Use of Monoclonal Antibodies To Identify Serotypes of Enterovirus Isolates

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Nonpoliovirus enterovirus infections cause a variety of diseases that are common in young children and adults. The “gold standard” for laboratory diagnosis of enteroviruses is cell culture isolation, followed by serotype identification by neutralization assay. These procedures are time-consuming and expensive. Rapid serotype identification of enteroviruses is important in differentiating nonpoliovirus enterovirus pathogens from vaccine strain polioviruses that can be shed for some time after vaccination. In the present investigation, we evaluated a rapid method for serotype identification of enteroviruses by indirect immunofluorescence assay (IFA) using commercially available monoclonal antibodies for polioviruses, coxsackieviruses type B, and six serotypes of commonly circulating echoviruses. Of 291 isolates of enteroviruses included in the study, 95 were polioviruses and 196 were nonpoliovirus enteroviruses. Two hundred thirty-four of these (38 polioviruses and 196 nonpoliovirus enteroviruses) were consecutively grown in the laboratory over a 5-year period. IFA identified the serotypes of 74% of the consecutive isolates and 71% of all enterovirus isolates by yielding a positive staining result. The levels of agreement in the identification of the enterovirus group between IFA and neutralization tests were 92% for polioviruses and 94% for echoviruses. Specificity was near 100% for polioviruses and coxsackieviruses type B and 94% for echoviruses. We conclude that IFA can be helpful as a preliminary test for serotype identification of enteroviruses. The results are most accurate when the test identifies the isolate as a poliovirus.

Nonpoliovirus enterovirus infections are a major cause of acute febrile illness in infants and young children, aseptic meningitis, respiratory tract diseases, including otitis media, and infections of numerous other organ systems (3, 7, 9, 16). The “gold standard” for the diagnosis of enterovirus infections is culture of the virus from specimens such as throat swabs, nasopharyngeal secretions, rectal swabs, or cerebrospinal fluids. Enteroviruses can be isolated from clinical specimens relatively rapidly: 42% of cultures yield a positive result within 3 days, and 85% of cultures yield a positive result within 7 days (5). It has been shown that early identification of enterovirus infection can affect patient management, for example, by allowing early withdrawal of antibiotics and early discharge (4, 6, 21). However, the cytopathic effect seen in enterovirus-positive cell cultures prior to serotype designation does not differentiate whether the virus is a poliovirus or nonpoliovirus enterovirus serotype. This differentiation is very important, especially when the affected child belongs to an age group to whom oral poliovirus vaccines are usually administered. In oral poliovirus vaccine recipients, the virus persists in the throat for 1 to 3 weeks and is excreted in the feces for 1 to 6 weeks or longer (15). Therefore, a positive culture for enteroviruses from these sites does not indicate enterovirus disease unless it is confirmed by serotyping results.

The standard method for differentiation between polioviruses and nonpoliovirus enteroviruses is neutralization (10). The technique is cumbersome, expensive, and time-consuming; therefore, this method is not generally available in the diagnostic virology laboratory (12, 14, 19). Another potentially useful method for differentiation between polioviruses and nonpoliovirus enteroviruses uses a set of PCR primers that is specific for three poliovirus serotypes (1), but this method has not been put into clinical use. Other techniques for the rapid detection of enteroviruses, such as nucleic acid hybridization (11) and the more widely used PCR assay (17), do not differentiate these two virus subgroups.

Recently, monoclonal antibodies to selected types of enteroviruses have become commercially available for the detection of specific enterovirus serotypes by the indirect immunofluorescence assay (IFA). A preliminary study has shown that IFA was able to identify more than half of the enterovirus isolates tested (2), although only a small number of isolates were tested with poliovirus antibodies. The simplicity of this technique suggests that it may be helpful in the clinical laboratory for rapid differentiation between polioviruses and nonpoliovirus enteroviruses. In this study, we compared the results of the IFA and the standard neutralization technique for the serotype identification of enteroviruses. The primary purpose was to use the IFA to rapidly differentiate between polioviruses and nonpoliovirus enteroviruses.

MATERIALS AND METHODS

Specimens. Included in the study were a total of 291 enterovirus isolates. Of these, 234 were consecutive isolates grown from clinical specimens obtained from patients who received medical care at the University of Texas Medical Branch between 1991 and 1995. Of these, 196 were nonpoliovirus enterovirus isolates and 38 were poliovirus isolates. To increase the number of poliovirus isolates in this study, another 57 isolates of polioviruses consecutively identified between 1985 and 1990 were also included. Therefore, a total of 95 poliovirus isolates were included in this study. All the isolates had previously produced cytopathic effects in cell cultures.

