Enteroviruses and Chronic Fatigue Syndrome

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A thesis submitted to the Faculty of Medicine
of the University of Glasgow
for the degree of Doctor of Philosophy

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March, 1999

Acknowledgments

I would like to thank my supervisor, Dr. G.B. Clements for giving me the opportunity to carry out this work in the Regional Virus Laboratory and who, along with Dr. C. Gemmell, provided constructive criticism of this manuscript.
I would also like to acknowledge the M.E. Association and the Linbury Trust for providing the financial support for this project over the years.

My thanks go to Dr Donald Black of the Beatson Institute for Cancer Research for providing laboratory space in his department and to Mr Robert McFarlane for initial help with automated sequencing and for his expert technical advice with the cloning procedures.
I am also extremely grateful to Professor Jeffrey Almond and Dr David Evans who also generously provided laboratory space and expertise in the department of Animal and Microbial Sciences at Reading University and to Dr Ian Goodfellow who looked after me while I was there.
Acknowledgment must also go to Gwen Allardyce of the Scottish Centre for Infection and Environmental Health for statistical analysis (Odds ratio testing) in the early stages of the project.
I must also mention the staff of the Regional Virus Laboratory both past and present who made my time there an enjoyable and educational one. I also owe a great deal to Dr Daniel Galbraith who is always there with help and advice when I need it.

To my friends and family, who still have no idea what I actually do but who nonetheless have supported me throughout the years, I thank most sincerely and I dedicate this thesis to my late mother-in-law who was so proud of my achievements.

Finally, to my husband Andrew - you make everything worthwhile.

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LIST OF ABBREVIATIONS

AFP	acute flaccid paralysis
AHC	acute haemorrhagic conjunctivitis
ATP	adenosine-triphosphate
BDV	borna disease virus
cDNA	complementary DNA
CAR	coxsackie/adenovirus receptor
CDC	Centres for Disease Control
CDR	communicable disease report
CFIDS	chronic fatigue and immune dysfunction syndrome
CFS	chronic fatigue syndrome
CMV	cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CPHL	Central Public Health Laboratory
CSF	cerebrospinal fluid
DAF	decay accelerating factor
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EA	early antigen
EBV	Epstein-Barr virus
ECHO	enteric cytopathogenic human orphan
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMCV	encephalomyocarditis virus
EMEM	Earles minimum essential medium
EV	enterovirus
FBS	foetal bovine serum
FCS	foetal calf serum
FMDV	foot and mouth disease virus
GCG	Genetics Computer Group
HAV	hepatitis A virus
HCl	hydrochloric acid
HHV-6	human herpes virus 6
HPA	hypothalamic-pituitary-adrenal
HRV	human rhinovirus
HSV	herpes simplex virus

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CSF	cerebrospinal fluid
DAF	decay accelerating factor
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
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DTT	dithiothreitol
EA	early antigen
EBV	Epstein-Barr virus
ECHO	enteric cytopathogenic human orphan
EDTA	ethylenediaminetetraacetic acid
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GCG	Genetics Computer Group
HAV	hepatitis A virus
HCl	hydrochloric acid
HHV-6	human herpes virus 6
HPA	hypothalamic-pituitary-adrenal
HRV	human rhinovirus
HSV	herpes simplex virus

HTLV	human T-lymphotropic virus
ICAM	intercellular adhesion molecule
ICNV	international committee on the nomenclature of viruses
ICTV	international committee on the taxonomy of viruses
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPV	inactivated poliovirus vaccine
IPTG	isopropyl-1-thio-β-D-glactopyranoside
IRES	internal ribosome entry site
LDLR	low density lipoprotein receptor
LBM	Lim & Benyesh-Melnick
LPS	lipopolysaccharide
ME	myalgic encephalomyelitis
MEM	minimum essential medium
M-MLV	Moloney murine leukaemia virus
mRNA	messenger RNA
MRI	magnetic resonance imaging
NID	national immunization days
NK	natural killer
NPEV	non-polio enterovirus
NTR	non-translated region
OPA	One-Phor-All
OPV	oral poliovirus vaccine
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
PHA	phytohaemagglutinin
PIFS	post-infectious fatigue syndrome
PPM	parts per million
PV	poliovirus
PVFS	post-viral fatigue syndrome
PVR	poliovirus receptor
PWM	pokeweed mitogen
RGD	arginine/glycine/aspartic acid
RMK	rhesus monkey kidney
RNA	ribonucleic acid
RT	reverse-transcriptase
RVL	Regional Virus Laboratory

LIST OF PUBLICATIONS

Nairn, C. & Clements, G.B. (1999). A study of enterovirus isolations in Glasgow from 1977-1997. *Journal of Medical Virology*, accepted for publication.

Nairn, C., Galbraith, D.N., Taylor, K.W. & Clements, G.B. (1999). Enteroviruses in the serum of children at the onset of type 1 diabetes mellitus. *Diabetic Medicine*, accepted for publication.

Han, S., Clements, G.B., Nairn, C., Westmoreland, D., Llewelyn, M.B. & Fox, J.D. (1998). Molecular-based techniques for detection and analysis of enteroviruses in stool and serum samples. *Abstracts of the European Society for Clinical Virology*, Hamburg, Germany.

Galbraith, D.N., Nairn, C. & Clements, G.B. (1997). Evidence for enteroviral persistence in humans. *Journal of General Virology*, **78**, 307-312.

Riding, M.H., Galbraith, D.N., Nairn, C., Reid, D. & Clements, G.B. (1996). Genetic diversity of enteroviral meningitis: surveillance by direct sequencing of PCR products. *Abstracts of the Xth International Congress of Virology*, Jerusalem, Israel: 220.

Galbraith, D.N., Nairn, C. & Clements, G.B. (1995). Phylogenetic analysis of short enteroviral sequences from patients with chronic fatigue syndrome. *Journal of General Virology*, **76**, 1701-1707.

Nairn, C., Galbraith, D.N. & Clements, G.B. (1995). Comparison of coxsackie B neutralization and enteroviral PCR in chronic fatigue patients. *Journal of Medical Virology*, **46**, 310-313.

Clements, G.B., McGarry, F., Nairn, C. & Galbraith, D.N. (1995). Detection of enterovirus-specific RNA in serum: the relationship to chronic fatigue. *Journal of Medical Virology*, **45**, 156-161.

SUMMARY

Chronic Fatigue Syndrome is an illness characterized by severe, disabling fatigue of sudden onset that is not alleviated by rest, has been present for at least six months and causes a reduction in premorbid activity by at least 50%. Accompanying symptoms usually include myalgia, mood and sleep disturbance. Patients often describe the sudden onset of fatigue following a flu-like illness leading a number of investigators to propose an infectious cause.

Early serological evidence from Glasgow suggested an association between the enteroviruses (members of the *Picornaviridae*) and CFS in a high proportion of cases (Fegan *et al.*, 1983). This was followed by the use of PCR (polymerase chain reaction) assays to detect the highly conserved 5' non-translated region (NTR). This technique can detect the majority of the enteroviruses with the exception of echovirus 22 and 23. Initial work on muscle biopsies showed that 53% of biopsies from CFS patients were positive for enteroviral sequences compared to 15% of controls (Gow *et al.*, 1991). Obtaining muscle biopsies is not an easy task however and work prior to this thesis looked at testing serum samples as an alternative. In this initial group, 42% of CFS patients were positive by a nested PCR compared to 2% controls, demonstrating that serum samples were suitable to test for enteroviral sequences (Clements *et al.*, 1995). It has been suggested that enteroviruses persist in CFS patients, thus contributing to the chronic nature of the syndrome, but studies have not actually looked at enteroviral persistence over time. Rather, evidence has been based on a positive result from one sample only obtained from patients with a long history of illness and this does not provide direct evidence of persistence.

A prospective study was thus initiated to form the basis of this thesis to follow a group of patients with CFS and examine their serum for evidence of enteroviral sequences over time and to compare any positive PCR products by sequence analysis. Additionally, a questionnaire was developed to determine the clinical nature of the illness and to try to correlate this with the enteroviral status of the patient at a particular time.

Initial phylogenetic analysis of sequence derived from twenty serum positive samples showed that the sequences derived from CFS patients grouped apart from the known enteroviruses and from sequences derived from other clinical cases on a phylogenetic tree

(Galbraith *et al.*, 1995). The sequences were approximately 69-84% similar to the closest sequence of a coxsackievirus B3. However, the sequences could have been from known enteroviruses with no sequence data available, from known enteroviruses with variant 5' NTRs or from previously unknown enteroviruses. Further analysis at a later date with other available sequence data showed that the sequences grouped within the coxsackie/echovirus group although some were still 10-13% different. The variation observed was of the order of that seen between clinical isolates of the same serotype, thus the CFS patient sequences may have represented known enteroviruses with variant 5' NTRs. Without corroboration from other regions this tentative group could not be described further. The capsid region was chosen as an alternative target region since it is relatively conserved among the enteroviruses, containing the neutralizing regions which form the basis of the serotypes. Semi-nested priming could amplify the majority of the known enteroviruses to a sensitivity of 1 $TCID_{50}$, which was 100-fold less than the standard PCR. Testing of 55 samples previously positive by the standard PCR did not generate any positives. The use of other techniques (inverse PCR, long PCR and production of a cDNA library) to acquire additional sequence were also unsuccessful. Either the assays were not sensitive enough to detect the low levels of RNA present in the serum (as assessed by the standard PCR) or the genome is so different or deleted (perhaps due to the presence of defective-interfering particles) that amplification was not possible. Indeed, the discovery that 85% of enteroviral RNA samples lacked a poly-A tail may also point to there being atypical sequence present. Limited sample volume prevented this from being pursued further.

Persistence was examined by analyzing the 5' NTR sequence of two sequential samples obtained from sixteen patients. Many sequences, however, were more similar to those isolated in the same year than to their respective pair. Additionally, correlation with clinical data did not suggest a role for enteroviral persistence in the maintenance of the syndrome, although a positive correlation between severity of symptoms (based on ability to work and time spent walking for example) and the presence of enteroviral sequence was noted on analysis of first questionnaires from a cohort of 130 patients. This was not present on analysis of the second questionnaire from each patient. The average duration of fatigue for this group of patients was 3.9 years and thus questionnaire analysis proved difficult in terms of patients recalling features prior to their fatigue.

In a preliminary study, a group of patients who had a history of fatigue of approximately

six months was described. Enteroviral sequences were detected in the serum of 42% of CFS patients compared to 27% of acutely-ill patients and 2% of healthy control individuals. Additionally, a number of positives in the CFS patient group were detected after one round of PCR only, perhaps indicating a higher titre of enterovirus in these samples. While this study was being carried out there was an outbreak of echovirus 4 and this suggested therefore that the virus played a direct role in triggering the syndrome. Sequence analysis of PCR positives was not performed at this time to confirm this theory. Thus it may be useful to test patients shortly after the onset of fatigue where there is a history of a flu-like illness, when there is a greater chance of recovering a possible triggering agent, even though a formal diagnosis has not been made.

Consistently, a higher proportion of CFS patients than comparison individuals were positive for enteroviral sequences throughout this study (except in 1997) and thus this work does not rule out the enteroviruses as being involved in CFS. However, the methods employed did not elucidate any further sequence information nor provide a definitive answer regarding the role of these viruses in individuals with a long history of illness.

1. Introduction

1.1. Towards the concept of 'viruses'

By the end of the nineteenth century the existence of microorganisms such as bacteria, fungi and protozoa was well established. However, there were many infectious diseases at the time, such as measles and rubella, that could not be attributed to these agents and the search for alternative causes began.

In the late 1870s, Adolf Mayer discovered that tobacco mosaic disease could be transmitted to healthy tobacco plants by inoculating them with an extract of the diseased plant. He was unable to demonstrate the presence of any kind of disease agent, but continued to search for a bacterium as the cause. Dimitri Ivanofsky (1864-1920) also working on this disease confirmed some of the findings of Mayer, passing the infected plant material through a Chamberland filter (designed to retain most bacteria) and showing that it still retained infectivity. He suggested that the infective material might be a toxin produced by bacteria, but he did not pursue this idea. Also at this time, Martinus Beijerinck (1851-1931) demonstrated that the filtered plant material could be diluted and that it could regain its 'strength' after replicating in the living tissue of a plant. He showed that only living cells could be infected and postulated that the agent responsible must be incorporated into the living protoplasm of the cell and could not multiply outside the cell. He subsequently called the agent 'Contagium vivum fluidum', meaning contagious living liquid (Levine, 1996; Collier, 1998).

Together, Mayer, Ivanofsky and Beijerinck developed the idea of the existence of a filterable agent that was too small to observe in the light microscope, but was able to cause disease by multiplying in living cells. This agent was subsequently called a 'virus', from the Latin for slimy liquid or poison.

This idea of a filterable agent was accepted by many individuals who began to look in diseased tissues for these agents and in 1898 to 1899, Loeffler and Frosch described and isolated foot and mouth disease virus (FMDV), the first filterable agent from animals.

Work by Frederick Twort in 1915 and that of Felix d'Herelle at the Pasteur Institute on *Shigella* led to the discovery of viruses that killed bacteria. D'Herelle named these bacteriophages, and developed the plaque assay in 1917 for determining virus titre. From this date, bacteriophage studies were pivotal in the development of molecular biology, being used as models for the study of replication of other viruses.

Tobacco Mosaic Virus (T.M.V.) continued to be studied with a view to purifying the virus and identifying the biochemical nature of the particle. Crystallization of the virus was achieved in 1935 by Wendell Stanley, followed a few years later by X-ray crystallography of the T.M.V. crystals. The structure was confirmed in 1939 when the first electron micrographs of any virus were taken. Brenner and Horne described negative staining of viruses for electron microscopy in 1959 and this led to a great increase in knowledge regarding virus structure and it is still an important technique today.

It was however, the development of cell culture techniques (from culturing single cells by Sanford in 1948 to developing the HeLa cell line by Gey in 1952) that was crucial to the advancement of virology as a science. Discovering that poliovirus could replicate in non-neuronal human tissue (Enders *et al.*, 1949) led to the development of the poliovirus vaccine since the virus could be grown in cell culture whereas previously, viruses such as smallpox, rabies and influenza which were used for vaccines had been grown in animals or embryonated hen eggs. Poliovirus was the first animal virus to be subject to the plaque assay and this led the way for detailed study of this virus. Today many hundreds of viruses have been described (Levine, 1996).

1.2. Classification of Viruses

Early virus classification systems were based on what were regarded as common pathogenic properties (for example viruses causing hepatitis i.e. hepatitis A, hepatitis B and yellow fever were grouped together as 'hepatitis viruses'), common organ tropisms (for example respiratory viruses would have included those now known as influenza viruses, rhinoviruses and adenoviruses) and common ecological characteristics (for example, the arboviruses would have included agents now known as togaviruses, bunyaviruses and rhabdoviruses).

In the 1950's there was sufficient biochemical and morphological data on the nature of the virus particles to influence the classification of viruses. This eventually led to the first groupings based on shared virion properties. From this basis, taxonomic groups were constructed and included the herpesvirus group, myxovirus group and poxvirus group. There were also an increasing number of new human and animal viruses discovered at this time. This led to the establishment in 1966 of the I.C.N.V. (The International Committee on Nomenclature of Viruses) at the International Congress of Microbiology in Moscow. The function of the ICNV was to classify hundreds of viruses isolated from humans, animals, plants, invertebrates and bacteria (The ICNV changed to the International Committee for the Taxonomy of viruses (ICTV) in 1974). In its report of 1995, a universal taxonomy scheme for viruses was described. It comprised one order (suffix -virales), 71 families (suffix -viridae), 11 subfamilies (suffix -virinae) and 164 genera (suffix -virus), including many floating genera and more than 4000 member viruses (Murphy *et al.*, 1995).

Classification of viruses into families is mainly based on virion morphology (size, shape, symmetry, presence of envelope), and the nature of the genome (DNA or RNA, single or double stranded, linear or circular, positive-sense or anti-sense). Lwoff, Horne and Tournier laid down the basis for this scheme in 1962. Other factors such as biological properties (host range and mode of transmission), physicochemical properties such as pH stability, and antigenic properties are also important in identifying types and strains within the families. The relationship between viral mRNA and the virus genome forms the basis of the Baltimore scheme for the grouping of viruses (Baltimore, 1971). Groupings include the positive-sense single stranded RNA viruses typified by the Picornaviridae; double stranded RNA viruses such as the Reoviridae and double-stranded DNA viruses which replicate in the cytoplasm i.e. Poxviruses, as opposed to the nucleus i.e. Herpesviruses.

Today the techniques of molecular biology are widely available and sequencing of the nucleic acid of a new virus is often carried out before other data are available. Hepatitis C virus (HCV) for example, shows high levels of sequence variability, and cannot be classified into serotypes using neutralization assays because of the absence of simple virus culture methods. Instead, comparison of nucleotide sequence data has led to classification into genotypes that differ from each other by approximately 30% over the entire genome (Mellor *et al.*, 1995; Simmonds *et al.*, 1993).

1.3. Picornaviridae

1.3.1. Members of the *Picornaviridae* family

The *Picornaviridae* are examples of the single-strand positive sense RNA viruses, officially classified in 1963. They are among the smallest (*pico*) RNA viruses known, comprising a diverse group of human and animal pathogens. The picornavirus genera are distinguished by a number of criteria including buoyant density in caesium chloride, acid stability and size of capsid proteins.

Within the genera, species are distinguished immunologically by the ability of specific antisera to neutralize only homotypic virus. Table 1 shows the six genera of the family and the type species for each genus. The genus name parechovirus was recently approved by the ICTV (Pringle, 1997; Mayo & Pringle, 1998) to include human echovirus types 22 and 23.

Rhinoviruses inhabit the upper respiratory tract and are one of the causative agents of the acute afebrile upper respiratory diseases, which group clinically as the common cold. They are acid labile and have a low optimal temperature of replication which reflects their adaptation to the nasopharyngeal region. There are now over 100 recognized human serotypes and they have a buoyant density of 1.4g/ml.

The cardioviruses are rarely associated with human disease having been recovered mainly from rodents, but their true natural host is unknown. They are acid stable, have a buoyant density of 1.34g/ml and are represented by two serotypes: the encephalomyocarditis (EMC) viruses (EMC virus, Columbia SK virus, ME virus, MM virus and mengovirus) and Theiler's Murine Encephalomyelitis Viruses (TMEV).

Aphthoviruses rarely infect humans and are named for the vesicular lesions they produce in cloven-footed animals especially cattle, goats, pigs and sheep. They have a buoyant density between that of the enteroviruses and the rhinoviruses. They are highly labile being rapidly inactivated at pH 7 or less.

The hepatoviruses have also only recently been considered to be a distinct genus within the picornavirus family. Previously, human hepatitis A virus was classified as enterovirus 72 because it shared similar physical properties with the enteroviruses, such as acid stability, a buoyant density of 1.34g/ml and a similar genome and particle size. However, when the complete sequence of the hepatitis A genome was determined, it was found to share little homology with the enteroviruses at the molecular level and was thus reclassified (Melnick, 1990; Rueckert, 1996).

GENUS	TYPE SPECIES
Enterovirus	poliovirus 1
Rhinovirus	human rhinovirus (HRV)-1A
Cardiovirus	encephalomyocarditis virus (EMC)
Aphthovirus	foot and mouth disease virus (FMDV) O
Hepatovirus	human hepatitis A virus (HAV)
Parechovirus	echovirus 22

Table 1: The 6 genera within the *Picornaviridae* family and the type species for each genus.

1.3.2. Virus Structure

Virus structures have been determined by X-ray crystallography and at least one member from each picornavirus genus has been examined. Poliovirus is one of the most extensively characterized viruses but all the picornaviruses share similar features. They are non-enveloped, icosahedral particles of approximately 24-30nm in diameter. The protein capsid protects the RNA from nucleases, recognizes particular target cell receptors, determines the antigenicity of the virus particle and is involved in delivering the RNA through the cell membrane. The mature capsid is approximately 5nm thick and is composed of 4 types of protein- VP1, VP2, VP3 and VP4, one copy of each making up a sub-unit or protomer, the nomenclature following that of Rueckert and Wimmer (1984). These protomers are organized into pentameric units held together by a urea-sensitive binding domain. Twelve pentamers, held together by an acid-sensitive binding domain, associate to form the complete protein capsid (Figure 1A).

Proteins VP1, VP2 and VP3 are exposed at the viral surface and are composed of approximately 250 amino acids. They are relatively compact, each consisting of an eight stranded anti-parallel beta-barrel with two flanking helices, the wedge-shaped folding pattern of which is conserved in the capsid proteins of icosahedral eukaryotic RNA viruses. One anti-parallel sheet is made up of strands B, I, D and G and the other is made of strands C, H, E and F (Figure 1B), the two sheets forming the front and back of the barrel. The β sheets are joined at one end by 4 loops, the differences in size and conformation of which make the capsid proteins different. These loops cover the surface of the virion and mostly determine the antigenic sites of the virus. Five copies of VP1 surround each five-fold axis of the icosahedral particle, while VP2 and VP3 alternate around the three-fold axis. The beta barrels of VP1 are arranged in such a way that 3 loops are exposed and form a peak at the 5-fold axis of the pentamer. In the case of poliovirus and human rhinovirus, the major neutralizing antigenic sites reside in the exposed BC loop of VP1, whereas in aphthovirus the major sites reside in the GH loop (Rueckert, 1996).

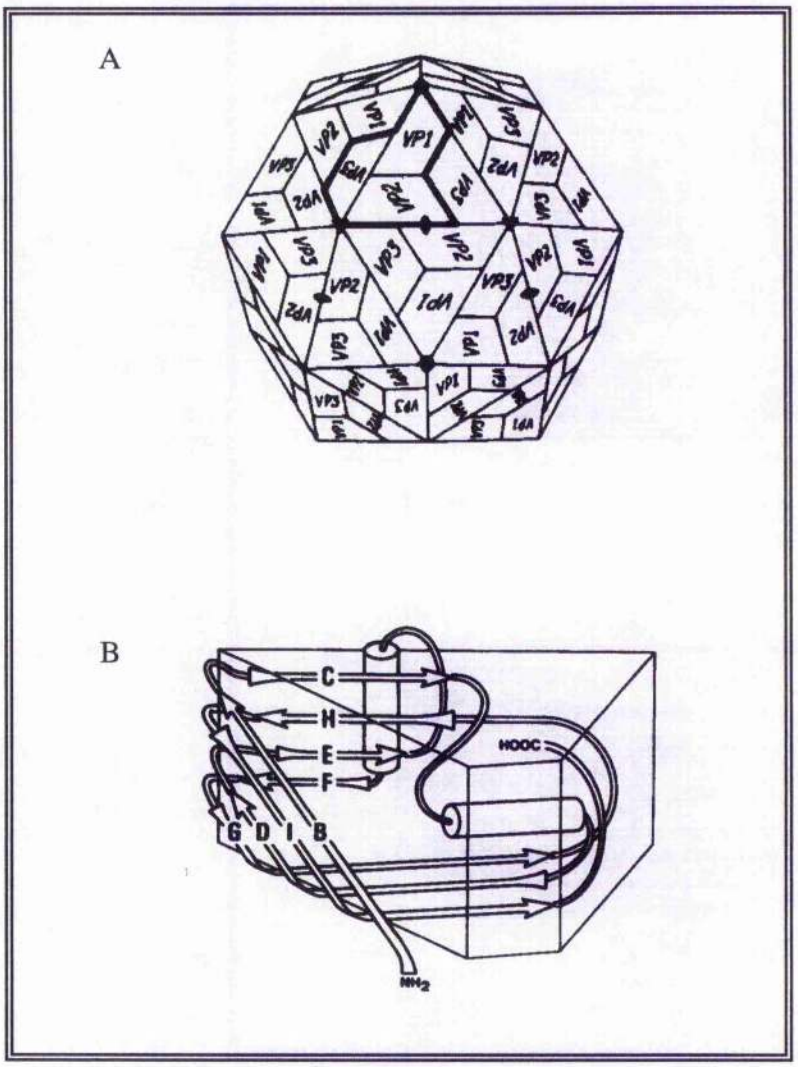


Figure 1: Picornavirus capsid structure showing VP1, VP2 and VP3 icosahedral arrangement (A). β-barrel structure of capsid protein (B) (Hellen & Wimmer, 1995).

The peak at the 5-fold axis is surrounded by a deep canyon in the human enteroviruses and rhinoviruses, but not in the cardioviruses and aphthoviruses. This canyon is inaccessible to antibodies because of steric hindrance but is thought to be the acceptor site for receptor binding. Indeed, poliovirus mutants which are resistant to soluble receptor (srr) have been identified. Mutations at the canyon surface at the interface between protomers, probably reduce binding affinity by interfering with receptor contact. A hydrophobic pocket of VP1 has also been identified, accessible through a pore in the canyon floor. In poliovirus types 1 and 3 this pocket is occupied by a sphingosine-like molecule (pocket factor) which is thought to stabilize the virus during transit from one host cell to the next. Mutations near the pocket may affect the positioning of this molecule and thus modulate the receptor contact region on the surface. This pocket factor can be displaced by anti-viral WIN compounds (from Sterling-Winthrop) which bind in the hydrophobic pocket and result in conformational changes in the canyon floor, also affecting the receptor binding process (Racaniello, 1995).

Of the four proteins, VP1 shows the greatest sequence variability probably because of its involvement in receptor interactions. VP4, containing 70 amino acids, is not exposed at the viral surface but is confined to the interior in close association with the RNA and thus shows the least variability (Rueckert, 1990). The N' terminus of each VP4 is covalently bound to a myristic acid residue (Chow *et al.*, 1987). The myristate sequences penetrate the pentameric apex, possibly forming a framework for assembly of the capsid.

1.3.3. Genome Organization

The first picornaviral RNA to be completely sequenced was poliovirus type 1 (Kitamura *et al.*, 1981; Racaniello & Baltimore, 1981) and subsequent sequencing of other members has shown a common genome organization (Figure 2). The length of the genome varies between 7209 bases (HRV14) and 8450 bases (FMDV). At the 5' end, there is a covalently attached protein of approximately 22 amino acids known as the VPg (virion protein, genome linked) which may play a role in the packaging of viral RNA or in the initiation of viral RNA synthesis (Grandien *et al.*, 1989). The next stretch, the 5' non-translated region (NTR), of between 600 and 1200 bases in length is highly conserved and

capable of forming secondary and tertiary interactions. It also contains the internal ribosomal entry site (IRES) the importance of which is discussed in section 1.3.4. The 5' NTR is followed by a long open reading frame (ORF), which is translated into a single large protein (polyprotein) that codes for the structural capsid proteins (P1 region), and the non-structural proteins (P2-P3 regions). Sequence variation is highest in VP1, which codes for the major antigenic sites, whilst variation in the non-structural proteins is considerably less. This is because many of the non-structural proteins are enzymes and thus extreme variation may be deleterious. In contrast, variation in the capsid proteins may be advantageous, enabling the virus to evade the immune responses of the host. At the 3' end, a second NTR ends with a variable length polyadenosine (poly-A) tract. Cardioviruses and aphthoviruses differ slightly from this organization encoding a leader (L) protein before the coding region that contains a polycytidylate (poly-C) tract. Additionally, the 2A protein of FMDV is very small (16 amino acid residues compared to approximately 140) and the genome has three individual copies of the VPg protein in tandem (Rueckert, 1996).

Phylogenetic analysis of the nucleotide sequences (partial and complete) of the picornaviral genomes can be used to present the genetic relationships of the family. Figure 3, based on nucleotide sequence comparisons of the P1 capsid region, shows the genera clustering according to their original classification, with the enteroviruses most closely related to the rhinoviruses and more distantly related to the aphthoviruses, cardioviruses and hepatoviruses.

The recently described parechoviruses, would cluster apart from the rhinovirus, enterovirus, apthovirus and cardiovirus group, much like the hepatoviruses, based on amino acid sequence identity of the VP1 capsid region (Ghazi *et al.*, 1998). Compared to the enteroviruses, the average amino-acid sequence identity of the parechoviruses was less than 20% (Oberste *et al.*, 1998).

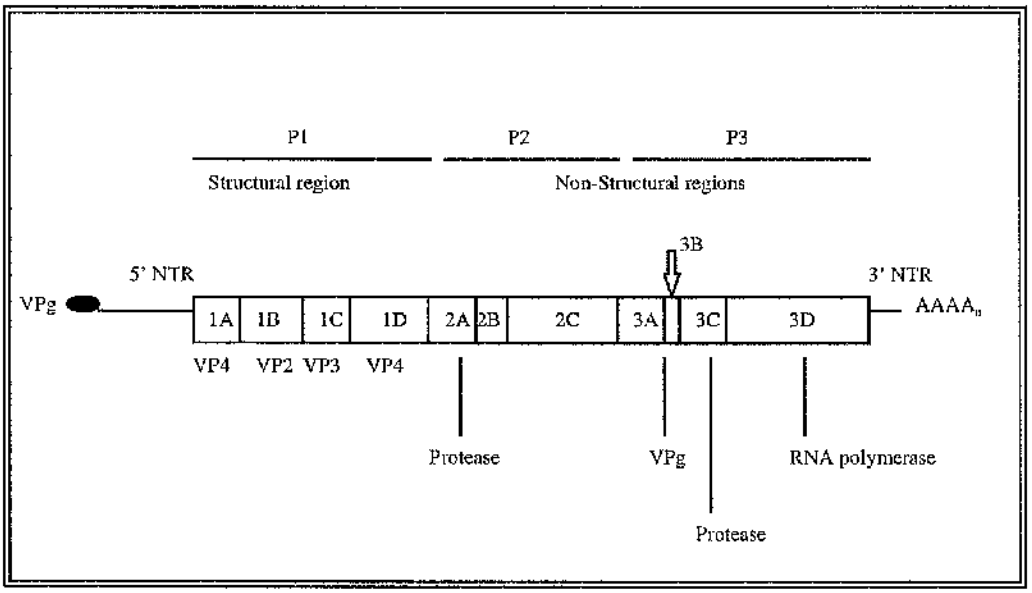


Figure 2: Diagrammatic representation of the genome organization of the *Picornaviridae*. The products of specific regions are indicated where known. Adapted from Field's "Virology" (Rueckert, 1996).

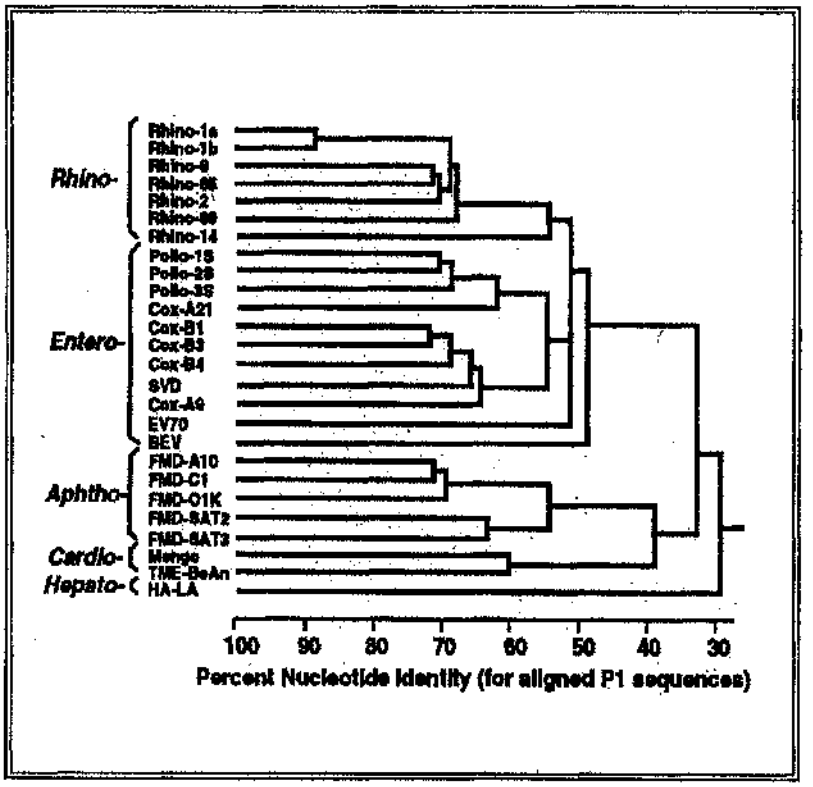


Figure 3: Phylogenetic clustering of members of the *Picornaviridae* based on sequence of the P1 capsid region (Rueckert, 1996).

1.3.4. Features of the 5' NTR

The 5' NTRs of the picornaviruses have highly conserved primary and secondary structures, and play a vital role in RNA replication and translation of viral proteins. Manual and computer assisted folding procedures have been used to predict the secondary structure for the 5' NTR of poliovirus RNA (Rivera *et al.*, 1988; Pilipenko *et al.*, 1989; Skinner *et al.*, 1989). Sub-optimal folding algorithms also showed conservation of secondary structure among the other enteroviruses and rhinoviruses which was supported by phylogenetic data (Le & Zuker, 1990; Poyry *et al.*, 1992). The nucleotide sequence variation observed in this region of all sequenced enteroviruses shows compensatory base changes, in general, that support the stem-loop structures (Poyry *et al.*, 1992, Poyry *et al.*, 1996). The aphthoviruses, cardioviruses and hepatoviruses have a different folding arrangement (Figures 4A and 4B). At the 5' end, approximately 90 nucleotides form a cloverleaf-like structure that is essential for replication and which also functions as a binding site for the viral polymerase (3CD) and a host protein with a relative molecular weight of 36,000 (Andino *et al.*, 1990, 1993).

The next section of the 5' NTR, a highly folded region of approximately 450 nucleotides long is known as the internal ribosome-binding site (IRES). Deletion experiments have been used to define the IRES borders at 130 and 600 nucleotides encompassing domains II to V and part of VI. Deletion experiments and mutation analysis have also been used to identify regions of importance in the IRES. For example, if position 200 in domain III is affected, the recombinant virus has a temperature sensitive phenotype and protein synthesis is affected detrimentally. Domain IV is a highly complex multiple stem-loop structure the details of which differ in several of the proposed models. Internal deletions and insertions can result in viruses with defective translation activity. In domain V, nearly all mutations that disrupt base pairing are lethal to virus replication and translation in vitro. A mutation (C to U) at position 472 has been identified as a major attenuating mutation of Sabin type 3 vaccine strain (Evans *et al.*, 1985). Similarly at position 480 in sabin type 1, an A to G mutation has been shown to contribute to attenuation (Kawamura *et al.*, 1989).

The downstream 5' NTRs of seven echoviruses with different neurovirulent phenotypes have been amplified and sequenced (Romero & Rotbart, 1995). The polypyrimidine tract of echoviruses 2, 4, 6, 9, 11 and 12 was shown to be twenty nucleotides in length, supporting the idea of a critical distance for efficient translation initiation. The

echoviruses with neurovirulent phenotypes, that is, those frequently causing infection of the central nervous system (CNS), possessed the same sequence as each other, also seen in highly neurovirulent poliovirus. The less neurovirulent types (echoviruses 2 and 12) were slightly different in this sequence which may decrease their ability to initiate translation, enabling the host to limit their spread to the CNS.

Picornavirus replication occurs in the cell cytoplasm, beginning with the attachment of the virus to its cellular receptor on the surface of the target cell. The details of the replication cycle, uncovered using poliovirus type 1/Mahoney as the model virus, have been described extensively elsewhere (Rueckert, 1996) and will not be expanded upon in this thesis. One important aspect of the replication cycle worth mentioning further is the mechanism of translation initiation. Picornaviruses lack the 7-methyl-guanosine cap structure found at the 5' end of eukaryotic cellular mRNA. In addition to the extensive secondary structure they have an unusually long 5' NTR and they contain multiple AUG codons all of which can be inhibitory to the initiation process. The 5' NTR is therefore not an easy target for the conventional initiation process and inefficient translation would be expected (Belsham & Sonenberg, 1996). In an *in vitro* cell-free translation system such as the rabbit reticulocyte lysate, enteroviral RNAs are very inefficient and additional aberrant internal translation products are generated. However, if cytoplasmic extracts from HeLa cells are added, the aberrant translation initiation is suppressed and viral protein synthesis is stimulated. Conversely, RNA from EMCV and FMDV is extremely efficient when translated in rabbit reticulocyte lysate systems and replicates efficiently and therefore must require different *trans*-acting factors from the enteroviruses.

Picornavirus translation initiation must therefore occur in a cap-independent manner and this involves the binding of ribosomes to the IRES. Both IRES types contain a highly conserved polypyrimidine tract located 20 to 25 nucleotides upstream of a conserved AUG triplet at the 3' end of the IRES. If this spacing is altered in any way then translation initiation is affected in a negative manner (Pilipenko *et al.*, 1992). Translation initiation of enterovirus and rhinovirus genomes does not usually occur at this AUG. It is likely that the ribosomes are transferred probably by a scanning mechanism to the correct AUG initiation codon, approximately 150 nucleotides downstream. With FMDV, approximately 30% of ribosomes initiate translation at the AUG at the 3' end of the IRES while the rest initiate at the next AUG downstream and almost all the cardiovirus ribosomes initiate translation at the AUG at the 3' end of the IRES.

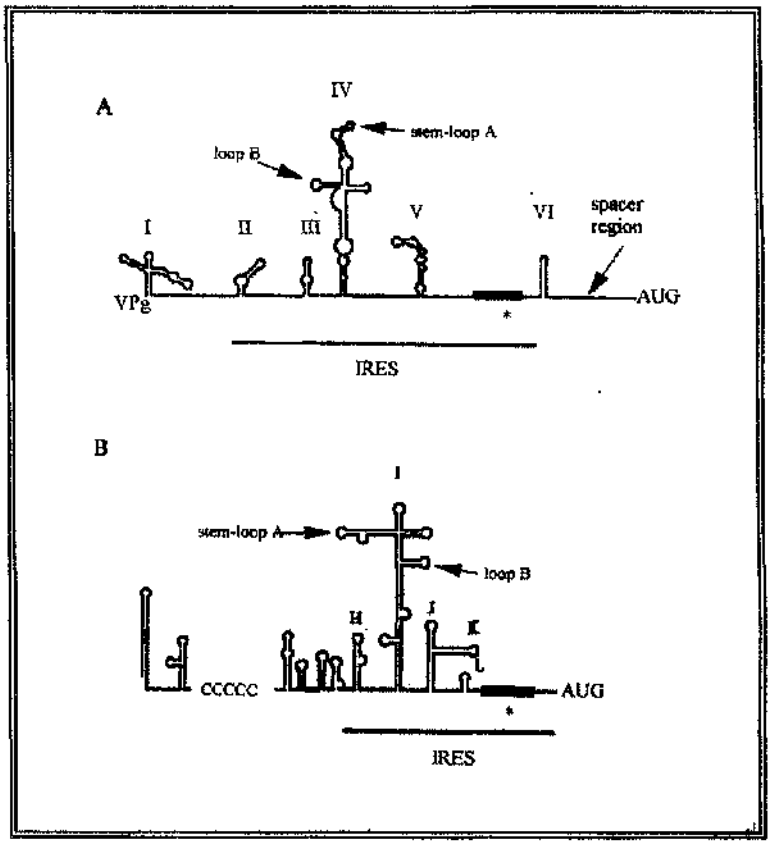


Figure 4: Secondary structure prediction for the enterovirus and rhinovirus 5' NTR elements (A) and the cardiovirus and aphthovirus 5' NTR elements (B) (Belsham & Sonenberg, 1996). Numbering of regions in A is according to the convention of Harber & Wimmer (1993). The polypyrimidine tract is indicated by *.

1.4. Enterovirus Genus

1.4.1. Background and properties of the enteroviruses

In 1908, Landsteiner and Popper described the transmission of poliomyelitis to monkeys by inoculation of a faecal extract from a patient with paralytic disease. Forty years later, Dalldorf and Sickles inoculated suckling mice with faecal samples from suspected poliomyelitis patients from the town of Coxsackie, New York. The mice became paralysed, but not from poliovirus infection. Polioviruses are now known to have a restricted host range in laboratory animals with most types infecting and causing flaccid paralysis in monkeys and chimpanzees only.

This new virus induced flaccid paralysis and widespread myositis of skeletal muscle in suckling mice and the isolates became the first prototypes of the group A coxsackieviruses. Other strains were identified that produced a different pathology causing spastic paralysis in suckling mice with some types producing pancreatitis, necrosis of brown fat pads, myocarditis, endocarditis and hepatitis in both suckling and adult mice. These became the prototypes of the group B coxsackieviruses (Godman *et al.*, 1952).

Virus types that were not pathogenic for new-born mice or neutralized by poliovirus antisera were also identified (Robbins *et al.*, 1951). These were named echoviruses (Enteric Cytopathogenic Human Orphan viruses) because at the time they could not be associated with any disease, but they now form the largest enterovirus subgroup. Some of the isolates initially reported as being 'novel' echoviruses had not been identified correctly. Echovirus 10 was actually a reovirus, echovirus 28 was found to be rhinovirus type A1 and echovirus 34 was shown to be a variant of coxsackievirus A24. Echovirus 22 although identified as a picornavirus in terms of genome organization, has little sequence identity with the other enteroviruses. The cytopathic effect observed is incomplete, compared to other enteroviruses, as is that of echovirus 23 which shares identity with echovirus 22 in certain parts of the genome. In addition analysis of the protein composition of echovirus 22 showed that VP0 does not undergo processing to VP2 and VP4 and that VP3 contains an apparent insertion of approximately 25 amino acids at its amino terminus. This has led to their recognition as an independent group of picornaviruses and to their reclassification (Auvinen & Hyypia, 1990; Hyypia *et al.*, 1992; Stanway *et al.*, 1994; Pringle; 1997; Mayo

& Pringle, 1998).

Classification of new enterovirus types as echoviruses was based on their failure to produce any disease in suckling mice or monkeys, but there are some variants that exhibit animal pathogenicity. A number of echoviruses produce asymptomatic infections in monkeys, and some echovirus 9 strains can cause paralysis in new-born mice leading to their early classification as coxsackievirus A23. Conversely, some strains such as coxsackie A9 lack pathogenicity and therefore resemble the echoviruses.

Thus with 67 types recognized all subsequently identified enteroviruses were designated 'enterovirus' and given a consecutive number starting with 68.

The human enteroviruses are distinguished from each other by neutralization of infectivity by specific antibodies (Table 2). Simian, porcine, bovine and insect enteroviruses have also been identified, but it is unusual for humans to have recognizable infections with the animal enteroviruses. Enteroviruses are stable over a pH range of 3-10 and are insensitive to ether, chloroform and deoxycholate. They have a buoyant density in caesium chloride of 1.32-1.35g/ml and a sedimentation co-efficient of 150s-165s. Treatment with 0.3% formaldehyde, 0.1N hydrochloric acid or free residual chlorine at a level of 0.3-0.5 ppm (parts per million) causes rapid inactivation that can be prevented in the presence of extraneous organic material (Melnick, 1990). Enteroviruses are also thermolabile and can be destroyed rapidly at 50°C unless magnesium chloride is present to inhibit the inactivation. This has led to the use of magnesium chloride as a stabilizer in poliovirus vaccines (Melnick *et al.*, 1961).

Human Enterovirus species	**Types**
poliovirus	types 1, 2 & 3
coxsackievirus group A	types A1-A22 & A24
coxsackievirus group B	types B1-B6
echovirus	types 1-9, 11-27, 29-33
enterovirus	types 68-71

Table 2: Human enteroviruses and types in each species.

1.4.2. Transmission and clinical features

Enteroviruses are transmitted mainly via the faecal-oral route, but infection can also be passed by droplets and aerosols from coughs and sneezes. Virus is generally shed for long periods in stools (up to a month or more) thus contaminating the sewage system where the virus can survive the chlorination process. In the United States the average concentration of enteroviruses in sewage is approximately 100 plaque forming units (pfu)/litre, a figure which may be 100 times higher in less developed countries. Enterovirus survival in the marine environment is aided by adsorption to sediment material, which can ultimately lead to the contamination of domestic water and of molluscs and crustaceans (Melnick, 1990).

The portal of entry for enteroviruses in humans is thought to be the alimentary tract via the mouth. Once acquired, the virus multiplies in the lymphoid tissue of the pharynx and gut and reaches the lymph nodes within a day. A minor viraemia occurs by the third day (primary viraemia) and then the virus can spread to several secondary sites and replicate there. This leads to a major viraemia occurring usually between the 3rd and 7th day that generally coincides with the appearance of clinical symptoms. Secondary spread of the virus thus occurs via the bloodstream to other susceptible sites including the spinal cord, meninges, myocardium and skin, depending on the infecting enterovirus. If a high level of multiplication of virus occurs as it spreads through the CNS, motor neurons are destroyed and paralysis occurs. Virus is usually excreted in the stools for several weeks and is present in the pharynx one to two weeks after infection in an individual with a clinical or sub-clinical infection. Enteroviruses can also be recovered from pharyngeal washings, spinal cord and cerebrospinal fluid (CSF), brain, heart, blood, urine, conjunctivae and lesions of the skin and mucous membranes.
Multiplication of one type of enterovirus in the alimentary tract may interfere with the replication of another which may explain why live poliovaccine can fail in patients with concurrent enteroviral infections (Parks *et al.*, 1967).

Enterovirus type 70, in contrast to the other enteroviruses, is spread by fomites and by direct inoculation of the conjunctiva from contaminated fingers. Incubation times are relatively short (12 to 72 hours) and replication occurs preferentially at 33-35°C, due to their adaptation to conjunctiva temperatures (Jin-Murphy, 1973; Melnick, 1990).

Enteroviruses are not predominantly associated with enteric disease but they are so named because the human alimentary tract is the main site of replication. Single types can cause a number of distinct clinical syndromes while a given clinical syndrome can be associated with many different enterovirus types, as outlined in Table 5. For an enterovirus to be associated with a particular disease, homotypic antibodies must be present; evidence for infection by other likely candidates must be negative; the virus must be recoverable from body fluids or target organs and the recovery rate from patients with the illness must be significantly higher than from healthy persons of similar age and socio-economic setting in the same area at the same time. Infections with the enteroviruses however, are characterized by a high proportion of sub-clinical manifestations. In the case of poliovirus for example, recognized clinical illness occurs in approximately 1% of infections (Melnick, 1990). The outcome of enteroviral infection is dependent on a number of factors including the age of the individual and the serotype as will be discussed later.

Table 3: Clinical syndromes and associated enteroviruses (modified from Grandien *et al.*, 1989).

Clinical syndromes	Associated enterovirus type
paralysis	polio 1, 2, 3 , coxsackievirus A7, A9 (infrequent), coxsackie B2-B5 (infrequent), echovirus 2, 4, 6, 9, 11, 30, possibly 1, 7, 13, 14, 16, 18, 31
aseptic meningitis	polio 1, 2, 3, coxsackie A2, A4, A7, A9, A10, coxsackie B1-B6, echoviruses except 12, 24, 26, 29, 32, 33, EV 71
undifferentiated febrile illness	polio 1, 2, 3, coxsackie B1-B6
herpangina	coxsackievirus A2, A3, A4, A5, A6, A8, A10
acute lymphatic or nodular pharyngitis	coxsackievirus A10
exanthem	coxsackievirus A4, A5, A6, A9, A16, echovirus 2, 4, 6, 9, 11, 16, 18, possibly 1, 3, 5, 7, 12, 14, 19, 20
hand foot and mouth disease	coxsackievirus A5, A10, A16, enterovirus type 71
pneumonitis of infants	coxsackievirus A9, A16
common cold	coxsackievirus A21, A24
hepatitis	coxackievirus A4, A9, coxsackievirus B5
infantile diarrhoea	coxsackievirus A18, A20, A21, A22, A24
acute haemorrhagic conjunctivitis	coxsackievirus A24, enterovirus type 70
pleurodynia	coxsackievirus B1-B5
severe systemic infection in infants meningoencephalitis	coxsackie B1-B5, enterovirus type 71
pericarditis, myocarditis	coxsackie B1-B5, echovirus 1, 6, 9, 19
respiratory illness	coxsackievirus B4, B5, echovirus 4, 9, 11, 20, 25, probably 1, 2, 3, 6, 7, 16, 19, 22, enterovirus type 68
rash	coxsackievirus B5
epidemic myalgia	Echovirus 1, 6, 9
encephalitis, Guillain-Barre syndrome, ataxia	Echovirus 2, 6, 9, 19, possibly 3, 4, 7, 11, 14, 18, 22

1.4.3. Epidemiology of the enteroviruses

Poliovirus infections were established throughout most of the world's populations up to the late nineteenth century, surviving in endemic form because of a continuous supply of susceptible infants. Antibodies to all three types were present almost universally in women of childbearing age producing high levels of passive immunity in babies at birth. Consequently, most infants were protected by maternal antibody at the time of their first poliovirus infection. However, subsequent improvement in hygiene and sanitation in industrialized countries in the late nineteenth and early twentieth centuries led to a delay in the exposure of infants to poliovirus. This meant that the virus was encountered later in life when the disease was more likely to be paralytic in nature and the pattern of disease changed. Instead of being relatively uncommon and endemic, large epidemics of poliomyelitis began to occur (Minor & Bell, 1990). Poliomyelitis is no longer a major problem in developed countries due to the widespread vaccination programmes using the live attenuated oral poliovirus vaccine (OPV) developed by Albert Sabin in 1961 and the inactivated vaccine (IPV) developed by Salk (Salk, 1953; Sabin, 1957; Sabin & Boulger 1973). Due to the efforts of the World Health Organization (WHO) global eradication programme, established in 1988, the number of reported cases of poliomyelitis has been reduced by 89%. As of mid-May 1998, 4116 cases with onset during 1997 were reported worldwide (WHO, 1998).

