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Diagnosis of Enterovirus Infection by Genus-Specific PCR and Enzyme-Linked Immunosorbent Assays

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PCR for the diagnosis of enterovirus infections is resource intensive but is increasingly used due to wide availability. Enzyme-linked immunosorbent assays (ELISAs) that detect heterotypical antibodies against enterovirus immunoglobulin M (IgM), IgA, and IgG were compared with reverse transcription-PCR by using primers specific to the 5′ untranslated regions of 60 enterovirus species. The ELISAs were less sensitive than the PCR, and only the ELISA for IgM was highly specific. When retrospective diagnosis is important or when specimens are unsuitable for PCR, the ELISA has a limited role if PCR is not available.

Enteroviruses are among the most common causes of infections in humans, which are often asymptomatic (12, 13). There are 64 *Enterovirus* serotypes that infect humans, and these are classified into five species (6). Laboratory diagnosis is important because enteroviruses can cause serious infections (3, 12), antiviral therapy may be used (16) if more virulent serotypes such as enterovirus 71 are identified, and epidemiological surveillance is important for managing outbreaks (3, 11).

Diagnosis of enterovirus infection is possible by using virus isolation, nucleic acid testing (NAT), and serological tests, including complement fixation (CF), neutralization, and enzyme-linked immunosorbent assays (ELISAs). Viral culture and neutralization tests are time-consuming, while CF may give false-negative results. NAT methods are now widely available (14); multicenter evaluations support the efficacy of in-house and commercially available enterovirus NAT methods (9, 15) and their improved sensitivity relative to that of viral culture (1, 17, 19, 24). ELISA may provide an alternative to NAT, with benefits of lower cost and a reduced need for experienced personnel and dedicated laboratories. ELISA can be used for retrospective diagnosis, can be simply automated, and may be effective in the investigation of outbreaks. ELISA methods that test for homotypical antibodies are impractical unless there is a clinical suspicion of the presence of one particular serotype, whereas heterotypical antibody assays allow detection of most enterovirus serotypes. Heterotypical ELISA methods use antigens from a limited number of enteroviruses (2, 4) or synthetic peptide antigens (10, 18) but detect antibodies that are directed against epitopes shared by multiple enterovirus serotypes. There are limited data from studies comparing ELISA with PCR; most studies have compared ELISA with viral culture or CF (2, 20). The aim of this study was to evaluate a commercially available heterotypical enterovirus ELISA (Genzyme Virotech, Russelsheim, Germany) for the detection of enterovirus-specific immunoglobulin A (IgA), IgM, and IgG antibodies and to compare the results with those of reverse transcription (RT)-PCR and CF.

Plasma and stool samples were collected from 205 children with type 1 diabetes and from 160 healthy controls from 1997 to 1999 (median age, 8.2 years; range, 0.7 to 15.8 years). Most children were asymptomatic, without evidence of enterovirus infection, at the time of testing. The samples were collected to investigate the prevalence of enterovirus RNA in children at the onset of diabetes compared with that in healthy controls. The purpose of this analysis was to evaluate the sensitivities and specificities of ELISAs for the entire cohort. Informed consent was obtained from the parents or guardians of the participants, and the study was approved by the hospital's ethics committee. All samples were tested by using RT-PCR, and plasma samples were tested by using ELISA. A further 27 serum samples collected from 1995 to 1999 from patients at the Prince of Wales Hospital, Randwick, New South Wales, Australia, with clinically suspected enterovirus infections and elevated CF titers (in 23 samples), were tested by using ELISA. Eight samples had high CF titers (1:128 to 1:1,024), seven had titers of 1:64, eight had titers of 1:32, and four had titers of $<$1:8.