Cells. Human diploid fibroblast (MRC-5) and rhesus monkey kidney cells were purchased from Browwhittaker (Walkersville, Md.). All the cell tubes were

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Monoclonal antibodies. The monoclonal antibody reagents were commercially prepared and were purchased from Chemicon International (Temecula, Calif.). Three monoclonal antibody blends were used: monoclonal antibodies for each of polioviruses, coxsackieviruses, and echoviruses. The poliovirus monoclonal antibody blend was for poliovirus serotypes 1, 2, and 3. The coxsackievirus type B monoclonal antibody blend detected coxsackieviruses B1, B2, B3, B4, B5, and B6. The echovirus monoclonal antibody blend was for common serotypes of echoviruses (serotypes 4, 6, 9, 11, 30, and 34).

Controls. Prepared slides containing both positive control wells (cells infected with poliovirus, coxsackievirus type B, or echovirus) and negative control wells (uninfected cells) were purchased from Chemicon International. These control slides were stained with the monoclonal antibodies, and the viruses in the control wells identified by IFA each time, together with the laboratory-prepared experimental slides, to control for quality and accuracy of identification.

Cell inoculation and harvesting. Clinical isolates of enteroviruses had been stored at −70°C. Each isolate was assigned an identification number and was regrown in MRC-5 cells incubated at 36°C. Monolayers were inspected daily for the cytopathic effect. Isolates that failed to grow in MRC-5 cells were reinoculated in rhesus monkey kidney cells by the same methods. Harvesting was done when 50% of the cells were observed to have a cytopathic effect. During harvesting, the cell medium was removed and the remaining cell monolayer was washed with phosphate-buffered saline. The cells were scraped off the tube and the cell suspension was spotted onto three wells of a glass slide. The slides were air dried and fixed with acetone.

Specimen preparation for IFA. The monoclonal antibody reagents were directly applied to specific wells on each slide, according to the manufacturer’s instructions. The slides were incubated for 30 min at 37°C, and then the fluorescent conjugate (goat anti-mouse immunoglobulin G fluorescein isothiocyanate-labeled antibody) was applied to all wells. After another 30 min of incubation, the slides were rinsed with phosphate-buffered saline. After mounting, the slide wells were examined under a fluorescence microscope.

Identification of enterovirus. Serotype identification by standard neutralization techniques was kindly performed by the Medical Virology Branch, Bureau of Laboratories, Texas Department of Health, Austin. These neutralization results were used as the gold standard in this study. For IFA, all procedures including reading of slides and initial identification of enteroviruses were performed by an assigned investigator (A.S.R.) who was blinded to the neutralization results. A bright apple green fluorescence in one of the three wells identified the enterovirus serotype of the test isolate. Positive specimens were compared to the specimens on the control slides for accuracy of identification. Another investigator (L.M. or T.C.) who was unaware of the neutralization test results at the time of reading confirmed all immunofluorescent readings. In case of disagreement (5 of 291, 1.7%), the final reading followed that of the experienced investigators (L.M. or T.C.). The fluorescence results were then compared to the neutralization test results previously determined by the Texas State Laboratory.

RESULTS

The numbers and serotypes of the 291 enterovirus isolates included in the study are presented in Table 1. The clinical specimens from which the enteroviral isolates were cultured are described in Table 2. Because the monoclonal antibody blends contained only antibodies to specific serotypes of enteroviruses, not all of the isolates were expected to have a positive staining result with these blends. Given the serotypes identified by the neutralization method, we predicted that IFA with the monoclonal antibody blends would identify 95 of 90 poliovirus isolates, 26 of 34 coxsackievirus isolates, and 107 of 161 echovirus isolates (Table 1). A comparison of the results of the neutralization assay and IFA is presented in Table 3. Overall, IFA identified 208 of 291 (71.4%) isolates by yielding positive staining results, and the IFA results for 239 of 291 (82.1%) of the isolates were in agreement with the neutralization test results. Twenty-two poliovirus isolates were IFA negative (3 type 1 polioviruses, 10 type 2 polioviruses, and 9 type 3 polioviruses). One coxsackievirus type A21 isolate stained with the coxsackievirus type B blend, and 5 coxsackievirus type A9 isolates stained with the echovirus blend. For echoviruses of six serotypes included in the echovirus monoclonal antibody blend, seven isolates were IFA negative; these were echovirus type 6 (n = 2), echovirus type 11 (n = 2), and echovirus type 30 (n = 3). One echovirus type 11 isolate stained with both the poliovirus and the echovirus monoclonal antibody blends. Of 54 isolates of echovirus for which antibodies were not included in the blend, eight had a positive staining result for echovirus: echovirus type 1 (n = 3), echovirus type 7 (n = 2), echovirus type 8 (n = 1), and echovirus type 14 (n = 2). Enterovirus 71 isolate did not stain with any monoclonal antibody blend.

