THE SEROLOGICAL DIAGNOSIS OF ENTEROVIRUS INFECTIONS

THESIS

FOR THE

DEGREE OF MASTER OF SCIENCE

IN THE FACULTY OF MEDICINE

UNIVERSITY OF GLASGOW.

DECEMBER 1990.

BY

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ACKNOWLEDGMENTS.

CIS. UK Ltd. Mr. Alan Baines, Managing Director.
For financial support.

Professor Morag C. Timbury. Formerly of Glasgow University and the Regional Virus Laboratory, Ruchill hospital.
For encouragement and the use of premises at the Regional Virus Laboratory, Ruchill Hospital.

Dr. Eleanor J. Bell. Formerly of Glasgow University and the Enterovirus (Scotland) Reference Laboratory, Ruchill Hospital.
For inspiration, encouragement and many useful discussions concerning various aspect of the work.

Professor J.E. Banatvala. Virology department St.Thomas Hospital London.
For the time spent in his unit, his advice on aspects of the ELISA test and for providing the initial control sera.

Dr L. Greenham. Department of Microbiology Bristol University.
For supervision and help during the writing up of this thesis.

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DECLARATION.

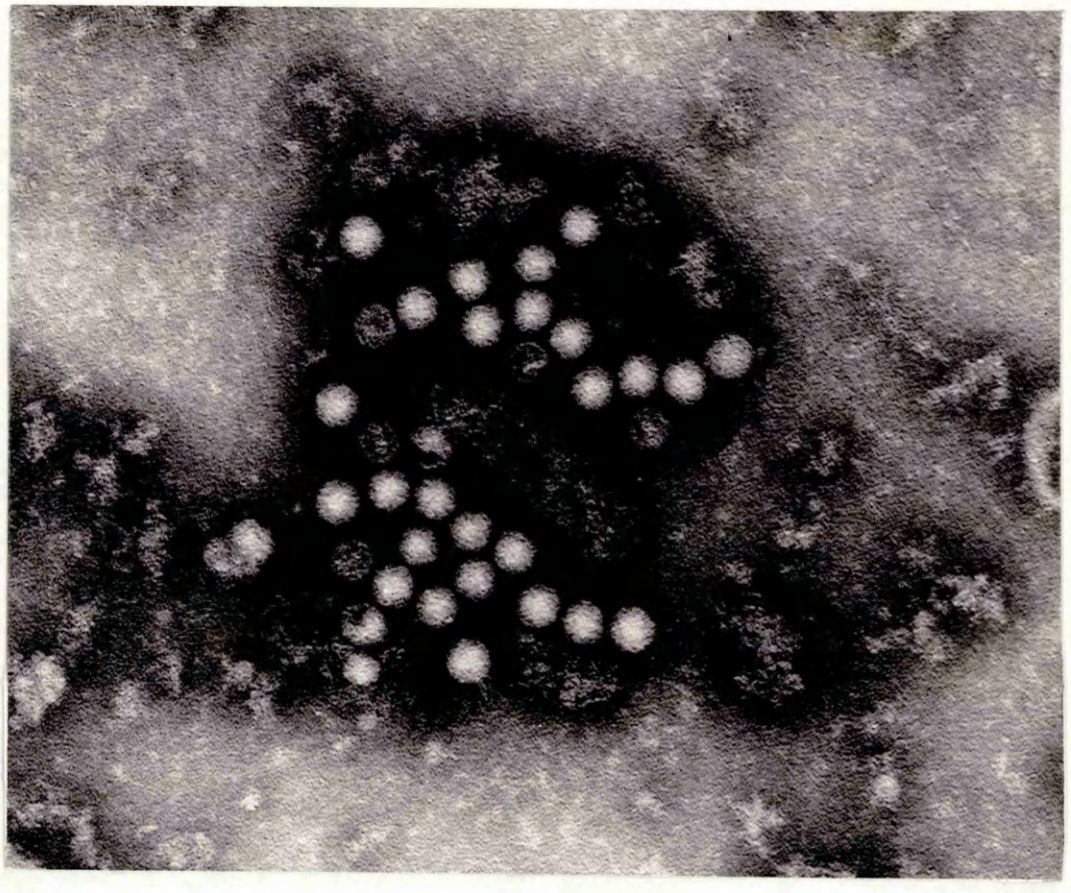
I declare that the following thesis embodies the results of my own special work, that it has been composed by myself and that it does not include work forming part of a thesis presented successfully for a degree in Glasgow or any other University.

November 1990. Robert A. McCartney.

LIST OF ABBREVIATIONS.

A.M.	Aseptic meningitis.
CAV	Coxsackie A virus.
CBV	Coxsackie B virus.
CF.	Complement fixation.
CNS.	Central nervous system.
CPE.	Cytopathic effect.
CSF.	Cerebral spinal fluid.
D antigen	"Empty" or incomplete virus particles.
ECG	Electrocardiogram
EcV	Echo virus.
ELISA	Enzyme linked immunosorbent assay.
EDTA	Ethylenediamine tetraacetic acid / Versene
Ev.	Enterovirus.
HAV	Hepatitis A virus.
ME	Myalgic encephalomyelitis.
MEM	Minimal essential medium.
mRNA	Messenger ribonucleic acid.
N antigen	Natural complete virus particle.
NT(s)	Neutralisation test(s).
OD	Optical density.
OPD	O-phenylenediaminedihydrochloride.
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction.
PVS	Post viral (fatigue) syndrome.
RIA	Radio immunoassay.
RNA	Ribonucleic acid.
TCID	Tissue culture infectious dose.
VP	Viral protein.

ELECTRON MICROGRAPH OF AN ENTEROVIRUS.



Coxsackie B virus serotype 2.

Magnification factor of 200,000.

By courtesy of Mr. V. Annette of Bristol Public Health Laboratory.

SUMMARY.

A micro-neutralisation test for the detection of Coxsackie B specific antibody in patients serum is described. Patients with suspected cardiac and other illnesses with possible Coxsackie B virus etiology were examined. In 1983 34% had titres of >256, in the better selected patients tested in 1984 the figure was 46%

In the absence of virus isolation or the detection of significant rising titres the interpretation of static neutralising titres is both difficult and controversial. Detection of specific IgM in such patients, implying recent infection, appeared to be a possible answer to this problem.

A u - antibody capture enzyme linked immuno-sorbent assay(ELISA) for the detection of Coxsackie B specific IgM antibody is described. This assay can readily be used for testing large numbers of sera and has the advantage over neutralisation tests in that a positive result represents recent or active infection.

A regimen for the serological diagnosis of Coxsackie B virus infections using this ELISA as a screening test followed by neutralisation tests to confirm the positive results was introduced. Seven hundred and sixty patients and 304 healthy adult controls were tested. The percentage positive by

ELISA in each of the clinical categories of myopericarditis(33%), chest pain(22%), myalgic encephalomyelitis(31%), myalgia/Bornholm(19%) and controls(9%) was similar to that reported when neutralisation tests alone were used, although results for the individual patient may be different.

The duration of detection of CBV IgM in each of the clinical categories was measured. In acute illness such as Bornholm disease the CBV IgM was detected for 1 - 2 months, in myopericarditis for 3 - 6 months. Persisting CBV IgM was detected for more than one year in some patients with M.E.

Cross reactions in this CBV IgM ELISA were investigated. Heterotypic responses were observed from patients with hepatitis A infections and in those with proven systemic Coxsackie A virus and echovirus infections. No cross reactions were observed in sera from the 5 cases of poliovirus infections investigated. The exploitation of these cross-reactions in the rapid diagnosis of aseptic meningitis, a major cause of which are enteroviruses is reported.

Forty five patients with suspected aseptic meningitis were investigated. A total of 10(22%) patients had an enterovirus isolated from the CSF(6), faeces(4) or both(6). Thirty(67%) were found to have enterovirus specific IgM in their serum, including all 10 patients

from whom an enterovirus was isolated. Twenty one patients were confirmed as having clinically diagnosed aseptic meningitis, 18(86%) of these were positive in the CBV IgM ELISA.

Two major differences in the CBV IgM response between patients with aseptic meningitis and those with cardiac and other illnesses of suspected CBV aetiology were noted. Firstly, a higher frequency of heterotypic reactions were observed, and secondly, stronger positive CBV IgM responses were detected more often in the aseptic meningitis patients.

CHAPTER 1

INTRODUCTION.

Historical background.

The name Picornavirus was proposed by the Enterovirus Study Group and accepted by the International Nomenclature Committee (1963) to designate a group of agents including the following:-

A. Picornaviruses of human origin.

1. Enteroviruses *
   a). Polioviruses 1-3.
   b). Coxsackie A viruses 1-22, 24.
   c). Coxsackie B viruses 1-6.
   d). Echoviruses 1-9, 11-27, 29-34.
   e). Enteroviruses 68-72.
2. Rhinoviruses.

* Enteroviruses.

This subgroup was named in 1957 by the Committee on the Enteroviruses, National Foundation for Infantile Paralysis,(1) to bring together polioviruses, Coxsackie viruses and echoviruses, all viruses for which the human alimentary tract is the natural habitat.

Although a crippling disease retrospectively recognisable as paralytic poliomyelitis appears in records of early antiquity, it began to be described as a clinical entity only in the late 1700's and early 1800's. It became the subject of intensive study only after increasingly severe epidemics ocurred in Europe and North America.

Experimental work became possible following the successful transmission of the disease to monkeys in 1908 by Lansteiner and Popper.(2). During the next forty years it was shown that the virus was regularly present in the stools of patients, that subhuman primates could be infected by the alimentary route, and that strains could be adapted to growth in laboratory rodents, permitting an expansion of laboratory studies. Significant antigenic differences among poliovirus strains were documented, resulting in their initial separation into three serological types.

The discovery by Enders et al(1949) (3) of poliovirus proliferation in primate cells of non neural origin did much to facilitate the study of the enteroviruses. Prior to this, virus isolations were slow, expensive and difficult. More frequently than not at this time, clinical symptomatology rather than specific aetiology contributed to the diagnosis of particular diseases, poliomyelitis included.

In 1948, during the investigation of a poliomyelitis outbreak in the town of Coxsackie in New York State, viruses isolated from the faeces of two children were found to induce paralysis in newborn mice and hamsters (4). The isolation of further viruses with similar properties was soon reported (5 & 6). Pathogenicity for newborn mice was used to define this new group of viruses which were named Coxsackieviruses. The group was later divided into group A (24 serotypes) and group B (6 serotypes) Coxsackieviruses.

Following the introduction of in vitro cell cultures many new viruses were isolated from faecal material. It became clear that some of these isolates were neither polioviruses nor Coxsackieviruses since they differed in respect of both serology and pathogenicity for newborn mice. At first they lacked an association with any specific disease and were thus described as Orphan viruses, and in 1955 were given the acronym ECHOviruses, derived from the description Enteric Cytopathogenic Human Orphan.

Of the 33 serotypes assigned to this group, type 10 was reclassified as reovirus type 1, and type 28 was found to be a rhinovirus. Echovirus type 9 was found to be identical to Coxsackie A virus 23.

The difficulties of assigning new enteric picornaviruses to one of the four groups led to the

recomendation that new enterovirus serotypes should be given numbers only (7). To date five new enterovirus serotypes have been accepted and designated enterovirus 68 to 72.

In the current classification, enteroviruses are accorded generic status within the Picornaviridae Family as are rhinoviruses and a number of animal picornaviruses.

Characteristics of Enteroviruses.

The enteroviruses share many clinical, epidemiological, physical and biochemical features.

Typically enteroviruses are small 20-27 nM icosahedral particles containing a single molecule of single stranded ribonucleic acid (RNA) enclosed in a protein coat made up of four distinct polypeptides designated viral protein (VP) 1 ,VP2, VP3, and VP4. The virions are non enveloped, resistant to lipid solvents such as ether and to inactivation by low pH.

Viral replication occurs in the cytoplasm of infected cells, with the viral RNA acting as a single polycistronic molecule of messenger RNA (mRNA) translated into a single protein which is subsequently cleaved to give the four structural proteins and thus purified RNA is infectious in its own right. Following replication mature progeny virus particles are released by lysis of the infected cell.

The non-polio enteroviruses (NPEV) have been divided into groups based upon their initial pathogenicity for newborn mice, viz.:-

Group A Coxsackieviruses (CAV) typically produce lethal paralysing infections with severe,generalised myositis of skeletal muscle.

Group B Coxsackieviruses (CBV) produce scattered focal myositis, but more severe damage to the nervous system, brown fat, pancreas and other viscera including the heart.

Echoviruses(EcV) are, by definition, nonpathogenic for newborn mice.

Although this form of classification has held up remarkably well for 30 years it has now been replaced by a numbering system, with new viruses of the enterovirus (EV) genus being numbered from 68 onwards.

Enteroviruses are transmitted most frequently by the faecal - oral route, but in conditions of improving hygiene the respiratory route becomes more important and, indeed is a more sensitive route. They have been implicated in a wide range of diseases of man with a heterogenicity across the classic groupings.

For example, (a) paralytic poliomyelitis can be caused by CBVs, by some CAVs, most notably CAV type 7, rarely by some EcVs and also as a sequel to EV 71 infection.

(b) Acute muscle disease (Bornholm disease) is mainly caused by CBV, sometimes by CAV and occasionally by EcV(especially Ecv type 6).

(c) Skin rash and oropharyngeal lesions (hand foot and mouth disease) are caused mainly by CAV (types 4,9 10 & 16) but may also be caused by CBV (types 1-5) and EcV type 9.

(d) Acute upper respiratory infections can be caused by most EVs eg. (CAV type 21).

(e) Aseptic meningitis can be caused by most enteroviruses.

(f) Viral heart muscle disease is particularly associated with CBVs.

(g) Acute haemorrhagic conjunctivitis is associated with CAV type 24, and EV type 70.

(h) Acute, life threatening disseminated viral infection of neonates is associated with EcV types 7 and 11.

(i) Myalgic encephalomyelitis may be associated with CBV(types 1-5).

(j) Less frequently, enteroviruses have been associated with hepatitis, pharyngitis and encephalitis.

Infection with an enterovirus is usually subclinical and until recently there was little evidence for persistent infections occurring in humans.

Serology.

During the last ten years there has been an increase in direct antigen detection by immunological methods and in serological tests for the diagnosis of recent virus infection, as alternatives to virus isolation procedures. This change in emphasis has been due to a number of factors. There has been dissatisfaction with the time taken to isolate and identify viruses. Cell culture techniques are expensive in terms of staff time and resources, which , when at a premium, tend to be deployed in areas which have the most immediate impact on patient management.

This change in emphasis towards serology has also been seen in the diagnosis of enterovirus infections, for which virus isolation has been the cornerstone of diagnosis for many years. Serological tests, notably neutralising antibody studies on patients from whom an EV had been isolated from the gastrointestinal tract, had a role in determining the systemic nature of infections.

In the investigation of viral heart disease (myopericarditis), which is generally regarded as a late manifestation of Coxsackie B virus infection, patients presented at a time long after virus shedding

had ceased and the detection of rising titres of antibody was unusual. In these cases association of a disease syndrome with a particular virus was made on the finding of elevated antibody titres. While this type of epidemiological evidence is useful in the wider context of virus-disease association, in individual patients it may give a misleading diagnosis as up to 8% of the well population have antibody levels of this magnitude to one or more CBV serotype.

This situation was far from ideal, so attention turned to the possibility of detecting enterovirus specific IgM in order to diagnose recent infection. Work in this field has centred on the group B Coxsackieviruses(CBV) since a) there are only six serotypes to contend with, and b) there was good epidemiological evidence of their association with serious disease of man, particularly heart disease.