National immunization days (NID) have been carried out in all but 4 poliomyelitis endemic countries. In 1997, approximately 450 million children under 5 years of age in 80 countries were immunized. Reported cases in China dropped from 5000 in 1990 to zero in 1995 following an immunization day. Surveillance systems for acute flaccid paralysis (AFP) have also been established in many countries and have shown that transmission of wild poliovirus has been interrupted in a number of areas throughout the world. Poliovirus transmission now occurs primarily in South Asia and Sub-Saharan Africa and is most intense in highly populated countries like India. The eradication programme has led to the certification of the Americas (in 1994) as poliomyelitis-free, the probable interruption of poliovirus transmission in the Western Pacific region and the restriction of poliovirus transmission to a single area in Europe (Bland & Clements, 1998; WHO, 1998). Vaccine associated paralytic poliomyelitis can occur, albeit at a very low rate, following

reversion of the oral poliovirus vaccine (OPV) to virulence within the individual. The risk in the USA was estimated to be one case of paralytic poliomyelitis per 2.5 million doses administered. In England and Wales an average of three cases of paralytic poliomyelitis per year occurred between 1985 and 1991 (Joce *et al.*, 1992) and Sabin vaccine was associated with 13 out of 21 cases in both recipients and contacts. Of the remaining cases, 5 were imported and in 3 the source was not discovered.

Infection with the non-polio enteroviruses (NPEV), that is the coxsackieviruses, echoviruses and higher enteroviruses, still occurs frequently in different areas of the world, in different climates and at different time intervals. Information regarding cases and outbreaks of infection is extensive with literature from the general medical and scientific press along with reports from the WHO, the CDC and the CDR (Communicable Disease Report) in the United Kingdom. The global surveillance data reported a total of 59,776 non-polio enterovirus infections from 1975 to 1983, 64% of which were due to the echovirus group. In tropical climates enteroviruses are isolated throughout the year whereas in temperate climates they are most common in the summer and autumn when warm weather favours their spread, although they can be present at low levels in the winter and spring. In the United States from June to October, the average number of enterovirus isolations was 6.6 times higher than the monthly average for the other seven months of the year, with 82% of the total isolates recovered during these peak months (Moore, 1982).

A number of factors influence the frequency and type of symptomatic infection in the population including age, sex, serotype, virulence, portal of entry, immunocompetence and previous exposure.

Age is one of the most important determinants of the course of enteroviral infection. Infection with coxsackie B viruses for example, is generally more severe in new-borns than in older children and adults, whereas poliovirus infection is more likely to lead to paralysis in adults. A difference in the rate of occurrence of enteroviral disease between males and females has also been noted (1.5:1 to 2.5:1 ratio) generally for the more severe disease with cardiac or CNS involvement.

Low socio-economic status is also a factor in the early acquisition of infection. Active surveillance of infection in young children in West Virginia in 1951 to 1953 showed the isolation rate in the lower socio-economic setting to be 2 to 7 times higher than in the

higher socio-economic setting. These figures may be influenced by the greater number of individuals per household and per room in the former group. Overcrowding, poor sanitation and hygiene combine to maximize the spread of enteroviral infection (Minor & Bell, 1990).

Physical exertion is another important risk factor. In the 1940s it was observed that physical exertion while incubating poliovirus was associated with higher incidence and greater extent of paralysis (Russell, 1947).

There tends to be a particular serotype of NPEV that is endemic circulating among a small number of non-immune individuals many of whom are young children. In some isolated communities such as the Eskimos the whole population may lack antibody to particular serotypes. When a serotype is absent from an area for a number of years a population of susceptible individuals builds up. If that virus is then introduced into the population, a wave of infection occurs in all age groups. Data from the UK and Ireland in the 1960's and early 1970's shows evidence of these waves of infection. In 1969 an epidemic of echovirus 9 accounted for 40% of the isolates reported that year, with 25% to 33% of the cases occurring in Scotland. From isolation data collected in the Regional Virus Laboratory in Glasgow over 20 years (1957 to 1976), echovirus 9 was shown to be the predominant virus at 3-4 yearly intervals (Grist *et al.*, 1978). In the UK in 1971, 60% of echoviruses were type 4, previously responsible for approximately 5% of cases. The Glasgow data show similar figures with a higher number of echovirus 4 isolates in 1971-1972 compared to the previous years where there had been none. In 1990, in the largest outbreak to be reported in this country and to have been confirmed by virus isolation, echovirus 4 was isolated from over 400 individuals, with 80% of these cases presenting with meningism (Gallacher *et al.*, 1993). This virus accounted for 80% of the isolates that year compared to only 10 isolates in the previous 17 years.

In 1970 a large epidemic of acute conjunctivitis was observed in Singapore with over 60,000 cases reported. The agent responsible was identified as a variant of coxsackievirus A24. At approximately the same time, a pandemic of acute haemorrhagic conjunctivitis (AHC) occurred in Africa, South East Asia, Japan and India caused by an unidentified enterovirus, subsequently named enterovirus 70. AHC almost always occurs as epidemics and these two virus types are responsible for the majority of outbreaks. In 1981, a

pandemic of AHC occurred with over 30 million cases worldwide. Oligonucleotide fingerprint analysis showed that isolates obtained from different areas of the world were closely related, whereas isolates from the epidemic of 1969, though related to each other, differed from the pandemic strains by many nucleotides. This data suggested that one basic genotype is in circulation worldwide at any one time (Kew *et al.*, 1983).

1.4.4. Traditional methods of enterovirus detection

Traditionally, enteroviral diagnosis relies on isolation in cell culture and subsequent serotyping by neutralization with intersecting pools of antisera (LBM pools) (Lim & Benyesh-Melnick, 1960). However, no single cell line is optimal for all of the enterovirus serotypes and laboratories generally use a combination of a simian cell line (such as Rhesus Monkey Kidney, RMK) and a human diploid cell line (such as MRC-5). Successful virus isolation is however difficult. Certain serotypes, especially within the coxsackievirus A group, grow poorly or not at all in cell culture; some samples may contain substances which are toxic to the cells; the virus itself may be antibody-neutralized or replication defective; virus titres may be too low for detection; the virus may not be viable due to delays in transit and specimen handling or virus shedding may have ceased (Nicholson *et al.*, 1994; Rotbart & Romero, 1995). A high level of expertise is required to recognize cytopathic effect (c.p.e.) and the process is time consuming: mean times for the isolation of enterovirus from CSF samples have been reported as ranging from 3.7 to 8.2 days (Chonmaitree *et al.*, 1982; Jarvis & Tucker, 1981).

The difficulties of virus isolation have highlighted the need for alternative diagnostic strategies. The large number of serotypes means that serological methods are limited to individual cases where a particular virus is suspected. Ideally, paired sera are used to test for rising titres of enterovirus specific antibody, but the tests can be of low sensitivity and specificity due to poor recognition by reference antisera. Extensive cross-reactivity can also occur between the many serotypes such as echovirus types 1 and 8, which have recently been reclassified as a single serotype (Hyypia *et al.*, 1997).

Antigenic relationships also exist between coxsackievirus types A3 and A8; A11 and A15; A13 and A18; echovirus types 6 and 30 and types 12 and 29 (Morens *et al.*, 1991). Antigenic variants have also been identified which may have arisen from mutation or

antigenic drift. This can lead to the appearance of prime strains, which are neutralized poorly or not at all by antibody to the prototype strains.

A number of alternative techniques to neutralization have been described including an ELISA for the detection of coxsackievirus A antigens (Yolken & Torsch, 1981), a micrometabolic inhibition test for estimating levels of coxsackie B1 to B6 neutralizing antibodies (Bell & McCartney, 1984) and a μ-antibody capture ELISA for detecting coxsackievirus B specific IgM (King *et al.*, 1983). There are disadvantages with these approaches including the high cost of antisera and monoclonal antibodies due to the testing of each sample with all serotypes and controls, and the difficulties in interpreting high static antibody titres.

1.4.5. Molecular methods of detection

The most direct method of detection is to look for the virus itself or a sub-component of it and advances in molecular biological techniques have now made this possible.

1.4.5.1. Hybridization studies. The initial cloning and sequencing of the genome of poliovirus type 1 led to the development of nucleic acid hybridization assays using molecular probes to detect a wide range of enterovirus serotypes (Kitamura *et al.*, 1981).

A cDNA probe of the 3' end of coxsackievirus B3 RNA (Nancy strain) was used to detect enteroviruses in infected cells (Hyypia *et al.*, 1984). The test was specific (Herpes simplex virus (HSV) type 1, Adenovirus type 2 and measles virus were negative) and could detect nucleic acids from approximately 5000 infected cells. This sensitivity was adequate for samples that had undergone cell culture procedures but not for samples tested directly probably because of low levels of virus in the sample or RNase activity which can be present in stool specimens.

Cova *et al.* (1988) described the use of RNA probes corresponding to the VP1 capsid region and 5' NTR of poliovirus type 1 to detect enteroviral RNA in stool specimens or infected cell lysates. The 5' NTR riboprobe was much more efficient than the corresponding cDNA probe, detecting $10^{3.5}$ to 10^{5} $TCID_{50}$ of poliovirus compared to not less

than $10^{4.5}$ $TCID_{50}$. The VP1 riboprobe detected all three poliovirus serotypes and coxsackievirus A21 indicating a close relationship between these serotypes, whereas the 5' NTR probe also detected coxsackieviruses B1, B3, B4, A7, A9, A21, echoviruses 9, 11, 33 and HRV-2 due to the conserved nature of this region. Of a panel of positive specimens, the VP1 probe detected 50% of poliovirus infected stools, all the poliovirus infected cell lysates but did not react with the other enteroviruses. The 5' NTR probe picked up 16/18 positive enterovirus samples including poliovirus types 1, 2 and 3 from crude cell extracts. One advantage of using riboprobes was that a large amount of probe could be synthesized with a high specific activity, compared to the cDNA probes

Rotbart and co-workers (1988) prepared synthetic probes for the 5' NTR of coxsackievirus B3 or the protease gene of echovirus 9 labelled with either ^{32}P or alkaline phosphatase for use in blot assays. They were able to detect a wide range of human enteroviruses but with a sensitivity of only 10^2 to 10^5 $TCID_{50}$ of target virus.

The limiting factor in hybridization assays is that clinical specimens may contain low levels of enterovirus (as low as 1-10 titratable virus per ml in CSF from cases of aseptic meningitis (Wilfert & Zeller, 1985). Consequently, many assays are not sensitive enough to be of use in the diagnostic laboratory.

1.4.5.2. Detection of VP1 antigen. Another potential diagnostic tool described by Yousef and colleagues (1987a), used a monoclonal antibody (5-D8/1) which reacted with a single peptide (VP1) common to all the enteroviruses tested. The antibody was used in both immunofluorescence (IF) and enzyme immunoassay (EIA) tests, with no non-specific reactions, but the latter was more sensitive. Antibody 5-D8/1 was unable to neutralize coxsackievirus types B1, B3 and B5, since neutralizing antibodies tend to be serotype specific, but it did have complement fixation activity. Clinically, the antibody was used in dot-blot enzyme immunoassays to identify 122/130 field isolates (Yousef *et al.*, 1987b). Unidentified isolates were subsequently shown to have low viral titres and could be picked up by an indirect IF assay. Evaluation of the commercial antibody for the identification of enterovirus in primary culture cell lysates by indirect IF, compared to seroneutralization and the polymerase chain reaction (PCR) was carried out (Trabelsi *et al.*, 1995). All 39 serotypes tested were reactive with the antibody. Cell lysates from primary culture of 61 clinical isolates were also tested by indirect IF which detected all strains that could be

typed and those that could not. PCR of the 5' NTR produced the same results. The advantage of the antibody is that it can also detect echovirus types 22 and 23, which most PCR tests cannot.

1.4.5.3. Polymerase Chain Reaction (PCR). The problem of sensitivity encountered with hybridization reactions has been overcome to a large extent by the development of the Polymerase Chain Reaction (PCR). This is a relatively new technique, (original protocols by Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis & Faloona 1987), which allows the amplification of a segment of DNA that lies between two regions of known sequence. Two synthetic oligonucleotides are used as primers in a series of reactions catalysed by a thermostable DNA polymerase enzyme, enabling very small quantities (from as low as 1ng) of mRNA to be amplified and detected (Gilliland *et al.*, 1990). A second set of primers that lie within the amplified region can be used to amplify the first round product, increasing the sensitivity, a process known as 'nested' PCR. Enteroviruses have an RNA genome, which has to be reverse-transcribed to complementary DNA (cDNA), to act as the template for PCR. RT-PCR has become the tool favoured by investigators looking for enteroviruses in various clinical syndromes and it has been refined into a rapid and sensitive technique. The conserved 5' NTR is an ideal target for the use of a single group-specific PCR. Primers can also be designed to be serotype-specific depending on the requirements of the investigator, for example, a coxsackievirus A specific PCR has been described (Gjoen & Bruu, 1997).

a. PCR and Hybridization. Zoll and colleagues (1992) described one of the first protocols for the detection of almost all the human enteroviruses. Using one primer set based on the 5' NTR, 60/66 serotypes were amplified, the exceptions being echovirus types 16, 22 and 23 and coxsackievirus types A11, A17, A24. A second primer set which amplified a larger fragment of the 5' NTR confirmed these results. Hybridization of the amplified products with an internal probe resulted in 55/60 positive results. Coxsackievirus types A1, A12, A20, A21 and A22 were not detected. A coxsackie B3 cDNA clone was used to estimate the detection limit of the PCR and a product was observed from 0.1fg of the clone that corresponded to approximately 10 genome equivalents. Other viruses including 10 different rhinovirus types, human cytomegalovirus (CMV) and herpes simplex 1 and 2 were not detected by these primers. Twelve enterovirus positive stools were positive by PCR as were the corresponding throat

swabs from 6 of these patients, thus proving the applicability of group-specific PCR for diagnostic use.

b. Semi-Nested and Nested PCR. A semi-nested PCR of the 5' NTR, with a sensitivity of 1fg of RNA, was used to investigate a number of clinical syndromes including suspected meningitis and encephalitis (Leparc *et al.*, 1994). The results showed that the enteroviral PCR was positive in 35% of CSF samples and 30% of throat swabs compared to 15% positives by culture of both sample types. Testing of faecal samples resulted in 50% positives by enteroviral PCR versus 43% by culture, which reflects the high level of enterovirus shedding in faeces, compared to other biological samples. Thoren and Widell (1994) also using a semi-nested PCR, with a sensitivity of 0.01 $TCID_{50}$ showed that PCR of serum could be used to detect enterovirus in patients with suspected enteroviral meningitis. They suggested that it should be used in conjunction with PCR of CSF, as it was less sensitive when used on its own (52% sensitivity for serum alone compared to 71% for CSF alone and 86% for PCR of serum and CSF samples combined).

Compared to the traditional isolation and typing of viral agents, PCR only produces a positive or negative result for the virus of interest, with no information regarding the serotype. This is generally acceptable since knowing the serotype is not important in the clinical management of the patient and it is useful for epidemiological purposes. Kammerer *et al.* (1994) used a nested PCR with a sensitivity of $5x10^{-2}$ pfu/ml to successfully amplify the 33 prototypes tested, except echovirus 22 which produced smaller sized bands. The PCR product was then subjected to digestion with restriction endonucleases designed to identify different serotypes. PCR was carried out on 37 specimens from patients with neurological disease, 43% of which were positive and 117 heart tissue specimens, 10% of which were positive. Restriction enzyme analysis provided conclusive information on the typing of 14/28 of the positives, and for those that could not be typed, subsequent sequencing showed that mutations had occurred at the restriction site, resulting in the altered cleavage pattern seen.

c. Commercial PCR Kits. Rotbart *et al.* (1994) developed a PCR assay that uses a single enzyme for both the RT and PCR stages, and detects the amplified product in a simple microwell plate format with colour detection. Uracil *N*-glycosylase (UNG) is also incorporated to prevent amplification of carry-over product. Twenty-seven isolates

(including coxsackievirus A and B types, echovirus types and enterovirus type 70 and 71) were detected at a sensitivity of ≤1 $TCID_{50}$ and there was no cross-reactivity with other non-enterovirus or bacterial agents. With archival CSF specimens, the sensitivity and specificity of PCR versus culture plus clinical diagnosis was 97.4% and 100% respectively. The PCR can also be performed in 5 hours and thus has the potential to be a good diagnostic aid. The system is now available commercially as the Roche Amplicor Enterovirus Kit. Yerly *et al.* (1996) compared this kit with standard cell culture techniques for CSF samples from aseptic meningitis cases. After 2-6 days of cell culture, only 34% of CSF specimens were positive for enterovirus compared to 66% positives with the kit. The PCR positive results were independent of the time delay between the onset of symptoms and the collection of the sample which is an advantage as delays will inevitably occur in the clinical situation. In this case, samples collected more than 24 hours after onset were culture negative. An in-house single round PCR was also used (specificity confirmed by southern blotting) and was found to be more sensitive than the kit (it was positive for 3 additional samples). In-house PCR systems also generally include an additional PCR to control for the extraction procedure by amplifying for example, a 'housekeeping' gene. This PCR control has to be positive for the 'test' PCR to be valid. In the case of the commercial kit an extraction control is not included, therefore it is difficult to assess whether negative samples are genuinely negative.

Even with these latest developments virus isolation is still performed in the routine virology laboratory with confirmatory neutralization and IF tests. PCR is slowly emerging as a useful addition to these tests and is being integrated into the modern diagnostic laboratory. This has resulted in the increased detection of enteroviruses in clinical syndromes such as aseptic meningitis, where currently, non-polio enteroviruses account for 80-92% of all cases from which an aetiological agent is identified. In general, of the 25-33% of CSF specimens negative for virus in tissue culture, two thirds will be positive for enterovirus by PCR (Chonmaitree *et al.*, 1982).

1.4.6. Molecular relationships among the human enteroviruses

Molecular techniques have allowed a number of the enteroviruses to be sequenced and although not used in the routine identification of the enteroviruses, genome sequencing can be useful for epidemiological purposes.

Analysis of the sequence of the capsid region as a whole (Hyypia *et al.*, 1997) and of the individual proteins (Poyry *et al.*, 1996; Huttunen *et al.*, 1996; Pulli *et al.*, 1995; Dahllund *et al.*, 1995; Poyry *et al.*, 1994), has led to the identification of 5 clusters of enteroviruses (Table 5). Analysis of the 3D RNA polymerase region of a number of enteroviruses produces the same clustering (Poyry *et al.*, 1996; Dahllund *et al.*, 1995).
In the capsid coding region, the amino acid similarity is at least 71% within a cluster and ranges from 53% to 68% between clusters, whereas amino acid similarity in the 3D protein can be up to 90% within a cluster.
In contrast, phylogenetic analysis of the highly conserved 5' NTR reveals the presence of only two genetic clusters, one containing the polioviruses, coxsackievirus A21, coxsackievirus A24 and enterovirus 70, and the other containing enteroviruses from clusters A and B with a minimum nucleotide identity of 77% within each cluster (Hyypia *et al.*, 1997).

Interestingly, one enterovirus of lower animals, Swine Vesicular Disease Virus (SVDV) is shown to group in cluster B alongside the coxsackie B-like enteroviruses. SVDV has been shown to be closely related antigenically to coxsackie B5. SVDV can be neutralized by coxsackie B5 antisera and vice versa and it has been suggested that coxsackie B5 gave rise to SVDV through transmission and adaptation to swine (Brown *et al.*, 1973; Graves, 1973). However, sequence analysis by Zhang and colleagues (1993) showed that SVDV was not any more closely related to coxsackievirus B5 than to the other coxsackie types, except in the capsid region.

Cluster	**Enterovirus types**
A Coxsackie A16-like	Coxsackie types A2, A3, A5, A7, A8, A10, A12, A14, A16, enterovirus type 71
B Coxsackie B-like	Coxsackie types B1, B3, B4, B5, coxsackie A9, enterovirus 69, echovirus types 1 to 9, 11 to 17, 19-21, 24, 26, 27, 29, 30, 31, 32, 33, SVDV
C Poliovirus -like	Poliovirus types 1, 2, 3, coxsackie types A1, A11, A13, A15, A17, A18, A19, A20, A21, A22, A24
D Enterovirus 70-like	Enterovirus types 68, 70
E BEV- like	Bovine enterovirus types 1, 2a, 2b

Table 4: Genetic clusters of the enteroviruses based on the amino acid sequence of the capsid protein region and the 3D RNA polymerase region

The molecular relationships observed suggest that the present subgroup classification for enteroviruses should be revised. This is especially true when considering the coxsackie A viruses which occur in several of the clusters rather than as a particular subgroup. Revised subgroup classification is also indicated when the receptor specificity of the enteroviruses is considered (Table 5). A number of receptors belong to the immunoglobulin (Ig) superfamily, members of which are involved in cell-cell recognition and adhesion. Others are integrins, a family of adhesion receptors comprised of at least 7 different β sub-units and 14 different α sub-units, that associate to form at least 16 heterodimers (Bergelson *et al.*, 1992). The three poliovirus types use a common specific receptor (PVR), whereas the coxsackie A types use a variety of molecules for cell entry, some of which are also receptors for other enterovirus types, for example ICAM-1 is also used by the major rhinovirus group.

Molecular techniques such as nucleic acid hybridization and PCR have also been useful in investigating the role of enteroviruses in heart muscle disease (myocarditis and dilated cardiomyopathy) (Bowles *et al.*, 1986) and have confirmed the association originally established by retrospective serology (Grist *et al.*, 1974). Similarly, enteroviruses have been implicated in the initiation of juvenile-onset type-1 diabetes mellitus and Chronic Fatigue Syndrome (CFS) by epidemiological and serological data. However, in many studies the associations have not been independently confirmed. The conflicting evidence for the involvement of enteroviruses in CFS will be discussed in the following section.

Table 5: Enteroviruses and their receptor molecules.

Virus	Receptor Molecule	Description
Poliovirus type 1-3 (Mendelsohn *et al*, 1989, Shepley & Racaniello, 1994)	Poliovirus receptor (PVR) Lymphocyte homing receptor (CD44)	Integral membrane protein (Krah and Crowel, 1982) of Ig super family. Extracellular portion predicted to fold into 3 domains. 100 kDa glycoprotein (Shepley et al, 1988).
Coxsackievirus types B1-B6 (Bergelson *et al*, 1997)	Coxsackie/Adenovirus Receptor(CAR)	46 kDa protein. Shown to be 365 amino acid transmembrane protein with a short leader, a 225 amino acid extracellular domain (with two predicted Ig-like domains) and a 107 amino acid intracellular domain.
Coxsackievirus A types 13, 15, 18, 20, 21 (Colonno 1987, Pulli *et al*., 1995); HRV (Major group~90 serotypes) (Greve 1989)	Intercellular Adhesion Molecule 1(ICAM-1)	90 kDa polypeptide member of Ig superfamily that acts as a ligand for IFA-1, an integrin. Contains 5 predicted Ig-like domains.
Coxsackievirus type A9, echovirus 22 (Roivainen *et al*., 1994), FMDV (8 subtypes) (Berinstein *et al*., 1995)	Integrin $\alpha_v\beta_3$	Vitronectin receptor. Recognises RGD (arginine-glycine-aspartic acid) tripeptide insertion at C-terminal end of VP1
Echovirus type 1 (8) (Bergelson *et al*, 1992, 1993)	Integrin VLA (very late antigen) -2 ($\alpha_2\beta_1$)	Mediates attachment to collagen and laminin and participates in tumour metastasis in vivo.
Echovirus types 3, 6, 7, 11, 12, 13, 20, 21, 29, 33 (Ward *et al*, 1994; Bergelson *et al*, 1994); Enterovirus 70 (Karnauchow *et al*, 1996)	Decay Accelerating Factor (DAF) (CD55)	A 70 kDa glycosyl phosphatidyl-inositil (GPI)- anchored surface membrane protein. 5 extracellular domains are predicted including 4 short consensus repeats (SCR) of approx. 60 amino acids and a C-terminal serine-threonine rich region of approx. 70 amino acids.
Human Rhinovirus (Minor group ~10 serotypes) (Hofer *et al*, 1994)	Low density lipoprotein receptor (LDLR)	Identical to human α-2 macroglobulin receptor. Contains two non-covalently asssociated polypeptides of approx 420 and 85kDa. (Strickland *et al*., 1990)
EMC virus (Huber *et al*, 1994)	Vascular Cellular Adhesion Molecule 1 (VCAM-1)	Member of Ig super family. Restricted to endothelial cells.

1.5. Enteroviruses and Chronic Fatigue Syndrome

1.5.1. Chronic Fatigue Syndrome (CFS)

CFS is a chronic illness characterized by severe disabling fatigue of greater than six months duration with a combination of symptoms that features self (patient)-reported impairments in concentration and short-term memory, sleep disturbance and musculoskeletal pain. (Sharpe *et al.*, 1991). Diagnosis is confirmed only after alternative medical and psychiatric causes of chronic fatiguing illness have been excluded. This is not a new illness. Reviews of the medical literature have uncovered reports of syndromes resembling CFS described as febricula, neurasthenia and DaCosta's syndrome in the 18th, 19th and early 20th centuries respectively (Straus, 1991). Chronic brucellosis, hypoglycemia and candidiasis have been blamed for many cases over the years as investigators sought a name for this illness (Evans, 1947; Straus, 1991; Anderson *et al.*, 1986).

In the summer of 1934 an epidemic of poliomyelitis struck Los Angeles with many of the patients treated in the County Hospital (Gilliam, 1938). Within a few weeks, a large number of cases of similar illness began appearing among the staff, particularly the nurses, suggesting the presence of a transmissible agent, but examination of the CSF showed nothing abnormal in 53 out of 59 cases. Approximately 200 cases were reported, an attack rate of 4.4%, with no deaths and most of the patients fully recovered.

Fourteen years later (1948-1949) a similar epidemic occurred in Iceland with over 1000 cases, nearly half of them in children (Sigurdsson *et al.*, 1950) leading to the term 'Iceland disease' or 'Akureyri disease' to describe the outbreak. The label of benign myalgic encephalomyelitis (M.E.) was introduced in 1956 in an article in the Lancet (Ramsay & O'Sullivan, 1956) describing an outbreak at the Royal Free Hospital in London. Between July 13th and November 24th 1955, 292 hospital staff were affected by an illness which amongst other things, included CNS involvement. Eighty-seven percent of those affected were admitted to hospital. At approximately the same time, medical staff saw similar cases occurring sporadically in North-West London. The majority of cases recovered, but severe disability persisted in at least four patients. The epidemiology of the outbreak suggested that the disease was spread by case to case contact and had an incubation period of 5 to 6 days. Symptoms of malaise, headache and sore throat are common to the

prodromal phase of many infections but laboratory investigations proved negative for isolation and for antibodies to the agents tested. Other investigations were unremarkable (Medical Staff of the Royal Free, 1957). In these three outbreaks, the attack rate per 100 cases was higher in women than men (6.4 against 1.6 in Los Angeles; 8.3 against 5.1 in Iceland and 10.4 against 2.8 at the Royal Free). Subsequent outbreaks of similar infection were thus labeled as M.E., Royal Free or Iceland Disease: an outbreak of 'Iceland disease' was reported in children in New York in 1953 (White & Burtch, 1954) and more recently Fegan *et al.* (1983) reported on an epidemic of M.E. in a rural practice in Ayrshire, Scotland. The term M.E. however literally means a distinct pathological process of inflammation of the brain and spinal cord with concomitant muscle pain. There is no evidence for this in these patients and the use of this term should therefore be avoided.

In the 1950's, sporadic cases of the illness were also described and because of the frequent reporting of flu-like symptoms at onset these were labeled as post viral fatigue syndrome (PVFS) (Newham & Edwards, 1979). Several studies in the 1980's of such sporadic cases revealed that in some, chronic fatigue began during a bout of infectious mononucleosis. Additionally, some patients had abnormal levels of antibody to Epstein Barr Virus (EBV) (Tobi *et al.*, 1982; Dubois *et al.*, 1984; Jones *et al.*, 1985; Straus *et al.*, 1985). The syndrome was therefore given yet another name- chronic EBV syndrome or chronic mononucleosis syndrome. A workshop organized by the Centres for Disease Control (CDC) was held in 1987 to develop a consensus on the features of this syndrome and this revealed doubt about there generally being a causal relationship with EBV. The meeting led to the renaming of the illness as chronic fatigue syndrome (CFS), to describe a syndrome characterized by chronic fatigue but making no assumptions regarding the actiology or pathology of the disease. A case definition was also developed for research purposes to enable associated risk factors and laboratory abnormalities to be recognized (Holmes *et al.*, 1988). The CDC definition states that for diagnosis, two major criteria must be present, i.e., new onset persistent or relapsing fatigue that does not resolve with bed-rest, reduces the average daily activity by 50% of premorbid levels and has been present for at least 6 months, together with the exclusion of other disorders that would cause similar symptoms such as malignancy or auto immune disease. At least six symptoms are required from a list including headache, sore throat and myalgia together with two physical criteria such as low-grade fever and non-exudative pharyngitis, or alternatively eight or more symptoms. This definition is often modified in practice by

researchers because some of the criteria are difficult to comply with, resulting in inconsistencies between studies. In an attempt to address this problem, the definition was revised in 1994 (Fukuda *et al.*, 1994). Cases including untreated hypothyroidism, malignancies, major depressive disorder, eating disorders, alcohol and substance abuse are excluded at the outset. Distinctions are then made between CFS and idiopathic chronic fatigue. For classification as CFS, patients must have unexplained persistent or relapsing chronic fatigue of new or definite onset that is not alleviated by rest, is not the result of ongoing exertion and results in substantial reduction in previous levels of occupational, educational and social activities. In addition, there must be the concurrent occurrence of four or more symptoms which have persisted or recurred during six months of illness from a list which includes memory impairment, difficulty in concentration, sore throat, muscle pain, joint pain, headache and non-refreshing sleep. Idiopathic chronic fatigue is described as unexplained chronic fatigue that fails to meet CFS criteria. Other disorders that do not necessarily exclude patients from a CFS diagnosis include fibromyalgia, somatoform disorders, anxiety disorders and neurasthenia, but it is important that such conditions are noted.

Other countries use slightly different criteria for diagnosis. In the United Kingdom, the Oxford Criteria (Sharpe *et al.*, 1991) state that fatigue must be the principal symptom, being severe, disabling, affecting physical and mental functioning and being present for at least six months for at least 50% of the time. The syndrome should also be of definite onset and symptoms such as myalgia, mood and sleep disturbance may be present. Patients with established medical conditions that produce chronic fatigue should be excluded along with those with diagnoses of schizophrenia, manic depressive illness, substance abuse, eating disorders and proven organic brain disease. The Oxford criteria also include a subtype of CFS called post-infectious fatigue syndrome (PIFS) where there is also definite evidence of infection at onset or presentation corroborated by a laboratory, and the syndrome is present for at least six months after the onset of infection.

Fatigue, as a symptom is extremely common in the community. Buchwald *et al.*(1987) and Kroenke *et al.* (1988) reported that between 21% and 24% of patients seeking medical care through a hospital based out-patient clinic had fatigue as their chief complaint. Only a fraction of these (2-5%) fulfil the criteria for CFS, with depression and anxiety the underlying causes of most of the cases of chronic fatigue (Komaroff & Buchwald, 1998).

It is difficult to obtain prevalence figures for CFS because of the effects of selection bias for example. According to the Royal College Report (1996), the population point prevalence of CFS is 0.1%-0.9% using the CDC criteria that excludes patients with psychiatric disorder, and 2.6% in primary care according to the Oxford criteria.

The problems surrounding CFS are numerous including the difficulty of diagnosis; the different names used to describe it; the lack of objective biological markers; the debate about an organic or psychiatric cause and the fact that some medical professionals still do not believe in its existence. The illness has often been dismissed as hysteria by sceptics but was fully investigated in 1978 by a symposium at the Royal Society of Medicine. Their findings indicated that there was little evidence in favour of a hysterical phenomenon with the majority supporting an organic basis for the illness (Ramsay, 1978).

In 1996, a joint working group of the Royal colleges of physicians, psychiatrists and general practitioners published a report on CFS in response to a request from the Chief Medical Officer. This report highlighted the problems encountered in the study of the illness and provided a summary of a number of research topics including studies on virology, muscle function and immunology, psychiatry and neuropsychiatry, prognosis and management. A number of these will be expanded on below.

1. Virological Studies. The reason that many investigators began to look towards viruses as potential triggers of CFS was that many patients reported that the onset of fatigue had followed symptoms of a flu-like illness. Early studies linked EBV with CFS (Tobi *et al.*, 1982; DuBois *et al.*, 1984; Jones *et al.*, 1985; Straus *et al.*, 1985) due to the presence of elevated levels of antibody to viral capsid antigen (VCA) and early antigen (EA). A fatigue state has been shown to exist after infection with EBV: a prospective study of 250 patients with either glandular fever or ordinary upper respiratory tract infection, showed that glandular fever was a significant risk for both acute and chronic fatigue syndromes (White *et al.*, 1995; 1998). However, other studies of patients and evidence from seroepidemiologic surveys showed that many patients with clinical evidence of CFS had no serological evidence of prior EBV infection and that EBV titres in patient and control groups often overlapped, or were present alongside elevated titres of antibody to other viruses such as herpes simplex and measles (Holmes *et al.*, 1987; Sumaya, 1991). EBV is therefore not thought to be the aetiologic agent in most CFS cases (Horwitz *et al.*, 1985; Hellinger *et al.*, 1988; Sumaya, 1991). The other most common agents studied in

association with CFS, especially in the U.K. are the enteroviruses and these will be discussed in the following section (1.5.2.).

Human Herpes virus-6 (HHV-6) infects B- and T-lymphocytes and most adults have been infected with the virus (over 85% prevalence rate in the U.S.). Evidence linking HHV-6 with CFS has been reported by a number of individuals looking at elevated antibody titres and the detection of HHV-6 antigen and DNA in peripheral blood mononuclear cells (PBMC).

Buchwald and co-workers (1992) detected replicating HHV-6 in peripheral blood lymphocytes of 70% of patients compared to 20% of controls, and this was confirmed by the detection of the virus by specific monoclonal antibodies to HHV-6 proteins and by PCR assays for HHV-6 DNA. Elevated levels of HHV-6 early antigen-specific IgM were found in more patients than controls by Patnaik *et al.* (1995) perhaps indicating active replication of the virus in CFS. In an Italian study, fresh PBMCs from heparinized blood samples were used for virus isolation studies. Typical HHV-6 cytopathic effect was found in 73.1% of CFS patients but not in controls and DNA was detected by PCR in 61.5% of patients versus 11.7% of controls (Zorzenon *et al.*, 1996).

However, in four clusters of CFS in the U.S. there was no difference in the number of positives for HHV-6 antibody between patients and controls. The mean titre of antibody was higher for patients than controls but was not statistically significant (Levine *et al.*, 1992). Both HHV-6 and EBV are capable of establishing latency after primary infection and it has been suggested that they are merely reactivated in CFS patients possibly because of immune dysfunction, rather than being the causative agent.

The detection of HTLV-II (human T-lymphotropic virus) like viral sequences in the peripheral blood lymphocytes of patients with CFS suggested the possibility of retroviral involvement (DeFreitas *et al.*, 1991). Such sequences were not detected in the lymphocytes of healthy individuals. Other groups failed to confirm these findings (Levine *et al.*, 1992; Landay *et al.*, 1991; Gow *et al.*, 1992). Gow and colleagues found no difference between the patient and control populations by PCR of the *gag*, *pol*, *env* and *tax* regions of HTLV-I and HTLV-II. However, an endogenous *gag* band was observed in both the patient and control groups, using the same primers as the DeFreitas group. The authors suggested that this sequence represented an endogenous sequence rather than exogenous virus in their own and DeFreitas findings.

Borna disease virus (BDV) is a neurotropic RNA virus and the prototype for a newly described group of animal viruses. Nakaya *et al.* (1996) used nested PCR followed by hybridization to demonstrate the presence of BDV RNA in 3 out of 25 CFS patients. Together with the detection of antibodies to BDVp24 protein in 6 patients, the overall prevalence was 32% in Japanese CFS patients.

Kitani and colleagues (1996) looked at the seroprevalence of BDV in a larger patient group with 89 cases compared to 100 healthy control individuals. Antibody to BDV p24 protein was found in 30/89 patients (33.7%). BDV RNA was present in 12.3% of CFS patients compared to 4.7% of controls using nested PCR. Additionally, in one Japanese family, 3 out of 5 developed CFS almost simultaneously. BDV RNA was detected in the peripheral blood lymphocytes from all 3, suggesting a close association of BDV infection with Japanese CFS patients.

In the U.K., Gow and colleagues (1997a) reported on the testing of brain samples, PBMCs, serum and CSF samples for BDV from CFS patients, matched controls and individuals with depressive disorder (not all samples were obtained from all patients). All samples were tested by nested PCR followed by hybridization and produced only one positive result from the CSF of a patient with CFS. Western blotting and IF were also carried out on the serum from 21 CFS patients and 13 controls. Two patients were positive by both tests. From this data there did not appear to be a significant association between BDV and CFS in these patients but it is possible that BDV is more prevalent in Japan, thus accounting for the results observed there.

Establishing a link with a particular virus is difficult because viral infections are common throughout the year and chance associations are difficult to exclude. Thus reliable markers of recent infection must be used to test patients and appropriately matched controls.

2. Immunological Studies. CFS has been referred to as Chronic Fatigue and Immune Dysfunction Syndrome (CFIDS) by some individuals (DeFreitas *et al.*, 1991; Ojo-Amaize *et al.*, 1994) suggesting involvement of the immune system. Numerous studies have reported various immunological abnormalities but interpretation is complicated by the lack of standardization of methods among laboratories, the heterogeneity of patient groups and by the lack of concurrent testing of patients and controls (Klonoff, 1992).

Evidence for abnormal humoral immunity was reported by Lloyd *et al.* (1989) who compared 100 CFS patients and 100 healthy age and sex matched controls. Reduced immunoglobulin levels were observed in 56% of patients, with levels of IgG_3 and IgG_1 subclasses particularly affected. IgG_3 is thought to play a major role in virus neutralization. Additional studies have also shown IgG_1 (Read *et al.*, 1988) and IgG_3 (Linde *et al.*, 1988) subclass deficiencies.

T-cell proliferation after exposure to phytohaemagglutinin (PHA) is a common marker for T-cell function. In general, CFS patients' lymphocyte response *in vitro* to PHA is significantly lower than that of controls and the production of γ-interferon (IFN) following mitogen stimulation is also decreased in most patients compared with controls. Klimas *et al.* (1990) also observed a significant reduction in lymphoproliferative responses after PHA and PWM (pokeweed mitogen) stimulation. Over 80% of the study population had values of ≥ 1SD (standard deviation) below the normal mean. Impaired T-cell function was also observed *in vivo*, with reduced delayed type hypersensitivity skin responses in 88% of patients (Lloyd *et al.*, 1989). Klimas *et al.* (1990) also noted a 67% elevation of CD8 cells co-expressing HLA-DR markers. Interferon or other cytokines may have induced these cell surface antigens. Once activated, these cells can continue to produce cytokines. Cytokines such as interleukin (IL)-6 are pro-inflammatory and may be responsible for changes in mood and cognitive function, appetite disturbance, myalgia and fatigue, symptoms observed frequently in patients with CFS (Landay *et al.*, 1991; Straus *et al.*, 1988). Recently, Gupta *et al.* (1997) studied the response of adherent (monocytes) and non-adherent (lymphocytes) mononuclear cells to PHA or lipopolysaccharide (LPS) in CFS patients and matched controls. Spontaneous production of IL-6 was significantly higher from both cells in CFS patients than controls, while spontaneous production of IL-10 was lower than that of controls. Production of TNF (tumour necrosis factor)-α from non-stimulated adherent monocytes was also higher in patients than controls.

Another immunological abnormality frequently reported has been low NK (natural killer) cell cytotoxicity. NK cells form part of the innate immune system that acts against tumour cells and virus-infected cells. Klimas reported that the number of NK cells was elevated, but killing of tumour cells was diminished. The NK cell defect may be due to the impaired ability of mononuclear cells to produce γ-IFN, which enhances NK cell cytotoxicity and cellular antigen presentation to lymphocytes. The function of NK cells has been found to be increased (Gold *et al.*, 1990), decreased (Kibler *et al.*, 1985; Caligiuri

et al., 1987) and normal (Borysiewicz *et al.*, 1986), as measured by cytolytic activity against a number of different target cell lines.

The Royal College report draws attention to the difficulties in interpreting these abnormalities, many of which can be non-specific in nature. Additionally the illness is often not of comparable severity or duration in different patients and comparison may be difficult. Other variables such as age, sex and co-existent stress or infection could also affect immune status. Where there are no signs of a specific illness or laboratory findings, the immunological abnormalities observed may also be attributed to neuro-immunological influences. Immune function has been shown to change in association with mental states ranging from life stress to psychotic illness. For example, exam stress can alter salivary IgA concentrations and depression has been associated with altered T-cell function (Cohen & Williamson, 1991). Attention should therefore not be focused solely towards an organic cause for the illness.

Though there is evidence of a disturbed immune system in many cases, whether this is primary or secondary to the development of CFS is unknown. Certain individuals may be at risk of developing CFS because of an inherently hyper-responsive immune system, responding to certain infections (e.g. viral) with sustained and inappropriate cytokine release (interferons and interleukins). This release may disrupt neurotransmitter function and result in the symptoms of CFS (Straus *et al.*, 1988). Anxiety or depression (either present before the CFS or as a result of the illness) may magnify the response or cause a prolonged cycle of disturbed immune function, inappropriate cytokine release, further symptoms, depression and continued disturbed immune function. Latent viruses, of the herpes group for example, may also be reactivated in such circumstances.

The immune dysfunction could also be the result of a primary cause such as a viral trigger via a 'hit and run' mechanism, where the virus is cleared after infection but the immune system does not recover as normal. Alternatively, the virus or infecting agent may be defective resulting in abnormal persistence within the host. A low grade infection may result and this in turn could cause chronic low level activation of the immune system.

3. Other Studies. Patients consistently report muscle pain and muscle weakness on exercise. However, there is no consistent evidence of a primary disorder of muscle and no convincing evidence of any changes in muscle structure or function other than those that have resulted from inactivity in CFS patients. Behan *et al* (1991) reported mitochondrial

abnormalities in the muscle of CFS patients, with diffuse or focal atrophy of type II fibres and mitochondrial degeneration in 80% of cases. These results were disputed in a blinded study where there was no difference between CFS patients and normal controls (Plioplys & Plioplys, 1995).

Magnetic resonance imaging (MRI) and single-photon emission computed tomography (SPECT) have been used to study the involvement of the CNS in CFS patients with both techniques highlighting abnormalities. MRI has shown spotted areas of high signal in the cerebral white matter more often in CFS patients than in control subjects (Buchwald *et al.*, 1992). SPECT abnormalities have also been reported to occur more often in CFS than in control subjects and it has been postulated that they represent reduced blood flow and/or dysfunction of neuronal cells (Komaroff & Buchwald, 1998). However, other groups did not find the same abnormalities and the Royal College report suggests that normal variations should be more clearly established to provide a background level against which the abnormalities can be compared (Royal College report, 1996).

The role of neuro-endocrine dysfunction has also been investigated in CFS. Subtle abnormalities of the HPA (hypothalamic-pituitary-adrenal) axis have been reported and it is possible that previous depressive illness may alter the reactivity of the HPA axis to subsequent infection or psychosocial stress (Demitrack *et al.*, 1991). Psychiatric disorders and especially depression have been shown to predispose patients to CFS: depression is diagnosed in CFS patients 2 to 3.3 times more frequently than those with other chronic illnesses; a higher lifetime prevalence (24-50%) of prior psychiatric disorder is reported in CFS patients and depression and stress have also been suggested as being able to influence infection (Klonoff, 1992). A number of investigators have suggested that CFS does not have any organic basis and is purely psychiatric in origin. However, while some of the symptoms of CFS could reflect a primary psychiatric disorder many are not characteristic of psychiatric illness such as sore throat, arthralgias or post-exercise malaise. In addition individuals with CFS tend not to suffer from guilt or lack of motivation which are indicators of major depression and trials of Fluoxetine (Prozac) have been unsuccessful (Komaroff & Buchwald, 1998). These findings suggest that the symptoms of CFS are not an atypical manifestation of major depression and that psychiatric disease cannot fully explain CFS.

CFS has also been shown to be associated with allergies: a premorbid history of inhalant, food or drug allergies has been reported by approximately 65% of CFS patients. Additionally, cutaneous reactivity to inhalants or foods occurs in 50% of CFS patients compared to 20-30% of the general population. There is also a positive correlation between allergy severity and the magnitude of the EBV serological response. Whether allergy predisposes to EBV infection and then CFS or whether EBV predisposes to allergies is unknown (Klonoff, 1992).

There is a plethora of research on CFS which cannot be fully described in this manuscript and as such only the main areas have been discussed. A general consensus on CFS is that it is multifactorial in origin involving the complex interaction of predisposing factors (past psychological illness, somatic attribution style, history of fatigue, disturbed immune function), triggering factors (viral illness, life events, stress, depression/anxiety) and maintenance factors, that is those factors which could maintain and prolong the illness such as the patients response to fatigue (time off work, bed rest), effects of inactivity (fatigue) and the response of the doctor (raising sick notes) for example. It is the role of the enteroviruses in CFS which will be explored in this thesis.

1.5.2. Enteroviruses and CFS - evidence for an association

The evidence for an association between enterovirus infection (particularly the coxsackie B group) and CFS has accumulated over a number of years from serological assays, nucleic acid hybridization analysis and more recently PCR studies. Serological analysis has focused on the detection of elevated neutralizing antibody titres to coxsackievirus types B1 to B5. Elevated titres were observed in 82% of M.E. cases (Fegan *et al.* 1983), 76% of suspected M.E. cases (Keighley & Bell, 1983), 47% of cases in a retrospective survey (Calder & Warnock, 1984), 70% of PVFS cases (Behan *et al.*, 1985) and 46% of patients in a subsequent study by Calder *et al.* (1987). In this latter study, 65 patients who were initially positive were re-tested at 6 months and one year later. Of these, 55% were still unwell and high antibody titres persisted in all but two of the patients. These figures were significant when compared to a group of control individuals. Testing of 950 normal adults in the West of Scotland for coxsackie B virus neutralizing antibody showed only 4% of samples to have titres of ≥512 and 10% to be 256 (Fegan *et al.*, 1983).

Neutralization titres are not a reliable means of determining recent enteroviral infection since antibody levels can remain elevated in an individual for months to years. A more relevant assay would be to test for specific IgM, which is indicative of recent infection. A μ-antibody capture ELISA was used to show that 31% of 118 patients and 37% of 290 patients respectively, were positive for IgM compared to 9% of controls (McCartney *et al.*, 1986; Bell *et al.*, 1988). Additionally, IgM was detected more than one year later in a number of patients (McCartney *et al.*, 1986).

However, Miller *et al.* (1991) also tested sera from 53 patients and 49 controls for IgM using the capture ELISA method and 122 patients and 120 controls for IgG neutralizing antibody using the micrometabolic inhibition method at entry into the study. The results showed no significant difference between patients and controls for either measurement (24.4 % versus 22.6% positive respectively for IgM; 56.2% versus 55.3% positive respectively for IgG). After six months the figures for IgG were relatively unchanged, whereas the percentage of IgM positives had decreased in both patient and control groups but were still not significantly different from each other.

Yousef and colleagues (1988) attempted virus isolation from concentrated faecal samples by direct culture and after acid dissociation, to investigate 76 patients with PVFS and 30

matched neighbourhood controls. Enterovirus was detected in 17 (22%) patients and 2 (7%) controls. One year later the 17 isolation positives were re-tested and the same virus type was isolated from 5 (29%) patients. In addition, 13 (76%) had detectable IgM and 9 (53%) were positive for VP1 antigen in serum using the monoclonal antibody 5-D8/1 (Yousef *et al.*, 1987a). These results were suggestive of a chronic infection with enteroviruses in PVFS patients. In addition, there was a correlation between clinical improvement and disappearance of VP1 antigen and IgM complexes. In an additional group of 87 PVFS patients, enteroviral antigen was detected in the serum of 51% of patients and IgM circulating immune complexes were found in 74% of samples. All the patients were re-tested after 4 months and 89% were still positive, while no controls were positive for enteroviral antigen. This difference in frequency of detection of VP1 antigen between patients and controls was deemed significant (Yousef et al., 1988).

Halpin and Wessely (1989) confirmed this association, albeit at a lower frequency. Thirty percent (9/30) of patients with chronic unexplained fatigue were positive for VP1 antigen compared with 12% (4/43) of neurological case controls, a result which was just significant. However, Lynch and Seth (1989) comparing 20 patients with PVFS and 20 controls with depression could not distinguish the groups on the basis of VP1 antigen detection, questioning the specificity and sensitivity of the test.

More recently, molecular techniques have been used to study enteroviruses. Archard *et al.* (1988) used an enterovirus specific probe prepared from coxsackievirus B2 RNA in quantitative slot-blots to test for RNA in skeletal muscle biopsy specimens. Ninety-six patients with a history of PVFS of up to 20 years were tested. Twenty were positive for enterovirus with hybridization signals more than 3 standard deviations greater than the mean of normal muscle controls. The remaining 76 gave low, background values which were statistically indistinguishable from the normal muscle controls. Increased serum creatine kinase levels, used as a marker of muscle damage, were present in 11 out of 96 patient cases, 9 of whom were positive for enteroviral RNA.

Gow and colleagues (1991) also tested muscle biopsies of 60 patients with PVFS and 41 controls undergoing routine surgery from the same catchment area. Routine investigations did not reveal any significant abnormalities and muscle samples showed no evidence of inflammation or necrosis. Mild to moderate, non-specific atrophy of type 2 fibres was present in the biopsy samples, which is consistent with a chronic illness. Of the 60

patients and 41 controls, 12 and 6 respectively had neutralizing antibody titres of ≥ 256 to one of the coxsackie B viruses (types B1 to B5). A single round PCR of the 5' NTR followed by hybridization produced 32/60 (53%) positive results in the patient group, compared to 6/41 (15%) control group positives, results which were statistically significant. Based on the PCR results, the authors suggested that there was a persistent enterovirus infection in the muscle of some patients with PVFS and that this interfered with cell metabolism, causing the chronic fatigue.

