Coxsackievirus B1-Based Antibody-Capture Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin G (IgG), IgM, and IgA with Broad Specificity for Enteroviruses

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An antibody-capture enzyme-linked immunosorbent assay (ELISA) with coxsackievirus B1 as the antigen was evaluated for detection of immunoglobulin G (IgG), IgM, and IgA antibodies and showed broad specificity for enteroviruses. In total, 116 serum or cerebrospinal fluid samples from 62 patients were tested by ELISA and the complement fixation test (CFT). Additionally, 15 serum samples that contained poliovirus-specific IgM antibody were tested. Serum samples from 200 healthy blood donors were used for standardization of the assays. The sensitivity of the ELISA varied with time of serum sampling, with a relatively low sensitivity when serum was collected within 3 days after the onset of symptoms (23%; 5 of 22) but good sensitivity when serum was collected later (83%; 20 of 24). The sensitivity was better than that of the CFT. The ELISAs were broadly reactive as concluded from typing of virus isolates that were simultaneously obtained. The assay did, furthermore, detect antibody against poliovirus type 3. Sera that contained rheumatoid factor, antinuclear antibody, or cardiolipin antibody (by the Venereal Disease Research Laboratory test) did not react in this ELISA. Nonspecific reactivity did occur, however, in cases of infectious mononucleosis and in Mycoplasma pneumoniae infection. The enterovirus-specific ELISA is found to be simple to perform, more sensitive than the CFT, and far less laborious than the neutralization test.

Enteroviruses are common pathogens that can cause a variety of symptoms ranging from mild respiratory infection to severe central nervous system disease. Enteroviruses may furthermore play a role in chronic diseases such as postviral fatigue syndrome (1, 12, 14, 37), chronic myocarditis/dilated cardiomyopathy (8), polymyositis/dermatomyositis (7, 38), postpolio syndrome (30), and insulin-dependent diabetes mellitus (2, 20, 34). The diagnosis of the acute infection is mainly based on virus isolation and serological tests such as virus neutralization or complement fixation (CF). Virus isolation is not successful in cases of the chronic diseases mentioned above, and a relationship with enteroviruses can be investigated only by molecular techniques (39) or serology. Enteroviruses include 69 serotypes. Hence, a neutralization test is laborious as long as the virus type is not known. CF tests (CFTs) are useful but of limited diagnostic value, particularly in a presumed persistent phase of infection. Enzyme-linked immunosorbent assays (ELISAs) for the diagnosis of enteroviral infections based on either antibody capture (3, 22, 29) or an indirect technique (6, 19) have been described previously. These tests made use of either a single serotype (6) or multiple serotypes (3, 19) as the antigen. Tests for immunoglobulin G (IgG) antibody were shown to be of limited value because of the presence of anamnestic antibody from earlier infections (6).

We describe an antibody-capture ELISA based on a heat-inactivated coxsackievirus B1 antigen for the detection of enterovirus-specific IgM, IgA, and IgG antibodies. We have deliberately chosen the antibody-capture ELISA because false reactions due to the interference with rheumatoid factors seldom occur and competition between antibodies of various isotypes is avoided. The antibody-capture ELISAs are highly sensitive, with exception of the IgG-capture assay. A high level of nonspecific IgG in serum reduces the sensitivity of this assay to a relatively low level. The advantage of this low sensitivity is that the assay will not detect low concentrations of IgG class antibodies from past infections but only increased levels from recent infections or from persistent infections. The capture principle leads to an increased sensitivity (also for IgG antibody) when cerebrospinal fluid (CSF) is tested (32). In this report, we describe the performance of this ELISA for the diagnosis of acute enterovirus infection with routine samples.