The first test for CBV-specific IgM to be evaluated was based on immunodiffusion techniques (8 & 9). Using high titre antigen preparations containing a mixture of intact and empty capsids, two distinct lines of precipitation were observed,one being IgM antibody precipitating intact virus and the second IgG reacting with empty capsid.

Initial studies (8) showed that this technique could be used for the detection of specific IgM to all CBVs

except CBV2. IgM was found in the sera of 77 of 79 patients from whom CBV1,3,4 or 6 had been isolated. It was noted that not all IgM antibody was homotypic for the virus isolated but that heterotypic reactions occurred in 22% of patients. CBV specific IgM was also found in 21/86 (24%) of patients with CNS disease from whom CBVs had not been isolated. The test was then used to detect the presence of CBV specific IgM in a group of 259 patients with pericarditis(148 cases), myocarditis(92 cases) and pleurodynia(19 cases) and a control group of 259 patients with a diagnosis of viral or mycoplasma pneumonia (8). CBV specific IgM was detected in 27%, 25%, 37% and 8% respectively in patients with pericarditis, myocarditis, pleurodynia and controls. The author noted that some sera reacted with more than one CBV. 80% gave monotypic reactions, 16% reacted with two serotypes, and one serum reacted with each of serotypes 3, 4, & 5.

Minor et al(10) used the same basic principle in a counter-immunoelectrophoresis test in an attempt to improve the sensitivity and specificity of the test. Concentrated antigen was again required and it was found necessary to use recent CBV isolates rather than prototype strains which gave inconsistent results. Twenty nine patients from whom a CBV had been isolated and who produced ≥4-fold rise in

neutralising antibody titres were tested. Twenty(69%) of these were positive for CBV IgM antibody; 19(65%) produced a monotypic homologous response and one(CBV5 isolate) a heterotypic response. Again, patients infected with CBV2 produced poorer results in this assay; only three of seven patients infected with this virus were positive and one of these exhibited a heterotypic reaction only. In controls studied at the same time, 2(9%) of 22 patients from whom an Ecv or a CAV had been isolated (both Ecv type 6) and one (2%) of 50 patients from whom a non-enterovirus had been isolated were positive in this assay.

It has been suggested that these methods should have been exploited as routine diagnostic procedures. There are several reasons why this did not happen. 1) The preparation of high titre antigen on a regular basis for large numbers of tests made the test very expensive.

2) Current trends in routine diagnostic virology laboratories are towards enzyme immunoassays with all the attendant instrumentation, and staff are poorly motivated in relation to what is perceived as outdated technology. 3) The work load in laboratories using neutralisation tests was so large that the use of IEOP was not practicable.

In 1980 El-Hagrassy, Banatvala & Coltart (11)

described a μ-antibody capture enzyme-linked immunosorbent assay(ELISA) for the detection of CBV specific IgM. The antigen used was a pool of CBV 1-5 antigens and each sample was also tested against normal(control) antigen; the result test was assessed on the difference between the two readings. Ten sera known to contain CBV IgM (by neutralisation tests on density gradient fractions) were all positive by this ELISA test. Ten of 12 (83%) sera from patients from whom a CBV had been isolated and/or showed a >4-fold increase in CBV neutralising antibody titres were also positive. As a control group 46 patients with recent infections other than CBV were tested, and none was found positive.

Dorries and ter Meulen(12 & 13) described an indirect solid phase radio-immunoassay (1980) and subsequently an ELISA (1983) for the detection of CBV specific IgM using individual antigens as opposed to a pooled virus . They found that the reactions of sera in their system gave three patterns of results, homotypic, type-predominant, and heterotypic. In the homotypic pattern sera gave a positive result with only one CBV serotype which was the same as that isolated. In the type-predominant group there was a strong reaction with the isolated serotype and weaker reactions against other serotypes. In the heterotypic response,

moderate to strong positive reactions were found against all serotypes regardless of the isolated serotype.

On further investigation of their test the authors found that the antigens which they used contained a mixture of intact or native virions and incomplete pro-virions in the form of capsids of VP0 (polypeptide precursor of VP2 & VP4), VP1 and VP3. Reactions of these separated polypeptides with sera showing homotypic and heterotypic responses indicated that both the type and group specific antigenic determinants were located on the VP1. These results are in agreement with many other workers who have shown that type specific reactions in serological tests for enteroviruses require the use of purified intact native virus. Dorries and ter Meulen also demonstrated a cross-reaction in their test with serum from a patient with an acute CAV 9 infection. This, with the report by Minor et al (10) of two cross-reacting sera from patients with Ecv 6 infections, were the first reports of cross -reactions with other members of the enterovirus group.

Morgan-Capner & McSorley (14) described a u-antibody capture radio-immunoassay (MACRIA) for the detection of CBV4 and CBV5 antibody. Their results, although on small numbers of sera, are interesting in

that they give further evidence of the occurrence of cross-reactions within the enterovirus group. Ten of 20(50%) cases of CBV1,2,3,5 & 6 infections gave positive results in their CBV4 assay, and 7 of 18(39%) CBV1,2,3,4, & 6 infections reacted in the CBV5 assay. More significantly, 9 of 13(69%) cases from whom a CAV or EcV had been isolated gave a positive reaction in both the CBV4 and CBV5 assays.

From the foregoing it was quite clear that further work was required to develop the CBV IgM test for use in a routine diagnostic laboratory, and to determine the extent of, and the problems caused by, the apparent cross reactions and to find a solution to such problems. A further question might be added : could any detectable cross-reactions be used in a diagnostic manner?

The following chapters set out attempts to test these questions, and put forward a working hypothesis for the serological diagnosis of enterovirus infections in man.

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CHAPTER 2.

NEUTRALISATION TESTS.

Introduction.

The neutralisation test (NT) is at present the most commonly used technique for the serological diagnosis of Coxsackie B virus (CBV) infections. The test is based on the measurement of neutralising antibody in patients serum, i.e. antibody capable of inhibiting virus infectivity in vivo and in vitro.

Neutralising antibody appears early in the illness (0-3 days) reaching a peak after 14 days, and thereafter high levels of neutralising antibody may persist for months or years. Their decrease is not necessarily associated with recovery from the presenting illness (1, 2). Thus it is possible to diagnose a current infection by demonstrating a fourfold or greater rise in antibody titre between sera taken in the acute and convalescent phases of illness.

The majority of patients with suspected CBV infections present too late in their illness to demonstrate significant rises in neutralising antibody titre, and thus virus diagnosis depends on the interpretation of static high titres often in single serum samples. Grist et al (3), reported an association of high CBV titres with recent infection in their study of acute myocarditis and non-bacterial pericarditis. They regarded titres of ≥512 as diagnostically significant since only 4% of the sample of the healthy adult population sampled showed this level of antibody. Using this criterion they found that at least 33% of acute myocarditis and 14% of pericarditis cases were associated with a recent CBV infection.

This chapter presents the results obtained in 1983-84 using NT for the serological confirmation of diagnosis of CBV infections in patients with suspected CBV associated viral heart disease.

Neutralisation Tests.

1 Patients.

Blood samples from 2226 patients were tested in 1983 and from 1430 in 1984. The majority of these patients were located in the West of Scotland, the remainder in East Scotland and in various parts of England.

The clinical classification of patients into the various designated groups was based entirely on information provided by many clinicians on the request forms accompanying specimens. Because the numbers were too great and the sources so varied no routine follow - up enquiry was pursued.

Materials and methods.

Cell cultures

(a) Vero cells.

Continuous African Green Monkey Kidney(ATCC No.CCL81) were obtained from Flow Laboratories Ltd., Irvine, Scotland.The passage number varied in the sequential experiments.

(b) Media

The composition of the culture media (1-4) used for the growth and maintenance of the cell lines is shown in Table 2.1. The phosphate buffered saline(PBS) used throughout was Dulbecco A Saline without added calcium and magnesium (4,5).

(c) Preparation of cell cultures

Monolayers of Vero cells were grown in 120cm^2 plastic tissue culture flasks (Nunc) in Medium No.1. Screw-capped flasks were incubated at 37°C and confluent monolayers were achieved in 2-4 days.

Table 2.1.

CELL CULTURE MEDIA FOR NEUTRALISATION TESTS.

	Constituents.				
Medium No.	EMEM	FCS	P+S	$MgCl_2$	199
1	100ml	5ml	1ml	0ml	0ml
2	100ml	5ml	1ml	1ml	0ml
3	100ml	0.5ml	1ml	1ml	0ml
4	0ml	5ml	1ml	1ml	100ml

EMEM = Eagles minimum essential medium (Earle's salts) with 2.5% $NaHCO_3$

FCS = Foetal calf serum.

P+S = Penicillin at 100iu/ml plus streptomycin at 100ug/ml.

$MgCl_2$ = 2M magnesium chloride.

199 = Medium 199.

Sub-Cultivation.

Confluent monolayers of Vero cells were washed twice with sterile PBS. They were then flooded with a solution of 0.25%(1:250) trypsin/0.2% ethylene diamine tetra acetic acid(EDTA). After 1-2 minutes this solution was poured off and the flasks incubated at 37°C until the cells became detached from the plastic (3-5 mins.). The detached cells were suspended in 10ml of medium No.1 and mixed by aspiration until a single cell suspension was obtained. The cell suspension was reseeded into 120cm² flasks at a "split" ratio of 1:4(i.e., 1 flask into 4 flasks), giving confluent monolayers in 2-4 days. Cells showing abnormal growth characteristics, such as very slow growth, or "stringy" appearance, proved unsuitable in the NT and were discarded.

(d) Preparation of cell suspension for the NT

Confluent monolayers of healthy Vero cells were washed and trypsinised as in **(c)** above. The cells were then resuspended in PBS (20 ml per donor flask) and centrifuged at 1500 rpm in a swing-out rotor on a bench centrifuge. The PBS was removed and the pellet of cells resuspended in medium No.2 (10 ml per flask). The cells from all the flasks were then pooled and a total and viable cell count made.

(e) Cell count.

The cell suspension from **(d)** was diluted 1 in 10 in 0.4% trypan blue stain. This is a supravital stain which is only taken up by dead cells within 2 minutes at room temperature. An improved Neubauer haemocytometer was charged with this and total and viable cell counts performed. An excess of dead cells (>10%) indicated that the cell suspension was unsuitable for use in the NT, though in practice this was rare. The suspension was finally adjusted to give a concentration of 1.5×10^5 cells per ml in medium No.2. 100ul of this suspension was added to each well of the microtitre plates in the N.T.

PREPARATION OF VIRUS STOCKS.

(a) Source

Coxsackie B virus type 1, strain PO 49683

Coxsackie B virus type 2, strain Ohio - Red 50207

Coxsackie B virus type 3, strain Nancy 50531

Coxsackie B virus type 4, strain Dalldorf 51196

Coxsackie B virus type 5, strain 53112.

The above Coxsackie B viruses were donated by Dr. E.J.Bell, Regional Virus Laboratory, Ruchill Hospital, who received them from the Central Public Health Laboratory, Colindale, London. The viruses were passaged in primary primate cells, then adapted to grow in Vero cells at the Regional Virus Laboratory, Ruchill

Hospital.

(b) **Propagation.**

CBV types 1-5 were grown in confluent monolayers of Vero cells in 120 cm^2 tissue culture flasks. 0.5ml of seed virus was added to approximately 50ml of Medium No.3. Confluent monolayers of Vero cells were washed once in PBS and 50ml of virus suspension was added to each flask. The flasks were then incubated at 37°C until all the cells exhibited a typical enterovirus cytopathic effect (CPE) in 2-3 days. The flasks were then frozen at -20°C and thawed, three times. The cell culture fluids were then centrifuged at 3000 rpm for 20 min in a bench centrifuge to remove cellular debris. The supernates were aliquoted in 0.5 ml volumes and stored at -20°C.

(c) Virus titration.

(i) Logarithmic dilutions of clarified suspension of CBV 1-5 from 10^{-1}-10^{-8} in Medium No.4 were prepared.

(ii) 0.025 ml (25ul) of each virus dilution was added to one column of 4 wells commencing with the highest dilution on the right hand side of the plate.

(iii) 25 ul of Medium No.4 was added to each test well to represent and replace the serum dilution used in the neutralisation test. The plate was then covered with a loose lid and incubated at room temperature for 90 mins.

Figure.2.1

VIRUS TITRATION.

		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
CBV 1										
	1	+	+	+	+	−	−	−	−	
	2	+	+	+	+	−	−	−	−	$TCID_{50}$
	3	+	+	+	−	−	−	−	−	$= 10^{4}$.
	4	+	+	+	−	−	−	−	−	
CBV 2										
	5	+	+	+	+	−	−	−	−	
	6	+	+	+	+	−	−	−	−	$TCID_{50}$
	7	+	+	+	+	−	−	−	−	$= 10^{4.5}$
	8	+	+	+	+	−	−	−	−	
CBV 3										
	9	+	+	+	+	+	+	−	−	
	10	+	+	+	+	+	+	−	−	$TCID_{50}$
	11	+	+	+	+	+	−	−	−	$= 10^{6}$
	12	+	+	+	+	+	−	−	−	
CBV 4										
	1	+	+	+	−	−	−	−	−	
	2	+	+	+	−	−	−	−	−	$TCID_{50}$
	3	+	+	−	−	−	−	−	−	$= 10^{3}$
	4	+	+	−	−	−	−	−	−	

Figure. 2.1. continued.

VIRUS TITRATION.

CBV 5	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
5	+	+	+	+	+	+	+	-	
6	+	+	+	+	+	+	-	-	$TCID_{50}$
7	+	+	+	+	+	+	-	-	$= 10^{7}$
8	+	+	+	+	+	+	+	-	
9	4	3	2	1					
10	4	3	1	1					
11	4	3	1	1					
12	4	3	2	1					
CELL CONTROL	100%	50%	25%	12.5%					

+ = Cytopathic effect (cpe)

- = No cpe (100% cells).

4 = Confluent cell layer.

3 = 75% confluency.

2 = 50% confluency.

1 = 25% confluency.

(iv) 100 ul of Vero cells at a concentration of 1.5 x 10^5 cells/ml in Medium No.2 was then added to each test well.

(v) Cell controls were prepared in quadruplicate wells, giving cell concentrations of 100%, 50%, 25% and 12.5 % of test cell suspension in medium No.2, and 50ul of medium No.4 was then added to each control well to replace the virus/serum mixtures volumes.

(vi) The microtitre plates were then sealed with non-toxic plate sealing tape (Titertek) and incubated at 37°C for 64-70 hours.

(vii) Each well of the microtitre plate was then examined microscopically for CPE using an inverted microscope (Olympus IMT-1) with a X 4 objective and X 10 eyepieces.

(viii) Cell controls These were expected to be confluent at 100% and 75% dilutions, half confluent at 25% and sparse at 12.5%.

(ix) Readings. Each well showing 1+ (approximately 10% of cells infected) or greater CPE was scored as positive. Those showing no CPE or microplaques only were scored as negative. The final dilution of virus was expressed as tissue culture infective dose 50 ($TCID_{50}$), i.e., the concentration of virus capable of producing a 1+ or greater CPE, under these test conditions, in 50% of the inoculated

cultures as calculated by Karber's formula :

$TCID_{50}$ = L - d(S - 0.5), where: L = $\log_{10}$ of the lowest dilution (-1 here); d = $\log_{10}$ difference between each dilution (1 here) and S = sum of the positive results. The $TCID_{50}$ for the 5 CBV serotypes varied from 10^{-3} - 10^{-7}. In the neutralisation tests viruses were used at 100 $TCID_{50}$. (see Figure. 2.1).

Positive control sera.