In some patients, a continuing humoral immune response against viral antigens is not detected, even when enteroviral RNA is present in the affected tissue (Archard *et al.*, 1988; Bowles *et al.*, 1989). Together with the failure to isolate infectious virus or detect virus specific antigen, this suggests that progression to disease is associated with some kind of selection of defective virus. Cunningham *et al* (1990) used single-stranded virus specific RNA hybridization probes to compare the relative amounts of viral RNA species in a possible persistent infection *in vivo*, to those in a cytolytic infection *in vitro*. In the coxsackie B2 productive infection, positive strand RNA was synthesized in approximately 100-fold excess over the negative strand. However, where enteroviral RNA was detected in the skeletal muscle of CFS cases, production of equal amounts of positive and negative strands of enteroviral RNA was noted. This suggested that enteroviral persistence in muscle was due to a defect in the control of viral RNA synthesis.

It is not easy or practicable to obtain muscle biopsies from large groups of individuals and the possibility of using alternative samples was explored. With access to a well-defined group of individuals with CFS, through the Department of Infectious Diseases at Ruchill Hospital, a study using blood and stool samples to test for enteroviral sequences was undertaken.

The initial findings of this study are described by Clements *et al.* (1995), in which a group of 118 chronic fatigue syndrome (CFS) patients (181 samples of serum and/or buffy coat and/or stool) was tested for the presence of enteroviral sequences by nested PCR. The patient group comprising 77 females and 41 males, was matched as closely as possible to two comparison groups. The first comparison group (A) was composed of 101 patients (114 samples of serum and/or stools) with symptoms suggestive of an acute enteroviral disease such as headache, rash and/or pyrexia but no fatigue. This group was matched by

age, sex and date of specimen receipt. The second comparison group (B) comprised 126 individuals from whom serum had been obtained for occupational health or ante-natal screening purposes. This group was assumed to be healthy and was age matched but not sex matched because the group was predominantly female.

Enteroviral sequences were detected in 41% of serum samples, 27% of buffy-coat samples and 48% of stool samples of CFS patients. Where patients provided both serum and buffy coat specimens, 53% of the PCR results were in concordance. In the comparison groups, 27% of the serum samples from acutely ill patients and 2% of the healthy patients were positive for enteroviral specific sequences.

Analysis of the data revealed a significant difference at the 95% level between the findings on CFS serum samples and either comparison group. There was, however no significant difference between the CF stool results and group A stool results (28%). In the CFS group the results using serum and buffy coat were pooled and patients categorized as positive or negative, and on this basis 50/118 (42%) were enteroviral PCR positive. When the patients in the study were examined by sex and the duration of symptoms, it became apparent that the excess of females observed in the group overall was only seen in patients with a history of less than 2 years of fatigue. The majority of patients described symptoms commencing after an acute infection which is suggestive of a post-infectious fatigue syndrome. The presence of enteroviral specific sequences in a significant number of patients with chronic fatigue syndrome points to some role for the virus in this syndrome and provides additional evidence to confirm and extend the studies on muscle biopsies. The study also demonstrated that serum samples could be used as an alternative to muscle biopsies for the detection of enteroviral sequences in patients with CFS.

1.6. Summary and aims of the study

Enteroviruses are established aetiological agents of a wide variety of clinical syndromes, many of which involve the musculo-skeletal system. They have also been implicated in a number of chronic diseases, enteroviral RNA being detected in affected tissues in some cases of chronic heart disease for example. For a number of years there has also been accumulating evidence of an association between enteroviruses and Chronic Fatigue Syndrome (CFS) although much of this evidence has come from the United Kingdom only and has not been confirmed in studies from other countries. In the U.K., initial serological data has been supported recently by molecular detection of enteroviral RNA in muscle biopsies and serum samples. Although enteroviruses do not persist normally in the course of infection, defective replication of enteroviruses in some CFS patients has been suggested as a mechanism of persistence, thus contributing to the chronic nature of the syndrome. This evidence has also been disputed by other groups. Most studies have been based on the detection of enteroviral RNA by PCR, with sampling at one time point only which cannot provide direct evidence for persistence. No long-term follow-up studies have been carried out to look at enteroviral PCR status over time in relation to the clinical illness or to examine the type of enterovirus present in these patients.

The main aims of this study were therefore as follows:

1. To evaluate PCR-based technologies for the detection of enteroviral genomes in samples from CFS patients. This would be achieved initially using nested PCR to detect the 5' non-translated region of the enterovirus genome in serum samples obtained from CFS patients and comparison patients.
2. To determine the genetic sequence of the enterovirus(es) associated with CFS. This would be achieved by sequencing the PCR products and carrying out phylogenetic analysis of the sequences in the context of the enterovirus group as a whole.
3. To determine whether enteroviruses are associated with the syndrome by following a cohort of individuals with CFS from a well-defined population of patients over time.
4. To correlate the clinical status of the CFS patient, by means of a questionnaire, with the presence or absence of enteroviral sequences over time.

2. Materials and Methods

2.1. Materials

2.1.1. Cell Lines

MRC-5 (human embryonic lung fibroblasts) and RMK (Rhesus monkey kidney) cell lines (both obtained from Bio-Whittaker at a concentration of 2.5 x 10^5 in Earles Minimum Essential Medium (EMEM) with 10% foetal bovine serum (FBS) were propagated in 75cm² flasks in Medium 199 (with GlutaMAX™) supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin solution (10,000 units penicillin/10,000µg streptomycin/ml), 1% fungizone (250 µg/ml), 1% MEM vitamin solution (all Gibco BRL). Cells were incubated at 37°C in 5% CO_2 and passaged once before use.

2.1.2. Clinical Specimens

Specimens included in the 'Study' group were obtained from patients who had been diagnosed with Chronic Fatigue Syndrome (CFS) according to the Oxford Criteria (Sharpe *et al.*, 1991). Patients attended the outpatient clinic of the Infectious Disease Unit, Ruchill Hospital, or their general practitioner where a specimen of heparinized venous blood and/or a throat swab was obtained. Throat swabs were maintained in viral transport medium (VTM) (Gibco BRL) supplemented with 1% penicillin/streptomycin solution (Gibco BRL). The serum fraction was removed from whole blood by centrifugation at 2500 rpm for 5 minutes. Both samples were stored at -20°C until required.

The 'Comparison' Group was composed of patients who did not have CFS, and whose blood samples had been received at the Regional Virus Laboratory, either for ante-natal or for occupational health reasons. Samples with sufficient volume for testing were chosen at random each month to provide a background level of enterovirus detection throughout the years of the study. Again, the serum fraction was removed and stored at -20°C until required.

2.1.3. Oligonucleotide Primers

Oligonucleotides were supplied from three sources during the course of the study: Oswell DNA Services (UK), Cruachem (UK) and Gibco-BRL. The sequences of the primers (shown 5' to 3') are presented in Table 6. All primers were used at a working concentration of approximately 40 pmoles/μl.

Primer Name	Nucleotide Sequence (from 5'-3')	Position	Length	% G+C content	Tm
P1+	cggtacctttgtgcgcctgt	63-82	20	60	77.5
P1-	acaggcgcacaaaggtaccg	82-63	20	60	77.5
P4+	ctgaatgcggctaatcctaac	457-477	21	47.6	71.4
P4-	ttaggattagccgcattcag	476-457	20	45	70.2
P6+	gcacttctgttacccc	168-183	16	50	58.6
P6-	ggtaacagaagtgcttgatc	181-162	20	45	63.7
P9+	gtgcgaagagtctattgagc	416-435	20	50	66.7
P9-	tcaatagactcttcgcac	433-416	18	44	59.5
A+	tggctgcttatggtgacaat	581-600	20	45	66.2
A-	attgtcaccataagcagcca	600-581	20	45	66.2
B-	tctgggaacttccaccacca	1199-1180	20	55	75.0
prot1+	aactccaccctgcagattga	5173-5192	20	50	72.0
prot2-	acggcccacctatcatagat	5468-5449	20	50	70.4
prot3-	agcattctcttggtgggtgt	5768-5749	20	50	71.0
Y-	cgcaccgaatgcggagaatttacc	7398-7375	24	54	84.5
ABL1+	cagcggccagtagcatctgactt	n/a	23	56	79.7
ABL2-	tgtgattatagcctaagacccggag	n/a	25	48	77.2
poly -T	ttcgcgaggttaacgtcgact$_{(14)}$	3' poly (A)	34	32.4	74.9

Table 6: Oligonucleotide sequences and positions relative to the coxsackievirus B3 genome (5' to 3') (GenBank accession number M33854). Forward and reverse primers are denoted as '+' and '-' respectively. %G+C relates to the number of guanidine and cytosine residues. The Tm recorded is that according to the manufacturer.

2.1.4. Extraction and PCR reagents

All reagents for the extraction and PCR procedures were stored as small volume aliquots (where appropriate) and either used as supplied or diluted in DEPC (diethyl pyrocarbonate) (Sigma) treated water (Baxter Healthcare Limited). DEPC (0.01%) was added to the water which was left to stand overnight before autoclaving. Aerosol resistant tips (Alpha laboratories) were used at all times and micro-centrifuge tubes (0.5ml thin-walled from Advanced Biotechnologies and 1.5ml from Sarstedt) were autoclaved before use.

2.1.5. Patient Questionnaire

In conjunction with the infectious disease consultants at Ruchill Hospital, a self-administered patient questionnaire was developed (see Appendix). Patient details including previous medical history, present symptoms and PCR status were stored using the data manager software package DataEase version 4.5 (1992) supplied by DataEase U.K. Limited.

2.2. *Coxsackievirus B neutralization assay*

Study and comparison sera were diluted 1/16 in phosphate buffered saline (PBS) (Sigma) and heat inactivated at 56°C for 30 minutes. Aliquots (25µl) of each sample were transferred to 5 microtitre plates (one for each of coxsackievirus B types 1 to 5) and double-diluted 8 times in medium 199 (Gibco BRL). Aliquots (25µl) of the coxsackievirus stock suspensions (types B1 to B5) at 100 $TCID_{50}$ were added to the diluted sera and the plates incubated at room temperature to allow neutralization of the virus to occur. After the incubation, 100µl aliquots of Vero cell suspension (at 1.5 x 10^5 cells per ml) were added to each of the wells and incubated with carbon dioxide (CO_2) at 37°C for 3 days. Control wells were included to test that the serum alone was not toxic to the cells. The end point was taken as the dilution below that which shows cytopathic effect (i.e. the lowest dilution of serum that just neutralizes the virus). In accordance with the routine laboratory method of reporting, titres less than 256 were interpreted as not significant, titres of 256 were suggestive of infection and those of 512 and above were indicative of recent infection (Grist *et al.*, 1974).

2.3. Preparation of Control RNA

2.3.1. Preparation of negative control RNA

MRC-5 cells (Biowhittaker) were cultured as monolayers in 75cm² flasks and maintained in Medium 199 (with GlutaMAX™) with 0.5% FBS, 1% penicillin/streptomycin and 1% fungizone at 37°C in 5% CO_2. When the cells were confluent the medium was decanted, the cell layer washed twice with 10mls PBS, and 1ml of trypsin/EDTA (Gibco) was added. After approximately 1 minute the trypsin was removed and the tubes maintained at room temperature until the cells began to detach. Fresh medium (approximately 10mls) was then added and the cells removed to a universal container for centrifugation at 3000rpm for 5 minutes. The medium was decanted and the cell pellet washed twice with PBS before being subjected to the RNA extraction procedure as described in 2.4.

2.3.2. Preparation of Positive Control RNA

Coxsackievirus type A9 was used as the positive control virus throughout this investigation. All other enterovirus types mentioned in the text were prepared following the same procedure. MRC-5 cells were seeded with an aliquot of coxsackievirus A9 positive cell culture fluid (previously identified by the RVL by traditional typing methods (Grist *et al.*, 1979; Minor & Bell, 1990)) and incubated until a cytopathic effect (c.p.e.) of approximately 75% was evident. The viral titre of the tissue culture fluid was determined according to the method of Grist *et al.* (1974). Dilutions of the cell culture fluid to 10^{-7} were made in 0.5% medium 199. Four culture tubes of MRC-5 monolayers were seeded with 200µl of each dilution and incubated in the stationary sloped position at 37°C. After approximately 6 days, the tubes were examined for c.p.e and scored accordingly. The titre was determined according to the Karber formula (Karber, 1931). The titred cell culture fluid was maintained in 1ml aliquots at -70°C.

2.4. Extraction of RNA

Two commercial methods were used in this study, the Glassmax™ RNA Microisolation Spin Cartridge system and TRIzol™ (Total RNA Isolation) reagent, both supplied by Gibco BRL.

2.4.1. Glassmax™ System

RNA was extracted using a modified version of the manufacturer's instructions. Serum or throat swab sample (in VTM) (200µl) was added to 450µl of ice-cold denaturing solution [guanidinium isothiocyanate/2-mercaptoethanol (10%)], 25µl RNAguard ribonuclease inhibitor (1/20 dilution) (Pharmacia), and 25µl calf-liver ribosomal RNA (1/100 dilution) (Sigma) in a 1.5ml micro-centrifuge tube and vortexed. To this, ethanol (420µl; 100%) (BDH) was added, the mixture vortexed and then centrifuged at maximum speed for 5 minutes in a micro-centrifuge. The nucleic acid pellet was retained and resuspended in 450 µl binding solution (6M sodium iodide) and 40µl of 3M sodium acetate solution pH 5.5 (supplied). The resultant solution was vortexed and transferred to a Glassmax cartridge which was centrifuged at maximum speed for 1 minute. The nucleic acid bound to the cartridge was then washed three times with RNA wash buffer and twice with 80% (v/v) ethanol. After a final centrifugation to remove the last traces of ethanol, the nucleic acid was eluted by centrifugation with 25µl DEPC-treated water heated to 65°C.

2.4.2. TRIzol™ (Total RNA Isolation) Reagent

This reagent consists of a mono-phasic solution (pH 4-5) of phenol and guanidine isothiocyanate. TRIzol (1000µl) and 200µl of sample (or virus infected cell culture fluid) were combined in a 1.5ml micro-centrifuge tube and incubated at room temperature for 5 minutes. Chloroform (200µl) was added, the mixture vortexed and incubated for a further 2 minutes before centrifugation at 12,000g for 15 minutes. The upper aqueous phase containing the RNA was transferred to a fresh tube and mixed with an equal volume of isopropyl alcohol and 15µl calf-liver ribosomal RNA (1/40 dilution) for 10 minutes at -20°C. Following centrifugation at 12,000g for 15 minutes, the RNA precipitate formed a gel-like pellet on the sides and bottom of the tube. The supernatant was removed and the pellet washed with 75% ethanol, and then dried briefly under vacuum. The RNA was dissolved in 20µl DEPC-treated water and incubated for 10 minutes at approximately 55°C. The RNA from both methods was stored at -70°C until required. The integrity of the extracted RNA was determined by visualizing the 18s and 28s ribosomal bands following agarose gel electrophoresis.

2.5.1. Reverse Transcription of RNA

For each reaction, 4µl 5x first strand buffer (250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$) (Gibco BRL), 2µl 10mM deoxynucleoside triphosphates (dNTPs) (Advanced Biotechnologies), 1µl RNAguard (1/20 dilution), 1µl $pd(N)_6$ random hexamers (1/100 dilution) (Pharmacia), 1µl Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (Gibco BRL) and 1µl 0.1M dithiothreitol (DTT) (Gibco BRL) were mixed with 10µl sample or control RNA and incubated at room temperature for 10 minutes. The reaction mixture was then incubated at 37°C for 45 minutes followed by 95°C for 5 minutes, before being cooled on ice. The cDNA was either amplified immediately by PCR or stored at -20°C until required. The use of random hexamers enabled the same cDNA sample to be used as a template in a number of different PCR reactions using different primers.

2.5.2. Polymerase Chain Reaction (PCR)

With the introduction of new thermocyclers and the use of thin-walled tubes, the PCR protocols originally described in Clements *et al.* (1995) and Galbraith *et al.* (1995) were modified resulting in smaller reaction volumes and shorter cycling times.

For a standard PCR amplification using P1+ and P4- or ABL1 and ABL2 control primer combinations (Table 6: p52) , a 50µl reaction mixture was prepared containing 5µl of 10x Reaction Buffer IV (200mM $(NH_4)_2SO_4$, 750mM Tris-HCl (pH 9.0), 0.1% (w/v) Tween) and 3µl magnesium chloride (25mM) (both Advanced Biotechnologies), 1µl dNTPs (10mM), 40 pmoles each primer, 0.25µl Thermoprime plus DNA polymerase (5 units/µl) (Advanced Biotechnologies) and 10µl cDNA (prepared as described in 2.4.1.). The mixture was overlaid with a drop of mineral oil (Sigma) to prevent evaporation and subjected to the following conditions on an Omnigene Thermocycler (Hybaid): 1 minute at 94°C, followed by thirty-five cycles of 50 seconds at 94°C, 50 seconds at 55°C and 1 minute, 10 seconds at 72°C, followed by a final extension step of 72°C for 10 minutes.

For the 'nested' PCR using P6+ and P9- primers (Table 6: p52), 2µl of first round product was amplified for 25 cycles in a 50µl reaction volume as described above. Reaction

conditions for other primer combinations will be described in the text where appropriate.

2.6. Monitoring for contamination in the RT-PCR process

In this study all reasonable precautions were taken within the laboratory to minimize the risk of contamination. Separate rooms (and safety cabinets in some areas) were used for extraction of RNA, preparation of reagent mastermixes, cycling of RT and first round mixes, cycling of second round mixes and gel electrophoresis of PCR products. In each room, dedicated pipettes and specimen racks, aerosol resistant tips, protective clothing, gloves and storage facilities were available. Only six samples were processed together alongside one positive (coxsackievirus A9 infected MRC-5 cells) and one negative (MRC-5 cells) control. A 'no template' control was included at the RT and first round stage. A second PCR was run simultaneously using control primers ABL1/ABL2. These amplify ableson tyrosine kinase messenger (m)RNA and are used to ensure that RNA has been successfully extracted from the samples (Gow *et al.*, 1991). In approximately 99% of serum samples an amplification product was observed using the ABL primers. If all the controls were amplified as expected, then the sample results were accepted. Any positive results were repeated from the extraction stage.

Surfaces in each working area within the laboratory were swabbed with cotton-tipped swabs twice over the study period. These surfaces included: bench tops, pipettes, specimen racks, fume cabinet surfaces, centrifuges, freezer handles and door handles. Two swabs were obtained from each surface: one was placed into water for direct PCR amplification to detect PCR product and one was placed into VTM for extraction and RT-PCR amplification to detect viral RNA.

For direct PCR amplification, 10µl of the swab solution was amplified using P6+ and P9- primers in a standard 50µl reaction as described in 2.5.2.

The VTM solution (200µl) was extracted using TRIzol™ as described previously (2.4.2). The resulting RNA was subjected to RT-PCR as described in section 2.5.

2.7. *Agarose Gel Electrophoresis*

PCR products (10µl) were combined with 2.5µl loading buffer (prepared using a concentrated solution of Ficoll® 500 (Pharmacia) and Orange G dye (Gurr Microscopy) in 0.5x Tris/boric acid/EDTA (TBE) buffer). Products were run alongside 350ng of 100 base pair ladder (Gibco BRL) on a 1.5% agarose (w/v) (SeaKem®, Flowgen) horizontal gel containing 10µg ethidium bromide (Sigma) in 0.5x TBE running buffer for approximately 2 hours at 80 volts. Bands were visualized under U.V. light and images recorded with a gel documentation system (Sony).

Predicted PCR product sizes

P1+/P4-	414 base pairs	P6+/P9-	264 base pairs
P4+/B-	750 base pairs	A+/B-	600 base pairs
prot1+/prot3-	595 base pairs	prot1+/prot2-	295 base pairs
ABL1/ABL2	220 base pairs	P4+/prot2-	5000 base pairs

2.8. *'Purification' of PCR Products*

PCR products were separated from the unincorporated PCR components using the Geneclean II® kit (Stratech Scientific). A much simpler and more rapid method was later adopted using Microspin™ S-400 HR columns (Pharmacia).

2.8.1. Geneclean II® kit

In a micro centrifuge tube, 20µl PCR product, 30µl Tris-EDTA Buffer (pH 8.0) (Sigma) and 150µl Sodium Iodide (NaI) stock solution were combined before adding 6µl Glassmilk® silica matrix (Stratech Scientific) and mixing continuously for 10 minutes. The DNA/Glassmilk® complex was then pelleted and the supernatant removed. The pellet was washed three times with New Wash (made according to manufacturer's instructions). Any remaining liquid was carefully removed and the pellet resuspended in 20µl sterile DEPC-treated water. After incubating at 50°C for 3 minutes the mixture was centrifuged and the supernatant (approximately 20µl) containing the eluted DNA was removed to a fresh tube.

2.8.2. Microspin™ S-400 columns

The column resin was resuspended by vortexing, the cap was loosened and the bottom closure snapped off. The column was then placed in a 1.5ml micro-centrifuge tube and centrifuged at 3000 r.p.m. in a micro-centrifuge (MSE Micro Centaur) for 1 minute to remove the storage buffer. After placing in a clean 1.5ml microcentrifuge tube, 30µl of PCR product was then placed carefully on the top of the sephacryl resin which was centrifuged again for 2 minutes to elute the product.

2.9. Sequencing of PCR products

A number of enterovirus isolates in tissue culture fluid were available for sequencing. Isolates were either identified in the Regional Virus Laboratory or obtained from reference collections (Table 7). RNA extraction and RT-PCR procedures were as described previously.

Initially, manual chain termination sequencing of samples and a few echovirus isolates (echo 3, 4, 7, 9, 11, 20) was performed using the Sequenase® version 2.0 DNA sequencing kit (United States Biochemical). This technique was later replaced by automated fluorescent sequencing.

All fragments were sequenced using both forward and reverse primers, using the same set that had generated the PCR product. For example, the 264 base pair product from the nested PCR was sequenced using P6+ and P9- primers.

Clinical Isolates	Code	Location/month/year
Coxsackie A1	Reference strain	Unknown
Coxsackie A5	Swartz strain	USA/8/1964
Coxsackie A7	M708	USA/7/1964
Coxsackie A9	8558	Monklands/12/1994
Coxsackie A11	ET911	USA/10/1960
Coxsackie A15	ETL69	USA/5/1961
Coxsackie A20	M1165	USA/4/1966
Coxsackie A22	Reference strain	Unknown
Coxsackie B2	4048	Glasgow/4/1994
Coxsackie B2	49	Edinburgh/7/1992
Coxsackie B2	50	Edinburgh/8/1992
Coxsackie B2	51	Edinburgh/9/1992
Coxsackie B2	53	Edinburgh/9/1992
Coxsackie B2	54	Edinburgh/11/1992
Coxsackie B2	55	Edinburgh/11/1992
Coxsackie B2	56	Edinburgh/12/1992
Coxsackie B3	3629	Dumfries/5/1997
Coxsackie B3	3748	Dumfries/5/1997
Coxsackie B3	4413	Dumfries/6/1997
Coxsackie B3	4541	Dumfries/6/1997
Coxsackie B3	4625	Dumfries/6/1997
Coxsackie B3	5027	Dumfries/6/1997
Coxsackie B3	6220	Dumfries/8/1997
Coxsackie B3	6687	Glasgow/8/1997
Coxsackie B3	8985	Stirling/10/1997
Coxsackie B3	1996QC	CPHL/6/1996

Table 7: Clinical isolates typed and sequenced in the RVL. Sources of isolates are indicated where known.

Clinical Isolates	Code	Location/month/year
Coxsackie B5	6237	Dundee/5/1961
Coxsackie B5	6242	Dundee/5/1961
Coxsackie B5	2868	Dumfries/4/1996
Coxsackie B5	2907	Dumfries/4/1996
Coxsackie B5	6093	Glasgow/8/1996
Coxsackie B6	Reference strain	Unknown
Echovirus 2	Reference	Unknown
Echovirus 3	6291	Dumfries/11/1993
Echovirus 4	1990	Glasgow/3/1990
Echovirus 5	E5	Glasgow/U/1994
Echovirus 6	4283	Glasgow/6/1994
Echovirus 6	4566	Glasgow/7/1994
Echovirus 6	4730	Glasgow/7/1994
Echovirus 6	4989	Glasgow/7/1994
Echovirus 7	1788	Dumfries/3/1994
Echovirus 9	6518	Glasgow/11/1993
Echovirus 11	2350	Lanarkshire/2/1989
Echovirus 19	E19	Glasgow/3/1980
Echovirus 20	645	Glasgow/1/1994
Echovirus 25	E25	Glasgow/3/1980
Echovirus 30	6392	Ayrshire/7/1966
Echovirus 30	6398	Ayrshire/7/1966
Echovirus 30	6548	Ayrshire/11/1975
Echovirus 30	8117	Monklands/10/1995
Echovirus 30	8293	Monklands/11/1995
Echovirus 30	3786	Dumfries/5/1996
Echovirus 30	4313	Dumfries/6/1996
Echovirus 30	4844	Glasgow/6/1997

Table 7 (cont): Clinical isolates typed and sequenced in the RVL. Sources of isolates and year of isolation are indicated where known.

2.9.1. Manual sequencing

2.9.1.1. Chain termination sequencing using the Sequenase version 2.0 DNA sequencing kit. In a 0.5ml microcentrifuge tube, 5µl of primer and 10µl 'geneclcaned' PCR product (as described in 2.8.1.) were incubated at 95°C for 2 minutes. After a 10 second spin the mix was incubated at 70°C for 3 minutes, 95°C for 45 seconds and 37°C for 10 minutes before cooling on ice. An aliquot of this (8µl) was added to 2µl reaction buffer (5x), 1µl 0.1M DTT, 2µl labelling mix (1/10 dilution with water) and 1µl Redivue [^{35}S] dATP (Amersham International plc). Finally 2µl sequenase enzyme (1/8.5 dilution with enzyme dilution buffer) was added and the reaction mixed thoroughly. Four tubes were prepared containing 2.5µl of each termination mix (ddG, ddA, ddT, ddC) and pre-warmed to 37°C for 1 minute. Aliquots of the labelling reaction mixture (3.5µl) were then added to each of the four tubes, centrifuged for a few seconds and incubated at 37°C for 5 minutes. Stop solution (4µl) was added and the tubes refrigerated until required for electrophoresis.

2.9.1.2. Denaturing Acrylamide Gel Electrophoresis. Two glass plates were thoroughly cleaned with ethanol and one treated with Repelcote water repellent (BDH) in a safety cabinet. The plates and 1mm spacers were placed together and bound with electrical tape. A 6% acrylamide gel was prepared by adding 24ml concentrate, 66ml diluent and 10ml buffer (Sequagel™ sequencing system, National Diagnostics) followed by 300µl ammonium persulphate (25%) (w/v) (Sigma) and 40µl TEMED (Sigma). The solution was poured between the glass plates and a sharkstooth comb placed in upside down. Once the gel had set, the tape and the comb were removed and the plates loaded into the gel electrophoresis system (Gibco BRL) buffered by 0.5x TBE (Gibco BRL). The comb was repositioned with the teeth touching the top of the gel, and the gel was pre-run for 30 minutes at approximately 1500 volts. The radioactive samples were denatured at 95°C for 5 minutes and 3µl of each mix loaded onto the gel in the order G, A, T, C. The gel was run for 2.5-3.0 hours at a constant 1500 volts and then soaked in acetic acid (5%) (BDH) /methanol (15%) (v/v) (BDH) for 15 minutes. After washing in water for 10 minutes, the gel was transferred to 4mm chromatographic paper and dried under vacuum at 80°C for 1.0-1.5 hours. The dried gel was placed in a film cassette in contact with photographic film (Kodak) for 2-5 days, and the film developed in an automatic processor in the hospital X-Ray department. The nucleotide sequence was determined and transferred manually to the computer database (UNIX system) for analysis.

2.9.2. Automated Sequencing

Initially, sequencing was carried out by Mr Robert McFarlane of the Beatson Institute for Cancer Research (results presented in Galbraith *et al.*, 1995, 1997). Subsequent sequencing reactions were performed by the author and run on gels by staff of the Molecular Biology Support Unit (M.B.S.U.) of Glasgow University.

Both laboratories used the ABI 373 DNA sequencer (Applied Biosystems) and accompanying software for base calling of sequences.

Template DNA (6µl) (prepared as described in 2.8.2.) and 3.2 pmoles of primer were mixed and added to 8µl reaction premix (ABI PRISM™ Dye terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer) in a 20 µl volume. The reaction was overlaid with a wax pellet (PCR Gem 50, Perkin Elmer) and incubated through 25 cycles of 96°C for 30 seconds, 50°C for 20 seconds and 60°C for 4 minutes. After cycling the product was removed from under the wax layer to a fresh tube. To this, 50µl ethanol (100%) and 2µl sodium acetate (3M: pH 4.5) (Sigma) was added and the reaction incubated at -70°C for 15 minutes. After centrifugation at maximum speed in a microcentrifuge for 15 minutes, the pellet was washed with 70% ethanol (v/v) and then air-dried. Samples were then sent to the M.B.S.U. for electrophoresis. Raw data was returned on disc and transferred to the database (UNIX system) for analysis. The forward and reverse sequences were aligned and any ambiguities corrected manually.

2.10. Inverse PCR

This is a method for obtaining the sequence of flanking regions of a known sequence, by using primers in the reverse orientation to those that will amplify the known sequence (see Figure 22: p123). The first stage involves the production of double-stranded cDNA from the RNA template. A specific primer in the RT reaction will produce a cDNA fragment of a specific size whereas a poly-T primer will attach to the poly-A tail and produce full-length cDNA. In the latter case, a restriction enzyme can then be used to cut the DNA into fragments of appropriate size for ligation. A low DNA concentration favours circularization of product rather than concatemerization, and fragments between 300 base pairs and 3 kilobases can be ligated. The circular DNA can then act as a template for PCR, or can be linearized with a second restriction enzyme prior to PCR.

Protocols for inverse PCR were based on the methods described by Ochman and colleagues

(1990) and Zeiner and Gehring (1994).

2.10.1. Preparation of double stranded cDNA using specific internal primer

Coxsackievirus A9 was used as the control in the development of this technique. RNA extracted as described in 2.4.2, was used as a template for double stranded cDNA synthesis using a kit provided by Boehringer Mannheim. Essentially, 9µl sample RNA was copied into cDNA according to the manufacturer's instructions except for the omission of the radioactive tracer. The final reaction mix was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) before precipitating with 2 volumes of 100% ethanol. After washing with 70% (v/v) ethanol, the pellet was resuspended in 10µl sterile water.

A short double stranded cDNA fragment (approximately 600 base pairs) was produced by using primer A- (Table 6: p52) in the initial first strand synthesis reaction. The resulting blunt ended fragment was then self-ligated in a 100µl reaction containing 10 units T4 DNA ligase. The ligation reaction was divided into two aliquots, one which was heat inactivated at 65°C for 20 minutes and one which was extracted with an equal volume of phenol/ chloroform/isoamylalcohol followed by precipitation with 2 volumes of 100% ethanol. The resulting pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 20µl sterile water.

2.10.2. Preparation of full length double-stranded cDNA using a poly-T primer

Full-length double stranded DNA was obtained using the poly-T primer (Table 6: p52) to initiate first strand synthesis following the protocol outlined above. Hae II (Gibco BRL) was used to digest the double stranded cDNA in a 15µl reaction containing 10 units of enzyme and 5µl cDNA in 2x One Phor All buffer (OPA buffer, Pharmacia: 10x buffer contains 100mM Tris Acetate pH7.5, 100mM magnesium acetate and 500mM potassium acetate) for 2 hours at 37°C. Hae II cuts at positions 39, 1207 and 2294 of the coxsackie A9 genome, producing fragments of 38, 1168, 1087 and 5206 base pairs. Following inactivation of the enzyme at 65°C for 20 minutes, 10µl of the reaction was included in a 100µl reaction containing 10 units of T4 DNA ligase (Boehringer) and 1mM ATP (Pharmacia) in 1x OPA buffer. This was incubated overnight at 15°C and then inactivated

at 65°C for 20 minutes. Linearization of the 1168 base pair cDNA fragment was accomplished using 10 units of Xmn I (Promega), which cuts at position 229 of coxsackievirus A9, in a 50µl reaction in 1x OPA buffer at 37°C overnight.

2.10.3. Inverse PCR

Aliquots (10µl) of double stranded cDNA template (either in circle or linear form), prepared as described above were amplified in 50µl reactions using P6- and P9+ primers (Table 6: p52) under the following conditions: 94°C for 2 minutes followed by 35 cycles of 94°C for 50 seconds, 55°C for 50 seconds, 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. A second 'nested' PCR was also carried out under the same conditions (for 25 cycles) using primers P4+ and P1- (Table 6: p52).

Aliquots of the template were also amplified using the standard P1+/P4- and P6+/P9- primer combinations (Table 6: p52) as described in section 2.5.2 as a control to ensure that the template was present and intact.

All PCR products were visualized by gel electrophoresis on 1% agarose gels.

2.11. Poly A polymerase reaction

Adenosine residues were added to the 3' end of RNA in a 20µl reaction containing 6.5µl sample RNA; 4µl of buffer (250µl REACT 1 buffer (50mM Tris-HCl, 10mM magnesium chloride pH 8.0)) (Gibco BRL), 125µl sodium chloride (5M), 125µl acetylated BSA (10mg/ml) (Promega)); 1µl RNAguard; 2.5µl ATP (10mM) (Pharmacia); 5µl manganese chloride (10mM) (Sigma) and 1µl poly A polymerase enzyme (Gibco BRL) (method kindly provided by Daniel Bailey, Reading University). The reaction was centrifuged for a few seconds in a microcentrifuge and incubated at 37°C for 15 minutes. The final reaction products were extracted with phenol/chloroform /isoamylalcohol (25:24:1), followed by precipitation with 2 volumes of 100% ethanol and 1/10 volume of sodium acetate (3M, pH 5.2). After washing with 70% ethanol (v/v) the pellet was dried and resuspended in 11µl DEPC-treated water. Samples treated in this way were subjected to RT-PCR using primers P1+/P4- and P6+/P9- following the standard protocol of 2.5 except that poly-T primer was used in place of the random hexamers in the RT reaction.

PCR products were then visualised by agarose gel electrophoresis according to method 2.7.

2.12. *Long distance PCR*

As an alternative to designing internal primers capable of amplifying fragments of the genome of different enterovirus types, it is possible to take advantage of the conserved sequence at the 5' end and the poly-A tail at the 3' end of the viral genome. A primer designed to bind to the poly-A tail can be used to prime the RT reaction and produce full-length cDNA. A combination of a 5' primer and a primer at the extreme 3' end (Y-) could then be used to amplify the cDNA. Primers prot1+, prot2- and prot3- were also designed at this stage to bind to a relatively conserved region of the 2C protease among the coxsackie and echoviruses, with a view to amplifying longer regions of the genome.

Long distance PCR was carried out following the methods of Gow *et al.*, (1996) and Lindberg *et al* (1997). Positive control coxsackievirus A9 (dilutions 10^{-1} to 10^{-5}) was reverse transcribed using a 3' end primer (i.e. poly-T) and SUPERSCRIPT™ II (Gibco BRL) reverse transcriptase. The sample RNA (10µl) was heated to 70°C for 10 minutes with 1µl RNAguard (1/20 dilution) and 1µl of the chosen primer, before quick chilling on ice. The contents were collected by centrifugation before adding 4µl of 5x first strand buffer, 2µl DTT (0.1M) and 1µl dNTPs (10mM) and incubating at 42°C for 2 minutes. To this, 1µl of the reverse transcriptase was added, mixed and incubated at 42°C for 50 minutes, before inactivating at 70°C for 15 minutes. The RNA component in the RNA-cDNA hybrid was then removed by incubating with 2 units of RNase H (Gibco BRL) at 37°C for 20 minutes.

Different combinations of forward and reverse primers (eg. P1+ and prot3-) were then used in a first round reaction of 50µl containing 1x reaction buffer IV, 2.5 units Thermoprime plus DNA polymerase (both Advanced Biotechnologies), and 0.06 units Deep Vent DNA polymerase (New England Biolabs). The reaction was incubated for 2 minutes at 94°C, followed by 15 cycles of 50 seconds at 94°C, 50 seconds at 55°C and 68°C for 5 minutes, and then 15 cycles as above with an additional 15 seconds on each successive extension cycle. The amplification was terminated by a final extension at 72°C for 10 minutes. A second PCR was performed on these products using nested (eg. P4+ and prot2-) or semi-nested primers (eg. P1+ and prot2-), under the same conditions as above except that 25 cycles (10 of these with the additional extension time) were performed. Gel electrophoresis was performed on 0.7% (w/v) agarose gels as described in section 2.7.

2.13. Cloning Protocol

This protocol was performed at the Beatson Institute for Cancer Research using the ZAP-cDNA® synthesis kit and the ZAP-cDNA® Gigapack® III gold cloning kit (Stratagene®) according to the manufacturers instructions except where indicated. Ingredients of solutions and agar used are described fully in the manufacturer's protocol.

2.13.1. Preparation of cDNA

cDNA was prepared using 20µl sample RNA (poly-(A)$^+$) according to the manufacturer's instructions using [α-^{32}P]dGTP at the second strand synthesis stage. After size fractionation through sepharose CL-2B gel filtration medium, cDNA fractions were extracted with phenol/chloroform [1:1(v/v)] and then precipitated with ethanol. The cDNA pellets were resuspended in sterile water, the volume dependent on the amount of radioactivity detected (3.5µl if less than 30 counts per second/ 5µl if greater than 30 counts per second). Quantitation of the cDNA was carried out by the ethidium bromide plate assay described in 2.15.

2.13.2. Ligation into vector and packaging into host bacteria

Ligation of the sample cDNA (2.5µl) into the Uni-ZAP XR vector (Lambda/pBluescript phagemid) was carried out as described in the manufacturer's protocol. Ligated DNA (3µl) was then packaged as described and the phage supernatant stored at 4°C until required.

2.13.3. Plating, titering and amplification of the library

The packaged reaction was titered with XL1-Blue MRF' host bacteria on NZY agar (Gibco) plates using IPTG (isopropyl-1-thio-β-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue/white selection. Aliquots of each library containing ~5 x 10^6 pfu of bacteriophage were then amplified according to the manufacturer's protocol, and the titre checked using host cells and serial dilutions of the amplified library.

2.13.4. Performing plaque lifts

Approximately 250,000 pfu (previously incubated with 4ml host cells) was plated onto 240mm² NZY agar plates and incubated at 37°C for approximately 8 hours. Hybond-N (22x22cm, Amersham) membranes were used to perform the lifts in duplicate according to the protocol. Denaturation, neutralization and rinsing of the membranes were carried out as follows. Whatman® paper was placed in three trays and soaked in 1.5 M sodium chloride/0.5M sodium hydroxide solution, 1.5 M sodium chloride/0.5 M Tris-HCl (pH 8.0) solution and 2x SSC buffer respectively. Membranes were placed on the paper (lift side up) for the recommended time and then blotted briefly before undergoing cross-linking using the autocrosslink setting on a Stratalinker® UV crosslinker.

2.13.5. Hybridizing and screening

Prehybridization of the membranes was carried out in prehybridization buffer (see appendix 2) for at least 2 hours in a shaking water bath at 65°C. Radioactive probe was prepared using High Prime solution (Boehringer Mannheim), [α^{32}P] dCTP (Redivue, Amersham International, plc) and 11µl template DNA according to the manufacturer's instructions. The reaction was stopped by incubating with an equal volume of 0.4M sodium hydroxide (NaOH) for 5 minutes. The probe was then added to approximately 50mls prehybridization solution and incubated with the filters overnight at 65°C. The membranes were washed (for 20 minutes) in decreasing concentrations of SSC (containing 0.1% SDS), from 2x SSC to 0.2 SSC and then exposed to film (Kodak) at -70°C, the exposure time depending on the level of radioactivity observed. The film was developed in an automatic processor.

The duplicate developed films were then matched to the original plate and areas of positive hybridization on both were noted. Any positive plaques were picked into SM buffer for further screening.

A number of probes were available for screening the membranes: one of approximately 5kB was obtained from the long PCR of coxsackievirus A9 (primers P4+/prot2-); one encompassed approximately 300 base pairs of the protease region and was produced by semi-nested PCR using primers prot1+, prot2- and prot3- and the specific 264 base fragment generated by the standard PCR (primers P6+/P9-) from the sample being screened. In addition, the control probe consisted of the PCR product from the ABI1/ABL2 PCR.

2.14. *Ethidium Bromide Plate Assay - Quantitation of cDNA*

Agarose (100ml of 0.8% (w/v)) in TAE buffer (1x) was prepared and cooled before adding 10µl of ethidium bromide stock solution (10mg/ml) and mixing. This was poured into 100mm petri dishes using approximately 10ml per plate and allowed to harden. Hind III digested Lambda DNA (Gibco BRL) (500ng/µl) was used as the standard and diluted in 100mM EDTA to cover the range from 200 to 10ng/µl. Duplicates of the standard (0.5µl) were each spotted onto the surface of the plates by capillary action, immediately before spotting the cDNA samples adjacent to the standards. After 10 to 15 minutes at room temperature the plate was inverted and photographed using a UV lightbox. Estimations of the concentration of the samples were made by comparing the intensities of the spots to the standards.

2.15. *Molecular Computing*

Sequence information was stored in the UNIX database maintained by the University of Glasgow. Sequence data was analysed using the University of Wisconsin Genetics Computer Group (UW/GCG) package, version 7.0 (Devereux *et al.*, 1984) accessed via UNIX.

2.15.1. Phylogenetic trees

Alignment of sequences was carried out using PILEUP (GCG). Programs COMPACT and TOPHYLIP (A.Wright, University of Glasgow) were then used to truncate the sequences. Trees were generated using parsimony analysis (PHYLIP/DNAPARS).

2.15.2. Dendrograms

Alignment of sequences was carried out using PILEUP. A distance analysis method (DISTANCES) using one of six distance correction methods (uncorrected, Jukes-Cantor, Kimura 2-parameter, Jin-Nei gamma, Tajima-Nei and Tamura) was used to compare the aligned sequences. GROWTREE using either the neighbour joining or UPGMA methods was then used to generate the dendrogram plot (UW/GCG).

The programs to create the phylogenetic trees were not available after 1995 and therefore method 2.15.2 was used after this date for all sequence comparisons.

The program FASTA was used to compare newly generated sequence data to sequences in the GenBank database to identify that with most sequence identity.

The program GAP was used to obtain the percentage identity between two nucleotide sequences based on the whole sequence available. For amino-acid comparisons, percentage identity (at the level of the nucleotide sequence) and percentage similarity (at the level of the amino acid) can be calculated.

2.16. Statistical methods

2.16.1. Chi-square analysis

1. Data in the form of a 2x2 contingency table (Table 8).

Test statistic $\chi^2 = n(ad-bc)^2/(a+c)(b+d)(a+b)(c+d)$

2. Data with more than two categories of classification (Table 9).

The expected frequency for each cell in the table is calculated as follows:-

Expected = total of row x total of column/total

Test statistic $\chi^2 = \sum$ (observed value-expected value)^2/expected value

In both cases the degrees of freedom = (r-1)(c-1) where r = rows and c = columns

2.16.2. Comparing two population means

Comparison of two means from populations A and B that are not normally distributed requires the following test statistic:-

$$z = (x_1-x_2) - (\mu_1 - \mu_2)/ \sqrt{(\sigma_1^2/n_1 + \sigma_2^2/n_2)}$$

-where x is equal to the mean and σ is equal to the standard deviation.

The mean of population A is larger than the mean of B if the null hypothesis (Ho) that μ_1 is less than or equal to μ_2 can be rejected, where μ_1 and μ_2 are parameters for the data sets A and B respectively.

Statistical methods taken from Daniel (1983).

Table	x	y	Total
x	a	b	a+b
y	c	d	c+d
Total	a+c	b+d	n

Table 8: 2x2 contingency table.

Second Category	First Category					
	1c	2c	3c	..	(n)c	
1r						Total 1r
2r						Total 2r
..						..
(n)r						Total (n)r
	Total 1c	Total 2c	Total3c	..	Total (n)c	TOTAL

Table 9: Two-way classification of data values

3. Results

3.1. Standardization of PCR methods

3.1.1. Titration of coxsackievirus A9

Coxsackievirus type A9 (8558) was chosen as the positive control for this study. The viral titre was determined as described in 2.3.2., the results presented in Table 10. The titre of the virus was determined to be 10^6 $TCID_{50}$ per inoculum volume (200µl). RNA extracted from this stock was used as the positive control in the standardization of all PCR assays.

3.1.2. Sensitivity and specificity of the 'standard' enterovirus PCR

The standard enterovirus RT-PCR described in 2.5 was already established in the laboratory and conditions optimized before this project was undertaken. The sensitivity was measured using a ten-fold dilution series (in sterile DEPC-treated water) of titred coxsackievirus A9 RNA to a final dilution of 10^{-10}. This series was amplified using primers P1+/P4- and P6+/P9- (Table 6: p52) according to method 2.5. Both first and second round products were visualized by gel electrophoresis as described in 2.7. Figure 5A shows that the 'nested' PCR was 100-fold more sensitive than one round of PCR detecting the coxsackievirus A9 control to a dilution of 10^{-8} (equivalent to 0.01 $TCID_{50}$) compared to 10^{-6} (1 $TCID_{50}$) for the first round. The diffuse banding observed in lanes 1 to 6 is a result of an overload of virus at these high dilutions, which gradually reduces as the dilutions decrease. The nested PCR primers were shown to amplify all the enterovirus types (59 types: viral titres unknown) available for testing in the laboratory with the exception of echovirus 22. Coxsackievirus types A6, A24, and echovirus types 23, 29 and 32 were not available for testing.

Rhinoviruses (untyped) and unrelated viruses such as cytomegalovirus (CMV) and herpes-simplex virus (HSV) were not amplified (Figure 5B) and are shown alongside five examples of enterovirus amplification products, confirming that the primers are enterovirus-group specific.

Dilution of Coxsackievirus A9	Proportion of infected cultures
10^{-1}	4/4 =1
10^{-2}	4/4 =1
10^{-3}	4/4 =1
10^{-4}	4/4 =1
10^{-5}	4/4 =1
10^{-6}	2/4 =0.5
10^{-7}	0/4 =0

Note: Calculation of the 50% tissue culture infective dose ($TCID_{50}$). This was determined using the Karber formula (Karber, 1931), where L= the negative log of the lowest dilution, d= the difference between the log dilution steps and S = the sum of the proportions of 'positive' tests.

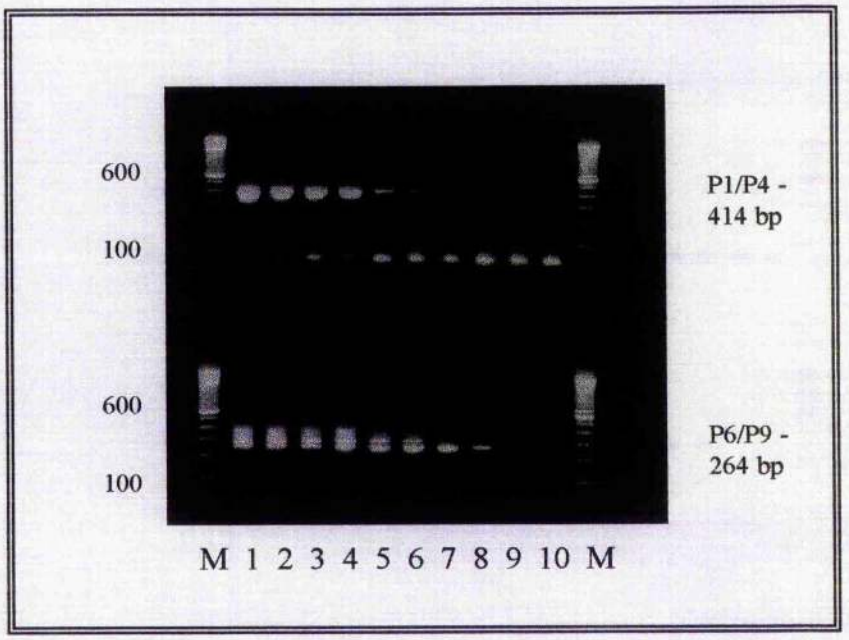
$$\log TCID_{50} = L\text{-}d\ (S\text{-}0.5)$$

$$-1\text{-}1(5.5\text{-}0.5)$$

$$-1\text{-}5 = -6$$

Table 10: Results of titration of coxsackievirus A9 showing dilution of virus and number of tubes with cytopathic effect (c.p.e.).

A.



B.

