of mycoviruses as biocontrol agents (Sasaki *et al.*, 2006).

For successful anastomosis to occur in fungi it is necessary that the interacting isolates are vegetatively compatible i.e. in the same vegetative compatibility group (VCG). In ascomycete fungi, this process is controlled by a series of gene loci, known as *vic* (vegetative incompatibility) or *het* (heterokaryon incompatibility) loci. Conversely the existence of

VCGs in many fungi provides a natural barrier to horizontal transmission of mycoviruses. If the two hyphae fuse that are vegetatively incompatible an incompatibility reaction follows involving isolation of the fusion cell by septal plugging followed by its vacuolisation, organelle degeneration and DNA fragmentation (Glass and Kaneko, 2003).

Interspecies transfer of mycoviruses in Aspergilli has only been reported using protoplast fusion (Van Diepeningen *et al.*, 1997; 1998) and dsRNA transfer *via* protoplast fusion is limited by vegetative incompatibility (Lhoas, 1970; Liang *et al*., 1983; Varga *et al.*, 1994; Van Diepeningen *et al.*, 1997). In the experiments reported here protoplast fusion was induced with PEG as originally reported for yeast (Das *et al.*, 1989) and modified for *Aspergillus* spp. (Van Diepeningen *et al.*, 1998; 2006).

Another approach is to transfect protoplasts with purified virions using PEG which has been successfully used for a mycoreovirus (Hillman *et al*., 2004) and a partitivirus of *Rosellinia necatrix* (Sasaki *et al*., 2006; Chiba *et al*., 2009). It is believed that particles of dsRNA viruses contain all the enzymatic activities necessary for transcription and the synthesis of viral mRNA, leading to the onset of infection (Chiba *et al*., 2009). The successful transfection of AfuPV into a virus-free, *A. fumigatus* strain is reported here. Attempts to launch mycovirus infections in fungi have utilised a number of procedures including the production of infectious cDNA clones or the use of *in vitro* synthesized transcripts derived from virus cDNA clones, both of which have been used successfully (Choi and Nuss, 1992; Chen *et al*., 1994).

The results obtained (sections 7.1.1, 7.1.2.1 and 7.1.2.2) illustrate successful attempts using different procedures to transfer three different *A. fumigatus* mycoviruses (see Chapter 3 for further details) to the yellow pigmented and hygromycin resistant *A. fumgatus* isolate Af-237y. These results confirm the tractability of transfer of the mycoviruses investigated between different isolates of *A. fumigatus*. In this study AfuCV from *A. fumigatus* isolate A-56 with a sectored phenotype was successfully transferred by all methods investigated which included hyphal anastomosis, protoplast fusion and direct transfection with purified virus. The uncharacterised *A. fumigatus* mycovirus dsRNAs were also successfully transferred by protoplast fusion whereas hyphal anastomosis was unsuccessful. However, the possibility that the transfer might have occurred should not be excluded. It is possible the dsRNA titre was too low to be visualised by ethidium bromide staining. Moreover, due to lack of any sequence

data for these dsRNAs it was not possible to design oligonucleotide primers for a sensitive assay like RT-PCR to diagnose virus infection. In further experiments transfection of Af-237y *A. fumigatus* protoplasts with AfuPV virions was achieved but was unsuccessful with AfuPV dsRNA. Infection of the Af-237y isolate of *A. fumigatus* with AfuPV resulted in a change in pigmentation and sectoring of the recipient strain. After selection of individual colonies from AfuPV trasnfected spores, isogenic lines were produced to investigate the effects that mycovirus infection had on the host fungus. These lines consisted of uninfected yellow isolate (starting strain; Af-237y) and AfuPV transfected isolate (Af-237y-88). The isogenic lines were compared on two different media (ACM and MM) and mycelial growth rate and biomass production were compared. The data obtained from both lines were analysed using Student's t-test. The results showed that the differences between the virus-infected and virus-free isolate of *A. fumigatus* were highly significant. The results suggested that AfuPV conferred hypovirulence to *A. fumigatus*. This result is dissimilar to that obtained for isogenic, virus-infected and virus-free lines of *A. niger,* where host fitness was impaired differentially in rich media (Van Diepeningen *et al*., 2006). Any hypovirulent effects of mycovirus infection with AfuPV in *A. fumigatus* were not apparently masked by an abundance of available nutrients in rich ACM as compared to MM.

It is quite unusual for partitiviruses to cause phenotypic changes and virulence attenuation in their hosts as they are normally associated with latent infections (Ghabrial *et al.*, 2005). However recent reports of the incidence of a partitivirus in the cultivated mushroom *Flammulina velutipes* associated with abnormally slow growth and the production of brown discoloured fruiting bodies (Magae and Sunagawa, 2010) are similar to the hypovirulence effects reported here for AfuPV, The *Rosellinia necatrix* isolate W8 also shows a hypovirulent phenotype and it produces irregular colony margins and slow growth on PDA (Sasaki *et al.*, 2005) confirming that partitiviruses can cause such effects.

7.4 Conclusions

The results obtained in this chapter demonstrate successful attempts to transfer *A. fumigatus* mycoviruses to the yellow pigmented and hygromycin resistant *A. fumgatus* isolate Af-237y using different procedures. However, protoplast fusion and transfection were the most efficient methods to transfer the *A. fumigatus* mycoviruses. In further experiments transfection of Af-237y *A. fumigatus* protoplasts with AfuPV virions was achieved but was

unsuccessful with AfuPV dsRNA. Infection of the Af-237y isolate of *A. fumigatus* with AfuPV resulted in a change in pigmentation and sectoring of the recipient strain. In order to investigate the effects of AfuPV infection on *A. fumigatus*, isogenic lines comprising the wild-type, virus-free, yellow isolate Af-237y and a virus-infected isolate Af-237y-88, regenerated from single-spores derived from Af-237y protoplasts transfected with purified AfuPV particles at an efficiency of >75% as determined by RT-PCR, were compared. AfuPV infection of Af-237y resulted in an abnormal phenotype on agar plates consisting of aconidial sectors and light pigmentation as compared to the virus-free isolate which was more pigmented and uniform. However several similar phenotypes were found in the AfuPV infected isogenic lines with decreased and variable amounts of pigmentation and sectoring appearing to be proportional to increased virus concentration, but this was not quantified (see Figs. 7.9 and 7.11). AfuPV infection significantly lowered the host fitness of *A. fumigatus* isolate Af-237y as determined by growth rate on solid media and hyphal biomass in broth.

Chapter 8

Conclusions and future work

8. Conclusions and future work

The current study has revealed 3 groups of mycoviruses that infect *A. fumigatus* which include a chrysovirus, a partitivirus and an uncharacterised dsRNA-containing virus. In current investigation 366 isolates (clinical and environmental) of *A. fumigatus* were screened for the presence of dsRNAs, 24 of which were found to contain dsRNA elements. In this screening of *A. fumigatus* isolates, mixed infections of these three viruses were also found. Representative strains of the chrysovirus and the partitivirus have been characterised and fully sequenced (Jamal *et al*., 2010; Bhatti *et al*., 2011a respectively). However, the most frequently occurring virus found in *A. fumigatus* is the uncharacterised dsRNA-containing virus. It would be a worthwhile study to characterise it so that the relationship of this virus can be studied in relation to origin and function of other known viruses. Random cloning and cDNA library construction techniques are being employed to obtain cDNA clones of this virus.

The successful characterisation of isolate 88 partitivirus (AfuPV) revealed the presence of a bipartite genome (Ghabrial *et al.*, 2008b; Bhatti *et al.*, 2011a). The larger segment, dsRNA-1, encodes an RdRP, whereas the smaller segment, dsRNA-2, encodes a CP. It was also revealed that the AfuPV genome, like other members of *Partitiviridae* family, possesses conserved motifs (III-VIII; Fig 5.4) and first two motifs are not well defined in the RdRP genome. The sequence also showed that 5' and 3' UTRs vary in length, however terminal conservation for both dsRNAs has been found. Phylogenetic analysis revealed that the RdRP gene of AfuPV is closely related to *Botryotinia fuckeliana* partitivirus-1 (BfPV-1) whilst the CP is closely related to *Discula destructiva* virus 1 (DdV-1).

In order to elucidate the effects of the partitivirus, attempts were made to completely cure the AfuPV infection which apparently has no effect on the health of its host. For this purpose a range of cycloheximide concentrations (0.1 mM- 150 mM) were used. The treatment with cycloheximide resulted in partially cured isolate (F-4) in which the smaller component of the AfuPV (CP; dsRNA2) was eradicated. However, presence of larger component (RdRP; dsRNA1) confirms the AfuPV infection was only partially cured. This investigation resulted in an interesting conclusion that replication of RdRP gene is not affected by eradication of CP. Following the failure of curing experiment successful efforts were made to transfer AfuPV into uninfected isolate (Af-237y) to elucidate the role of AfuPV under the same genetic background. Initially, different methods to transfer the virus were investigated to

broaden the possibilities to transfer *A.fumigatus* mycoviruses. The isogenic lines produced after successful transfection of AfuPV infection were further investigated in terms of biomass production and mycelia growth rate on two different media (MM and ACM). Results showed that the mycelia growth and biomass yield was significantly lower in the AfuPV transfected isolate in comparison with the uninfected yellow isolate (Af-237y). Less pigmentation and sectored morphology suggested that the transfected AfuPV infection is conferring a hypovirulent effects on the fitness of *A. fumigatus* isolate Af-237y.

Many dsRNA mycoviruses found during this study were apparently associated with symptomless infections but an increasing number have been identified that cause phenotypic changes and/or reduce the virulence in their fungal hosts which now includes AfuCV and AfuPV. Modulation of fungal gene expression and alteration of phenotypic traits as a consequence of mycovirus infections are little understood, with the exception of the chestnut blight fungus (*Cryphonectria parasitica*)/hypovirus system (Deng *et al*., 2007; Ghabrial and Suzuki, 2009).

Initial results obtained from AfuPV suggest that the AfuPV infection confers hypovirulence to the transfected host. Further experimentation in murine models will be essential to elucidate the role of AfuPV in pathogenicity. The hypovirulence found in AfuPV can be used as a potential source of biological control to control *A. fumigatus* infection. It would be of great interest to broaden the host range of mycoviruses so as to utilise their potential as a biological control agent not only for controlling opportunistic human pathogen such as *Aspergillus fumigatus* but also other pathogenic fungi. The information regarding host range mycoviruses is inadequate because of lack of information about the suitable inoculation procedures. It would be of great significance to make use of these viruses and use them as therapeutic agents against human and plant pathogenic fungi. There is evidence to support the idea of the transfer of mycovirus between related and non related fungi. The most effective method used so far includes protoplast fusion and protoplast transfection. Both these methods are considered to be a very important tools for transferring mycoviruses intra-specifically and inter-specific fungal isolates as it overcomes the main obstacle (vegetative incompatibility) in their transmission.

Transfer of mycoviruses between *A. niger and A. nidulans via* protoplast fusion has been successfully reported (van Diepeningen *et al*., 1998) and transmission of *Fusarium boothi* dsRNA virus to other *Fusarium* species and into *Chryphonectria paracitica* were not only

successful but also attenuated the fungi (Lee *et al.,* 2011). Transmission of mycoviruses through transfection approach is a new route to transfer mycoviruses which has been successfully reported in *A. fumigatus* (Bhatti *et al.,* 2011a) where a novel partitivirus of isolate 88 to an uninfected isolate generated a hypovirulent response. The hypovirulence symptoms induced after mycovirus suggest that transferring mycoviruses between related and non related species is very flexible and suggests that there is no adaptation between a particular host and mycovirus (van Diepeningen *et al*., 2006). Successful transfection has also been reported for somatically incompatible fungal isolates with purified virus particles of two mycoviruses, the partitivirus and the mycoreovirus from the white root rot fungus *Rosellinia necatrix.* Their experimental host range was also extended with in subclasses of class Sordariomycetes (Kanematsu *et al.,* 2010). Thus, finding these novel mycoviruses associated with hypovirulence in the *A. fumigatus* and finding the way to transfer to uninfected isolates *via* protoplast transfection has directed this investigation towards more interesting future research. Extension of host range of these mycoviruses will be of great importance for utilizing the potential of these novel mycoviruses as a biological control agent against *A. fumigatus* and other pathogenic fungi.

A detailed study should also be conducted in virus-infected isolates to search for transposons or other elements, which can be used in research for vaccine development against aspergillosis. There is an urgent need to expand the host range of dsRNA mycoviruses. Mycoviruses lack extracellular modes of transmission and genetic manipulation/modification of those structures involved in dsRNA mycoviruses binding to the target fungus may be done so that a chimeric virus may be generated that is able to selectively infect fungi *via* an extracellular mode of transmission.

The mechanism of RNA silencing (antiviral defense mechanism) is poorly understood in mycoviruses except in *C. parastica* where, CHV-1 infection can confer attenuation to its host (*C. parastica*). It is hypothesised that the down-regulation and variation in phenotype are due to dsRNA interactions with fungal transcription mechanisms. These mechanisms include the potential generation of siRNAs cleaved from mycovirus dsRNAs which may play a regulatory role in gene expression. It is also considered that the accumulation of low levels of *A. niger* virus-derived siRNA have been detected previously by northern blotting (Hammond *et al*., 2008) and it will be very interesting to investigate similar aspects within *A. fumigatus* mycoviruses, described above, by comparing the isogenic lines (virus-infected and

virus-free) and dectecting the levels of siRNAs to better understand the RNA silencing mechanism in *A. fumigatus*.

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9. References

Ahn, I. P. and Lee, Y. H., (2001). A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. *Mol. Plant. Micro. Int.,* 14, 496-507.

Anderson, M. J., Brookman, J. L. and Denning, D. W., (2003). In *Genomics of Plants and Fungi* (eds Prade, R.A. and Bohnert, B.J.) pp. 1-39. Marcel Dekker Inc. N.Y., U.S.A.

Aoki, N., Moriyama, H., Kodama, M., Arie, T., Teraoka, T. and Fukuhara, T., (2009). A novel mycovirus associated with four double-stranded RNAs affects host fungal growth in *Alternaria alternata*. *Virus Res*., 140, 179-187.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J., (1990). Basic local alignment search tool. *J. Mol. Biol*., 215, 403-410.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25, 3389–3402.

Banks, G. T., Buck, K. W., Chain, E. B., Himmelweit, F., Marks, J. E., Tyler, J. M., Hollings, M., Last, F. T. and Stone, O. M., (1968). Viruses in fungi and interferon stimulation. *Nature*, (London) 218, 542-545.

Banks, G. T., Buck, K. W., Chain, E. B., Darbyshire, J. E., Himmelweit, F., Ratti, G., Sharpe, T. J. and Planterose, D. N., (1970). Antiviral activity of double stranded RNA from a virus isolated from *Aspergillus foetidus. Nature,* 227, 505-507.

