

Antibody capture radioimmunoassay (MACRIA) for coxsackievirus B4 and B5-specific IgM

BY P. MORGAN-CAPNER AND C. MCSORLEY

*Department of Medical Microbiology, King's College Hospital Medical School,
Denmark Hill, London SE5 8RX*

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SUMMARY

An antibody capture radioimmunoassay was established for the detection of coxsackievirus B4 and B5-specific IgM. A significant feature of the assay was the use of an unrefined coxsackievirus B (CBV) antigen. The antigen was prepared by freeze thawing, ultrasonication and low speed centrifugation of infected Vero cells with no purification or concentration of the antigen being performed. Results of sera tested were expressed as a serum ratio (SR) by comparison with a low positive control serum. To establish an SR indicating positivity in the assays, 100 antenatal sera collected in late February were tested. The mean SR was calculated and the mean plus three standard deviations was taken as the minimum SR indicating positivity. Although resulting in a relatively insensitive assay, such a value was required to exclude sera giving a low level of reactivity which may be due to residual enterovirus-specific IgM resulting from a remote infection.

The homologous CBV-IgM assay was positive in four cases of CBV4 infection and six cases of CBV5 infection. For the CBV4 IgM assay, ten of 20 (50%) sera from infections with CBV other than CBV4 were positive and nine of the 13 (69%) sera from infections with echoviruses or coxsackieviruses A were positive. Five of 18 (27%) sera with an elevated CBV neutralization titre were positive in the CBV4-IgM assay. For the CBV5-IgM assay seven of 18 (39%) sera from infections with CBV other than CBV5 were positive and nine of 13 (69%) sera from infections with echoviruses or coxsackieviruses A were positive. The nine sera that were positive from this group in the CBV5-IgM assay were the same nine as were positive in the CBV4-IgM assay. Two of the 18 (11%) sera with an elevated CBV neutralization titre were positive in the CBV5-IgM assay. These two sera were also positive in the CBV4-IgM assay and had an elevated monotypic CBV4 neutralization titre. None of the sera giving positive results gave significant reactivity when tested with control antigen. Twelve rheumatoid factor containing sera and 46 sera from other infections were negative in both assays. Of 24 sera from confirmed CBV infection, seven gave a positive monotypic CBV4 or 5-IgM response, ten were positive in both assays and seven were negative in both assays. The positive results seen with sera from cases of heterologous enterovirus infection may result from an anamnestic IgM response or, more likely, IgM directed against enterovirus cross-reacting antigens. The absence of homologous neutralizing antibody at a dilution of 1:20 in nine of 20 sera that gave a positive CBV-IgM

result and the presence of positive results for CBV4 and 5-IgM in a 14 month old infant who had echovirus 7 infection indicates that the IgM need not be directed against neutralizing antigens.

Thus the CBV4 and 5-IgM assays developed appeared to be specific for an enterovirus infection but because of the cross-reactivity were not type-specific or group-specific.

INTRODUCTION

Infection with enteroviruses is often subclinical but may result in a wide range of clinical syndromes from minor febrile illnesses to life-threatening conditions such as paralysis and myocarditis. Confirmation of a virus infection is best made by isolation of the infecting enterovirus from tissue, CSF, throat or faeces, although in the latter two sites the virus may merely be a transient passenger and not aetiologically implicated in the illness being investigated. However, the appropriate specimens are often not taken or are taken too late in the evolution of the illness for the virus to be isolated. There is, therefore, a requirement for serological tests for the diagnosis of recent enterovirus infection.

The existence of at least 67 serotypes (Grist, Bell & Assaad, 1978) and the lack of a reliable serological test utilizing a group antigen has resulted in the quantitation of neutralizing antibody against poliovirus and coxsackievirus B (CBV) being the only serological test readily available. Such tests are only likely to reveal a diagnostic rise in titre if the first serum is collected early in the illness (Grist & Bell, 1974). Therefore a presumptive diagnosis is often made on the basis of an elevated neutralizing antibody titre. However, elevated CBV neutralizing antibody titres may be found in people that are clinically well with no history of a recent illness and have been found to persist at elevated levels for months or years (Grist & Bell, 1974). It has also been demonstrated that anamnestic rises in neutralizing titre occur to CBV serotypes other than that responsible for the illness (Grist & Bell, 1974).

An established serological principle for the diagnosis of recent virus infection is the demonstration of virus-specific IgM. Techniques for the detection of CBV-specific IgM have been described by a number of authors. Schmidt, Lennette & Dennis (1968) showed that the demonstration of a reduction in neutralizing titre by treatment with 2-mercaptoethanol was unreliable but that serum fractionation by sucrose density gradient ultracentrifugation, with neutralization titres being performed on the fractions obtained, appeared to be reliable for the three sera they evaluated. In 1973, Schmidt, Magoffin & Lennette described the diagnostic use of an immunodiffusion test for the diagnosis of recent infection. IgM antibody reacts with the intact virion of CBV and was found to form an immunoprecipitation line distinct from that produced by IgG. However, such an assay was unreliable for the detection of CBV2-specific IgM and the antigens used required concentration to achieve a high titre. Their assay did demonstrate that an appreciable incidence of cross-reactivity occurred with positive immunodiffusion lines occurring to heterologous CBV serotypes in addition to that with the homologous, infecting CBV. They also demonstrated another problem of evaluating a CBV-specific IgM assay in that they found positive results in 8% of their control group. Minor *et al.*

(1979) modified this technique by using counterimmunoelectrophoresis (CIE) but the assay did not overcome the problems associated with immunodiffusion.

MacWilliam & Cooper (1974) attempted detection of CBV-specific IgM by immunofluorescence using CBV-infected monolayers but were unsuccessful. Methods of detection of CBV-specific IgM by indirect solid-phase radio-immunoassay (Dörries & ter Meulen, 1980) and indirect enzyme-linked immunosorbent assay (Katze & Crowell, 1980*a*) have been described. Both assays required the purification of virus antigen prior to coating the solid phase and such assays are likely to give false positive results with sera containing rheumatoid factor.