MATERIALS AND METHODS

Patients. Routine serum samples were collected during an 8-month period (May to December 1992) from patients with symptoms of respiratory infection, gastroenteritis, meningitis, pleuritis, and myocarditis. One hundred sixteen samples from 62 patients were tested for enteroviral antibodies by CFT and ELISA. A stool specimen, a throat swab, or CSF was obtained from 35 of 62 patients for viral culture. For 14 patients, cultures remained negative for enterovirus; enterovirus was isolated from 21 patients. Enteroviruses were typed by the use of pools of neutralizing sera (17). The types of enteroviruses that were isolated were coxsackievirus A9 (once), coxsackievirus B5 (four times), echovirus 6 (once), echovirus 9 (twice), echovirus 11 (three times), echovirus 19 (once), echovirus 30 (eight times), and poliovirus type 3 (once). Additionally, 15 serum samples that were obtained during a recent Dutch epidemic of poliomyelitis type 3 were kindly provided by the National Institute of Public Health and Environmental Protection. The latter sera were all taken from asymptomatic contacts of patients with poliomyelitis. All sera contained poliovirus type 3-specific IgM antibodies.
that were detected by a poliovirus type-specific ELISA (23, 27, 36).

Control sera. Sera collected in March 1991 from 200 healthy blood donors were used as controls. To investigate the specificity of the assay, rheumatoid factor IgM-positive serum samples (n = 20), serum samples with antinuclear antibodies (n = 21), syphilitic serum samples (positive by the Venereal Disease Research Laboratory test and the Treponema pallidum microhemagglutination test) (n = 20), serum samples with heterophile antibodies (Monospot; Ortho Diagnostics Systems, Raritan, N.J.) (n = 20), and Mycoplasma pneumoniae IgM- and IgA-positive serum samples (n = 15) were tested as well. Serum samples were kept at −20°C until analysis.

Preparation of antigen. Coxsackievirus B1 was grown in buffalo green monkey cells. Buffalo green monkey cells were tested as well. Serum samples were kept at −20°C until analysis. Wilson and Nakane (35). The conjugated viral and control noninfected cell cultures. The protein contents of the viral conjugated with horseradish peroxidase as described by Lowry et al. (21). The antigens were denatured the virions and convert antigenicity from type nema

Preparation of antigen. Coxsackievirus B1 (Tucson strain [28]) at a multiplicity of infection of 0.5. The cultures were maintained in 150 ml of minimal essential medium with 3% fetal bovine serum, 100 U of penicillin per ml, and 50 µg of gentamicin per ml. The roller bottles were incubated at 36°C. Virus was harvested when complete cytopathic effect was observed, usually after 2 to 3 days. Cells were freeze-thawed three times and together with the culture medium firmly shaken with 10% chloroform for 15 min and centrifuged at 2,000 × g for 30 min. The supernatant was centrifuged through a 30% (wt/wt) sucrose cushion at 150,000 × g for 3 h. The pellets were resuspended in a final volume of 6 ml of phosphate-buffered saline (PBS) and heated at 56°C for 60 min in order to denature the virions and convert antigenicity from type specific (N antigen) to group specific (H antigen) (19, 24). Control antigens were prepared in the same way by using noninfected cell cultures. The protein contents of the viral antigen and the control antigen preparations were determined as described by Lowry et al. (21). The antigens were conjugated with horseradish peroxidase as described by Wilson and Nakane (35). The conjugated viral and control antigens were suspended in PBS containing 0.005% mercaptoethanol and 2% fetal bovine serum. The labelled antigens were stored in small aliquots at 4°C.