Viral RNA was extracted from 200 μl of plasma or a 20% stool suspension by a simplified guanidinium thiocyanate method as previously described (23). Enterovirus RNA was detected by a single-step nested RT-PCR, with the following primers from the highly conserved 5′ untranslated region (UTR) of the enterovirus genome: EV1 (CAA GCA CTT CTG TTT CCC CGG), EV2 (TCC TCC GGC CCC TGA ATG CG), EV3 (ATT GTC ACC ATA AGC AGC CA), and EV4 (CAC YGC ATG GCC AAT CCA A). The PCR method detected viral RNA from 60 of 64 human enterovirus serotypes, results that are similar to previous findings from studies that used primers EV1, EV2, and EV3 (25). Coxsackievirus strains A1, A4, A8, and A13 were not tested because the

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TABLE 1. Comparison of ELISA IgA, IgM, and IgG results, stratified by enterovirus RT-PCR results

ELISA	No. of ELISA-positive patients/no. of PCR-positive patients	Sensitivity[a] (%)	No. of ELISA-positive patients/no. of PCR-negative patients (%)	*P* value[b]	Specificity[c] (%)
IgA	20/68	29	70/297 (24)	0.3	76
IgM	19/68	28	11/297 (4)	<0.001	96
IgG	37/68	54	102/297 (34)	0.002	66
IgM or IgG	48/68	71	107/297 (36)	<0.001	64
IgA or IgG	42/68	62	137/297 (46)	0.02	54
IgA or IgM	32/68	47	78/297 (26)	0.001	74
IgA, IgM or IgG	50/68	74	141/297 (47)	<0.001	53

[a] Number of true positives divided by total number of PCR-positive patients.
[b] Determined by the χ^2 test for the difference in the proportion of ELISA-positive results in PCR-positive and PCR-negative patients.
[c] Number of true negatives divided by total number of PCR-negative patients.

isolates could not be obtained. The PCR method did not detect samples containing rhinovirus or hepatitis C.

First-round RT-PCR was performed with one tube containing 10 μl of RNA, a 0.5 μM concentration each of primers EV1 and EV4, 0.2 mM deoxynucleoside triphosphate, 10 mM dithiothreitol, *Taq* 10× PCR buffer, 2.0 mM $MgCl_2$, 1.5 U of *Taq* polymerase (Promega, Madison, Wis.), and 4 U of avian myeloblastosis virus reverse transcriptase (Promega) under standard conditions to avoid the risk of contamination and inhibition (8). The reactions were performed with a GeneAmp model 2400 or 9600 thermal cycler (Perkin-Elmer, Shelton, Conn.). First-strand cDNA synthesis was performed for 40 min at 42°C, followed by denaturation at 95°C for 3 min, and then 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. The second-round reaction mixture contained 5 μl of the first-round PCR amplicon, a 0.5 μM concentration each of primers EV2 and EV3, and the same reagents and PCR cycling conditions as for the first-round PCR, with the omission of dithiothreitol and reverse transcriptase. The PCR products were separated on a 2% agarose gel stained with ethidium bromide and visualized under UV light. Negative (water) and positive (enterovirus 70 cultured in LLC-MK_2 cells, diluted 1:1,000) controls were included in all reactions from the extraction step and were run in parallel with test samples.

IgA, IgM, and IgG antibodies against the enterovirus group were detected with the Genzyme Virotech (Russelsheim, Germany) ELISA kit according to the manufacturer's instructions. The ELISAs provided semiquantitative and qualitative detection of cross-reacting heterotypical antibodies against enteroviruses. Coxsackievirus B5- and echovirus 24-infected A549 cells were used in the preparation of antigens. The samples were centrifuged for 5 min at 1,000 × *g*, and 10 μl of supernatant was removed and diluted 1:100 in phosphate-buffered saline (pH 7.2). The serum samples were preincubated with rheumatoid factor removal reagent to prevent false positives due to rheumatoid factor activity and false negatives due to IgG-versus-IgM competition. Serum samples containing anticardiolipin and antinuclear antibodies were also tested, and the presence of these antibodies in the samples did not influence the detection of enterovirus antibodies. Negative, positive, buffer, and cutoff control samples were run in parallel with test samples on each 96-well plate. The samples were considered positive when the optical density reading at 450/620 nm was at least 10% greater than the cutoff control value. Serological testing for antibodies to the enterovirus group was performed by using standard CF methods (5). CF titers were considered weakly positive if the titers were ≥1:32 and strongly positive if the titers were ≥1:128. Statistical comparisons between ELISA results for PCR-positive and -negative samples were performed by using χ^2 tests, assuming borderline samples to be negative.