IgM capture antibody (MACRIA) techniques have been established and used with success for the diagnosis of hepatitis A (Flehmig *et al.* 1979) and rubella (Mortimer *et al.* 1981). These assays have proved to be sensitive, specific and unaffected by the presence of rheumatoid factor. As the patient's specific-IgM selects the antigen prior to the detection of bound antigen, an advantage of this type of assay should be that minimal prior purification of antigen is required. El-Hagrassy, Banatvala & Coltart (1980) described an IgM-capture assay for the detection of CBV-specific IgM but established their assay as a CBV group test and did not examine sera from cases of infection with other enteroviruses. Their assay had an enzyme-labelled indicator antibody. We describe here the development and evaluation, both in homologous CBV infection and in heterologous enterovirus infection, of CBV4 and CBV5-specific IgM assays based on an ¹²⁵I-labelled indicator antibody.

MATERIALS AND METHODS

Sera

Negative sera

The negative control serum used in both assays was a pool of five sera in equal quantities. These sera were collected from adults and had low (≤ 40) or undetectable (< 20) neutralizing antibody titres against CBV1 to CBV5 which had not altered in later sera collected from the same persons.

The assays were established and evaluated with 100 antenatal (ANC) sera collected in late February when recent enterovirus infection would have been unlikely as such infections are more prevalent in summer and autumn in a temperate climate (Grist, Bell & Assaad, 1978).

To determine the possible effect of rheumatoid factor, 12 sera containing rheumatoid factor detectable by latex agglutination were evaluated.

Forty-six convalescent sera from serologically confirmed cases of infection with other agents (herpes simplex 5, adenovirus 5, measles 1, mumps 3, *Mycoplasma pneumoniae* 5, cytomegalovirus 2, Epstein-Barr virus 4, hepatitis A 7, influenza A 2, influenza B 2, varicella-zoster 5 and rubella 5) were examined.

Presumed enterovirus-specific IgM-containing sera

The control positive serum for the CBV4-specific IgM assay which was used during its development and evaluation was obtained 18 days after the onset of a febrile illness and rash in an adult male from whom CBV4 was isolated from a throat swab. The control positive serum for the CBV5 assay was collected 10 days after

Table 1. *Sera from cases of confirmed enterovirus infection*

Virus group	Method of diagnosis	Virus strain diagnosed	No. of sera	
Coxsackievirus B	Isolation of virus	CBV2	4	
		CBV4	3	
		CBV5	6	
		CBV6	4	
	Rise in neutralizing antibody	Monospecific	CBV4	1
			CBV3	2
		Multispecific	CBV1 and 4	1
			CBV1 and 2	1
			CBV3 and 5	1
			CBV2, 3 and 5	1
Coxsackievirus A	Isolation of virus	CAV9	1	
		CAV16	1	
	Rise in neutralizing antibody	CAV16	1	
Echovirus	Isolation of virus	EV7	2	
		EV9	1	
		EV11	2	
		EV17	2	
		EV30	3	

CBV, coxsackievirus B; CAV, coxsackievirus A; EV, echovirus.

the onset of meningism and fever in a 7-year-old male from whose faeces CBV5 was isolated.

Thirty-seven appropriately timed sera (taken approximately 1–3 weeks after the onset of the illness) were available from confirmed cases of enterovirus infection (Table 1). Sequential sera were available from one case of Bornholm disease in a 45-year-old female from whom CBV5 was isolated, a young adult male with a rash and fever from whom CBV4 was isolated and a 14-month-old male infant with myocarditis from whom echovirus type 7 was isolated.

Possible enterovirus infection

Eighteen sera from cases of possible CBV infection as suggested by clinical symptoms and with a neutralization titre ≥ 160 to at least one of the viruses CBV1–5 were available. For evaluation these sera were divided into three groups:

(1) Raised monospecific CBV neutralization titre to the CBV of the IgM assay (CBV4:13, CBV5:0).

(2) A raised neutralization titre to more than one CBV serotype, but including the CBV of the IgM assay (CBV4:4, CBV5:1).

(3) Raised neutralization titres to CBV other than the CBV of the IgM assay (CBV4:1, CBV5:17). Also included was the serum of the mother of a neonate who had a confirmed CBV4 infection.

*Serological tests**Coxsackievirus B neutralization test*

CBV neutralization titres were performed in flat-bottomed microtitre plates (Falcon^(R), Becton Dickinson & Co., USA). Sera were diluted 1:20 in growth medium (Eagles MEM with 7% fetal calf serum) and inactivated for 30 min at 56 °C. 25 µl volumes of sera were diluted in duplicate from 1:20 to 1:1280 in the microtitre plates before adding 25 µl of 100 TCID₅₀ of the appropriate CBV. This was prepared by infecting Vero cells, freezing and thawing three times when at least 95% cytopathic effect (cpe) had been obtained, and diluting as necessary in growth medium. After incubation at RT for 1 h, 50 µl of Vero cells at 2×10^5 cells/ml were added to each well, the plates sealed with Scotch^(R) pressure-sensitive adhesive tape and then incubated at 37 °C for 4 days prior to reading. The neutralization antibody titre was taken as that dilution of serum with which 50% inhibition of cpe occurred. Each batch of tests was accompanied by appropriate cell and serum controls, back titrations of CBV and confirmation of their identity with standard control antisera.

M-antibody capture radioimmunoassay (MACRIA)

The MACRIA technique was based on that described by Mortimer *et al.* (1981) for the detection of rubella-specific IgM. Briefly, polystyrene beads coated with anti-µ were incubated in a dilution of the patient's serum. After washing, any of the patient's IgM specific for the CBV antigen used was reacted with that antigen and any antigen still bound after further washing was detected by incubation with ¹²⁵I-labelled anti-CBV antibody. After washing again the bound reactivity was counted. The components of the assay were evaluated in order that a maximum differentiation in bound radioactivity occurred between the negative and positive control sera (T/N ratio).