Blawid, R., Stephan, D. and Maiss, E., (2008). *Alphacryptovirus and Betacryptovirus* : In *Encylopedia of Virology* 3rd edn.. Mahy, B. W. J. and Van Regenmortel, M. H. V. (eds.), (Elsevier Academic Press). pp, 98-104.

Bennett, J. W., (2010). An overview of the genus *Aspergillus*. In: *Aspergillus* molecular biology and genomics. (eds.) Machida, M. and Gomi, K. Caister Academic Press. UK. pp, 1-17.

Bhatti, M. F., Bignell, E. M. and Coutts, R. H. A., (2011a). Complete nucleotide sequences of two dsRNAs associated with a new partitivirus infecting *Aspergillus fumigatus*. *Arch. Virol.,* 156, 1677-1680.

Bhatti, M. F., Jamal, A., Petrou, M. A, Cairns, T. C., Bignell, E. M. and Coutts, R.H.A., (2011b). The effects of dsRNA mycoviruses on growth and murine virulence of *Aspergillus fumigatus*. *Fungal Genet. Biol.* (in press).

Bodey, G. P. and Vartivarian, S., (1989). Aspergillosis. *Eur. J. Clin. Microbiol. Infect. Dis*., 8, 413-437.

Border, D. J., Buck, K. W., Chain, E. B., Kempson-Jones, G. F., Lhoas, P. and Ratti, G., (1972). Viruses of *Penicillium* and *Aspergillus* species. *Biochem. J.,* 127, 4-6.

Bozarth, R. F., (1972). Mycoviruses: A new dimension in microbiology. *Environ. Health Perspect.,* 2, 23-39.

Brooke, A. G., (1994). Industrial fermentation and *Aspergillus* citric acid. In: The genus *Aspergillus*: from taxonomy and genetics to industrial application, Powell, K.A., Renwick, A., and Peberdy, R.F. (eds.) (New York: Plenum Press), pp, 122-134.

Bruenn, J. A., (1993). A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. *Nuc. Acids Res*., 21, 5667-5669.

Buck, K. W., (1978). Semi-conservative replication of double-stranded RNA by a virion-associated RNA polymerase. *Biochem. Biophys. Res. Commun*., 84, 639-645.

Buck, K. W., (1986). Fungal virology-an overview. In *Fungal Virology* (ed Buck, K. W.,), pp. 1-84, CRC Press, Boca Raton, USA.

Buck, K. W., (1988). From interferon induction to fungal viruses. *Eur. J. Epidemiol*., 4, 395-399.

Buck, K. W., (1996). Comparison of the replication of positive-stranded RNA viruses of plants and animals. *Adv. Virus Res.* 47, 159-251.

Buck, K. W., (1998). Molecular variability of viruses of fungi. In *Molecular variability of fungal pathogens,* (eds Bridge, P. D., Couteaudier, Y. and Clarkson, J. M.). pp. 53-72, Wallingford, UK: CAB International.

Buck, K. W. and Ratti, G., (1975). Biophysical and biochemical properties of two viruses isolated from *Aspergillus foetidus*. *J. Gen. Virol*., 27, 211-224.

Buck, K. W., Girvan, R. F., Ratti, G., (1973). Two serologically distinct double-stranded ribonucleic acid viruses isolated from *Aspergillus niger*. *Biochem. Soc. Trans.*, 1, 1138-1140.

Cansado, J., Longo, E., Agrelo, D. and Villa, T. G., (1989). Curing of the killer character of *Saccharomyces cerevisiae* with acridine orange. *FEMS Microbiol. Lett.*, 65, 233-237.

Caston, R. J., Trus, B. L., Booy, F. P., Wickner, R. B., Wall, J. S. and Steven, A. C., (1997). Structure of L-A virus: a specialized compartment for the transcription and replication of double-stranded RNA, *J. Cell Biol.* 138, 975-985.

Caston, J., Ghabrial, S., Jiang, D., Rivas, G., Alfonson, C., Roca, R., Luque, D. and Carrascosa, J., (2003). Three-dimensional structure of *Penicillium chrysogenum* virus: a double-stranded RNA virus with a genuine T=1 capsid. *J. Mol. Biol*., 331, 417-431.

Chazalet, V., Debeaupuis, J. P., Sarfati, J., Lortholary, J., Ribaud, P., Shah, P., Cornet, M. Vu Thien, H., Gluckman E., Brucker, G., and Latge, J. P., (1998). Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. *J. Clin. Microbiol*., 36, 1494-1500.

Chen, B., Choi, G. H. and Nuss, D. L., (1994). Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science*, 264, 1762-1764.

Cheng, J., Jiang, D., Fu, Y., Li, G., Peng, Y. and Ghabrial, S. A., (2003). Molecular characterization of a dsRNA totivirus infecting the sclerotial parasite *Coniothyrium minitans*. *Virus Res*., 93, 41-50.

Chiba, S., Salaipeth, L., Lin, Yu-H., Sasaki, A., Kanematsu, S. and Suzuki, N., (2009). A novel bipartite double-stranded RNA mycovirus from the white root rot fungus *Rosellinia necatrix*: molecular and biological characterization, taxonomic considerations, and potential for biological control. *J. Virol*., 83, 12801-12812.

Choi, G. H. and Nuss, D. L., (1992). Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science,* 257, 800-803.

Chung, C. T., Suzanne, L. N. and Roger, H. M., (1989). One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci*. USA., 86, 2172-2175.

Coenen, A., Kevel, F. and Hoekstra, R. F., (1997). Factors affecting the spread of double stranded RNA viruses in *Aspergillus nidulans*. *Gen. Res.*, 69, 1-10.

Coffin, R. S. and Coutts, R. H. A., (1992). DsRNA cloning and diagnosis of beet pseudo-yellows virus by PCR and nucleic acid hybridization. *Intervirology,* 33, 197-203.

Coutts, R. H. A. and Livieratos, I. (2003). A rapid method for sequencing the 5' and 3'-termini of double-stranded RNA viral templates using RLM-RACE. *J. Phytopathology,* 151, 525-527.

Coutts, R. H. A., Covelli, L., Di Serio, F., Citir, A., Acikgoz, S., Hernandez, C., Ragozzino, A. and Flores, R., (2004). Cherry chlorotic rusty spot and Amasya cherry diseases are associated with a complex pattern of mycoviral-like double-stranded RNAs. II. Characterization of a new species in the genus *Partitivirus*. *J. Gen. Virol*., 85, 3399-3403.

Crawford, L. J., Osman, T. A. M., Booy, F. P., Coutts, R. H. A., Brasier, C. M. and Buck, K. W., (2006). Molecular characterization of a partitivirus from *Ophiostoma himal-ulmi*, *Virus Genes,* 33, 33-39.

Curie, J. N., (1917). The citric acid fermentation of *Aspergillus niger*. *J. Biol. Chem*., 31, 15-37.

Dalzoto, P. R., Blanco, C. G., Cordeiro, V. K., Ribeiro, J. Z., Kitajima, E. W. and Azevedo, J. L., (2006). Horizontal transfer and hypovirulence associated with double-stranded RNA in *Beauveria bassiana*. *Mycol. Res*., 110, 1475-1481.

Das, A., Gokhale, D. V. and Peberdy, J. F., (1989). Protoplast fusion and genetic recombination in intra- and inter isolate crossing in *Aspergillus niger*. *Enzyme Microb. Technol.*, 11; 2-5.

Dasbach, E. J., Davies, G. M., and Teutsch, S. M., (2000). Burden of aspergillosis-related hospitalizations in the United States. *Clin. Infect. Dis.*, 31, 1524-1528.

De Guido, M. A., Minafra, A., Santomauro, A. *and* Faretra, F., (2007). Molecular characterization of mycoviruses in *Botryotinia fuckeliana. XIV International Botrytis Symposium, Cape Town [abstract]*.

Deng, F., Allen, T. D., Hillman, B. I., Nuss, D. L., (2007). Comparative analysis of alterations in host phenotype and transcript accumulation following hypovirus and mycoreovirus infections in the chestnut blight fungus. *Eukaryotic Cell.* 6, 1286-1298.

Denning, D. W., (1998). Invasive aspergillosis. *Clin. Infect. Dis.* 26, 781-803.

Derouin, F., (1994). Special issue on aspergillosis. *Pathol. Biol*., 42, 625-736.

Diaz-Ruiz, J. and Kaper, J., (1978). Isolation of viral double-stranded RNAs using a LiCl fractionation procedure. *Prep. Biochem*., 8, 1-17.

Dieleman-van Zaayen, A., (1967). Virus-like particles in a weed mould growing on mushroom trays. *Nature*, 216, 595-96.

Dryden, K. A., Wang, G., Yeager, M., Nibert, M.L., Coombs, K. M., Furlong, D. B., Fields, B. N. and Baker, T. S., (1993) Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J. Cell Biol.* 122, 1023-1041.

Elias, K. S., Cotty, P. J., (1996). Incidence and stability of infection by double-stranded RNA genetic elements in *Aspergillus* section *Flavi* and effects on aflatoxigenecity. *Can. J. Bot.*, 74, 716-725.

Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U. & Ball, L. A., (2005). *Virus Taxonomy, VIIIth Report of the ICTV*. Elsevier/Academic Press, London.

Feinberg, A. P. and Vogelstein, B., (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem*., 137, 266–267.

Fink, G. R. and Styles, C. A., (1972). Curing of a killer factor in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*., 69, 2846-2849.

Fukuhara, T., Moriyama, H., Pak, J. K., Hyakutake, T. and Nitta, T., (1993). Enigmatic double-stranded RNA in Japonica rice. *Plant Mol. Biol.*, 21, 1121-1130.

Fulbright, D. W., (1984). Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. *Phytopathology***,** 74**,** 722–724.

Frisvad, J. C., Rank, C., Nielsen, K. F. and Larsen, T. O., (2008). Metabolomics of *Aspergillus fumigatus. Med. Mycol.*, 1, 1-19.

Ghabrial, S. A., (1980). Effects of fungal viruses on their hosts. *Ann. Rev. Phytopathol.*, 18, 441-61.

Ghabrial, S. A., (1994). New developments in fungal virology. *Adv. Virus Res*., 43, 303-388.

Ghabrial, S. A., (1998). Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes*, 16**,** 119-131.

Ghabrial, S. A., (2001). Fungal viruses. In *Encyclopaedia of plant pathology*, (eds O. Maloy and T. Murray) pp. 478-483. New York Wiley.

Ghabrial, S. A., (2008). Chrysoviruses: In *Encyclopedia of Virology* 3rd edn. Mahy, B. W. J. and Van Regenmortel, M. H. V. (eds.), (Elsevier Academic Press, the Netherlands) pp, 503-513.

Ghabrial, S. A, and Hillman, B. I., (1999). Partitiviruses-fungal (*Partitiviridae)*. In: Encyclopedia of Virology, 2nd Edn., Granoff, A. and Webster R.G. (eds.), Elsevier/ Academic Press, San Diego. pp. 1477–1151.

Ghabrial, S. A. and Suzuki, N., (2008). Fungal viruses; In *Encylopedia of Virology* 3rd edn.. Mahy, B. W. J. and Van Regenmortel, M. H. V. (eds.), (Oxford: Elsevier): pp, 284–291.

Ghabrial, S. A. and Suzuki, N., (2009). Viruses of plant pathogenic fungi. *Ann. Rev. Phytopath.*, 47, 53-384.

Ghabrial, S. A., Jiang, D. and Caston, J. R., (2005). *Chrysoviridae*. In: *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*. Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U. and Ball, L. A. (eds.), Elsevier Academic Press, San Diego, pp, 591-595.

Ghabrial, S. A., Ochoa, W. F., Baker, T.S. and Nibert, M. L., (2008a). Partitiviruses: In *Encylopedia of Virology* 3rd edn.. Mahy, B. W. J. and Van Regenmortel, M. H. V. (eds.), (Elsevier Academic Press, the Netherlands). pp, 68-75.

Ghabrial, S. A., Ochoa, W. F., Baker, T. S. and Nibert, M. L., (2008b). Partitiviruses: general features. In: Mahy B. W. J, van Regenmortel M. H. V. (eds) *Encyclopedia of Virology, third edition*. Elsevier/Academic Press, London, pp 503-513.

Gitlin, L., Andino, R., (2003). Nucleic acid-based immune system: the antiviral potential of mammalian RNA silencing. *J. Virol*., 77, 7159-7165.

Glass, N. L. and Kaneko, I., (2003). Fatal attraction: Nonself recognition and heterokaryon incompatibility in filamentous fungi. *Eukaryot. Cell*, 2, 1-8.

Go, S. J., Cha, D. Y. and Wessels, J. G. H., (1992). Symptoms of virus infected oyster mushrooms, *Pleurotus florida*. *Kor. J. Mycol*., 20, 229-233.

Goldbach, R., Bucher, E. and Prins, M., (2003). Resistance mechanisms to plant viruses: an overview. *Virus Res*., 92, 207-212.

Golubev, W, I., Pfeiffer, I., Churkina, L. G. and Golubeva, E. W., (2003). Double-stranded RNA viruses in a mycocinogenic strain of *Cystofilobasidium infirmominiatum*. *FEMS Yeast Res*., 3, 63-68.

Gorman, C. M., Fuller, H. T. and Dyer, P. S., (2009). Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus. Nature*, 457, 471-474.

Grimes, J. M., Burroughs, J. N., Gouet, P., Diprose, J. M., Malby, R., Zientara, S. Merten, P. C. P. and Stuart, D. I., (1998). The atomic structure of the blue tongue virus core. *Nature,* 395, 470-478.

Gruenwedel, D.W. and Davidson, N., (1966). Complexing and denaturation of DNA by methyl mercuric hydroxide. I. Spectrophotometric studies. *J. Mol. Biol*. 21, 129-144.

Haas, H., (2003). Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage. *Appl. Microbiol. Biotechnol.*, 62, 316-330.

Haines, J., (1995). *Aspergillus* in compost: straw man or fatal flaw. *Biocycle*, 6, 32-35.

Hammond, T. M., Andrewski, M. D., Roossinck, M. J. and Keller, N. P., (2008). *Aspergillus* mycoviruses are targets and suppressors of RNA silencing. *Eukaryotic Cell* 7, 350-357.

Herrero, N., M. S. Sánchez, and Zabalgogeazcoa, I., (2009). Mycoviruses are common among different species of endophytic fungi of grasses. *Arch. Virol.* 154, 327–330.

Hillman, B. I., Foglia, R. and Yuan, W., (2000). Satellite and defective RNAs of *Cryphonectria hypovirus* 3, a virus species in the family *Hypoviridae* with a single open reading frame. *Virology*, 276, 181-189.

Hillman, B. I. and Suzuki, N., (2004). Viruses of the chestnut blight fungus, *Cryphonectria parasitica*. *Adv. Virus. Res*., 63, 423-472.

Hillman, B. I., Supyani, S., Kondo, H. and Suzuki, N., (2004). A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. *J. Virol*., 78, 892–898.

Ho, P. L. and Yuen, K. Y., (2000). Aspergillosis in bone marrow transplant recipients. *Crit. Rev. Oncol. Hematol*., 34, 55-69.