The numbers of true-positive, true-negative, false-positive,
and false-negative values and the sensitivity, specificity, and positive and negative predictive values for each major enterovirus group are presented in Table 4. Data for the three isolates that stained with more than one antibody blends (two poliovirus isolates and one echovirus isolate) were excluded from the calculation. The sensitivity was the highest for IFA staining of echoviruses (93.9%) and lowest for IFA staining of polioviruses (73.1%). However, IFA for poliovirus had 100% specificity. The positive and negative predictive values were also high for all three enterovirus groups, ranging from 88.6 to 100%.

To determine the usefulness of IFA for the serotype identification of enterovirus isolates consecutively grown in our clinical laboratory over a 5-year period, the results for 234 isolates grown between 1991 and 1995 were separately analyzed. Only 38 polioviruses were isolated during this period. Of these, 36 poliovirus isolates were identified by IFA as poliovirus. One poliovirus type 1 isolate stained with both coxsackievirus and echovirus antibodies, and a poliovirus type 2 isolate did not stain positively. The numbers of nonpoliovirus enteroviruses were as given above. Therefore, IFA identified 173 of 234 (73.9%) isolates by yielding positive staining results, and the results of IFA for 215 isolates agreed with the neutralization test results (91.9%).

**DISCUSSION**

We have shown in a clinical laboratory setting that IFA with commercially available monoclonal antibody blends rapidly identified the serotypes of about 74% of enteroviruses consecutively grown over a 5-year period; the level of agreement between the IFA and neutralization test results was 92%. In this setting, polioviruses accounted for approximately 16% of the total number of enterovirus isolates. Of 234 enterovirus isolates grown in our laboratory during the 5-year period between 1991 and 1995, 38 were polioviruses. This proportion of polioviruses has been constant in our laboratory. Between 1982 and 1987, polioviruses accounted for 67 of 421 (15.9%) of the enterovirus isolates identified in our laboratory (5).

Because the number of poliovirus isolates during the 5-year period was low, we have also studied poliovirus isolates previously grown from 1985 to 1990. IFA with monoclonal antibody blends available for all three types of enteroviruses was used to test 196 nonpoliovirus enterovirus isolates and 95 poliovirus isolates. IFA for polioviruses gave the lowest sensitivity (73%) but the highest specificity (100%). We have called the result for one echovirus type 11 isolate which had a positive staining result for both poliovirus and echovirus indeterminate and excluded the data for that isolate from our calculations for the sake of simplicity. This result, if counted, would have been the only false-positive result for poliovirus and would have reduced the specificity by 0.5%. While IFA for the echoviruses included in the monoclonal antibody blend gave the highest sensitivity (93%), the specificity was lower (90%; see footnote c of Table 3), mainly because the monoclonal antibodies in the blend cross-reacted with other nonpoliovirus enteroviruses (other echovirus serotypes not included in the blend and coxsackievirus). Only 3 of 18 (17%) of the false-positive results were for polioviruses. The misidentification of a poliovirus as an echovirus could potentially cause overdiagnosis of an enterovirus disease in vaccine strain poliovirus shedders. IFA for coxsackievirus type B had a sensitivity of 85%, with a high specificity of 99.6%. The false-positive result came from cross-reactivity with one isolate of coxsackievirus type A21. The indeterminate result was for one poliovirus type 1 isolate which stained positively with both coxsackievirus and echovirus monoclonal antibody blends; if the result were counted as a false-positive result, the specificity would have been reduced by 0.4%.

In order to differentiate between polioviruses and nonpoliovirus enteroviruses, monoclonal antibodies for only three types of polioviruses are needed. In a study comparing neutralization assay results to IFA results for the identification of polioviruses in stool samples, using individual monoclonal antibodies to three poliovirus serotypes, Cohen-Abbo et al. (8) reported...

**TABLE 3. Comparison of results between neutralization and IFA methods**

<table>
<thead>
<tr>
<th>Neutralization assay result</th>
<th>No. of isolates identified by IFA with monoclonal antibodies</th>
<th>Poliovirus</th>
<th>Coxsackievirus</th>
<th>Echovirus</th>
<th>IFA negative</th>
<th>Indeterminate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polioviruses (n = 95)</td>
<td>68</td>
<td>0</td>
<td>3</td>
<td>22</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Coxsackievirus type A (n = 8)</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Coxsackievirus type B (n = 26)</td>
<td>0</td>
<td>22</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Echoviruses, in blend (n = 107)</td>
<td>0</td>
<td>0</td>
<td>99</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Echoviruses, others (n = 54)</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>47</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Enterovirus type 71 (n = 1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* a One isolate stained with both coxsackievirus and echovirus antibody blends. The second isolate stained with both poliovirus and echovirus antibody blends.
* b Coxsackievirus types B2 and B4.
* c Echovirus serotypes 4, 6, 9, 11, 30, and 34; sensitivity, 93%; specificity, 90%.