(a) Source

Enterovirus typing sera raised in monkeys were used as positive control sera. These antisera were donated by Dr. E.J.Bell, who received them from the American Type Culture Collection, Maryland, USA. They had already been characterised and their homotypic and heterotypic neutralising titres estimated. Titres ranged from 1/100 - 1/400.

(b) Standardisation of positive control sera.

Serial doubling dilutions of heat inactivated antisera from 1/20 - 1/1280 were prepared as follows:

(i) 25 ul of medium No.4 was added to two rows of 8 wells for each antiserum in a flat well microtitre plate, and 25 ul of a 1/10 dilution of each antiserum in PBS, previously inactivated at 56°C for 30 mins, were added to the first pair of wells in each of 2 rows. Using microtitre diluting loops (Titertek) the

antisera were diluted through a series of seven wells, giving dilutions of 1/20 - 1/1280 in duplicate. The eighth well in each row held serum at a 1/20 dilution which served as a serum toxicity control.

(ii) 25 ul of CBV at 100 $TCID_{50}$ per 0.025ml in medium No.4 was added to each well of the serum titration, and 25 ul of medium No.4 was added to the serum controls.

(iii) Virus controls at 100, 10, 1, and 0.1 $TCID_{50}$ were set up at the same time. The plates were covered with a loose lid and incubated at room temperature for 90 mins.

(iv) 100 ul of Vero cells at a concentration of 1.5×10^5 cells/ml in medium N0.2 were added to each well, including the virus controls.

(v) Cell controls at 100, 50, 25 and 12.5% were also set up. The plates were sealed with non toxic tape and incubated at 37°C for 64-70 hrs.

(vi) The plates were then examined microscopically for evidence of either virus growth or toxicity.

(c) Reading positive control serum titrations.

(i) Cell controls: 100% cell control needed to show a complete monolayer, with confluence gradually decreasing.

(ii) Virus controls: these should show virus dilutions to be no more than one log_{10} step over or under the expected result.

(d) Antiserum. Providing the cell and virus controls are acceptable, the titre of the positive control serum is taken as the highest dilution of serum giving complete inhibition of viral CPE.

In the neutralisation test the positive control sera were double diluted from 4 times titre to 1/4 titre thus giving a measure of the sensitivity of the test.

(e) Patients sera.

Sera for testing were diluted 1/16 in PBS then inactivated at 56°C for 30 mins. These dilutions were normally stored overnight at 4°C before being tested. The sera were then titrated for testing from 1/32 - 1/512 giving final dilutions of 1/64 - 1/1024 in the serum/virus mixture.

Coxsackie B1-5 Neutralisation Tests Proper

(a) Preparation of plates

Eight sera could be tested in duplicate against one virus on a single microtitre plate. Sera were normally tested in batches of 32 giving a total of 22 plates, including 2 control plates, per batch. Serum controls at 1/64 were included in the CBV 1 plate. Cell controls were included in all other plates to check for toxicity of the plate. In order to reduce the risk of cross contamination only one virus serotype was used on any one test plate, but were combined in the control plates.

(b) Preparation of virus dilutions.

On the day before the test, virus dilutions containing 100 $TCID_{50}$ were prepared in medium No.4, and further dilutions in 2 ml volumes at 10, 1 and 0.1 $TCID_{50}$ were also made. These were stored at 4°C prior to use.

(c) Protocol.

(i) Doubling dilutions of patients' sera from 1/32-1/512 were made in medium No.4 in 25 ul volumes in flat bottom microtitre plates using microdiluter loops. Doubling dilutions of positive control sera for each virus from 4 times titre-1/4 titre were prepared in duplicate on a separate control plate.

(ii) 25 ul of virus at 100$TCID_{50}$ was added to the appropriate test and control plates, and virus titrations at 100,10,1 and 0.1 $TCID_{50}$ were also included.

(iii) Medium No.4 was added to the serum controls and to the virus titration to keep all volumes constant.

(iv) The plates were then incubated at room temperature for 90 minutes.

(v) 100 ul of Vero cells in medium No.2 at a concentration of 1.5 x 10^5 cells/ml were added to each test and control well. Cell controls containing 100%, 50%, 25% and 12.5% dilutions of cells were also prepared, and 50 ul of medium No.4 was added to the

wells of the cell controls to replace the serum/virus mixtures.

(vi) The plates were then sealed with non toxic plate sealing tape and incubated at 37°C for 64-70 hours.

(d) Reading the neutralisation test.

Each well was examined microscopically for evidence of virus growth.

(i) Cell controls.

The 100% must show a confluent cell monolayer, gradually reducing to show no more than a few plaques at 12.5%.

(ii) Virus controls.

These must show the strength of each virus to be no more than one $\log_{10}$ step over or under the expected result.

(iii) Positive serum controls.

These should be within one dilution step of the expected result. Those showing neutralisation titres one step lower than this when the virus control is at the highest acceptable level are regarded as readable and the patients' serum titres adjusted accordingly. If the virus is showing low infectivity and the positive serum controls do not show an end point then the test results are invalid.

(iv) Patients' sera.

All serum controls should show confluent monolayers.

Occasionally, single wells may be toxic, giving the impression of virus growth, but if only one of the duplicate serum controls is toxic results were accepted. If both serum controls were toxic the serum was retested after further heat inactivation.

The end point of the patients' serum titration was taken as the highest dilution giving complete inhibition of viral CPE. This may be adjusted later by one dilution step either way depending on the results of the virus and positive serum controls.

Results.

Two relevant points of background information regarding the circulation of CBV in our community have already been established. Firstly there has been no major epidemic of CBV infection since 1965, when Coxsackie B5 was predominant both in Scotland and elsewhere in the United Kingdom (6,7). Secondly, CBV antibody studies of normal population groups between 1973 and 1978 showed that 10% had titres of 256 and 4% titres of $\geq$512 to one or more of the group B Coxsackieviruses (8), as revealed by recent studies in 1980-81 by O'Neil et al. (9). My own figures for 1984 in Table 2.3 indicate that this antibody status has remained relatively unchanged. The table lists the CBV

serotypes isolated during 1983 and 1984, and shows it is not unusual for one serotype to predominate in any particular year. Bell and McCartney (7) listed the serotypes isolated in the same community in the years 1972-1983 and although overall CBV types 4 and 2 were predominant there was no discernable pattern in the frequency of occurrence of any serotype.

Table 2.3 lists the total number of patients tested serologically in each of the two years and the percentage found positive by the criteria already given. The percentage positive for 1983, 34%, is similar to those recorded in previous years (7). In 1984 this figure rises to 46%. The reason for this apparent increase in positives in 1984 was that this laboratory was no longer able to cope with the escalating demands by clinicians for CBV serology. In order to reduce the number tested stricter criteria for the selection of patients for testing were imposed on clinicians. By this means the number of sera tested was reduced by over one third, and those selected were therefore more likely to produce a positive result.

Table 2.2.

CBV ISOLATES 1983 - 1984.

	B1	B2	B3	B4	B5	TOTAL
1983	10	0	1	3	0	13
1984	0	1	0	0	15	16

Table 2.3.

CBV SEROLOGY RESULTS 1983 - 1984.

		% POSITIVE		
	No. of	ANTIBODY TITRES OF:		TOTAL %
	patients	≥512	256	≥256
1983	2222	17	17	34
1984	1430	27	18	46
	Controls*			
1984	87	5	12	17

* Sera obtained from healthy adults in the West of Scotland, and donated by the Scottish serum bank, Ruchill hospital.

Significant rising or falling titres (≥4 fold) were found in only a few cases, in 6 in 1983 and 6 in 1984, most of whom had acute chest pain (pleurodynia) or acute myocarditis. This is not an unexpected result since cardiac disease is thought to be a late manifestation of viral infection (9, 10), but it indicates how difficult it is to obtain serum early enough to demonstrate significant rising antibody titres.

Table 2.4.

CBV serological results in various clinical categories 1983 - 1984.

	1983				1984			
Clinical category	No. tested	(%) ≥512	(%) 256	TOTAL (%) ≥256	No. tested	(%) ≥512	(%) 256	TOTAL (%) ≥256
Myo/Per	313	18	14	32	352	33	22	55
C.P.	597	16	17	33	374	23	19	42
Mya/Bor	254	22	19	41	164	24	15	39
M.E.	389	16	24	40	210	35	15	50
Misc	682	12	14	26	330	22	20	42

Myo/Peri = Myopericarditis. C.P. = Chest pain.

Mya/Bor = Myalgia/Bornholm. M.E. = Myalgic - encephalomyelitis

Misc = Miscellaneous.

Since the beginning of the late 1970's, interest has concentrated on patients with suspected viral heart disease. These were predominantly males in the 40-60 year age group. The results of studies on these patients, tested in 1983 and 1984, and classified on their admission diagnosis, are given in Table 2.4. Again, differences in the percentage positives between the two years are seen, particularly in the cardiac groups (myopericarditis and chest pain). The 1983 figures are similar to those found in routine testing in the years 1975-82 (7). The 1984 figures are much closer to those found in a six year controlled study of cardiac patients (3), in which 42%, 33% and 13% were found to have titres of $\geq$256 in the chest pain, myocarditis and pericarditis groups respectively. This difference between the 1983 & 1984 results is attributable to the better selection of patients rather than an increase in circulating CBV's in the community.

In 1980 there was a revival of interest in the possible viral aetiology of myalgic encephalo - myelitis (ME), previously known as Royal Free Disease after the outbreak in that London hospital in 1975 (12, 13). As a result the range of patients tested was widened to include those with such diverse symptomatology as general myalgia, chest pain with palpitations but without ECG changes, vertigo,

labyrinthitis, paraesthesia and extreme fatigue.

Requests for laboratory investigation of patients with non-cardiac disease now exceeded those with suspected cardiac illness. The percentage positives in the ME group with titres ≥256 were 40% in 1983 and 50% in 1984, again reflecting the better selection of cases by clinicians. In our 1983 study of 54 well documented cases of ME. 59% were found to have titres of ≥256 (8).

Selecting titres of ≥512 to one or more CBV, the most frequent response detected was to CBV 4 (Table 2.5), which, over the preceding years, had been the serotype most frequently isolated, although not as often as the serological results might imply.The figures for 1984 reflect better the circulating viruses, with increases over 1983 in CBV 1, 3 and 5 serotypes despite the fact that 33% fewer sera were examined. Since 1979 CBV 4 and, to a lesser extent, CBV 2 antibody responses have been those most commonly encountered (7). On the few occasions when a CBV was isolated, and paired acute and convalescent sera were available for study, the titre of the heterotypic antibody response equalled or even exceeded those of the homotypic antibody (Table 2.6). Thus in the absence of virus isolation, prediction of the infecting serotype in patients with multiple rising or static

high titres is almost impossible.

Table 2.5.

Frequency of CBV serotype responses* 1983 -1984.

	Serotypes					Dominant CBV serotype isolated in
Year	B1	B2	B3	B4	B5	the comunity
1983	18	134	17	255	3	CBV 1.
1984	70	53	35	180	47	CBV 5.

* = titres $\geq$ 512.

Table 2.6.

Examples of homotypic and heterotypic antibody to CBV infections.

Patient	Virus isolated from faeces	Antibody titres in acute(A) and convalescent(C) serum. CBV serotypes				
			B1	B2	B3	B4	B5
F26 years	CBV 1	A	<64	<64	<64	<64	<64
chest pain		C	256	512	256	512	256
M13 years	CBV 2	A	<64	<64	<64	64	<64
sore throat		C	<64	>1024	>1024	64	<64
M25 years	CBV 5	A	<64	<64	<64	<64	<64
meningitis		C	<64	<64	<64	<64	256

Foot note.

The acute and convalescent sera were a minimum of 7 days apart.

Discussion.

The CBV microtitre neutralisation test permits the testing of large numbers of sera not possible by conventional tube culture methods. This test gives accurate reproducible results provided adequate cell, serum and virus controls are included. The main problems are associated with the virus sensitivity of the cell cultures which may change after repeated subcultivations, while cell lines, although theoretically capable of an infinite number of subcultures, generally become unusable after 30-40 possibly due to mycoplasma infection from FCS or the experimenter/tissue culturist.

Antibody responses in the individual patient vary markedly and the clinician must interpret the virological results with careful regard to the patient's illness and onset date. Some patients may only achieve maximum titres of 128 (especially in the case of CBV 1, 3 and 5 serotypes), which fall short of the minimum "positive" level, and yet such titres may be relevant for such patients. In contrast, detection of high titres may be purely coincidental. Heterotypic antibody responses cause similar problems. In these neutralisation tests it has been observed that high titres to CBV 4 and CBV 2 appear together regularly, and a similar but less frequent cross

reaction was observed between CBV 1 and 3. CBV 5 showed the highest frequency of monotypic response. To some extent this phenomenon is due to cross reactions between common antigens on different viruses. Another explanation may be that because CBV 4 is dominant throughout the world, infection is acquired early in life with, or more often without, symptoms. Later infection with another CBV results in an anamnestic boost (recall) of the old CBV 4 antibody. This type of response is common in poliovirus and influenza virus infections.

In general, however, serological diagnosis based on high static titres have proved useful in clarifying the role of CBV in adult heart disease (8, 9). The findings reported here have been confirmed independently by other investigators (9, 14).

The association of Coxsackie B viruses with certain adult heart diseases is now well recognised. Other diseases, such as diabetes, pancreatitis and myalgic encephalomyelitis (ME), are now receiving closer scrutiny in respect of such an association. A similar pathology to many of these illnesses is seen when mice are infected with the viruses , so an associated role in human disease would not be totally unexpected.

Although serological testing for CBV neutralising antibody is time consuming and its limitations in respect of accurate virus diagnosis in the individual patient are considerable, the demand by clinicians for this service has increased annually. There is no specific antiviral therapy available, but some patients, often adults in the 30-40 year age group who are struck down by an illness mimicking a heart attack or with debilitating ME, apparently derive considerable psychological benefit if a presumptive diagnosis of CBV infection is made. Clinicians report that such a virus diagnosis often prevents these patients from becoming cardiac or psychiatric "cripples" and that they accept their illness with a much more positive attitude. The fact that stable high neutralising titres are so difficult to interpret, especially in chronic relapsing illnesses, has led to the postulation that detection of Coxsackie B specific IgM antibody would help in virological diagnosis for many patients. Detection of specific IgM in such patients implies recent infection or perhaps persisting viral antigen.

Several workers have devoted much time and effort towards the development of CBV specific IgM assays, using the modern virological technique of enzyme linked immunosorbent assay (ELISA) and radioimmunoassays (RIA) (15, 16, 17). All have detected nonspecific cross-reactions, some not only internally within the CBV group, but also externally with other enteroviruses. Attempts to apply some of these techniques in routine diagnostic testing are described in the following chapters.

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CHAPTER 3

The μ antibody capture ELISA for the detection of Coxsackie B virus (CBV) specific IgM antibody.

I. INTRODUCTION.

Humoral immunity following viral infection is dependent on the production of IgG, IgM and IgA antibodies. IgG and IgM are found circulating in the blood stream and are the main classes of antibodies detected by viral serology. IgA is a secretory antibody found at the site of viral replication and is not normally used as a marker of viral infection. Viral specific IgG antibody usually persists for life whereas antibody of the IgM type is short lived and characterises primary infection. It is possible that persistence or reactivation of virus in an active antigenic form may also be accompanied by a prolonged or recrudescent IgM antibody response. Thus tests for the detection of viral specific IgM offer the possibility of a rapid specific diagnosis on a single serum specimen sampled early after the onset of illness.