Hollings, M., (1962). Viruses associated with a die-back disease of cultivated mushrooms. *Nature,* 196, 962-65.

Hospenthal, D. R., Kwon-Chung, K. J. and Bennett, J. E., (1998). Concentrations of airborne *Aspergillus* compared to the incidence of invasive aspergillosis: lack of correlation. *Med. Mycol*., 36, 165-168.

Howard, D. H., **(**1999). Acquisition, transport, and storage of iron by pathogenic fungi. *Clin. Microbiol. Rev.* 12, 394-404.

Howitt, R. L. J., Beever, R. E., Pearson, M. N. and Forster, R. L. S., (2006) Genome characterization of a flexuous rod-shaped mycovirus, *Botrytis virus X*, reveals high amino acid identity to genes from plant potex-like viruses. *Arch. Virol.*, 151, 563-579.

Huismans, H., Van Dijk, A. A. and Els, H. J., (1987) Uncoating of parental bluetongue virus to core and subcore particles in infected L cells. *Virology.*157, 180-188.

Jamal, A., Bignell, E. M. and Coutts, R. H. A., (2010). Complete nucleotide sequences of four dsRNAs associated with a new chrysovirus infecting *Aspergillus fumigatus. Virus Res*., 153, 64-70.

Jiang, D. and Ghabrial, S. A., (2004). Molecular characterization of *Penicillium chrysogenum virus*: reconsideration of the taxonomy of the genus *Chrysovirus*. *J. Gen. Virol*., 85, 2111-2121.

Kanaani, H., Hargreaves, M., Ristovski, Z. and Morawska, L., (2008). Fungal spore fragmentation as a function of airflow rates and fungal generation methods. *Atmos. Envir*., 43, 3725–3735.

Kanematsu, S., Sasaki, A., Onoue, M., Oikawa, Y. and Ito, T., (2010). Extending the fungal host range of a partitivirus and a mycoreovirus from Rosellinia necatrix by inoculation of protoplasts with virus particles. *Phytopathology*, 100, 922-930.

Kim, J. W. and Bozarth, R. F., (1985). Intergeneric occurrence of related fungal viruses: the *Aspergillus ochraceus* virus complex and its relationship to the *Penicillium stoloniferum* virus S. *J. Gen. Virol*., 66, 1991-2002.

Kim, J. W., Choi, E. Y. and Kim, Y. T., (2006). Intergeneric relationship between the *Aspergillus ochraceous virus F* and the *Penicillium stoloniferum* virus S. *Virus Res*., 120, 212–215.

Klich, M. A., (2006). Identification of clinically relevant *Aspergilli*. *Med. Mycol*., 44, 127-131.

Koltin, Y. and Day, D. R., (1975). Specificity of *Ustilago maydis* killer proteins. *Appl. Microbiol*. 30, 694-696

Krappmann, S., (2006). Tools to study molecular mechanisms of *Aspergillus* pathogenicity. *Trends Microbiol*. 14, 356-364.

Kurup, V. P. and Kumar, A., **(**1991). Immunodiagnosis of aspergillosis. *Clin. Microbiol. Rev.* 4**,** 439-456.

Labbé, M., Charpilienne, A, Crawford, S. E., Estes, M. and Cohen J., (1991). Expression of rotavirus VP2 produces empty core-like particles. *J Virol.* 65, 2946-2952.

Laemmli, U., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227, 680-685.

Latge, J. P., (1999). *Aspergillus fumigatus* and Aspergillosis. *Clin. Microbiol. Rev.,* 12, 310-350.

Latge, J. P., (2003) *Aspergillus fumigatus*, a saprophytic pathogenic fungus, *Mycologist,* 17, 56-61.

Lee, U., Hong, J. S., Choi, J. K., Kim, K. C., Kim, Y. S., Curtis, I. S., Nam, H. G. and Lim, P. O., (2000). *Broad bean wilt virus* causes necrotic symptoms and generates defective RNAs in *Capsicum annuum*. *Phytopathology,* 90, 1390-1395.

Lemke, P. A. and Nash, C. H., (1974). Fungal viruses. *Bacteriol. Rev.,* 38, 29-56.

Liang, P., Chen, K. and Liu, H., (1983). The transfer of dsRNA virus through protoplast fusion in *Penicillium chrysogenum. Acta Mycol. Sin.,* 2, 102-109.

Lhoas, P., (1970). Use of heterokaryosis to infect virusfree isolates of *Aspergillus niger*. *Aspergillus Newsletter,* 11, 8-9.

Lin, S. J., Schranz, J. and Teutsch, S. M., (2001). Aspergillosis case-fatality rate: systematic review of the literature. *Clin. Infect. Dis.,* 32, 358-366.

Liu, W., Duns, G. and Chen, J. (2008). Genomic characterization of a novel partitivirus infecting *Aspergillus ochraceaus*. *Virus Genes,* 37, 322-327.

Magae, Y. and Sunagawa, M., **(**2010). Characterization of a mycovirus associated with the brown discoloration of edible mushroom, *Flammulina velutipes***.** *Virology Journal* 7, 342.

Marquez, L. M., Redman, R. S., Rodriguez, R. J. and Roossinck, M. J., (2007). A virus in a fungus in a plant: Three-way symbiosis required for thermal tolerance. *Science,* 315, 515-523.

Martins, M. K., Furlaneto, M. C., Sosa-Gomez, D. R. Faria, M. R. and Fungaro, M. H., (1999). Double-stranded RNA in the entomopathogenic fungus *Metarhizium flavoviride*. *Curr. Gen.,* 36, 94–97.

McCabe, P. M., Pfeiffer, P. and Van Alfen, N. K., (1999). The influence of dsRNA viruses on the biology of plant pathogenic fungi. *Trends Microbiol.,* 7, 377-381.

Mertens, P., (2004.) The dsRNA viruses. *Virus Res.,* 101, 3-13.

Mertens, P.P.C., Wei, C. and Hillman, B., (2005). *Reoviridae-Mycoreovirus*. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (eds.) *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses,* Elsevier, San Diego, pp. 556-560.

Milne, R. G. and Cristina M., (1999) *Cryptoviruses (Partitiviridae)*: In Encyclopedia of Virology (2nd Edn), Granoff, A.and.Webster, R.G., (eds), (Elsevier Academic Press, Torino, Italy). pp, 312-315.

Mindich, M., and Bamford, D. H., (1988). Lipid-containing bacteriophages. *In* The Bacteriophages. Vol. 2. R. Calendar, editor. *Plenum Publishing Corp*., New York. 475-520.

Nagy, P. and Simon, A., (1997). New insights into the mechanisms of RNA recombination. *Virology*, 235, 1-9.

Nakatsukasa-Akune, M., Yamashita, K., Shimoda, Y., Uchiumi T., Abe, M., Aoki, T., Kamizawa, A., Ayabe, S., Higashi, S. and Suzuki, A., (2005). Suppression of root nodule formation by artificial expression of the TrEnodDR1 (coat protein of White clover cryptic virus 1) gene in *Lotus japonicus*. *Mol Plant Microbe Interact* 18, 1069-1080.

Nemeroff, M. E. and Bruenn, J. A., (1986). Conservative replication and transcription of *Saccharomyces cerevisiae* viral double-stranded RNA *in vitro*. *J. Virol.,* 57, 754-758.

Nuss, D. L., (1992). Biological control of chestnut blight: an example of virus-mediated attenuation of fungal pathogenesis. *Microbiol. Rev*., 56, 561-576.

Nuss, D. L., (2005). Hypovirulence: mycoviruses at the fungal-plant interface. *Nat. Rev. Microbiol.,* 3, 632-642.

Nuss, D. L. and Koltin, Y., (1990). Significance of dsRNA genetic elements in plant pathogenic fungi. *Annu. Rev.Phytopath*., 28, 37-58.

Ochoa, F. W., Havens, W. M., Sinkovits, R. S., Nibert, M. L., Ghabrial, S. A., Timothy, S. and Baker, T. S., (2008). Partitivirus structure reveals a 120-subunit, helix-rich capsid with distinctive surface arches formed by quasi symmetric coat-protein dimers. *Cell*, 16, 776-786.

Palukaitis, P., Rezaian, A. and Garcia-Arenal, F., (2008). Satellite nucleic acids and viruses: in *Encylopedia of Virology* 3rd edn. Mahy, B. W. J. and Van Regenmortel, M. H. V. (eds.), (Elsevier Academic Press, the Netherlands). pp, 526-535.

Patricia, M., McCabe, P. P. and Neal, K. V. A., (1999). The influence of dsRNA viruses on the biology of plant pathogenic fungi. *Trends in Microbiol*., 7, 377-381.

Paris, S., Boisvieux-Ulrich, E., Crestani, B., Houcine, O., Taramelli, D., Lombardi, L. and Latgé, J. P., (1997). Internalization of *Aspergillus fumigatus* conidia by epithelial and endothelial cells. *Infection and Immunity*, 65, 1510-1514.

Park, Y., Chen, X. and Punja, Z. K., (2006). Molecular and biological characterization of a mitovirus in *Chalara elegans* (*Thielaviopsis basicola*). *Phytopathology*, 96, 468-479.

Pearson, M. N., Beever, R. E., Boine, B. and Arthur, K., (2009) Mycoviruses of filamentous fungi and their relevance to plant pathology. *Mol. Plant Path.*, 10, 115-128.

Pitt, J. I., **(**1994). The current role of *Aspergillus* and *Penicillium* in human and animal health. *J. Med. Vet. Mycol.*, 32, 17-32.

Polashock, J. J. and. Hillman, B. I., (1994). A small mitochondrial double stranded (ds) RNA element associated with a hypovirulent isolate of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T-dsRNA and W-dsRNA. *Proc. Nat. Acad. Sci. (USA)*, 91, 8680-8684.

Pullen, M. E., (1968). Virus diseases in root crops sugar beet. In *The incidence and spread of viruses. Rothamsted Experimental Station Report* for 1967, part 1, pp. 124-125.

Pullen, M. E., (1969). Virus-like particles in sugar beet. In *Virus diseases of grasses and cereals. Rothamsted Experimental StationReport* for 1968, part 1, p. 127.

Punja, Z., K., (1995). Influence of double-stranded RNAs on growth, sporulation, pathogenecity, and survival of *Chalara elegans*. *Can. J. Bot*., 73, 1001-1009.

Ramanan, N., and Wang, Y., (2000). A high-affinity iron permease essential for *Candida albicans* virulence. *Science*, 288, 1062-1064.

Rao, J. R., Nelson, D. W. A. and McClean, S., (2007).The enigma of double-stranded RNA (dsRNA) associated with mushroom virus X (MVX). *Curr. Issues Mol. Biol*., 9, 103-122.

Raper, K. B. and Fennell, D. I., **(**1965). *Aspergillus fumigatus group*, In the genus *Aspergillus* (eds K. B. Raper and D. I. Fennell) pp. 238–268. The William & Wilkins Co., Baltimore, Maryland.

Ratledge, C. and Dover, L. G., (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.*, 54, 881-941.

Ratti, G. and Buck, K. W., (1972). Virus particles in *Aspergillus foetidus*: a multicomponent system. *J. Gen. Virol.*, 14, 165-175.

Rawlinson, C. J., Hornby, D., Pearson, V., Carpenter, J. M., (1973). Virus-like particles in take-all fungus, *Gaeumannomyces graminis. Ann. Appl. Biol.*, 74, 197-209.

Rementeria, A., Lopez-Molina, N., Ludwig, A., Vivanco, B. A., Bikandi, J., Pontón, J. and Garaizar, J., (2005). Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev. Iberoam. Micol*., 22, 1-23.

Rickner, D. L., Stobbs, D. W. and Aim, G., (1993). The effect of virus disease on oyster mushroom production. *Micologia Neotropical Aplicada*, 6, 73-79.

Ro, H. S., Kang, E. J., Yu, J. S., Lee, T. S., Lee, C. W. and Lee, H. S., (2007). Isolation and characterization of a novel mycovirus, PeSV, in *Pleurotus eryngii* and the development of a diagnostic system for it. *Biotech. Lett*., 29, 129-135.

Robinson, H. L. and Deacon, J. W., (2002). Double-stranded RNA elements in *Rhizoctonia solani* AG 3. *Mycol. Res*., 106, 12-22.

Romaine, C.P. and Schlagnhaufer, B., (1995). PCR analysis of the viral complex associated with La France disease of *Agaricus bisporus*. *Appl. Environ. Microbiol.*, 61, 2322-2325.

Rong, R., Rao, S., Scott, S. W., Carner, G. R., and Tainter, F. H., (2002). Complete sequence of the genome of two dsRNA viruses from *Discula destructiva*. *Virus Res*. 90, 217-224.

Ruijter, G. J. G., Kubicek, C. P. and Vissler, J., (2002). Production of organic acids by fungi. In: The mycota vol. X. Industrial applications, Osiewacz, H.D. (ed.) (Heidelberg: Springer-Verlag), pp, 213-230.

Rytel, M. W., Shope, R. E. and Kilbourne, E. D., (1966). An antiviral substance from *Penicillium funiculosurm V*. Induction of interferon by helenine. *J. Exp. Med.*, 123, 577-84.

Saitou, N. and Nei, M., (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

Sasaki, A., Miyanishi, M., Ozaki, K., Onoue, M. and Yoshida, K., (2005). Molecular characterization of a partitivirus from the plant pathogenic ascomycete *Rosellinia necatrix*. *Arch. Virol*., 150, 1069-1083.

Sasaki, A., Kanematsu, S., Onoue, M., Oyama, Y. and Yoshida, K., (2006). Infection of *Rosellinia necatrix* with purified viral particles of a member of *Partitiviridae* (RnPV1-W8). *Arch. Virol*., 151, 697-707.

Schmidt, F. R., Lemke, P. A., and Esser, K., (1986). Viral influences on aflatoxin formation by *Aspergillus flavus*. *Appl. Microbiol. Biotechnol*., 24, 248-252.

Schrettl, M., Bignell, E., Kragl, C., Joechl, C., Rogers, T., Arst, H. N. Jr., Haynes, K., Haas, H., (2004) Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. *J. Exp. Med.,* 200, 1213-1219.

Sevag, M. G., Lackman, D. B. and Smolens, J., (1938). Isolation of components of streptococcal nucleoproteins in serologically active form. *J. Biol. Chem.* 124, 425-436.

Sharzei, A., Banihashemi, Z. and Afsharifar, A., (2007). Presence of a double-stranded RNA mycovirus in *Fusarium oxysporum f. sp. Melonis*. *Iran. J. Plant Pathol*., 43, 9-26.

St-Germain, G. and Summerbell, R., (1996). *Identifying filamentous fungi - A clinical laboratory handbook,* 1st ed. Star Publishing Company, Belmont, California.

Sui, C., Wei, J. H., Zhan, Q. Q. and Zhang. J., (2009). First Report of *Broad bean wilt virus 2* infecting *Bupleurum chinense* in China. *Plant Disease* 93, 844-844.