The solid phase. Polystyrene beads, 6.4 mm (Northumbria Biologicals, Cramlington, U.K.) were coated with anti-µ (Dako, Copenhagen, Denmark) according to the method of Sexton, Hodgson & Morgan-Capner (1982). Immediately prior to use the beads were washed once in phosphate buffered saline (PBS) and incubated for 3 h in PBS containing 1% bovine serum albumen.

Serum incubation phase. The serum was diluted in PBS containing 0.05% Tween 20 (PBST) in the wells of plastic trays (Abbott Laboratories, Basingstoke, UK). The optimum dilution for the assay was assessed by evaluating various serum dilutions ranging from 1:10 to 1:10⁷. The dilution selected for use was 1:200 as this was the highest dilution before a significant drop in T/N ratio occurred. There was no effect on background binding of incorporating 20% normal rabbit serum (NRS) or 20% control antigen in the serum diluent. Prior to incubation with the antigen the beads were washed four times with PBST. An Abbott pentawash system was used for all washing steps.

Antigen incubation phase. The CBV1-5 were obtained from Epsom Public Health Laboratory, UK, and their serotype confirmed by neutralization with antisera provided by the Division of Microbiological Reagents and Quality Control of the Public Health Laboratory Service, Colindale, UK.

CBV antigen was prepared by infecting Vero cells. A monolayer of confluent Vero

cells in a 75 cm³ flask (Lux Scientific Corporation, USA) was changed to 20 ml Eagle's maintenance medium containing no fetal calf serum. 0.5 ml of CBV containing 10⁵ to 10⁶ TCID₅₀ was added to the flask and incubated for 24–48 h at 35° until at least 95% cpe was present. Various products and treatments of this antigen were assessed in order to obtain the best results. Supernatant virus, both before and after low-speed centrifugation (7200 g/10 min), freezing and thawing with and without low-speed centrifugation, Minicon-B15 (Amicon Ltd., High Wycombe, UK), concentration to one tenth of the initial volume, ultrasonication (60 s) and high-speed centrifugation 115000 g/1 h) with reconstitution of the pellet in PBST to original volume, were all assessed. The final method used was to freeze and thaw three times, ultrasonicate and remove cell debris by low-speed centrifugation. The supernatant was decanted off and a few crystals of sodium azide added for preservation. Control antigen was prepared from uninfected Vero cells by a similar method.

The dilution of antigen for use was assessed by using various dilutions of antigen from neat to a dilution of 1:20 in PBS containing 0.2% Tween 20 (PBS 0.2% T) in an assay for CBV-specific IgM using a dilution series of the positive serum in negative serum from neat to 1:20. As a result of this evaluation the antigen was used at a dilution of 1:2. The optimum duration of the antigen incubation phase was established at 48 h after assessing time periods from 24 to 96 h.

Prior to the addition of the ¹²⁵I-labelled anti-CBV serum the beads were again washed four times with PBST.

Anti-coxsackievirus B ¹²⁵I-labelled antibody

The anti-CBV4 and CBV5 were hyperimmune rabbit sera obtained from the Division of Microbiological Reagents and Quality Control of the Public Health Laboratory Service, Colindale, UK. Their neutralizing antibody titres against the homologous CBV were 3200. The IgG fractions were prepared by gel filtration on DE52 gel (Whatman Ltd., Maidstone, UK) by the method of Tedder (1981). This IgG preparation was labelled with ¹²⁵I by the iodogen method of Salacinski *et al.* (1979) in the ratio of 1 mCi ¹²⁵I to 60 µg protein. Free iodine was separated from iodinated protein by fractionation on a Sephadex G25 column (Pharmacia Ltd., Hounslow, UK). The radio-iodinated protein was absorbed on a column of rabbit serum proteins linked to Sepharose 4B (Pharmacia Ltd.) and stored in 0.2 M-Tris, 0.9% NaCl buffer containing 5% BSA and azide.

The optimum input counts of ¹²⁵I-labelled CBV antiserum was established by evaluating a dilution series of the label from 50000 counts to 400000 counts/60 s/200 µl and 200000 counts/60 s/200 µl was found to be optimum.

Using the principles established by Mortimer *et al.* (1981) the label was diluted in PBS 0.2% T. They stressed the importance for reducing background binding of incorporating, in the label diluent, normal serum proteins of the same species as the immunoglobulin components within the assay. To this end NRS was incorporated into the diluent and levels from 10–30% assessed. As background binding was progressively reduced, with resulting higher T/N ratios, by increasing the NRS content, the label diluent finally chosen contained 30% NRS. As it was impossible to ensure that any normal human serum did not contain enterovirus-specific antibodies, this component was not incorporated in the level diluent.

Table 2. Effect of using different coxsackievirus B antigens with radiolabelled anti-CBV5 indicator antibody label

	Test: negative ratio				
	CBV1 Ag.	CBV2 Ag.	CBV3 Ag.	CBV4 Ag.	CBV5 Ag.
Confirmed CBV5 infection	1.0	0.8	1.0	0.7	3.7
Confirmed CBV2 infection	1.2	0.8	0.8	0.7	1.0
Confirmed CBV2 infection	0.9	0.8	0.8	0.7	3.3

However, negative control serum in the diluent in addition to NRS revealed no significant difference in T/N ratio. Unsuccessful attempts were also made to reduce background binding by pre-absorbing the ^{125}I label with control antigen and by incorporating control antigen at levels of 10–50% into the label diluent.

Established assay procedure

The results obtained from the evaluations described above led to the following procedure being adopted:

- (1) Beads were coated with rabbit anti- μ in NHCl and blocked with 1% BSA prior to use.
- (2) Coated beads were added to the serum diluted 1:200 in PBST in duplicate in the wells of Abbott trays.
- (3) The beads were incubated for 3 h at 37 °C prior to washing four times with PBST.
- (4) To each bead was added 200 μl of CBV antigen diluted 1:2 in PBS 0.2% T. The antigen was prepared by infecting Vero cells and when greater than 95% cpe was present freezing and thawing three times, ultrasonating for 60 s and clarifying by centrifugation (7200 g/20 min).
- (5) After 48 h at 4 °C the beads were washed four times with PBST.
- (6) To each bead was added 200 μl of ^{125}I -labelled anti-CBV, diluted in PBS 0.2% T containing 30% normal rabbit serum, such that the counts were 200000/60 s/200 μl .
- (7) After 3 h at 37 °C the beads were washed four times with PBST and bound radioactivity measured by counting for 10 min in an NE 1600 gamma counter (Nuclear Enterprises, Edinburgh, UK). An empty cassette was counted for 10 min and the background counts subtracted from the counts obtained with each bead before averaging the duplicate counts obtained.