The technique known as ELISA was pioneered by Engvall et al.(1,2) and Van Weemen and Schuurs (3,4). It depends on the linkage of soluble antigens or antibody to a solid phase without altering significantly their immunological specificity or reactivity. Then soluble antibody or antigen, conjugated with an enzyme such as horseradish peroxidase or alkaline phosphatase, can be used for detection with the enzyme activity being demonstrated after the addition of chromogenic substrates.

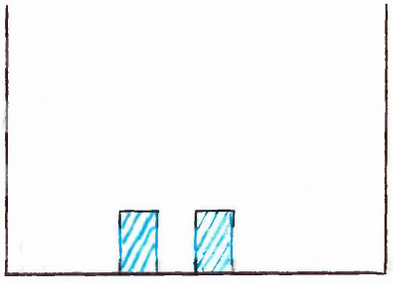
Various techniques employing this phenomenon have been described both for the detection of viral antigens and antibody.

The technique described in this chapter was developed by King et al. (5) for use in the evaluation of the role of CBV in the aetiology of juvenile onset diabetes mellitus.

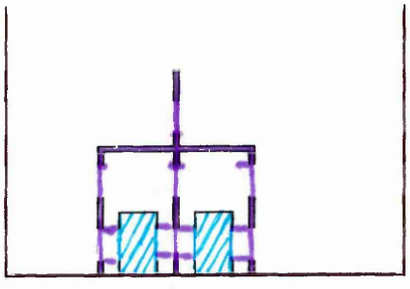
The scientific basis of the test is an amalgam of the two classical solid phase immunoassays, namely, indirect u antibody capture and the sandwich technique, which is summarised in Figure.3.1.

Figure 3.1

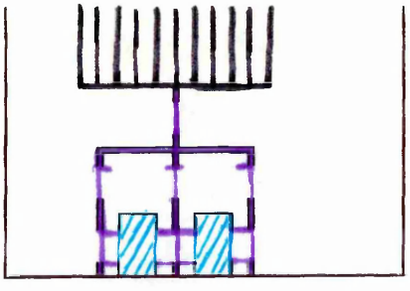
Antibody capture ELISA for the detection of CBV specific IgM.



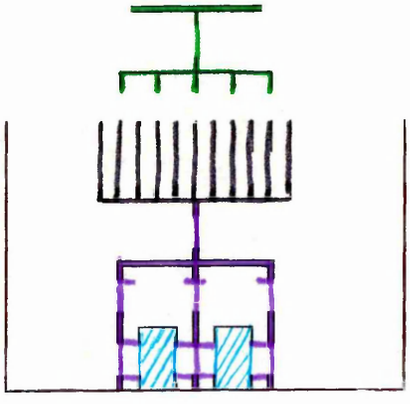
Wells of the plate coated with sheep anti - human IgM (u chain specific).



Patient's serum is added; any IgM antibody present is captured on to the solid phase.

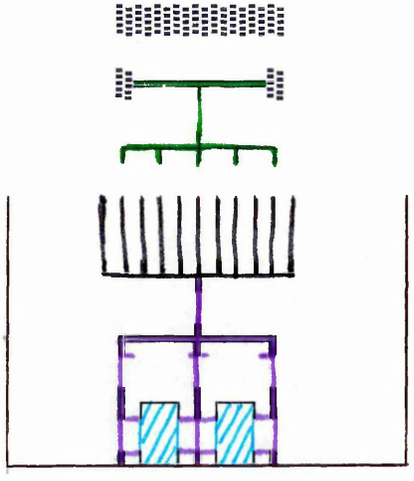


CBV single serotype is added. This will attach to any specific IgM antibody present.

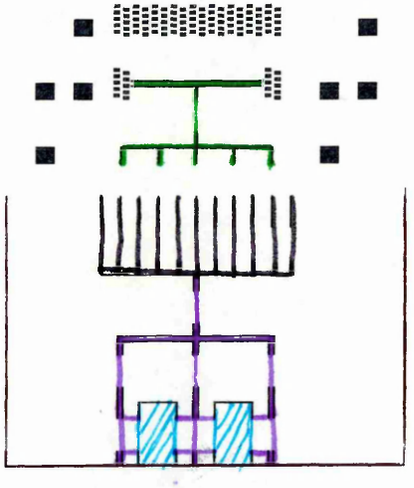


Mouse anti - CBV antiserum is added. This will link to any CBV present.

Figure 3.1 continued.



Goat-anti-mouse serum(IgG) conjugated to horseradish peroxidase is added; this will link to any mouse IgG present.



O - phenylenediamine 2HCl + H_2O_2 substrate is added. This will form a yellow colour in presence ofthe horseradish peroxidase. The intensity of the colour produced is directly proportional to linked goat-anti-mouse IgG, hence to the mouse anti-CBV IgG absorbed to its specific antigen.

Antihuman IgM u - chain specific, is coated onto the surface of microtitre wells. Patient's serum is added and any IgM present, regardless of specificity, will be captured by the anti - u antibody.

CBV is then added, and this will attach to any homologous CBV-specific IgM which has been captured onto the solid phase, making this a specific test for the virus. The presence of bound virus is detected using a mouse anti-CBV antiserum and a goat anti - mouse horseradish peroxidase conjugate in the classical patients serum-antigen-antiserum-antispecies serum sandwich. The intensity of the produced colour is therefore directly proportional to the amount of CBV specific IgM in the patient's serum.

II MATERIALS AND REAGENTS.

Microtitre plates.

These were ELISA grade flat bottomed polycarbonate microtitre plates (Dynatech Ltd.).

Coating antibody.

This was antihuman IgM (u chain specific), raised in sheep and affinity purified, and supplied in liquid form with 0.1% azide preservative, aliquoted and stored at -20°C. (Scottish Antibody Production Unit; SAPU.).

CBV antigens.

CBV serotypes 1-5 complement fixing antigens with minimum CF titres of 1 in 8, as determined by chessboard titration were used. These were supplied as a frozen suspension of live virus grown in Vero cells (M.A. Bioproducts, Maryland, USA), and stored at -70°C

Control antigen.

The antigen was prepared from uninfected Vero cells in exactly the same way as the CBV antigens and also supplied by M.A. Bioproducts.

CBV antisera.

The positive control antisera to CBV types 1-5 were prepared for complement fixation tests (CFT) with minimum CFT titres of 1 in 8 as determined by chessboard titration.(M.A. Bioproducts). All sera were raised in mice. These were aliquoted and stored at -20°C.

Conjugate.

This was horseradish peroxidase-conjugated antimouse serum raised in goats and absorbed with human serum proteins (Sigma Ltd.). It was aliquoted and stored at -20°C

Substrate.

This was crystaline O-phenylenediaminedihydrochloride (OPD),(Sigma Ltd.). It was stored dessicated at -20°C For use 100mg was dissolved in 100ml of substrate buffer(see below) and 0.8ml of 10 vol H_2O_2 added before use. The substrate was always prepared immediately before use.

Acid.

1N H_2SO_4 analar grade (BDH Ltd.) was employed for stopping development of the colour.

Buffer Solutions.

All chemicals used in the preparation of buffers were of Analar grade (BDH Ltd, Burntfield avenue, Thornliebank, Glasgow).

Coating buffer.

This was carbonate-bicarbonate buffer at pH 9.6. Its recipe was: 1.159g Na_2CO_3, 2.93g of $NaHCO_3$ and 0.2g of NaN_3 dissolved in a total of 1000ml of deionised water. Its pH was then checked and adjusted to pH 9.6 with carbonate or bicarbonate if required. If the working pH varied by more than 0.4 units, fresh buffer was prepared. The prepared buffer was stored at room temperature for up to 14 days.

Washing/serum buffer.

One tablet of phosphate buffered saline (Dulbecco's Solution A; Oxoid Ltd.) was dissolved in 100ml of deionised water and 0.1ml of Tween 20 (BDH Ltd.) added. The buffer was stored at room temperature for up to 14 days.

Test buffer.

Washing buffer with added 25% lamb serum (Gibco Ltd.), was freshly prepared daily from stocks of washing buffer. The lamb serum was stored at -20°C.

Substrate buffer.

This was phosphate citrate buffer at pH 5.0, prepared to the following recipe: 24.3ml of 0.1M citric acid and 25.7ml of 0.2M Na_2HPO_4 were added to 50ml of deionised water, and this was stored at 4°C for up to 14 days.

III STANDARDISATION OF TEST REAGENTS.

Patients' sera and CBV antisera were used at fixed dilutions of 1 in 400(5) The conjugate was used at the manufacturer's recommended optimal working dilution, usually 1 in 1000. The coating antibody and CBV antigens were titrated against each other to determine their optimum working dilutions. Each new batch of antigen was standardised using the determined working dilutions of the other reagents.

Coating antibody / CBV antigen titration.

(a) Serial doubling dilutions of coating antibody were prepared in coating buffer from 1/50 - 1/1600, and 100ul of each dilution was transferred into 4 columns of 12 wells of a microtitre plate (see Figure 3.2). The plates were covered with a loose fitting lid and incubated in a humidity chamber at 4°C over night.

Figure 3.2
Layout of plates for coating antibody/ CBV antigen titration

COATING ANTIBODY DILUTIONS

ANTIGEN DILUTION	1/50	1/100	1/200	1/400	1/800	1/1600
TTC	P P N N 1/5	P P N N 1/5	P P N N 1/5	P P N N 1/5	P P N N 1/5	P P N N 1/5
TTC	1/10	1/10	1/10	1/10	1/10	1/10
TTC	1/15	1/15	1/15	1/15	1/15	1/15
TTC	1/30	1/30	1/30	1/30	1/30	1/30

T = Test well
C = Control well

Steps
1. Add coating antibody diluted 1/50 - 1/1600.
2. Add known positive and negative patients serum at 1/400 to columns P(positive) and columns N(negative) respectivly.
3. Add CBV antigen to Test well rows and control antigen and control antigen to Control well rows at the dilutions shown.
4. Add homologous mouse anti-CBV serum at 1/400 to all wells
5. Add goat anti-mouse HRP conjugate at 1/1000 to all wells
6. Add substrate to all wells.
7. Stop the reaction after 10 - 20 mins and read plates.

The plates were then washed three times in washing buffer, as described below.

Washing procedure.

The microtitre plates were held upside down over a sink and the contents forcibly shaken out. All the wells were then filled with washing buffer and each plate allowed to soak for 60 seconds. This process was repeated three times. Following the third wash the plates were forcibly slapped on to absorbent paper towels to remove all excess liquid from the wells. The plates were **not** allowed to dry out before the next set of reagents were sequentially added, as follows:

(b) A known negative and positive human serum (originally gifted by Professor Banatvala, St. Thomas' Hospital, London) were diluted 1/400 in serum buffer; 100 ul of each serum was added to two columns of 12 wells for each dilution of coating antibody.

The plates were incubated in a humidity chamber for 3 hours at 37°C, and then washed as before.

(c) CBV antigens and control antigens were diluted 1/5, 1/10, 1/15 and 1/30 in test buffer, and 100 ul of each dilution of CBV antigen was added to 4 columns of 2 wells for each dilution of coating antibody, while 100 ul of the appropriate dilution of control antigen was added to the third well of each column.

Thus each microtitre plate was divided into 8

sections of 12 wells, each section representing one dilution of CBV antigen tested against one dilution of coating antibody using duplicate positive and negative human sera.

The plates were then incubated overnight at 4°C in a humidity chamber, and then washed as before. Next 100 ul of a 1/400 dilution of the appropriate CBV antiserum in test buffer was added to all test and control wells. The plates were incubated in a humidity chamber at 37°C for 2 hours and washed.

Conjugate, at its working dilution of 1/1000 in test buffer , was added in 100 ul amounts to all the test and control wells. The plates were again incubated in a humidity chamber at 37°C for 3 hours, and then washed.

Substrate was prepared as above and 100 ul added to all the test and control wells, while 100 ul of substrate was also added to one row of eight wells of a fresh microtitre plate to be used as blanks for the plate reader. The plates were then incubated in the dark at room temperature for 15 minutes. The reaction was stopped by adding 100 ul of HCl to all the wells, including the blanks.

Plate readings.

Using a Titertek Multiskan MC Spectrophotometer on a dual wavelength programme the plates were read at A_{450} using an interference filter of A_{640}. The reading was presented on a scale between 0-2.000. The result for each serum was taken as the mean of the two CBV antigen readings minus the reading of the control well. Computation of the final results was done on a BBC micro computer interfaced with the Multiskan, the commercially purchased programme giving a printout in numerical sequence of readings on an Epsom FX-80 dot matrix printer. (Format BOB, Figures 3.3 and 3.4).

Results.

Figure 3.5 illustrates results obtained when titrating coating antibody and CBV 5 antigen. The graph shows the absorbency values of each dilution of CBV 5 (positive serum means) plotted against the varying dilutions of coating antibody. The peak of each coincides with a coating antibody dilution of 1/400. This dilution was thereafter used in the subsequent routine ELISA test.

Figure 3.3.
Antihuman IgM/CBV 5 Titration.
Print out of results.

FORMAT BOB 12/05/86.

SAMPLE NUMBER	SAMPLE NAME	BLOCK	AVERAGE VALUE	No.OF WELLS	STD DEV	ANTIGEN DILUTION	
1	1	H1-H2	0.625	2	n<3		
2	1C	H3-	0.007	1	n<3		
3	2	G1-G2	0.614	2	n<3		
4	2C	G3-	0.008	1	n<3	1/5	
5	3	F1-F2	0.009	2	n<3		
6	3C	F3-	0.007	1	n<3		
7	4	E1-E2	0.009	2	n<3		A
8	4C	E3-	0.008	1	n<3		N
9	5	H4-H5	0.395	2	n<3		T
10	5C	H6-	0.005	1	n<3		I
11	6	G4-G5	0.398	2	n<3		H
12	6C	G6-	0.007	1	n<3	1/10	U
13	7	F4-F5	0.010	2	n<3		M
14	7C	F6-	0.006	1	n<3		A
15	8	E4-E5	0.008	2	n<3		N
16	8C	E6-	0.006	1	n<3		Ig
17	9	H7-H8	0.231	2	n<3		M
18	9C	H9-	0.008	1	n<3		
19	10	G7-G8	0.237	2	n<3		1/
20	10C	G9-	0.007	1	n<3		50
21	11	F7-F8	0.009	2	n<3	1/15	
22	11C	F9-	0.008	1	n<3		
23	12	E7-E8	0.011	2	n<3		
24	12C	E9-	0.007	1	n<3		
25	13	H10-H11	0.110	2	n<3		
26	13C	H12-	0.007	1	n<3		
27	14	G10-G11	0.106	2	n<3		
28	14C	G12-	0.008	1	n<3	1/30	
29	15	F10-F11	0.008	2	n<3		
30	15C	F12-	0.009	1	n<3		
31	16	E10-E11	0.008	2	n<3		
32	16C	E12-	0.008	1	n<3		
33	17	D1-D2	0.698	2	n<3		
34	17C	D3-	0.010	1	n<3		
35	18	C1-C2	0.664	2	n<3		
36	18C	C3-	0.007	1	n<3	1/5	
37	19	B1-B2	0.011	2	n<3		
38	19C	B3-	0.009	1	n<3		
39	20	A1-A2	0.011	2	n<3		
40	20C	A3-	0.006	1	n<3		

Figure 3.3 continued.