**TABLE 4. Sensitivity, specificity, positive, and negative predictive values of IFA method, with the neutralization method used as the “gold standard”**

<table>
<thead>
<tr>
<th>Antibody blend</th>
<th>No. of isolates with the following result:</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True positive</td>
<td>True negative</td>
<td>False positive</td>
<td>False negative</td>
<td></td>
</tr>
<tr>
<td>Poliovirus</td>
<td>68</td>
<td>195</td>
<td>0</td>
<td>25</td>
<td>73.1</td>
</tr>
<tr>
<td>Coxsackievirus type B</td>
<td>22</td>
<td>261</td>
<td>1</td>
<td>4</td>
<td>84.6</td>
</tr>
<tr>
<td>Echovirus</td>
<td>107a</td>
<td>164</td>
<td>10</td>
<td>7</td>
<td>93.9</td>
</tr>
</tbody>
</table>

* a Including eight echovirus isolates of serotypes not in the echovirus monoclonal antibody blend.
95.9% (187 of 195) agreement between the two methods. The monoclonal antibodies used in that study, however, were prepared by the National Institute of Biologic Standards in the United Kingdom and are of limited availability. Our IFA results with commercially available reagents have shown a nearly 100% positive predictive value for identification of polioviruses. In other words, if an isolate is identified by IFA as a poliovirus, it is very likely to be accurate. If only the blend of monoclonal antibodies to polioviruses is used and the IFA staining result is negative, the probability that the isolate is not poliovirus is about 89%. In this case, the use of monoclonal antibody blends for coxsackieviruses B and echoviruses may enhance the possibility of serotype identification of the enterovirus isolate. However, negative staining with all available monoclonal antibody blends does not exclude the possibility that the enterovirus is one of the serotypes included in the IFA.

We have previously reported a rapid method for the differentiation between poliovirus and nonpoliovirus enteroviruses by reverse transcription and PCR amplification in which we used a single set of primers specifically for the three poliovirus serotypes (1). The sensitivity and specificity of the method were 100 and 96%, respectively. This method, however, has not been put to clinical use because of technical limitations in our clinical virology laboratory. Although IFA is a less sensitive test, the availability of the reagents, the rapidity and ease of the test, and the ability to identify specific coxsackievirus and echovirus serotypes make it a more practical method for clinical applications. This IFA protocol is being used in our laboratory. This allows us to report a preliminary typing result for approximately 75% of the enterovirus isolates; the final neutralization typing by the Texas State Laboratory is not generally available until weeks later.

Bastis et al. (2) have used monoclonal antibody blends for coxsackieviruses type B and echoviruses from the same commercial source to test 465 enterovirus isolates. The antibodies identified 251 (54%) of the isolates tested, and the IFA results agreed with the neutralization assay results for 465 (97%) of the isolates tested. Additionally, for 45 isolates (14 polioviruses and 31 nonpoliovirus enteroviruses), IFA was performed with individual monoclonal antibodies for each of the three poliovirus serotypes. The IFA results agreed 100% with the results of the neutralization method for the latter group of isolates. Our results differ in the proportion of the isolates whose serotypes were identified by IFA (71 versus 54%) and the sensitivity of IFA for poliovirus (73 versus 100%). The differences in the types of enteroviruses isolated and the limited number of poliovirus isolates tested with poliovirus antibodies in the study of Bastis et al. (2) may explain these differences in the results.

While these studies of rapid methods for the identification of enterovirus serotypes have been in progress, there has also been a strong interest in the rapid detection of enteroviruses from clinical specimens. Numerous nucleic acid hybridization methods with cDNA probes, RNA probes, or oligonumeric probes for the detection of enteroviruses in body fluids have been reported (19). The sensitivities of these tests with actual clinical specimens is only 33% or less. The most promising development in the direct detection of enteroviruses has been the application of PCR for the universal detection of enterovirus serotypes (18). The results obtained by this method are highly reproducible (20), and use of the test in the clinical setting has provided a means for the rapid diagnosis of enteroviral meningitis, with significant potential cost savings (13). This procedure, which will soon be commercially available, has great potential for the rapid diagnosis of viral infections and for improving patient management. The primers used in the test, however, are suitable for the detection of 60 of 68 enterovirus serotypes, including all polioviruses. Therefore, this PCR will best be used with clinical specimens for which contamination from vaccine strain polioviruses is unlikely, such as cerebrospinal fluid specimens. Application of the PCR assay to other clinical specimens will not give a specific answer to whether the enterovirus detected is the nonpoliovirus enterovirus pathogen or a vaccine strain poliovirus.

In summary, the IFA procedure with blends of monoclonal antibodies directed at polioviruses, coxsackieviruses type B, and six common serotypes of echoviruses identified the serotypes of 74% of consecutive clinical enterovirus isolates. The level of agreement between IFA and the neutralization assay in this setting was 92%.

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