Specificity of ^{125}I indicator antibody

To determine the specificity of the hyperimmune rabbit sera used as the ^{125}I -labelled indicator antibody for detecting only bound CBV of the type against which it was prepared, the following evaluation was performed with the anti-CBV5 indicator antibody. Three sera (two from CBV2 infections, one from a CBV5 infection) were evaluated with individual CBV1 to CBV5 antigens. Positive T/N ratios were seen only with CBV5 antigen in the CBV5 infection serum and one of the CBV2 infection sera (Table 2). Neither of the CBV2 infection sera gave a positive result if CBV2 antigen was used.

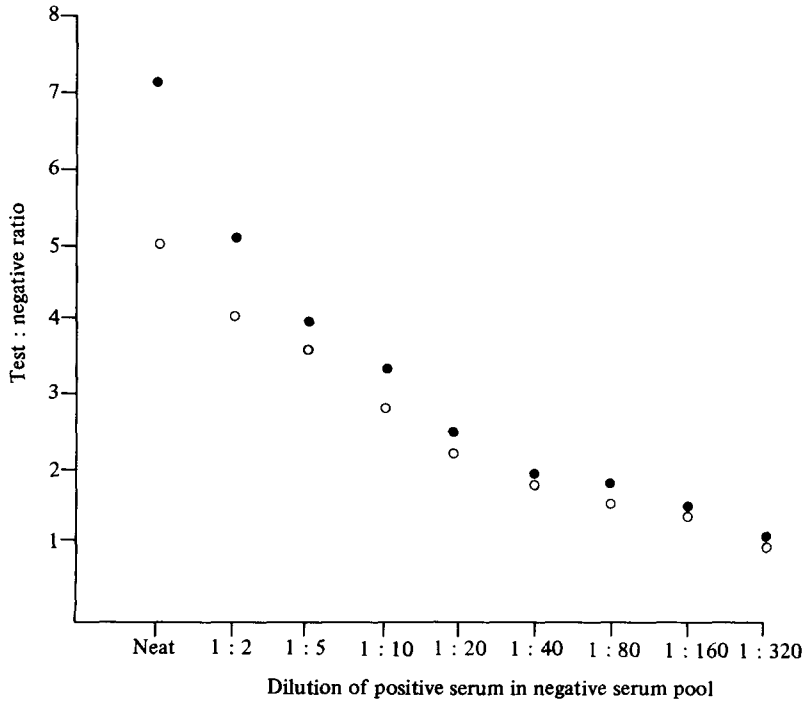


Fig. 1. Evaluations of dilutions of the positive serum in negative serum for the coxsackievirus B5-specific IgM assay (duplicate assays, ● and ○).

Sensitivity of the assay

The sensitivity of the assay was investigated by diluting the CBV5-IgM positive serum in control negative serum from neat to 1:320. A progressive decline in T/N ratio occurred such that at a dilution of 1:20 the T/N ratio was 2 to 3 (Fig. 1). Similar results were seen with the CBV4-IgM positive control serum.

Expression of results

Each assay included the negative control serum, the positive control serum and a low positive serum obtained by diluting the positive serum 1:20 in negative control serum. In order to minimize batch-to-batch variation in the assay, results for test sera were expressed as a serum ratio (SR) by comparing the counts obtained with the serum being evaluated with the counts obtained with the low positive control serum.

RESULTS

Fig. 2 shows the results of the CBV4-IgM assay for sera in the various groups. The majority of the 100 ANC sera gave SR values between 20 and 50% (column A, Fig. 2). The mean value was 40% and the mean plus 3 standard deviations (SDs) was 97%. This latter value was taken to indicate positivity in the assay. Only three of the 100 ANC sera give values above 97%. The three sera from patients from whom CBV4 was isolated, the serum from a patient showing a monotypic rise in neutralization titre to CBV4 and the serum of the mother of a neonate with CBV4 infection, gave SRs between 150 and 220% (column B, Fig. 2).