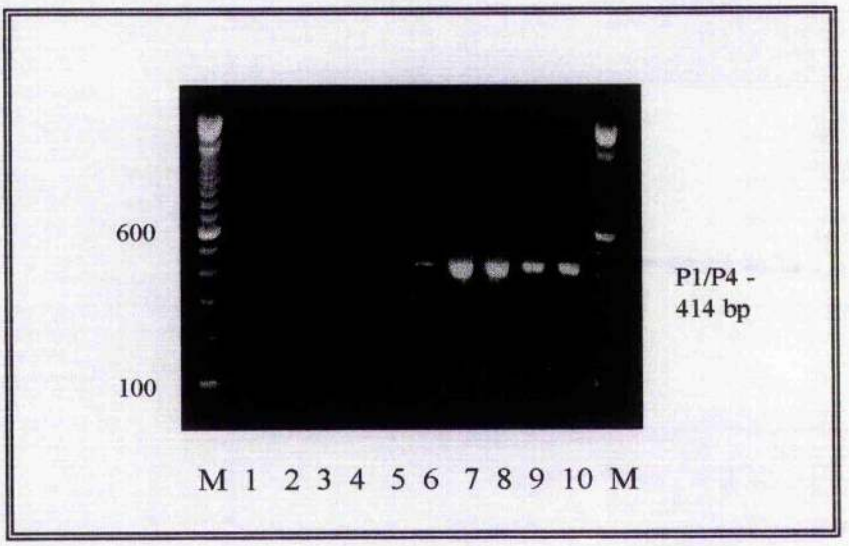


Figure 5: A. Agarose gel showing PCR products from first (top) and nested (bottom) rounds of the standard EV-PCR on a dilution series of coxsackievirus A9. Lane M-100bp DNA ladder, Lanes 1 to 10 - dilutions 10^{-1} to 10^{-10} of coxsackievirus A9. B. Agarose gel showing PCR products from a first round standard EV-PCR on a series of viruses. Lane M- 100bp DNA ladder. Lanes 1- MRC-5 control, 2- Rhinovirus untyped, 3- Rhinovirus untyped, 4- CMV, 5- HSV, 6- coxsackievirus A9 8558, 7- coxsackievirus B3 3629, 8- coxsackievirus B5 2907, 9- echovirus 4 1990, 10- echovirus 6 4566.

3.1.3. Monitoring for contamination

3.1.3.1. Swabs in VTM. These swabs were used as a monitor of viral RNA contamination. Two out of 30 swabs were positive. These were from the outside door handles of two laboratories where RNA extraction and PCR cycling were carried out independently.

3.1.3.2. Swabs in water. These swabs were used as a monitor of PCR product contamination. Two out of 30 swabs were positive. These were from the pipette used for loading agarose gels, and the surface of the centrifuge used in the gel electrophoresis room. On a few occasions contamination was observed in the routine screening PCR where all or most of the samples were positive along with the negative controls. When this occurred, all reagents in use were discarded, laboratory equipment and surfaces were swabbed with hydrochloric acid (1M) and the RNA extraction and RT-PCR was repeated with fresh aliquots of reagent. Contamination occurred approximately 3 times over the study period.

3.2. Comparison of coxsackie B neutralization and PCR

Serum samples from 100 chronic fatigue patients and 100 healthy comparison individuals were tested for the presence of enteroviral RNA by PCR and the presence of coxsackievirus type B neutralizing antibodies (Nairn *et al.*, 1995). The coxsackie B neutralization assay (method 2.2) results are shown in table 11A. Titres equal to or above 256 were regarded as evidence of recent infection and were scored as positive. Following these criteria, 34% of the study group and 41% of the comparison group were positive for antibody to one or more of the coxsackie B viruses, a result which was not significant (computed χ^2 value=1.045, 95% χ^2 value with 1 degree of freedom =3.84). In addition, statistical analysis showed that the presence or absence of any particular coxsackie virus was not associated with either study or comparison group. In 12 individuals, there was evidence of more than one serotype of coxsackie B antibody in the serum sample. This could be a result of cross-reactivity of the neutralizing antibodies or of infection by several serotypes.

Of the 100 study group samples, 42 were positive for enteroviral sequences after two

rounds of PCR, compared to only 9 from the comparison group. Statistical analysis showed a significant difference at the 95% level between the groups with respect to positive enterovirus PCR (computed χ^2 value=28.60, 95% χ^2 value with 1 degree of freedom =3.84).

Table 11B shows a comparison between the neutralization results and the PCR results. In the study group, 66% of results were concordant and in the comparison group, 58 results correlated. Of the samples that did not correlate, the majority in the study group were PCR positive/coxsackie B antibody negative (21/34). This was probably a result of the PCR detecting enterovirus types other than coxsackie B. In the comparison group the majority were PCR negative/antibody positive (37/42).

The neutralization assay therefore did not differentiate the CFS study group from the healthy comparison group whereas the PCR results did differentiate the two groups. PCR was thus chosen as the method for screening a prospective group of patients with CFS for the presence of enteroviral RNA.

A.

Coxsackie B antibody titre	Coxsackie B antibody type	Study group	Comparison group
≥256	coxsackie B1	1	2
≥256	coxsackie B2	6	16
≥256	coxsackie B3	2	1
≥256	coxsackie B4	16	10
≥256	coxsackie B5	2	7
≥256	coxsackie B2+B4	3	3
≥256	coxsackie B3+B4	1	0
≥256	coxsackie other*	3	2
≤128	negative	66	59
Total		**100**	**100**

* Other refers to either coxsackie antibody types B2+B4+B5 (2) or B1+B4 (1) in the study group and either coxsackie antibody types B4+B5 (1) or B1+B2 (1) in the comparison group.

B.

	Enteroviral PCR positive	Enteroviral PCR negative	Total
Study group			
Coxsackie B antibody positive	21	13	34
Coxsackie B antibody negative	21	45	66
Total	**42**	**58**	**100**
Comparison group			
Coxsackie B antibody positive	4	37	41
Coxsackie B antibody negative	5	54	59
Total	**9**	**91**	**100**

Table 11: (A) Results of coxsackievirus type B neutralization test comparing the study and comparison patients. (B) Comparison of coxsackie B neutralization test with enteroviral PCR analysis for the study and comparison groups.

3.3. *Sequence analysis of the 5' non-translated region*

In this initial analysis, serum and or throat swab samples were obtained from individuals with a diagnosis of CFS that fulfilled the Oxford Criteria (Sharpe *et al.*, 1991). Patients attended the outpatient clinic of the Infectious Disease unit at Ruchill Hospital, between January 1992 and January 1994. Serum samples from a group of comparison individuals were tested simultaneously as were echovirus isolates (types 3, 4, 7, 9, 11 and 20) identified in the R.V.L.

RNA was extracted from the samples using the 'Glassmax' extraction kit as described in 2.4.1 and reverse transcribed following the method outlined in 2.5.1. Nested PCR amplification using primers P1+ /P4- followed by P6+/P9- and a separate control PCR using primers ABL1/ABL2 were carried out in 100µl volumes as described in 2.5.2. Nested products and 'ABL' products were run out on a 1.5% agarose gel as described in 2.7.

Within this study group, 44/238 serum samples and 29/175 throat swab samples from patients with CFS were enteroviral PCR positive. In the comparison group, 3/130 serum samples were positive. PCR products of the correct size were also observed in all cases for the ABL control PCR. The echoviruses were also successfully amplified.

The following PCR products were purified using the Gene-clean kit according to method 2.8.1 and sequenced in both directions using primers P6+ and P9- as described in 2.9.1.

1. Twenty products derived from samples obtained from CFS cases (CFS 1-20).
2. Three products derived from samples obtained from non-CFS comparison cases (Non-CFS 1, 2 & 7).
3. Four products derived from samples obtained from patients complaining of anterior chest pain with a presumptive diagnosis of myocarditis (Non-CFS 3-6).
4. Six echovirus isolates (types 3, 4, 7, 9, 11 and 20) (Table 7).

Additionally, two other samples obtained from CFS patient 7 were positive for enteroviral sequences, a throat swab obtained at the same time as the serum sample together with a second serum sample obtained eight months later. A sequence comparison of these three

Additionally, two other samples obtained from CFS patient 7 were positive for enteroviral sequences, a throat swab obtained at the same time as the serum sample together with a second serum sample obtained eight months later. A sequence comparison of these three sequences with coxsackievirus B3 (Klump *et al.*, 1990) is shown in Figure 6. The overall comparison shows a 99.05% identity between the two serum samples taken 10 months apart, 98.10% identity between the throat swab and the serum samples taken at the same time and 87.68% identity between the first serum sample and the published coxsackievirus B3 sequence.

The sequences obtained were closely related to each other and were similar to the coxsackievirus B3, being derived from the highly conserved 5' NTR. Further phylogenetic comparisons were thus made with other members of the enterovirus genus (Galbraith *et al.*, 1995). Figure 7 shows the phylogenetic tree produced by parsimony analysis of the CFS patient sequences in relation to the non-CFS and published sequences available in the GenBank database and the echoviruses from the R.V.L. The analysis showed three groupings of viruses. Group I comprised poliovirus types 1, 2 and 3, enterovirus type 70 and coxsackieviruses A21 and A24. Group II contained the coxsackie B virus types 1, 3, 4 and 5, coxsackievirus A9 and echoviruses 3, 4, 7, 9, 11 and 20, 6/7 non-CFS patients and one CFS patient. The third group (III) comprised 19/20 sequences obtained from the CFS patients and one non-CFS patient.

Another method of illustrating the relationships between sequences uses a dendrogram format which produces a numerical measure of relationships. Figure 8 shows a dendrogram of nine representative sequences, three from each of groups I, II and III. The dendrogram shows that the sequences from each group map together, as predicted from the phylogenetic tree.

Analysis of the 264 base pair fragment demonstrated that the CFS patient sequences were similar to the known published enteroviral sequences (see Table 12 for comparison with coxsackievirus B3) and unrelated to all other available sequences in the GenBank database (sequence homology less than 50%) at this time. Although the primers used in this study were designed to amplify the enterovirus genus only, it was possible that other members of the *Picornaviridae* may have been amplified due to the conserved nature of the genome at the 5' NTR.

```
                          193                                                238
Sample taken  30/08/93    ATCAATAAACTGCTCACGCGGTCGAAGGAGAAAACCTCCGTTACCC
Sample taken  02/06/94    ------------------------------------------------
Throat swab   30/08/93    -------TT--------------------------------------
Coxsackievirus B3         -------G----------T----------G---T-----T---

                                                                     309
GACTGACTACTTCGAGAAACCCAGTAACACCATGGAGATTGCGAAGCGTTTCGCTCAGCACACCCCCAGTG
------------------------------------------------------------------------
------------------------------------------------------------------------
--G-CA----------A-----T---------G----AG----AG--T--------------TA--------

                                                                     380
TAGATCAGTCCGATGAGTCACCGCATTCCCCACAGGCGACTGTGGCGGTGGCTGCGTTGGCGGCCTGCCCA
------------------------------------------------------------------------
--------G-------------------------------------------------------------
--------GT----------------------G------C-------------------------------

                          403
TGGGGCAACCCATGGGATGCTTC
--------------CS------
----------------A-------
-----A-----------C---CT
```

Figure 6: Partial 5' NTR sequences derived from CFS patient 7. Differences between the patient sequences and a published coxsackievirus B3 sequence are shown. S refers to a mixed population of both G and C nucleotides at this position. The numbers refer to the nucleotide positions of the complete genome of coxsackievirus B3 (GenBank Accession Number M33854).

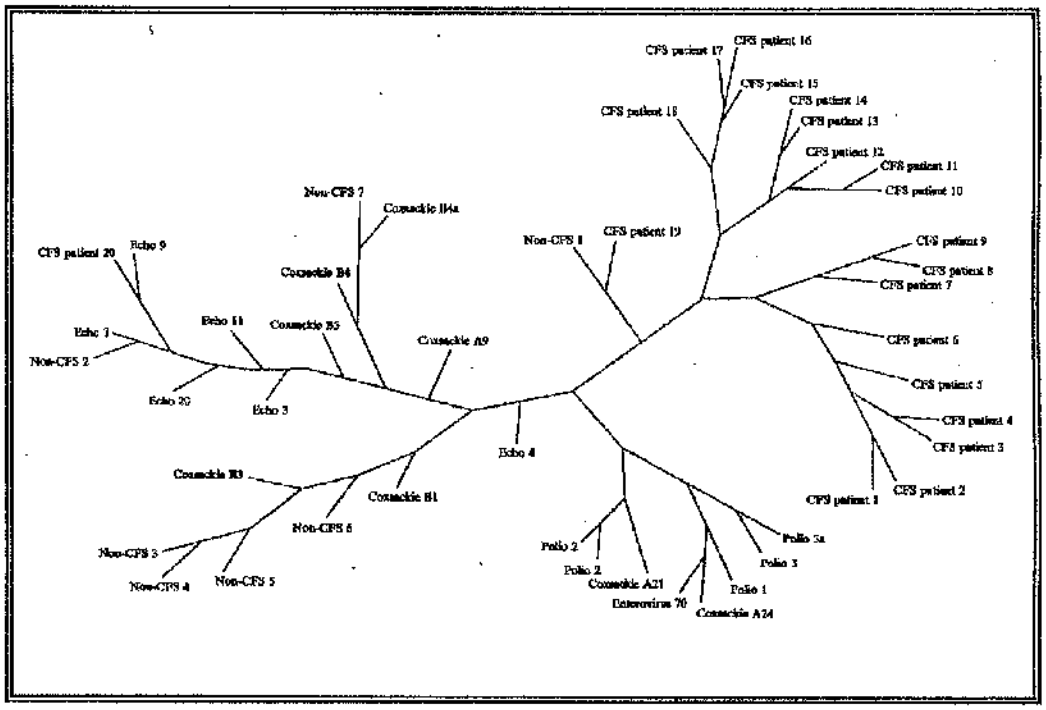


Figure 7: Phylogenetic tree produced by parsimony analysis (PHYLIP/DNAPARS) based on the sequence of a short region of the 5' NTR. GenBank sequences and accession numbers are as follows: coxsackievirus A9 (D00627), coxsackievirus A21 (D00538), coxsackievirus A24 (D90457), coxsackievirus B1 (M16560), coxsackievirus B3 (M33854), coxsackievirus B4 (X05690), coxsackievirus B5 (X67706), enterovirus 70 (D00820), poliovirus 1 (J02281), poliovirus 2 (D00625, M12197), poliovirus 3 (K01392), poliovirus 3a (X04468). Echoviruses were isolated and typed in the Regional Virus Laboratory, Glasgow. CFS and non-CFS patient sequences were derived as described in the methods.

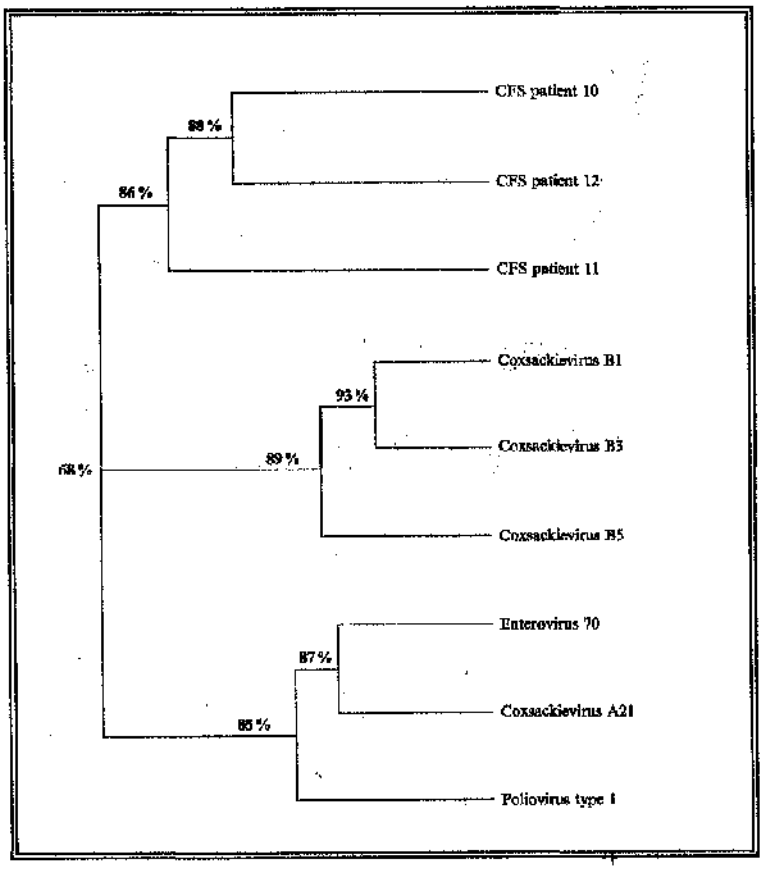


Figure 8: Dendrogram produced using the programs PILEUP and FIGURE from nine enteroviral sequences extracted from figure 7. The numbers indicate the degree of identity between the sequences at each branch of the dendrogram. GenBank sequences and accession numbers are as follows: coxsackievirus B1 (M16560), coxsackievirus B3 (M33854), coxsackievirus B5 (X67706), enterovirus 70 (D00820), coxsackievirus A21 (D00538), poliovirus 1 (J02281).

CFS patient no.	Identity (%)	CFS patient no.	Identity (%)
1	75.8	11	78.3
2	78.1	12	76.6
3	75.9	13	81.4
4	75.5	14	84.3
5	82.0	15	83.9
6	79.7	16	78.1
7	84.2	17	68.9
8	81.3	18	72.1
9	83.1	19	84.1
10	78.9	20	74.8

Table 12: Comparison of CFS patient nucleotide sequence with coxsackievirus B3 (GenBank Accession No. M33854). Figures were generated by FASTA analysis (refer to methods).

A consensus sequence from the CFS patient sequences was obtained and compared with other *Picornaviridae* including swine vesicular disease virus (SVDV; accession no. D00435), Theiler's murine encephalomyelitis virus (TMEV; accession no. X56019), foot and mouth disease virus (FMDV; accession no. M32257), mengovirus (accession no. L22089), echovirus 22 (accession no. L02971), hepatitis A virus (accession no. K02990), encephalomyocarditis virus (EMC; accession no. M22457) and human rhinovirus 1B (accession no. D00239). The CFS consensus sequence was found to be unrelated to any of these viruses (sequence identity less than 50%) other than SVDV (83.2% sequence identity). Phylogenetic analysis of the capsid region of a number of enteroviruses has shown that SVDV groups alongside the coxsackie-like viruses (Hyypia *et al*, 1997).

Reproducibility of sequence analysis in this series of samples was established by carrying out two independent RNA extractions on a single serum sample and amplifying by PCR in the same laboratory, followed by sequencing the amplicons in two different laboratories. One amplicon was sequenced at the R.V.L. where the study was carried out while the other was sequenced at a second laboratory using automated fluorescent sequencing (Beatson Institute of Cancer Research, Glasgow). The sequence from the two laboratories was identical with the exception of a single base change.

3.4. Prospective Follow-up study

Patients who had previously presented to the out-patient clinic at Ruchill Hospital with a diagnosis of CFS and new patients who fulfilled the Oxford criteria (Sharpe *et al.*, 1991) were recruited to the follow-up study. A sample of clotted blood was obtained from the patient and a health questionnaire (see appendix) was completed at or as near as possible to the time of blood sampling. Patients were contacted (via their general practitioner) at twelve-month intervals and asked to complete another questionnaire and to provide an additional blood sample. All blood samples were tested for the presence of enteroviral sequences by the standard PCR methods described (designated EV-PCR). Information from the questionnaires was maintained on DataEase version 4.5.

3.4.1. Features of patient group

Over the study period, 585 questionnaires were completed. Within this group, 333 individuals completed one questionnaire, 159 completed two, 68 completed three and 25 completed four questionnaires. General information regarding age and sex distribution, occupations etc. was obtained from the first questionnaire. The age distribution of both male and female patients presented in Figure 9 shows an approximately normal distribution. The age and sex of the patients within each of the four questionnaire groups is presented in Table 13 and shows a female to male predominance throughout. The number of general practitioners involved was approximately 230.

A broad range of occupations was recorded (bus driver, civil servant, pharmacist for example) with no one occupation over-represented. Forty-eight per cent of patients were married, 37% were single and 47% had children.

In the 6 months prior to the onset of fatigue, 84 (25%) reported receiving prescribed medication (mainly antibiotics) from their general practitioner; 68 (20%) reported allergies (for example hay fever and penicillin); 20 (6%) had been immunized and 33 (10%) had experienced health problems while on holiday. One hundred and seventy patients (51%) reported the occurrence of a major change in life, which included job-related and personal events. Eighty-one percent reported an acute illness prior to the onset of fatigue. Different combinations of symptom category were reported and as such patients could not be divided into distinct groups.

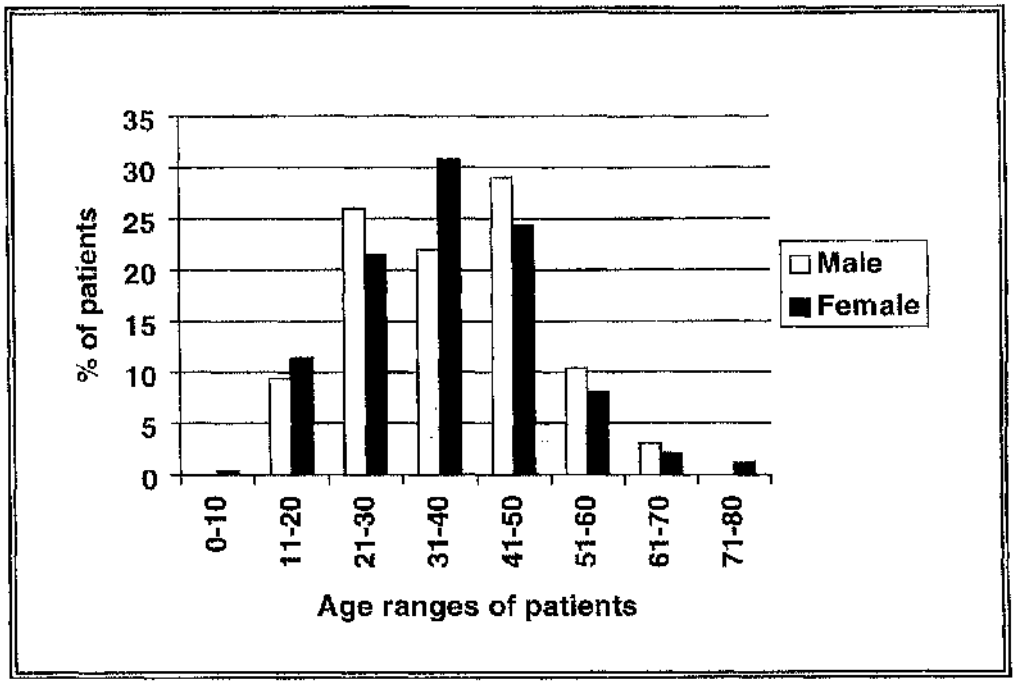


Figure 9: Age distribution (from 0 to 80 years) of male (n=96) and female (n=237) patients with a diagnosis of CFS. Data obtained from the first questionnaire.

Questionnaires completed	Total	Female (%)	Male (%)	Age range in years (mean)
1	333	237 (71%)	96 (29%)	9-72 (36.6)
2	159	113 (71%)	46 (29%)	14-73 (40.3)
3	68	49 (72%)	19 (28%)	16-74 (42.1)
4	25	18 (72%)	7 (28%)	18-70 (46.6)

Table 13: Total number and age range of patients completing 1, 2, 3 and 4 questionnaires divided into female and male categories.

Individuals were also asked about their stress levels prior to fatigue: high (30%), middling (44%) and low (25%) levels were reported. Similarly, mood prior to fatigue was assessed: good (47%), middling (36%) and poor (15%) responses were noted. Physical activities such as gardening, sports and socializing were given up by 78% of the study group.

The majority of individuals (90%) reported suffering from their condition for more than 50% of the time. The month of onset of fatigue for the patients is shown in Figure 10, alongside the monthly distribution of enterovirus isolates reported in Glasgow, for the years 1994 to 1997. The figure clearly shows that the monthly distribution of the onset of fatigue does not coincide with the seasonal distribution of the enteroviruses, which shows the majority of isolates in the summer/autumn months. The distribution of onset of fatigue is more evenly spread throughout the year with small peaks in December and February. The average duration of fatigue was 3.9 years within a range of 6 months to 9 years or greater. The greatest proportion (63%) reported suffering from their illness for less than 3 years, while 7.6% had suffered for greater than 9 years. The duration of fatigue for both male and female patients is shown in Figure 11, showing there is little difference between the sexes. The ten most common symptoms reported by patients are shown in Figure 12, with daytime drowsiness reported most frequently. Symptoms that were not reported frequently included being unable to recognize objects by sight (6%), difficulty swallowing foods (11%) and appearance of any recent allergies (11%).

Of these 333 individuals, 36% were positive for enteroviral sequences by PCR analysis. The patients were thus split into two groups (positive and negative) based on the PCR results and the questionnaire responses were compared between the groups. In all cases, there was no difference between the groups in terms of month of onset of fatigue, duration of fatigue, symptoms reported and so on.

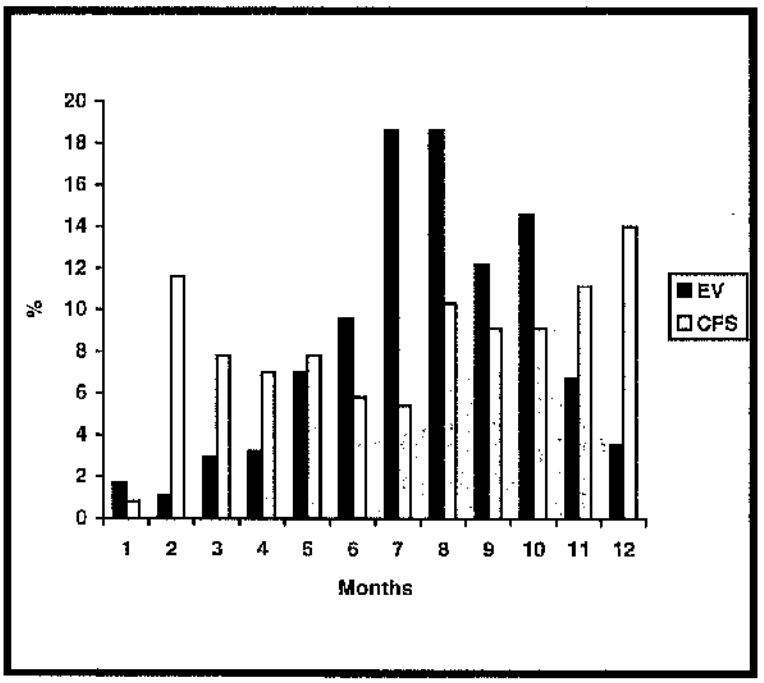


Figure 10: Monthly distribution of enterovirus isolates (coxsackievirus A types (n=37), coxsackievirus B types (n=117) and echovirus types (n=189)) reported in Glasgow for the years 1994 to 1997 compared to the distribution of the month of onset of fatigue for CFS patients (n=242).

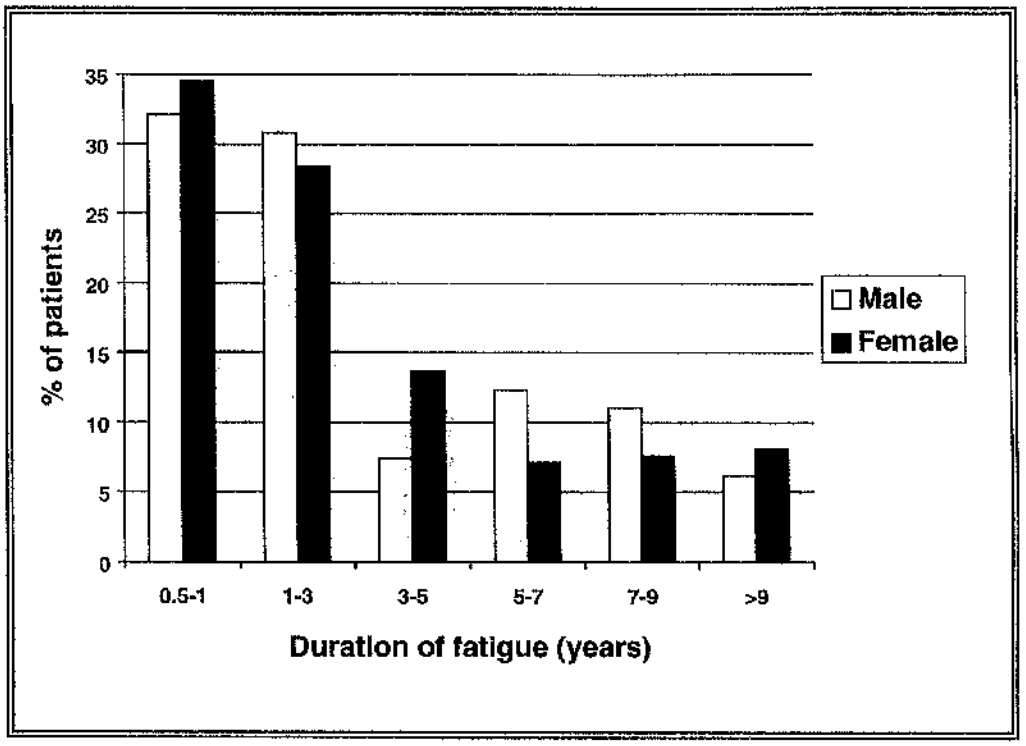


Figure 11: Distribution of the duration of fatigue (years) for both male (n=96) and female (n=237) CFS patients.

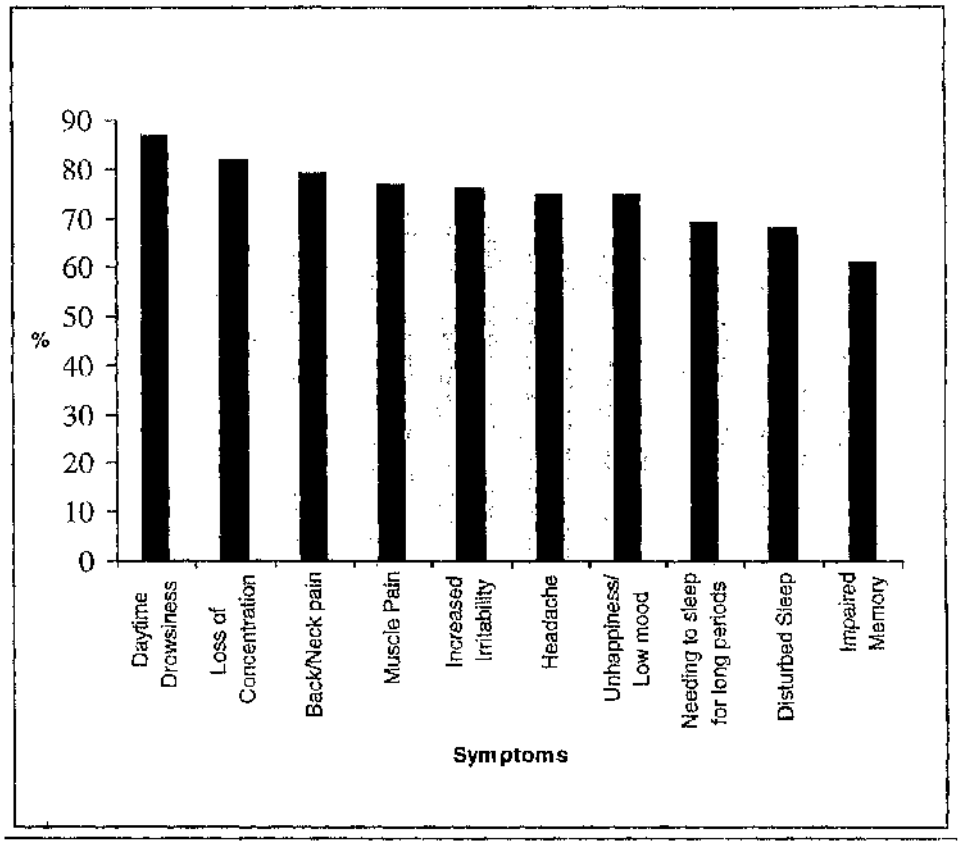


Figure 12: The ten most common symptoms reported by CFS patients

3.4.2. Analysis of patients over time

Following completion of the follow-up study, 130 patients were identified where a completed questionnaire and corresponding blood sample, obtained over two time points, were available for analysis. This cohort consisted of 95 females (age range 15-73 years: mean age 39.3 years) and 35 males (age range 14-67 years: mean age 43.1 years). The female to male ratio and the mean age in this group was similar to that observed for the patient group as a whole.

3.4.2.1. *Analysis of data derived from questionnaire 1 (Q 1).* The overall EV-PCR results for this group were as follows: sixty-seven patients (51.5%) were EV-PCR positive and sixty-three (48.5%) were EV-PCR negative. To test the hypothesis that the presence of enterovirus is related to CFS, a number of features were identified which might reflect the severity of illness in a patient, for example, ability to work. The EV-PCR positive and negative groups were then compared with respect to these features.

Feature 1 - Employment. Individuals were asked about their current employment status, ticking one of seven choices including at work/school or retired on health grounds for example. The employment status of individuals in both EV-PCR groups is shown in Table 14. From Q1, of those EV-PCR positive, 25% were at work or school whereas 42% were on sick leave. Conversely, 43% of the EV-PCR negative group were at work or school whereas 28% were on sick leave. To examine whether PCR positivity has an effect on work status the results in each group were divided into two categories, those able to work and those not able to work (discounting the three 'unknowns') (Table 15).

A χ^2 value for this 2x2 contingency table could then be calculated, according to method 2.16.1. The computed value for χ^2 was 7.4. The 95% χ^2 value with one degree of freedom equalled 3.84. The computed value was therefore significant at the 95% level indicating a relationship between enteroviral PCR status and ability to work from Q1, that is, those individuals who were EV-PCR positive were less likely to be working than those who were EV-PCR negative.

Q1: feature 1 – employment	EV-PCR +	EV-PCR -	**Total**
At work/school	17 (25.4%)	27 (43%)	44
Housewife/unemployed	5 (7.5%)	8 (12.7%)	13
Retired	2 (2.9%)	1 (1.6%)	3
Retired on health grounds	15 (22.4)	6 (9.5%)	21
Sick leave	28 (42%)	18 (28.6%)	46
Unknown	0	3 (4.7%)	3
Total	**67**	**63**	**130**

Table 14: Feature 1 - employment status from questionnaire 1 of EV-PCR positive and EV-PCR negative CFS patients.

Q1 – ability to work	EV-PCR +	EV-PCR -	**Total**
Able to work	24	36	60
Not able to work	43	24	67
Total	67	60	**127**

Table 15: 2x2 contingency table comparing ability to work and EV-PCR status for CFS patients from questionnaire 1.

Feature 2 - Domestic Work. For those individuals not in employment, the severity of illness was measured by the ability to carry out normal domestic work in the home, answering either yes or no. The results are shown in Table 16. Only 28% of those who were EV-PCR positive were able to carry out their normal domestic duties, whereas 44% of those who were EV-PCR negative were able to carry out their domestic work. These percentages are very similar to those observed for the two groups regarding employment status. The χ^2 value for this data was 3.37 which was not significant at the 95% level (χ^2 value of 3.8), but was significant at the 90% level (χ^2 value of 2.7).

Taking these two features together for the first questionnaire, the results would suggest that EV-PCR positivity was related to the severity of illness as measured by the ability to work, attend school or carry out domestic work.

Feature 3 - Physical activity. The physical activity of each patient was measured by the amount of time they spent outside during the day, how much of this time they spent walking and how far they could walk (questions 3, 4 and 5 of the questionnaire). For the latter question, if the response was "a few yards", this was scored as 0 miles. If the response was "unlimited", this was scored as an arbitrary 10 miles. The scores for the patients in each group were totaled and a mean value calculated, shown in Table 17. The mean time spent outside and the mean time spent walking was no different between the groups, but the distance walked by those in the EV-PCR negative group was almost double that of the positive group, although the distances are small. The distribution of the distance walked for the positive and negative groups is shown in Figure 13. The maximum distance walked by individuals in the EV-PCR positive group was 4 miles compared to some individuals in the EV-PCR negative group who could walk an unlimited distance.

The mean distance walked was compared between the two groups using hypothesis testing: sampling from populations that are not normally distributed (2.16.2). The test statistic ($z = 1.99$) was greater than the critical value of 1.645 (where $\alpha=0.05$ in a one-tailed test) and thus the null hypothesis (that the EV-PCR negative group mean is less than or equal to the EV-PCR positive group mean) was rejected. The mean distance walked was therefore significantly different between the two groups.

Q1: feature 2 – domestic work	EV-PCR +	EV-PCR –	**Total**
Yes	19 (28%)	26 (44%)	45
No	48 (72%)	33 (56%)	81
Total	**67**	**59**	**126**

Table 16: Feature 2 - ability to carry out domestic work from questionnaire 1 of EV-PCR positive and EV-PCR negative CFS patients.

Q1: feature 3 – physical activity	EV-PCR +	EV-PCR –
Mean time spent outside (hours)	3.0	3.0
Mean time spent walking (hours)	0.8	1.0
Mean distance walked (miles)	1.0	1.8

Table 17: Feature 3 - physical activity from questionnaire 1 of EV-PCR positive and EV-PCR negative CFS patients.

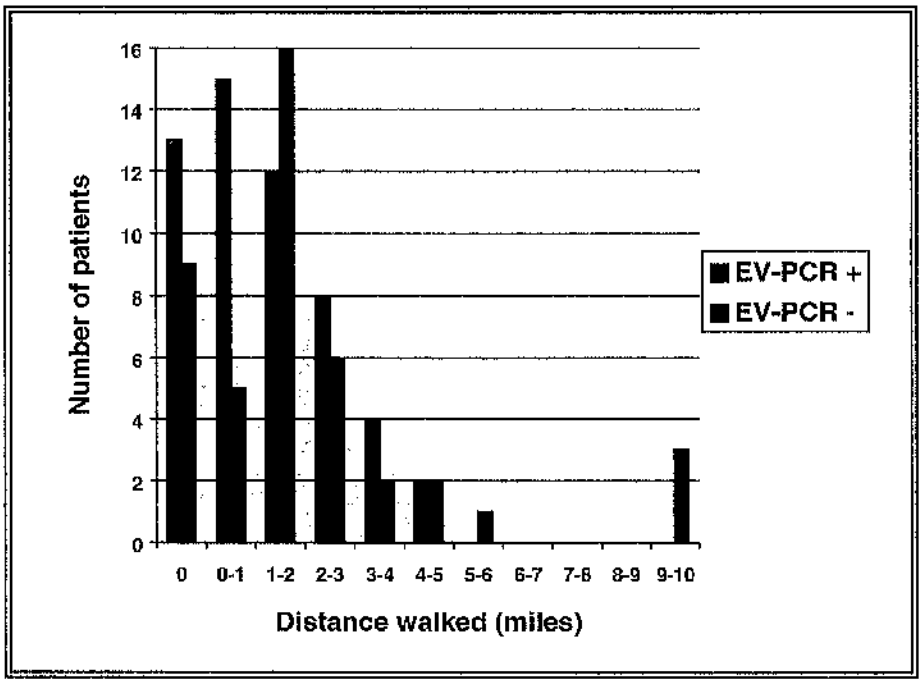


Figure 13: Distribution of distance walked (miles) comparing EV-PCR positive and EV-PCR negative CFS patients.

Feature 4 – Time off work. Individuals in employment were asked to say how much time (greater than two weeks) they had taken off work in the previous year.
Those individuals EV-PCR positive at the time of their first questionnaire had taken an average of 5.4 months off during the previous year. Those individuals EV-PCR negative had taken an average of 3.7 months off during the previous year.

The distribution of time off work, comparing EV-PCR positive and EV-PCR negative individuals is shown in Figure 14. Only 11% of individuals who were EV-PCR positive had taken no time off work in the previous year compared to 27% of the EV-PCR negative group. Conversely, 27% of the EV-PCR positive group had taken more than 10 months off work, compared to 14% of the negative group. The same test statistic used for the distance walked was calculated to compare the means of the two groups. The test statistic ($z = 1.85$) was greater than the critical value of 1.645 (where $\alpha = 0.05$ in a one tailed test) and thus the null hypothesis was rejected. The means of the two groups were therefore significantly different.

In summary, from the data derived from the first questionnaire there was a significant difference between those individuals who were EV-PCR positive and those EV-PCR negative with respect to the features examined. Those individuals with an EV-PCR + result were less likely to be at work/school, less likely to be able to carry out domestic work, less able to walk distances and more likely to have taken time off work.

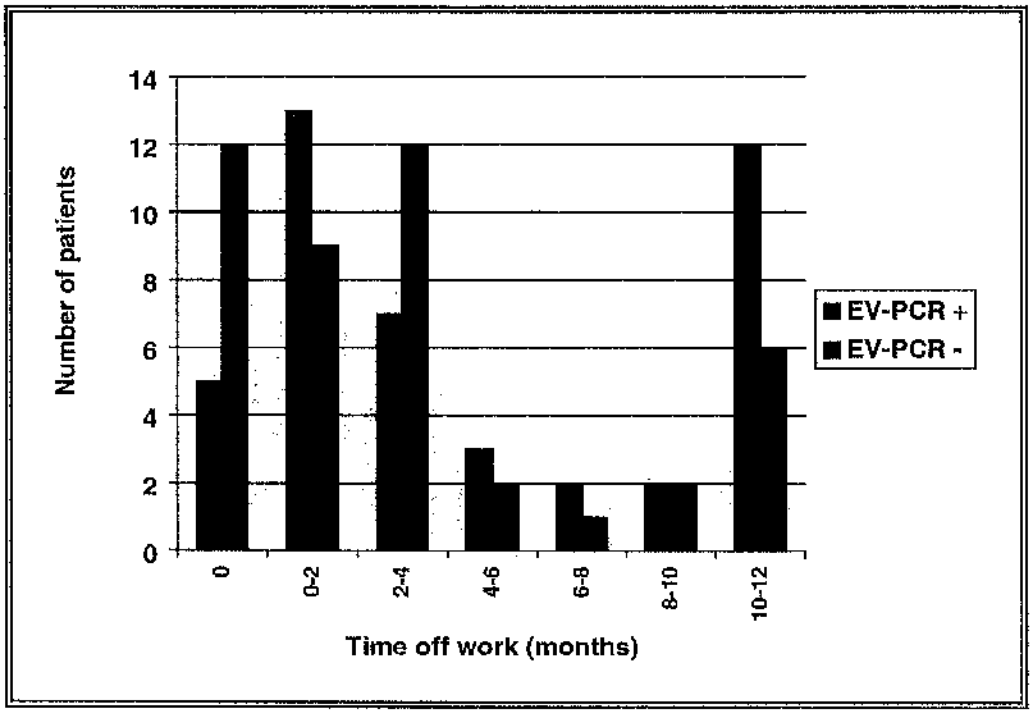


Figure 14: Distribution of time off work (months) comparing EV-PCR positive and EV-PCR negative CFS patients.

3.4.2.2. *Analysis of data derived from second questionnaire (Q 2).* After 12 months the EV-PCR results for the patient cohort were as follows: 21 (16%) patients were positive and 109 were negative. The patients were sub-divided into 4 groups based on the PCR results at the two time points: - EV-PCR +/+, EV-PCR +/-, EV-PCR -/- and EV-PCR -/+ for Q1/Q2. Table 18 shows the numbers of patients in each group. Only 9.2% of patients were positive over the two time points. A comparison of sequence data obtained from ten of these patients is presented in section 3.4.3. The four groups identified here can be compared where possible for the features analysed previously. In some instances, the small sample numbers made it impossible to apply any statistical analysis to the findings.

Feature 1 -- Employment. The employment status of individuals EV-PCR positive initially, then positive or negative twelve months later is shown in Table 19. After the twelve months, a greater proportion of the group were at work/school than previously (37% compared to 25%), fewer individuals were on sick leave (10% compared to 42%) and there was an increase in the number who had retired on health grounds (22% to 34%). Comparing between the EV-PCR +/+ and +/- groups, 25% of the former were at work/school compared to 40% of the latter. There was no significant difference between the groups reporting sick leave and retired on health grounds.

Table 20 shows the employment status of those individuals EV-PCR negative initially then positive or negative after 12 months. Over the twelve months, there was no change in the number of individuals at work/school, the percentage of individuals on sick leave had reduced from 28.6% to 15.8% and the percentage retired on health grounds had increased from 9.5% to 20.6%.

Comparing between the EV-PCR -/- and -/+ groups, a greater proportion of individuals in the former was at work or school (46%) compared to the latter (22%). The percentage retired on health grounds was no different between the two groups and the percentage on sick leave was greater in the -/- group.

The ability to work was compared among these four groups (EV-PCR +/+, +/-, -/+ and -/-) according to method 2.16.1 producing a χ^2 value of 1.41. This value was not significant at the 95% level with three degrees of freedom (χ^2 value of 7.815) or the 90% level with three degrees of freedom (χ^2 value of 6.261).

	PCR **positive** Q2	PCR **negative** Q2	**Total**
PCR **positive** Q1	12 (9.2%) **+/+**	55 (42.3%) **+/-**	67
PCR **negative** Q1	9 (6.9%) **-/+**	54 (41.5%) **-/-**	63
Total	21	109	**130**

Table 18: EV-PCR results over 12 months for 130 CFS patients.

Q2: feature 1 – employment status	**EV-PCR +/+**	**EV-PCR +/-**	**Total**
At work/school	3 (25%)	22 (40%)	25 (37%)
Housewife/unemployed	4 (33%)	4 (7.2%)	8 (12%)
Retired	0	2 (3.6%)	2 (3%)
Retired on health grounds	4 (33%)	19 (34.5%)	23 (34%)
Sick leave	1 (8%)	6 (10.9%)	7 (10%)
Unknown	0	2 (3.6%)	2 (3%)
Total	**12**	**55**	**67**

Table 19: Feature 1 - employment status from questionnaire 2 comparing individuals EV-PCR positive initially then positive or negative after 12 months (+/+ or +/-).

Q2: feature 1 – employment status	**EV-PCR -/-**	**EV-PCR -/+**	**Total**
At work/school	25 (46%)	2 (22.2%)	27 (43%)
Housewife/unemployed	8 (14.8%)	4 (44.4%)	12 (19%)
Retired	1 (1.8%)	0	1 (1.6%)
Retired on health grounds	11 (20.4%)	2 (22.2%)	13 (20.6%)
Sick leave	9 (16.7%)	1 (11.1%)	10 (15.8%)
Total	**54**	**9**	**63**

Table 20: Feature 1 - employment status from questionnaire 2 comparing individuals negative initially, then negative or positive after 12 months (-/- or -/+).

Feature 2- Domestic work. The ability of the individual to carry out domestic work was also assessed after twelve months. Firstly, 33% of those individuals in the EV-PCR +/+ group were able to carry out their normal domestic work compared to 42% in the EV-PCR +/- group. Of those individuals with two negative results (-/-), 56% were able to carry out their domestic work, compared to 67% of those in the -/+ group.

These four groups were compared for their ability to carry out domestic duties, resulting in a χ^2 value of 3.84. This value was not significant at either the 90% (χ^2 value of 6.251) or the 95% level (χ^2 value of 7.815).

Taking these two features into account, severity of illness as measured by employment status and ability to carry out domestic work, was not related to EV-PCR positivity after 12 months.

Feature 3 - Physical Activity. The physical activity of the patients in each of the four groups is shown in Tables 21 and 22. Within the groups, those who remained positive (+/+) spent 1 hour less outside than they did twelve months previously, but could on average walk further (2.3 miles compared to 1.0 mile). Those in the +/- group, spent one hour more outside and could also walk further than before (1.8 miles compared with 1.0 mile).

Those individuals in the -/+ group spent an average of one hour longer out of the home than they did twelve months previously. They spent slightly less time walking, but could walk further (2.2 miles compared to 1.8 miles). Those individuals with two negative results also spent an average of one hour longer outside the home, but the time spent walking and the distance walked remained the same.

Feature 4 - Time off work. The time taken off work in the year prior to the second sample being taken is shown in Table 23. The group with most time off (9.3 months) was EV-PCR positive at the two time points (EV-PCR +/+) while the group with least time off (4.1 months) had two negative results (EV-PCR -/-) over the time period. Only 4 people in the EV-PCR +/+ group answered this in questionnaire 2 compared to 35 of the EV-PCR -/- group and therefore it is difficult to assess the significance of the result.

Q2: feature 3 - physical activity	EV-PCR +/+	EV-PCR +/-
Time spent outside (hours)	2.0	4.0
Time spent walking (hours)	0.6	0.9
Distance walked (miles)	2.3	1.8

Table 21: Feature 3 - physical activity after 12 months comparing individuals in the +/+ and +/- groups.

Q2: feature 3 - physical activity	EV-PCR -/-	EV-PCR -/+
Time spent outside (hours)	4.0	4.0
Time spent walking (hours)	1.0	0.8
Distance walked (miles)	1.8	2.2

Table 22: Feature 3 - physical activity after 12 months comparing individuals in the -/- and -/+ groups.

Q2: feature 4 - time (months) off work prior to second sample	Positive Q2	Negative Q2
Positive Q1	9.3	6.9
Negative Q1	7.0	4.1

Table 23: Feature 4 - Time taken off work by CFS patients in the year prior to obtaining the second sample.

Of this cohort of 130 individuals, 65 completed a third questionnaire and provided a blood sample for analysis, 7 (11%) of which were EV-PCR positive. Only 19 completed a fourth questionnaire and supplied a blood sample, none of which was EV-PCR positive. These questionnaires were not subjected to further analysis since the numbers in the PCR groups were too small for statistical analysis.

The groups providing either 1, 2, 3 or 4 questionnaires were compared to identify any changes in the populations responding at each call-back. The age range of the patients narrowed slightly over the 4 questionnaires with the mean age increasing from 36.6 years for those providing one questionnaire to 46.6 years for those providing four (Table 13). The duration of fatigue was also examined (Table 24). The average duration of fatigue increased, in general, by one year, with each subsequent questionnaire submitted, as would be expected. There was however a shift in the distribution of the duration of fatigue. Only 7.6% who provided one questionnaire had suffered for greater than 9 years compared to 21.7% of those who had provided four questionnaires. These figures would suggest that those continuing to submit questionnaires had been ill for longer, thus enriching the study cohort.

Questionnaire	Duration of fatigue (years)	Mean duration of fatigue (years)	% suffering for <3 years	% suffering for >9 years
Q 1	0.5 - >9	3.9	63	7.6
Q 2	1 - >9	5.2	35	15.6
Q 3	1.5 - >9	6.4	19.7	23
Q 4	3 - >9	7.2	0	21.7

Table 24: Duration of fatigue (range and mean). The % of patients suffering for fewer than 3 years and greater than 9 years for patients providing 1, 2, 3 and 4 questionnaires is shown.