41	21	D4-D5	0.450	2	n<3		
42	21C	D6-	0.007	1	n<3		A
43	22	C4-C5	0.428	2	n<3		N
44	22C	C6-	0.007	1	n<3	1/10	T
45	23	B4-B5	0.011	2	n<3		I
46	23C	B6-	0.006	1	n<3		
47	24	A4-A5	0.009	2	n<3		H
48	24C	A6-	0.006	1	n<3		U
49	25	D7-D8	0.292	2	n<3		M
50	25C	D9-	0.008	1	n<3		A
51	26	C7-C8	0.260	2	n<3		N
52	26C	C9-	0.008	1	n<3	1/15	Ig
53	27	B7-B8	0.008	2	n<3		M
54	27C	B9-	0.008	1	n<3		
55	28	A7-A8	0.008	2	n<3		1/
56	28C	A9-	0.008	1	n<3		100
57	29	D10-D11	0.133	2	n<3		
58	29C	D12-	0.008	1	n<3		
59	30	C10-C11	0.128	2	n<3		
60	30C	C12-	0.009	1	n<3	1/30	
61	31	B10-B11	0.008	2	n<3		
62	31C	B12-	0.007	1	n<3		
63	32	A10-A11	0.008	2	n<3		
64	32C	A12-	0.006	1	n<3		

Figure 3.4.

Antihuman IgM/CBV 5 titration.

Antigen		Antihuman IgM.					
dilution.		1/50	1/100	1/200	1/400	1/800	1/1600
1/5	p	618	688	733	861	761	695
	p	606	657	788	862	743	589
	n	002	002	005	006	005	006
	n	001	005	006	006	006	006
1/10	p	390	443	427	551	441	348
	p	391	421	500	546	453	347
	n	004	005	004	005	005	006
	n	002	003	005	005	006	006
1/15	p	223	284	289	319	261	182
	p	230	252	302	313	264	184
	n	004	000	002	002	006	004
	n	001	000	003	001	005	006
1/30	p	103	115	125	139	110	076
	p	098	119	132	139	112	084
	n	001	001	001	001	004	007
	n	000	002	000	002	003	004

p = positive control serum.

n = negative control serum.

Mouse antiserum used at 1/400

Conjugate used at 1/1000

All OD readings given in the following tables, graphs and figures other than those which are actual computer or reader printouts are expressed as units on a scale 0 - 2000.

Unit = OD reading x 1000.

Figure 3.5
Anti human IgM/CBV 5
Positive control sera.

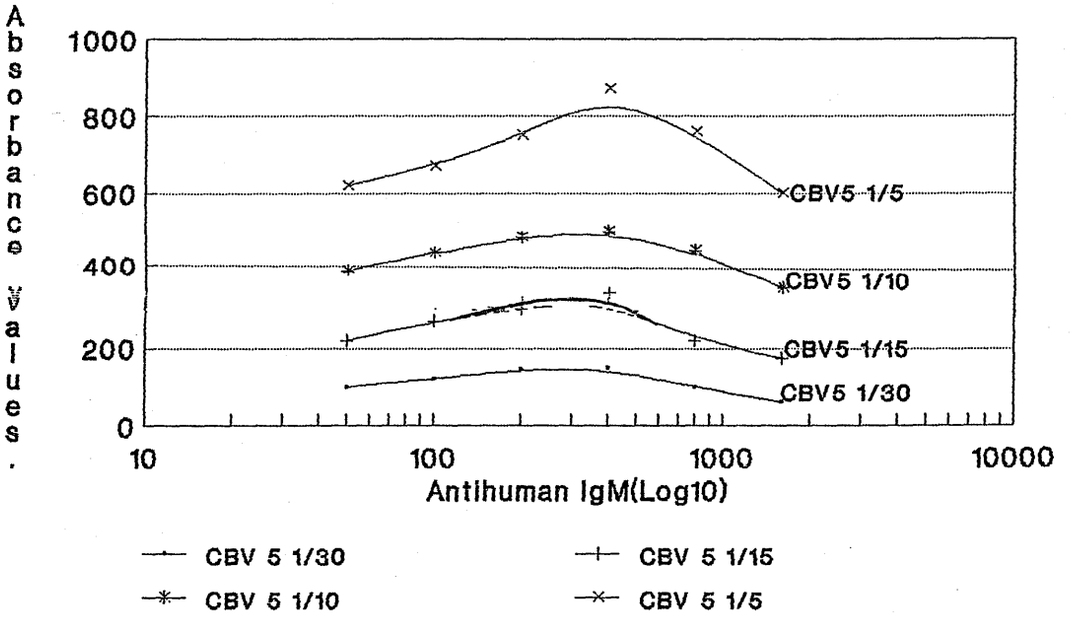
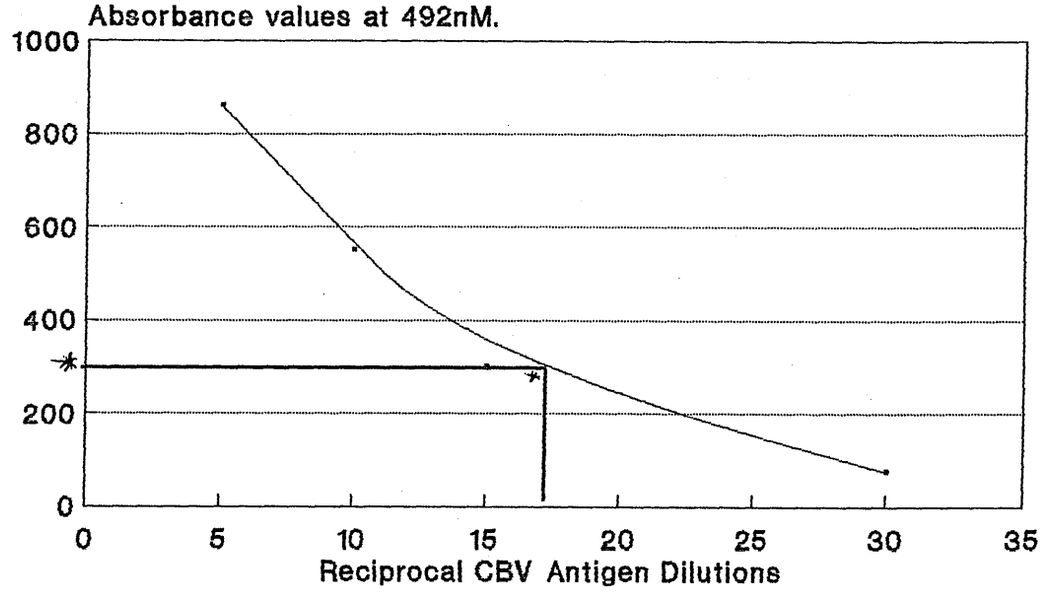


Figure 3.6.
Antihuman IgM(1/400)/CBV 5 titration



The solid horizontal line* marks the optimal OD for this serum of 0.300 giving a CBV 5 antigen dilution of 1/17.

Using coating antibody at 1/400, Figure 3.6 shows the absorbency values of the positive serum versus the different CBV 5 concentrations. The optimal absorbency value of this particular serum is 0.300 +/- 20% (as predicted from repeat routine testing). Reading from the graph, a dilution of between 1/14 and 1/17 would give the desired result. Consequently a dilution of 1/15 was selected for use in the routine ELISA test.

Frequent testing of numerous batches of commercial antigens showed that most had an optimal using dilutions of 1/15, test dilutions lower than this were not found. Because of this it was decided to use the commercial antigens at 1/15 without pretesting, saving approximately £100 per batch of 5 CBV antigens purchased. Only when positive and negative control sera gave inconsistent results were the CBV antigens re-titrated.

Coating antibody was much more variable, and consequently each batch was titrated against a single CBV antigen before use in the routine ELISA test.

2.PREPARATION OF IN-HOUSE CBV ANTIGENS.

Due to the high cost of commercial CBV antigens (a cost of £800 for 5ml each of CBV 1-5, plus 5ml of control antigen) it was decided that the preparation of in-house antigens should be attempted.

I Materials and reagents.

Cell cultures

Vero cells were grown in 175 cm^2 plastic tissue culture flasks (NUNC), as described in Chapter 2.

PBS

This was Dulbecco's phosphate buffered saline solution A with 0.1% phenol red indicator (GIBCO Ltd).

Medium

Eagle's MEM with 5% lamb serum* and 1% penicillin/streptomycin solution was employed. The antibiotic solution incorporated gave 10,000 iu penicillin / 10,000ug streptomycin per ml. (GIBCO Ltd).

Salt solution

This was Dulbecco's solution B (Oxoid Ltd), which contained calcium and magnesium ions.

Seed virus

This was cell lysates of Coxsackie B virus types 1-5 supplied as complement fixing antigens (M.A. Bioproducts).

Footnote. *Lamb serum was chosen as it was used in the ELISA test as a blocking agent, thus its use here would reduce the risk of non-specific reactions in the ELISA test with in-house antigens.

Preparation.

Confluent monolayers of Vero cells were washed three times with PBS and inoculated with 0.5ml of seed virus in 20ml of PBS (containing 1% salt solution). The virus was allowed to absorb onto the cells for 2 hours at 37°C. Control antigen was prepared in parallel using 20ml of PBS/1% salt solution only. The inocula were removed and replaced by 20ml of maintenance medium, and the flasks were reincubated at 37°C until complete (100%) cytopathic effect was observed (18-36 hours). The flasks were then frozen to -20°C and thawed at room temperature three times. The resultant preparation was aliquoted and stored at -70°C.

Standardisation.

Antigens, prepared and stored as described above, were tested at varying dilutions against known positive and known negative human sera in duplicate. All other reagents were used at their optimal working dilutions. Incubation times , temperatures and washing procedures were all as previously described in this chapter.

One microtitre plate was sufficient for each antigen titration. The dilutions ranged from 1/2 - 1/24 . The layout of a typical test (CBV 1 and CBV 4 antigen titrations) is shown in Figure 3.7. The print-out obtained for the CBV 1 antigen in given in

Figure 3.8. The final results were then transferred to worksheets (Figure 3.9).

The average OD value for the positive serum at each of the CBV antigen dilutions was then calculated and a graph of the OD values against CBV antigen dilutions plotted (Figure 3.10). The optimum CBV antigen concentration was taken as the highest dilution giving the expected OD value for the positive serum used in the test. Figure 3.10 shows the graphs obtained for CBV 1 batch RH 1/86 and CBV 4 batch RH 1/86. The expected OD value for each serum was 0.500 ± 20%, which gave working dilutions of 1/6 and 1/10 for CBV 1 RH 1/86 and CBV 4 RH 1/86 respectively.

Figure 3.7

Titration of In - House CBV Antigens.

ANTIGEN DILUTIONS		H	G	F	E	D	C	B	A		ANTIGEN DILUTIONS
	1	T	T	T	T	T	T	T	T		
1/2	2	T	T	T	T	T	T	T	T	1/12	
	3	C	C	C	C	C	C	C	C		
	4	T	T	T	T	T	T	T	T		
1/4	5	T	T	T	T	T	T	T	T	1/16	
	6	C	C	C	C	C	C	C	C		
	7	T	T	T	T	T	T	T	T		
1/8	8	T	T	T	T	T	T	T	T	1/20	
	9	C	C	C	C	C	C	C	C		
	10	T	T	T	T	T	T	T	T		
1/10	11	T	T	T	T	T	T	T	T	1/24	
	12	C	C	C	C	C	C	C	C		
		P	P	N	N	P	P	N	N		
		1/400 coating antibody				1/400 coating antibody					

T = CBV Antigen
C = Control Antigen
P = Positive serum
N = Negative serum

Figure 3.8

Titration of In-House CBV Antigens .
Print out of results.

FORMAT BOB 15/09/86.

SAMPLE NUMBER	SAMPLE NAME	BLOCK	AVERAGE VALUE	No.OF WELLS	STD DEV	CBV ANTIGEN DILNS	
1T	1	H1-H2	0.677	2	n<3	P	
2C	1C	H3-	0.009	1	n<3	P	
3T	2	G1-G2	0.656	2	n<3	P	
4C	2C	G3-	0.011	1	n<3	P	1/2
5T	3	F1-F2	0.018	2	n<3	N	
6C	3C	F3-	0.008	1	n<3	N	
7T	4	E1-E2	0.017	2	n<3	N	
8C	4C	E3-	0.007	1	n<3	N	
9T	5	H4-H5	0.562	2	n<3	P	
10C	5C	H6-	0.002	1	n<3	P	
11T	6	G4-G5	0.522	2	n<3	P	
12C	6C	G6-	0.000	1	n<3	P	1/4
13T	7	F4-F5	0.013	2	n<3	N	
14C	7C	F6-	0.000	1	n<3	N	
15T	8	E4-E5	0.012	2	n<3	N	
16C	8C	E6-	0.001	1	n<3	N	
17T	9	H7-H8	0.389	2	n<3	P	
18C	9C	H9-	0.011	1	n<3	P	
19T	10	G7-G8	0.382	2	n<3	P	
20C	10C	G9-	0.010	1	n<3	P	1/8
21T	11	F7-F8	0.013	2	n<3	N	
22C	11C	F9-	0.009	1	n<3	N	
23T	12	E7-E8	0.011	2	n<3	N	
24C	12C	E9-	0.009	1	n<3	N	
25T	13	H10-H11	0.312	2	n<3	P	
26C	13C	H12-	0.016	1	n<3	P	
27T	14	G10-G11	0.318	2	n<3	P	
28C	14C	G12-	0.013	1	n<3	P	1/10
29T	15	F10-F11	0.013	2	n<3	N	
30C	15C	F12-	0.014	1	n<3	N	
31T	16	E10-E11	0.010	2	n<3	N	
32C	16C	E12-	0.011	1	n<3	N	

Fig 3.8 continued.

33T	17	D1-D2	0.344	2	n<3	P
34C	17C	D3-	0.008	1	n<3	P
35T	18	C1-C2	0.346	2	n<3	P
36C	18C	C3-	0.011	1	n<3	P 1/12
37T	19	B1-B2	0.014	2	n<3	N
38C	19C	B3-	0.010	1	n<3	N
39T	20	A1-A2	0.012	2	n<3	N
40C	20C	A3-	0.009	1	n<3	N
41T	21	D4-D5	0.227	2	n<3	P
42C	21C	D6-	0.002	1	n<3	P
43T	22	C4-C5	0.224	2	n<3	P
44C	22C	C6-	0.002	1	n<3	P 1/16
45T	23	B4-B5	0.011	2	n<3	N
46C	23C	B6-	0.002	1	n<3	N
47T	24	A4-A5	0.011	2	n<3	N
48C	24C	A6-	0.001	1	n<3	N
49T	25	D7-D8	0.207	2	n<3	P
50C	25C	D9-	0.009	1	n<3	P
51T	26	C7-C8	0.202	2	n<3	P
52C	26C	C9-	0.010	1	n<3	P 1/20
53T	27	B7-B8	0.012	2	n<3	N
54C	27C	B9-	0.009	1	n<3	N
55T	28	A7-A8	0.011	2	n<3	N
56C	28C	A9-	0.008	1	n<3	N
57T	29	D10-D11	0.184	2	n<3	P
58C	29C	D12-	0.010	1	n<3	P
59T	30	C10-C11	0.182	2	n<3	P
60C	30C	C12-	0.010	1	n<3	P 1/24
61T	31	B10-B11	0.011	2	n<3	N
62C	31C	B12-	0.011	1	n<3	N
63T	32	A10-A11	0.010	2	n<3	N
64C	32C	A12-	0.011	1	n<3	N

T = CVB Antigen
C = Control antigen
P = Positive Serum
N = Negative Serum

FORMAT BOB = Self programmed Format on BBC microcomputer.

Figure 3.9.

Titration of In-House CBV Antigens

RH 1/86 CBV1 and CBV4 Titration.