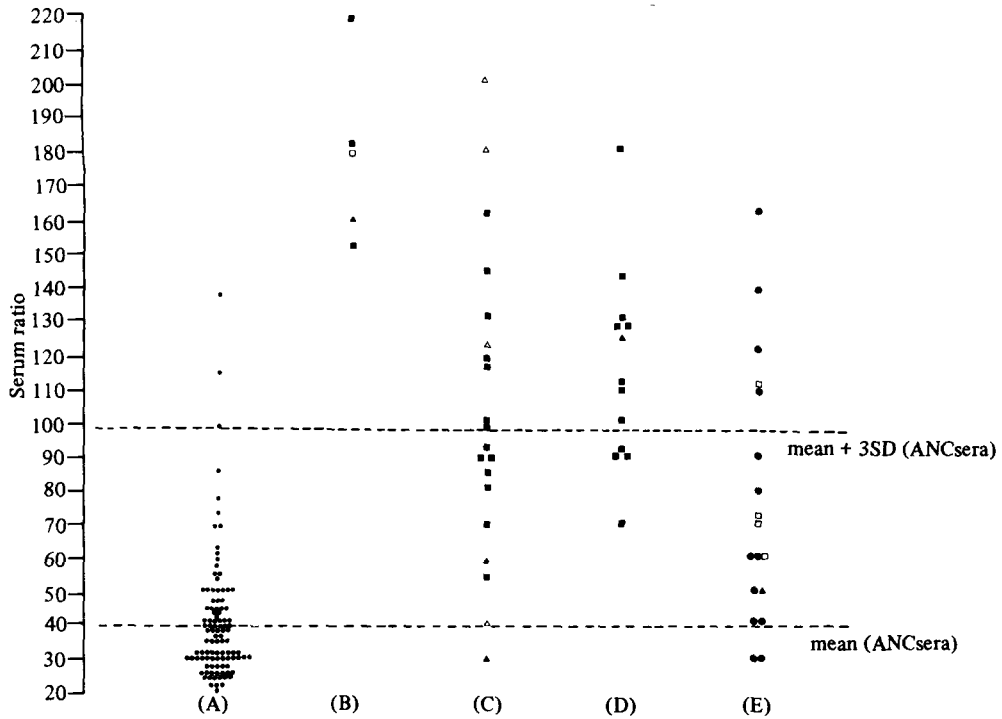


Fig. 2. Coxsackievirus B4-specific IgM serum ratios for (A) 100 antenatal sera (●), (B) coxsackievirus B4 infections, (C) other coxsackievirus B infections, (D) coxsackievirus A and echovirus infections and (E) elevated coxsackievirus B neutralizing antibody titre (≥ 160). Cases in groups B–D were diagnosed by virus isolation (■), by neutralizing antibody rise to one serotype (▲), by neutralizing antibody rise to more than one serotype (△) and the mother of a neonate with coxsackievirus B4 infection (□). Cases in group E had a raised homologous titre only (●), raised heterologous (monotypic) titre only (▲) and a raised titre to more than one serotype including the homologous serotype (□). The mean serum ratio and the mean plus 3 standard deviations for the 100 ANC sera are indicated by the lower and upper dashed lines respectively.

The 20 sera from cases of confirmed CBV1, 2, 3, 5 or 6 infection gave SR values ranging from 30 to 200%, with ten (50%) giving values above 97% (column C, Fig. 2). Thirteen sera from cases of coxsackievirus A or echovirus infection gave SRs between 70 and 180% with nine (69%) giving SRs greater than 97% (column D, Fig. 2). There was no significant difference in the incidence of sera giving an SR value $> 97\%$ between these two groups ($0.50 > P > 0.10$). When cases of possible CBV infection with elevated neutralization antibody titres were examined, the SRs ranged from 30 to 165% and five of 18 (27%) had SRs above 97% (column E, Fig. 2). The five positive sera all had an elevated neutralization titre to CBV4 with one having, in addition, a raised neutralization titre to CBV2.

The results obtained with CBV5-IgM assay are shown in Fig. 3. The SRs for the 100 ANC sera largely group in the 20–75% range with a mean value of 50% (column A, Fig. 3). The mean plus 3 SD value was 143% and again this value was taken to indicate positivity in the assay. Only three sera gave values above this level and it was notable that the same ANC serum gave the highest SR in both the

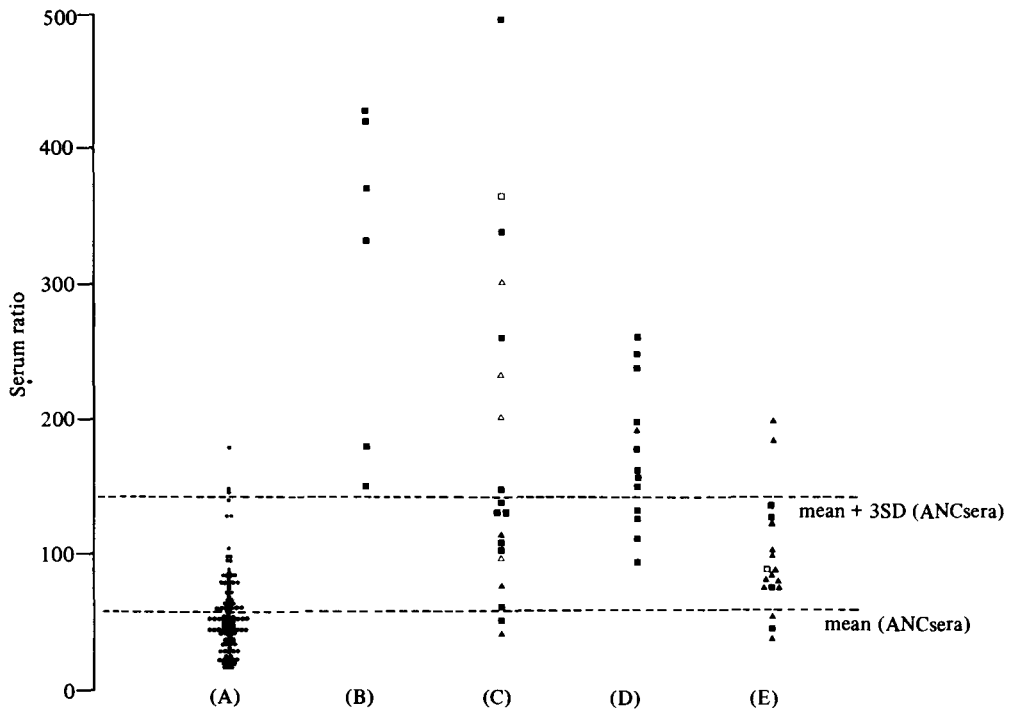


Fig. 3. Coxsackievirus B5-specific IgM serum ratios for (A) 100 antenatal sera (●), (B) coxsackievirus B5 infections, (C) other coxsackievirus B infections, (D) coxsackievirus A and echovirus infections and (E) elevated coxsackievirus B neutralizing antibody titre (≥ 160). Cases in groups B–D were diagnosed by virus isolation (■), neutralizing antibody rise to one serotype (▲), neutralizing antibody rise to more than one serotype (△) and the mother of a neonate with coxsackievirus B4 infection (□). Cases in group E had a raised heterologous (monotypic) titre only (▲), raised titre to more than one serotype including homologous serotype (□) and raised titre to more than one serotype excluding homologous serotype (■). The mean serum ratio and the mean plus 3 SDs for the 100 ANC sera are indicated by the lower and upper dashed lines respectively.