3.4.3. Analysis of PCR positives over time

As part of the prospective study, screening new CFS cases for enteroviral sequences and following up those already tested, patients who had two enterovirus PCR-positive samples at two different time points were identified. These products were sequenced as described in 2.8.2 and 2.9.2. Ten of these are from the 12 patient samples identified as EV-PCR+ at least twelve months apart (Table 18). The remaining 6 pairs of sequences identified were not part of the clinical follow-up because the patients did not provide two completed questionnaires for analysis.

Table 25 shows the GAP comparison statistics for the maximum available sequence of the pairs obtained from individual patients. Five of the sixteen pairs of sequences [patients 2(HA), 4(MO), 5(PA), 6 (TI) and 10 (HV)] demonstrated a level of identity of 97.5% or greater with samples taken up to 24 months apart. In the case of patient 6, the 0.4% difference equates to 1 base pair change within the region analysed.

Figure 15 presents the pairs of sequences from the sixteen patients compared to a consensus sequence of 250 bases. The sequence comparison of patients 1 to 8 has been described previously (Galbraith *et al.*, 1997). The additional pairs were identified as the study progressed. From the data derived from the original eight sequences it was concluded that there was evidence for enteroviral persistence in patients TI, PA, MO and HA, based on the presence of unique base pairs that were different from the consensus sequence. For example, pair TI at bases 174 and 241, pair HA at 318 and pair PA at 196 (where there is an additional cytosine). A comparison using the pairs TI, PA, MO and HA was made with 34 sequences derived from additional CFS patients from whom a single sample was available (comparison not shown). Comparing these sequences with pair TI, one contained guanine at position 241, but did not have the feature at 174 and furthermore was dissimilar at seven other bases. None of the 34 sequences in the comparison contained the inserted cytosine present in pair PA. In the case of pair MO, the features were present in one of the comparison sequences, and in pair HA the thymine at position 318 was present in two of the 34 sequences. However, the rest of these three individual sequences showed at least ten differences from the consensus sequence.

Patient (code)	Interval between samples (months)	Percentage identity
1 (CR)	26	92.00
2 (HA)*	10	98.20
3 (HO)	40	70.60
4 (MO)*	5	97.50
5 (PA)	8	97.50
6 (TI)	24	99.60
7 (MC)	12	90.00
8 (HMa)	41	89.50
9 (HMb)*	12	84.2
10 (HV)*	12	98.3
11 (MCK)*	12	88.4
12 (MCN)*	12	95.8
13 (WI)*	12	87.8
14 (SE)*	12	89.6
15 (MCG)*	12	85.9
16 (HP)*	12	88.7

Table 25: GAP comparison statistics for pairs of enteroviral sequences obtained from individual CFS patients. * indicates the pairs included in the clinical follow-up as EV-PCR positive over time (Table 17).

```
     174                                                       214
Con  CTGTTACCCC GGACTGAGTA TC AATAAACT GCTCACGCGG TCGAAGGAGA
TI93 A.......... .......... .. ........ .......... ..........
TI95 A.......... .......... .. ........ .......... ..........
PA93 ....CT..... .......... ..C........ .......... ..........
PA94 .......... .......... ..C........ .......... ..........
CR92 .....T.... .......... .. ........ .......... ..........
CR94 .......... .......... .. ....G... .......... CT........
MO93 .......... .......... .. ........ .......... ..........
MO94 .......... .......... .. ........ .G........ ..........
HA93 .......... .......... .. ........ .......... ..........
HA94 .......... .......... .. ........ .......... ..........
MC93 .......... .......... .. ........ .......... ..........
MC94 .......... .......... .. ....GG.. A......T.. CT........
HM90 .......... .......... .. ........ .......... ..........
HM94 .......... ....C..... .. ....GG.. ...TG..... CT........
HM95                                    ..A.. CT........
HO91 .......... .......... .. ..A.G... .......... .T........
HO94 .......... ........A. CG ..G.G... .....AAG.. C.........
HP94 .......... .......... .. ........ .......... ..........
HP95 .......... ....C..... .. ....GG.. ...TG..... CT........
WI95 .......... .......... .. ........ .......... ..........
WI96 .......... ....C..... .. ....GG.. ...TG..... CT........
MCG95 ......... .......... .. ........ .......... ..........
MCG96 ......... .......NN. .. ....NG.. .......... CT.....N.N
MCK94                          .. .......... ..........
MCK95                  .. .. ....GG.. ...TG..... CT........
HV94                         .GG.. ...TG..... CT........
HV95                         .GG.. ...TG..... CT........
MCN94 .......... .......... .. ....GG.. ...TG..... CT........
MCN95 .......... .......... .. ....GG.. ....T..... CT........
SE95 .......... ....C..... .. ....G... .......... .T........
SE96 .......... .......... .. .....G.. .......... CT........

     224                                                       264
Con  AAACG TCCGT TACCCGGCTA ACTACTTCGA GAAACCCAGT AACACCATGG
TI93 ..... ..... .......G.. .......... .......... .......C..
TI95 ..... ..... .......G.. .......... .......... .......C..
PA93 ..... ..... ......A..G .......... .......... ..........
PA94 ..... ..... ......A..G .......... .......... ..........
CR92 ..... ..... ......A..G .......... .......... ..........
CR94 ..... .T... .........C. .T........ .......... .C........
MO93 ..... ..... .......... .......... .......... .......C..
MO94 ..... ..... .......... .......... .......... .......C..
HA93 ..... ..... ......A..G .......... .......... ..........
HA94 ..... ..... ......A..G .......... .......... ..........
MC93 ..... ..... ......A..G .......... .......... ..........
MC94 .....T..T.. .........C. .T........ ......T... .........A
HM90 ..... ..... ......A..G .......... .......... ..........
HM94 ..... .T... .........C. .T........ A......... .C..T.....
HM95 ...A. ....C ........... .G.......T ........... .N........
HO91 ..G.. .T... ..T.....C. .......... ......T... .......G...
HO94 GG... GGAAA A....AC..G G......... .......... ...T...C..
HP94 ..... ..... .......... .......... .......... .......C..
HP95 ..... .T... .........C. .T........ A......... .C..T.....
WI95 ..... ..... .......... .......... .......... .......C..
WI96 ..... .T... .........C. .T........ A......... .C..T.....
MCG95 ..... ..... .. ........ .......... .......... .......C..
MCG96 ..... .T... ..T.....C. .T........ .....TT... .C....G..A
MCK94 ..... ..... .......... .......... .......... .......n..
MCK95 ..... .T... .........C. .T........ A......... .C..T.....
HV94 .G... .T... .........C. .T........ A.......C. .C..T.....
HV95 ..... .T... .........C. .T........ A......... .C..T.....
MCN94 ..... .T... .........C. .T........ ......T... ..........
MCN95 ..... .T... .........C. .T........ .......... .C........
SE95 ..G.. .T... ..T....... .......... ......T... .........A
SE96 ... . .T... ..T.....C. .T........ ......T... .C........
```

```
     274                                                        314
Con  AGGTTGCGAA GCGTTTCGCT CAGCACACCC CCAGTGTAGA TCAGGCCGAT
TI93 .AA.......  .......A..  ......G...  .TG.......  ..........
TI95 .AA.......  ..........  ......C...  .TC.......  ..........
PA93 ..A.......  ..........  ..........  ..........  ..........
PA94 ..T.......  ..........  ..........  ..........  ..........
CR92 ..A.......  ..........  ..........  ..........  ..........
CR94 .........C  .T........  .C.....A..  .TG.......  .....T....
MO93 .AA.......  .......A..  ......G...  .TG.......  ..........
MO94 .AA.......  ..........  ......G...  .TG.......  ..........
HA93 ..A.......  ..........  ..........  ..........  ....T.....
HA94 ..A.......  ..........  ..........  ..........  ....T.....
MC93 ..A.......  ..........  ..........  ..........  ..........
MC94 .A......C.  .T........  .C.....A..  ..........  .....T....
HM90 ..A.......  ..........  ..........  ..........  ..........
HM94 .........G  .T........  .C.....A..  ..........  .....T....
HM95 ..........  ...CA.....  ..........  .......T..  .........T.
HO91 .A.....AG.  .T........  ......TA..  ..........  .....T....
HO94 .NA.......  .T....AT.A  .TCA.AC...  .TG.......  .....A....
HP94 .AA.......  .......N..  ......G...  .TG.......  ..........
HP95 .........G  .T........  .C.....A..  ..........  .....T....
WI95 .AA.......  ..........  ......G...  .TG.......  ..........
WI96 .........G  .T........  .C.....A..  ..........  .....T....
MCG95 .AA......  ..........  ......G...  .TG.......  ..........
MCG96 .A......C.  ..........  .CA....A..  .........T.  .....T....
MCK94 .A.......  .T........  ......G...  .TG.......  .....T....
MCK95 .........G  .T........  .C.....A..  ..........  .....T....
HV94 .........G  .T........  .C.....A..  .........N.  .....T....
HV95 .........G  .T........  .C.....A..  ..........  .....T....
MCN94 .........T.  .T........  .CA....A..  ..........  .....T....
MCN95 .........N.  .T........  .C.....A..  .........N  .....T....
SE95 .A..A...G.  .T........  ......TT..  ..........  .....T....
SE96 .........C.  ..........  .C........  .......T..  ......T...

     324                                                        364
Con  GAGTCACCGC GTTCCCCACA GGCGACTGTG GCGGTGGCTG CGTTGGCGGC
TI93 ..........  ..........  ..T.......  ..........  ..........
TI95 ..........  ..........  ..T.......  ..........  ..........
PA93 ..........  A.........  ..........  ..........  ..........
PA94 ..........  A.........  ..........  ..........  ..........
CR92 ..........  A.........  ..........  ..........  ..........
CR94 ..........  AA........  ..........  ..........  ..........
MO93 ..........  ..........  ..T.......  ..........  ..........
MO94 ..........  ..........  ..T.......  ..........  ..........
HA93 ..........  A.........  ..........  ..........  ..........
HA94 ..........  A.........  ..........  ..........  ..........
MC93 ..........  A.........  ..........  ..........  ..........
MC94 ..........  A........G  ..G...C...  ..........  ..........
HM90 ..........  A.........  ..........  ..........  ..........
HM94 ..........  .........G  ......C...  ..........  ..........
HM95 ..........  A.........  .......NN.  .T........  T....TT...
HO91 ..........  A........G  ......C...  ..........  ..........
HO94 ..........  ..........  ..T.......  A.....A...  ..........
HP94 ..........  ..........  ..T.......  ..........  ..........
HP95 ..........  .........G  ......C...  ..........  ..........
WI95 ..........  ..........  ..T.......  ..........  ..........
WI96 ..........  .........G  ......C...  ..........  ..........
MCG95 ..........  ..........  ..T.......  ..........  ..........
MCG96 ..........  AA...T...G  ......C...  ..........  ..C.......
MCK94 ..........  ..........  ..T.......  ..........  ..........
MCK95 ..........  .........G  ......C...  ..........  ..........
HV94 ..........  .........G  ......C...  ..........  ..........
HV95 ..........  .........G  ......C...  ..........  ..........
MCN94 ..........  A........G  ..T...C...  ..........  ..........
MCN95 ..........  A.........  ..........  ..........  ..........
SE95 ..........  .........G  ......C...  ..........  ..........
SE96 ...C......  .........G  ......C...  ..........  ..C.......
```

```
    374                                                          414
Con   CTG CCCATGG GGCAACCCAT GGGACGCTTC AATATGGACA TGGTGCGAAG
TT93  ... ....... .......... C......... .......... ..........
TT95  ... ....... .......... C......... .......... ..........
PA93  ... ....... .......... ....T..... ......CC.. ..........
PA94  ... ....... .......... ....T..... ......CG.T A.........
CR92  ... ....... .......... ..A.T..... .......... ..........
CR94  ... ....... .......... .A........ ....CT.... ..........
MO93  ... ....... .......... .......... .......... ..........
MO94  ... ....... .....A.... C......... .......C.. ..........
HA93  ... ....... .......... ....T..... .......G.. ..........
HA94  ... ....... .......... ....T..... ......CN.T AT........
MC93  ... ....... .......... ....T..... .......... ..........
MC94  ... ....... .AT....... .......... ....CT.... ..........
HM90  ... ....... .......... ....T..... ......ACAT ..........
HM94  ... ....... .......... .......C.. ....CT.... ..........
HM95  ... ....... C.T....... C...T..... ...C.C.... ..........
HO91  ... ....... ..A....... .........CT ....CA.... ..........
HO94  ..AA.....T. .....T.... ...C...... G...CC.CG. ..........
HP94  ... ....... .......... .......... .......... ..........
HP95  ... ....... .......... .......... ....CT.... ..........
WI95  ... ....... .......... .......... .......... ..........
WI96  ... ....... .......... .......... ....CT.... ..........
MCG95 ... ....... .......... .......... .......... ..........
MCG96 ... ....... ..T....... .......... ....CT.... ..........
MCK94 ... ....... .......... .......... .......... ..........
MCK95 ... ....... .......... .......... ...GCT.... ..........
HV94  ... ....... .......... .......... ....CT.... ..........
HV95  ... ....... .......... .......... ....CT.... ..........
MCN94 ... ....... .......... .......... ....CT.... ..........
MCN95 ... ....... .......... ...N...... ....CT.... ..........
SE95  C
SE96  ... ...G... .........C ...N...... ....CT.... ..........
```

Figure 15: Nucleotide sequences of the partial 5' NTR of enterovirus isolates from CFS patients as described in Table 25. GenBank accession numbers for samples 1 to 8 are X96897-X96912. Differences between the sequences and a consensus sequence (Con) are shown. Unique bases referred to in the text are highlighted in bold. The nucleotide positions are based on the complete genome of coxsackievirus B3 (GenBank Accession no. M33854). N represents a mixture of bases at this position.

The two sequences from HO91 and HO94 were only 70.6% identical. This strongly suggests that these sequences were derived from two different enteroviruses. The sequence derived from the serum of patient 3 (HO91) was 99% identical to a coxsackievirus B3 sequence (accession no. M33854) and the other (HO94) was most similar to echovirus 6 (accession no. U16283).

When the sequences of a further eight pairs were included in the comparison, TI, PA, MO and HA still had unique differences but there were no such unique bases in the remaining pairs. Overall the CFS sequences were very similar, for example MC94, HM94 and HV94 at positions 200/201, 214/215, 242 and 245, though none of the sequences were identical.

On further examination, it was discovered that sequences from samples isolated in the same year were often more alike each other than they were to their consecutive pair. For example, CR94 was compared with MCN94, MC94 and HM02, generating identities of greater than 94%. These figures were equal to (MCN) or greater than the identities with the respective pair. Similarly, HA93 was compared with other sequences from 1993 and MCG93 shared 99.2% identity, and comparing WI96 to other 1996 samples showed identities greater (91%) than with the paired sample.

The first samples of each of the four pairs of sequences that suggested persistence were compared with all the sequences in the database by FASTA analysis to determine the sequence closest in identity. Only two of them, TI93 and PA93 were most like their respective pair (TI95 and PA94). Additional evidence for a lack of persistence is observed with patient HM who provided 3 samples that were PCR positive over time. Comparison of the sequence shows no pattern of evolution of sequence over time with base changes from the consensus occurring at random, for example, the CT at position 214/215 is present in the two later sequences, and not in the first, the A at position 334 is present in the first and last sequences but not the second.

3.4.4. Reliability of direct sequencing of RT-PCR products

Five sequences of coxsackievirus A9 control RNA were obtained by RT-PCR and direct sequencing using P1+ and P4- primers. Coxsackievirus A9 sequences 1 and 2 were derived from PCR products obtained from RNA extracted from one aliquot of control while samples 3 to 5 were derived from PCR products obtained from RNA extracted from separate aliquots of the control. Four consensus sequences of 350 base pairs and one of 311 were obtained. The sequence was identical in all cases with the exception of one base pair at position 109 in sequence 4 (Figure 16). The coxsackievirus A9 control used in this study was 84% similar to the published sequence.

3.4.5. Analysis of enteroviral PCR results over time

This study was conducted over 8 years in total, with work in the last 6 years carried out by the author. A total of 1716 specimens from CFS patients was tested for the presence of enteroviral sequences by PCR during this period. In addition 441 specimens from a group of comparison individuals were tested. Overall, 22% of CFS patients were enteroviral PCR positive and 5.2% of comparison individuals were positive. The percentages within the years did not vary greatly for the comparison group, but did differ for the patient group (Figure 17). The difference between 1990 and 1997 was quite marked with 52.2% positives in 1990 compared to 0% in 1997. PCR of control specimens was not carried out in 1997.

As part of a study on enterovirus isolations in Glasgow over a 21-year period, the number and type of enteroviruses isolated by the routine laboratory for the study period described in this thesis was determined (Nairn & Clements, 1999). The number of isolates and the predominant enterovirus in each year are shown in Table 26.

```
                109                                                   158
cox A9-1     ttagaagtta tactccacgg ccaacagcag gcatggtaca ccaatcatgt
cox A9-2     .......... .......... .......... .......... ..........
cox A9-3     .......... .......... .......... .......... ..........
cox A9-4     a......... .......... .......... .......... ..........
cox A9-5     .......... .......... .......... .......... ..........

                159                                                   208
cox a9-1     catggtcaag cacttctgtc tccccggact gagtatcaat aaactgctca
cox a9-2     .......... .......... .......... .......... ..........
cox a9-3     .......... .......... .......... .......... ..........
cox a9-4     .......... .......... .......... .......... ..........
cox a9-5     .......... .......... .......... .......... ..........

                209                                                   258
cox a9-1     cgcggtcgaa ggagaaaacg tccgttaccc ggctaactac ttcgagaaac
cox a9-2     .......... .......... .......... .......... ..........
cox a9-3     .......... .......... .......... .......... ..........
cox a9-4     .......... .......... .......... .......... ..........
cox a9-5     .......... .......... .......... .......... ..........

                259                                                   308
cox a9-1     ccagtaacac cacggaaatt gcgaagcgtt tcgctcagca cgcccctggt
cox a9-2     .......... .......... .......... .......... ..........
cox a9-3     .......... .......... .......... .......... ..........
cox a9-4     .......... .......... .......... .......... ..........
cox a9-5     .......... .......... .......... .......... ..........

                309                                                   358
cox a9-1     gtagatcagg ccgatgagtc accgcgttcc ccacaggtga ctgtggcggt
cox a9-2     .......... .......... .......... .......... ..........
cox a9-3     .......... .......... .......... .......... ..........
cox a9-4     .......... .......... .......... .......... ..........
cox a9-5     .......... .......... .......... .......... ..........

                359                                                   408
cox a9-1     ggctgcgttg gcggcctgcc catggggcaa cccatgggac gcttcaatat
cox a9-2     .......... .......... .......... .......... ..........
cox a9-3     .......... .......... .......... .......... ..........
cox a9-4     .......... .......... .......... .......... ..........
cox a9-5     .......... .......... .......... .......... ..........

                409                                                   458
cox a9-1     ggacatggtg cgaagagtct attgagctag ttagtagtcc tccggccect
cox a9-2     .......... .......... .......... .......... ..........
cox a9-3     .......... .
cox a9-4     .......... .......... .......... .......... ..........
cox a9-5     .......... .......... .......... .......... ..........
```

Figure 16: Alignment of sequences derived from 5 separate PCR products of coxsackievirus A9 control using the program PILEUP. The numbers refer to the nucleotide positions of coxsackievirus B3 (GenBank accession no. M33854).

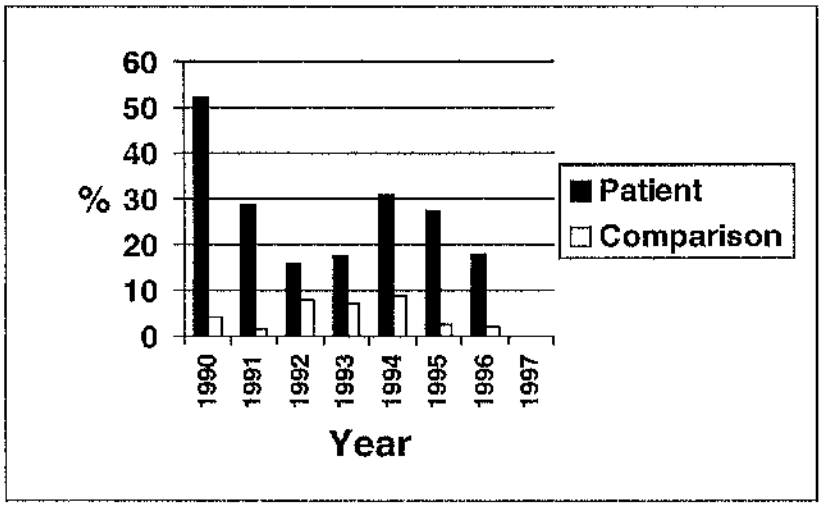


Figure 17: Percentage of enterovirus PCR positives from 1990 to 1997 for the patient and comparison groups.

Year	Total enterovirus isolations (number)	Predominant enterovirus type(s) (% of total for year)
1990	525	echo 4 (86)
1991	186	echo 30 (34)
1992	110	echo 30 (13.6), echo 11 (14.5)
1993	89	coxsackie A9 (21)
1994	156	echo 6 (19)
1995	118	coxsackie A9 (18.6)
1996	116	coxsackie B5 (25.8), echo 30 (23)
1997	79	coxsackie B3 (24)

Table 26: Number and predominant type of enteroviruses isolated during the years 1990-1997.

The most striking feature in this period, in fact in the whole 21-year analysis was the outbreak of echovirus 4 in 1990 (Gallacher *et al.*, 1993) which contributed 86% of the enterovirus isolates that year. This coincided with the highest proportion of enterovirus PCR positives (52%) over the study period. Fourteen out of 22 patients who were PCR positive in 1990 reported a date of onset of fatigue, following a flu-like illness, of the end of 1989 or beginning of 1990. All the positive PCR results came from samples obtained from August to December of that year. For one patient, an echovirus 4 was isolated 2 months prior to an enterovirus positive PCR result. However, no other isolation data was available for this group of patients and no sequence studies were undertaken at this time to determine the relatedness of the PCR products to the echovirus 4 isolates.

3.4.6. Comparison of CFS sequences with clinical isolates

Representative clinical isolates identified in the laboratory in the years of the study (Table 7) were sequenced as described in 2.9.2 and compared to the CFS sequences described previously. In addition, earlier isolates and multiple isolates suspected to have come from outbreaks were included to measure the amount of variation that would be expected for a particular serotype. Other sequences accessed from GenBank, which were not available at the time of the initial analysis, were also included.

Ten CFS patient sequences (from analyses 3.3 and 3.4.3.) were taken as representative of the group, and Figure 18 shows the dendrogram produced from the extended analysis. The enteroviruses grouped into two main clusters, one containing the polioviruses, some coxsackie A viruses and EV70, and the other containing the remainder of the sequences including those derived from the patients with CFS and the enteroviruses isolated and typed in the RVL. This clustering of enteroviruses has been observed previously and has been reviewed by Hyypia *et al.* (1997).

Figure 18: Dendrogram showing the relationships between the CFS patients, clinical isolates and published enteroviruses based on the 5' NTR sequence. GenBank accession numbers (from top to bottom of the dendrogram) are as follows: poliovirus 1A (J02281), poliovirus 1B (U01150), poliovirus 2A (X00595), poliovirus 2B (D00625), poliovirus 2C(M12197), poliovirus 3A (X04468), poliovirus 3B (X01076), poliovirus 3C (K01392), coxsackievirus A24 (D90457), coxsackievirus A21 (D00538), enterovirus 70 (D00820), echovirus 7A (L76400), echovirus 9A (X84981), coxsackievirus B5 (X67706), echovirus 6A (U16238), echovirus 9B (X92886), coxsackievirus B2 (Y09512), echovirus 25A (X90724), echovirus 5 (X89535), echovirus 2 (X89532), echovirus 12A (X79047), echovirus 7B (L76401), coxsackievirus B3A (M33854), coxsackievirus B3B (M88483), coxsackievirus B3C (U57056), coxsackievirus B1 (M16560), echovirus 25B (X90722), coxsackievirus A16 (U05876), echovirus 6B (L76399), echovirus 8 (X89539), echovirus 3 (X89533), enterovirus 71 (U22521), coxsackievirus A9 (D00627), echovirus 4 (X89534), echovirus 1 (L76395), echovirus 11 (X80059), coxsackievirus B4A (D00149), coxsackievirus B4B (X05690), echovirus 27 (L76396), echovirus 30 (L76398). The remaining sequences represent the enteroviruses isolated and typed in the RVL and those derived from patients with CFS (MCN94, TI93, HM01, CR92, SE95, HO91 highlighted in pink and CFS9, 13, 15, 16 highlighted in green). The dendrogram was generated by the programs PILEUP, DISTANCES and GROWTREE. The numbers represent the percentage identity (calculated by the program GAP) of the sequences at that point of the branch.

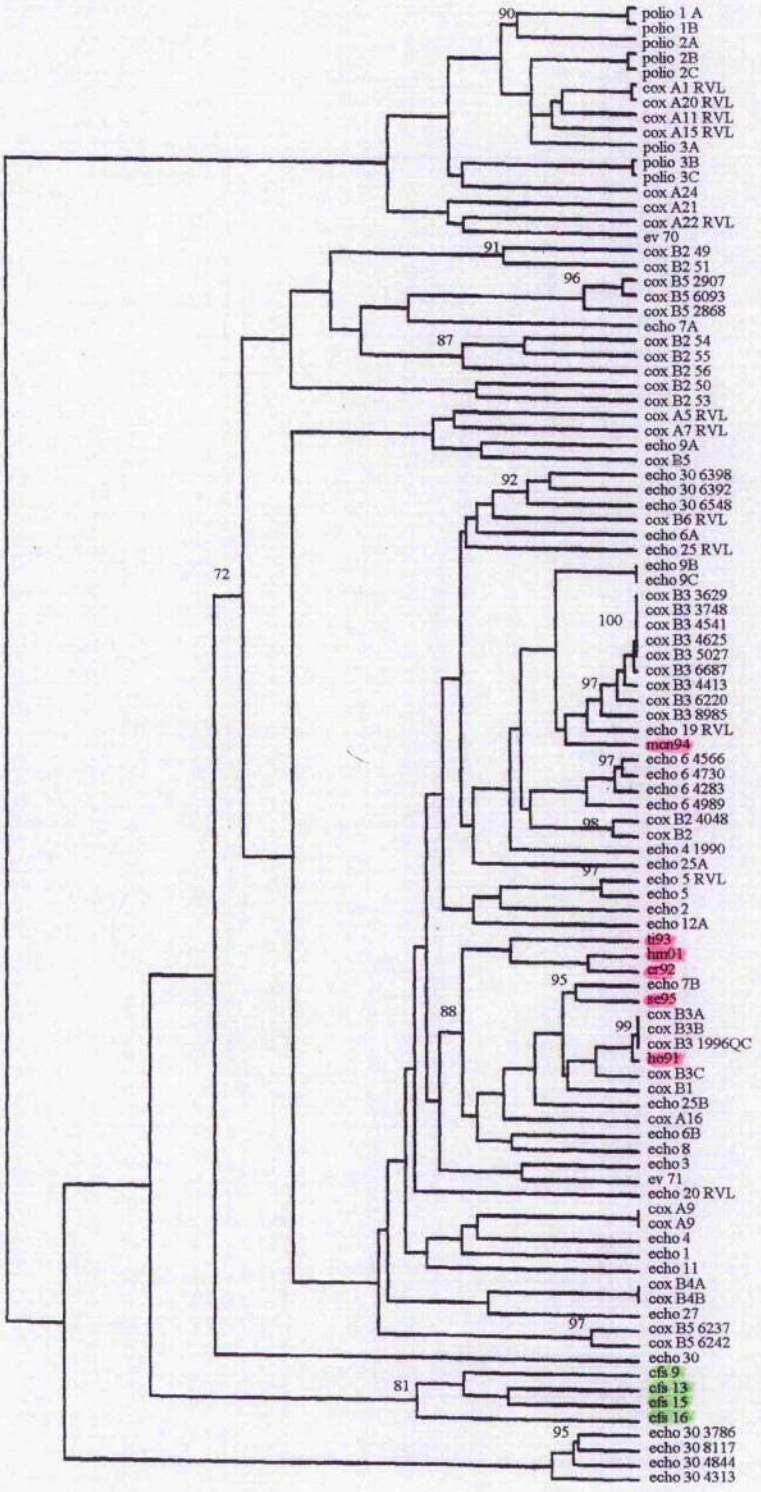

90
polio 1 A
polio 1B
polio 2A
polio 2B
polio 2C
cox A1 RVL
cox A20 RVL
cox A11 RVL
cox A15 RVL
polio 3A
polio 3B
polio 3C
cox A24
cox A21
cox A22 RVL
ev 70
91
cox B2 49
cox B2 51
96
cox B5 2907
cox B5 6093
cox B5 2868
echo 7A
87
cox B2 54
cox B2 55
cox B2 56
cox B2 50
cox B2 53
cox A5 RVL
cox A7 RVL
echo 9A
cox B5
92
echo 30 6398
echo 30 6392
echo 30 6548
cox B6 RVL
echo 6A
echo 25 RVL
72
echo 9B
echo 9C
cox B3 3629
100
cox B3 3748
cox B3 4541
cox B3 4625
cox B3 5027
cox B3 6687
97
cox B3 4413
cox B3 6220
cox B3 8985
echo 19 RVL
mon94
97
echo 6 4566
echo 6 4730
echo 6 4283
echo 6 4989
98
cox B2 4048
cox B2
echo 4 1990
97
echo 25A
echo 5 RVL
echo 5
echo 2
echo 12A
ti93
hm01
er92
95
echo 7B
se95
88
cox B3A
99
cox B3B
cox B3 1996QC
ho91
cox B3C
cox B1
echo 25B
cox A16
echo 6B
echo 8
echo 3
ev 71
echo 20 RVL
cox A9
cox A9
echo 4
echo 1
echo 11
cox B4A
cox B4B
echo 27
97
cox B5 6237
cox B5 6242
echo 30
cfs 9
81
cfs 13
cfs 15
cfs 16
95
echo 30 3786
echo 30 8117
echo 30 4844
echo 30 4313

Figure 18

The CFS patient sequences from Figure 7 (CFS patients 9, 13, 15, 16) still grouped together, separately from the known enteroviral sequences, the closest match being echovirus 6B, with a percentage identity of 87.4%. The CFS patient sequences from the follow-up study grouped within the major enterovirus cluster, MCN94 grouping alongside echovirus 19 with an identity of 95.6% and SE95 grouping beside echovirus 7B also with an identity of 95%. HM01, CR92 and TI93 clustered together, with identities of 88-90% to echovirus 6B which was the most similar enteroviral sequence. HO91 clustered with the coxsackie B3 isolates as suggested by the previous FASTA analysis.

Clinical isolates of echovirus type 5 (echo 5 RVL) and coxsackievirus A9 (cox A9 RVL) grouped alongside their respective serotype. Nine sequences derived from coxsackie B3 isolates obtained in 1997 (cox B3 3629, 3748, 4541, 4625, 5027, 6687, 4413, 6220, 8985) clustered together on the dendrogram with identities ranging from 97.2% and 100%. However, all the isolates differed from the published coxsackie B3 sequences (cox B3A and cox B3B) by up to 15%. Similarly, four echovirus 6 sample sequences (echo 6 4566, 4730, 4283, 4989) isolated within one month of each other in 1994 clustered apart from the published sequences (echo 6A and echo 6B) with differences of up to 20%.

Other groups of multiple isolates of coxsackie B2, coxsackie B5 and echovirus 30 types did not cluster with their respective serotype obtained from GenBank apart from one coxsackievirus B2 isolate (4048) which was 98.6% identical to the published coxsackievirus B2. The remaining coxsackie B2 sequences were spread throughout the dendrogram with identities between isolates ranging from 76.9 to 93.5 %. The coxsackie B5 sequences formed two clusters, one containing the 1961 isolates (6237 and 6242) and the other containing the recent 1996 isolates (2868, 2907 and 6903). Similarly, the echovirus 30 isolates formed two clusters, one containing the early isolates of 1966 and 1975 (6392, 6398 and 6548) and one containing the recent isolates from 1995 to 1997 (3786, 4313, 4844 and 8117). Only one echovirus type 4 isolate (echo 4 1990) from the meningitis outbreak in 1990 was available for sequencing and included in the comparison. This sequence was only 82% identical to the published echovirus 4 sequence and did not group alongside it on the dendrogram. Three patients with a date of onset of symptoms in 1990 or early 1991 (CR, SE and CFS 15) were identified and their sequences compared with the echovirus 4 isolate from 1990. Identities ranged from 78% to 88% only.

3.5. Obtaining additional sequence information

3.5.1. Amplifying other regions of the genome

Sequence analysis of the 5' NTR does not consistently group the enteroviruses according to their serotype and thus determining whether one sequence is like another is difficult. An alternative region was sought for amplification that would provide a more accurate indication of the genetic type of the virus. From the available sequence information in GenBank, enterovirus sequences were aligned by the program PILEUP, and regions of similarity were sought as targets for PCR amplification. One such region spanned bases 580 to 1199 (numbering according to coxsackie B3: accession no. M33854), encompassing the end of the 5' NTR, all of the VP4 capsid protein and part of the VP2 capsid protein. Phylogenetic analysis of sequences from the capsid region has been shown to group the enteroviruses according to their serotype (Hyypia *et al.*, 1997).

Primers A and B (Table 6) were designed to amplify this region and in conjunction with primer 4+, a semi-nested PCR was developed. Primers 4+ and B- were used in a first round reaction (conditions as EV-PCR with primers P1+ and P4-) producing a band of 750 base pairs. This was followed by a second round using primers A+ and B- (conditions as EV-PCR with primers P6+ and P9-, except that an annealing temperature of 60°C was used) producing a fragment of approximately 620 base pairs. The sensitivity of this optimized reaction was measured using the titred coxsackie A9 stock as described in 3.1.2.

Gel electrophoresis of the PCR products was performed as described earlier (see Figure 19A for results). The first round was sensitive to 100 $TCID_{50}$ whereas the second round was sensitive to 1 $TCID_{50}$. This is 100 times less sensitive than the standard PCR. This lowering of sensitivity with larger products has been observed elsewhere (Arola *et al*, 1996). However, it was necessary to design consensus primers which would amplify the majority of the enteroviruses, in order to screen the samples and these were the best that were available. These primers were tested against all the enteroviruses available in the laboratory. The majority of the enteroviruses were amplified with the exception of coxsackieviruses A2, A4, A8, A12, A14, A16 and echovirus 22. As described for the standard PCR, some types were not available for testing. Amplification of a small number

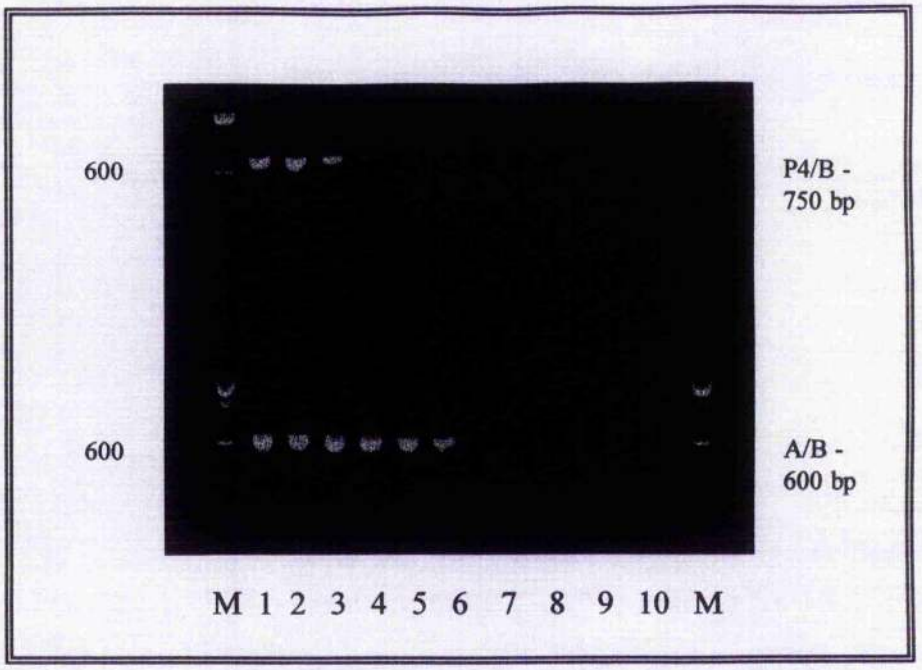
of untyped rhinovirus isolates using these primers produced a smaller product (approximately 500 bases) due to the presence of a short 5' NTR (approximately 620 bases) compared to the enteroviruses. Again unrelated virus types such as CMV and HSV did not amplify (Figure 19B).

A number of the amplicons from the standard enteroviruses (Table 7) were sequenced according to the methods outlined previously, to provide comparisons for the prototype sequences in the database and the sequences derived from the patients. A dendrogram illustrating the relationships of these sequences in this region is shown in Figure 20. This figure shows that in general, multiple sequences of the same serotype cluster together. For example, the coxsackie B2 and echovirus 30 sequences which were spread throughout the dendrogram based on the 5' NTR, clustered with their respective serotype when part of the capsid region was analysed.

Samples from CFS patients previously positive for enteroviral sequences using primers directed towards the 5' NTR, were tested using the primers for the capsid region. Out of 55 RNA samples tested, no specific amplicons of the desired size were observed and no consistent pattern was observed (Figure 21). Some samples produced a number of different sized bands. Increasing the annealing temperature to reduce the non-specificity did not alter the banding pattern observed. Altering other parameters such as starting template concentration and use of hot-start did not affect the results either. Other samples did not produce any bands at all, and altering the parameters to reduce the specificity did not affect the results.

A third region of the genome was identified as a possible target for PCR spanning the region from 5173 to 5768 bases which encompasses all of the VPg region and part of the 3C protease. Using a combination of primers prot1+, prot2- and prot3- in a semi-nested PCR, a product of approximately 300 base pairs was generated using coxsackievirus A9 as a control. However, the RT reaction required priming with the poly-T primer rather than the random hexamers for this reaction to work efficiently and as will be discussed later this approach could not be used with the majority of clinical samples and was not pursued.

A.



B.

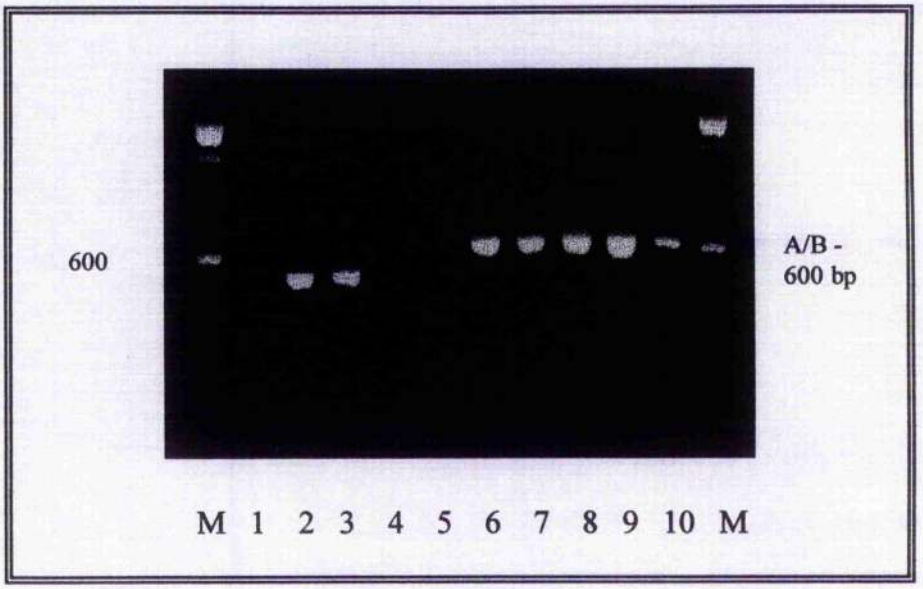
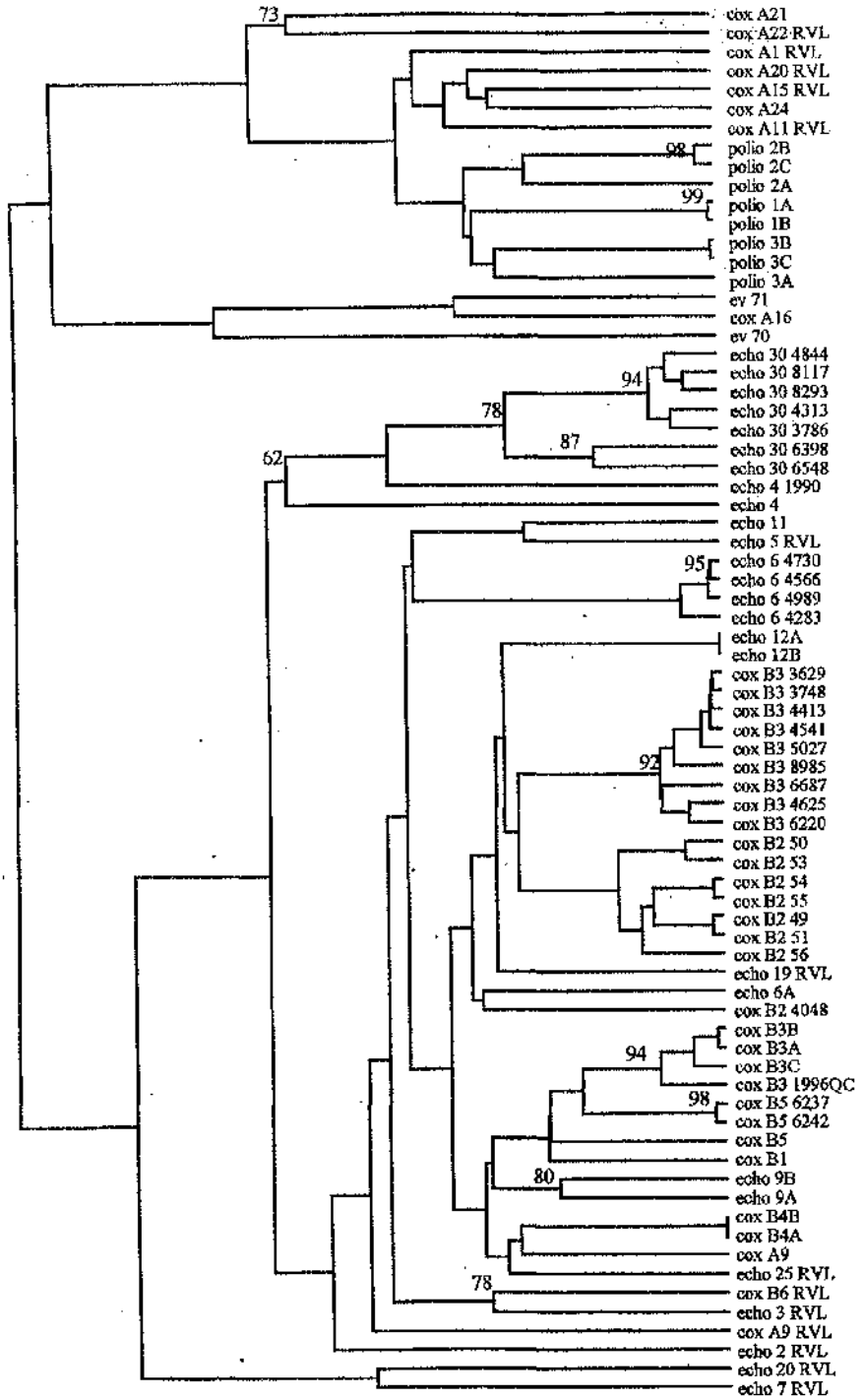


Figure 19: A. Agarose gel showing PCR amplification products of part of the capsid region of a dilution series of coxsackievirus A9. Top- first round (primers 4+/B-), Bottom- semi-nested (primers A+/B-). M- 100bp DNA ladder, Lanes 1 to 10 – coxsackievirus A9 dilution series 10^{-1} to 10^{-10}. B. Agarose gel showing PCR amplification products of part of the capsid region for a series of virus types. Lane M - 100bp DNA ladder; lane 1- MRC-5 negative control; lanes 2 and 3 - rhinovirus (untyped); lane 4- CMV; lane 5- HSV; lane 6- coxsackievirus A9 8558; lane 7- coxsackievirus B3 3629; lane 8- coxsackievirus B5 2907; lane 9- echovirus 4 1990, lane 10- echovirus 6 4566.

Figure 20: Dendrogram showing the relationships between the clinical isolates and published enteroviruses based on part of the capsid region. GenBank accession numbers (from top to bottom of the dendrogram) are as follows: coxsackievirus A21 (D00538), coxsackievirus A24 (D90457), poliovirus 2B (D00625), poliovirus 2C (M12197), poliovirus 2A (X00595), poliovirus 1A (J02281), poliovirus 1B (U01150), poliovirus 3B (X01076), poliovirus 3C (K01392), poliovirus 3A (X04468), enterovirus 71 (U22521), coxsackievirus A16 (U05876), enterovirus 70 (D00820), echovirus 4 (X89534), echovirus 11 (X80059), echovirus 12A (X79047), echovirus 12B (X77708), echovirus 6A (U16238), coxsackievirus B3B (M88483), coxsackievirus B3A (M33854), coxsackievirus B3C (U57056), coxsackievirus B5 (X67706), coxsackievirus B1 (M16560), echovirus 9B (X92886), echovirus 9A (X84981), coxsackievirus B4B (X05690), coxsackievirus B4A (D00149), coxsackievirus A9 (D00627). The remaining sequences represent the enteroviruses isolated and typed in the R.V.L. (designated with the suffix -RVL). The dendrogram was generated with the programs PILEUP, DISTANCES and GROWTREE. The numbers represent the percentage identity (calculated by the program GAP) of the sequences at that point of the branch.


cox A21
cox A22 RVL
cox A1 RVL
cox A20 RVL
cox A15 RVL
cox A24
cox A11 RVL
polio 2B
polio 2C
polio 2A
polio 1A
polio 1B
polio 3B
polio 3C
polio 3A
ev 71
cox A16
ev 70
echo 30 4844
echo 30 8117
echo 30 8293
echo 30 4313
echo 30 3786
echo 30 6398
echo 30 6548
echo 4 1990
echo 4
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echo 6 4730
echo 6 4566
echo 6 4989
echo 6 4283
echo 12A
echo 12B
cox B3 3629
cox B3 3748
cox B3 4413
cox B3 4541
cox B3 5027
cox B3 8985
cox B3 6687
cox B3 4625
cox B3 6220
cox B2 50
cox B2 53
cox B2 54
cox B2 55
cox B2 49
cox B2 51
cox B2 56
echo 19 RVL
echo 6A
cox B2 4048
cox B3B
cox B3A
cox B3C
cox B3 1996QC
cox B5 6237
cox B5 6242
cox B5
cox B1
echo 9B
echo 9A
cox B4B
cox B4A
cox A9
echo 25 RVL
cox B6 RVL
echo 3 RVL
cox A9 RVL
echo 2 RVL
echo 20 RVL
echo 7 RVL
73
98
99
94
78
87
62
95
92
94
98
80
78

Figure 20

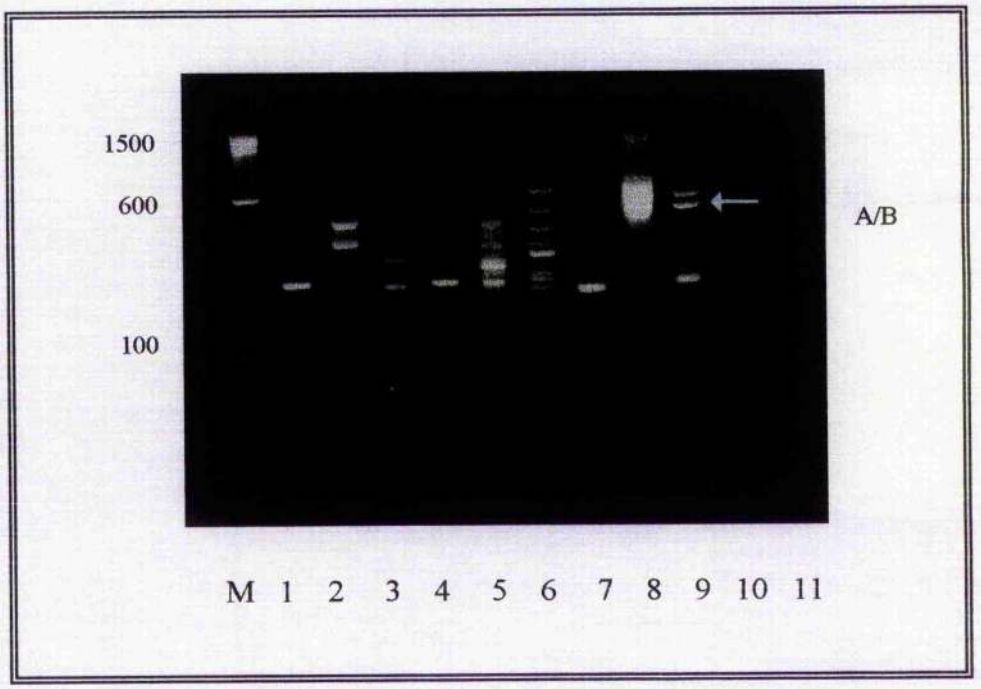

Figure 21: Agarose gel showing amplification products of semi-nested PCR using primers 4+/B- then A+/B-. Lane M- 100bp DNA ladder; lanes 1 to 7 and 9 - CFS samples; lane 8- coxsackievirus A9 positive control; lane 10- 1st round PCR no-template control; lane 11- 2nd round no-template control. The expected 600 base pair product is indicated by an arrow.