Capture ELISA technique. The antibody-capture ELISAs for detection of enterovirus IgG, IgM, and IgA were carried out essentially as described previously (32, 33). Polyethylene terephthalate glycol microtiter plates (96-well PETG assay plates, no. 6595; Costar Europe, Badhoevedorp, The Netherlands) were coated with 125 µl of goat antihuman IgG (Cappel Laboratories, Cochranville, Pa.), goat antihuman IgA (Cappel), or monoclonal antihuman IgM (kindly provided by J. P. Coutelier, Universite Catholique de Louvain and Institute of Cellular and Molecular Pathology, Brussels, Belgium) diluted in Tris buffer (pH 9.0). The optimal dilutions were determined by checkerboard titration and were 1:500 for anti-IgG and 1:1,000 for anti-IgM and anti-IgA antibodies. The plates were incubated overnight at 4°C, washed four times with wash buffer (0.01 M PBS [pH 7.2] with 0.05% Tween 20), and shaken dry. Next, 100 µl of patient serum diluted at 1:100 in PBS-Tween with 2% fetal bovine serum and 0.005% mercaptoethanol (PFT-M) was added and the plates were incubated at 37°C in a humidified atmosphere for 2 h. All sera were tested in duplicate. Plates were washed again four times and shaken dry, and 100 µl of horseradish peroxidase conjugated antigen diluted in PFT-M was added. The optimal dilution of conjugated antigen was determined by block titration and was 1:1,000 for the IgG assay and 1:1,500 for the IgM and IgA assay. After overnight incubation at 4°C, the plates were washed again and 100 µl of substrate solution was added. The substrate solution was prepared immediately before use by dissolving 4 mg of orthophenylene diamine per ml in 0.05 M citrate buffer (pH 5.2) and then adding 0.045% hydrogen peroxide (final concentration). The reaction was stopped after exactly 10 min by the addition of 100 µl of 3 M H₂SO₄. The Abs₅₄₀ was read (Titertek Multiskan; Flow Laboratories, Irvine, United Kingdom). The buffer control was used as a blank. A strongly positive serum sample, a cutoff serum sample, and a negative control serum sample were included in each test. The cutoff was chosen on the assumption that at most 5% of the serum samples from healthy blood donors were reactive. A serum sample was considered positive when the absorbance was at least twice the absorbance of the cutoff serum for IgM and IgA or at least the same as cutoff value for IgG. The ELISA with the control antigen was performed in a similar way.

CFT. The CFT was performed according to the microtitrate technique described by Casey (9). The enterovirus antigen in the CFT consisted of a mixture of antigens including coxsackieviruses B1 to B5 and A9 and echoviruses 4, 6, 9, 14, 24, and 30 (Behringwerke AG, Marburg, Germany). The titer is expressed as the reciprocal of the highest dilution showing 50% hemolysis. The CFT was considered positive when a fourfold increase of antibodies between acute and convalescent sera was found or when the titer was >128.

Neutralization test. Sera from some of the patients were tested for neutralizing antibodies as described by Melnick et al. (25). IgM was prepared by a fractionation-concentration technique after gel chromatography as described by Inouye and Kono (15). Because of the limited amount of serum, the IgM fraction was tested only against the virus strain against which the highest neutralizing antibody titer was found in unfracionated serum.

RESULTS

Standardization of enterovirus-specific ELISA. Two hundred serum samples from healthy blood donors were tested for enterovirus IgG, IgM, and IgA antibodies. From the outcome, cutoff values were determined on the assumption that at most 5% of the serum samples were reactive. This is illustrated for the IgG assay in Fig. 1. Serum with reactivity

FIG. 1. Results of the IgG-capture assay with serum samples from 200 healthy blood donors.
at the cutoff level as well as a strongly reactive serum sample and a negative serum sample were selected for standardization of the assays. The strongly positive serum sample that was used was derived from a patient with a culture-proven enteroviral infection who also had a high titer in the CFT (titer, >256). The A_90 of this serum sample was greater than five times the absorbance of the cutoff serum sample for the IgM and IgA ELISA and greater than three times the absorbance of the cutoff serum sample for the IgG ELISA.

To reveal any nonspecific reactions, all 200 donor serum samples were tested against the horseradish peroxidase-labelled control antigen made from uninfected cells. None of the sera reacted with the control antigen.