Suzuki, K., Ikeda, K.I., Sasaki, A. Kanemastsu, S., Matsumoto, N and Yoshida K., (2005). Horizontal transmission and host virulence attenuation of totivirus in violet root rot fungus *Helicobasidium mompa. J. Gen. Plant Pathol*., 71, 161-168.

Szewczyk, E., Nayak, T., Oakley, C. E., Edgerton, H., Xiong, Y., Talesh, N. T., Osmani, S. A. and Oakley, B. R., (2007). Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nature Protocols*, 1, 3111-3119.

Tavantzis, S. M., (2008). Partiviruses of Fungi. In " Encyclopedia of Virology" 3rd edn., Mahy, B. W. V. and Van Regenmortel, M. H. V. (eds), (Elsevier Academic Press, San Diego, CA., U.S.A). pp, 63-68.

Thomas, J. J., Bakhtiar R. and Siuzdak, G., (2000). Mass spectrometry in viral proteomics, *Acc. Chem. Res.* 33, 179-187.

Treton, B. Y., Le Dall, M. T. and Heslot, H., (1987). UV induced curing of the double stranded RNA virus of the yeast *Yarrowia lipolytica*. *Curr. Genet*., 12, 37-39.

Van Diepeningen, A. D., Debets, A. J. M. and Hoekstra, R. F., (1997). Heterokaryon incompatibility blocks virus transfer among natural isolates of black *Aspergilli. Curr. Genet.*, 32, 209-17.

Van Diepeningen, A.D., Debets, A. J. M. and Hoekstra, R. F., (1998). Intra- and interspecies virus transfer in Aspergilli *via* protoplast fusion. *Fungal Genet. Biol*., 25, 171-180.

Van Diepeningen, D. A., Debets, A. J. M. and R. F. Hoekstra., (2006). Dynamics of dsRNA mycoviruses in black *Aspergillus* populations. *Fungal Genet. Biol.*, 43, 446-452.

Van Diepeningen, A. D., Varga, J., Hoekstra, R. F. and Debets, A. J. M., (2008). Mycoviruses in the Aspergilli. In: Varga J, Samson RA (eds), Aspergillus in the genomic era. Wageningen Academic Publishers, Wageningen, the Netherlands, pp 133-176.

Van de Sande, W., Lo-Ten-Foe, J., Van Belkum, A., Netea, M., Kullberg, B. and Vonk, A., (2010). Mycoviruses: future therapeutic agents of invasive fungal infections in humans? *Eur. J. Clin. Microbiol. Infec.t Dis.* 29, 755-763

Varga, J., Kevei, F., Vagvolgyi, C., Vriesema, A. and Croft, J. H., (1994). Double-stranded RNA mycoviruses in section Nigri of the *Aspergillus* genus. *Can. J. Microbiol*., 39, 325-329.

Varga, J., Rinyu E., Kevei, E., Toth, B. and Kozakiewicz, Z., (1998). Double-stranded RNA mycoviruses in species of *Aspergillus* sections *Circumdati* and *Fumigati*. *Can. J. Microbiol.*, 44, 569-574.

Varga, J., Toth, B., Szencz, S., Molnar, J., Fekete, C. S., Szabo, L., (2001). Double-stranded RNA elements and virus-like particles in Aspergilli. *Acta Biol. Hung*., 52, 355-363.

Varga, J., Toth, B. and Vagvolgyi, C., (2003). Recent advances in mycovirus research. *Acta Microbiol. Immunol. Hung*., 50, 77-94.

Vicentefranqueira, R., Moreno, M. A., Leal, F., Calera, J. A., (2005) The zrfA and zrfB genes of *Aspergillus fumigatus* encode the zinc transporter proteins of a zinc uptake system induced in an acid, zinc-depleted environment. *Eukaryot Cell*, 4, 837-848.

Warn, P. A., Sharp, A., Anderson, M. J. and Denning, D. W., (2006). Single-spored cultures of the sequenced strain of *Aspergillus fumigatus* (Af293) have significantly different mortality rates in a murine model of IPA. In: Proceedings of the second advances against aspergillosis conference. P301 (p.134).

Wasylnka, J. A. and Moore, M. M., (2002). Uptake of *Aspergillus fumigatus* conidia by phagocytic and non phagocytic cells *in vitro*: quantitation using isolates expressing green fluorescent protein. *Infection and Immunity,* 70, 3156-3163.

Wickner, R.B., (1996). Double-stranded RNA viruses of *Saccharomyces cerevisiae*. *Microbiol. Rev.* 60, 250-265.

Wickner, R. B., Wang, C. C. & Patterson, J. L., (2005). *Totiviridae*. In *Eighth Report of the International Committee on Taxonomy of Viruses*, (eds C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger and A. L. Ball) pp. 571-580. London: Elsevier/Academic Press.

Xie, J., Wei, D., Jiang, D., Fu, Y., Li, G., Ghabrial, S. and Peng, Y., (2006). Characterization of debilititation-associated mycovirus infecting the plant-pathogenic fungus *Sclerotonia sclerotium. J. Gen. Virol*., 87, 241-249.

Yamada, T., Seno, M., Shiraishi, T., Kato, H., An, C., Yoshida, T. and Oku, H., (1991). Isolation and characterization of double-stranded RNAs from the pea pathogen, *Mycosphaerella pinodes, Plant Cell Physiol.*, 32, 65-71.

Zuker, M., (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*., 31, 3406-3415.

10. Appendix Chapter 3

Table 3.1 List of *Aspergillus fumigatus* isolates screened for presence of dsRNA elements.

Serial No.	Isolate No.	Accession Number	Age and sex of the Patient	Hospital [a]
Clinical Isolates				
1	A-49	40010	Unknown	HH
2	A-50	40020	-do-	-do-
3	A-51	40023	-do-	-do-
4	A-52	40027	-do-	-do-
5	A-53	40042	-do-	-do-
6	A-54	40047	-do-	WM
7	A-55	40069	-do-	HH
8	A-56	40093	-do-	-do-
9	A-57	40096	-do-	-do-
10	A-58	40117	-do-	-do-
11	A-60	40125	-do-	-do-
12	A-61	40134	-do-	-do-
13	A-62	40141	-do-	-do-
14	A-63	40155	-do-	-do-
15	A-64	40159	-do-	-do-
16	A-65	40171	-do-	-do-
17	A-66	40190	-do-	-do-
18	A-67	40192	-do-	-do-
19	A-68	40194	-do-	-do-
20	A-69	40215	-do-	-do-
21	A-70	40221	-do-	-do-
22	A-71	40223	-do-	-do-
23	A-72	40235	-do-	-do-
24	A-73	40275	-do-	-do-
25	A-74	40298	-do-	-do-
26	A-75	40303	-do-	-do-
27	A-76	40317	-do-	-do-

28	A-77	40339	-do-	-do-
29	A-78	40350	-do-	-do-
30	A-79	40357	-do-	-do-
31	A-80	40359	-do-	-do-
32	A-83	40402	-do-	-do-
33	A-84	40424	-do-	-do-
34	A-85	40425	-do-	-do-
35	A-86	40433	-do-	-do-
36	A-87	40439	-do-	-do-
37	A-88	40443	-do-	-do-
38	A-89	40455	-do-	-do-
39	A-90	40470	-do-	-do-
40	1	MI-07- 40776	33 Male	-do-
41	2	MI-07-40798	85	HH
42	3	MI-07-40907	33 Male	-do-
43	4	MI-07-40899	81 Female	WM
44	5	MI-07-40919	62 Male	HH
45	6	MI-07-40949	96 Female	WM
46	7	MI-07-40801	81	EH
47	8	40051	Unknown	-do-
48	9	40924	39 Female	XCH
49	10	MI-07-40914	63 Female	WM
50	11	40630	Unknown	XCH
51	12	40081	-do-	HH
52	12A	40695	-do-	CW
53	13	MI-07-40924	50 F	CW
54	14	MI-07-40804	32 Male	STM
55	15	MI-07-40800	51 Male	CW
56	16	244	Unknown	HH
57	17	836	-do-	-do-
58	18	213	-do-	-do-
59	19	212	-do-	-do-

60	20	40283	34 Male	STM
61	21	40018	53 Male	-do-
62	22	40019	43 Female	-do-
63	23	40020	43 Female	-do-
64	24	40581	Unknown	-do-
65	25	40400	-do-	CW
66	26	41092	76 Male	HH
67	27	40046	68 Male	-do-
68	28	40048	72 Female	-do-
69	29	40050	37 Female	-do-
70	30	40064	73 Male	XCH
71	31	40086	45 Male	HH
72	32	40089	53 Male	-do-
73	33	40102	63 Female	-do-
74	34	40110	82 Female	XCH
75	35	40128	78 Male	HH
76	36	40138	75 Male	-do-
77	37	40160	45 Male	STM
78	38	40175	77 Male	HH
79	39	40177	44 Male	-do-
80	40	40180	74 Male	XCH
81	41	40190	61 Female	HH
82	42	40191	82 Male	XCH
83	43	40209	4 month, Female	CW
84	44	40256	80 Female	XCH
85	45	40258	71 Male	-do-
86	46	40260	52 Male	HH
87	47	40262	36 Female	-do-
88	48	40264	75 Female	-do-
89	49	H1208044	Unknown	-do-
90	50	40284	63 Female	-do-
91	51	40285	43 Female	-do-

92	52	40296	68 Female	-do-
93	53	40299	71 Female	-do-
94	54	40300	78 Female	-do-
95	55	40304	68 Female	-do-
96	56	40319	62 Male	CW
97	57	40322	52 Male	HH
98	58	40323	78 Female	-do-
100	59	40324	59 Male	-do-
101	60	40340	76 Female	CW
102	61	40342	61 Female	HH
103	62	40343	74 Male	-do-
104	63	40348	47 Female	XCH
105	64	40384	82 Male	HH
106	65	40396	59 Male	-do-
107	66	41144	51 Female	-do-
108	67	40398	64 Male	-do-
109	68	40879	43 Male	WM
110	69	40880	24 Female	-do-
111	70	40895	35 Male	-do-
112	71	40904	37 Female	HH
113	72	40920	71 Male	-do-
114	73	40921	42 Male	-do-
115	74	40922	37 Female	-do-
116	75	MI-07-40803	45 Female	WM
117	76	40936	41 Male	HH
118	77	40939	52 Female	-do-
119	78	40969	54 Female	-do-
120	79	41025	80 Female	-do-
121	80	41218	48 Female	XCH
122	81	41026	64 Male	WM
123	82	41035	20 Male	-do-
124	83	41046	42 Male	HH

125	84	41050	35 Female	-do-
126	85	41058	45 Female	-do-
127	86	41065	46 Male	-do-
128	87	41076	33 Female	-do-
129	88	41249	72 Male	-do-
130	89	41080	34 Female	-do-
131	90	41084	34 Male	-do-
132	91	40034	57 Female	-do-
133	92	41093	46 Male	XCH
134	93	41094	41 Male	HH
135	94	41100	58 Female	EH
136	95	40750	77 Female	HH
138	96	40755	81 Female	WM
139	97	40853	53 Female	HH
140	98	40865	29 Female	-do-
141	100	40869	33 Male	-do-
142	101	40874	46 Female	-do-
143	102	40978	74 Female	WM
144	103	420	63 Female	-do-
145	104	40992	28 Female	-do-
146	105	41042	41 Male	CW
147	106	41047	74 Female	WM
148	107	41050	40 Male	HH
149	108	41082	63 Male	-do-
150	109	41101	62 Female	-do-
151	110	41102	62 Female	-do-
152	111	41104	62 Male	-do-
153	112	41110	64 Female	-do-
154	113	41115	62 Male	XCH
155	114	41121	58 Male	HH
156	115	41124	76 Female	CW
157	116	40385	57 Male	HH

158	117	41146	58 Male	-do-
159	118	41148	43 Female	-do-
160	119	41150	62 Female	-do-
161	120	41170	55 Male	-do-
162	121	41171	63 Female	-do-
163	122	41177	46 Female	CW
164	123	612	70 Male	HH
165	124	41184	53 Female	-do-
166	125	41185	67 Male	-do-
167	126	41187	48 Male	XCH
168	127	41194	46 Female	CW
169	128	41212	72 Male	HH
170	129	40989	33 Male	-do-
171	130	41219	81 Female	-do-
172	131	41222	67 Male	-do-
173	132	41225	63 Male	-do-
174	133	41227	81 Female	-do-
175	134	41228	81 Female	-do-
176	135	41233	63 Male	-do-
177	136	41234	81 Female	-do-
178	138	41067	35 Male	EH
179	139	40077	Unknown	HH
180	140	40079	-do-	-do-
181	141	40080	-do-	-do-
182	142	40099	-do-	-do-
183	143	40100	-do-	-do-
184	144	40119	-do-	-do-
185	145	40122	-do-	-do-
186	146	40253	-do-	-do-
187	147	40263	-do-	-do-
188	148	40279	-do-	-do-
189	149	40315	Unknown	-do-

190	150	40328	-do-	-do-
191	151	40344	-do-	-do-
192	152	40358	-do-	-do-
193	153	40365	-do-	-do-
194	154	40366	-do-	-do-
195	155	40367	-do-	-do-
196	156	40378	-do-	-do-
197	157	40397	-do-	-do-
198	158	40399	-do-	-do-
199	159	40031	43 Male	CW
200	160	40401	Unknown	HH
201	161	40403	-do-	-do-
202	162	40404	-do-	-do-
203	163	40415	-do-	-do-
204	164	40417	-do-	-do-
205	165	40418	-do-	-do-
206	166	40421	-do-	-do-
207	167	40436	-do-	-do-
208	168	40453	-do-	-do-
209	169	40454	-do-	-do-
210	170	40457	-do-	-do-
211	171	40459	-do-	-do-
212	172	40460	-do-	-do-
213	173	40461	-do-	-do-
214	174	40489	-do-	-do-
215	175	40515	-do-	-do-
216	176	40516	-do-	-do-
217	177	40590	-do-	-do-
218	178	40589	-do-	-do-
219	179	40609	-do-	-do-
220	180	40627	-do-	-do-
221	181	40651	-do-	-do-

222	182	40693	-do-	-do-
223	183	40705	-do-	-do-
224	184	40659	-do-	-do-
225	185	40670	-do-	-do-
226	186	40668	-do-	-do-
227	187	40628	-do-	-do-
228	188	40633	-do-	-do-
229	189	40524	-do-	-do-
230	190	40554	-do-	-do-
231	191	40022	29 Male	-do-
232	192	40587	-do-	-do-
233	193	MI-07-40911	73 Male	-do-
234	194	MI-07-40929	61 Female	-do-
235	195	MI-07-40916	65 Male	WM
236	196	40964	75 Female	HH
237	197	40725	Unknown	HH
238	198	40728	-do-	-do-
239	199	40743	-do-	-do-
240	200	40760	-do-	-do-
241	201	40762	-do-	-do-
242	202	40765	-do-	-do-
243	203	40777	-do-	-do-
244	204	40820	-do-	-do-
245	205	40855	-do-	-do-
246	206	40863	-do-	-do-
247	207	40635	-do-	-do-
248	662	H668018	-do-	-do-
249	674	H987406	-do-	-do-
250	675	H648546	-do-	-do-
251	677	H1120969	-do-	-do-
252	708	H1107194	-do-	-do-
253	710	H816041	-do-	-do-