CBV4-IgM and CBV5-IgM assays (138 and 175% respectively). The six sera from cases of CBV5 infection gave SRs between 150 and 440% (column B, Fig. 3). The 18 sera from cases of CBV infection other than CBV5 gave SRs between 40 and 500% with seven (39%) having SRs above 143% (column C, Fig. 3). The 13 sera from cases of infection with other enteroviruses gave SRs ranging from 95 to 260% and nine (69%) gave SRs above 143% (column D, Fig. 3). The nine sera that gave positive results in the CBV5-IgM assay were those nine that had also given a positive result in the CBV4-IgM assay. The 18 sera from possible cases of CBV infection gave SRs ranging from 35 to 200% but only two (11%) gave values above 143% (column E, Fig. 3). These two sera were also positive for CBV4-IgM and had a raised monotypic CBV4 neutralization titre.

The sera which gave a positive result in the CBV4 or CBV5-IgM assays were all re-tested using control antigen diluted 1:2 in PBS 0.2% T and the result compared with that obtained with the test antigen. In no serum was significant reactivity seen with the control antigen.

No positive results were seen with the rheumatoid factor containing sera or with

Table 3. *Coxsackievirus B4 and B5-IgM in confirmed cases of enterovirus infection*

Infection	Positive result for CBV IgM with			
	CBV4 only	CBV5 only	Both CBV4 and 5	Neither
Confirmed CBV4	3*	—	1	—
Confirmed CBV5	—	2**	4	—
Confirmed CBV1–3, CBV6, or multitypic neutralization titre rise	1‡	1‡	5	7
Confirmed echovirus or coxsackievirus A	—	—	9	4

* Neonate, 13-year-old and 26-year-old; ** 1-year-old and 4-year-old; ‡ CBV6 infections.

Table 4. *Association of coxsackievirus B-IgM and presence of coxsackievirus B neutralizing antibody in confirmed cases of enterovirus infection*

Neutralizing antibody at a dilution of 1:20 to		Positive result for CBV IgM with			
CBV4	CBV5	CBV4 only	CBV5 only	Both CBV4 and 5	Neither
+	—	1	1	6	2
—	+	0	1	0	0
+	+	0	0	5	0
—	—	0	0	2	2

sera collected from cases of infection due to other agents. It is interesting to note that none of the sera from the seven cases of hepatitis A (all hepatitis A IgM positive) were positive in the CBV4 or CBV5-IgM assays.

Table 3 illustrates the relation between the results of the CBV4-IgM and CBV5-IgM assays in confirmed enterovirus infection. Of the four cases of CBV4 infection, three gave a positive result in the CBV4-IgM assay but not in the CBV5-IgM assay whilst one gave a positive result in both assays. The three sera giving a monotypic CBV4-IgM positive result were obtained from a neonate, a 13-year-old and a 26-year-old. Two of the six sera from CBV5 infections gave positive results in the CBV5 assay only but the other four gave positive results in both the CBV4 and 5 assays. The two sera giving a monotypic CBV5-IgM positive result were obtained from a 1-year-old and a 4-year-old child. Of the 14 sera from cases of infection with CBV other than CBV4 or 5 or having a multitypic neutralization titre rise, five (two cases CBV2, three cases multispecific neutralizing antibody rise-CBV1 and 4, CBV 3 and 5, CBV 2, 3 and 5) gave a positive result in both assays, seven gave a negative result in both assays and two sera gave a positive monotypic CBV-IgM result. These latter two sera were from two cases of CBV6 infection. For these two sera the results obtained were very close to the SR value taken to indicate positivity (one serum SR CBV4-IgM 120%, CBV5-IgM 142%; one serum SR CBV4-IgM 86%, CBV5-IgM 147%). The 13 sera from cases of coxsackievirus A and echovirus infection gave a positive result in both assays in nine cases with the remaining four being negative in both assays.

A selection of 20 sera from cases of proven enterovirus infection were tested for the presence of CBV4 and CBV5 neutralizing antibody at a serum dilution of

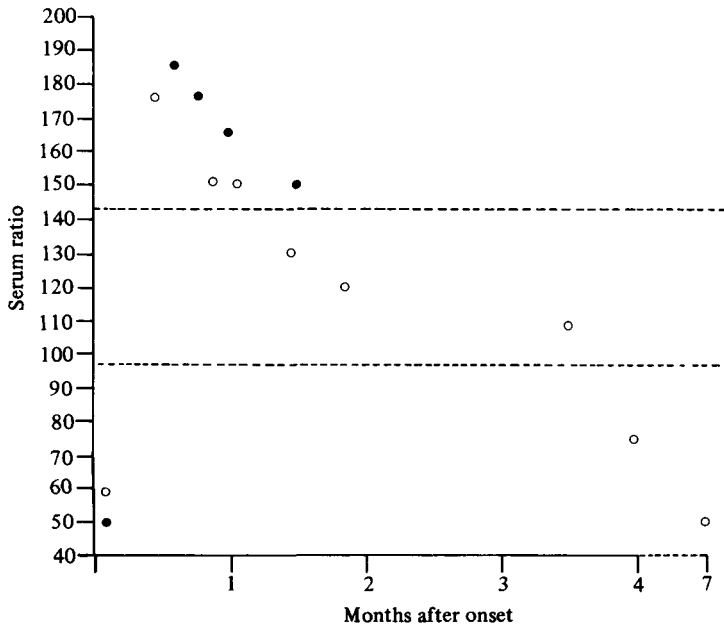


Fig. 4. The CBV5-specific IgM response (○) in a case of Bornholm disease in an adult female (CBV5 isolated from throat swab) and the CBV4-specific IgM response (●) in a case of febrile illness with a rash in an adult male (CBV4 isolated from throat swab). The dashed lines indicate the mean plus 3 SD for the SRs of 100 ANC sera in the CBV4-IgM (lower line) and CBV5-IgM assays (upper line).