**Patients.** One hundred sixteen serum samples from 62 patients were tested for enteroviral antibodies by CFT and ELISA. A stool specimen, a throat swab, or CSF was obtained from 35 patients for viral culture. For 14 patients, culture remained negative for enterovirus, and enterovirus was isolated from 21 patients. Data for these 21 patients are represented in Table 1. A recent enterovirus infection was diagnosed in 7 patients by CFT (33%) and in 14 patients by ELISA (67%; IgG 6 times, IgM 10 times, and IgA 6 times). In seven patients, no enteroviral antibodies were detected by ELISA. In two of them (patients 5 and 14), such antibodies were detected by CFT. Two patients were eventually diagnosed as also having a bacterial infection at the time of blood sampling (patients 5 and 6). Fifteen of the 21 patients presented with meningeal signs (Table 1, patients 7 to 21). Enterovirus was isolated either from CSF (n = 9), from a throat swab (n = 2), or from a fecal sample (n = 4). In 5 of 15 patients, recent enteroviral infection was diagnosed by CFT (33%), and in 10 patients, it was diagnosed by ELISA (67%; IgG five times, IgM seven times, and IgA four times). Five patients presented with meningeal signs during a small epidemic of echovirus 30 meningitis (patients 17 to 21). Sera from these patients were also tested for neutralizing antibodies against echovirus 30. All patients had a neutralizing antibody titer of ≥128. After fractionation of these serum samples, neutralizing antibodies of the IgM class could be detected in all samples as well. In 2 of the 14 patients whose cultures were negative for enterovirus, a recent enterovirus infection was diagnosed by serology. The clinical diagnosis for these two patients was Bornholm disease and myocarditis (Table 2, patients 26 and 28). Among the remaining 12 of 14 culture-negative patients, other infections were documented for 6: parainfluenza virus (n = 2), herpes simplex virus (n = 1), varicella-zoster virus (n = 1), ureaplasma infection (n = 1), and streptococcus infection (n = 1);
in three cases no infection was documented, and in three cases a noninfectious cause was found. All serum samples from these patients were negative by ELISA and CFT.

For 27 patients, no material for culture was available; thus, only serologic tests were performed. For 21 of these patients, enterovirus-specific serology was negative. In five of these patients there was serologic evidence for other viral infections, namely, by herpes simplex virus (n = 1), parainfluenza virus (n = 3), and respiratory syncytial virus (n = 1). For six patients, serology indicated a past or recent enterovirus infection. Data for four of these patients are presented in Table 2 (patients 22, 24, 25, and 27). One patient presented with convulsions, and serologic results indicated a recent enterovirus infection: high titers were determined by CFT, and the patient was positive for both IgM and IgA antibodies. In one patient, IgM antibodies against enterovirus were detected. However, in this patient there were also IgM antibodies against cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and varicella-zoster virus, which means that these antibody responses were probably nonspecific.

Seven patients with cardiomyopathy, myocarditis/pericarditis, or pleuritis (Bornholm disease) as a clinical diagnosis are represented in Table 2. One patient died of cardiomyopathy at birth (patient 23). The high titer in the CFT probably represents maternal antibodies. Among the remaining six patients, recent enteroviral infection was diagnosed in four by CFT (67%) and in all by ELISA (100%; IgG four times, IgM four times, and IgA four times). For three patients, serum was also tested for neutralizing antibodies against coxsackieviruses B1, B2, B3, B4, and B5. Two patients had neutralizing antibodies against coxsackievirus B4, and one patient had neutralizing antibodies against coxsackievirus B1. In these samples neutralizing antibodies of the IgM class could be detected, which indicates recent infection.

Fifteen serum samples that were drawn from asymptomatic contacts of patients with poliomyelitis were tested by the coxsackievirus B1-based antibody-capture ELISA and by CFT. All sera contained poliovirus type 3-specific IgM antibodies as determined at the National Institute of Public Health and Environmental Protection. The results are summarized in Table 3. All sera were positive by the IgM ELISA, 12 serum samples were positive by the IgA ELISA, and 9 serum samples were positive by the IgG-capture ELISA. Seven serum samples were positive by all three ELISAs. Nine serum samples were positive by CFT (titer, >256).

**Specificity of the assays.** The results for the control sera with known antibody specificity are summarized in Table 4. Of the 20 serum samples with rheumatoid factors of the IgM type, only 1 was reactive in the IgM and IgA ELISA. Three of 21 serum samples with antinuclear antibodies reacted in the IgM ELISA, but none of them reacted in the IgA ELISA. One of 20 Venereal Disease Research Laboratory test-positive serum samples reacted with enteroviral antigen in the IgM ELISA as well as in the IgA ELISA. Unfortunately, reactivity to the enteroviral antigen was encountered in half (10 of 20) of the Monospot-positive sera and in the majority (10 of 15) of sera containing IgM and IgA antibodies against *M. pneumoniae*. All sera that reacted with the enteroviral antigen in the ELISA were also tested with the control antigen and by CFT. None of the sera reacted with the control antigen in the IgG and IgA ELISAs. In the IgM