SERUM	Antigen Dilution	ANTIGEN B1	ANTIGEN B4
P		668	829
P	1/2	645	853
N		010	018
N		010	017
P		560	788
P	1/4	522	759
N		013	015
N		011	015
P		378	568
P	1/8	372	552
N		004	014
N		002	014
P		296	504
P	1/10	305	490
N		-001	013
N		001	014
P		336	430
P	1/12	335	427
N		004	014
N		003	013
P		225	346
P	1/16	222	354
N		009	013
N		010	014
P		198	264
P	1/20	192	277
N		003	011
N		003	012
P		174	239
P	1/24	172	234
N		000	011
N		-001	011

P=POSITIVE SERUM

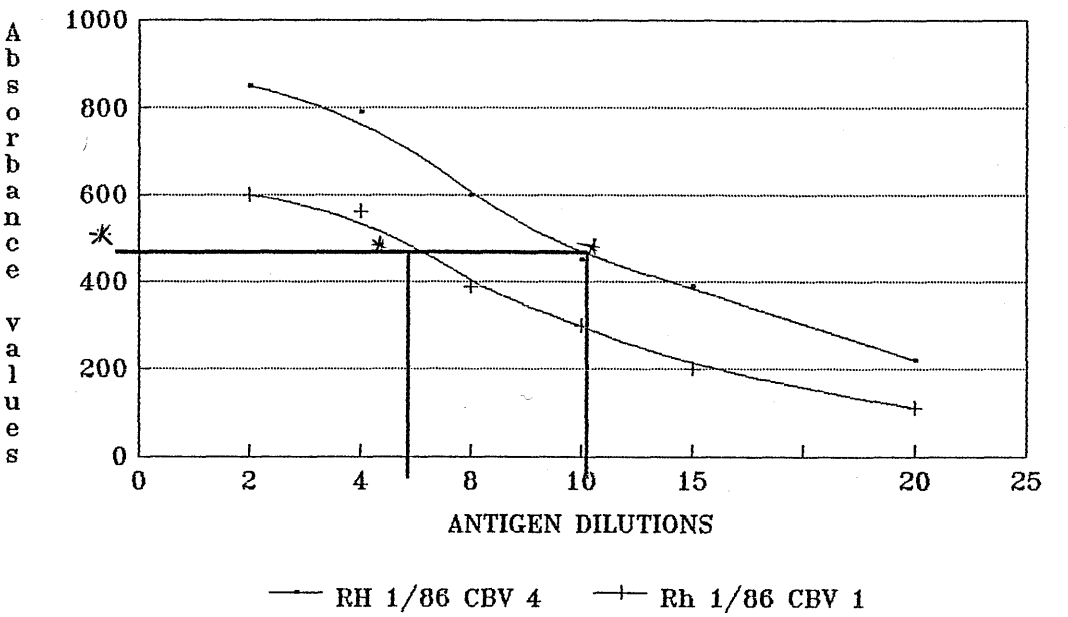
N=NEGATIVE SERUM

Antihuman IgM used at 1/400.

Mouse anti CBV serum at 1/400

Conjugate (SIGMA) at 1/1000.

Figure 3.10
Titration of In-house CBV antigens
RH 1/86 CBV 1 and RH 1/86 CBV 4



Foot Note

The solid horizontal lines* mark the optimal OD readings for the positive sera used; giving dilutions of 1/5 for CBV 1 and 1/10 for CBV 4.

Evaluation of in-house CBV antigens.

A panel of 16 sera known to give a reasonable array of positive(+ to +++), weak positive(±) and negative(-) results with commercial antigens in routine testing was prepared (Fig 3.11, A and B). Each of these sera was tested in duplicate against the in-house CBV antigens, prepared in this laboratory, used at their optimal dilutions. The results obtained were then compared with those obtained with commercial antigens, because a good correlation (100%) with the positive and negative sera with a variety of antigens was essential. Weak positive reactions (±) were less reliable and an 80% or better correlation was acceptable. In-house CBV antigens meeting these requirements were aliquoted in volumes sufficient for a single batch of tests and stored at -70°C until required.

Figure 3.11A

Standardisation of In - House antigens.

Panel of Quality Control sera.

I.D. No.	Expected B1 result, Commercial Antigen	B1 reading, In - house Antigen	B1 result, In - house Antigen
1	-	001	-
2	-	011	-
3	-	002	-
4	-	000	-
5	-	003	-
6	+	031	+
7	+++	282	++
8	-	003	-
9	++	233	++
10	+	057	+
11	-	003	-
12	+/-	018	Eq
13	-	002	-
14	-	003	-
15	-	002	-
16	-	003	-

Figure 3.11A (continued)

Standardisation of In - House antigens.

Panel of Quality Control sera.

I.D. No.	Expected B1 result, Commercial Antigen	B1 reading, In - house Antigen	B1 result, In - house Antigen
1	-	001	-
2	-	009	-
3	-	003	-
4	-	001	-
5	-	002	-
6	+	033	+
7	+++	284	++
8	-	002	-
9	++	233	++
10	+	049	+
11	-	000	-
12	+/-	019	Eq
13	-	002	-
14	-	003	-
15	-	000	-
16	-	004	-

Figure 3.11B.

Standardisation of In - House antigens.

Panel of Quality Control sera.

I.D. No.	Expected B4 result, Commercial Antigen	B4 reading, In - house Antigen	B4 result, In - house Antigen
1	-	009	-
2	-	015	-
3	+	049	+
4	-	010	-
5	-	007	-
6	+++	615	+++
7	++	375	++
8	-	018	Eq
9	+	168	++
10	+	071	+
11	-	008	-
12	+/-	024	+/-
13	-	007	-
14	-	005	-
15	-	009	-
16	-	004	-

Figure 3.11B (continued)

Standardisation of In - House antigens.

Panel of Quality Control sera.

I.D. No.	Expected B4 result, Commercial Antigen	B4 reading, In - house Antigen	B4 result, In - house Antigen
1	-	005	-
2	-	012	-
3	+	044	+
4	-	008	-
5	-	003	-
6	+++	579	+++
7	++	340	++
8	-	006	-
9	+	164	++
10	+	070	+
11	-	007	-
12	+/-	021	+/-
13	-	006	-
14	-	004	-
15	-	009	-
16	-	005	-

3. THE SELECTION AND USE OF CONTROL SERA.

The selection of good control human serum for ELISA tests is critical if the test system is to be successful. Three known positive sera (one high, one medium and one low) and five negative control sera were titrated against each CBV antigen tested. Earlier experiments had shown that one set of control sera per batch of tests , titrated in a separate plate, was inadequate because of between-plate variation, and variations in reading times. Total confidence in the results was only gained by including controls on each plate(ie; within-plate controls).

Initially CBV IgM positive control sera were obtained from Professor Banatvala of St.Thomas' Hospital London. These were used as controls in ELISA tests done with selected sera with known rising titres (>4 fold) or high (>512) titres provided by the conventional neutralisation tests. From the results, sera giving a variety of positive responses to one (monotypic) or more (heterotypic) CBV serotypes were selected for use as control sera. Such sera were:
1. those giving a monotypic response and absorbency values of between 0.300 - 0.600 to each CBV serotype (B 1 - 5)= high positive control: 2. a serum that gave absorbency values of between 0.070 - 0.150 to all 5 CBV serotypes (heterotypic) = medium positive control:

3. one serum for each CBV serotype that gave absorbency values of between 0.030 - 0.050 (monotypic) = low positive control. Each positive control serum selected was retested in duplicate against CBV 1-5 to confirm the reproducibility of results before being used in the test proper. All the positive control sera were stored at 4°C for up to 4 weeks.

Sera from the above tests which gave absorbency values of between 0.007 - 0.015 against all 5 CBV serotypes were then pooled. This negative control pooled sera was first retested in quintuplicate against all 5 CBV serotypes to ensure that it was consistently negative, and then aliquoted in 0.2ml volumes and stored at -20°C.

A continuing supply of positive control sera was obtained by careful selection from the routine tests on patients' sera. This avoided undesirable variations in levels of IgM which might result from long - term storage of any selected sera. Control sera in use at any one time were used in all titrations of CBV antigens and other reagents.

4 THE DEVELOPED ROUTINE TEST METHOD.

Day 1.

Coating of plates. Anti human IgM was diluted to the test dilution in coating buffer, and 100ul was added to all wells of 5 microtitre plates. This allowed 24 test sera and 8 control sera to be tested against all 5 CBV serotypes, employing one antigen per plate. The plates were incubated overnight at 4°C in a humidity chamber. Plates may be left at this stage for up to 72 hours before further use without adversely affecting the final results of the test.

Day 2.

Worksheets were prepared with lists of the control and patients sera to be tested . A 1/400 dilution of each test and control serum was made in washing buffer (0.025ml sera + 10ml buffer). Coated plates from **day** 1 were washed three times in washing buffer and 100ul of each diluted serum was added to a column of 3 wells on each plate (see Figure.3.12). The plates were then covered and incubated at 37°C for three hours.

CBV and control antigens were diluted to test dilutions in test buffer. The plates were washed (as above) and 100 ul of a CBV antigen was added to the first 2 of the 3 serum wells on its appropriate plate

and 100ul of control antigen was added to the third well of each column on all 5 plates. The plates were then incubated at 4°C overnight in a humidity chamber.

Day 3.

CBV antiserum dilutions were prepared in test buffer. The plates were washed (as above) and 100ul of antiserum was added to all the wells of the appropriate plates (see Figure 3.11C). The plates were then incubated for 2 hours at 37°C in a humidity chamber.

Conjugate was next diluted to its working dilution in test buffer. The plates were washed (as above) and 100ul of diluted conjugate was added to every well. The plates were then incubated at 37°C for 3 hours in a humidity chamber.

At the end of this incubation period the substrate was prepared and stored in the dark at room temperature until used (for a maximum period of 10 - 15 minutes). The plates were then washed (as above) and 100ul of substrate was added to every well together with a column of 8 wells in a new blank plate. The plates were incubated at room temperature in the dark for 15 minutes. All reaction was stopped by then adding 100ul of 1N H_2SO_4 to every well, including those in the new blank plate.

The plate reader and computer were next prepared for use. The reader was zeroed using the blank plate

before the readings of the test plates were made. The raw data were processed using the computer programme and the final results were transferred to the worksheets.

Further batches of patients sera were readily tested on a rotational basis e.g., start Day 2 - finish Day 4; start Day 3 - finish Day 5; coat Day 5(for 72 hours) - finish Day 2.

Figure 3.12.

Routine test method.

P1	P2	P3	N	N	N	N	N	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
P1	P2	P3	N	N	N	N	N	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
P1	P2	P3	N	N	N	N	N	C	C	C	C	C	C	C	C	S	S	S	S	S	S	S	S
X1	X2	X3	X4	X5	X6	X7	X8	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
1	2	3	4	5	6	7	8	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
1	2	3	4	5	6	7	8	C	C	C	C	C	C	C	C	S	S	S	S	S	S	S	S
X9	10	11	12	13	14	15	16	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
X9	10	11	12	13	14	15	16	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
X9	10	11	12	13	14	15	16	C	C	C	C	C	C	C	C	S	S	S	S	S	S	S	S
17	18	19	20	21	22	23	24	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
17	18	19	20	21	22	23	24	T	T	T	T	T	T	T	T	S	S	S	S	S	S	S	S
17	18	19	20	21	22	23	24	C	C	C	C	C	C	C	C	S	S	S	S	S	S	S	S
			A								B						C	

P = Positive control(1 - 3)sera.

N = Negative control sera.

X = Patients sera (1 - 24)

T = CBV antigen(1 - 5)

C = Control antigen.

S = Coxsackie B antiserum. (Homologous; one per plate).

A = patients serum added to plate.

B = Coxsackie B and control antigen added to plate.

C = mouse - anti - Coxsackie B virus serum added to plate.

Note for figure 3.12. One plate was used for each virus serotype CBV1 - 5. Positive and negative control sera were included in every plate, and five plates were required to test 24 patients sera against all 5 viruses.

Determination of results

The mean reading of the 5 negative control sera plus 3 standard deviations was used as a basis for determining a cut off value. A minimum cut-off of 0.020 was invariably set.

Sera giving a reading lower than this were considered negative, while sera with a reading higher than the cut-off but less than two times this value were regarded as weak (±) positive and were re-tested to ensure reproducibility. Those sera giving a value greater than two times the cut-off were regarded as positive and were further graded from 1+ to 4+ depending on their test absorbency value, according to the following scale :-

1+ = 0.040 to 0.100

2+ = 0.101 to 0.500

3+ = 0.501 to 1.000

4+ = 1.001 to 2.000

Discussion.

The detection of viral specific IgM antibody by the ELISA technique is gradually replacing the more cumbersome haemagglutination inhibition, complement fixation and neutralisation tests as the standard technique of coxsackie B diagnostic viral serology.

The Coxsackie B IgM ELISA test described in this chapter has the potential for replacing the current neutralisation test, and the test protocol may also prove similarly useful in investigating the role of other enteroviruses in other disease syndromes.

The test described here can readily be used for testing large numbers of patients' sera and has the advantage over neutralisation tests in that (a) a positive result represents a recent or an active infection, (b) only one serum sample per patient needs to be tested when obtained at the correct time in the illness, (c) results can be available within 48 hours of receipt of the sample at the test laboratory.

The high cost of the ELISA reagents needs to be weighed against the more labour intensive neutralisation tests. Further, ELISA tests offer speedy and significant results. The cost of the tests can be reduced dramatically by preparing "in-house" antigens, since the relatively crude preparations described in this chapter have proved both useable and reliable.

Further purification of the antigens, involving high speed clarification and sedimentation followed by fractionation on sucrose and caesium chloride gradients have shown no significant advantage over the described unpurified in-house antigens. Further, the time and cost of reagents used to purify antigens has tended to cancel out any financial advantage offered over commercial preparations.

Freeze-dried antigen preparations, supplied by many commercial firms for use in the Coxsackie B virus complement fixation test, were found to be unsuitable for the ELISA test since they failed to provide positive test results when they were titrated against positive sera.

The application of this CBV IgM ELISA for the routine diagnosis of Coxsackie B infections is described in detail in chapter 4.

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CHAPTER 4

Use of the u - antibody capture ELISA in the routine diagnosis of CBV infections.

I. Introduction.

Coxsackie B viruses (CBV) are normally endemic in the United Kingdom. They are associated with a wide spectrum of illness including aseptic meningitis, Bornholm disease, and myopericarditis. Isolation of virus from cases other than aseptic meningitis and Bornholm disease has generally been unsuccessful, and the identification of a possible viral cause has depended upon serological testing. Neutralisation tests (NT), and, to a lesser extent, complement fixation tests (CFT) have been the two main techniques used for the serological diagnosis of CBV infections. The more recent development of an enzyme-linked immunosorbent assay (ELISA) technique for the detection of CBV specific IgM (1) has added a new impetus to the serological investigation of CBV infections. I have evaluated this newly developed technique as it would apply to the daily routine of a serology laboratory and used it for the serological diagnosis of patients with cardiac and other illnesses

of suspected CBV aetiology.

II Materials and methods.

Patients studied. Most of these patients were from the West of Scotland, with others from East Scotland and occasionally from England. The clinical classification of patients was based entirely on information provided by hospital clinicians and general practitioners on requested-information cards accompanying specimens to the laboratory. Single convalescent sera were most commonly submitted for CBV investigations, the majority being from adults in the 25-55 year age group. Follow-up serum samples were requested from certain patients in order to monitor the duration of CBV IgM antibody. Sera from controls were obtained from apparently healthy adults in the West of Scotland, sampled over the same period of time.