Enterovirus RNA was detected in 19% (68 of 365) of all samples tested (cases, 30% [$n = 62$]; controls, 4% [$n = 6$]). Enterovirus RNA was detected in 17% (61 of 365) of the plasma samples and in 21% (29 of 135) of the stool samples. All three ELISAs showed lower sensitivies for the diagnosis of enterovirus infection than did PCR, and only the ELISA for IgM had a high specificity for detecting infection (96%), as shown in Table 1. There were no statistically significant differences in the sensitivities of the three tests for children with diabetes compared with those for the healthy controls (for IgM, 27 versus 33%; for IgA, 29 versus 33%; and for IgG, 55 versus 50%). The PCR-positive subjects were significantly more likely to have a positive ELISA result for IgM or IgG than were the PCR-negative subjects (Table 1). When children whose stool samples alone were positive for enterovirus RNA were excluded from the analysis, IgM and IgG were still significantly associated with the PCR results (26% of PCR-positive plasma samples versus 5% of PCR-negative plasma samples were IgM positive [$P < 0.0001$] and 53% of PCR-positive plasma samples versus 35% of PCR-negative plasma samples were IgG positive [$P = 0.01$]). The ELISA also had a lower sensitivity than did CF (Table 2). Of the samples with high CF titers (≥1:128), none were IgM positive and seven of eight were either IgA or IgG positive.

The measurement of heterotypical enterovirus IgA, IgM, and IgG antibodies by ELISA was a more insensitive assessment of infection than was RT-PCR or CF in this study. Only 28% of PCR-positive samples and one sample (4%) with an elevated CF titer were positive for IgM. The higher sensitivity of the ELISA for IgG (54%) was at the expense of reduced specificity (66%), and the IgG values were in the borderline range for approximately one in five PCR-positive and -negative cases. The sensitivities of all three tests were low for both diabetic and nondiabetic children, suggesting that these results have wider applicability. Furthermore, the regulation of the antibody response to enterovirus antigens is not disturbed in children with preclinical diabetes (18) and is unlikely to be so in children with recent-onset diabetes. Our findings are also

TABLE 2. Comparison of ELISA IgA, IgM, and IgG results, stratified by CF results

ELISA	No. of ELISA-positive patients/no. with elevated CF titer[a]	Sensitivity (%)	No. of ELISA-positive patients/no. with low CF titer[b]	Specificity (%)
IgA	9/23	39	1/4	75
IgM	1/23	4	1/4	75
IgG	12/23	52	1/4	75
IgM or IgG	12/23	52	0/4	100
IgA or IgG	18/23	78	1/4	75
IgA or IgM	9/23	39	0/4	100
IgA, IgM or IgG	18/23	78	1/4	75

[a] An elevated CF titer was ≥1:32.
[b] A low CF titer was <8.

comparable with those of a recent study of patients with enteroviral meningitis, in which the measurement of IgM antibodies by ELISA had a sensitivity of 34% compared with that of PCR (21).

These results highlight the different roles of NAT and ELISA in the diagnosis of enterovirus infections. While detection of enterovirus RNA is a sensitive measure of acute or recent infection, ELISA is a measure of antibody response to an earlier infection with an enterovirus. This was clearly demonstrated by the discordance between the PCR and IgG results, since 34% of PCR-negative subjects had evidence of past enterovirus infection.

The ELISA used in this study, which measures heterotypical antibody responses, relies on cross-reactivity between multiple enterovirus serotypes. False-negative tests could therefore have resulted from poorly or partly induced heterotypical antibody responses. The antigen mixture used in the kit may not have covered the range of enteroviruses infecting the populations tested; alternatively, younger children may have had a higher proportion of homotypical antibodies present (7). However, heterotypical antibody responses were common in Japanese children predominantly less than 10 years of age who were studied during an epidemic of enteroviral meningitis (22).

Because serology has an unacceptably lower sensitivity than does NAT, as these data show, its role in diagnosing enterovirus infections remains unresolved. Our results strongly support the use of NAT in preference to serology in the diagnosis of enterovirus infections in children and for population screening. NAT of enteroviruses has been available for more than 10 years, is reproducible (15), and should be the investigation tool of choice for the diagnosis of enterovirus infections in children.

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