254	726	H1055053	-do-	-do-
255	711	H816041	-do-	-do-
256	729	H10343308	-do-	-do-
257	738	H811313	-do-	-do-
258	742	H1130101	-do-	-do-
259	748	H1132109	-do-	-do-
260	761	H1030707	-do-	-do-
261	762	H479172	-do-	-do-
262	781	H942862	-do-	-do-
263	783	H1201171	-do-	-do-
264	784	H969774	-do-	-do-
265	785	H483538	-do-	-do-
266	793	01039026	-do-	-do-
267	802	CX902428	-do-	-do-
268	803	01382830	-do-	-do-
269	804	H1201884	-do-	-do-
270	805	H366790	-do-	-do-
271	809	A926543	-do-	-do-
272	834	H668018	-do-	-do-
273	862	CC330286	-do-	-do-
274	863	H872948	-do-	-do-
275	864	H1129626	-do-	-do-
276	867	H235352	-do-	-do-
277	883	H1200138	-do-	-do-
278	884	H142507	-do-	-do-
279	887	H1004724	-do-	-do-
280	902	H982531	-do-	-do-
281	903	H1101746	-do-	-do-
282	904	H479735	-do-	-do-
283	905	H1201454	-do-	-do-
284	932	01060439	-do-	-do-
285	934	00720992	-do-	-do-

286	954	H101261	-do-	-do-
287	9A	01949120	-do-	-do-
288	10A	01947371	-do-	-do-
289	36A	H959114	-do-	-do-
290	37A	GP	-do-	-do-
291	1A	40017	81 Female	-do-
292	50	H1202392	-do-	-do-
293	51	GP	-do-	-do-
294	219	40818	46 Male	CW
295	220	41267	64 Male	XCH
296	221	41268	32 Female	HH
297	222	41269	67 Male	-do-
298	223	41274	74 Female	-do-
299	224	41285	72 Male	-do-
300	225	41286	69 Male	-do-
301	226	41293	64 Female	-do-
302	227	41304	61 Female	-do-
303	228	41310	65 Female	-do-
304	229	41321	77 Male	-do-
305	230	41329	53 Male	-do-
306	231	41346	68 Female	XCH
307	232	41347	67 Female	XCH
308	233	40423	71 Male	HH
309	234	40451	66 Male	-do-
310	235	40452	59 Male	CW
311	236	40462	89 Male	HH
312	237	40464	63 Female	-do-
313	238	40465	59 Male	-do-
314	239	40496	66 Male	-do-
315	240	40497	46 Female	-do-
316	241	40528	46 Female	-do-
317	242	40542	64 Male	-do-

318	243	40543	52 Male	-do-
319	244	40544	44 Male	-do-
320	245	40560	77 Female	-do-
321	246	40571	77 Female	-do-
322	247	40579	82 Female	-do-
323	248	40584	71 Male	-do-
324	249	40603	85 Female	-do-
325	250	40607	34 Male	-do-
326	251	40626	59 Male	XCH
327	252	40638	66 Male	HH
328	253	40639	71 Female	-do-
329	254	40642	59 Male	-do-
330	255	40645	47 Female	-do-
331	256	40662	74 Male	-do-
332	257	40763	Unknown	-do-
333	258	41179	21 Female	-do-
Environmental Isolates				
334	1	1811	---	HH
335	Env 1	X No 1	---	-do-
336	Env 2	X No 2	---	-do-
337	Env 3	X No 4	---	-do-
338	Env 4	X No 5	---	-do-
339	Env 5	2694	---	-do-
340	Env 6	10069	---	-do-
341	Env 7	2859	---	-do-
342	Env 8	3124	---	-do-
343	Env 9	3130	---	-do-
344	Env 10	3135	---	-do-
345	Env 11	3233	---	-do-
346	Exeter 7.3	---	---	---
347	Exeter 10.2	---	---	---
348	Exeter 16.1	---	---	---

349	Exeter 22.4	---	---	---
350	Exeter 193	---	---	---
351	Exeter 2.2	---	---	---
352	Exeter 3.1	---	---	---
353	Exeter 4.2	---	---	---
354	Exeter 6.2	---	---	---
355	Exeter 8.3	---	---	---
356	Exeter 9.1	---	---	---
357	Exeter 11.7	---	---	---
358	Exeter 14.5	---	---	---
359	Exeter 15.2	---	---	---
360	Exeter 21.1	---	---	---
361	Exeter 23.1	---	---	---
362	Exeter 25.1	---	---	---
363	Exeter 15.2	---	---	---
364	Exeter 21.1	---	---	---
365	Exeter 23.1	---	---	---
366	Exeter 25.1	---	---	---

[a] Hospital of isolation: WM=West Middlesex; HH=Hammersmith; XCH=Charing Cross; CW=Chelsea and Westminster; STM=St Marys.

Publications

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ANNOTATED SEQUENCE RECORD

Complete nucleotide sequences of two dsRNAs associated with a new partitivirus infecting *Aspergillus fumigatus*

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Introduction

Viruses are ubiquitous in all major groups of filamentous fungi, and those with RNA genomes are now classified into 10 families, of which four include double-stranded RNA (dsRNA) viruses and the remaining six include single-stranded (ss) RNA viruses [10]. Most mycoviruses cause cryptic infections, but some cause phenotypic alterations including hypovirulence and debilitation [10]. In the genus *Aspergillus*, dsRNA mycoviruses are common in several asexual species, where they vary widely both in number and composition, but are rare in sexual *Aspergilli* [6, 13, 14]. Documentation of *Aspergillus* mycoviruses concerns cataloguing the presence or absence of dsRNA elements in 0-13% of the species examined [13], and until recently, the molecular characterisation of *Aspergillus* mycoviruses was restricted to a partitivirus found in *A. ochraceus* [9]. However, in a recent screen of 366 *A. fumigatus* (Nakazawa) isolates, in contrast to a previous study [14], dsRNA elements were discovered in 6.6% of the isolates, including a chrysovirus named Aspergillus fumigatus chrysovirus, the complete genome of which was cloned, sequenced and analysed [7]. We now report that the dsRNA profile found in another group of virus-infected *A. fumigatus* isolates parallels that found in the genus *Partitivirus* [5], and the complete bipartite genome of one strain has been cDNA cloned, sequenced and analysed. Phylogenetic analysis was performed to determine its evolutionary relationship status with other members of the family *Partitiviridae*.

Provenance of the virus material

All 366 *A. fumigatus* isolates were grown and screened for the presence of dsRNAs as before [7], removing traces of DNA and ssRNA with DNase1 and S1 nuclease, respectively [4], followed by electrophoresis through 1% agarose gels containing TAE and ethidium bromide. Virus particles were purified from the frozen mycelia of several *A. fumigatus* isolates found to contain dsRNA elements [7], one of which, isolate 88, contained two dsRNA elements whose sizes were consistent with those comprising a partitivirus, which was named Aspergillus fumigatus partitivirus-1 (AfuPV-1; Fig. 1a). Methyl mercuric hydroxide–denatured AfuPV-1 dsRNAs 1 and 2 were used for reverse transcription-PCR (RT-PCR) amplification with random primers, cDNA cloning and sequencing as before [4]. Gaps in the sequence were closed by RT-PCR using targeted priming with oligonucleotides pairs and a single-primer, genome-walking protocol [4]. All DNA manipulations were performed according to standard protocols [11]. An RLM-RACE PCR procedure was used to determine the 5′- and 3′-terminal sequences of the dsRNAs [3]. All clones were sequenced in triplicate.

Sequence similarity searches of the GenBank, Swissprot and EMBL databases were conducted using BLAST [1]. Sequence alignments and phylogenetic analysis were

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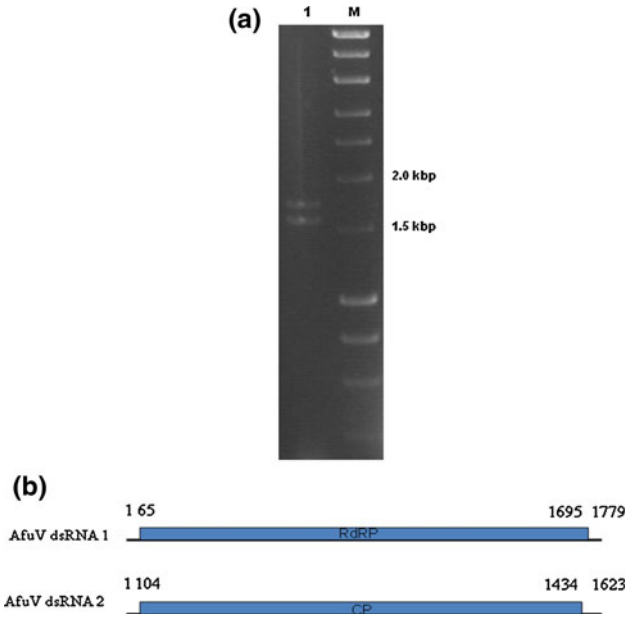

Fig. 1 (**a**) Occurrence of partitivirus dsRNAs in *Aspergillus fumigatus*. Agarose gel electrophoresis of dsRNAs purified from *A. fumigatus* isolate 88: Lane 1, *A. fumigatus* isolate 88 dsRNAs; Lane M, DNA marker showing 1.5- and 2-kbp bands; (**b**) Schematic representation of the monocistronic genomic organisation of *Aspergillus fumigatus* partitivirus-1 (AfuPV-1) dsRNA1 and dsRNA2. The RdRP ORF (nt positions 66-1694 on dsRNA1) and the CP ORF (nt positions 105-1433 on dsRNA2) are represented by rectangular boxes

performed using CLUSTAL_X [12] and the Fast Fourier Transform MAFFT program L9INS-1 [8]. A bootstrap test was conducted with 1000 re-samplings for the neighbor-joining (NJ) trees. Searches for amino acid signatures and protein motifs were conducted using the programs included in the ExPASy proteomics tools (http://www.expasy.org/tools/).

Sequence properties

The dsRNA extracted from purified AfuPV-1 virions was resolved into two bands by agarose gel electrophoresis (Fig. 1a), and cDNA cloning and sequence analysis revealed the presence of two distinct dsRNA segments in association with purified virions. Northern hybridisation analysis using radioactive cDNA probes specific for each of the two dsRNA segments showed that each segment has unique sequences that are encapsidated in isometric particles, as revealed by electron microscopy (data not shown). The complete sequence of dsRNA1 is 1779 base pairs (bp) long (GenBank database accession number FN376847), and dsRNA2 is 1623 bp long (accession number FN398100; Fig. 1b). Sequence analysis of AfuPV-1 dsRNA1 cDNA indicated that it contains one major ORF from nucleotide (nt) 66 to 1694, which potentially encodes a protein of 542 amino acid (aa) residues with a molecular mass of 63 kDa. The major ORF of AfuPV-1 dsRNA2, from nt 105 to 1433, corresponding to 442 aa residues, putatively encodes a protein with a estimated molecular mass of 48 kDa (Fig. 1b).

The 5′-UTRs flanking the major ORFs of the plus strands of dsRNA1 and dsRNA2 are respectively 65 and 104 nt long (Fig. 1b) with a sequence identity of ~60%. The differences mainly arise from the size differences in their 3′-proximal regions, and the identical stretches include the terminal motif 5′-CGCAAAAG-3′ (Fig. S1a in Supplementary Material). The AfuPV-1 5′-UTRs were closely related to the similarly sized 5′-UTRs of Botryotinia fuckeliana partitivirus-1 (BfPV-1) dsRNA1 (61 nt and 61% identical) and dsRNA2 (101 nt and 51% identical). Distinct from most partitiviruses, the AfuPV-1 5′-terminal regions were devoid of CAA repeats, which are characteristic for members of the family *Partitiviridae* [5], and the regions were not significantly A/U rich. The 3′-UTRs of the plus strands of AfuPV-1 dsRNA1 and dsRNA2 differ in length even more than the 5′-UTRs (85 and 190 nt, respectively), and there was little sequence conservation between them apart from the terminal motif 5′-AUCCA-3′ (Fig. S1b).

The deduced aa sequence of the major ORF of AfuPV-1 dsRNA1 contains characteristic sequence motifs found in putative RNA-dependent RNA polymerases (RdRPs) of mycoviruses [2], and we conclude that dsRNA1 encodes the AfuPV-1 RdRP. A comparison of the aa sequence of the putative AfuPV-1 RdRP with sequences in the NCBI database using the BLASTP algorithm revealed highly significant similarities to the RdRP of viruses in the family *Partitiviridae* (Table S1). The RdRP sequences of four fungal partitiviruses, namely those of BfPV, Discula destructiva virus 1 (DdV1), Discula destructiva virus 2 (DdV2) and ophiostoma partitivirus 1 showed 70–80% aa sequence identity to AfuPV-1 without any gaps. Based on the complete RdRP aa sequences of different members of the family *Partitiviridae*, we generated a phylogenetic tree by the NJ method (Fig. 2a). This phylogram gives further support for the strong relatedness of the viruses mentioned above. The tree has three major branches: one contains cryptoviruses and several fungal partitiviruses, the second branch contains fungal partitiviruses and the third branch contains AfuPV-1 and partitiviruses that infect ascomycetous fungi (Fig. 2a), essentially coincident with the current taxonomy of the genus [5].