ELISA tests. The u-antibody capture technique described in chapter 3 was used. Plates were coated with sheep anti-human IgM (u-chain specific), and each serum tested at a dilution of 1/400 in duplicate against viral antigens CBV 1-5 and in parallel with control antigen. Three positive control sera (high, medium and low) and five negative control sera were included for each CBV antigen. The result for each serum was taken as the mean absorbence value of the two

test wells minus the absorbence of the control antigen well. The cut-off point was taken as the negative serum control mean plus three standard deviations. Sera giving a reading higher than this, but below a value of 2 times the cut off were regarded as weak positive (±); sera with readings of >2 times the cut off were regarded as positive and graded 1+ to 4+ (see page 96).

Neutralisation tests. Initially sera were tested by neutralisation test (NT). The microtitre neutralisation test described in chapter 2 was then used. The end points of the serum titrations were determined by microscopic examination for cytopathic effect rather than by colour change. Static titres of ≥512 or a four fold rise or fall in titre were regarded as indicative of recent CBV infection while titres of 256 were only suggestive of this. Both positive and negative sera were selected for CBV IgM estimations. In later test series sera were first screened by ELISA and only positives were tested by NT.

III. Results.

During 1984, before use of the CBV IgM ELISA test system, 1430 patients and a control group of 87 healthy adults were tested by conventional NT. The results (Table 4.1) reveal that the % positives (titres ≥256) in each clinical category was higher than those

recorded for equivalent disease categories studied during 1983 (2).

Table. 4.1.

Results of CBV Neutralisation Tests on 1430 Patients and 87 controls Tested in 1984.

		CBV antibody titres		
Clinical category	**Number of patients tested**	% ≥512	% 256	TOTAL % ≥256
Myopericarditis	352	33	22	55
Chest pain	374	23	19	42
M.E.*	210	35	15	50
Myalgia/Bornholm	164	24	15	39
Miscellaneous**	330	22	20	42
Total	1430	27	18	46
Healthy control adults	87	5	12	17

* M.E. = Myalgic Encephalomyelitis.

** Includes neurological illness, fatigue, muscle aches, etc. for whom CBV tests were specifically requested by clinicians.

This increase in positives reflected not only a better selection of patients by clinicians but also more strict criteria applied when selecting for testing. There was no significant increase in circulating CBVs, and the controls showed no significant change in antibody status (5% with titres of $\geq$512, 12% with titres of 256) compared with control groups studied during the last 10 years (2,3,4).

Sera with NT titres of $\geq$256 and titres of $\leq$128, selected from routine CBV NT were studied for the presence of CBV IgM by ELISA. These included patients in various clinical categories presenting with acute and chronic illnesses. A total of 273 sera were tested by ELISA; 122 were found to be CBV IgM positive, of which 101 (83%) were also positive ($\geq$256) by NT. Only 8% of these were CBV IgM positive without confirmatory NT results (Table 4.2). On the basis of these results, a regimen was adopted of first screening sera for CBV 1-5 specific IgM by ELISA and then testing by NT only those which were found to be positive.

Table 4.2.

Comparison of results on 273 sera from patients with suspected CBV infections tested in 1984.

	NT positive (≥256)	NT negative (≤128)	Total
CBV IgM positive	101(37%)	21(8%)	122(45%)
CBV IgM negative	94(34%)	57(21%)	151(55%)
Total	195(71%)	78(29%)	273(100%)

Between January and August 1985, 760 patients with suspected CBV infections were studied. The results are shown in Table 4.3. The percentage positive for each clinical category was similar to those listed in Table 4.1 when NT titres of $\geq$512, indicative of recent infection, were compered.

Sera from 304 healthy adults in the West of Scotland were tested by ELISA at the same time, of which 132 (43%) were male and 173 (57%) female, with 206 (68%) aged 20-49 years, and were similar inage and sex to the various disease groups. Only 26 (9%) of this control group were positive.

Details of the combined ELISA and NT findings in the 191 patients and 26 controls found positive in the CBV IgM screen are listed in Table 4.4. A strong correlation between positive CBV IgM and high NT titres was seen in the myopericarditis, chest pain, and myalgia/Bornholm categories (65 (90%), 40 (91%) and 11 (92%) respectively) These illnesses are now recognised as having a CBV aetiology. The corresponding figure for the group with myalgic encephalomyelitis (ME) was 30 (83%). These results were compared with the control group, where only 2 (8%) of the CBV IgM positive sera were also NT positive.

A matching monotypic or heterotypic response by both test systems occurred in approximately 60-66% of

all disease categories studied, with the exception of the miscellaneous group, which included illnesses less likely to have a CBV aetiology.

Cross-reactions between this CBV IgM ELISA test and other enterovirus infections were also investigated. The results of these tests are shown in Table 4.5.

Table 4.6 lists the variety of responses observed by CBV ELISA and by NT, and includes examples of the interpretation of results commonly found when using this diagnostic regimen.

Sequential sera were available for study from 52 patients with acute or chronic illnesses. The duration of CBV IgM detection varied from 1 to 2 months in Bornholm disease, 3-6 months in myo/pericarditis (with recovery) and at least 1 year in some patients with relapsing ME.

Table 4.3.

CBV IgM results in 760 patients and 304 controls tested in 1985.

Clinical category	Number of patients tested	Number of CBV IgM positives(%)
Myopericarditis	220	72(33%)
Chest pain	198	44(22%)
M.E.*	118	36(31%)
Myalgia/Bornholm	62	12(19%)
Miscellaneous**	162	27(17%)
Total	760	191(25%)
Healthy control adults	304	26(9%)

* M.E. = Myalgic Encephalomyelitis.

** Includes neurological illness, fatigue, muscle pain etc, for when CBV tests were specifically requested by clinicians.

Table 4.4.

Correlation of CBV IgM positive and Neutralisation test results on 191 Patients and 26 Controls.

Clinical categories.

	Myo	C/P	M.E.	My/B	Misc	Ctr
No. of Patients.	72	44	36	12	27	26
IgM Pos/Neut Pos*.	90%	91%	83%	92%	78%	8%
Matching monotypic IgM and NT.	31%	27%	33%	33%	19%	0%
Matching heterotypic IgM and NT.	31%	32%	33%	33%	26%	0%
IgM positive non matching NT.	28%	32%	17%	25%	33%	8%
IgM positive NT negative.	10%	9%	17%	8%	22%	92%

Myo = Myopericarditis. M.E = Myalgic Encephalomyelitis

C/P = Chest pain. My/B = Myalgia/Bornholm.

Misc = Miscellaneous. Includes neurological illness, fatigue, muscle aches and pains etc. Where CBV tests had been specifically requested by clinicians.

Ctr. = Controls. Health adult sera.

* NT positive = titres $\geq$256.

Table 4.5.

CBV IgM ELISA CROSS REACTIONS.

Test serum	No of Sera.	CBV IgM Pos
HAV IgM Positive	14	14
Echovirus (CSF isolate)	12	12
Poliovirus NT >512/R T	5	0
		HAV IgM Pos
CBV IgM positive	60	0

HAV = Hepatitis A virus.

A unique one-way cross with Hepatitis A virus (HAV; Enterovirus 72) IgM positive sera (Abbott Laboratories HAVAB-M test) was demonstrated. Of 14 HAV IgM positive sera all gave CBV IgM positive reactions in this ELISA test. In contrast, of 60 randomly selected CBV IgM positive sera from this study, none was found to be positive in the HAVAB-M test system. Heterotypic responses in the ELISA test were observed in all of the patients with proven systemic Coxsackie A or echovirus infections. No cross - reacting responses were observed in the 5 cases of poliovirus infection investigated.

Table 4.6

CBV IgM and NT Responses Observed and Interpretation of the Results.

Patient	Interval After Onset	CBV IgM and Neutralising Antibody titres(NT) B1	B2	B3	B4	B5	Comments
1. W.H. 36M	6 dy	-	+	-	-	-	Rising B2 IgM and NT
Joint pains		<64	<64	<64	<64	<64	indicate current
myalgia.	30 dy	-	++	-	-	-	infection B2/B4
		<64	256	<64	128	<64	cross reactions
							are not uncommon
2. S.R. 37F	5 dy	-	-	-	-	-	Rising B1,B3,& B5 NT
Chest		<64	<64	<64	256	<64	IgM indicate current
and arm	15 dy	+	-	+	-	+	infection; B4 NT
pain.		≥1024	<64	512	512	≥1024	suggests infection
							earlier in life.
3. J.M. 59M	8 dy	++	-	-	+	-	Falling B1 IgM & NT
Muscle		≥1024	<64	<64	256	<64	indicate infection
bone	22 dy	-	-	-	-	-	in past weeks; note
aches		512	<64	<64	256	<64	cross reaction with B4

Table 4.6 continued

Patient	Interval After Onset	CBV IgM and Neutralising Antibody titres(NT) B1	B2	B3	B4	B5	Comments
4. H.M. 37M	? dy	+	-	-	-	-	Static B1 IgM & NT
Pericarditis		256	64	<64	512	128	indicates recent
	31 dy	+	-	-	-	-	infection.Note B4 &
		256	64	<64	512	128	B5 anamnestic NT
							responses.
5. A.S. 38M	2 dy	-	-	-	-	-	Slow rise in B5 (with
Myalgia,		<64	<64	<64	64	<64	anamnestic B2)IgM but
chest pain.	12 dy	-	-	-	-	-	negative NT response
		<64	<64	<64	64	<64	indicates current
	34 dy	-	±	-	-	++	enterovirus infection
		<64	<64	<64	64	<64	4th serum requested
							to detectpossible late B5 NT rise.
6. S.M. 15F	7 wk	-	-	-	-	-	Negative IgM but high
Pericarditis.		<64	512	<64	128	<64	static B2 NT; no
	4 mo	-	-	-	-	-	evidence of recent
		<64	512	<64	128	<64	enterovirus infection

Table 4.6 continued.

Patient	Interval After Onset	CBV IgM and Neutralising Antibody titres(NT) B1	B2	B3	B4	B5	Comments
7. S.T. 40M	12 dy	-	±	-	+	-	
Persistent		<64	<64	<64	512	<64	Persisting B4 IgM &
fatigue for	20 dy	-	±	-	+	-	NT; ? persisting B4
4.5 months		<64	<64	<64	512	<64	over this 5 month
	11 wk	-	-	-	+	-	period.
		<64	<64	<64	512	<64	
	5 mo	-	±	-	+	-	
		<64	<64	<64	512	<64	
8. A.M. 51M	13 dy	-	-	-	-	-	Negative IgM but
myocarditis.		1024	512	<64	256	<64	rising B5 NT. ? No
	23 dy	-	-	-	-	-	Igm response or
		512	512	<64	256	512	present only at low (<1/400) levels.
9. K.E. 32F	?	-	-	-	+	-	B4 IgM & NT indicate
Asymptomatic		<64	<64	<64	512	<64	recent infection; ?
but delivered							related to baby's
baby who died							cardiomyopathy.(No
with cardiomyopathy.							serum from baby).

Table 4.6 continued.

Patient	Interval After Onset	CBV IgM and Neutralising Antibody titres(NT) B1	B2	B3	B4	B5	Comments
10. E.R. 29F Pericarditis.	8 dy	+ 512	++ ≥1024	- 256	+ 128	+ ≥1024	Heterotypic IgM and matching high NT response indicate recent CBV infection.
11. J.P. 32F Myalgic Encephalomyelitis.	10 mo	+ <64	+ ≥1024	+ 256	+ <64	+ 128	Heterotypic IgM & matching high B2 NT; since acute serum was not available interpretation is difficult but IgM/NT evidence suggests recent CBV infection.
12. C.L. 15F Aseptic meningitis.	4 dy	+ <64	++ <64	- <64	- 64	++ 64	Heterotypic IgM with negative NT indicates recent enterovirus infection.

IV. **Discussion.**

Until recently, the only practical quantitative assessment of CBV antibodies was by (NT). This test is labour intensive, and the interpretation of static high titres in the individual patient difficult. Although quantitation of antibodies by ELISA requires detailed evaluation, by introducing the u-antibody capture CBV-specific IgM ELISA technique, it was hoped to provide a rapid, reliable and more precise serological diagnosis. To evaluate this ELISA technique for the routine diagnostic laboratory the test was performed on 273 sera selected from routine NT runs, and both NT positive and negative sera were included in the tests. Eighty three percent of those sera positive by CBV IgM tests were also NT positive, and such results were considered to indicate recent CBV infection. Only 8% of the selected sera were CBV IgM positive but NT negative, thereby suggesting a recent infection by other non-CBV members of the enterovirus group. On the basis of these observations, I introduced a CBV serological diagnostic regimen of screening patients for CBV 1-5 specific IgM by ELISA. Only those positive in this assay had a confirmatory CBV 1-5 NT done on them, therebymaking a more rapid diagnosis possible. Within 3 days of receipt of a serum sample one of two possible reports could be issued: (1) the serum is CBV IgM positive, indicating a current/recent

enterovirus infection. The NT results will follow; (2) the serum is CBV IgM negative, indicating no evidence of a recent enterovirus infection at the time this sample was taken.

Between January and August 1985, 760 patients with various illnesses of suspected CBV origin were tested. Most were adults in the 25-55 year age group. Thirty three percent of those with suspected myopericarditis were found to be CBV IgM positive, which is almost certainly an underestimate due to the inclusion of patients with less well defined clinical diagnosis (5). Since the association of CBV infections with myopericarditis is well recognised, this figure provided a positive control against which to assess serological results from other clinical categories. A negative control figure was obtained by testing sera from 304 healthy adults sampled over the same time scale, of which only 26 (9%) of these were CBV IgM positive.

Previous studies in an outbreak of ME (6) suggested that the CBV group played an important role in this debilitating illness. This was confirmed here, with 36 (31%) of 118 patients with suspected ME recorded as positives in the CBV IgM test. The percentage positive in the other disease categories was lower, possibly reflecting less well defined

clinical diagnoses. Nevertheless, the positives were still appreciably higher than those in the negative control group.

The CBV IgM ELISA results obtained here show the range of both monotypic and heterotypic responses reported by others (1,7). Monotypic responses are most common in children under seven years of age (8). Although the type of illness investigated in this study involved mainly adults, matching monotypic ELISA and NT results were not uncommon, being observed in approximately one third of all cases excluding the miscellaneous group. Matching heterotypic responses in both tests were also seen in one third of those same categories. Only 8-10% CBV IgM positive sera were negative by NT in the myalgia / Bornholm, chest pain, and myopericarditis groups, implying recent infection with other enteroviruses. The corresponding figure for the healthy adult group was 92%, only 2 of the 26 ELISA positive sera having elevated but non-matching NT antibody responses.

Cross reactions of this CBV IgM ELISA test with other members of the enterovirus group have been documented by various workers (1,7). The studies described here confirmed the unique one-way cross between this test and the hepatitis A virus (HAV) IgM. CBV IgM responses were also observed in patients with

systemic Coxsackie A and echovirus infections, but not in the few poliovirus infections tested. Despite the disadvantages of such cross reactions, they appear to offer a scant problem in routine testing. Most of the patients were in the older age groups, where HAV infections are less common, while the categories of illnesses which were studied had a greater probability of being CBV-related than other-virus-related.

The duration of the detectable CBV IgM in sera was varied. In acute illnesses with rapid recovery, for example, in Bornholm disease, CBV IgM levels fell within 1-2 months; in the myopericarditis group the levels fell after 3-6 months. On the other hand persistent CBV IgM levels were detected for more than one year in some patients with myalgic encephalomyelitis.

Detection of monotypic CBV IgM with matching high monotypic NT response was regarded as indicating a recent primary infection with that CBV serotype while heterotypic but matching positive ELISA and NT results suggested a recent infection with an unspecified member of the CBV group. Interpretation of heterotypic positive but non matching responses by both ELISA and NT tests was more difficult, but did not exclude recent Coxsackie infection (A or B serotypes).