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Clinical diagnosis symptoms and signs</th>
<th>Virus isolate</th>
<th>Material</th>
<th>No. of days after onset</th>
<th>CFT titer</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>Neutralizing antibody titer</th>
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<tr>
<td>22</td>
<td>1 yr</td>
<td>Cardiomyopathy</td>
<td>NT</td>
<td>Serum</td>
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<td>NT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>23</td>
<td>1 day</td>
<td>Cardiomyopathy</td>
<td>NT</td>
<td>Serum</td>
<td>+44</td>
<td>64</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>24</td>
<td>20 yr</td>
<td>Pleuritis</td>
<td>NT</td>
<td>Serum</td>
<td>+4</td>
<td>128</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>25</td>
<td>25 yr</td>
<td>Pleuritis</td>
<td>NT</td>
<td>Serum</td>
<td>+14</td>
<td>128</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>NT</td>
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<tr>
<td>26</td>
<td>37 yr</td>
<td>Myopericarditis</td>
<td>Negative</td>
<td>Serum</td>
<td>+12</td>
<td>&gt;256</td>
<td>+</td>
<td>++</td>
<td>++</td>
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<tr>
<td>27</td>
<td>26 yr</td>
<td>Pleuritis</td>
<td>NT</td>
<td>Serum</td>
<td>+3</td>
<td>&gt;256</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>28</td>
<td>28 yr</td>
<td>Bornholm disease</td>
<td>Negative</td>
<td>Serum</td>
<td>+11</td>
<td>&gt;256</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>CVB4, 512</td>
</tr>
<tr>
<td>29</td>
<td>9 yr</td>
<td>Poliovirus type-specific IgM</td>
<td>1,2,3</td>
<td>Serum</td>
<td>+6</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CVB4, 128</td>
</tr>
<tr>
<td>30</td>
<td>17 yr</td>
<td>Poliovirus type-specific IgM</td>
<td>1,2,3</td>
<td>Serum</td>
<td>+16</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CVB4, 64</td>
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</table>

**TABLE 2. Patients with cardiomyopathy, myocarditis/pericarditis, or pleuritis**

* Abbreviations: NT, not tested; CVB1 and CVB4, coxsackieviruses B1 and B4, respectively. Symbols: -, negative (IgG < the cutoff; IgM and IgA < 2x the cutoff); +, positive (IgG, 1x to 2x the cutoff; IgM and IgA, 2x to 5x the cutoff); ++, strongly positive (IgG > 2x the cutoff; IgM and IgA > 5x the cutoff).

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Poliovirus type-specific IgM</th>
<th>CFT titer</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
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<tbody>
<tr>
<td>29</td>
<td>9 yr</td>
<td>3</td>
<td>&gt;256</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>17 yr</td>
<td>3</td>
<td>4</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<td>31</td>
<td>10 yr</td>
<td>3</td>
<td>256</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>15 yr</td>
<td>3</td>
<td>&gt;256</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>11 yr</td>
<td>3</td>
<td>8</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td>9 yr</td>
<td>3</td>
<td>16</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>35</td>
<td>18 yr</td>
<td>1,2,3</td>
<td>&gt;256</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>23 yr</td>
<td>1,2,3</td>
<td>64</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>7 yr</td>
<td>3</td>
<td>64</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>38</td>
<td>9 yr</td>
<td>3</td>
<td>&gt;256</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<tr>
<td>39</td>
<td>17 yr</td>
<td>3</td>
<td>&gt;256</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<td>3</td>
<td>&gt;256</td>
<td>++</td>
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<td>+</td>
</tr>
<tr>
<td>41</td>
<td>31 yr</td>
<td>1,2,3</td>
<td>&gt;256</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>42</td>
<td>36 yr</td>
<td>3</td>
<td>&gt;256</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td>31 yr</td>
<td>1,2,3</td>
<td>&gt;256</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**TABLE 3. Sera taken during recent Dutch epidemic of poliomyelitis type 3**

* Symbols: -, negative (IgG < the cutoff; IgM and IgA < 2x the cutoff); +, positive (IgG, 1x to 2x the cutoff; IgM and IgA, 2x to 5x the cutoff); ++, strongly positive (IgG > 2x the cutoff; IgM and IgA > 5x the cutoff).