1:20. These were seven cases from whom CBV was isolated (CBV2:1, CBV4:2, CBV5:1, CBV6:3), three cases showing a rise in CBV neutralizing antibody titre (CBV3, CBV1 and 2 and CBV2, 3 and 5), one case of coxsackievirus A9, two cases of coxsackievirus A16 and seven cases of echovirus infection (echovirus 7, 2; echovirus 11, 2; echovirus 17, 1; echovirus 30, 2). The results are shown in Table 4 where the presence of neutralizing antibody is correlated with the results of CBV4 and CBV5-IgM assays. One serum gave a monotypic CBV5-IgM positive result and six sera gave positive CBV4 and 5-IgM results but did not have CBV5 neutralizing antibody present at a dilution of 1:20. Two sera gave a positive CBV4 and 5-IgM result but did not contain CBV4 or 5 neutralizing antibody present at a dilution of 1:20. Therefore nine sera gave a positive result with a CBV-IgM assay but did not have homologous neutralizing antibody present at a dilution of 1:20. However, the two cases of confirmed CBV4 infection and the one case of CBV5 infection all had homologous neutralizing antibody present.

The CBV5-IgM response in a 45-year-old woman who had Bornholm disease from whom CBV5 was isolated is shown in Fig. 4. The acute serum taken 3 days after onset is negative but the second serum taken at 12 days is positive. The level progressively declines over the ensuing weeks having fallen below the level indicating positivity by the sixth week. A similar response is also shown in Figure 4 for the CBV4-IgM response in the case of a rash with fever in a young adult from whom CBV4 was isolated.

Fig. 5 demonstrates the CBV4 and CBV5-IgM response in a 14-month-old infant who had echovirus 7 isolated from a faeces collected 4 days after onset. Serum

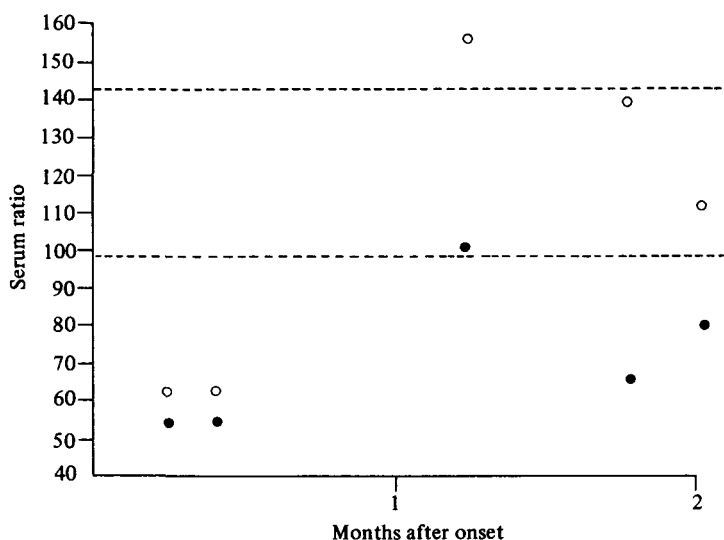


Fig. 5. The CBV4 and CBV5-specific IgM responses in a case of myocarditis in a 14-month infant (echovirus 7 isolated from stool). ● CBV4-specific IgM, ○ CBV5-specific IgM. The dashed lines indicate the mean + 3 SD for the SRs of 100 ANC sera in the CBV4-IgM (lower line) and CBV5-IgM assays (upper line).

collected 14 days after onset was negative for CBV4 and CBV5-IgM but serum collected 40 days later was positive in both assays. The levels then gradually declined. The neutralization titres remained at < 20 for CBV4 and CBV5.

DISCUSSION

The MACRIA assay established for the detection of CBV IgM was similar to that established for the detection of rubella-specific IgM (Mortimer *et al.* 1981). The various components of the assay were evaluated so as to give maximum differentiation between a positive and a negative serum, the test:negative (T/N) ratio. Various treatments of the crude antigen were assessed in order to obtain the best results without losing significant antigen volume that would result in the requirement to produce very large volumes of untreated antigen. The method of antigen preparation finally selected was simple, comprising release of intracellular virus by freezing, thawing and ultrasonication with the removal of cell debris by low-speed centrifugation. It is probable that purification and concentration of the antigen would result in higher T/N ratios with positive sera. However, simply increasing T/N ratios may not help to distinguish positive sera if increased ratios are also seen with presumed negative sera due to background levels of enterovirus-specific IgM. This would merely result in an increase in the serum ratio which needs to be taken to indicate positivity (see later). Various attempts were made to reduce background binding, thereby increasing T/N ratios, by incorporating NRS or control antigen in the serum or ^{125}I label diluent. The only significant effect was seen with the addition of NRS to the label diluent.

Even with optimally collected sera, the T/N ratios obtained were never

greater than 10 and ratios > 2 could only be obtained to a serum dilution of 1:20. The development of a monoclonal detector antibody should considerably increase these ratios as it has done for the rubella MACRIA (Tedder, Yao and Anderson, 1982). However, for the reasons stated above in relation to antigen purification, it may be that such an improvement in T/N ratios would not help to differentiate positive from negative sera. Indeed, this has been the case with the rubella monoclonal antibody as compared to the previously used hyperimmune rabbit antiserum (unpublished observations). An improvement in discrimination is not likely if there is a lack of specificity of the reaction between IgM and the viral antigen.

The specificity of the ^{125}I -labelled anti-CBV detector antibody for the homologous CBV antigen was shown by evaluating three sera with CBV antigens 1–5 and ^{125}I -labelled anti-CBV5. Of the two sera from CBV2 infections one serum was positive with CBV5 antigen but negative with CBV2 antigen, one serum was negative with both CBV2 and CBV5 antigens. If there had been cross-reactivity for the CBV antigen of the labelled anti-CBV5, a reaction would have been seen with at least the CBV2 antigen. This specificity of the ^{125}I -labelled anti-CBV5 for CBV5 antigen has also previously been established in an antigen detection system (Teare, 1982). It is also noteworthy that no sera gave significant reactivity if control antigen was used in the assay.

The sensitivity of the assay was investigated by evaluating a dilution series of the positive serum in negative serum. There was a progressive decline in T/N ratios with increasing dilution so that at a dilution of 1:20, T/N ratios of 2–3 were seen. On the basis of this evaluation, a dilution of 1:20 of the positive serum was taken as a low positive control for testing in each assay run. Each serum tested was compared with this low positive control, the result being expressed as a serum ratio.