3.5.2. Alternative strategies

3.5.2.1. Inverse PCR. Coxsackievirus A9 was used as the control to optimize the inverse PCR reaction. The generation of full-length cDNA followed by restriction enzyme digestion was carried out as described in 2.10.2. Varying amounts of cDNA were then amplified by inverse PCR as described in 2.10.3. Gel electrophoresis of the products showed smearing in each of the wells with no specific bands of the expected size observed.

The reaction was repeated, priming the cDNA synthesis specifically with primer A- according to method 2.10.1, using 10^{-2} and 10^{-3} dilutions of coxsackievirus A9. This would produce a double-stranded cDNA template of approximately 600 base pairs.
Enterovirus PCR using the standard primers was performed on this template to ensure that it was intact after processing. A product of the correct size was observed in all samples except for the 10^{-2} dilution that was subjected to the phenol/chloroform extraction procedure. Thus the template was intact for all but one sample.
The inverse PCR products generated using these templates are shown in Figure 23. Again, the 10^{-2} dilution did not produce any amplification product. Although bands were observed for each of the other reactions around the expected size (approximately 190 base pairs), there were multiple bands present which were not distinct and sequencing of the products was not possible. This procedure was therefore deemed unsuitable for use with clinical specimens since it was unable to generate useful sequencing material.

3.5.2.2. Long PCR of the enteroviral genome. A full length cDNA template for long PCR can be created by priming the RT reaction with a poly-T primer which binds to the poly-A tail of the enterovirus genome. Subsequent PCR with extreme 5' and 3'primers can then generate fragments which will be near full-length. Before using the 3' negative sense primers in the long PCR, they were used to prime the synthesis of cDNA in a standard RT reaction to ensure that they would bind. The standard 5' NTR PCR using primers P1+ and P4- was then performed to ensure that the cDNA had been successfully generated. Out of 22 standard viruses tested, a PCR product of the correct size was generated for all virus types when the poly-T primer was used to prime the RT reaction

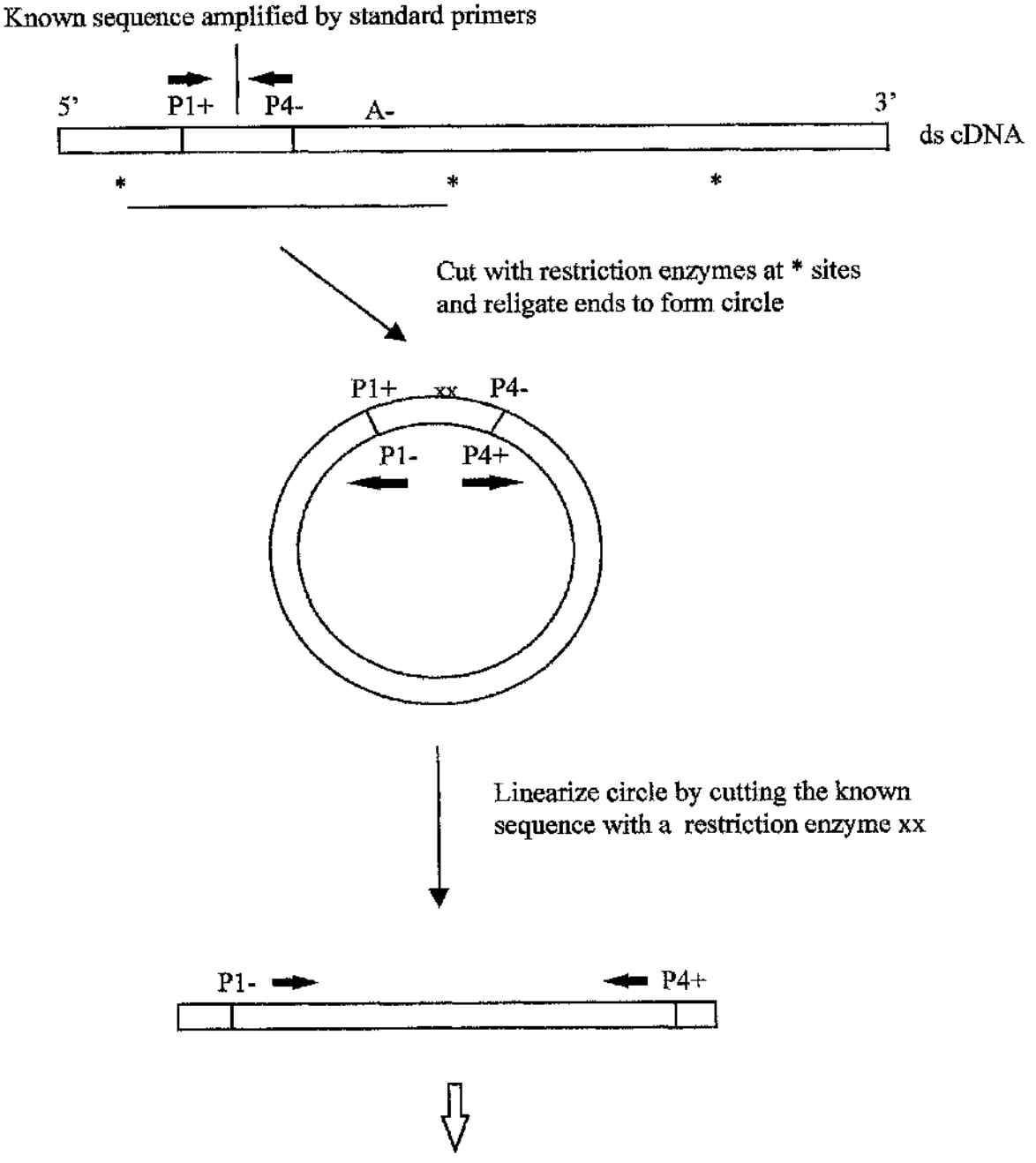

Figure 22: Diagrammatic representation of the inverse PCR reaction, showing orientation of forward (P1+ and P4-) and reverse (P1- and P4+) primers.

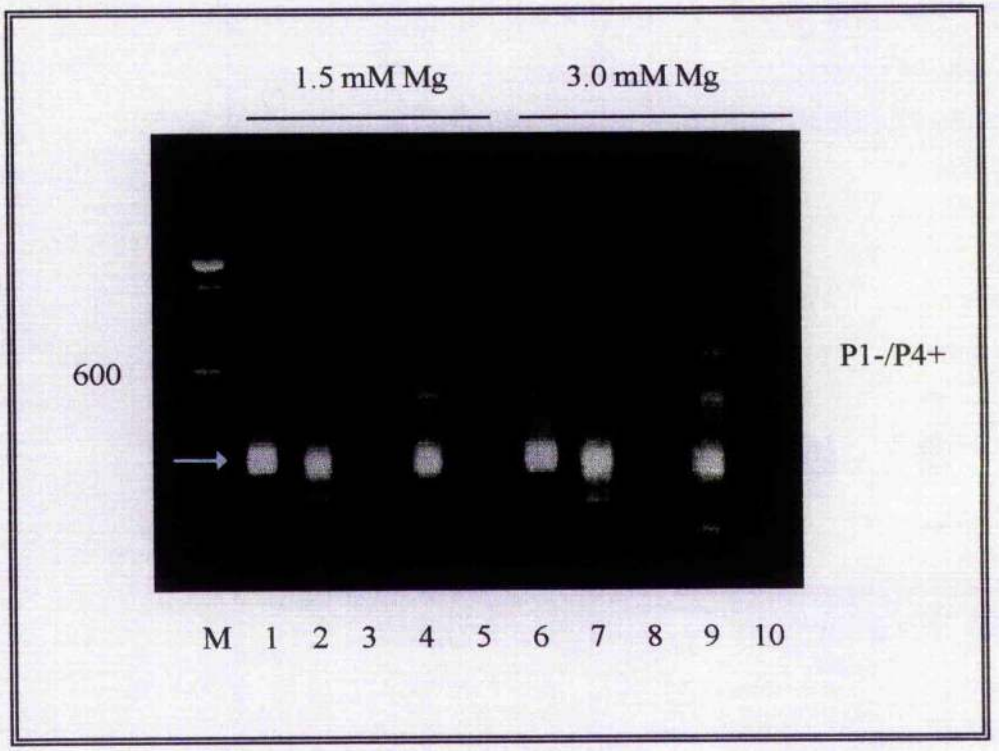

Figure 23: Agarose gel electrophoresis showing products of inverse PCR. Lane M - 100bp DNA ladder, Lanes 1 to 5 – Inverse PCR using 1.5mM $MgCl_2$, Lanes 6 to 10 – Inverse PCR using 3.0mM $MgCl_2$. Lanes 1 and 6 –coxsackie A9 10^{-2} (heat-inactivated); lanes 2 and 7 – coxsackie A9 10^{-3} (heat inactivated); lanes 3 and 8 – coxsackie A9 10^{-2} (chloroform extracted); lanes 4 and 9 – coxsackie A9 10^{-3} (chloroform extracted) lanes 5 and 10 – no template control. The expected 190 base pair product is indicated by an arrow.

PCR products were generated from the RNA of 18 different virus types, including coxsackievirus types B1 to B5, A5, A9, A19 and A22, echovirus types 4, 6, 7, 9, 18, 19, 25 and 30 and enterovirus 69 when primers Y-, prot2- and prot3- were used in the RT reaction. The primers were thus considered to be appropriate for use in the amplification of clinical samples.

Amplification of the entire genome was carried out according to method 2.12 using coxsackievirus A9 as the standard. Because clinical samples are likely to contain low levels of enterovirus, as evidenced by the standard EV-PCR, it was necessary to design a nested PCR. A number of different primer combinations were used to determine the greatest sensitivity. Priming of cDNA synthesis using the poly-T primer followed by a first round PCR with primers P1+ and Y- and a nested PCR with primers P4+ and prot2 produced the best sensitivity (see Figure 24A). Long PCR using these primers was therefore possible but the sensitivity was low (to 10^{-3}) and would therefore not be sufficient for the detection of enteroviral sequences in the clinical samples from CFS patients.

3.5.2.3. Construction of a cDNA library. RNA molecules can be labile in their natural form and difficult to amplify. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then inserted into a self-replicating lambda vector (UNI-ZAP XR vector). This can then be used to infect host bacteria which can be grown and screened in hybridisation assays for any insert of interest. The cDNA library generated represents the information encoded in the messenger RNA of the sample and is much easier to manipulate than the parental RNA. The procedure makes use of the poly-A tail of the RNA to prime the cDNA synthesis. It was therefore important to ensure that samples for cloning could be primed in this way.

Previous positive RNA samples were screened using poly-T to prime the reverse transcriptase reaction followed by a standard enterovirus PCR using primers P1/P4 and P6/P9. Out of 83 previous positives, only 13 produced a positive band, suggesting that the primer was unable to bind to the A-tail.
If the poly-A tail was reduced or absent in these samples then the primer would not bind. Adenosine residues (from ATP) can however, be added to the 3' end of RNA by the enzyme poly-A polymerase (see Method 2.11).

Four samples were chosen which could not be primed with the poly-T primer. Adenosine residues were added using the polymerase enzyme and the resulting product was reverse transcribed using the poly-T primer and then amplified using the standard 5' NTR enterovirus primers. The resulting agarose gel (Figure 24B) clearly shows that the samples without the tail have not amplified, whereas the same samples with the added tail have been amplified, suggesting that the poly-T primer is binding to the additional adenosine residues.

For the cloning procedure, two samples with sufficient volume were selected. Five 200 µl aliquots were processed according to method 2.4.2, with the exception that each RNA sample was dissolved in 6 µl of DEPC-treated water. The aliquots were then combined to produce a more concentrated starting material. An aliquot of RNA (10µl) was used in the standard PCR reaction priming with poly-T to ensure that the sample was still positive. Two samples were used in the cloning procedure (2.13) one that could be primed with the poly-T primer (sample A) and one that required treatment with poly-A polymerase (sample B).

After size fractionation, four fractions of each cDNA were phenol/chloroform extracted and quantitated by an ethidium-bromide plate assay (2.14). Three fractions from sample A contained 50ng/µl. Each cDNA (100ng) was ligated into the Uni-ZAP vector (2.14.2) and packaged reactions were titred with XL1-Blue MRF' host bacteria with blue/white selection. The ratio of insert to background should be at least 10 times, but in this case the ratio was only 2:1. The library was amplified producing titres of 6.1 x 10^6 pfu/µl to 1.4 x 10^7 pfu/µl in the fractions. Approximately 500,000 pfu of each fraction was screened with different radioactive probes according to methods 2.13.4 and 2.13.5.

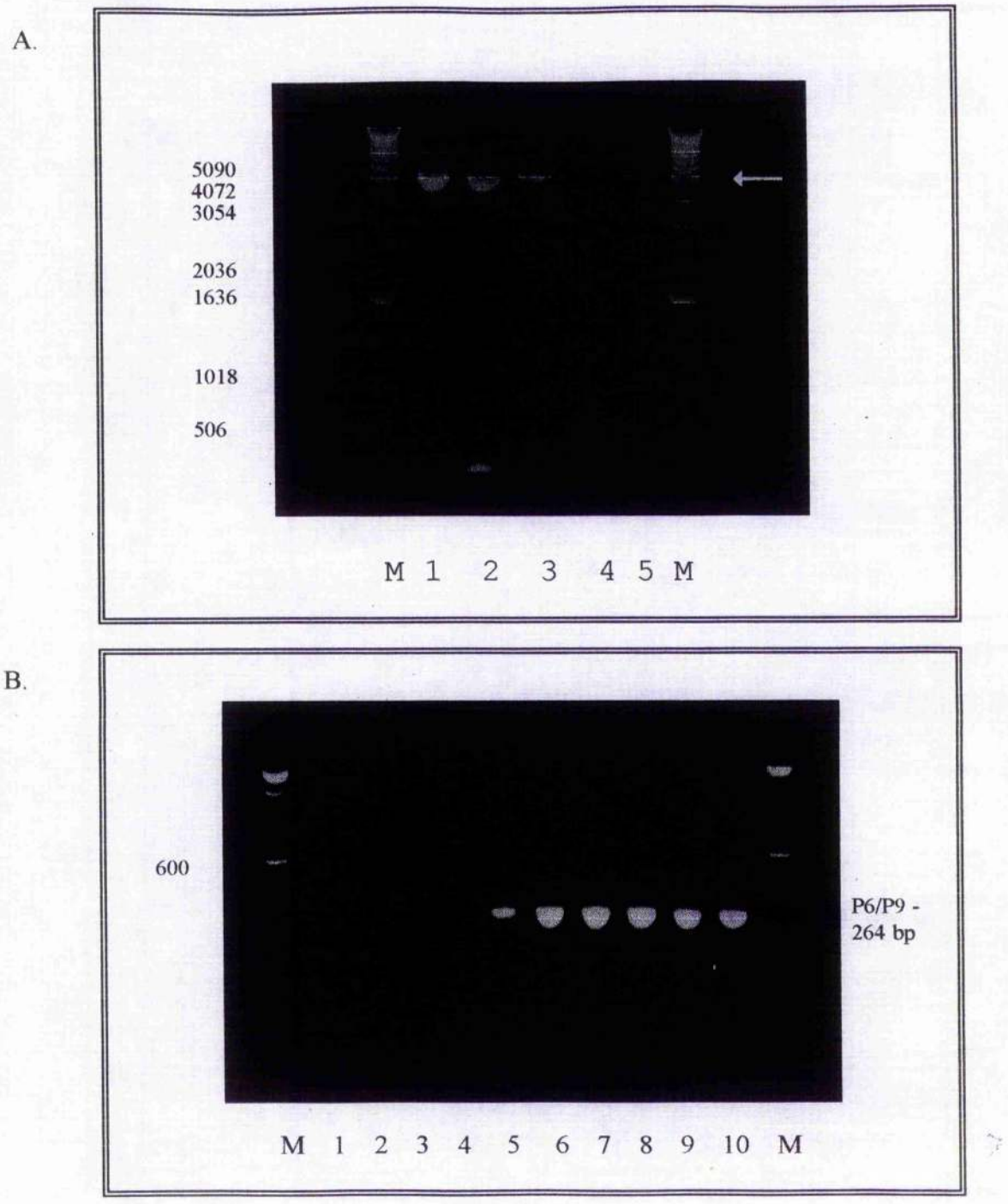

Figure 24: A. Agarose gel electrophoresis of long PCR products from semi-nested PCR. Lane M - 1kb DNA ladder; lanes 1 to 4: coxsackievirus A9 10^{-1} to coxsackievirus $A9^{-4}$ (primers P1+/prot2-: expected product size 5000 base pairs approx.); lane 5 - coxsackievirus $A9^{-1}$ (primers P1+/prot3-: expected product size 5300 base pairs approx.). B. Agarose gel showing PCR products obtained using primers P6+/P9- from samples with (lanes 6-10) and without (lanes 1-5) treatment with poly-A polymerase. Lanes 1-4 and lanes 6-9 represent four samples from CFS patients. Lanes 5 and 10 are products of the control, coxsackievirus A9. Expected PCR products are indicated by an arrow.

There were no positive hybridization spots with either the long coxsackie A9 based probe or the protease probe. With the standard P6+/P9- product a possible positive plaque was observed which was picked into SM buffer and used to infect new XL1-blue cells. The new plates were then screened as before, producing 2 possible positives at this stage. These were picked into SM buffer and the process repeated once more, resulting in 100% positive plaques on the plate. A number of plaques were picked and screened by PCR using primers T3 and T7 to determine if an insert was present. The samples were all positive at the same size with these primers suggesting that there was no insert in the vector and that the hybridization had been non-specific. This was confirmed by sequencing of the purified pBluescript plasmid after excision from the lambda/pBluescript phagemid vector.

Similar results were obtained for sample B. The initial ratio of insert to background plaques was low, and subsequent screening did not produce any genuine positives.

As a control, all the filters used in the screening process were screened with a PCR product obtained by amplification with the ABL1/2 primers. None of these were positive, suggesting that the library did not contain enough of the serum RNA of interest to begin with to enable it to be detected by hybridization methods.

4. Discussion

4.1. Chronic Fatigue Syndrome

Chronic Fatigue Syndrome (CFS) is a poorly understood condition characterized by chronic, debilitating fatigue of sudden onset that is defined as lasting longer than six months. Common associated findings are myalgia, sore throat, poor concentration, sleep disturbance and mood disorders but the presence of other somatic complaints often makes the syndrome difficult to distinguish from other organic, psychiatric and poorly understood disorders. For example, fibromyalgia is a common rheumatologic condition characterized by chronic myalgia, fatigue, disrupted sleep and headaches that occurs mostly in women and may follow a viral illness, much like CFS (Buchwald & Garrity, 1994). Likewise, patients with depressive illness often have no energy, their concentration is impaired and they are under-active and depressive illnesses are also twice as common in women as in men (Kendell, 1991). There is also an overlap of symptoms between CFS and somatisation disorder where patients present with physical symptoms that are likely to be associated with depression or anxiety, rather than physical disease.
However, having said this, the symptoms that define CFS (according to Fukuda *et al.*, 1994) are reported much more frequently by patients with CFS than by healthy subjects or by patients with other diseases that produce chronic fatigue (Komaroff & Buchwald, 1998).

Data from early investigations of CFS was difficult to compare because of the variety of names used by different investigators for the condition and the failure to apply uniform diagnostic criteria. There was also a lack of objective measures of illness severity and in some cases control groups were not tested. This led to the introduction of the Oxford criteria (Sharpe *et al.*, 1991) and the CDC criteria (Holmes *et al.*, 1988, revised by Fukuda *et al.*, 1994) for the diagnosis of CFS for research purposes which meant that clinical groups could then be well-defined. Comparison of data between studies was therefore possible, affording a better understanding of the nature of the illness.

The research on CFS has provided many contradictions with few agreed and consistent

findings regarding the nature of the illness. What has been consistent across studies is the mean age at diagnosis (35-41 years) and the preponderance of women. It is acknowledged however that many more women than men consult their general practitioner on a regular basis and this may account for the difference observed. Other common features include a belief in a physical rather than a psychological cause of illness by the patient and a high frequency of depression, which is significant (2-3.3 times more frequent) when compared to patients with other chronic physical illnesses (Wessely & Powell, 1989). Findings that have not been independently confirmed among studies include various immunological and virological abnormalities. The presence of these abnormalities does however point to an organic rather than a psychiatric basis for the disease although it is likely that elements of both are involved. The variety of symptoms and abnormalities observed thus suggest that the illness is multifactorial.

The reporting of a sudden onset of CFS following a flu-like illness by many patients, suggested involvement of an infectious agent, especially where epidemics of CFS occurred in a number of individuals in the same geographical area over a short time span. For example, in Lake Tahoe, 150 patients were diagnosed predominantly in 1985 with chronic mononucleosis-like syndrome (Barnes, 1986). Additionally, there were 'outbreaks' of a similar illness in two other communities within a radius of 100 miles at about the same time (Komaroff, 1988). Similar outbreaks have been described in Los Angeles (Gilliam, 1938), London (Ramsay & O'Sullivan, 1956) and Scotland (Fegan *et al.*, 1983). A good history of an acute onset has been used to distinguish the CFS patient from other patients presenting with a complaint of chronic fatigue who are normally vague about the date of onset. One difficulty in using these criteria is that patients are not diagnosed until at least six months after onset of illness and thus a confirmed laboratory diagnosis of infection at onset of fatigue is generally not possible. In cases where corroboration of a viral illness by laboratory data is available the illness is classified as a post-infectious/viral fatigue syndrome (PIFS/PVFS) (Sharpe *et al.*, 1991).

Immunological abnormalities have been observed in CFS patients although interpretation of results is complicated by lack of standardization of tests compounded with the failure of studies to use similar patient groups. The most consistent findings have included IgG deficiencies, impaired T-cell function and low NK cell cytotoxicity. It has thus been suggested that CFS is associated with an immunological disturbance. This can then permit

infection by or the reactivation of latent and persistent infectious agents, particularly viruses, in the host. Viruses may contribute directly to the illness by damaging certain tissues and indirectly by eliciting an immune response. Alternatively, a viral 'hit and run' mechanism has been proposed based on the lack of recovery of an infectious agent (Levy, 1994). Here the virus enters the host and infects the target cells causing immune abnormalities and the production of inflammatory cytokines, which in turn produce the symptoms observed in CFS. As has been stated earlier, the Royal College report which brought together research topics from a wide variety of areas warned against over-interpreting these immunological abnormalities because of lack of standardization of test methods (Royal Colleges, 1996).

The search for a common viral agent as a trigger or precipitating factor has included the study of herpesviruses, retroviruses and enteroviruses. Evidence for and against the involvement of these virus types has been presented. Much of this evidence has been based on retrospective serological data which cannot be linked directly to the illness. Enterovirus neutralizing antibodies for example, can be detected for months to years after the initial infection. Elevated titres of antibodies to a number of different viruses have been detected in different groups of CFS patients and this was suggested to be the result of a nonspecific polyclonal immune response. To test this hypothesis, antibody titres to 18 common viruses (EBV, HHV-6, HSV-1, HSV-2 and 14 enteroviruses) were measured simultaneously in 20 patients with CFS and compared with age and gender matched controls (Manian, 1994). Antibody titres to EBV viral capsid antigen were elevated in 55% of cases compared to 15% of controls but for the 14 enteroviruses, antibodies to only coxsackievirus types B1 and B4 were present at significant titres. Additionally there was no correlation between elevated titers of EBV viral capsid antigen IgG and IgG to HHV-6, HSV-1 and HSV-2 or antibody to coxsackie types B1 and B4. Although only 20 patients and controls were sampled, the data did not support the idea that elevation of viral antibody titres in CFS patients was due to a non-specific polyclonal immune response.

More direct evidence for the involvement of a particular virus in the syndrome can be provided by isolation of the virus or detection of viral sequences. Again, conflicting data have been presented. Retroviral sequences (DeFreitas *et al.*, 1991), HHV-6 replication (Buchwald *et al.*, 1992) and enteroviral sequences (Archard *et al.*, 1988) have been reported in significant numbers of CFS patients compared to control groups, but subsequent investigations have not independently confirmed these results (Secchiero *et al.*,

1995; Swanink *et al.*, 1994).

4.2. Enteroviruses and Chronic Fatigue Syndrome

In the United Kingdom, researchers have focused attention on the association of enteroviruses with CFS. Investigations centred on the detection of coxsackie B virus neutralizing antibodies, which were present in significant proportions of individuals with CFS compared to healthy control groups (Fegan *et al.*, 1983; Keighley and Bell, 1983; Bell & McCartney; 1984; Calder *et al.*, 1984; Behan *et al.*, 1985). Serological criteria based on raised antibody titres had proved useful in demonstrating the role of coxsackie B viruses in some cases of adult heart disease (Bell *et al.*, 1983; O'Neill *et al.*, 1983). Early studies indicated a link between high antibody titre and recent infection, therefore titres of 256 were suggestive while titres of 512 and above were indicative of recent infection (Grist *et al.*, 1974). A disadvantage of using this as a measure of infection is that neutralizing antibody can remain elevated for many years in an individual and interpretation of static titres then becomes difficult.

An ELISA for the detection of coxsackie B virus specific IgM was developed as a more useful marker of recent infection (King *et al.*, 1983). McCartney *et al.*, (1986) showed that a significant proportion of CFS patients (31% of 118 and 37% of 290) were positive for coxsackie B-specific IgM compared to healthy controls (9% positive), while Miller *et al.*, (1991) found no significant difference between 53 patients and 49 controls (24.4% versus 22.6% positives respectively). It must also be noted that cross-reactivity of the IgM test with other enteroviruses (coxsackie A and echovirus types) and hepatitis A virus has been observed (King *et al.*, 1983; McCartney *et al.*, 1986). This test has not been routinely used since for diagnostic purposes.

Although serological testing only provides indirect evidence for the presence of enteroviruses, additional molecular studies have provided further direct evidence for the association of enteroviruses in patients with CFS. Direct isolation of the virus has also been shown, although only in a few studies. Using acid dissociation culture of stool samples, Yousef *et al.* (1988) isolated enterovirus from 17 patients and 2 controls. This technique removes bound neutralizing antibody from any virus present which would normally hinder isolation. Isolates of coxsackievirus types B1-5 and echovirus 1, 9 and 11

were recorded and after 12 months, five patients were still positive, yielding the same virus type. More recently, Vedhara *et al.*, (1997) isolated non-polio enterovirus using acid-culture from two CFS patients on day zero of a poliovirus vaccine trial. Studies from the Netherlands (Swanink *et al.*, 1994) on 76 patients and 76 controls and from Sweden (Lindh *et al.*, 1996) on 82 samples from 34 patients failed to isolate virus using acid-culture methods. In routine cell culture systems, 25% to 35% of specimens from patients with typical enterovirus infections of any serotype are negative (Chonmaitree *et al.*, 1982), so it is not surprising that many virological studies of CFS patients do not yield any positive isolation results. Indeed, isolation of virus from affected tissue in chronic heart disease, where a positive association with enteroviruses has been suggested, is rarely successful (Morgan-Capner *et al.*, 1984). In addition to the problems of virus isolation in general, discussed in section 1.4.4., the nature of the chronic illness is such that the virus may already have been cleared by the time the investigations are carried out.

Both Swanink and Lindh also failed to detect enterovirus in CFS patients using PCR techniques apart from one positive sample in the former study from a CFS patient. Neither group included an extraction control in the PCR to show that the RNA had been successfully extracted from each sample. It is known that stool samples can contain inhibitors of the PCR process (Wilde *et al.*, 1990) and these must be removed during the RNA isolation process. Negative results should therefore be accompanied by a positive control PCR result indicating that the RNA extraction procedure has been successful. Additionally, the Swedish study did not include any control samples and did not supply any information regarding the sensitivity of the PCR (Swanink could detect approximately 20 pfu).

Concurrent testing of comparison samples for enterovirus by nested PCR in the present study showed an overall uniform background level of detection of 5.2% for the years 1990 to 1996. It is therefore surprising that all the samples (except one) tested by both groups discussed above were negative. Perhaps these results reflect a difference in the epidemiology of the enteroviruses in different countries and as such cannot be used as evidence to discount the data from the U.K.

Another marker of enteroviral infection which has been used is the VP1 antigen, a capsid protein common to all enteroviruses which can be detected by 5-D8/1, a monoclonal antibody. VP1 antigen was detected in 51% of CFS patients compared to 0% of controls

in a study by Yousef *et al.* (1988). This finding was confirmed by Halpin and Wessely (1989) but disputed by Lynch and Seth (1989) and Swanink *et al.*, (1994) who both found no difference in the detection of the antigen between patients and controls. The test has not been widely used since in the investigation of CFS patients.

Early studies using molecular techniques described the detection of enteroviral specific RNA in muscle biopsies of a significant number of CFS patients compared to controls (Archard *et al.*, 1988; Bowles and Archard, 1990; Cunningham *et al.*, 1991). Additionally, Cunningham showed that where enteroviral RNA was detected in biopsies from CFS patients, the amounts of positive and negative strands were approximately equal, whereas in a lytic infection positive strand is synthesized in approximately 100-fold excess. This could be the result of a defect in the control of viral RNA synthesis and together with the failure to isolate enterovirus in conventional cell culture this suggested that the enterovirus detected in CFS patients was in some way unusual.

Further advances led to the development of the polymerase chain reaction which was capable of amplifying specific DNA sequences from low levels of starting material. Many investigators thus began developing PCR protocols and this technique began to be favoured over hybridization and serological methods to study enterovirus involvement in CFS.

The data discussed herein was derived from studies conducted over approximately 7 years. The preliminary data indicating a positive relationship between enteroviral sequences and CFS by PCR was based on samples collected during 1990 and 1991 (Clements *et al.*, 1995; Nairn *et al.*, 1995). Phylogenetic analysis was performed on sequences derived from samples obtained during 1992 and 1994 (Galbraith *et al.*, 1995) and the follow-up study which included an investigation into enteroviral persistence (Galbraith *et al.*, 1997) and a clinical evaluation of the patients' illness, was carried out on samples collected between 1994 and 1997.

4.3.1. Contamination

PCR is a process capable of amplifying and detecting a target DNA molecule present only once in a sample of 10^5 cells (Saiki *et al.*, 1988). In approximately 35 cycles of PCR a target can be amplified by a factor of more than 10 million. This sensitivity means that even a small amount of contamination of target DNA in the starting material will result in a positive reaction product. This problem is more pronounced when nested-PCR is used since this is more sensitive than one round of PCR.

In this study although all reasonable precautions were taken within the laboratory (see methods), contamination was observed occasionally where samples, negative virus controls and no template controls were positive. These instances were obvious and the results were disregarded. Sequencing of PCR positives throughout the study showed that none of the samples were identical in sequence, although many were very similar, and at no time was the coxsackievirus A9 positive-control sequence detected in any of the positive clinical samples.

Critics have suggested that the significant numbers of PCR positives reported in Clements *et al.*, (1995) and Galbraith *et al.*, (1995) were a result of contamination since this work has not been replicated elsewhere. However, samples were only recorded as positive if all the controls in that particular run amplified as predicted and if repeated extraction and RT-PCR produced a second positive result. Additionally, if contamination from PCR product carry-over due to poor technique was responsible for the positive results observed, more positives would have been expected in the control group. Furthermore, there were no positives at all with the VP4/VP2 capsid PCR.

4.3.2. Sensitivity and specificity of PCR

The majority of published PCR protocols for enterovirus detection target the 5' non-translated region (NTR), a highly conserved stretch of approximately 750 base pairs,

common to the majority of the enteroviruses (Stanway, 1990). Many of the reported PCR protocols describe a single round PCR followed by hybridization, which can be time consuming and often requires the use of radioactive probes. Additionally, under stringent reaction conditions for high specificity, hybridization requires almost perfect homology between the probe and the product (Severini *et al.*, 1993). Nested PCR is suited to the sensitive and specific detection of a small number of templates against a large background of cellular RNA (Erlich *et al.*, 1991). It is as sensitive as PCR plus hybridization, more rapid and does not require perfect homology except at the 3' ends of primers. PCR also allows for subsequent sequencing of the positive product for identification purposes. A number of nested and semi-nested PCR protocols for the 5' NTR have been described. Severini *et al.*, (1993) described the detection of coxsackievirus types B1 to B6, poliovirus types 1 to 3, and echovirus types 9, 19 and 31 using a nested PCR. The sensitivity of the reaction was such that close to a single molecule, in as much as 1mg of tissue could be detected. Kammerer *et al.* (1994) tested 33 prototype viruses, including the poliovirus and coxsackie B types, coxsackie A types 5, 7, 9, 16 and 21, echovirus types 1 to 9, 11,12, 22, 24 and 33 and rhinovirus types 1, 2b, 14 and 89. With the exception of echovirus 22 which was not amplified, all the virus types were detected to a sensitivity of 0.05 PFU/ml. Nicholson *et al.* (1994) used a nested PCR which recognized all poliovirus and coxsackie B isolates, coxsackieviruses A9 and A16 and echovirus types 4, 6, 7, 11, 18 and 30, to a sensitivity of 0.1 $TCID_{50}$.

In the present study, coxsackievirus types A6, A24 and echovirus types 23, 29 and 32 were not available for testing but all other enteroviruses (59 types) were amplified using the nested primers, with the exception of echovirus 22. Echovirus types 22 and 23 have been shown to differ considerably from the other enteroviruses in terms of their genome sequence, structural proteins and lack of host-cell shut-off. Echovirus 22 has recently been classified as the type member of a sixth genus, parechovirus (Mayo & Pringle, 1998). Many PCR protocols have thus failed to detect these serotypes (Hyypia *et al.*, 1989; Olive *et al.*, 1990; Chapman *et al.*, 1990) while others have reported the amplification of smaller sized bands (Kammerer *et al.*, 1994) or the need for additional cycles for detection (Rotbart, 1990). In the latter study echovirus 2 also needed an additional 25 cycles for amplification compared to 9 other serotypes of the same titre. Using the microwell detection system 27 serotypes were detected at $\leq$ 1 $TCID_{50}$ with the exception of coxsackie A7 which was detected at 1 $TCID_{50}$ (Rotbart *et al.*, 1994). The decreased sensitivity

observed with these few serotypes is probably a result of differing homologies between the primers and the target sequence. The standard enteroviral nested PCR described in this thesis detected coxsackievirus A9 control to a dilution of 10^{-8}, which was equivalent to 0.01 $TCID_{50}$. For this virus type, the sensitivity was similar to other nested protocols that expressed the sensitivity in these terms. Endpoint sensitivities for other types were not determined.

4.3.3. PCR testing in CFS

PCR was used initially in investigations of CFS to test muscle biopsies and the association with enteroviruses was confirmed with a significant proportion of patients positive (53%) compared to controls (15%) (Gow *et al.*, 1991). Many individuals with CFS report muscle pain and fatigue on exercise: the detection of enteroviral sequences in muscle tissue may provide evidence for a direct association with chronic fatigue syndrome. The same group in a later publication on the results of a larger study, showed no difference in the number of enterovirus positive samples between patients with CFS and those with other neuromuscular disorders (Gow *et al.*, 1994). However, in a further publication it was stated that the methods used in the 1994 study were not as specific as had been hoped, suggesting that there were a number of false-positives in the series of samples (Behan *et al.*, 1996). Whether these were in the patient or control groups (or both) was not discussed but six of the CFS patient positives were shown to be enteroviral in origin by direct sequencing of the PCR products. The accuracy of the results previously reported by others and by Gow *et al* (1994) would therefore appear to be in question and there may be no convincing evidence of enterovirus positivity in CFS cases. Other groups also failed to find a significant difference between patients and controls (Leon-Monzon & Dalakas, 1992; Swanink *et al.*, 1994; Lindh *et al.*, 1996), the latter two reports having been discussed previously. However, focal localisation is a feature of some enterovirus infections (Kandolf *et al.*, 1987) and if viral particles are sparsely distributed in the muscle then they may escape detection and false-negative results may arise (Leon-Monzon & Dalakas, 1992). Examination of multiple biopsies might prevent this but it is difficult to justify obtaining multiple biopsy samples in control patient groups. Would it therefore be possible to use samples other than muscle biopsies from these patients? Serum samples were thus selected and with access to individuals with chronic fatigue syndrome, they were

tested for the presence of enterovirus by a nested PCR (Clements *et al.*, 1995) previously developed in this laboratory (Gow *et al.*, 1991). The results showed a significant difference between the patient and comparison groups for enteroviral RNA sequences and demonstrated that serum could be used as an alternative to muscle biopsies for detecting enteroviral sequences in CFS patients. Obtaining a serum sample is a less invasive procedure than retrieving a muscle biopsy, and consequently obtaining comparison samples is less of a problem. Enteroviruses were also detected from stool samples in 48% of patients versus 28% of controls but this result was not significant using the 'Odds ratio' test.

In the Regional Virus Laboratory in Glasgow, serum samples from CFS patients were routinely tested for the presence of coxsackievirus B neutralizing antibodies (types B1 to B5) because of the historical data indicating an association with CFS. The merits of this test (and other serological assays) have been mentioned previously. Using the same methods, the neutralization assay was used to test serum samples from CFS patients and compare the results with the detection of enteroviral sequences by PCR. The tests were performed on 100 CFS and 100 comparison individuals (Nairn *et al.*, 1995). Testing for coxsackievirus B6 antibodies was not carried out since this serotype is rarely found in Glasgow, with only two isolates reported in the 1977-1997 period (Nairn & Clements, 1999). The results showed that it was not possible to distinguish between patients in the CFS and comparison groups using the neutralization test, since equal numbers in both groups were positive for one or other of the antibodies (34% versus 41% respectively). The PCR results however, were significantly different between the two groups (42% versus 9% for patients and comparison individuals respectively). In the patient group, sixty six results were concordant. Of those that did not correlate the majority were PCR positive/coxsackie B antibody negative (21/34), and would have been the result of the PCR detecting enterovirus other than the coxsackie B serotype. In the comparison group, 58 results were concordant and the majority that were not, were PCR negative/coxsackie B antibody positive (37/42). The detection of neutralizing antibodies in 'healthy' patients is not surprising since these are known to persist for months to years following infection. The antibody test is therefore not an appropriate measure of recent infection and does not provide information regarding the current enteroviral status of the patient. PCR, on the other hand provides evidence for the direct detection of enteroviral RNA sequences at a given time point and can be used as a marker of enteroviral infection.

Thus PCR was shown to be a suitable and sensitive technique for the detection of enteroviruses in serum and from these two studies the association of enteroviruses with CFS, previously shown by serological testing and by PCR of muscle biopsies, was strengthened. This prompted the initiation of a study to examine new patients and to analyze the sequence of the enterovirus present by direct sequencing of any positive PCR products.

4.4. Phylogenetic analysis of enteroviral sequences

PCR was used to screen samples from new patients with CFS presenting to the Infectious Disease (ID) unit of Ruchill Hospital for the presence of enteroviral sequences. Initially, twenty PCR products (13 from serum and 7 from throat swab samples) were subjected to manual sequencing and compared to sequences from the GenBank database and laboratory isolate sequences using phylogenetic analysis.

Phylogenetic analysis refers to the comparison of sequence data and the construction of phylogenetic trees or dendrograms that graphically depict the relationships of sequences. It can be used to compare two sequences to determine how similar they are, to trace the source of transmission of a particular virus, or to identify an unknown sequence by comparison with sequences available in the various databases. There are numerous methods for constructing phylogenetic trees from molecular data (Felsenstein 1988; Miyamoto and Cracraft, 1991). They can be classified into distance methods and discrete-character methods. Distance methods include the neighbour-joining method proposed by Saitou and Nei (1987) and the unweighted pair-group method with arithmetic means (UPGMA: Sneath and Sokal, 1973). There are two major groups of discrete character methods - maximum parsimony and maximum likelihood methods. No one method has been shown to be optimal for inferring phylogenetic trees from alignments. Therefore, the consensus of opinion in the literature has been to carry out several different methods of comparison and if they yield the same or a virtually identical tree, then this is thought to be a reliable representation of the sequence relationships present (Saitou and Imanishi, 1989).

To ensure that the sequences obtained by manual sequencing were accurate the reliability of the procedure was measured by comparing the manual-derived sequence with one derived from automated sequencing from a single sample. Manual sequencing relies on the operator distinguishing bands across four lanes of a gel and this can be inaccurate at the top of the gel where bands are packed closely together. However, only one nucleotide difference was observed between the two sequences, suggesting that the sequence obtained by the manual methods was reliable and representative of that present in the original sample.

Phylogenetic analysis of the 20 sequences derived from the CFS patients showed that all but one of the sequences were separate and distinct from the known enteroviruses and from other enteroviruses and non-CFS sequences derived from laboratory specimens. The sequences were also illustrated in dendrogram format and this confirmed the phylogenetic tree groupings. Further sequence comparisons showed that a consensus CFS sequence was not related to other animal picornaviruses (<50% homology) with the exception of SVDV (approximately 83% similar) which is closely related to the coxsackie B viruses. However, at this time there were only a few enteroviruses out of the whole group with sequence data available for this region (poliovirus types 1-3, coxsackievirus types A9, A21, A24, B1, B3, B4, B5 and enterovirus type 70) thus it is possible that the CFS related sequences were derived from known enteroviruses whose sequence had not been determined. Alternatively, the CFS sequences may have indicated the presence of novel enteroviruses or represented sequences from known enteroviruses with variant 5' NTRs. The 5' NTRs of enteroviruses and rhinoviruses have highly conserved primary and secondary structures that have been confirmed as being vital for RNA replication and translation of viral proteins. Indeed the initiation of translation is mediated by a region within the 5' NTR known as the internal ribosome entry site (IRES) (Rohll *et al.*, 1994). The 5' NTR also contains determinants of neurovirulence. The base at nucleotide 472 of poliovirus 3 is the major determinant of virulence, and in a study of seven different echoviruses, the less virulent types (serotypes 2 and 12) exhibited variations in this region compared to the virulent types (Romero & Rotbart, 1995). Similarly, in a study of enterovirus 71 isolates, variations in the 5' NTR were revealed between isolates associated with hand, foot and mouth disease and those associated with aseptic meningitis (Zheng *et al.*, 1995).

Thus if an altered 5' NTR is a feature of the enteroviruses found in patients with chronic

fatigue syndrome and inefficient translation and replication result then this might lead to an atypical pattern of infection and result in a persistent infection.

An atypical sequence was also obtained from one non-CFS comparison patient (non-CFS 1). No clinical information was available for this patient. The other non-CFS sequences were dissimilar to the CFS patient sequences and grouped with the sequences of the known enteroviruses. This is to be expected from non-CFS sequences 2 to 6 which were derived from patients with a presumptive diagnosis of myocarditis. Enteroviruses, particularly the coxsackie B virus type, have been shown to be associated with human viral heart disease (Kandolf & Hofschneider, 1989). Controlled studies of patients with suspected viral heart disease showed that at least half the cases of acute myocarditis and one third of the cases of non-bacterial pericarditis were associated with coxsackie B virus infection (Grist and Bell, 1974). More recent summary data has reported the detection of enterovirus sequences in 45% of hearts by slot-blot hybridization and 25% by PCR or *in situ* hybridization (Martino *et al.*, 1995).

Gow and colleagues (1994) published a short enteroviral sequence identified from the muscle biopsy of a CFS patient. Although only 83 base pairs in length it was also distinct from the known enteroviruses (at least 20% difference in nucleotide identity from coxsackieviruses B1 and B3). Another group showed that sequences obtained from stool samples from CFS patients in Cardiff were closely related (>90%) to those described by Galbraith *et al.*, (1997) (Han *et al.*, 1998). Both these studies thus provided corroborating evidence for the presence of atypical enteroviral sequences associated with CFS. However, all the sequences analysed were short and without further corroboration from other regions of the viral genome, preferably those coding for the capsid proteins which give an indication as to the type of enterovirus, the atypical group identified here could not be defined further.

Among the 20 CFS patient sequences subjected to initial phylogenetic analysis were two sequences from patient 7 derived from samples obtained 10 months apart. These sequences differed by less than 1% suggesting that the virus may have persisted in this patient. Persistence has been suggested as being involved in the progression and maintenance of the fatigue syndrome because enteroviral antibodies and RNA have been detected in patients who have suffered with the syndrome over a long period (McCartney *et*

al., 1986; Archard *et al.*, 1988; Cunningham *et al.*, 1990; Bowles *et al.*, 1993). Indeed, the study by Cunningham *et al.*, (1990) provided evidence for abnormal replication and was interpreted as enteroviral persistence. The evidence for (Muir & Archard, 1994) and against (Melchers *et al.*, 1994) enteroviral persistence has been hotly debated. However, in most studies evidence for the presence or absence of persistence has been based on analysis at one time point only. None of the studies have looked at detecting the virus (viral sequences) over time. A follow-up study was thus implemented to examine patients over time with respect to their clinical condition (with the aid of the questionnaire) and their enteroviral PCR status.

4.5. Follow-up Study

4.5.1. Correlation of enteroviral status with clinical data

If enteroviruses act as trigger for CFS via a 'hit and run' mechanism then they should be detected shortly after the onset of fatigue, before they are cleared by the immune system. If they trigger the syndrome and then persist in the host in some way, then they might be expected to be detected throughout the course of the illness. If the virus acts as a precipitating factor in causing an exacerbation of symptoms, either through exogenous infection or from 'persistent infection' then the presence of virus may be associated with the clinical course of the illness.

There have been no attempts to follow CFS patients over time to look at the clinical course of CFS in relation to the presence or absence of the enteroviruses (or any other viruses), although one group did look at the effects of enteroviral infection in CFS patients by exposing the patients to live poliovirus vaccine (Vedhara *et al.*, 1997). In this study 7 CFS patients receiving vaccine were compared to 7 CFS patients receiving placebo and 9 control patients receiving vaccine in a double-blind study. Patients were monitored at intervals up to 8 weeks post-immunization by providing faecal samples for virus isolation studies, blood samples for immunological analysis and undergoing behavioural assessment. Administration of the vaccine was not associated with exacerbation of symptoms in CFS patients, the behavioural differences present between the groups did not change post-immunization, the number of cognitive failures did not increase and psychomotor function did not decrease. Additionally, there were no immunological abnormalities in the immune response to poliovirus although the reduced levels of CD28 and CD38 cells observed in

CFS patients compared to the control patients prior to immunization were maintained through the study. There were also no differences in cytokine release, T-cell responses, neutralizing antibodies or secretory IgA. Interestingly, acid culture of stool samples did produce differences between the groups, with polioviruses, non-polio enteroviruses and unclassified enteroviruses isolated from the CFS-vaccine group compared to only one poliovirus isolate in the control group and none in the CFS-placebo group. In addition, circulating enterovirus-like sequences were detected by PCR in the first serum sample tested of two CFS patients. Thus the data supported the hypothesis that persistent enteroviral shedding following infection was a feature of CFS (Yousef *et al.*, 1988).

The few long term follow-up studies that have been reported have mainly looked at predictors of outcome and have had a psychiatric basis. For example, Sharpe *et al.*, (1992) followed up patients at 6 weeks to 4 years and showed that for the first year, prognosis was poor but long term, the percentage of patients functionally impaired (in terms of walking, social activities, hobbies and occupation) reduced, although most still complained of fatigue. Age, sex and marital status was not related to functional impairment but belief in a viral cause, coping skills, changing job and joining a patient organization were related. Bonner *et al.*, (1994) studied 43 patients who were followed up after 4 years. This group of patients had been offered treatment in the form of cognitive behaviour therapy. Of the 23 who completed treatment, 87% were well compared to only 13% who had made a spontaneous recovery without receiving or completing treatment. Those that were still fatigued after 4 years (only 1/3) were likely to have had more somatic disorders, were more fatigued and had a previous psychiatric history when assessed. Wilson *et al.*, (1994) also showed psychiatric involvement, with a significantly higher rate of primary psychiatric diagnosis at follow-up in those patients who had not improved, but in this case there was no difference in premorbid psychiatric diagnoses at entry to the trial. A poor outcome for patients was also predicted for those with a high score on the disease conviction scale and those who did not believe that psychosocial factors played a role in their illness.

In the present study, patients were referred initially to the Infectious Disease Department at Ruchill Hospital by their General Practitioner. A number of patients were referred from the same practice and thus the interest and/or expertise of the G.P. in this field may have determined which patients were referred further and this does add some selection bias to the study group. A diagnosis of CFS was then confirmed by the Infectious Disease consultants and the patients were put forward for inclusion in the study. A psychiatric

evaluation was not performed on the patients, but they all fulfilled the Oxford Criteria which made them suitable for analysis. All patients were asked to complete a questionnaire at entry to the study and annually subsequently.

Patients were followed up to correlate clinical presentation (derived from the questionnaire data) with enteroviral PCR status over a four-year period. The number of patients participating each year decreased, due to a number of factors: failure to contact the G.P./patient due to a change in address, refusal of G.P. to take part, recovery of the patient and unwillingness to continue or general loss of interest in the study. Thus only 19 patients completed four questionnaires and provided four blood samples compared to 130 who completed two.

An overall view of the group regarding their health status prior to the illness and their clinical condition afterwards was gathered from the 333 'first' questionnaires completed by patients on first attendance at the clinic. There was nothing remarkable about the patient group prior to the onset of illness, for example travel abroad, immunizations, allergies etc. The age distribution showed an approximately normal distribution and the female to male preponderance observed in other studies was repeated here. The distribution of the month of onset of fatigue is interesting when compared to the distribution of isolation of enteroviruses in Glasgow (based on figures from 1994-1997). The peak of enterovirus isolation occurs in July and August while the onset of fatigue is fairly constant throughout the year with slight peaks in November/December and February when enteroviral activity is low. The majority of patients (81%) reported an acute illness (within 2 weeks) prior to the onset of fatigue and thus the peak of onset would be expected to match the peak of enterovirus activity if enteroviruses were a major factor in this illness. However, although some patients were definite about the date of onset (to the day) others could not remember so accurately, especially those who had been ill long-term and this may have altered the distribution.