The deduced sequence of the major ORF of AfuPV-1 dsRNA2 encodes a putative 442-aa protein with a molecular mass of 48 kDa. This predicted size is similar to that estimated by SDS-PAGE for the AfuPV-1 coat protein (CP) subunit derived from purified virions (data not shown) and is within the range (39-73 kDa) described

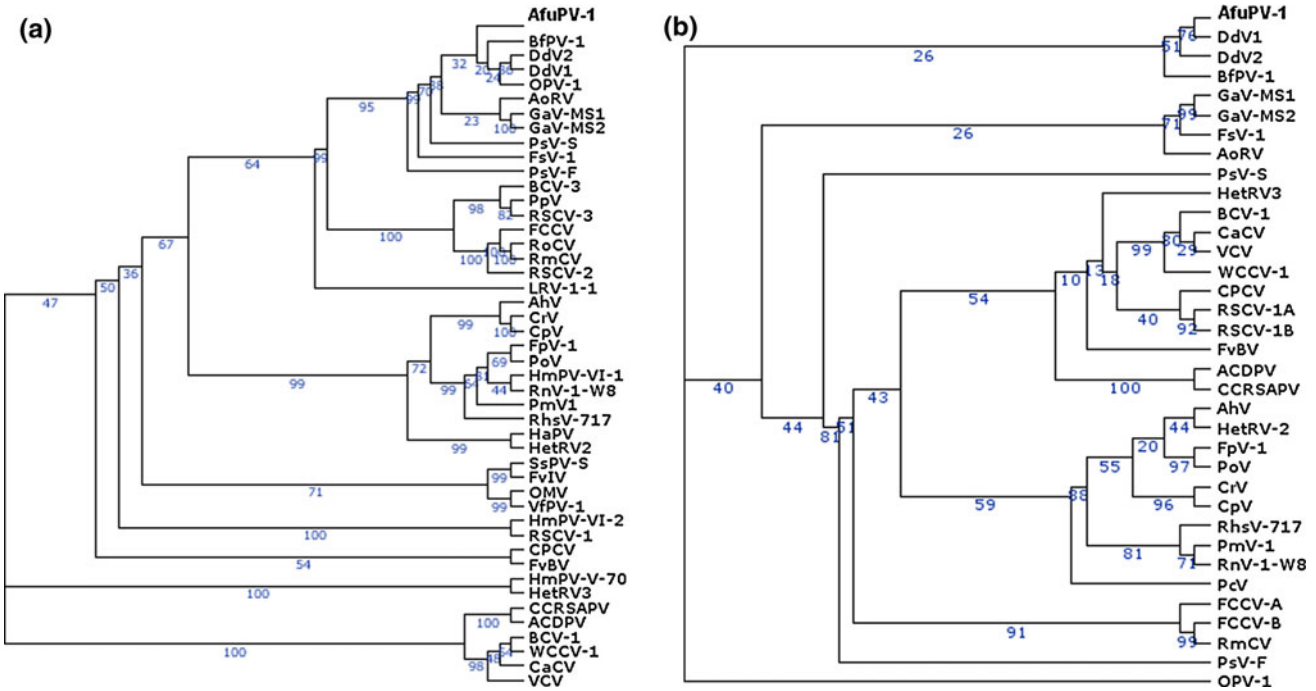

Fig. 2 Phylogenetic trees of the putative RdRP (**a**) and coat protein CP (**b**) aa sequences of members of the family *Partitiviridae* and AfuPV-1 (bold), generated by the NJ method. The numbers shown at branch points indicate bootstrap values. The datasets were subjected to 1,000 bootstrap replicates. Only complete RdRP and CP sequences taken from GenBank were analysed, and these are shown in Supplementary Table S1, which also includes the abbreviations for the individual viruses. The RdRP and CP trees were rooted with the leishmania RNA virus 1-1 (LRV-1-1; type member of the genus *Leishmaniavirus* in the family *Totiviridae*: M92355) RdRP, and the penicillium chrysogenum virus (PcV; type member of the genus *Chrysovirus* in the family *Chrysoviridae*: NC_007540) CP, respectively

for the CPs of members of the genus *Partitivirus* [5 and Table S1]. Multiple protein sequence alignment of the AfuPV-1 CP aa sequence showed a similar trend to that found for the AfuPV-1 RdRP sequences, and the CP sequences of DdV1, DdV2 and BfPV showed ~60% aa sequence identity. A phylogenetic tree indicating the relationships among representative, sequenced partitiviruses based on the full-length sequences of the CPs (Table S1; Fig. 2b) supported the relationships observed by phylogenetic analysis of the RdRP sequences (Fig. 2a). The high bootstrap values associated with these relationships support classification of AfuPV-1 as a new member of the genus *Partitivirus*, family *Partitiviridae*. Some isolates of *A. fumigatus* containing AfuPV-1 dsRNAs 1 and 2 also appeared to contain a smaller dsRNA element similar in size to those described for DdV1, DdV2 and BfPV, but these are considered to be satellite-like dsRNAs and not partitivirus genomic components [5] and were not investigated further.

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References

1. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
2. Bruenn JA (1993) A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. Nucleic Acids Res 21:5667–5669
3. Coutts RHA, Livieratos IC (2003) A rapid method for sequencing the 5′- and 3′-termini of dsRNA viral templates using RLM-RACE. J Phytopathol 151:525–527
4. Coutts RHA, Covelli L, Di Serio F, Citir A, Açıkgöz S, Hernández C, Alioti D, Ragozzino A, Flores R (2004) Cherry chlorotic rusty spot and Amasya cherry diseases are associated with a complex pattern of mycoviral-like double-stranded RNAs II. Characterization of a new species of genus *Partitivirus*. J Gen Virol 85:3399–3403
5. Ghabrial SA, Ochoa WF, Baker TS, Nibert ML (2008) Partitiviruses: general features. In: Mahy BWJ, van Regenmortel MHV (eds) Encyclopedia of Virology, 3rd edn. Elsevier/Academic Press, London, pp 503–513
6. Hammond TM, Andrewski MD, Roossinck MJ, Keller NP (2008) Aspergillus mycoviruses are targets and suppressors of RNA silencing. Eukaryot Cell 7:350–357
7. Jamal A, Bignell EM, Coutts RHA (2010) Complete nucleotide sequences of four dsRNAs associated with a new chrysovirus infecting *Aspergillus fumigatus*. Virus Res 153:64–70
8. Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518

9. Liu W, Duns G, Chen J (2008) Genomic characterization of a novel partitivirus infecting *Aspergillus ochraceus*. Virus Genes 37:322–327
10. Nuss DL (2010) Mycoviruses. In: Borkovich KA, Ebbole DJ, Momany M (eds) Cellular and molecular biology of filamentous fungi. American Society of Microbiology, Washington, DC, pp 145–152
11. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Publications, Cold Spring Harbor Laboratory, New York
12. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
13. van Diepeningen AD, Varga J, Hoekstra RF, Debets AJM (2008) Mycoviruses in the Aspergilli. In: Varga J, Samson RA (eds) Aspergillus in the genomic era. Wageningen Academic Publishers, Wageningen, pp 133–176
14. Varga J, Rinyu E, Kevei E, Toth B, Kozakiewicz Z (1998) Double-stranded RNA mycoviruses in species of *Aspergillus* sections *Circumdati* and *Fumigati*. Can J Microbiol 44:569–574

Supplementary Table 1. List of viruses in the family *Partitiviridae* with sequenced genomic dsRNAs

Virus [a]	Abbreviation	dsRNA segment no. (size in bp; encoded protein, size in kDa)	GenBank accession number
Genus: *Partitivirus*			
Aspergillus fumigatus partitivirus 1*	AfuPV-1	1 (1779; RdRP, 63)	FN376847
		2 (1623; CP, 48)	FN398100
Aspergillus ochraceus virus*	AoRV	1 (1754; RdRP, 62)	EU118277
		2 (1555; CP, 47)	EU118278
		3 (1220; unknown, 34)	EU118279
Atkinsonella hypoxylon virus*	AhV	1 (2180; RdRP, 78)	L39125
		2 (2135; CP, 74)	L39126
		3 (1790; satellite)	L39127
Botryotinia fuckeliana partitivirus-1*	BfPV	1 (1793; RdRP, 63)	AM491609
		2 (1566; CP, 48)	AM491610
		3 (1383; unknown, 14)	AM491611
Ceratocystis resinifera virus	CrV	1 (2207; RdRP, 77)	AY603052
		2 (2305; CP, 73)	AY603051
Ceratocystis polonica virus	CpV	1 (2315; RdRP, 77)	AY260756
		2 (2252; CP, 73)	AY247205
Discula destructiva virus 1*	DdV1	1 (1787; RdRP, 62)	NC_002797
		2 (1585; CP, 48)	NC_002800
		3 (1181; satellite)	NC_002801
		4 (308; satellite)	NC_002802
Discula destructiva virus 2*	DdV2	1 (1781; RdRP, 62)	NC_003710
		2 (1611; CP, 50)	NC_003711
Flammulina velutipes browning virus	FvBV	1 (1915; RdRP, 66)	AB465308
		2 (1730; CP, 60)	AB465309
Flammulina velutipes isometric virus	FvIV	1 (1919; RdRP, 69)	AB428575
Fusarium poae virus 1*	FpV-1	1 (2203; RdRP, 78)	NC_003884
		2 (2185; CP, 70)	NC_003883
Fusarium solani virus 1*	FsV-1	1 (1645; RdRP, 60)	D55668
		2 (1445; CP, 44)	D55669
Gremmeniella abietina virus MS1*	GaV-MS1	1 (1782; RdRP, 61)	NC_004018
		2 (1586; CP, 47)	NC_004019
		3 (1186; satellite)	NC_004020
Gremmeniella abietina virus MS2*	GaV-MS2	1 (1781; RdRP, 62)	NC_006444
		2 (1586; CP, 47)	NC_006445
		3 (1186; satellite)	NC_006446
Helicobasidium mompa partitivirus*	HmPV-V1-1	1 (2247; RdRP, 83)	AB110979
	-V1-2	1 (1776; RdRP, 63)	AB110980
	-V-70	1 (1928; RdRP, 70)	AB025903
Heterobasidion RNA virus 3	HetRV3-ec1	1 (1885; RdRP, 69)	FJ816271
		2(1826; CP, 57)	FJ816272
Heterobasidion RNA virus 2	HetRV2-pa1	1 (2290; RdRP, 85)	HM565953
		2 (2238; CP, 73)	HM565954
Heterobasidion annosum P-type partitivirus*	HaPV	1 (2325; RdRP, 87)	AF473549
Ophiostoma partitivirus 1	OPV-1	1 (1744; RdRP, 63)	AM087202
		2 (1567; CP, 46)	AM087203
Oyster mushroom isometric virus 2	OMV	1 (2038; RdRP, 70)	AY308801
Penicillium stoloniferum virus F*	PsV-F	1 (1677; RdRP, 62)	NC_007221
		2 (1500; CP, 47)	NC_007222
		3 (677; unknown)	NC_007223
Penicillium stoloniferum virus S*	PsV-S	1 (1753; RdRP, 62)	AM040148
		2 (1581; CP, 47)	AM040149
Pleurotus ostreatus virus	PoV	1 (2296; RdRP, 82)	NC_006961
		2 (2223; CP, 71)	NC_006960
Rhizoctonia solani virus 717*	RhsV-717	1 (2363; RdRP, 86)	NC_003801
		2 (2206; CP, 76)	NC_003802
Rosellinia necatrix virus 1-W8	RnV-1-W8	1 (2299; RdRP, 84)	NC_007537
		2 (2279; CP, 77)	NC_007538
Sclerotinia sclerotiorum partitivirus S	SsPV-S	1 (1874; RdRP, 68)	NC_013014
Genus: *Alphacryptovirus*			

Beet cryptic virus 1*	BCV-1	1 (2008; RdRP, 73)	EU489061
		2 (1783; CP, 53)	EU489062
Beet cryptic virus 3*	BCV-3	2 (1607; RdRP, 55)	S63913
Carrot cryptic virus*	CaCV	1 (1971; RdRP, 73)	FJ550604
		2 (1776; CP, 54)	FJ550605
Chondrostereum purpureum cryptic virus 1*	CPCV	1 (1920; RdRP, 68)	AM999771
		2 (1757; CP; 53)	AM999772
Fig cryptic virus	FCV	1 (1696; RdRP, 54)	FR687854
		2 (1415; CP, 38)	FR687855
Fragaria chiloensis cryptic virus*	FCCV	1 (1734; RdRP, 56)	NC_009519
		2 (1479; CPA, 39)	NC_009521
		2 (1465; CPB, 39)	NC_009520
Pyrus pyrifolia cryptic virus*	PpV	1 (1592; RdRP, 55)	AB012616
Raphanus sativus cryptic virus 1*	RSCV-1	1 (1866; RdRP, 67)	NC_008191
(or Radish yellow edge virus*)	(RYEV)	2 (1791; CPA, 56)	NC_008190
		3 (1778; CPB, 55)	EU285027
Raphanus sativus cryptic virus 2*	RSCV-2	1 (1717; RdRP, 55)	DQ218036
		2 (1521; unknown)	DQ218037
		3 (1485; unknown)	DQ218038
Raphanus sativus cryptic virus 3*	RSCV-3	1 (1609; RdRP, 55)	NC_011705
		2 (1581; unknown)	FJ461350
Rose cryptic virus*	RoCV	1 (1749; RdRP, 56)	NC_010346
Rosa multiflora cryptic virus*	RmCV	1 (1762; RdRP; 56)	EU024675
		2 (1475; unknown)	EU024676
		3 (1384; unknown)	EU024677
Vicia cryptic virus*	VCV	1 (2012; RdRP, 73)	NC_007241
		2 (1779; CP, 54)	NC_007242
White clover cryptic virus 1*	WCCV-1	1 (1955; RdRP, 73)	NC_006275
		2 (1708; CP, 54)	NC_006276

Unclassified viruses in the family *Partitiviridae*

Amasya cherry disease-associated partitivirus	ACDPV	1 (2002; RdRP, 73)	NC_006441
		2 (1839; CP, 55)	NC_006440
Black raspberry cryptic virus	BRCV	1 (1283: RdRP; 44) [b]	ABU55400
Cherry chlorotic rusty spot associated partitivirus	CCRSAPV	1 (2021; RdRP, 73)	NC_006442
		2 (1841; CP, 55)	NC_006443
Helicobasidium purpureum partitivirus	HpV	1 (486; RdRP, 19) [b]	AY949837
Heterobasidion annosum virus	HaV1	1 (1101; RdRP, 43) [b]	AF348136
Heterobasidion parviporum partitivirus	HpPV	1 (1025; RdRP, 34) [b]	CBJ23785
Nectria radicicola virus L1	NrV-L1	1 (1286; RdRP, 50) [b]	AF251278
Ophiostoma quercus partitivirus	OqV	1 (399; RdRP, 15) [b]	AM111099
Pepper cryptic virus	PCV	1 (1069; RdRP, 35) [b]	ABC96789
Picea sitchensis virus1	PsV	1 (1392; RdRP, 47) [b]	BT122981
Pinus sylvestris partitivirus	PsPV	1 (1078; RdRp, 41) [b]	AAY 51483
Pittosporum tobira cryptic virus-1	PCV-1	1 (676; RdRP, 26) [b]	GU595166
Primula malacoides virus 1	PmV1	1 (2390; RdRP, 84)	EU195326
		2 (2344; CP, 75)	EU195327
Vicia faba partitivirus 1	VfPV-1	1 (1915; RdRP, 67)	DQ910762

[a] An asterisk next to the virus name indicates it is presently recognised by the ICTV as a member or a tentative member in the family *Partitiviridae*. Family members or tentative members for which no sequence data is available are not included but partial sequences are included [b]. This table was adapted from that shown in [7].

Supplementary Fig S1. Alignment of the 5'-UTRs (Fig. S1a) and 3'-UTRs (Fig. S1b) of AfuPV-1 dsRNAs 1 and 2, both of which show similarities, indicated by asterisks, especially at the termini of the dsRNAs.