The CBV IgM ELISA test is rapid and reliable, and gives reproducible results when used for routine diagnostic purposes. Because all 5 CBV antigens are used, the test is expensive and currently costs £5 per specimen when commercial antigens are used. Screening sera with one antigen, or an antigen cocktail, to cut costs is not yet feasible but is clearly desirable since demands for the test are mounting. The clinician benefits from receipt of a rapid result, which is totally adequate for the purposes of the clinical management of the patient, though the identification of the actual CBV serotype is of more relevance to the virologist and/or epidemiologist studying the viral aetiology of specific diseases. To this end, a greater specificity in the test may be obtainable with the use of monoclonal antibodies rather than by resorting to tedious virus concentration and purification procedures.

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CHAPTER 5

u - ANTIBODY CAPTURE ELISA FOR THE RAPID DIAGNOSIS OF ENTEROVIRUS INFECTIONS IN PATIENTS WITH ASEPTIC MENINGITIS.

1. Introduction.

Non-bacterial aseptic meningitis (AM) continues to be a common sporadic or epidemic manifestation of enterovirus infections. Most of the 69 different enterovirus serotypes have been isolated from patients with AM, with the echoviruses being predominant (1).

The diagnosis of enterovirus infections, employing conventional virus isolation procedures, is usually not achieved until long after the patient has been discharged from hospital.(2). A survey carried out on 4073 patients with AM in the United States of America showed that as many as 3340 (82%) may remain virologically undiagnosed.(3).

Early detection of enterovirus specific IgM, which will indicate recent or current infection, provides an attractive alternative diagnostic tool. The CBV specific IgM ELISA test, as described in chapters 3 and 4, proved useful in the routine diagnosis of CBV infections in various clinical situations. However, this ELISA test has been shown to cross react with others of the enterovirus group, particularly Coxsackie A viruses and echoviruses. It was decided to exploit this disadvantage and apply this ELISA test to patients with aseptic meningitis.

II PATIENTS AND METHODS.

Patients.

Between August and November 1985, 45 patients admitted to Monklands District General hospital with suspected aseptic meningitis were virologically investigated. Twenty six were male and 19 were female, and their ages ranged from 11 months to 49 years (mean 21.7 years).

Virus isolation.

Samples of CSF and faeces, taken during the acute phase of illness, were inoculated into tube cultures (monolayers) of primary monkey kidney and MRC5 cells, according to the folowing protocol.

A 10% weight/volume emulsion of faeces in antibiotic-containing phosphate buffered saline (PBS;Dulbecco A with antibiotics) was prepared. This was allowed to stand at room temperature for 20 minutes then centrifuged at 2500 r.p.m. for a further 20 minutes. The resultant bacteria-free supernate was used to inoculate the cell cultures. CSF samples were inoculated untreated.

The inoculated tubes were examined every two days for evidence of virus growth. Negative cultures were maintained for fourteen days before being considered negative. Positive and suspect cultures

were frozen at -20°C and thawed once then 0.2ml of the fluid inoculated into two fresh tubes of the same tissue. Viruses thus isolated were aliquoted and stored at -20°C prior to identification.

Virus isolates were identified by neutralisation tests using pooled and monospecific antisera as described by Grist et al, 1979. (4).

Methods

ELISA tests were used with the collected paired sera. The first taken soon after admission, the second three to seven days later, and these were tested for the presence of CBV IgM antibody using the u - antibody capture ELISA test described in chapter 3.

Routine CF tests were also performed to exclude non-enteroviral causes of illness in these patients.

III Results.

Of the 45 patients studied, a total of 10(22%), yielded an enterovirus when using the conventional virus isolation procedures,(4 were isolated from faeces specimens only), whereas 30(67%) produced a positive CBV IgM response in the ELISA test(Table 5.1.)

Table 5.1

Results of Enterovirus studies on 45 Patients with suspected Aseptic meningitis.

Specimen	Total No. Examined	No. Positive(%)
CSF	45	6[a] (13)
Faeces	39	10[b] (26)
Sera	14 single	9 (64)
	31 pairs	21 (68)
CSF and Faeces	45(patients)	10 (22)
Sera	45(patients)	30 (67)

[a] All six patients with virus in the CSF had the same virus in their faeces.

[b] The isolates were B5(1), E5(5), E6(2), E7(1), and E11(7).

All patients from whom a virus was isolated, from CSF or faecal samples were found to be CBV IgM positive. Fourteen single serum samples and 31 paired sera were examined, 9 of the single sera were CBV IgM positive, these samples having been taken 1-7 days(mean 3.2 days) after admission. The 5 negative single sera had been sampled earlier in the illness, at 6 hours - 2 days (mean 1.05 days). Seventeen of the paired sera showed a significant rise, and 4 a

significant fall in CBV IgM levels. The acute phase sera of 16 of the 21 positive paired serum patients, taken on average 3 days after admission, gave a positive CBV IgM response, the 5 remaining patients whose acute phase sera were negative having been sampled earlier(mean 2.2 days) (Table 5.2.).

Table 5.2

CBV IgM Positive responses

21 paired sera.

	Acute CBV IgM		Convalescent CBV IgM	
	Positive	Negative	Positive	Negative
	16	5	18	3(FT)*
Mean days after onset of symptoms.	3 days (1-5days)	2.2 days (8hrs-4days)	(5-21days)	(13-36days)

* The three negative sera were from patients who gave positive results on the acute sample only.

FT = falling titres (i.e., from positive to negative)

Heterotypic CBV IgM responses were common in this group of patients (Table 5.3). No distinct pattern in the type of heterotypic response emerged either in relation to the age or to the sex of the patients or to the serotype of the virus isolated (3 patients had echovirus 5 isolated, 4 had echovirus 11 isolated).

Strong positive reactions in the ELISA test, as reflected by high OD values indicating high levels of IgM, were detected more frequently here than were obtained from patients with other illnesses with CBV etiology i.e., myopericarditis or Bornholm disease.

Table 5.3

Responses in 30 Patients with Aseptic Meningitis

CBV IgM positive.

	Monotypic	Heterotypic
No. of sera	7	23(77%)
Relative strength as reflected by OD values.	+	+++

On completion of these laboratory investigations, the final clinical diagnoses of these patients was revealed and compared with the virological findings, (Table 5.4). No virological diagnoses were made on the basis of the CF tests.

The group with aseptic meningitis had CSF lymphocyte cell counts ranging from 6 to 390 cells per ml (mean 98 cells).

Those with meningism had cell counts of <1 cells per ml. Although headache was a feature in most of the miscellaneous group of patients, none had meningism, and the one patient with a positive CBV IgM response in this group had xanthochromic staining of the CSF.

Table 5.4

Final clinical diagnosis and enterovirus results.

Clinical diagnosis.	No. Patients	No. Positive
Aseptic meningitis	21	18 (86%)
Meningism	16	11 (69%)
Miscellaneous	8	1 (12%)
Total	45	30 (67%)

Footnote.

Other viruses and microbes tested for by CF tests. Mumps (S and V), measles, herpes simplex, varicella zoster, cytomegalovirus, Mycoplasma pneumoniae and Leptospira *patoc.*

V DISCUSSION.

Enterovirus infections are usually under diagnosed, either because of the inadequacies of type and of timing of specimens submitted for investigation, or the sensitivity of the virus or antigen detection system used. Serological diagnosis has not hitherto been practicable because of the large numbers of enterovirus serotypes involved. The results reported in this chapter suggest that many of these difficulties can be readily overcome by using the u - antibody capture Coxsackie B virus IgM ELISA test. By exploiting the well recognised cross-reactivity of this test with the Coxsackie A virus and echovirus groups 5,6 (see chapter 3) an enterovirus infection was diagnosed in 67% of patients with suspected aseptic meningitis, compared with only 22% when using conventional virus isolation procedures. Not only did the described ELISA test increase the detection rate of enterovirus infections, but it also provided evidence of systemic infection in these patients as well. While isolation of virus from CSF is deemed diagnostic, isolation of virus from faeces is of considerably less significance, particularly in children.

Using this ELISA test a rapid result is possible, and a report may be issued within forty eight hours of receipt of the patient's serum at the laboratory. Although CBV IgM responses were detected in acute phase single serum samples, paired sera taken within a three to five day time interval gave a more reliable result. Significant rising or falling CBV IgM levels indicative of current or recent enterovirus infection were detected in 21 (68%) of the 31 paired sera examined here. Although no specific control group was included, sera from 304 apparently healthy adults living in the same conurbation were sampled between January and August 1985. When tested (see chapter 3) only 27 (9%) were CBV IgM positive, and none gave the high levels of CBV IgM seen in aseptic meningitis patients.

Chapter 3 describes the use of the CBV IgM ELISA test in routine diagnosis with more than 1000 patients with cardiac and other illnesses of suspected CBV aetiology. Two major differences in the CBV IgM response were noted in those patients with suspected aseptic meningitis. Firstly, a higher frequency of heterotypic cross reactions was observed, and these appeared not to be age related.

Studies by Banatvala et al (5) of juvenile onset diabetes mellitus have shown that monotypic responses

predominate in such children. In this study, of the seven children aged under ten years, 6 showed a heterotypic response. Secondly, strongly positive CBV IgM responses were detected more often in the aseptic meningitis patients. These differences in type and magnitude of response may be explained if echoviruses rather than Coxsackie B virus infections were being detected, a hypothesis supported by the viruses isolated.

Moreover, the acute onset and rapid spread of virus to the central nervous system in this group of patients may invoke a more rapid and stronger IgM response, in contrast to patients with enteroviral heart disease which is generally recognised as a late manifestation of virus infection.

The capability of this ELISA test to produce a rapid and reproducible result has added a new dimension to the serological diagnosis of enterovirus infections, especially because clinicians receive a report within two days of submission of specimens, a positive result being reassuring to the patient and helpful in clinical management. The limitation of the test in identifying the infecting serotype is of more significance for the virologist and epidemiologist than the clinician.

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CHAPTER 6.

CONCLUSIONS.

Serology is at the present time the most feasible way of investigating the possible CBV etiology of myopericarditis, ME and similar associated syndromes where the majority of patients seek medical advice after virus excretion has ceased, and hence where virus isolations are mostly unreliable.

Neutralisation tests are better suited to epidemiological surveys than diagnostic tests as the significance of static high titres is difficult to assess, and the detection of significant rising or falling titres is rare.

The introduction of the CBV IgM ELISA test which is described here has added a new dimension to the serological diagnosis of enterovirus infections. The detection of CBV specific IgM here has been considered to indicate a recent or persisting CBV infection, though the CBV IgM ELISA has not been found to be totally specific for CBV's, with cross reactions occurring with sera from patients with other proven non-polio enterovirus(NPEV) infections. However, the fact that enterovirus IgM is present is sufficient in many cases for a presumptive diagnosis to be made.

The probability of a CBV etiology can be increased by using a confirmatory neutralisation test. Sera giving matching positive IgM and high neutralisation test titres are considered, without doubt, the result of a specific CBV infection.

1. Current uses of the CBV IgM ELISA test.

The use of the CBV IgM ELISA on its own to diagnose current or recent CBV infections should be discouraged, since back-up neutralisation tests and constant monitoring of antibody levels in the community is required if the results are to be interpreted sensibly. The occurrence of a CBV epidemic, as happened in 1985, when CBV type 5 was predominant in the West of Scotland, does lead to increased levels of specific antibody in the community (1). This in turn lead to difficulties in the interpretation of the test results in relation to the cardiac- and ME-associated disease being investigated at that time.

A diagnostic serology laboratory regimen to screen patients sera, either as an aid to specific diagnoses or as part of studies into the etiology of diseases of unknown cause, by the CBV IgM ELISA test and with confirmatory NTs carried out on the positives, has been described in chapter 4. This type of regimen is best suited to larger laboratories where large numbers of sera are being tested, because on price alone the cost

per sample of screening small numbers would be prohibitive (see costs of tests table 6.1)

Table 6.1.

The breakdown of costs of the CBV IgM ELISA and the CBV neutralisation test(NT).

On a batch of 24 sera.

	Cost in £'s of:-	
REAGENTS/CONSUMABLES	ELISA	NT
Microtitre plates	3.50	9.20
Media/chemicals	15.00	17.00
Antigens	81.00(20.00)*	0.00
Antiserum	13.00	1.80
Conjugate	3.00	0.00
Stationary	1.50	1.00
Cells	0.00	20.00
Subtotal	117.00	49.00
Technical time	4.5 hrs	7.5 hrs
Cost (£6.83 per hour)	£30.73	£51.23
Total	£147.73	£100.23
Cost per sample	£6.16	£4.18

* The use of In-house antigens as described in chapter 3, pages 75-87 reduces the cost of ELISA reagents by £61.00, giving a Subtotal of £86.73. However it increases the technical time by 3 hours ie.£20.49 giving a final Total of £107.22, which is £4.46 per sample.

The approximate reagent cost of £5 per serum sample in the ELISA test adds up to £25000 when 5000 samples per year are being tested. This was the level of requests generated when the CBV IgM ELISA was introduced into routine use in the Regional Virus Laboratory, Ruchill. An increase in expenditure of this magnitude could not be met from the existing laboratory budget and financial support had to be secured from other sources i.e., other Health Authorities served by this laboratory.
The use of in-house antigens reduced the financial burden considerably.

The application of this CBV IgM ELISA, to the rapid diagnosis of aseptic meningitis taking full advantage of the reported cross-reactions, is the most exciting development reported here. This test has many advantages over conventional virus isolation, e.g.,

1. the results are available sooner, and normally within 48 hours of receipt of the specimen.
2. it is a more sensitive test in that a much higher rate of 67% positive diagnoses are achieved as compared with 22% when using conventional virus isolation procedures;
3. it is a more reliable marker of causal association of illness than is isolation from faeces, as a positive

result is consistent with a systemic infection and not merely viral carriage;

4. there is no need to use precious CSF samples to make a diagnosis.

There are a number of points worthy of consideration that accrue from using this test as a diagnostic tool when suspecting aseptic meningitis :-

1. it does not identify the serotype of the infecting virus, although this is of more interest to the virologist and epidemiologist than to the front line clinician;

2. it cannot detect poliovirus IgM and although aseptic meningitis resulting from poliovirus infection is rare in this country, isolation samples may still be additionally required to guard against this possibility, and they would certainly be needed if the CBV IgM ELISA test was used in countries where poliovirus infections were more common;

3. the test is very expensive unless large batches (minimum 24) of sera are tested, so that it would have to be in routine use for purposes other than aseptic meningitis to be financially feasible.

2. Future developments.

1. A single antigen screening test which would reduce costs and increase the use of the CBV IgM ELISA test in the diagnosis of aseptic meningitis is desirable. VP1 produced as a synthetic peptide would appear to be a feasible option, as a monoclonal antibody raised against VP1 has already been used to detect circulating immune complexes in patients with ME (2).

2. The use of the CBV IgM ELISA test in the investigation of the etiological agents of ME and other neuro-muscular diseases of unknown etiology and non-familial and clustering epidemiology, e.g., Motor Neuron disease, should be done in conjunction with the current research into non-cell culture based methods for the detection of viral antigens or nucleic acid sequences, e.g., polymerase chain reaction (PCR) for the detection of CBV specific RNA in tissue biopsies and other samples.

The association of CBV's with Postviral Fatigue Syndromes (PVS) is far from proven, but enough information in now available to suggest that such an association does exist. The application of techniques such as PCR and monoclonal antibodies for detecting circulating immune complexes containing VP1, to the investigation of PVSs should lead to a better

understanding of the role of enteroviruses in general and CBV's in particular, to these. The correlation of the results using this alternative technology with the CBV IgM ELISA results will clearly serve to establish the diagnostic value of the CBV IgM ELISA test.

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