* Vaccinated.
ELISA with the control antigen, some reactivity was found in the Monospot-positive sera and sera with antibodies against M. pneumoniae. However, reactivity with the control antigen was less prominent than reactivity with the enteroviral antigen. In 2 of 10 Monospot-positive serum samples and in 2 of 10 serum samples containing mycoplasmal antibodies, high titers were found by CFT, suggesting recent enterovirus infection or nonspecific reactivity occurring in CFT as well. All patient sera that were positive by ELISA with the enteroviral antigen were tested with the control antigen as well. None were reactive.

DISCUSSION

The aim of this study was to evaluate the sensitivity and specificity of a coxsackievirus B1-based antibody-capture ELISA as a simple test with broad reactivity for enteroviruses. It is known that cross-reactive epitopes are located on the empty capsids of enteroviruses. Heating to 56°C converts virions to empty capsids, thereby changing the antigenicity to group-specific reactivity (19, 24, 29). The present study confirms this broad specificity. During the development of these ELISAs we have made conjugated antigens of the 10 enterovirus types with the highest incidence in the Netherlands. These conjugates have been tested either separately or as antigen mixtures. So far, infections by the following known serotypes have been recognized: echoviruses 6, 7, 9, 11, 14, 19, 25, and 30; coxsackieviruses B1, B2, B4, and B5; and poliovirus type 3. A combination of various serotypes pooled with an antigen mixture did not further broaden reactivity for enteroviruses, nor did the use of other serotypes as the antigen (data not shown).

The cutoff values were arbitrarily chosen at the 95% confidence level of reactivity in a population of 200 healthy blood donors. In March (the time of collection of the donor sera), the incidence of acute enterovirus infections is low, but the exact prevalence was not known. This might have influenced the sensitivity and specificity of the tests.

Among the patients with culture-positive enterovirus infections, seven patients were missed by ELISA. In two of these patients (patients 5 and 6), there was also evidence of bacterial infection (Escherichia coli and Staphylococcus aureus). Therefore, the onset of enterovirus infection is not accurately known for these cases. It may be that the samples were drawn too early to find specific antibodies (patients 9 and 16). Unfortunately, there was no second sample. It may also be that the ELISA was falsely negative. Consistent with this possibility, enteroviral antibodies were detected in patients 5 and 14 by CFT but not by ELISA. Patient 8 suffered from neonatal meningitis and could have had a delayed antibody response, as seen more often with neonatal infections. Enteroviral antibodies were detected by ELISA in CSF from only two of eight patients. This is probably due to the time of sampling. CSF is usually taken at the onset of symptoms, when antibody production is just starting. Antibodies were indeed detected in two of three CSF samples that were taken more than 2 days after onset. Thus, ELISA with CSF may be useful when CSF is taken later than 2 days after onset, comparable to what is found in sera (see below). This has also been described by van Loon et al. (32), who did not detect antibodies against herpes simplex virus in CSF within 6 days after the onset of illness. In contrast, they found herpes simplex virus IgG and IgA in CSF from all patients with herpes simplex virus encephalitis more than 10 days after onset.

In patients with cardiomyopathy, myocarditis/pericarditis, or pleuritis, cultures are usually not performed or are performed with a considerable delay. When clinical symptoms become clear, cultures are usually negative. In such patients, only serology may reveal a relationship with enteroviral infection. We have tested serum samples from seven of such patients by the coxsackievirus group B1-based ELISA and CFT with the group B2 antigen, serum samples with the B5 antigen, and CFT with the B2 antigen (Table 2). For one patient, serology was negative by ELISA. This patient (patient 23) was born with cardiomyopathy and died at birth. Polymerase chain reactions with heart biopsies from this patient, taken at autopsy, were negative for enterovirus.

There is a discrepancy in IgM and IgA responses: nine patients had an IgM response but no IgA response, and three patients had an IgA response but no IgM response. This has been described before (26), but the reason for this discrepancy is not known.