(a)

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AfuPV-1-RNA1     (1) CGCAAAAGA----CCCUUGUCUUUUACCGCCUUGCGGG----------------------
AfPuV-1-RNA2     (1) CGCAAAAGGAACCCUUUUGACUCCAGUGUAUCUGGAGGGUUUACGCUAAAAUUUAUAAAA
                     ********     *  *** **          **  **

AfuPV-1-RNA1    (35) -----------------UUUUCUUGCCUCCUUCUCUUCUUGAGCGUAA
AfuPV-1-RNA2    (61) CCGUAAACUAAAUUAUUUCCUCGAGCCUCCUUCUCU----AAGCGACC
                                      *  **  ************     ****
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(b)

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AfuPV-1-RNA1 (1695) GAGUGUCUUCUUCUGCAGAACGCAGGU---------------------------------
AfuPV-1-RNA2 (1434) ACAUAGCCAGUACUUGAAGAAACCAGUUUAUCUGGUCAAGCCCAACCUUACCACUAGAUG
                       *  *   * **  *  *  *  **

AfuPV-1-RNA1 (1722) ----------------------------CUUCCUGUUUAUCAGGAGGAU------------
AfuPV-1-RNA2 (1494) UCUGGCCGUUUAUCGGUAGCGCUAGUGUUGCCUGUCUAUCAGGAAGACCCGUGUAUCGGG
                                              * ***** ******** **

AfuPV-1-RNA1 (1743) -----------------------------GUAACGU-GCCGUUUAUCGGCGC---UUUCC
AfuPV-1-RNA2 (1554) CCAUCUAUUUCCUAUCAAGGAGGUGGGUGAUAACGUGGCCGUUUAUCGGUACGUGUUCAC
                                                ****** ************  *   **  *

AfuPV-1-RNA1 (1770) GUUAUAUCCA
AfuPV-1-RNA2 (1614) UGUAAAUCCA
                       ** *****
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The effects of dsRNA mycoviruses on growth and murine virulence of *Aspergillus fumigatus*

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ABSTRACT

Some isolates of the opportunistic human pathogenic fungus *Aspergillus fumigatus* are known to be infected with mycoviruses. The dsRNA genomes of two of these mycoviruses, which include a chrysovirus and a partitivirus, have been completely sequenced and an RT-PCR assay for the viruses has been developed. Through curing virus-infected *A. fumigatus* isolates by cycloheximide treatment and transfecting virus-free isolates with purified virus, as checked by RT-PCR, isogenic virus-free and virus-infected lines of the fungus were generated whose phenotypes and growth have been directly compared. Mycovirus infection of *A. fumigatus* with either the chrysovirus or the partitivirus resulted in significant aberrant phenotypic alterations and attenuation of growth of the fungus but had no effect on susceptibility to common antifungals. Chrysovirus infection of *A. fumigatus* caused no significant alterations to murine pathogenicity.

1. Introduction

Viruses are ubiquitous in all major groups of filamentous fungi and those with RNA genomes are now classified into 10 families, of which four accommodate double-stranded RNA (dsRNA) viruses and the remaining six comprise single-stranded RNA viruses (Ghabrial and Suzuki, 2009). Most mycoviruses cause cryptic infections but some cause phenotypic alterations including hypovirulence and debilitation (Nuss, 2010). In the genus *Aspergillus* dsRNA mycoviruses occur in 0–13% of the species examined and are common in several asexual species, where they vary widely both in number and composition, but are rare in sexual *Aspergilli* (Hammond et al., 2008; van Diepeningen et al., 2008; Varga et al., 1998). In terms of *Aspergillus* mycovirus classification a third of all *A. foetidus* isolates examined contain a mixture of a totivirus, an uncharacterised unipartite RNA species and four polyadenylated dsRNAs (Ratti and Buck, 1972; Coutts, unpublished results), isolates of *Aspergillus niger* contain individual or mixed infections consisting of four polyadenylated dsRNAs, a totivirus, and a chrysovirus (Buck et al., 1973; van Diepeningen et al., 1998; Hammond et al., 2008), isolates of *Aspergillus flavus* contain a chrysovirus (Wood et al., 1974; Jamal, unpublished results) and *A. ochraceus* isolates contain a partitivirus, the genome of which has been fully sequenced (Liu et al., 2008). Evidence has been presented that at least 1 of 3 partially characterised *Aspergillus nidulans* dsRNA mycoviruses transferred from *A. niger* by protoplast fusion (van Diepeningen et al., 2006) is a target and a suppressor of RNA silencing which generates virus-derived, small interfering RNAs (siRNAs; Hammond et al., 2008).

Previously we reported that, in contrast to a previous study (Varga et al., 1998), in a screen of 366 clinical and environmental isolates of *Aspergillus fumigatus* for mycoviruses, 6.5% of the isolates were found to contain three distinct groups of dsRNA segments, which were occasionally present as mixtures (Jamal et al., 2010). Subsequently we have cloned and sequenced the complete genomes of 2 of these mycoviruses which are respectively members of the *Chrysovirus* genus (Jamal et al., 2010), and the *Partitivirus* genus (Bhatti et al., in press). The most common dsRNA profile found in 4% of the isolates, which has been noted previously in total nucleic acid extracts of the prototype *A. fumigatus* isolate AF293 (Warn et al., 2006), was that of a multipartite mycovirus consisting of at least four dsRNA segments which are yet to be cloned and sequenced. In this investigation isogenic lines of virus-free and virus-infected *A. fumigatus* for both chrysovirus and partitivirus infections have been compared with respect to phenotypic

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differences and effects on host fitness, quantified with respect to growth rate and biomass. The implications of these results regarding transmission limits for hosts and mycoviruses were discussed and the virulence and pathogenicity of selected isogenic lines of *A. fumigatus* in mice and their sensitivities to commercial fungicides were also examined. The aim of this investigation was to discover the influence that mycovirus infection has on the fitness and pathogenicity of the human pathogenic mould *A. fumigatus*.

2. Materials and methods

2.1. Culture of A. fumigatus isolates

A. fumigatus (Nakazawa) isolate A-56 infected with *A. fumigatus* chrysovirus (AfuCV; Jamal et al., 2010), isolate 88 infected with *A. fumigatus* partitivirus-1 (AfuPV-1: Bhatti et al., in press), a yellow pigmented, virus-free, isolate Af-237y (Aufauvre-Brown et al., 1998) and all other derived isolates were grown on *Aspergillus* complete medium (ACM) agar (Cove, 1966) or minimal medium (MM) agar (Pontecorvo et al., 1953) for 5 days. Spores were harvested from the cultures and used to inoculate 500 ml of ACM broth cultures which were then grown in flasks, incubated with shaking at 130 rpm at 37 °C. After 5 days growth the mycelium was harvested by gravity filtration on Miracloth (Calbiochem), washed with water, dried briefly on filter paper and stored at −80 °C until further processing.

2.2. Attempts to cure A. fumigatus of mycovirus infection

Spores ($n = 500$) of virus-infected *A. fumigatus* isolates A-56 and 88 were point-inoculated on ACM agar containing a range of concentrations of cycloheximide (0.01–150 mM) and incubated at 37 °C for 10 days. Spores were harvested and incubated for a further 7 days on ACM agar containing 0.01–150 mM cycloheximide. New spores were then collected, transferred to ACM recovery medium without cycloheximide, and incubated for a further 7 days prior to harvest and inoculation of ACM broth. After two weeks incubation total RNA extracts were isolated from the resultant fungal mycelial mats using the RNeasy Plant Mini kit (Qiagen, Crawley, UK). RT-PCR assays were then performed using oligonucleotide primers designed to generate amplicons representing the complete coding regions of the RNA dependent RNA polymerase (RdRP) and capsid protein (CP) genes predicted from the sequences of AfuCV dsRNAs 1 and 2 *viz*. CVRP-F (5′-ATGACATTCGGACATA CCAAC-3′) and CVRP-R (5′-TTAGCCATACTGCAAATTACT-3′) and CVCP-F (5′-ATGGCTATGGCAACATACAA-3′) and CVCP-R (5′- ACTT ACTCCTCTCTGTTGAAC-3′) respectively (Jamal et al., 2010); and the RdRP gene predicted from the sequence of AfuPV-1 dsRNA 1 (PVR-F (5′-ATGGAAGATTATACTCAAGATC-3′) and PVR-R (5′-GC CATAGGCGTAGAAGATTGAT-3′) (Bhatti et al., in press). RT-PCR amplification was performed as described previously and the products examined on agarose gels stained with ethidium bromide (Coutts et al., 2004).

2.3. Infection of virus-free A. fumigatus isolates with purified A. fumigatus mycoviruses

Protoplasts of *A. fumigatus* isolates Af-237y and A-56-C, which is an isogenic line of isolate *A. fumigatus* A-56 cured of virus infection following cycloheximide treatment, were generated from hyphae using a similar procedure to that originally described by Tang et al. (1992) as modified by Szewczyk et al. (2007). Partitivirus and chrysovirus virions were purified from the respective parental wild-type *A. fumigatus* isolates 88 and A-56 as described previously (Jamal et al., 2010) and used to inoculate protoplasts of the respective virus-free isolates in the presence of polyethylene glycol 6000 (10 μl at 0.05 μg/ml/2×10^7 protoplasts) substituting virions for transforming DNA (Szewczyk et al., 2007). This procedure is a similar protocol to one described previously for a mycovirus (Hillman et al., 2004). Spore-producing colonies were then isolated on an agar-based medium containing 5 mM ammonium tartrate, 1% glucose, 1 M sucrose and ACM salt solution (Cove, 1966). Following incubation at 37 °C for 36–48 h, individual colonies were inoculated into ACM broth and onto agar plates and incubated at 37 °C for 4 days. Total RNA extracts were prepared from the liquid cultures using the RNeasy kit as before and assayed for the presence of either virus using RT-PCR analysis as described in Section 2.2. Glycerol stocks of individual, spore-producing colonies were made and those testing positively for infection with either virus were passaged sequentially on ACM agar and re-tested by RT-PCR to initially assess any potential retention of virus on the surface of fungal mycelia during the inoculation and culture process and subsequently to confirm successful transfection.

2.4. Effects of virus infection on mycelial growth, colony morphology and biomass of A. fumigatus

Equal numbers of spores ($n = 500$) of isogenic lines of the *A. fumigatus* isolates A-56-C, A-56, Af-237y and Af-237y-88, the yellow isolate transfected with AfuPV-1 as described in Section 2.3, were centrally inoculated onto MM and ACM agar on Petri plates and incubated at 37 °C. The colony diameters of the four isolates were measured every 24 h over a period of 5 days and growth rate per time interval used to calculate the average growth rate per day. Time points were selected such that fungal growth had initiated before the first measurement, and the last measurement was made before the mycelium reached the Petri dish edge. This ensured that the measurements can be considered as estimates of axial growth rates in the exponential growth phase. All experiments were performed in triplicate.

To assess biomass production equal numbers of spores ($n = 3 \times 10^8$) of the four isolates were inoculated into 100 ml flasks containing 60 ml of ACM or MM broth and incubated at 37 °C on a rotary shaker (130 rpm) over a period of 5 days. The mycelium from individual cultures was harvested daily by filtration through Miracloth and the pellets dried at 37 °C until their weights were constant and biomass produced per time interval used to calculate the average growth rate per day. All experiments were performed in triplicate. Student's *t* test was used to analyse all of the data for significant differences in growth rate and biomass production.

2.5. Comparison of the effects of chrysovirus infection of isogenic lines of A. fumigatus on murine virulence

In order to assess fungal burden as a quantitative virulence criterion, quantitative PCR (qPCR) was performed on total genomic DNA extracted from homogenised whole lungs using a previously described procedure (Bergmann et al., 2009). For analysis of fungal burden in corticosteroid-treated animals immunosuppression was induced with subcutaneous injections of hydrocortisone acetate (112 mg/kg on days −3, −1, and +2). On Day 0 inocula containing 1.5×10^6 conidia were prepared from *A. fumigatus* isolates A56 and A56-C in 40 μl of saline. Spores were cultured on slants of solid ACM complete medium for 5 days at 37 °C prior to harvest. Spores were filtered through Miracloth and washed twice with saline prior to enumeration. Mice were anaesthetised by inhalation of isofluorane and infected by intranasal instillation. Bacterial infections were prevented by adding 1 g/L tetracycline and 64 mg/L ciproxicin to drinking water. Fungal burden was assessed by qPCR amplification using the oligonucleotides BT-F (GAGCCCTTTT CCGACCTGAT) and BT-R (GGAACTCCTCCCGGATCTTG) to amplify

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the beta tubulin (AFUA_7G00250) locus from the *A. fumigatus* genome, and the oligonucleotides Actin-F (CGAGCACAGCTTCTTT GCAG) and Actin-R (CCCATGGTGTCCGTTCTGA) to amplify the murine actin locus (NM_007393). Quantification of both gene targets was performed in triplicate, using independent infected animals. A modified mathematical model (incorporating adjustment for fungal inoculum) for relative quantification of *A. fumigatus* actin, and murine beta-tubulin was adopted for each mouse sample using the formula DCIB = $(E_{\text{target}})\Delta^{\text{Ct(fungal actin} - \text{murine tubulin)}}$ where DCIB is dose-corrected infection burden and E = efficiency of amplification of target gene and is equal to 2, based on the mean of 8 independent murine infections (Pfaffl, 2001; data not shown).

3. Results and discussion

3.1. Effects of mycovirus infection on the phenotype, growth and virulence of A. fumigatus

The generation of isogenic lines of *A. fumigatus*, with or without mycovirus infection, obtained by curing with cycloheximide or virus transfection, facilitated phenotype comparisons and quantification of fitness effects. Cycloheximide was first used to cure yeast of dsRNA virus infection (Fink and Styles, 1972) and subsequently used in a similar fashion to cure *Aspergillus* spp. of mycovirus infection (Elias and Cotty, 1996) and here with AfuCV. Only the highest concentration of cycloheximide of 150 mM investigated was successful in curing wild-type, *A. fumigatus* isolate A-56 of infection with AfuCV to produce isolate A-56-C but the treatment did cause a dramatic reduction in growth rate. However these cultures were rescued on cycloheximide-free media and shown to be virus-free by RT-PCR following six serial passages. The effects of virus infection on the fitness of *A. fumigatus* were quantified by comparing the growth of virus-free and virus-infected isogenic lines on solid and liquid media. To investigate the effects of AfuCV infection on *A. fumigatus*, isolate A56-C was compared with isolate A-56, regenerated from single-hyphal tip cultures following successful transfection of protoplasts of isolate A56-C with purified AfuCV virions at an infection efficiency of >90% as determined by RT-PCR. Growth of these *A. fumigatus* isogenic lines revealed that AfuCV infection resulted in an abnormal phenotype on agar plates characterised by the formation of aconidial sectors and darker green pigmentation as compared to the cured isolate which was pigmented light green, uniform and non-sectored (Fig. 1a). This phenotype was identical to that found for the original A-56 isolate prior to curing with cycloheximide (results not shown). Also whilst not significantly slower growing on agar-based culture media the hyphal biomasses of A-56-C produced in MM and ACM broth were significantly greater than those produced by the virus-infected, A-56 isolate in either medium (Table 1). The introduction of purified virus particles into protoplasts from the virus-free isolate of the fungus A-56-C resulted in a newly infected mycelium with the same morphology and virus composition as the original virus-infected isolate A56. Growth comparisons between the original A-56 isolate and the A-56-C isolate successfully transfected with AfuCV gave identical results (results not shown).