To establish an SR value that would indicate positivity for CBV-specific IgM, 100 ANC sera were tested. These were collected in late February so as to minimize the possibility of their containing CBV-specific IgM as the majority of such infections occur in the summer and autumn (Grist, Bell & Assaad, 1978). However, as later results showed, positive results were obtained with an appreciable percentage of sera from persons suffering infection with other enteroviruses. As there are at least 67 serotypes of enterovirus it therefore becomes extremely difficult, if not impossible, to totally exclude an enterovirus infection in the few months preceding the serum sample. Therefore it is extremely difficult to establish with certainty a group of negative sera.

Eighty-five of the ANC sera gave SRs in the CBV4-IgM assay of 50% or below with the remaining 15 giving a scatter of values up to 138%. In the CBV5-IgM assay, 89 ANC sera gave SRs of less than 90% with the remaining 11 giving a scatter of values up to 175%. The problem is the interpretation of those sera giving the higher SR values. These values may be due to low levels of enterovirus-specific IgM resulting from a remote enterovirus infection or due to the accepted scatter of reactivity of sera within any assay. However, as the assay was established with the aim of diagnosing recent CBV4 or CBV5 infection it is necessary to take into account the higher SR values occurring in a group of sera chosen because they are not likely to contain enterovirus-specific IgM. Therefore, an SR equivalent to the mean plus 3 SD of the 100 ANC sera was taken to be the minimum required to

indicate recent infection. Using this criterion three ANC sera gave a positive result in each assay. It has to be accepted that using such a high value to indicate positivity results in an insensitive assay. However, it is essential that sera with low levels of reactivity in the assay are not considered positive since it is unlikely that more than a few of the 100 ANC sera were taken from women who had had a recent enteroviral infection. One ANC serum gave the highest SR in both the CBV4 and CBV5-IgM assays and it seems likely that this woman had had a recent enterovirus infection. The relative insensitivity of the assay using such a value to indicate recent infection is confirmed by the results obtained with the CBV5-IgM assay in sequential sera from a case of Bornholm disease. By one month after the onset of the illness the SR falls below the value taken to indicate positivity but continues to fall in later sera, suggesting a progressive decline in the amount of detectable CBV5-IgM. However, the sera taken at 1–2 months would be considered CBV5-IgM negative by the criteria used.

The homologous assays were positive in all cases of CBV4 and CBV5 infection. The rate of positivity (39–50 %) obtained for sera from heterologous CBV infections agrees with the figure of 48 % obtained by Minor *et al.* (1979). However, they found a low level of reactivity (9 %) in sera obtained from infections with other enteroviruses whereas in this study 69 % of such sera gave a positive result. This discrepancy suggests that the CIE assay was more specific for CBV infection than MACRIA.

The positive results seen with sera from cases of heterologous enterovirus infection may be due to an anamnestic IgM response, as occurs with neutralizing antibody (Grist & Bell, 1974) or due to the patient's IgM reacting with antigenic determinants common to many enteroviruses. Katze & Crowell (1980*b*) showed that for CBV, a cross-reacting group antigen was present on the VP 1 protein but they did not examine other enteroviruses. From the established general characteristics of the primary and secondary immune response the first explanation would appear unlikely but does gain some support from the tendency of monotypic CBV-IgM responses to occur in younger patients. An anamnestic response is unlikely if regard is paid to the detection of specific-IgM in the absence of a significant neutralizing antibody titre. This finding suggests that the IgM may not be directed against neutralizing epitopes. The lack of specificity of the IgM/antigen reaction is also suggested by the positive results in CBV4 and CBV5-IgM assays of the sera from a 14 month infant with echovirus 7 infection. An apparent variability of specificity of the IgM/antigen reaction is shown by monotypic CBV4 or CBV5-IgM responses in two cases of CBV6 infection. However, the results obtained in the two assays were very close to the SR value taken to indicate positivity for both sera. The only possible solution to this problem of cross-reactivity would appear to be the preparation of purified type-specific antigens and appropriate specific detection antibody. However, even such a procedure may be of no avail if the problem does totally relate to the non-specificity of the IgM response.

The unreliability of an elevated neutralizing antibody titre for indicating recent CBV infection is indicated by the results obtained with such sera where supporting evidence in the form of detectable-specific IgM was present in only five of seventeen (29 %) cases with elevated CBV4 neutralizing antibody titres. The two positive results in the CBV5-IgM assay were possibly a result of recent CBV4 infection as

they were also positive for CBV4 IgM and had a monotypic elevated CBV4 neutralizing antibody titre.

The absence of positive results with sera containing rheumatoid factor confirms the resistance of M-antibody capture assays to interference by such sera. Rheumatoid factor containing sera commonly give problems in IgM assays where the antigen is attached to the solid phase. The specificity of the assay for IgM produced in response to an enterovirus infection gains support from the negative results with sera from cases of infection with other agents. It would obviously be of interest to examine further sera from cases of hepatitis A in view of the likely classification of hepatitis A virus as an enterovirus (Melnick, 1982). A larger number of sera from cases of infectious mononucleosis also require evaluation in view of the recent finding of rubella-specific IgM detectable by MACRIA during acute Epstein-Barr virus infection (Morgan-Capner, Tedder & Mace, 1983). However, the levels of rubella-specific IgM detected were low and such levels of enterovirus-specific IgM would be unlikely to be a problem in the assays described here.

Thus the IgM assays described appear to be specific for an enterovirus infection but because of the considerable levels of cross-reactivity such IgM assays were not type specific nor even specific for one group of enteroviruses. The results presented here suggest that the development of such a monotypic assay for IgM may prove impossible if the problem relates to cross-reactivity of the patient's IgM. However, these findings do not diminish the importance of developing an enterovirus group-specific IgM assay, even though such a test may always have to be run at an insensitive level to take account of background enterovirus infections (both subclinical and clinical) occurring within the community.

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