The ten symptoms reported most often (after fatigue and post-exertion weakness in muscles, which were virtually 100%) by this group of patients included many symptoms which are common secondary manifestations of depression or anxiety, including myalgia, sleep disorder and headache for example (Komaroff, 1993). Those symptoms that cannot easily be ascribed to a primary psychiatric illness including fevers, coughs, night sweats and joint pain were reported at lower frequencies by patients (14% for cough to 58% for

joint pain). Perhaps this indicates that in this group of patients the psychiatric component of the illness is greater than the physical component.

Of the 333 patients completing one questionnaire, 36% were enterovirus PCR positive. The patient responses to all of the questions were analysed (where possible) on the basis of the PCR results, thus two groups were identified for each question and the percentage in each group determined. In all cases, the percentages reporting a particular symptom or a specific type of illness at onset for example, mirrored the PCR results. Thus the patient responses to the questions were similar regardless of their PCR status. However, this analysis was performed on one group of samples only at one time-point, providing a snapshot only of a chronic illness.

Thus, patients were followed up at twelve monthly intervals to extend the questionnaire analysis and compare the data with the PCR results over the course of the illness. One hundred and thirty patients were compared in this analysis in which the severity of disease was measured objectively by a number of markers for example, ability to work (both in employment and in domestic terms) and physical activity. This was compared with enteroviral PCR status over time.

Analysis of data from the initial questionnaires and initial PCR testing of this cohort showed that 51.5% of the 130 patients were enteroviral PCR positive. Comparison of the PCR positive and negative groups showed a significant association between PCR positivity and severity of disease. That is, those individuals with a PCR positive result were less likely to be at work or school, less likely to be able to carry out their domestic work, less able to walk distances and more likely to have taken time off work. This suggested that the presence of the enterovirus was a major factor in influencing the severity of illness at this time.

The second set of questionnaires (obtained after twelve months) was similarly analysed. The number of patients who were enteroviral PCR positive had reduced to 16%. If the two groups of patients at this time were compared (21 positives and 109 negatives) there was no difference between the positive and negative groups in terms of ability to work, physical activity etc. Similarly, if the patients were divided into four groups based on their PCR status over time (EV-PCR +/+, +/-, -/+, -/-) there was no correlation between enteroviral positivity and ability to work or physical activity. The group with the most

time off work though had two enterovirus PCR positive results, whereas the group with least time off work had two negative results over time. However, the number of respondents in the PCR +/+ group was too low (4) for this result to be regarded as significant. After 12 months then, the continued presence of virus did not appear to play a part in the severity of illness.

From this data there was a positive correlation between overt clinical symptoms and enteroviral PCR status on examination of the first questionnaire from a cohort of patients. The mean duration of fatigue for this group of patients was 3.9 years (with no difference between the PCR negative and positive groups). Therefore, the virus could have triggered the syndrome and persisted for nearly 4 years, the exacerbation of symptoms being due to its reappearance in the circulation. Alternatively, the symptoms may have been precipitated by a recent episode of infection. With no pre-fatigue samples available for testing it is not possible to determine which of the two possibilities is correct. However, subsequent analysis of a second questionnaire at 12 months compared with PCR results did not support the positive correlation between symptoms and PCR status, while combined analysis of the sequential enteroviral PCR results compared with clinical symptoms, suggested that enteroviral persistence did not play a role in the maintenance of chronic fatigue syndrome. The condition of the majority of patients had not improved but only 16% of second samples were positive. However, the nature of the infection may be such that the enteroviral sequences are present in the individual at particular localized sites, for example the muscle (detection of which has been reported in other studies (Cunningham *et al.*, 1991, Gow *et al.*, 1991)) and shedding into the bloodstream may occur only at intervals. Therefore more frequent testing of patients would be required to maximize the chances of detecting the enteroviral sequences if this is occurring.

The use of self-administered questionnaires to obtain clinical information can be a problem. In this case, forms were completed at home by the patient, as closely as possible to the time of blood donation to enable a correlation of clinical symptoms and enteroviral PCR status to be made. This was not always possible owing to difficulties in obtaining appointments at health clinics to donate the sample and thus many questionnaires could not be included in this cohort, but in most of the 130 cases the difference was a matter of weeks at most. There are also difficulties associated in using questionnaires in CFS research due to the length of time since the onset of symptoms. Many patients simply

cannot remember details prior to their illness and they often misinterpret questions. In a number of cases a question with a 'yes or no' choice (for the list of current symptoms) was answered with 'sometimes' or 'occasionally'. Recall bias is also a feature of this syndrome with patients highly motivated to remember viral infections as many attribute this as the cause of their illness. Of all the patients who completed a first questionnaire in this study, approximately 50% answered 'yes' to ever having been diagnosed with a particular virus infection. The viruses involved were either coxsackieviruses or EBV in equal numbers apart from a few 'mystery' or 'ME' virus reports.

4.5.2. Enteroviral persistence

4.5.2.1 Evidence of enteroviral persistence *in vitro* and *in vivo*. Enteroviruses are known to be the aetiological agents in many acute conditions such as aseptic meningitis and poliomyelitis. With the exception of some cases of immunodeficiency it is not generally accepted that enteroviruses can cause persistent infections (Melchers *et al.*, 1995).

To persist in a host, the virus must be able to cause infection without being cytopathic and must be able to avoid detection and elimination by the immune system of the host. Avoidance of the immune system can be achieved by restricting viral gene expression for example as in the case of HSV, or by infection of tissues and cell types that are not readily accessible to the immune system such as the central nervous system, for example measles virus in the case of SSPE (sub-acute sclerosing pan-encephalitis). Non-lytic viruses are suited to persistence and can establish chronic infections but for lytic viruses, the cytopathic effect has to be limited. This can be achieved by a number of means including infection of non-permissive cells or by the virus adopting a strategy of non-lytic replication, by producing incomplete or defective viruses or generating mutants and variants (Torre *et al.*, 1991).

Persistent enterovirus infections have been demonstrated in cell lines and experimental animals. A persistent steady-state infection of cloned human WISH cells by echovirus 6 has been maintained for over 7 years with the production of defective virus particles (Gibson & Righthand, 1985). The virus particles only contained capsid proteins, VP0,

VP1 and VP3, thus processing of VP0 to VP2 and VP4 had not occurred. Most of the cells were infected and expressed viral antigen, and virus was released continuously without cellular destruction. The viral RNA was not able to produce a lytic infection when transfected into uninfected susceptible cells but could be used to convert uninfected cells to a persistently infected cell line (Righthand & Blackburn, 1989; Righthand, 1991).

Coxsackie B viruses have also been shown to establish persistent infections (McLaren *et al.*, 1993; Gow *et al.*, 1997b). An *in vitro* carrier cell model of persistent coxsackie B5 infection, where only a small proportion of cells carry the viral genome, was developed in rhabdomyosarcoma (RD) cells and maintained for over 100 passages. No cytopathic effect was detected, cellular protein synthesis was inhibited and two virus proteins (p33 and p39) had altered electrophoretic mobility suggesting that mutations had occurred. Coxsackie B viruses are widely accepted as aetiological agents of myocarditis and it has been suggested that dilated cardiomyopathy (DCM) is the result of a previous viral myocarditis, although there are numerous reports that discount the role of enteroviruses in DCM. The positive association has been supported by the detection of enteroviral RNA in the myocardium of patients with end-stage disease requiring transplantation (Bowles *et al.*, 1989). Persistence of enterovirus in the myocardium of patients with DCM has been shown to be a powerful indicator of poor prognosis (Why *et al.*, 1994). Conaldi *et al.*, (1997) looked at the interaction of coxsackieviruses with murine vascular endothelial cells. All 6 types infected the endothelial cells without producing any cytopathic effect, with the replication of coxsackie B types 3 and 5 persisting for an undefined period. Detection of virus in the cell supernatant and demonstration of the presence of the minus strand of the RNA indicated that continued virus replication was occurring. In this case only a small proportion of cells were involved, persistence being maintained through a carrier-state culture.

One example of enteroviral persistence *in vivo* is in patients with agammaglobulinemia who are susceptible to chronic enteroviral infection of the central nervous system, developing chronic meningoencephalitis as part of the syndrome. Wilfert *et al.*, (1977) showed that virus could be isolated from the CSF of 5 such patients. Echovirus types 9, 19, 30 and 33 were recovered for periods ranging from 2 months to 3 years. O'Neill *et al.*, (1988) described a case study where enterovirus was isolated from stool samples over a period of approximately 5 years. Coxsackievirus A15 was isolated up until 1984, then coxsackievirus A4 was isolated in 1985. Oligonucleotide fingerprint maps of 5 of each of

these showed that isolates of the same serotype were similar to each other but markedly different from the other serotype. Two isolates with identical prints were obtained from stools taken within eight days of each other. The degree of change was estimated at 1% of the genome over a period of 2 to 6 years. This case study showed that virus culture negative intervals were common in this syndrome although the signs and symptoms of the infection could persist for months to years. Rotbart *et al.* (1990) showed that by using PCR, enteroviral RNA could be detected in the CSF of patients with agammaglobulinemia through the culture negative periods. Leparc *et al.* (1994) also showed that while enteroviruses could not be isolated from CSF they could be detected by PCR in faeces and throat-swab samples.

4.5.2.2. Enteroviral persistence in patients with CFS. The majority of studies citing evidence for enteroviral persistence have based their findings on detecting enterovirus in one sample from patients who had been ill for a number of years. In the present study, persistence of enterovirus in CFS was assessed by identifying samples containing enteroviral RNA sequences, as detected by RT-PCR, from individual patients over time. The percentage identity between the sequences derived from the PCR amplicons was then determined. RT-nested PCR coupled with direct sequencing is a multiple step enzymatic process involving reverse transcriptase and *Taq* polymerase. These enzymes lack a 3' to 5' exonuclease activity and thus errors can occur during processing. Base substitution errors during reverse transcription have been measured at between 1/30000 to 1/2000 for every nucleotide polymerized (Preston *et al.*, 1988; Roberts *et al.*, 1989) and the mis-incorporation rate per nucleotide per cycle for *Taq* polymerase is estimated at $2x10^{-4}$, if constant over 30 cycles (Saiki *et al.*, 1988). It is generally believed that direct sequencing of PCR products generates reliable consensus sequences compared to sequencing cloned DNA of PCR products since the latter represents single amplicons that may contain random errors of the two enzymes. Base mis-incorporation in single amplicons should be masked by correct bases in the majority of the amplicons (Zhang *et al.*, 1997). Zhang assessed the reliability of direct sequencing by sequencing amplified products from coxsackie B3 infected mouse heart, on days 1 to 13 post inoculation and showed them to be identical to the published sequence of coxsackie B3 (Nancy strain). Three tissue samples from the same infected mouse heart were also processed independently and these sequences were identical to each other as well as to the published sequence. Thus coupling RT-PCR with direct sequencing of nested PCR products

generated accurate consensus sequence data.

RNA virus populations are thought to consist of a heterogeneous mixture of related genomes (quasispecies) rather than a single genome species (Domingo *et al.*, 1985). Thus in a virus stock consisting of quasispecies the population of genomes share a consensus sequence while differing from each other and the consensus sequence by one or a number of bases. The evidence for quasispecies has come from studies on clonal populations obtained at limiting dilution which have shown the occurrence of antigenic variants and mutant virus types. This is true for the enteroviruses: point mutations have been sequenced in antigenic variants of the Sabin type 1 vaccine strain derived from children post-immunization. Additionally, a single nucleotide change at position 472 in type 3 Sabin vaccine, which occurs on passage through the immunized individual, is associated with an increase in neurovirulence (Evans *et al.*, 1985).

An average nucleotide sequence then, results from assigning to each residue the nucleotide most frequently found at the corresponding position, and will in general coincide with the most abundant sequence. In the present study, the accuracy of automated sequencing following RT-PCR was determined by obtaining 5 independent sequences of the 5' NTR of the positive control virus coxsackie A9. Only one base pair difference in sequence 4 was noted which is probably not significant since the first few bases from either direction of a sequence usually contain several ambiguous base calls. This would suggest that sequencing of products is accurate and that the sequence obtained is representative of that present in the sample itself. Additionally, sequencing was carried out in both directions to confirm the bases identified.

Sixteen CFS patients provided two positive samples over time and these were sequenced and compared (Table 25 & Figure 13). Initial sequence comparisons based on 8 pairs of sequences suggested that 4/8 pairs (TI, PA, HA and MO) had evidence for enteroviral persistence based on a high sequence identity between the pairs and the presence of unique shared base pairs that were different from the consensus sequence (Galbraith *et al.*, 1997). Two very different enteroviral sequences were derived over time from one patient (HO) which was strong evidence for a re-infection with a different enterovirus type. This is not surprising since the samples were obtained over 3 years apart. A further eight pairs of sequences were included in the comparison and only one further pair (HV) had a high

enough sequence identity to suggest persistence, but it lacked any unique shared features. The remaining pairs were similar to each other but not at a high enough level to suggest that persistence had occurred. When the comparison was viewed as a whole, similarities were observed between sequences from different patients obtained in the same year. In fact, the percentage identities often exceeded those observed between consecutive pairs. Only two of the 'persistent' pairs identified initially (TI and PA) were closest in identity to their respective pair when compared to all the other CFS sequences and could be regarded as having persisted. Thus, if sequences from different patients are more similar to each other than to their respective pair then the high sequence identity in this region cannot be regarded as evidence for persistence. Additionally, comparison of three consecutive samples from one patient (HM) showed no pattern of evolution over time and thus no evidence for persistence.

From this follow-up data, only a minority of CFS patients provided two (or three) enteroviral positive samples over time, indicating that enteroviral persistence, as assessed by RT-PCR, is not a major factor in this syndrome. Within this small group, analysis of the 5' NTR enteroviral sequence of the positive samples did not provide an ideal means of assessing persistence because of the highly conserved nature of this region and the possibility that the variation is due to the quasispecies nature of the genome. Again corroboration from other regions of the genome is required, which might show that the same type had been detected over time.

Enteroviruses were consistently detected in a greater proportion of CFS patients than comparison patients throughout the study, although the proportion of CFS patients who were enterovirus PCR positive varied each year with 52.2% positives in 1990 compared to 0% (out of 136 samples tested) in 1997. These figures included new patients and those participating in the follow-up study. No control samples were processed for 1997 due to time constraints although controls for the years 1990 to 1996 had shown a mean background level of 5.2% that varied little over the years. Additionally, enteroviral PCR carried out in 1997 by staff of the routine diagnostic laboratory produced positive results in 6.5% of cerebrospinal fluid specimens and in a parallel study on juvenile-onset type 1 diabetes-mellitus, 27% of serum samples were positive for enteroviral sequences (Nairn *et al.*, 1999). Thus enteroviruses were still detectable from clinical samples by PCR in 1997 suggesting that the figure for the CFS patients was genuine and that at this time, there was

no evidence of enteroviral sequences in these individuals. This demonstrates the variation present within a sample population and underscores the need to submit patients to multiple testing over time rather than to single point testing.

Interestingly, the greatest proportion of CFS patient PCR positives over this study period was recorded in the same year (1990) as a large outbreak of echovirus 4. Sixty-four percent of PCR-positive patients reported a date of onset of fatigue of 1990 following a flu-like illness, suggesting that these patients were suffering from a post-infectious fatigue syndrome. For one patient with chronic fatigue, symptoms of meningitis accompanied by isolation of echovirus 4 were followed by persistent lethargy. A serum sample obtained two months later tested positive by PCR. According to the Oxford criteria, a patient must have persisting fatigue as a new symptom for at least six months before a diagnosis of CFS can be made. In this case, the criteria could not be met at the time of sampling although the patient had severe chronic fatigue. For many of the patients in this initial cohort (Clements *et al.*, 1995), PCR positive samples were obtained within months of onset of fatigue and as such many would not fulfil the Oxford criteria as was originally stated: the mean duration of fatigue was approximately six months. However, it would seem that because the patients were tested shortly after a presumed viral episode and shortly after the onset of fatigue, the triggering factor may have been detected in a high proportion of cases. Additionally, a number of the enterovirus positives were identified after one round of PCR only (5/88 serum samples, 2/32 stool samples and 6/62 buffy coat samples) reflecting a higher level of enteroviral RNA in the serum. In contrast, the comparison group samples required nested PCR for detection. Unfortunately, apart from the one report of an isolate from a CFS patient mentioned above, no other enteroviruses were isolated from these patients to confirm that the echovirus 4 outbreak had caused an outbreak of post-infectious fatigue syndrome. Additionally, sequencing of PCR products and echovirus 4 isolates which might have confirmed the relationship was not performed at this time.

All subsequent samples from CFS patients in the follow-up study required nested PCR for detection, suggesting a low level of enteroviral RNA in these samples. These patients were tested on average, approximately 4 years after the onset of fatigue, a figure which has been noted elsewhere. The mean duration of illness at presentation from 15 studies was 37.1 months (Klonoff, 1992).

In summary, PCR testing of CFS patients over a number of years showed that two different populations of CFS patients were present. Patients tested in 1990 and 1991 presented within months of the onset of fatigue and appeared to form an outbreak of post-infectious fatigue syndrome. A high percentage of these patients were enterovirus PCR positive and the timing coincided with a large outbreak of echovirus 4. This prompted the prospective study of patients attending the infectious disease clinic, for the presence of enteroviral sequences. These further samples were obtained from patients who had been ill for an average of 3.9 years. The percentage of PCR positives each year (16% to 31%) was still greater than that detected in the comparison patients (1.6% to 8.6%) but the difference was not as high as that observed in 1990-1991. Sequence analysis of a number of PCR products derived from the 5' NTR confirmed that the sequences were enteroviral-like but was unable to determine the serotype involved. The highly conserved nature of the 5' NTR made it difficult to determine whether persistence of sequence had occurred (apart from 2 cases) in a group of 16 patients who provided 2 samples over time.

4.6. Further comparison of CFS sequences

One of the questions raised from the initial phylogenetic analysis was the possibility that the atypical sequences observed were in fact known enteroviruses for which there was no sequence data available. To address this, a number of enteroviral sequences derived from clinical specimens in the laboratory were sequenced. These included one echovirus 4 isolate from the 1990 outbreak, which was 82% similar to the published echovirus 4 sequence. Also at this time, a number of additional sequences were obtained from the GenBank database, which were not available at the time of the initial comparisons. The four CFS sequences from the initial phylogenetic analysis still grouped apart from the rest, forming a cluster on their own with differences of approximately 13% from the closest match of echovirus 6. These CFS sequences were obtained by manual sequencing and as such contained a number of N's representing bases where a single nucleotide could not be assigned at these positions. This may be why they group together, apart from the remaining sequences. The other CFS sequences were spread throughout the coxsackie/echovirus-like group. Two sequences, MCN94 and SE95, were greater than 95% similar to echovirus 19 and echovirus 7 respectively and could probably be typed as

such. Others were still 10-12% different which makes them difficult to type as one serotype or another. Sequences derived from samples obtained from three patients with a date of onset in 1990 were compared with the 1990 echovirus 4 sequence but similarities ranged from 78 to 88% only.

The variation present naturally within groups of clinical isolates representing epidemic and endemic types was also examined. Coxsackie B3, which was dominant in 1997, had not been isolated in Glasgow since 1991 and 63% of the isolates were from one geographical region. The isolates were obtained within a five month period and differed from each other by very little (3%) but differed from the published coxsackie B3 isolates by as much as 15%, which may have been why this type caused a small outbreak after being out of circulation for 6 years. Similarly, echovirus 6 isolates from 1994 were very similar to each other but were up to 20% different from the published sequences. Other multiple isolates (echovirus 30 and coxsackie B5) representing enteroviruses isolated almost every year, clustered according to the time of isolation and showed considerable variation over time. High sequence identity between echovirus 30 isolates and coxsackievirus B1 isolates obtained in the same year has been observed elsewhere with the observation that prototype strains were significantly different (Drebot *et al.*, 1994; Diedrich *et al.*, 1995).

Since isolates of the same serotype can vary by up to 20% in the 5' NTR it is very likely that the CFS 5' NTR sequences observed were not 'atypical', but were from variants of the known enteroviruses. However, without corroboration using sequence from other regions, for example the capsid region which provides a more accurate indication of serotype (Arola *et al.*, 1996) this could not be confirmed.

4.7. Obtaining additional sequence

4.7.1. Standard PCR of the capsid region

The 5' NTR sequence of hepatitis C virus is very highly conserved and in many cases, different subtypes are indistinguishable, although major genotypes are reliably identified (Simmonds *et al.*, 1994). As an alternative, sequence from at least two separate coding regions, for example the E1 and NS-5 regions can be examined, identifying each of the 11 or 12 known types or serotypes. With the enteroviruses, the capsid coding region is suitable for amplification being relatively conserved among the different types and also because neutralizing antigens are present in this region and form the basis for serotyping. Therefore sequence information from this region should be more useful for distinguishing the serotype of the viral sequences identified, than that from the 5' NTR.

Primers for a semi-nested PCR were designed to amplify a portion of the capsid region (nucleotides 581-1191). Primer 4+ was the reverse sequence of the first round primer used in the standard PCR in this study, which has been shown to amplify all the known enteroviruses and sequences from CFS patients. Primer A+ was the reverse of primer 3 described by Zoll *et al.*, (1991) which had 100% homology with all the sequenced enteroviruses. As with the 5' NTR a number of clinical isolates were available for sequencing and analysis of this region produced a dendrogram in which individual serotypes grouped together in general. The reaction was optimized with coxsackievirus A9, but these conditions did not produce any correctly sized amplification products from samples obtained from CFS patients, even though they were positive for the 5' NTR sequence. Either the PCR was not sensitive enough, being 100-fold less sensitive than the standard 5' NTR-PCR or the sequence of this region is so different that the primers will not bind and therefore no amplification occurs.

The failure to amplify this region could also be explained by a deletion in the target sequence. Defective-interfering (DI) particles are naturally occurring deletion mutants that have been recognized for almost all groups of infectious viruses (Kuge *et al.*, 1986). The generation of DI particles of poliovirus type 1 (Mahoney) has been reported. These

initiated normal poliovirus replication but were unable to synthesize capsid proteins (Cole *et al.*, 1971), suggesting that they lacked the region encoding these proteins. Additionally, almost all the deletions were shown to be located at bases 1300 to 3100, the region coding for the capsid proteins. A similar result was observed with Sabin type 1 poliovirus with deletions between positions 1307 and 2630 (Kajigaya *et al.*, 1985). Secondary structure was discovered to be common to all the deletion sites and it was proposed that a portion of the template looped out due to structural interactions and that the polymerase bypassed this portion, resulting in the synthesis of deleted daughter chains.

In this study, the VP4/VP2 region amplified by primers A+/B- is slightly upstream (ending at position 1200) of the deletion sites referred to above but it is still possible that the lack of amplification observed with the clinical samples is explained by a deletion in this region. This would result in the production of defective particles which, lacking part of the capsid might be unable to establish a productive infection. Only one study has in fact been successful in isolating virus from a number of patients with CFS (Yousef *et al.*, 1988).

4.7.2. Inverse PCR

If other regions of the genome are so variable or absent that it is difficult to design primers that will anneal, then alternative techniques for amplification needed to be employed. It is possible to take advantage of the known sequence to which the standard primers will bind and design an inverse PCR reaction. In this study, primers in an inverse orientation to those which could amplify the 5' NTR region were used to amplify the flanking regions of the known 264 base pair sequence.

Preparation of the template for inverse PCR was attempted with various combinations of heat and chemical inactivation of enzymes all of which involved a number of different stages. No part of the process will result in 100% recovery of the DNA sample and thus the greater the number of steps to the protocol, the less DNA will be available for the PCR process. With clinical material, the starting copy number is low and the chances of retaining enough to have sufficient template for the PCR process are poor. The inverse PCR procedure was not successful. Even with coxsackievirus A9 as the control, no bands

were produced which were suitable for sequencing. It was difficult to optimize the system because of the lack of a positive control for the PCR reaction and time constraints prevented this being pursued further. Only a limited amount of enterovirus positive clinical material was available and it was decided not to use it in this system.

4.7.3. Long PCR

The complete nucleotide sequence of only a small number of picornavirus genomes is known. These have generally been elucidated by traditional cloning strategies that are time consuming and usually do not produce cDNA clones of full length. For example, Kandolf and Hofschneider (1985) screened nearly 5000 cDNA clones before isolating a full-length coxsackie B3 clone. Improvements in the PCR process have permitted the synthesis of amplicons up to 40kb. This is generally facilitated by the combination of thermostable DNA polymerases with and without proofreading activity (3' to 5' exonuclease activity). The limiting factor in RT-PCR is the reverse-transcription stage: standard RT-enzymes generally do not produce full-length cDNAs. The use of a recombinant reverse transcriptase enzyme which has been engineered without RNase H activity, which would normally degrade template RNA, enables the synthesis of full-length product to a reasonable efficiency (Lindberg *et al.*, 1997). Using a combination of RT and polymerase enzymes, long PCR has been achieved for hepatitis genomes (Tellier *et al.*, 1996), coxsackie B2 (Gow *et al.*, 1996) and 14 prototype strains of group B coxsackieviruses and echoviruses (Lindberg *et al.*, 1997). Nested long PCR of the hepatitis B genome was sensitive to 10^2 $TCID_{50}$ for the first round and 10 $TCID_{50}$ for the second which corresponded to 100 genome copies (Tellier *et al.*, 1996).

In this study approximately 5kb of the standard coxsackievirus A9 genome was amplified using a nested PCR system. The sensitivity of the nested reaction however was low, with the standard not detected below 10^3 $TCID_{50}$ and again this was unsuitable for use with clinical samples which contain less than 1 $TCID_{50}$ (based on the standard 5' NTR PCR). The problem with optimizing long PCR and especially a nested reaction is that it is time consuming with 35 cycles taking approximately 6 hours. These problems may be overcome by the recent introduction of the lightcycler system (Idaho Technologies) for PCR which uses glass capillary tubes as the reaction vessels. This allows the processes of

heating and cooling to be extremely rapid, and 35 cycles of standard PCR can be completed within 15 minutes. This would allow optimization of PCR to be carried out more quickly and because the volumes used in each reaction are small (less than 10µl) the cost of such a procedure would be minimized. It may then be possible to optimize the reaction to a level comparable with that described for hepatitis B.

One interesting fact that emerged from this procedure was that 85% of enterovirus positive clinical samples (by standard PCR) could not be primed with a poly-T primer. The subsequent addition of poly-A residues by enzymatic methods enabled priming to occur suggesting that the poly-A tail was either reduced or absent from the RNA in these samples. Alternatively, the genome may be present as fragments only due to degradation. Another group (Gow *et al.*, 1997) could only amplify 7/23 samples from muscle biopsies using a poly-T primer. The lack of a poly-A tail has been shown to have an effect on the infectivity of the virus. Early studies had shown that adenine sequences shorter than 20 nucleotides reduced the infectivity of virion RNA 20-fold (Spector & Baltimore, 1974). This was confirmed by Sarnow (1989) who showed that poliovirus RNAs with long homopolymeric adenine sequences at the 3' end were more infective than those with short adenine sequences. It was suggested that these RNA molecules were unstable or poor templates for translation or initiation of minus strand RNA synthesis. This might go some way to explaining why there is a distinct lack of success in isolating virus from CFS patients and why in most cases, nested PCR is required for the detection of RNA indicating a low level of infection. Again, because of limited sample volume, the implications of this finding could not be pursued further.

4.7.4. Construction and screening of a cDNA library

One final approach to obtain full length sequence was the cloning of cDNA derived from a positive sample, into a vector, creating a cDNA library which could then be screened for the appropriate cDNA of interest. The vector system used combines the high efficiency of lambda library construction and convenience of a plasmid system with blue-white colour selection and can accommodate DNA inserts up to 10kb in length. This process relies on the generation of full-length cDNA by priming with a poly-T primer and having sufficient RNA (optimum 5 µg) as starting material. As has been stated earlier, nested PCR is required to obtain a signal from most CFS samples and a number of samples could not be

primed by the poly-T primer. It was therefore difficult obtaining a sample that had sufficient volume to maximize the amount of RNA available that was also poly-A (+). If the samples had been received shortly after the onset of fatigue rather than after at least 6 months when CFS has been formally diagnosed, this might have improved the chances of picking up the virus at a higher level. As has been mentioned earlier, samples obtained in 1990 were received 2 to 6 months after the onset of illness and a number were positive after 35 cycles of amplification and did not require nested PCR for detection, reflecting a higher level of RNA in the sample. Subsequent samples which were in general obtained approximately 4 years after onset all required nested PCR for detection.

The cloning procedure involves the incorporation of ^{32}P at an early stage which means that subsequent stages must be carried out behind a protective screen and dedicated centrifuges and equipment must be used for safety reasons. The column used for size fractionation of the cDNA had to be assembled from various components and the column loaded with gel filtration medium. This was not easy to do and was very time consuming (previous versions of this kit provided a fractionation column). Again, working with the column behind a protective screen was restricting. After packaging of the cDNA/vector construct the number of recombinant plaques containing the insert should have been 10 to 100-fold above the background plaques, without the insert, but in this case the ratio was only approximately 2:1. Thus the recombinants were only present in 50% of plaques and the insert of interest was only present in very small quantities to begin with, making it very difficult to detect. The procedure was indeed completed without the identification of a positive plaque and screening the library with control PCR product representing the human ableson thymidine kinase mRNA produced no positives. This suggested that the library did not contain sufficient cDNA inserts at the beginning due to the low quantities of RNA present in the original sample. Thus this method was not sensitive enough to pick up the cDNA of interest.

Techniques such as subtractive hybridization can be used to enrich for the RNA species of interest (Diatchenko *et al.*, 1996). In theory, two populations of similar mRNA are used, with one containing RNA not expressed in the other (for example enterovirus positive RNA from a CFS patient and enterovirus negative RNA from a control patient). Both RNA species are converted into cDNA and then hybridized together. The hybrid sequences are then removed thus leaving behind cDNAs that are not hybridized and are

present in one sample only, which should be the enterovirus RNA (presuming the control RNA is negative for other viruses also). The population of mRNA is thus enriched for the enterovirus RNA and this might just provide enough starting material for the cloning techniques to work. This is a technique which could be tried in the future.

4.8. Conclusions

From the existing literature, what is clear about the illness described as Chronic Fatigue Syndrome (CFS) is that no one common factor can be considered to be the cause of all cases. Various aetiologies have been suggested but no consensus has emerged. It has proven difficult to design studies to address the problem because of the nature of the illness. Only studies set up with appropriate controls and employing recognized testing methods can be considered as valid and providing reliable results. This illness is almost certainly multifactorial in origin and as such different groups of patients examined at different times and locations, are likely to produce different results. Instead of dismissing data that is not confirmed by others, results should be viewed as pertinent data for the patients of study. For example, the conflicting evidence for the association of various viruses in a significant number of patients may be a result of epidemiological differences. Borna disease virus has been associated with CFS in Japan but not in the USA or UK while enteroviruses have been associated with CFS in the UK but not elsewhere.

Previous data from the UK had shown a significant association between enteroviruses and CFS based on serological and nucleic acid hybridization studies. Detection of enteroviral RNA in muscle biopsies also confirmed this association. A number of investigators had shown that the polymerase chain reaction was more sensitive than isolation and hybridization techniques for the detection of enteroviruses from clinical samples. This technique was optimized in this laboratory and a preliminary study on samples collected during 1990 and 1991 indicated that a significant proportion of patients with CFS were positive by PCR for enteroviral sequences compared with comparison patients. PCR was thus shown to be an appropriate technique for the detection of enterovirus in patients with CFS. Further investigation suggested that the trigger for CFS in this cohort may have been echovirus type 4, which was predominant in Glasgow that year and was responsible for a large outbreak of aseptic meningitis. This was true for one patient, with a confirmed echovirus 4 isolate followed by a persistent fatigue and the detection of enteroviral sequences in the serum approximately 2 months later. As such, this patient and a number of others (with a mean duration of fatigue of six months) did not fulfil the Oxford criteria of a minimum of six months fatigue for a formal diagnosis of CFS. This may be the reason why such a high proportion were enteroviral PCR positive, since they were tested

shortly after onset following an episode of flu-like illness. Additionally, a number of samples required only one round of PCR before enteroviral sequences were detected, which implies a greater quantity of viral sequences present in the original sample than those samples that required nested PCR for detection. With no sequence data from the PCR positives collected at this time or the echovirus 4 isolates it has not been possible to confirm that the echovirus 4 was the triggering agent in these cases.

Subsequent testing of new patients with CFS from 1992 onwards showed that enteroviruses were still detected by nested PCR in higher numbers (16-31%) compared to comparison samples (1.6-8.6%) although the figures were reduced. The majority of patients included in the follow-up study were on average tested 3.9 years after the onset of fatigue and if the virus was involved as a trigger via a 'hit and run' mechanism it would most likely have been cleared long before any testing was carried out. In 1997 there were no new PCR positive samples detected among the CFS patients. This was not the result of any changes in testing procedure since enteroviral PCR positive samples were still being detected by PCR in a parallel study on juvenile-onset type 1 diabetes mellitus (Nairn *et al.*, 1999). Additionally, results from the routine virological service in the laboratory showed that 6.5% of clinical samples were positive for enteroviral PCR.

Initial sequencing of 20 new enteroviral PCR positives demonstrated the presence of 'atypical' enteroviral sequences that on phylogenetic analysis grouped apart from the known enteroviral sequences and the non-CFS comparison sequences. This could be because the sequences represented 'novel' enteroviruses, or known enteroviruses for which no sequence data was available or known enteroviruses with abnormal 5' NTRs. The latter has implications for the functioning of the virus since this region contains the internal ribosome entry site (IRES). This area of highly structured RNA is important for the replication and translation functions of the genome and alterations can lead to reduced efficiency of both. 'Atypical' 5' NTR sequence has also been reported elsewhere (Han *et al.*, 1998).

Further sequence analysis of the 5' NTR of a number of clinical enterovirus isolates was carried out to examine the natural variation between isolates of the same serotype. The results showed that sequences from isolates of the same serotype collected within a short time span of each other were very closely related, whereas sequences from serotypes

isolated years apart differed greatly. The variation observed was of the same order as that between the CFS 'atypical' sequences and the known enteroviruses at the time. Therefore, this suggests that the 'atypical' sequences are probably known enteroviruses with variable 5' NTRs, and this is probably true for the sequences identified by Han *et al.* (1998). The 5' NTR is therefore not a suitable region for determining the type of the virus present because of the high degree of conservation.

Sequencing of other regions of the genome, especially those coding for the capsid proteins would give an indication of serotype. Primers were therefore designed to anneal to part of the VP4/VP2 region and could amplify the majority of the enterovirus types. Sequencing of clinical isolates and subsequent phylogenetic analysis showed that the different serotypes could be distinguished in this region. However, none of the samples that were previously positive using the standard 5' NTR primers were positive for the VP4/VP2 region. This could either have been due to a lack of sensitivity of this PCR (100-fold less sensitive than the standard), a high variability of sequence in this region that prevented the primers from annealing or a deletion in this region of the genome. Deletions downstream of the region amplified here have been demonstrated in defective-interfering particles which have been recognized for a number of virus types and have been shown to play a role in the establishment of persistent infection in tissue culture and *in vivo* (Holland & Villareal, 1975: Holland *et al.*, 1979; Holland *et al.*, 1980).

The use of other techniques including inverse and long PCR and the construction of a cDNA library all failed to elucidate any further sequence information from any of the CFS patients. It was thought that this was the result of sensitivity problems. The samples detected by one round of PCR only, from the 1990-1991 cohort may have provided better starting material for the less sensitive PCR of the other regions of the genome and cloning techniques which are also less sensitive than nested PCR. However, these results could also be due to the sequence being very different from the conventional viruses in the other regions studied. Indeed, the interesting discovery that a number of enteroviral RNA sequences had little or no poly-A tail at their 3' end points to some differences between these sequences and conventional enterovirus isolates. The inability to prime from the 3' end has also been reported by another group (Gow, 1997) and the lack of poly-A tail has been shown to relate to the infectivity of the virus. This may be the reason why the RNA is present at low levels in the serum, requiring nested PCR for detection and why isolation

of enteroviruses has been generally unsuccessful. Again, with very little sample volume available for these cases, this finding and its implications could not be pursued.

The idea of enteroviral persistence was explored in this thesis by identifying patients with more than one enterovirus PCR positive sample over time. Initial sequence analysis of eight pairs of sequences suggested that 4/8 had evidence for persistence based on a high level of sequence identity and the presence of unique shared features which were not observed in a consensus sequence. However, subsequently a further eight pairs of sequences were included in the analysis and this highlighted identities between samples obtained in the same year. These identities were in most cases greater than those observed between consecutive pairs, except for two patients whose sequences maintained their unique shared bases that were not observed in any other sequence. From this data, persistence was not evident in the majority of paired samples, but the 5' NTR was not an ideal region to explore this idea, being very highly conserved.
Comparison of clinical data derived from a patient questionnaire with enterovirus PCR results did however indicate that persistence did not contribute to the maintenance of CFS in the majority of patients, although data from the first questionnaire did support a relationship between enterovirus positivity and severity of symptoms as measured by ability to work and time spent walking etc.

Thus, the study of enteroviruses in a number of patients with chronic fatigue syndrome has proven difficult, mainly because of sensitivity problems and the small volumes of sample available for study. The results do not rule out a role for the enteroviruses in CFS, but the methods employed to study the enteroviruses and their nature did not provide a definitive answer. It has been shown that results of PCR testing for enteroviruses can vary over the years and therefore it is important that studies test patients more than once to gain more than a snapshot view of what is occurring. Testing patients shortly after the onset of fatigue may prove more fruitful in detecting potential triggering agents, even though the patients have not been formally diagnosed by this time. Additionally, patients may be able to recall more clearly their health etc. prior to the onset of fatigue. It is also important to treat every case of CFS as an individual illness, since the aetiology may involve a combination of environmental, psychological and biological factors. The heterogeneity of the patient group probably contributes to the great variety of data observed in this interesting syndrome.

Should the investigation of enteroviruses and CFS be pursued? Those individuals with a recent history of onset of symptoms following a flu-like illness would be worth investigating further to determine whether enteroviral sequences were present, whether there was evidence of active replication (by looking for the presence of the negative sense strand of the viral genome for example) and attempting to isolate the virus from stool samples after acid-culture since this has proved successful recently. Additionally, the analysis of the amounts of positive and negative strands could be carried out to attempt to repeat the work of Cunningham *et al.*, (1990) who concluded that the equal amounts of positive and negative strand were the result of a persistent infection. These individuals could also be monitored at more frequent intervals and clinical information obtained by interview rather than by questionnaire. For those cases, who have been ill for some time, it is difficult to determine (without a pre-fatigue sample) whether a viral sequence detected a number of years after onset has any bearing on the initiation of the syndrome.

Anderson, J.A., Chai, H. & Claman, H.N. (eds.) (1986). Candidiasis hypersensitivity syndrome. *Journal of Allergy and Clinical Immunology*, **78(2)**, 271-273.

Andino, R., Rieckhof, G.E. & Baltimore, D. (1990). A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA. *Cell*, **63**, 369-380.

Andino, R., Rieckhof, G.E., Achahoso, P.L. & Baltimore, D. (1993). Poliovirus RNA synthesis utilizes an RNP complex formed around the 5'-end of viral RNA. *EMBO Journal*, **12**, 3587-3598.

Archard, L.C., Bowles, N.E., Behan, P.O., Bell, E.J. & Doyle, D. (1988). Postviral fatigue syndrome: persistence of enterovirus RNA in muscle and elevated creatine kinase. *Journal of the Royal Society of Medicine*, **81**, 326-329.

Arola, A., Santti, J., Ruuskanen, O., Halonen, P. & Hyypia, T. (1996). Identification of enteroviruses in clinical specimens by competitive PCR followed by genetic typing using sequence analysis. *Journal of Clinical Microbiology*, **34(2),** 313-318.

Auvinen, P. & Hyypia, T. (1990). Echoviruses include genetically distinct serotypes. *Journal of General Virology*, **71**, 2133-2139.

Baltimore, D. (1971). Expression of animal virus genomes. *Bacteriological Reviews*, **35(3),** 235-241.

Barnes, D.M. (1986). Mystery disease at Lake Tahoe challenges virologists and clinicians. *Science*, **234**, 541-542.

Behan, P.O., Behan, W.M.H. & Bell, E.J. (1985). The postviral fatigue syndrome - an analysis of the findings in 50 cases. *Journal of Infection*, **10**, 211-222.

Behan, W., More, I. & Behan, P. (1991). Mitochondrial abnormalities in the post-viral fatigue syndrome. *Acta Neuropathologica*, **83**, 61-65.

Behan, W.M.H., Gow, J.W., Simpson, K. & Behan, P.O. (1996). Search for picornaviruses at onset of inflammatory myopathy. *Journal of Clinical Pathology*, **49**, 592-594.

Bell, E.J., Irvine, K.G., Gardiner, A.J.S. & Rodger, J.C. (1983). Coxsackie B infection in a general medical unit. *Scottish Medical Journal*, **28(2),** 157-159.

Bell, E.J. & McCartney, R.A. (1984). A study of coxsackie B virus infections, 1972-1983. *Journal of Hygiene*, **93**, 197-203.

Bell, E.J., McCartney, R.A. & Riding, M.H. (1988). Coxsackie B viruses and myalgic encephalomyelitis. *Journal of the Royal Society of Medicine*, **81**, 329-331.

Belsham, E.J. & Sonenberg, N. (1996). RNA-protein interactions in regulation of Picornavirus RNA translation. *Microbiological Reviews*, **60(3),** 499-511.

Bergelson, J.M., Shepley, M.P., Chan, B.M.C., Hemler, M.E. & Finberg, R.W. (1992). Identification of the integrin VLA-2 as a receptor for echovirus 1. *Science*, **255**, 1718-1720.

Bergelson, J.M., St John, N., Kawaguchi, S., Chan, M., Stubdal, H., Modlin, J. & Finberg, R.W. (1993). Infection by echoviruses 1 and 8 depends on the alpha 2 subunit of VLA-2. *Journal of Virology*, **67(11)**, 6847-6852.

Bergelson, J.M., Chan, M., Solomon, K.R., St. John, N.F., Lin, H. & Finberg, R.W. (1994). Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. *Proceedings of the National Academy of Sciences*, **91**, 6245-6248.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L. & Finberg, R.W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science*, **275**, 1320-1323.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P.W. & Baxt, B. (1995). Antibodies to the vitronectin receptor (integrin alpha(v) beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. *Journal of Virology*, **69(4)**, 2664-2666.

Bland, J. & Clements, J. (1998). Protecting the world's children: the story of WHO's immunization programme. *World Health Forum*, **19,** 162-173.

Bonner, D., Ron, M., Chalder, T., Butler, S. & Wessely, S. (1994). Chronic fatigue syndrome: a follow-up study. *Journal of Neurology, Neurosurgery & Psychiatry*, **57**, 617-621.

Borysiewicz, L.K., Haworth, S.J., Mundlin, J., Rickinson, A. & Sissons, J.G.P. (1986). Epstein Barr virus-specific immune defects in patients with persistent symptoms following infectious mononucleosis. *Quarterly Journal of Medixxx*, **58**, 111-121.

Bowles, N.E., Richardson, P.J., Olsen, E.G.J. & Archard, L.C. (1986). Detection of coxsackie-B-virus specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. *Lancet*, **i**, 1120-1123.

Bowles, N.E., Rose, M.L., Taylor, P., Banner, N.R., Morgan-Capner, P., Cunningham, L., Archard, L.C. & Yacoub, M.H. (1989). End-stage dilated cardiomyopathy: persistence of enteroviral RNA in myocardium at cardiac transplantation and lack of immune response. *Circulation*, **80**, 1128-1136.

Bowles, N.E. & Archard, L.C. (1990). Detection of enterovirus-specific RNA by the use of hybridisation probes. *Reviews in Medical Microbiology*, **1**, 175-184.

Bowles, N.E., Bayston, T.A., Zhang, H.-Y., Doyle, D., Lane, R.J.M., Cunningham, L. & Archard, L.C. (1993). Persistence of enterovirus RNA in muscle biopsy samples suggests that some cases of chronic fatigue syndrome result from a previous, inflammatory viral myopathy. *Journal of Medicine*, **24**, 145-160.

Brown, F., Talbot, P. & Burrows, R. (1973). Antigenic differences between isolates of Swine Vesicular Disease Virus and their relationship to coxsackie B5 virus. *Nature*, **245**, 315-316.

Buchwald, D., Sullivan, J.L. & Komaroff, A.L. (1987). Frequency of 'chronic active

Epstein-Barr virus infection' in a general medical practice. *Journal of the American Medical Association*, **257**, 2303-2307.

Buchwald, D., Cheney, P.R., Peterson, D.L., Henry, B., Wormsley, S.B., Geiger, A., Ablashi, D.V., Salahuddin, S.Z., Saxinger, C., Biddle, R., Kikinis, R., Jolesz, F.A., Folks, T., Balachandran, N., Peter, J.B., Gallo, R.C. & Komaroff, A.L. (1992). A chronic illness characterised by fatigue, neurologic and immunologic disorders, and active human herpesvirus type 6 infection. *Annals of Internal Medicine*, **116**, 103-113.

Buchwald, D. & Garrity, D. (1994). Comparison of patients with chronic fatigue syndrome, fibromyalgia and multiple chemical sensitivities. *Archives of Internal Medicine*, **154**, 2049-2053.

Calder, B.D. & Warnock, P.J. (1984). Coxsackie B infection in a Scottish general practice. *Journal of the Royal College of Practitioners*, **34**, 15-19.

Calder, B.D., Warnock, P.J., McCartney, R.A. & Bell, E.J. (1987). Coxsackie B viruses and the post-viral syndrome: a prospective study in general practice. *Journal of the Royal College of General Practitioners*, **37**, 11-14.

Caligiuri, M., Murray, C., Buchwald, D., Levine, H., Cheney, P., Peterson, D., Komaroff, A.L. & Ritz, J. (1987). Phenotypic and functional deficiency of natural killer cells in patients with CFS. *Journal of Immunology*, **139(10)**, 3306-3313.

Chapman, N.M., Tracy, S., Gauntt, C.J. & Fortmueller, U. (1990). Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridisation. *Journal of Clinical Microbiology*, **28(5)**, 843-850.

Chonmaitree, T., Menegus, M.A. & Powell, K.R. (1982). The clinical relevance of CSF viral culture. A 2-year experience with aseptic meningitis in Rochester, New York. *Journal of the American Medical Association*, **247**, 1843-1847.

Chow, M., Newman, J.F.E., Filman, D., Hogle, J.M., Rowlands, D.J. & Brown, F. (1987). Myristylation of picornavirus capsid protein VP4 and its structural significance. *Nature*,

327, 482-486.

Clements, G.B., McGarry, F., Nairn, C. & Galbraith, D.N. (1995). Detection of enterovirus-specific RNA in serum: the relationship to chronic fatigue. *Journal of Medical Virology*, **45**, 156-161.

Cohen, S & Williamson, G.M. (1991). Stress and infectious disease. *Psychological Bulletin*, **109(1)**, 5-24.

Cole, C.N., Smoler, D., Wimmer, E. & Baltimore, D. (1971). Defective-interfering particles of poliovirus. I. Isolation and physical properties. Journal of Virology, 7(4), 478-485.

Collier, L. (1998). A short history of research on viruses. In *Topley and Wilsons Microbiology and Microbial Infections (9th edition)*, ed. Collier, L., Balows, A. & Sussman, M. Vol. 4. Ch.1. London: Arnold.

Colonno, R.J. (1987). Cell surface receptors for Picornaviruses. *BioEssays*, **5(6)**, 270-274.

Conaldi, P.G., Serra, C., Mossa, A., Falcone, V., Basolo, F., Camussi, G., Dolei, A. & Toniolo, A. (1997). Persistent infection of human vascular endothelial cells by group B coxsackieviruses. *The Journal of Infectious Diseases*, **175**, 693-696.

Cova, L., Kopecka, H., Aymard, M. & Girard, M. (1988). Use of cRNA probes for the detection of enteroviruses by molecular hybridisation. *Journal of Medical Virology*, **24**, 11-18.

Cunningham, L., Bowles, N.E., Lane, R.J.M., Dubowitz, V. & Archard, L. (1990). Persistence of enteroviral RNA in chronic fatigue syndrome is associated with the abnormal production of equal amounts of positive and negative strands of enteroviral RNA. *Journal of General Virology*, **71**, 1399-1402.

Cunningham, L., Bowles, N.E. & Archard, L.C. (1991). Persistent virus infection of muscle in postviral fatigue syndrome. *British Medical Bulletin*, **47(4)**, 852-871.

Dahllund, L., Nissinen, L., Pulli, T., Hyttinen, V.-P., Stanway, G. & Hyypia, T. (1995). The genome of echovirus 11. *Virus Research*, **35**, 215-222.

Daniel, W.W. (1983). *Biostatistics: A foundation for analysis in the health sciences (Third edition)*. USA: John Wiley and Sons, Inc.

DeFreitas, E., Hilliard, B., Cheney, P.R., Bell, D.S., Kiggundu, E., Sankey, D., Wroblewska, Z., Palladino, M., Woodward, J.P. & Koprowski, H. (1991). Retroviral sequences related to human T-lymphotropic virus type II in patients with chronic fatigue immune dysfunction syndrome. *Proceedings of the National Academy of Sciences*, **88**, 2922-2926.

Demitrack, M.A., Dale, J.K., Straus, S.E., Lane, L., Listwak, S.J., Kruesi, M.J., Chrousos, G.P. & Gold, P.W. (1991). Evidence for impaired activation of the hypothalamic-pituitary-adrenal axis in patients with chronic fatigue syndrome. *Journal of Clinical Endocrinology and Metabolism*, **73(6)**, 1224-1234.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research*, **12**, 387-395.

Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D. & Siebert, P.D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences*, **93(12)**, 6025-6030.

Diedrich, S., Driesel, G. & Schreier, E. (1995). Sequence comparison of echovirus type 30 isolates to other enteroviruses in the 5' noncoding region. *Journal of Medical Virology*, **46**, 148-152.

Domingo, E., Martinez-Sala, E., Sobrino, F., de la Torre, J.-C., Portela, A., Ortin, J., Lopez-Gallindez, C., Perez-Brena, P., Villanueva, N., Najera, R., VandePol, S., Steinhauer, D., DePolo, N. & Holland, J. (1985). The quasispecies (extremely heterogenous) nature of

viral RNA genome populations: biological relevance- a review. *Gene*, **40**, 1-8.

Drebot, M.A., Nguan, C.Y., Campbell, J.J., Lee, S.H.S. & Forward, K.R. (1994). Molecular epidemiology of enterovirus outbreaks in Canada during 1991-1992: identification of echovirus 30 and coxsackievirus B1 strains by amplicon sequencing. *Journal of Medical Virology*, **44**, 340-347.

Dubois, R.E., Seeley, J.K., Brus, I., Sakamoto, K., Ballow, M., Harada, S., Bechtold, T.A., Pearson, G., & Purtilo, D.T. (1984). Chronic mononucleosis syndrome. *Southern Medical Journal*, **77(11),** 1376-1382.

Enders, J.F., Weller, T.H. & Robbins, F.C. (1949). Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. *Science*, **109**, 85-87.

Erlich, H.A., Gelfand, D.H. & Saiki, R.K. (1988). Specific DNA amplification. *Nature*, **331**, 461-462.

Erlich, H.A., Gelfand, D. & Sninsky, J.J. (1991). Recent advances in the polymerase chain reaction. *Science*, **252**, 1643-1651.

Evans, A.C. (1947). Brucellosis in the United States. *American Journal of Public Health*, **37,** 139-151.

Evans, D.M.A., Dunn, G., Minor, P.D., Schild, G.S., Cann, A.J., Stanway, G., Almond, J.W., Currey, K. & Maizel, J.V. (Jr.) (1985). A single nucleotide change in the 5' non-coding region of the genome of the Sabin type 3 poliovaccine is associated with increased neurovirulence. *Nature*, **314,** 548-550.

Fegan, K.G., Behan, P.O. & Bell, E.J. (1983). Myalgic encephalomyelitis - report of an epidemic. *Journal of the Royal College of General Practitioners*, **33**, 335 - 337.

Felsenstein, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics*, **22**, 521-565.

Fukuda, K., Straus, S.E., Hickie, I., Sharpe, M.C., Dobbins, J.G., Komaroff, A. & the International Chronic Fatigue Syndrome Study Group. (1994). The Chronic Fatigue Syndrome: a comprehensive approach to its definition and study. *Annals of Internal Medicine*, **121**, 953-959.

Galbraith, D.N., Nairn, C. & Clements, G.B. (1995). Phylogenetic analysis of short enteroviral sequences from patients with chronic fatigue syndrome. *Journal of General Virology*, **76**, 1701-1707.

Galbraith, D.N., Nairn, C. & Clements, G.B. (1997). Evidence for enteroviral persistence in humans. *Journal of General Virology*, **78**, 307-312.

Gallacher, K., Ghosh, K., Patel, A. & Walker, E. (1993). An outbreak of echovirus type 4 infections and its implications for diagnosis and management in general practice. *Journal of Infection*, **26**, 321-324.

Gibson, J.P. & Righthand, V.F. (1985). Persistence of echovirus 6 in cloned human cells. *Journal of Virology*, **54(1)**, 219-223.

Gilliam, A.G. (1938). Epidemiological study of an epidemic diagnosed as poliomyelitis occurring among the personnel of the Los Angeles County General Hospital during the summer of 1934. *US Public Health Bulletin* 1938, **40**, 1-90.

Gilliland, G., Perrin, S. & Bunn, H.F. (1990). Competitive PCR for quantitation of mRNA. *In PCR protocols: A guide to methods and applications*, ed. Innis, M.A., Gelfland, D.H., Sninsky, J.J. & White, T.J. Ch. 8. San Diego: Academic Press Inc.

Gjoen, K.V. & Bruu, A.-L. (1997). Specific detection of coxsackie viruses A by the polymerase chain reaction. *Clinical and Diagnostic Virology*, **8**, 183-188.

Godman, G.C., Bunting, H. & Melnick, J.L. (1952). The histopathology of coxsackievirus infection in mice. I. Morphologic observations with 4 different viral types. *American Journal of Pathology*, **28**, 223-257.

Gold, D., Bowden, R., Sixbey, J., Riggs, R., Katon, W.J., Asheley, R., Obrigewitch, R. & Corey, L. (1990). Chronic Fatigue. A prospective clinical and virologic study. *Journal of the American Medical Association*, **264**, 48-53.

Gow, J.W., Behan, W.M.H., Clements, G.B., Woodall, C., Riding, M. & Behan P.O. (1991). Enteroviral RNA sequences detected by polymerase chain reaction in muscle of patients with postviral fatigue syndrome. *BMJ*, **302**, 692-696.

Gow, J.W., Simpson, K., Schliephake, A., Behan, W.M.H., Morrison, L.J.A., Cavanagh, H., Rethwilm, A. & Behan, P.O. (1992). Search for retrovirus in the chronic fatigue syndrome. *Journal of Clinical Pathology*, **45**, 1058-1061.

Gow, J.W., Behan, W.M.H., Simpson, K., McGarry, F., Keir, S. & Behan, P.O. (1994). Studies on enterovirus in patients with chronic fatigue syndrome. *Clinical Infectious Diseases*, **18(Suppl 1)**, S126-129.

Gow, J.W., McGill, M., Behan, W.M.H. & Behan, P.O. (1996). Long RT-PCR amplification of full-length enterovirus genome. *Biotechniques*, **20**, 582-584.

Gow, J.W., de la Torre, J.C., Behan, W.M.H., Simpson, K., McGill, M., Dinan, T. & Behan, P.O. (1997a). Borna disease virus in Chronic Fatigue Syndrome. *Neurological Infections and Epidemiology*, **2**, 63-66.

Gow, J.W., Behan, W.M.H., Cash, P., Simpson, K. & Behan, P.O. (1997b). Genomic and template RNA transcription in a model of persistent enteroviral infection. *Journal of NeuroVirology*, **3**, 76-82.

Gow, J.W. (1997). Characterisation of persistent enterovirus infection in chronic fatigue syndrome. *Melvin Ramsay Society: 7th Annual Scientific and Medical Symposium.* Poster presentation.

Grandien, M., Forsgren, M. & Ehrnst, A. (1989). Enteroviruses and Reoviruses. *In Diagnostic procedures for Viral, Rickettsial and Chlamydial infections (Sixth edition),* ed. Schmidt, J.N. & Emmans, R.W. Ch. 16. Washington D.C.: American Public Health

Association Inc.

Graves, J.H. (1973). Serological relationships of swine vesicular disease virus and coxsackie B5 virus. *Nature*, **245**, 314-315.

Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E. & McClelland, A. (1989). The major human rhinovirus receptor is ICAM-1. *Cell*, **56(5)**, 839-847.

Grist, N.R. & Bell, E.J. (1974). A six year study of coxsackie virus B infections in heart disease. *Journal of Hygiene*, **73**, 165-172.

Grist, N.R., Ross, C.A. & Bell, E.J. (1974). *Diagnostic Methods in Clinical Virology (Second Edition)*. Oxford: Blackwell Scientific.

Grist, N.R., Bell, E.J. & Assaad, F. (1978). Enteroviruses in human disease. In *Progress in Medical Virology*, pp114-157. Karger: Basel.

Grist, N.R., Bell, E.J., Follet, E.A.C. & Urquhart, J.E.D. (1979). *Diagnostic Methods in Clinical Virology (Third Edition)*. Oxford: Blackwell Scientific.

Gupta, S., Aggarwal, S., See, D. & Starr, A. (1997). Cytokine production by adherent and non-adherent mononuclear cells in chronic fatigue syndrome. *Journal of Psychiatric Research*, **31(1)**, 149-156.

Halpin, D. & Wessely, S. (1989). VP-1 antigen in chronic postviral fatigue syndrome. *Lancet*, **1**, 1028-1029.

Han, S., Clements, G.B., Nairn, C., Westmoreland, D., Llewelyn, M.B. & Fox, J.D. (1998). Molecular-based techniques for detection and analysis of enteroviruses in stool and serum samples. *European Society for Clinical Virology*, Hamburg, poster.

Harber, J. & Wimmer, E. (1993). Aspects of the molecular biology of Picornaviruses. In *Regulation of gene expression in Animal Viruses*, ed. Carrasco, L., Sonenberg, N. &

Wimmer, E. New York: Plenum Press.

Hellinger, W.C., Smith, T.F., Van Scoy, R.E., Spitzer, P.G., Forgacs, P. & Edson, R.S. (1988). CFS and the diagnostic utility of antibody to Epstein-Barr virus early antigen. *Journal of the American Medical Association*, **260**, 971-973.

Hofer, F., Gruenberger, M., Kowalski, H., Machat, H., Huettinger, M., Kuechler, E. & Blaas, D. (1994). Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proceedings of the National Academy of Sciences*, **91**, 1839-1842.

Holland, J.J. & Villareal, L.P. (1975). Purification of defective interfering T particles of vesicular stomatitis and rabies viruses generated *in vivo* in the brains of newborn mice. *Virology*, **67**, 438-449.

Holland, J.J., Grabau, E., Jones, C.L. & Semler, B.L. (1979). Evolution of multiple genome mutations during long term persistent infection with vesicular stomatitis virus. *Cell*, **16**, 495-504.

Holland, J.J., Kennedy, S.I., Semler, B.L., Jones, L., Roux, L. & Grabau, E. (1980). Defective interfering RNA viruses and the host cell response. In *Comprehensive Virology*, ed. Fraenkel-Conrat, H. & Wagner, R.R. Vol. 16. New York: Plenum press.

Holland, J.J., de la Torre, J.C. & Steinhauer, D.A. (1992). RNA virus populations as quasispecies. *Current Topics in Microbiology and Immunology*, **176**, 1-20.

Holmes, G.P., Kaplan, J.E., Stewart, J.A., Hunt, B., Pinsky, P.F. & Schonberger, L.B. (1987). A cluster of patients with a chronic mononucleosis-like syndrome: is Epstein-Barr virus the cause? *Journal of the American Medical Association*, **257**, 2297-2302.

Holmes, G.P., Kaplan, J.E., Gantz, N.M., Komaroff, A.L., Schonberger, L.B., Straus, S.E., Jones, J.F., Dubois, R.E., Cunningham-Rundles, C., Pahwa, S., Tosato, G., Zegans, L.S., Purtilo, D.T., Brown, N., Schooley, R.T. & Brus, I. (1988). Chronic fatigue syndrome: a working case definition. *Annals of Internal Medicine*, **108**, 387-389.

Horwitz, C.A., Henle, W., Henle, G., Rudnick, H. & Latts, E. (1985). Long-term serological follow-up of patients for Epstein-Barr virus after recovery from infectious mononucleosis. *Journal of Infectious Diseases*, **151,** 1150-1153.

Huber, S.A. (1994). VCAM-1 is a receptor for encephalomyocarditis virus on murine vascular endothelial cells. *Journal of Virology*, **68(6),** 3453-3458.

Huttunen, P., Santti, J., Pulli, T. & Hyypia, T. (1996). The major echovirus group is genetically coherent and related to coxsackie B viruses. *Journal of General Virology*, **77,** 715-725.

Hyypia, T., Stalhandske, P., Vainionpaa, R. & Pettersson, U. (1984). Detection of enteroviruses by spot hybridisation. *Journal of Clinical Microbiology*, **19(3)**, 436-438.

Hyypia, T., Horsnell, C., Maaronen, M., Khan, M., Kalkkinen, N., Auvinen, P., Kinnunen, L. & Stanway, G. (1992). A distinct picornavirus group identified by sequence analysis. *Proceedings of the National Academy of Sciences*, **89**, 8847-8851.

Hyypia, T., Hovi, T., Knowles, N.J. & Stanway, G. (1997). Classification of enteroviruses based on molecular and biological properties. *Journal of General Virology*, **78,** 1-11.

Jarvis, W.R. & Tucker, G. (1981). Echovirus type 7 meningitis in young children. *American Journal of Diseases of Children*, **135**, 1009-1012.

Jin-Murphy, M. (1973). Acute haemorrhagic conjunctivitis. *Lancet*, **1**, 545-546.

Joce, R., Wood, D., Brown, D. & Begg, N. (1992). Paralytic Poliomyelitis in England and Wales, 1985-1991. *BMJ*, **305,** 79-82.

Jones, J.F., Ray, C.G., Minnich, L.L., Hicks, M.J., Kibler, R. & Lucas, D.O. (1985). Evidence for Epstein-Barr virus infection in patients with persistent unexplained illness: elevated anti-early antigen antibodies. *Annals of Internal Medicine*, **102,** 1-7.

Kajigaya, S., Arakawa, H., Kuge, S., Koi, T., Imura, N. & Nomoto, A. (1985). Isolation and characterization of defective-interfering particles of poliovirus Sabin 1 strain. *Virology*, **142(2),** 307-316.

Kammerer, U., Kunkel, B. & Korn, K. (1994). Nested PCR for specific detection and rapid identification of human picornaviruses. *Journal of Clinical Microbiology*, **32(2)**, 285-291.

Kandolf, R. & Hofschneider, P.H. (1985). Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. *Proceedings of the National Academy of Sciences*, **82(14),** 4818-4822.

Kandolf, R. & Hofschneider, P.H. (1989). Viral Heart Disease. *Springer Seminars in Immunopathology*, **11**, 1-13.

Kandolf, R., Ameis, D., Kirschner, P., Canu, A. & Hofschneider, P.H. (1987). In situ detection of enteroviral genomes in myocardial cells by nucleic acid hybridisation: an approach to the diagnosis of viral heart disease. *Proceedings of the National Academy of Sciences*, **84**, 6272-6276.

Karber, G. (1931). Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. *Archives of Experimental and Pathological Pharmacology*, **162**, 480-483.

Karnauchow, T.M., Tolson, D.L., Harrison, B.A., Altman, E., Lublin, D.M. & Dimock, K. (1996). The HeLa cell receptor for enterovirus 70 is decay-accelerating factor (CD55). *Journal of Virology*, **70(8)**, 5143-5152.

Kawamura, N., Kohara, M., Abe, S., Komatsu, T., Tago, M., Arita, M. & Nomoto, A. (1989). Determinants in the 5' non-coding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. *Journal of Virology*, **63**, 1302-1309.

Keighley, B.D. & Bell, E.J. (1983). Sporadic myalgic encephalomyelitis in a rural practice. *Journal of the Royal College of General Practitioners*, **33**, 339-341.

Kendell, R.E. (1991). Chronic fatigue, viruses and depression. *Lancet*, **337**, 160-162.

Kew, O.M., Nottay, B.K., Hatch, M.H., Hierholzer, J.C. & Obijeski, J.F. (1983). Oligonucleotide fingerprint analysis of enterovirus 70 isolates from the 1980 to 1981 pandemic of acute hemorrhagic conjunctivitis: evidence for a close genetic relationship among Asian and American strains. *Infection and Immunity*, **41(2)**, 631-635.

Kibler, R., Lucas, D.O., Hicks, M.J., Poulos, B.T. & Jones, J.F. (1985). Immune function in chronic active Epstein-Barr virus infection. *Journal of Clinical Immunology*, **5**, 46-54.

King, M.L., Shaikh, A., Bidwell, D., Valer, A. & Banatvala, J.E. (1983). Coxsackie-B-virus-specific IgM responses in children with insulin dependent (juvenile onset, type 1) diabetes mellitus. *Lancet*, **1,** 1397-1399.

Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.J., Dorner, A.J., Emini, E.A., Hanecak, R., Lee, J.J., van der Werf, S., Anderson, C.W. & Wimmer, E. (1981). Primary structure, gene organisation and polypeptide expression of poliovirus RNA. *Nature*, **291**, 547-553.

Kitani, T., Kuratsune, H., Fuke, I., Nakamura, Y., Nakaya, T., Asahi, S., Tobiume, M., Yamaguti, K., Machii, T., Inagi, R., Yamanishi, K. & Ikuta, K. (1996). Possible correlation between Borna Disease Virus infection and Japanese patients with chronic fatigue syndrome. *Microbiology and Immunology*, **40(6)**, 459-462.

Klimas, N.G., Salvato, F.R., Morgan, R. & Fletcher, M.A. (1990). Immunological abnormalities in chronic fatigue syndrome. *Journal of Clinical Microbiology*, **28(6)**, 1403-1410.

Klonoff, D.C. (1992). Chronic Fatigue Syndrome. *Clinical Infectious Diseases*, **15**, 812-823.

Klump, W.M., Bergmann, I., Muller, B.C., Ameis, D. & Kandolf, R. (1990). Complete nucleotide sequence of infectious coxsackievirus B3 cDNA: two initial 5'uridine residues are regained during plus-strand RNA synthesis. *Journal of Virology*, **64(4),** 1573-1583.

Komaroff, A.L. (1988). Chronic fatigue syndromes: relationship to chronic viral infections. *Journal of Virological Methods*, **21**, 3-10.

Komaroff, A.L. (1993). Clinical presentation of Chronic Fatigue Syndrome. In *Chronic Fatigue Syndrome,* ed. Bock, G.R. & Whelan, J. **Ciba Foundation Symposium 173**. England: John Wiley & Sons Ltd.

Komaroff, A.L. & Buchwald, D.S. (1998). Chronic Fatigue Syndrome: an update. *Annual Reviews of Medicine*, **49**, 1-13.

Krah, D.L. & Crowell, R.L. (1982). A solid-phase assay of solubilized HeLa cell membrane receptors for binding group B coxsackieviruses and polioviruses. *Virology*, **118**, 148-156.

Kroenke, K., Wood, D.R., Mangelsdorff, A., Meier, N.J. & Powell, J.B. (1988). Chronic fatigue in primary care. *Journal of the American Medical Association*, **260(7)**, 929-934.

Kuge, S., Saito, I. & Nomoto, A. (1986). Primary structure of poliovirus defective-interfering particle genomes and possible generation mechanisms of the particles. *Journal of Molecular Biology*, **192**, 473-487.

Landay, A.L., Jessop, C., Lennette, E.T. & Levy, J.A. (1991). Chronic fatigue syndrome: clinical condition associated with immune activation. *Lancet*, **338(8769)**, 707-712.

Le, S.-Y. & Zuker, M. (1990). Common structures of the 5' non-coding RNA in enteroviruses and rhinoviruses. *Journal of Molecular Biology*, **216**, 729-741.

Leon-Monzon, M. & Dalakas, M.C. (1992). Absence of persistent infection with enteroviruses in muscles of patients with inflammatory myopathies. *Annals of Neurology*, **32**, 219-222.

Leparc, I., Aymard, M. & Fuchs, F. (1994). Acute, chronic and persistent enterovirus and poliovirus infections: detection of viral genome by semi-nested PCR amplification in

culture negative samples. *Molecular and Cellular Probes*, **8**, 487-495.

Levine, J.A. (1996). The Origins of Virology. In *Fields Virology (3rd Edition)*, ed. Fields, B.N., Knipe, D.M. & Howley, P.M. Vol. 1. Ch. 1. Philadelphia: Lippincott-Raven.

Levine, P.H., Jacobson, S., Pocinki, A.G., Cheney, P., Peterson, D., Connelly, R.R., Weil, R., Robinson, S.M., Ablashi, D.V., Salahuddin, S.Z., Pearson, G.R. & Hoover, R. (1992). Clinical, epidemiologic and virologic studies in four clusters of the chronic fatigue syndrome. *Archives of Internal Medicine*, **152**, 1611-1616.

Levy, J.A. (1994). Part III: Viral studies of chronic fatigue syndrome. *Clinical Infectious Diseases*, **18(Suppl 1)**, S117-S120.

Lim, K.A. & Benyesh-Melnick, M. (1960). Typing of viruses by combinations of antiserum pools: application to typing of enteroviruses (coxsackie and echo). *Journal of Immunology*, **84**, 309-317.

Lindberg, A.M., Polacek, C. & Johansson, S. (1997). Amplification and cloning of complete enterovirus genomes by long distance PCR. *Journal of Virological Methods*, **65**, 191-199.

Linde, A., Hammarstrom, L. & Smith, C.I. (1988). IgG subclass deficiency and Chronic Fatigue Syndrome. *Lancet*, **1(8590)**, 885-886.

Lindh, A., Samuelson, A., Hedlund, K.-O., Evengard, B., Lindquist, L. & Ehrnst, A. (1996). No findings of enterovirus in Swedish patients with chronic fatigue syndrome. *Scandinavian Journal of Infection*, **28**, 305-307.

Lloyd, A.R., Wakefield, D., Boughton, C.R. & Dwyer, J.M. (1989). Immunological abnormalities in the chronic fatigue syndrome. *Medical Journal of Australia*, **151**, 122-124.

Lynch, S. & Seth, R. (1989). Post viral fatigue syndrome and the VP-1 antigen. *Lancet*, **Nov 11**, 1160-1161.

McCartney, R.A., Banatvala, J.E. & Bell, E.J. (1986). Routine use of μ-antibody capture ELISA for the serological diagnosis of coxsackie B virus infections. *Journal of Medical Virology*, **19**, 205-212.

McLaren, J., Argo, E. & Cash, P. (1993). Evolution of coxsackie B virus during *in vitro* persistent infection: detection of protein mutations using two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis*, **14**, 137-147.

Manian, F.A. (1994). Simultaneous measurement of antibodies to Epstein-Barr virus, Human Herpesvirus 6, Herpes Simplex virus types 1 and 2, and 14 enteroviruses in chronic fatigue syndrome: is there evidence of activation of a non-specific polyclonal immune response? *Clinical Infectious Diseases*, **19**, 448-453.

Martino, T.A., Liu, P., Petric, M. & Sole, M.J. (1995). Enteroviral myocarditis and dilated cardiomyopathy: a review of clinical and experimental studies. In *Human Enterovirus Infections*, ed. Rotbart, H.A. Ch. 14. Washington, D.C.: ASM Press.

Mayo, M.A. & Pringle, C.R. (1998). Virus Taxonomy 1997. *Journal of General Virology*, **79(4)**, 649-657.

Medical staff of the Royal Free Hospital (1957). An outbreak of encephalomyelitis in the Royal Free Hospital Group, London, in 1955. *BMJ*, **2**, 895-904.

Melchers, W., Zoll, J., van Kuppeveld, F., Swanink, C. & Galama, J. (1994). There is no evidence for persistent enterovirus infections in chronic medical conditions in humans. *Reviews in Medical Virology*, **4**, 235 - 243.

Mellor, J., Holmes, E.C., Jarvis, L.M., Yap, P.L., Simmonds, P. & the International HCV Collaborative Study Group (1995). Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. *Journal of General Virology*, **76**, 2493-2507.

Melnick, J.L. (1990). Enteroviruses: Polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In *Fields Virology (2nd edition)*, ed. Fields, B.N. & Knipe, D.M. Vol. 1. Ch.

21. New York: Raven Press Ltd.

Melnick, J.L., Ashkenazi, A., Midulla, V.C., Wallis, C.T. & Bernstein, A. (1961). Immunogenic potency of $MgCl_2$ stabilised oral poliovaccine. *Journal of American Medical Association*, **185,** 406-408.

Mendelsohn, C.L., Wimmer, E. & Racaniello, V.R. (1989). Cellular receptor for poliovirus: molecular cloning, nucleotide sequence and expression of a new member of the immunoglobulin superfamily. *Cell*, **56**, 855-865.

Miller, N.A., Carmichael, H.A., Calder, B.D., Behan, P.O., Bell, E.J., McCartney, R.A. & Hall, F.C. (1991). Antibody to coxsackie B virus in diagnosing postviral fatigue syndrome. *BMJ*, **302**, 140-143.

Minor, P.D. & Bell, E.J. (1990). Picornaviridae (excluding Rhinovirus). In *Topley and Wilson's Principles of Bacteriology, Virology and Immunity (Eighth Edition),* ed. Parker, M.T. & Collier, L.H. Vol. 4. Ch. 4.16. London: Edward Arnold.

Miyamoto, M.M. & Cracraft, J. (1991). Phylogenetic analysis of DNA sequences. Oxford University Press, New York.

Moore, M. (1982). From the Centers for Disease Control: Enteroviral disease in the United States, 1970-1979. *Journal of Infectious Diseases*, **146**, 103-108.

Morens, D.M., Pallansch, M.A. & Moore, M. (1991). Polioviruses and other enteroviruses. *In Textbook of Human Virology (2nd edition),* ed. Belshe, R.B. Ch. 17. St Louis London, Mosby yearbook.

Morgan-Capner, P., Richardson, P.J., McSorley, P.J., Daly, K. & Pattison, J.R. (1984). Virus investigations in heart muscle disease. In *Viral Heart Disease*, ed. Bolte, H.D. Berlin: Springer-Verlag.

Muir, P. & Archard, L.C. (1994). There is evidence for persistent enterovirus infections in chronic medical conditions in humans. *Reviews in Medical Virology*, **4**, 245-250.

Mullis, K.B., Faloona, F., Scharf, S.J., Saiki, R.K., Horn, G.T. & Erlich, H.A. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symposium on Quantitative Biology*, **51**, 263-273.

Mullis, K.B. & Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. *Methods in Enzymology*, **155**, 335-350.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. & Summers, M.D. (eds.) (1995). Virus Taxonomy- the classification and nomenclature of viruses. *Sixth report of the International Committee on Taxonomy of Viruses*. Vienna & New York: Springer-Verlag.

Nairn, C., Galbraith, D.N. & Clements, G.B. (1995). A comparison of coxsackie B neutralisation and enteroviral PCR in chronic fatigue patients. *Journal of Medical Virology*, **46**, 310-313.

Nairn, C. & Clements, G.B. (1999). A study of enterovirus isolations in Glasgow from 1977-1997. Accepted for publication

Nairn, C., Galbraith, D.N., Taylor, K.W. & Clements, G.B. (1999). Enteroviruses in the serum of children at the onset of type 1 diabetes mellitus. Accepted for publication.

Nakaya, T., Takahashi, H., Nakamura, Y., Asahi, S., Tobiume, M., Kuratsune, H., Kitani, T., Yamanishi, K. & Ikuta, K. (1996). Demonstration of borna disease virus in peripheral blood mononuclear cells derived from Japanese patients with chronic fatigue syndrome. *Federation of European Biochemical Societies Letters*, **378**, 145-149.

Newham, D. & Edwards, R.H.T. (1979). Effort Syndromes. *Physiotherapy*, **65**, 52-56.

Nicholson, F., Meetoo, G., Aiyar, S., Banatvala, J.E. & Muir, P. (1994). Detection of enterovirus RNA in clinical samples by nested polymerase chain reaction for rapid diagnosis of enterovirus infection. *Journal of Virological Methods*, **48 (2-3)**, 155-166.

Ochman, H., Medhora, M.M., Garza, D. & Hartl, D.L. (1990). Amplification of flanking sequences by inverse PCR. In *PCR Protocols: A guide to Methods and Applications*, ed. Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. Ch. 27. San Diego: Academic Press, Inc.

Ojo-Amaize, E.A., Conley, E.J. & Peter, J.B. (1994). Decreased natural killer cell activity is associated with severity of chronic fatigue immune dysfunction syndrome. *Clinical Infectious Diseases*, **18(Suppl 1),** S157-S159.

Olive, D.M., Al-Mufti, S., Al-Mulla, W., Khan, M.A., Pasca, A., Stanway, G. & Al-Nakib, W. (1990). Detection and differentiation of picornaviruses in clinical samples following genomic amplification. *Journal of General Virology*, **71**, 2141-2147.

O'Neill, D., McArthur, J.D., Kennedy, J.A. & Clements, G. (1983). Coxsackie B virus infection in coronary care unit patients. *Journal of Clinical Pathology*, **36(6),** 658-661.

O'Neill, K.M., Pallansch, M.A., Winkelstein, J.A., Lock, T.M. & Modlin, J.F. (1988). Chronic group A coxsackievirus infection in agammaglobulinaemia: demonstration of genomic variation of serotypically identical isolates persistently excreted by the same patients. *Journal of Infectious Diseases*, **157(1),** 183-186.

Parks, W.P., Queiroga, L.T. & Melnick, J.L. (1967). Studies of infantile diarrhea in Karachi, Pakistan. II. Multiple virus isolations from rectal swabs. *American Journal of Epidemiology*, **85**, 469-478.

Patnaik, M., Komaroff, A.L., Conley, E., Ojo-Amaize, E.A. & Peter, J.B. (1995). Prevalence of IgM antibodies to human herpesvirus 6 early antigen (p41/38) in patients with chronic fatigue syndrome. *Journal of Infectious Diseases*, **172(5),** 1364-1367.

Pilipenko, E.V., Blinov, V.M., Romanova, L.I., Sinyakov, A.N., Maslova, S.V. & Agol, V.I. (1989). Conserved structural domains in the 5'-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. *Virology*, **168**, 201-209.

Pilipenko, E.V., Gmyl, A.P., Maslova, S.V., Svitkin, Y.V., Sinyakov, A.N. & Agol, V.I. (1992). Prokaryotic-like *cis* elements in the cap-independent internal initiation of translation on picornavirus RNA. *Cell*, **68**, 119-131.

Plioplys, A.V. & Plioplys, S. (1995). Electron-microscopic investigation of muscle mitochondria in chronic fatigue syndrome. *Neuropsychobiology*, **32(4)**, 175-181.

Poyry, T., Kinnunen, L. & Hovi, T. (1992). Genetic variation *in vivo* and proposed functional domains of the 5' noncoding region of poliovirus RNA. *Journal of Virology*, **66(9)**, 5313-5319.

Poyry, T., Hyypia, T., Horsnell, C., Kinnunen, L., Hovi, T. & Stanway, G. (1994). Molecular analysis of coxsackievirus A16 reveals a new genetic group of enteroviruses. *Virology*, **202**, 982 - 987.

Poyry, T., Kinnunen, L., Hyypia, T., Brown, B., Horsnell, C., Hovi, T. & Stanway, G. (1996). Genetic and phylogenetic clustering of enteroviruses. *Journal of General Virology*, **77**, 1699-1717.

Preston, B.D., Poiesz, B.J. & Loeb, L.A. (1988). Fidelity of HIV-1 reverse transcriptase. *Science*, **242**, 1168-1171.

Pringle, C.R. (1997). Virus Taxonomy 1997. *Archives in Virology*, **142(8)**, 1727-1733.

Pulli, T., Koskimies, P. & Hyypia, T. (1995). Molecular comparison of coxsackie A virus serotypes. *Virology*, **212**, 30-38.

Racaniello, V.R. (1995). Early events in infection: receptor binding and cell entry. In *Human Enterovirus Infections*,ed. Rotbart, H.A. Ch. 3. Washington, D.C.: ASM Press.

Racaniello, V.R. & Baltimore, D. (1981). Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. *Proceedings of the National Academy of Sciences*, **78**, 4887-4891.

Ramsay, A.M. (1978). Epidemic neuromyasthenia 1934-1977: current approaches. *Postgraduate Medical Journal*, **54**, 704-774.

Ramsay, A.M. & O'Sullivan, E. (1956). Encephalomyelitis simulating poliomyelitis. *Lancet*, **1**, 761-764.

Read, R., Spickett, G., Harvey, J., Edwards, A.J. & Larson, H.E. (1988). IgG1 subclass deficiency in patients with Chronic Fatigue Syndrome. *Lancet*, **letter 8579**, 241-242.

Righthand, V.F. (1991). Transmission of viral persistence by transfection of human cultured cells with RNA of a persistent strain of echovirus 6. *Microbial Pathogenesis*, **11**, 57-65.

Righthand, V.F. & Blackburn, R.V. (1989). Steady state infection by echovirus 6 associated with nonlytic viral RNA and an unprocessed capsid polypeptide. *Journal of Virology*, **63(12)**, 5268-5275.

Rivera, V.M., Welsh, J.D. & Maizel, J.V. (1988). Comparitive sequence analysis of the 5' noncoding region of the enteroviruses and rhinoviruses. *Virology*, **165**, 42-50.

Robbins, F.C., Enders, J.F. & Weller, T.H. (1951). Studies on the cultivation of poliomyelitis viruses in tissue culture. V. The direct isolation and serologic identification of the virus strains in tissue culture from patients with nonparalytic and paralytic poliomyelitis. *American Journal of Hygiene*, **54**, 286-293.

Roberts, J.D., Preston, B.D., Johnston, L.A., Soni, A., Loeb, L.A. & Kunkel, T.A. (1989). Fidelity of two retroviral reverse transcriptases during DNA-dependent DNA synthesis in vitro. *Molecular Cellular Biology*, **9**, 469-476.

Rohll, J.B., Percy, N., Ley, R., Evans, D.J., Almond, J.W. & Barclay, W.S. (1994). The 5'-untranslated regions of Picornavirus RNAs contain independent functional domains essential for RNA replication and translation. *Journal of Virology*, **68(7)**, 4384-4391.

Roivainen, M., Piirainen, L., Hovi, T., Virtanen, I., Riikonen, T., Heino, J. & Hyypia, T.

(1994). Entry of coxsackievirus A9 into host cells: specific interactions with $\alpha_v\beta_3$ integrin, the vitronectin receptor. *Virology*, **203**, 357-365.

Romero, J.R. & Rotbart, H.A. (1995). Sequence analysis of the downstream 5' nontranslated region of seven echoviruses with different neurovirulence phenotypes. *Journal of Virology*, **69(2)**, 1370-1375.

Rotbart, H.A., Abzug, M.J. & Levin, M.J. (1988). Development and application of RNA probes for the study of picornaviruses. *Molecular and Cellular Probes*, **2**, 65-73.

Rotbart, H.A. (1990). Enzymatic RNA amplification of the enteroviruses. *Journal of Clinical Microbiology*, **28(3)**, 438-442.

Rotbart, H.A., Kinsella, J.P. & Wasserman, R.L. (1990). Persistent enteroviral infection in culture-negative meningoencephalitis: demonstration by enzymatic RNA amplification. *Journal of Infectious Diseases*, **161**, 787-791.

Rotbart, H.A., Sawyer, M.H., Fast, S., Lewinski, C., Murphy, N., Keyser, E.F., Spadoro, J., Kao, S.-Y. & Loeffelholz, M. (1994). Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. *Journal of Clinical Microbiology*, **32(10)**, 2590-2592.

Royal Colleges of Physicians, Psychiatrists and General Practitioners (1996). Chronic Fatigue Syndrome. *Report of a joint working group of the Royal Colleges of Physicians, Psychiatrists and General Practitioners.*

Rueckert, R.R. (1990). Picornaviridae and their replication. In *Virology*, ed. Fields, B.N. & Knipe, D.M. p 507-548. New York: Raven Press.

Rueckert, R.R. (1996). Picornaviridae: The viruses and their replication. In *Fields Virology, (3rd edition)*, ed. Fields, B.N., Knipe, D.M. & Howley, P.M. Vol. 1. Ch. 21. Philadelphia: Lippincott-Raven.

Rueckert, R.R. & Wimmer, E. (1984). Systematic nomenclature of picornavirus proteins.

Journal of Virology, **50**, 957-959.

Russell, W.R. (1947). The pre-paralytic stage and the effect of physical activity on the severity of paralysis. *BMJ*, **2,** 1023-1029.

Sabin, A.B. (1957). Properties and behaviour of orally administered attenuated poliovirus vaccine. *Journal of American Medical Association*, **164,** 1216-1223.

Sabin, A.B. & Boulger, L.R. (1973). History of Sabin attenuated poliovirus oral live vaccine strains. *Journal of Biological Standardisation*, **1**, 15.

Saiki, R.K., Scharf, S.J., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. & Arnheim, N. (1985). Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia. *Science*, **230**, 1350-1354.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J. Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487- 491.

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

Saitou, N. & Imanishi, M. (1989). Realtive efficiencies of the Fitch-Margolish, maximum-parsimony, maximum-likelihood, minimum-evolution and neighbor-joining methods of phylogenetic tree reconstruction in obtaining the correct tree. *Molecular Biology and Evolution*, **6**, 514-525.

Salk, J.E. (1953). Studies in human subjects on active immunization against poliomyelitis. I. A preliminary report of experiments in progress. *Journal of the American Medical Association*, **151**, 1081-1098.

Sarnow, P. (1989). Role of 3' end sequences in infectivity of poliovirus transcripts made *in vitro*. *Journal of Virology*, **63(1)**, 467-470.

Secchiero, P., Carrigan, D.R., Asano, Y., Benedetti, L., Crowley, R.W., Komaroff, A.L., Gallo, R.C. & Lusso, P. (1995). Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. *Journal of Infectious Diseases*, **171(2),** 273-280.

Severini, G.M., Mestroni, L., Falaschi, A., Camerini, F. & Giacci, M. (1993). Nested polymerase chain reaction for high-sensitivity detection of enteroviral RNA in biological samples. *Journal of Clinical Microbiology*, **31**, 1345-1349.

Sharpe, M.C., Archard, L.C., Banatvala, J.E., Borysiewicz, L.K., Clare, A.W., David, A., Edwards, R.H.T., Hawton, K.E.H., Lambert, H.P., Lane, R.J.M., McDonald, E.M., Mowbray, J.F., Pearson, D.J., Petto, T.E.A., Preedy, V.R., Smith, A.P., Smith, D.G., Taylor, D.J., Tyrrell, D.A.J., Wessely, S., White, P.D., Behan, P.O., Rose, F.C., Peters, T.J., Wallace, P.G., Warrell, D.A. & Wright, D.J.M. (1991). A report- chronic fatigue syndrome: guidelines for research. *Journal of the Royal Society of Medicine*, **84**, 118-121.

Sharpe, M., Hawton, K., Seagroatt, V. & Pasvol, G. (1992). Follow up of patients presenting with fatigue to an infectious diseases clinic. *BMJ*, **305**, 147-52.

Shepley, M.P., Sherry, B. & Weiner, H.L. (1988). Monoclonal antibody identification of a 100kDa membrane protein in HeLa cells and human spinal cord involved in poliovirus attachment. *Proceedings of the National Academy of Sciences*, **85(20),** 7743-7747.

Shepley, M.P. & Racaniello, V.R. (1994). A monoclonal antibody that blocks poliovirus attachment recognizes the lymphocyte homing receptor CD44. *Journal of Virology*, **68(3),** 1301-1308.

Sigurdsson, B., Sigurjonssen, J., Sigurdsson, J.H., Thorkelsson, J. & Gudmundsson, K. (1950). A disease epidemic in Iceland simulating poliomyelitis. *American Journal of Hygiene*, **52,** 222-238.

Simmonds, P., Holmes, E.C., Cha, T.-A., Chan, S.-W., McOmish, F., Irvine, B., Beall, E., Yap, P.L., Kolberg, J. & Urdea, M.S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region.

Journal of General Virology, **74**, 2391-2399.

Simmonds, P., Smith, D.B., McOmish, F., Yap, P.L., Kolberg, J., Urdea, M.S. & Holmes, E.C. (1994). Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. *Journal of General Virology*, **75**, 1053-1061.

Skinner, M.A., Racaniello, V.R., Dunn, G., Cooper, J., Minor, P.D. & Almond, J.W. (1989). New model for the secondary structure of the 5' non-coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. *Journal of Molecular Biology*, **207**, 379-392.

Sneath, P.H.A. & Sokal, R.R. (1973). Numerical Taxonomy. Freeman, San Fransisco.

Spector, D.H. & Baltimore, D. (1974). Requirement of 3'-terminal poly (adenylic acid) for the infectivity of poliovirus RNA. *Proceedings of the National Academy of Sciences*, **71(8),** 2983-2987.

Stanway, G. (1990). Structure function and evolution of picornaviruses. *Journal of General Virology*, **71**, 2483-2501.

Stanway, G., Kalkkinen, N., Roivainen, M., Ghazi, F., Khan, M., Smyth, M., Meurman, O. & Hyypia, T. (1994). Molecular and biological characteristics of echovirus 22, a representative of a new Picornavirus group. *Journal of Virology*, **68(12)**, 8232-8238

Straus, S.E., Tosato, G., Armstrong, G., Lawley, T., Preble, O.T., Henle, W., Davey, R., Pearson, G., Epstein, J., Brus, I. & Blaese, R.M. (1985). Persisting illness and fatigue in adults with evidence of Epstein-Barr virus infection. *Annals of Internal Medicine*, **102,** 7-16.

Straus, S.E., Dale, J.K., Wright, R. & Metcalfe, D.D. (1988). Allergy and the chronic fatigue syndrome. *Journal of Allergy and Clinical Immunology*, **81**, 791-795.

Straus, S.E. (1991). History of chronic fatigue syndrome. *Review of Infectious Diseases*, **13(S1)**, S2-S7.

Strayer, D.R., Carter, W.A., Brodsky, I., Cheney, P., Peterson, D., Salvato, P., Thompson, C., Loveless, M., Shapiro, D.E., Elsasser, W. & Gillespie, D.H. (1994). A controlled clinical trial with a specifically configured RNA drug, poly(I).poly(C12U), in chronic fatigue syndrome. *Clinical Infectious Diseases*, **18(1),** S88-S95.

Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M. & Argraves, W.S. (1990). Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *Journal of Biological Chemistry*, **265(29),** 17401-17404.

Sumaya, C.V. (1991). Serologic and virologic epidemiology of Epstein-Barr virus: relevance to chronic fatigue syndrome. *Review of Infectious Diseases*, **13(Suppl 1),** S19-S25.

Swanink, C.M.A., Melchers, W.J.G., van der Meer, J.W.M., Vercoulen, J.H.M.M., Bleijenberg, G., Fennis, J.F.M. & Galama, J.M.D. (1994). Enteroviruses and the chronic fatigue syndrome. *Clinical Infectious Diseases*, **19**, 860-864.

Tellier, R., Bukh, J., Emerson, S.U., Miller, R.H. & Purcell, R.H. (1996). Long PCR and its application to hepatitis viruses: amplification of hepatitis A, hepatitis B and hepatitis C virus genomes. *Journal of Clinical Microbiology*, **34(12)**, 3085-3091.

Thoren, A. & Widell, A. (1994). PCR for the diagnosis of enteroviral meningitis. *Scandinavian Journal of Infectious Diseases*, **26**, 249-254.

Tobi, M., Morag, A., Ravid, Z., Chowers, I., Feldman-Weiss, V., Michaeli, Y., Chetrit, E.B., Shalit, M. & Knobler, H. (1982). Prolonged atypical illness associated with serologic evidence of persistent Epstein-Barr virus infection. *Lancet*, **1,** 61-64.

Torre, J.C. de la, Borrow, P. & Oldstone, M.B.A. (1991). Viral persistence and disease: cytopathology in the absence of cytolysis. *British Medical Bulletin*, **47**, 838-851.

Trabelsi, A., Grattard, F., Nejmeddine, M., Aouni, M. Bourlet, T. & Pozzetto, B. (1995).

Evaluation of an enterovirus group-specific anti-VP1 monoclonal antibody, 5-D8/1, in comparison with neutralization and PCR for rapid identification of enteroviruses in cell culture. *Journal of Clinical Microbiology*, **33(9)**, 2454-2457.

Vedhara, K., Llewelyn, M.B., Fox, J.D., Jones, M., Jones, R., Clements, G.B., Wang, E.C.Y., Smith, A.P. & Borysiewicz, L.K. (1997). Consequences of live poliovirus vaccine administration in chronic fatigue syndrome. *Journal of Neuroimmunology*, **75**, 183-195.

Ward, T., Pipkin, P.A., Clarkson, N.A., Stone, D.M., Minor, P.D. & Almond, J.A. (1994). Decay-accelerating factor CD55 is identified as the receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. *The EMBO Journal*, **13(21)**, 5070-5074.

Wessely, S. & Powell, R. (1989). Fatigue syndromes: a comparison of chronic 'postviral' fatigue with neuromuscular and affective disorders. *Journal of Neurology, Neurosurgery and Psychiatry*, **42**, 940-948.

White, D.M. & Burtch, R.B. (1954). Iceland Disease. A new infection simulating acute anterior poliomyelitis. *Neurology*, **4**, 505-516.

White, P.D., Thomas, J.M., Amess, J., Grover, S.A., Kangro, H.O. & Clare, A.W. (1995). The existence of a fatigue state after glandular fever. *Psychological Medicine*, **25(5)**, 907-916.

White, P.D., Thomas, J.M., Amess, J., Crawford, D.H., Grover, S.A., Kangro, H.O. & Clare, A.W. (1998). Incidence, risk and prognosis of acute and chronic fatigue syndromes and psychiatric disorders after glandular fever. *British Journal of Psychiatry*, **173**, 475-481.

WHO, Geneva. Expanded Programme on Immunisation (EPI) (1998). *Weekly Epidemiological Record*, **73**, 161-168.

Why, H.J.F., Meany, B.T., Richardson, P.J., Olsen, E.G.J., Bowles, N.E., Cunningham, L., Freeke, C.A. & Archard, L.C. (1994). Clinical and prognostic significance of detection of enteroviral RNA in the myocardium of patients with myocarditis or dilated cardiomyopathy. *Circulation*, **89**, 2582-2589.

Wilde, J., Eiden, J. & Yolken, R. (1990). Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse-transcritpase and polymerase chain reactions. *Journal of Clinical Microbiology*, **28**, 1300-1307.

Wilfert, C.M., Buckley, R.H., Mohanakumar, T., Griffith, J.F., Katz, S.L., Whisnant, J.K., Eggleston, P.A., Moore, M., Treadwell, E., Oxman, M.N. & Rosen, F.S. (1977). Persistent and fatal central-nervous-system echovirus infections in patients with agammaglobulinemia. *New England Journal of Medicine*, **296(26),** 1485-1489.

Wilfert, C.M. & Zeller, J. (1985). Enterovirus Diagnosis. In *Medical Virology IV*, ed. Maza, L.M. de la & Peterson, E.M. New Jersey: Lawrence Erlbaum Associates.

Wilson, A., Hickie, I., Lloyd, A., Hadzi-Pavlovic, D., Boughton, C., Dwyer, J. & Wakefield, D. (1994). Longitudinal study of outcome of chronic fatigue syndrome. *BMJ*, **308**, 756-759.

Yerly, S., Gervaix, A., Simonet, V., Caflisch, M., Perrin, L. & Wunderli, W. (1996). Rapid and sensitive detection of enteroviruses in specimens from patients with aseptic meningitis. *Journal of Clinical Microbiology*, **34(1)**, 199-201.

Yolken, R.H. & Torsch, V.M. (1981). Enzyme-linked immunosorbent assay for detection and identification of coxsackieviruses A. *Infection and Immunity*, **31(2)**, 742-750.

Yousef, G.E., Brown, I.N. & Mowbray, J.F. (1987a). Derivation and biochemical characterization of an enterovirus group-specific monoclonal antibody. *Intervirology*, **28**, 163-170.

Yousef, G.E., Mann, G.F., Brown, I.N. & Mowbray, J.F. (1987b). Clinical and research application of an enterovirus group-reactive monoclonal antibody. *Intervirology*, **28**, 199-205.

Yousef, G.E., Bell, E.J., Mann, G.F., Murgesan, V., Smith, D.G. & McCartney, RA. (1988). Chronic enterovirus infection in patients with postviral fatigue syndrome. *Lancet*,

Jan 23, 146-150.

Zeiner, M. & Gehring, V. (1994). Cloning of 5' cDNA regions by inverse PCR. *Biotechniques*, **17(6)**, 1050-1054.

Zhang, G., Wilsden, G., Knowles, N.J. & McCauley, J.W. (1993). Complete nucleotide sequence of a coxsackie B5 virus and its relationship to swine vesicular disease virus. *Journal of General Virology*, **74**, 845-853.

Zhang, H., Soteriou, B., Knowlson, S., Theodoridou, A. & Archard. L.C. (1997). Characterisation of genomic RNA of coxsackievirus B3 in murine myocarditis: reliability of direct sequencing of reverse transcription-nested polymerase chain reaction products. *Journal of Virological Methods*, **69**, 7-17.

Zheng, Z.-M., He, P.-J., Caueffield, D., Neumann, M., Specter, S., Baker, C.C. & Bankowski, M.J. (1995). Enterovirus 71 isolated from China is serologically similar to the prototype E71 BrCr strain but differs in the 5' noncoding region. *Journal of Medical Virology*, **47**, 161-167.

Zoll, G.J., Melchers, W.J.G., Kopecka, H., Jambroes, G., Van der Poel, H.J.A. & Galama, J.M.D. (1992). General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. *Journal of Clinical Microbiology*, **30(1)**, 160-165.

Zorzenon, M., Colle, R., Rukh, G., Barsanti, L.A., Botta, G.A. & Ceccherini-Nelli, L. (1996). Active HHV-6 infection in chronic fatigue syndrome patients from Italy: new data. *Journal of Chronic Fatigue Syndrome*, **2(1)**, 3-12.

Addendum to reference list

Ghazi, F., Hughes, P.J, Hyypia, T. & Stanway, G. (1998). Molecular analysis of human parechovirus type 2 (formerly echovirus 23). *Journal of General Virology*, **79(11)**, 2641-2650.

Hellen, C.U.T & Wimmer, E. (1995). Enterovirus structure and assembly. In *Human Enterovirus Infections*, ed. Rotbart, H.A. Ch. 14. Washington, D.C.: ASM Press.

Oberste, M.S., Maher, K. & Pallansch, M.A. (1998). Complete sequence of echovirus 23 and its relationship to echovirus 22 and other human enteroviruses. *Virus Research*, **56**, 217-223.