The sensitivity of the antibody-capture ELISA is good when the outcome of the IgG, IgM, and IgA assays are combined. The sensitivity ranges from 67% in cases of culture-proven enteroviral infections and meningitis (14 of 21) to 86% (6 of 7) in cases of myocarditis, pericarditis, or pleuritis. The sensitivity in the case of poliovirus type 3-specific IgM-positive sera was 100% (15 of 15). The sensitivity is clearly related to the time when serum samples are taken. When serum samples are taken within 3 days after the onset of symptoms, the sensitivity is low (5 of 22 [23%]). When serum is drawn later than 3 days after onset, the sensitivity is good (20 of 24 [83%]). The CFT with the antigen mixture is less sensitive than the ELISA (44 versus 81%), especially when only one serum sample is available. When the ELISA and CFT were combined, the sensitivity was 88%. This ELISA was found to be as sensitive as an antibody-capture ELISA using different antigens (3, 22). The sensitivity of the IgM ELISA alone is 58%. When IgM and IgA are combined, the sensitivity increases to 67%. The IgG-capture assay adds another 16% to the sensitivity. The relatively low sensitivity of the IgM and IgA ELISA may be due to the recurrent character of enterovirus infections. Recurrent enteroviral infection with different serotypes causes boosts in responses to common epitopes. This may result in diminished antibody responses of both IgM and IgA. For cytomegalovirus, as an example, it is known that IgM and IgA responses are less prominent or even absent in recurrent infection compared with the primary infection (31, 33).

Antibody-capture ELISAs have proven to perform excellently with CSF (32). This is because of the low background levels of nonspecific IgGs in CSF as long as the blood-brain barrier is not damaged. This is particularly so for the IgG-capture assay as is illustrated by patients 11 and 12 in

<table>
<thead>
<tr>
<th>Known antibodies</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid factor IgM</td>
<td>0/20</td>
<td>1/20</td>
<td>1/20</td>
</tr>
<tr>
<td>Antinuclear antibodies</td>
<td>1/21</td>
<td>3/21</td>
<td>0/21</td>
</tr>
<tr>
<td>VDRL and TP-MHA</td>
<td>0/20</td>
<td>1/20</td>
<td>1/20</td>
</tr>
<tr>
<td>Heterophile antibodies</td>
<td>1/20</td>
<td>10/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Mycoplasma IgM and IgA</td>
<td>0/10</td>
<td>10/15</td>
<td>2/15</td>
</tr>
</tbody>
</table>

* VDRL, antibodies detected by the Veneral Disease Research Laboratory test.
* TP-MHA, antibodies detected by the Treponema pallidum microhemagglutination test.
our study. It can be envisaged that the IgG-capture ELISA will also perform well in case of high antibody levels (hyperimmunization), as has been reported to occur in persistent infection (2, 7, 37). This has already been investigated by using poliovirus type-specific ELISAs for postpolio syndrome, with which we did not find evidence for viral persistence (23). Similar studies are underway for postviral fatigue syndrome, dilated cardiomyopathy, and polyomysitis (16).

As expected, there was no interference by rheumatoid factor. There was little cross-reactivity in sera with antinuclear antibodies and none in sera with positive Venereal Disease Research Laboratory test reactions. Unfortunately, there was significant cross-reactivity with sera that contained heterophile antibodies or M. pneumoniae IgM and IgA antibodies. This is probably due to polyclonal B-cell activation, which is common in Epstein-Barr virus infection and mycoplasmal infection (4, 5, 11). Cross-reactivity occurred mainly in the IgM ELISA and less in the IgA ELISA.

Pitfalls in our assay seem to be similar to those reported by others (13). The major disadvantage of this test is its cross-reactivity with Epstein-Barr virus and M. pneumoniae. However, the clinical symptoms of these infections are quite different from those of enteroviral infections, and therefore we do not think this will be a major problem in clinical practice. Perhaps the use of synthetic peptides, which has recently been described by Cello et al. (10), may reduce cross-reactivity. The great advantage of the test we described is that it is simple to perform, more sensitive than the CFT, and far less laborious than the neutralization test.

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