These observations are similar to those made previously on the effects of virus-infection in *A. nidulans* where an uncharacterised mycovirus (*Aspergillus* virus 1816), which is related to AfuCV (Jamal et al., 2010), was transferred from *A. niger* by protoplast fusion and caused sectoring and a reduced growth rate (van Diepeningen et al., 1998, 2006; Hammond et al., 2008). Dissimilar to the *Penicillium* and the *Cryphonectria* chrysoviruses (Jiang and Ghabrial, 2004 and Liu et al., 2007, respectively), which are associated with latent infections of their hosts (Ghabrial, 2008), *Aspergillus* virus 1816, AfuCV and *Magnaporthe oryzae* chrysovirus 1 (van Diepeningen et al., 1998; this study; Urayama et al., 2010, respectively) are all associated with disease phenotypes of their hosts, impaired growth and unusual pigmentation.

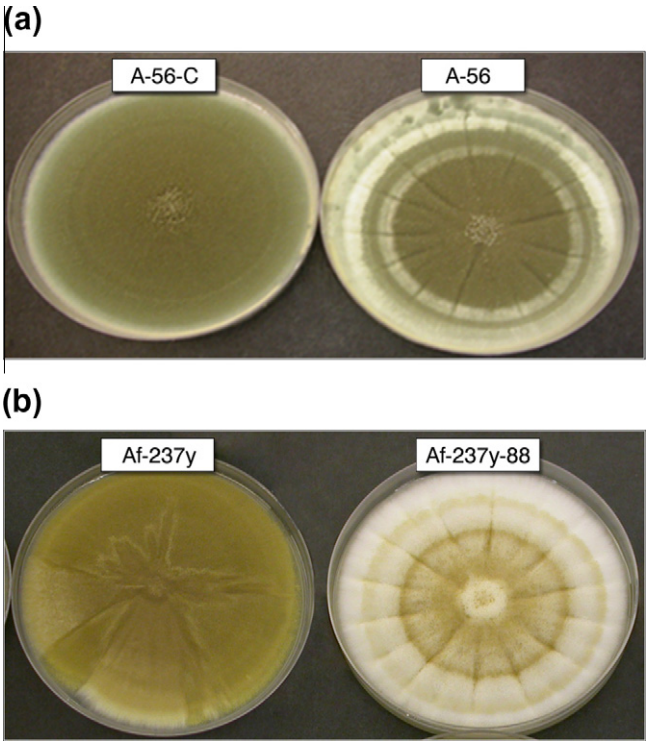

Fig. 1. Colony morphology of virus-free and virus-infected isogenic lines of *Aspergillus fumigatus*; (a) virus-free, cured A-56-C (left) and *Aspergillus fumigatus* chrysovirus (AfuCV) infected A-56 (right); (b) virus-free, Af-237y (left) and *Aspergillus fumigatus* partitivirus-1 (AfuPV-1) infected Af-237y-88 (right). Each strain was cultured on ACM at 37 °C for 5 days.

Table 1
Comparison of the growth rate and biomass of fungal isolates infected with AfuCV (A56) or AfuPV (Af-237y-88) and virus-free, isogenic lines A56-C and Af-237y respectively.

Fungal isolate	Radial growth (mm/day)[a]		Biomass (g dry weight/l)[b]	
	MM agar	ACM agar	MM broth	ACM broth
A56	6.75 ± 1.22	11.94 ± 0.45	3.20[c]	4.10[c]
A56-C	7.46 ± 1.14	11.94 ± 1.05	4.08	5.06
Af-237y-88	4.92 ± 0.82[c]	10.83 ± 0.33[c]	3.18[c]	4.33[c]
Af-237y	6.17 ± 0.77	12.00 ± 0.25	3.75	5.15

[a] Values of radial growth are the arithmetic means of three replicates (four/experiment) ± standard error of the mean.
[b] The values of biomass are arithmetic means of three replicative measurements.
[c] Statistically significant values; Independent samples *t*-test, $p < 0.05$.

As assessed by RT-PCR amplification assays, *A. fumigatus* isolate 88 could not be cured of AfuPV infection by cycloheximide treatment at a concentration of 150 mM and higher concentrations of the drug killed the fungus. Unsuccessful curing of mycovirus infection by treatment with cycloheximide is not an uncommon phenomenon (Ghabrial and Suzuki, 2009). Therefore to investigate the effects of AfuPV-1 infection on *A. fumigatus*, isogenic lines comprising the virus-free, yellow isolate Af-237y and a virus-infected isolate Af-237y-88 (Fig. 1b), regenerated from single-spores derived from Af-237y protoplasts transfected with purified AfuPV-1 virions at an efficiency of >75% as determined by RT-PCR, were compared.

AfuPV infection of the yellow *A. fumigatus* isolate resulted in an abnormal phenotype on agar plates consisting of aconidial sectors

and light pigmentation as compared to the virus-free isolate which was more pigmented and uniform (Fig. 1b). Several slightly different phenotypes were found in the AfuPV-1 infected isogenic lines with variable amounts of pigmentation and sectoring but following semi-quantitative RT-PCR analysis these were found to be not uniformly infected with virus whilst Af-237y-88 was (results not shown).

AfuPV infection significantly lowered the host fitness of *A. fumigatus* isolate Af-237y as determined by growth rate on solid media and hyphal biomass in broth (Table 1). Dissimilar to results obtained for isogenic, virus-infected and virus-free lines of *A. niger*, where host fitness was impaired differentially in rich media (van Diepeningen et al., 2006), the effects of mycovirus infection with either AfuCV or AfuPV-1 in *A. fumigatus* were not apparently masked by an abundance of available nutrients in rich ACM as compared to MM.

It is unusual for partitiviruses to cause phenotypic changes and virulence attenuation in their hosts as they are normally associated with latent infections (Ghabrial et al., 2008). However recent reports of the incidence of a partitivirus in the cultivated mushroom *Flammulina velutipes* associated with abnormally slow growth and the production of brown discoloured fruiting bodies (Magae and Sunagawa, 2010), similar to the effects reported here for AfuPV-1, confirms that partitiviruses can cause such effects. Interestingly a comparison of the susceptibility of all the virus-free and virus-infected isolates documented here to the common antifungals, amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, icafungin and anidulafungin, as assessed by minimal inhibitory concentration values (Petrou and Shanson, 2000) revealed no significant differences between the isolates (data not shown).

In the murine virulence assays dose-corrected infection burden (DCIB) values (see Section 2) of 4.68 and 4.51 were calculated for A-56-C and A-56 respectively, indicating no significant difference ($p = 0.7$) between the fungal burden of lungs infected with virus-free or chrysovirus-infected isogenic lines of *A. fumigatus* (Fig. 2). The statistical significance of variances between fungal burdens was calculated using a nonparametric Mann–Whitney *t*-test.

Although many dsRNA mycoviruses are apparently associated with symptomless infections, an increasing number of them have been identified that cause phenotypic changes and/or reduce the virulence in their fungal hosts (Ghabrial and Suzuki, 2009) including AfuCV and AfuPV-1. Modulation of fungal gene expression and alteration of phenotypic traits as a consequence of mycovirus infections are little understood with the exception of the chestnut blight fungus (*Cryphonectria parasitica*)/hypovirus system (Deng et al., 2007; Ghabrial and Suzuki, 2009). Down-regulation and variation in phenotype are hypothesised to be due to dsRNA interactions with fungal transcription mechanisms including the potential generation of siRNAs cleaved from mycovirus dsRNAs which may play a regulatory role. Accumulation of low levels of *A. niger* virus-derived siRNA have been detected previously by northern blotting (Hammond et al., 2008) and it will be interesting to investigate similar aspects with the *A. fumigatus* mycoviruses described here.

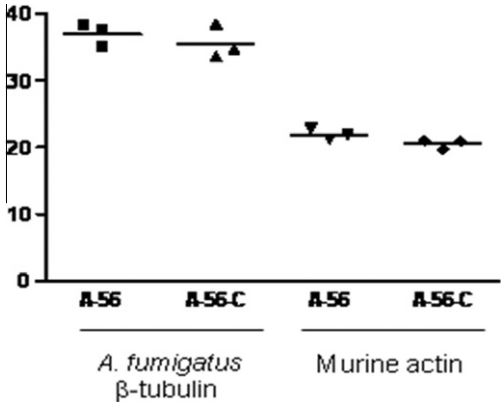

Fig. 2. Assessment of fungal burden, in a corticosteroid murine model using quantitative PCR. C_T values were measured for *A. fumigatus* β-tubulin and murine actin from total genomic DNA, extracted from homogenised lung tissue, following infection ($n = 3$ mice per *A. fumigatus isolate*) with a virus-infected isolate, A-56, and an isogenic, cured isolate, A-56-C.

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References

Aufauvre-Brown, A. et al., 1998. Comparison of virulence between clinical and environmental isolates of *Aspergillus fumigatus*. Eur. J. Clin. Microbiol. Inf. Dis. 17, 778–780.

Bergmann, A. et al., 2009. A regulator of *Aspergillus fumigatus* extracellular proteolytic activity is dispensable for virulence. Infect. Immun. 77, 4041–4050.

Bhatti, M.F., et al., in press. Complete nucleotide sequences of two dsRNAs associated with a new partitivirus infecting *Aspergillus fumigatus*. Arch. Virol.

Buck, K.W. et al., 1973. Two serologically distinct double-stranded ribonucleic acid viruses isolated from *Aspergillus niger*. Biochem. Soc.Trans. 1, 1138–1140.

Coutts, R.H.A. et al., 2004. Cherry chlorotic rusty spot and Amasya cherry diseases are associated with a complex pattern of mycoviral-like double-stranded RNAs II. Characterization of a new species of genus *Partitivirus*. J. Gen. Virol. 85, 3399–3403.

Cove, D.J., 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim. Biophys. Acta 113, 51–56.

Deng, F. et al., 2007. Comparative analysis of alterations in host phenotype and transcript accumulation following hypovirus and mycoreovirus infections in the chestnut blight fungus. Eukaryot. Cell 6, 1286–1298.

Elias, K.S., Cotty, P.J., 1996. Incidence and stability of infection by double-stranded RNA genetic elements in *Aspergillus* section *Flavi* and effects on aflatoxigenecity. Can. J. Bot. 74, 716–725.

Fink, G.R., Styles, C.A., 1972. Curing of a killer factor in *Saccharonzyces cerevisiae*. Proc. Natl. Acad. Sci. USA 69, 2846–2849.

Ghabrial, S.A., 2008. Chrysoviruses. In: Mahy, B.W.J., van Regenmortel, M.H.V. (Eds.), Encyclopedia of Virology, third ed. Elsevier Academic Press, The Netherlands, pp. 503–513.

Ghabrial, S.A., Suzuki, N., 2009. Viruses of plant pathogenic fungi. Ann. Rev. Phytopathol. 47, 353–384.

Ghabrial, S.A. et al., 2008. Partitiviruses: general features. In: Mahy, B.W.J., van Regenmortel, M.H.V. (Eds.), Encyclopedia of Virology, third ed. Elsevier Academic Press, The Netherlands, pp. 68–75.

Hammond, T.M. et al., 2008. *Aspergillus* mycoviruses are targets and suppressors of RNA silencing. Eukaryot. Cell 7, 350–357.

Hillman, B.I. et al., 2004. A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. J. Virol. 78, 892–898.

Jamal, A. et al., 2010. Complete nucleotide sequences of four dsRNAs associated with a new chrysovirus infecting *Aspergillus fumigatus*. Virus Res. 153, 64–70.

Jiang, D., Ghabrial, S.A., 2004. Molecular characterization of *Penicillium chrysogenum* virus: reconsideration of the taxonomy of the genus *Chrysovirus*. J. Gen. Virol. 85, 2111–2121.

Liu, Y.C. et al., 2007. Diversity of viruses in *Cryphonectria parasitica* and *C. nitschkei* in Japan and China, and partial characterization of a new chrysovirus species. Mycol. Res. 12, 1619–1628.

Liu, W. et al., 2008. Genomic characterization of a novel partitivirus infecting *Aspergillus ochraceus*. Virus Genes 37, 322–327.

Magae, Y., Sunagawa, M., 2010. Characterization of a mycovirus associated with the brown discoloration of edible mushroom, *Flammulina velutipes*. Virol. J. 7, 342.

Nuss, D.L., 2010. Mycoviruses. In: Borkovich, K.A. et al. (Eds.), Cellular and Molecular Biology of Filamentous Fungi. American Society of Microbiology, Washington, DC, USA, pp. 145–152.

Petrou, M.A., Shanson, D.C., 2000. Susceptibility of *Cryptococcus neoformans* by the NCCLS microdilution and Etest methods using five defined media. J. Antimicrobiol. Chemother. 46, 815–818.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29, E45.

Pontecorvo, G. et al., 1953. The genetics of *Aspergillus nidulans*. Adv. Gen. 5, 141–238.

Ratti, G., Buck, K.W., 1972. Virus particles in *Aspergillus foetidus*: a multicomponent system. J. Gen. Virol. 14, 165–175.

Szewczyk, E. et al., 2007. Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Prot. 1, 3111–3119.

Tang, C.M. et al., 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. 6, 1663–1671.

Urayama, S. et al., 2010. Mycoviruses related to chrysovirus affect vegetative growth in the rice blast fungus *Magnaporthe oryzae*. J. Gen. Virol. 91, 3085–3094.

van Diepeningen, A.D. et al., 1998. Intra- and inter-species virus transfer in aspergilli *via* protoplast fusion. Fungal Genet. Biol. 25, 171–180.

van Diepeningen, A.D. et al., 2006. Dynamics of dsRNA mycoviruses in black *Aspergillus* populations. Fungal Genet. Biol. 43, 446–452.

van Diepeningen, A.D. et al., 2008. Mycoviruses in the Aspergilli. In: Varga, J., Samson, R.A. (Eds.), *Aspergillus* in the Genomic Era. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 133–176.

Varga, J. et al., 1998. Double-stranded RNA mycoviruses in species of *Aspergillus* sections *Circumdati* and *Fumigati*. Can. J. Microbiol. 44, 569–574.

Warn, P.A., et al., 2006. Single-spore cultures of the sequenced strain of *Aspergillus fumigatus* (AF293) have significantly different mortality rates in a murine model of IPA. In: 2nd Advances Against Aspergillosis Meeting Syllabus P031.

Wood, H.A. et al., 1974. Proteinaceous virus-like particles from an isolate of *Aspergillus flavus*. J. Virol. 13, 532–534.

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