Rapid and Highly Sensitive Coxsackievirus A Indirect Immunofluorescence Assay Typing Kit for Enterovirus Serotyping\textsuperscript{V}

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We describe the development and evaluation of an indirect immunofluorescence assay (IFA) kit for rapid and sensitive detection of coxsackievirus A2, -4, -5, -6, and -10. This IFA kit was determined to have 95.9 to 100% sensitivity and 95.8 to 97.2% specificity. It also proved to be beneficial in reducing the number of enteroviruses that are untypeable in the clinical virology laboratory.

There are more than 90 serotypes (2, 13, 14) of human enteroviruses, and they cause a wide spectrum of acute febrile diseases among infants and children. They also cause aseptic meningitis; respiratory tract illnesses; herpangina; hand, foot, and mouth disease; otitis media; and infections of numerous other organ systems (8, 10, 14). CDC Taiwan implemented an enterovirus surveillance system after a devastating hand, foot, and mouth disease outbreak in 1998 caused 78 deaths and 405 severe cases (3). To strengthen the enterovirus surveillance and outbreak control, a virology laboratory network with 13 medical centers has been integrated with the system.

Clinical laboratories use one of two available diagnostic technologies for enterovirus serotyping: traditional and molecular (4, 8). The traditional method requires reference antisera and relies on virus culture and neutralization tests (5, 9) but is often laborious and time-consuming. The molecular methods that employ PCR and sequencing (11, 12) are faster and more accurate but are also less common in clinical laboratories because they require expensive equipment, special technology, and trained personnel. An indirect immunofluorescence assay (IFA) improved upon the traditional method and helped hospital laboratories simply and reliably deal with many clinical specimens (1, 6, 16). Although the molecular approaches are becoming the method of choice in specialized laboratories, for most clinical and hospital laboratories, as well as laboratories in some developing countries, the IFA might be a better choice if only a preliminary serotyping result is required.

Although IFA reagents provide considerable convenience in enterovirus diagnostics, coverage by commercial products is limited to only 19 serotypes (16). For example, in the detection of human enterovirus A species, the use of commercial reagents (e.g., those from Chemicon International, Temecula, CA) allows the diagnosis of coxsackievirus A16 (CVA16) and human enterovirus 71 (EV71) only; reagents for the others, including CVA2 to -8, -10, -12, and -14 as well as EV76 and EV89 to -92, are not yet available (13). When clinical laboratories conduct preliminary enterovirus screening tests by employing commercial IFA kits, many serotypes not yet covered by the reagents are reported as untypeable nonpolio enteroviruses (NPEV). An earlier study by Bastis et al. (1) showed that although commercial IFA reagents could identify more than half of the enterovirus isolates tested, a high percentage of isolates were nevertheless being reported as untypeable NPEV. In our virology laboratory network, CDC Taiwan used commercial IFA technology for its fast turnaround time and simple operation, and as a consequence, a high proportion of untypeable NPEV (20 to 78%) have been reported since 2002 (Fig. 1, upper panel).

To overcome this limited IFA commercial diagnostic coverage, we developed an IFA kit that supplements the commercial kit with the addition of antisera to CVA2, -4, -5, -6, and -10 (frequently encountered serotypes in Taiwan and Southeast Asia) and can identify the majority of common untypeable NPEV.

As to the methodology of antiserum preparation, we selected the specified virus strains from the American Type Culture Collection (Manassas, VA) based on the original rationale from Chemicon International. These strains were CVA2 Fleetwood strain, CVA4 High Point strain, CVA5 Swartz strain, CVA6 Gdula strain, and CVA10 Kowalik strain. The selected viruses were grown in rhabdomyosarcoma (RD) cells, and their 50% cell culture infective doses were determined before animal inoculations. In brief, we inoculated four New Zealand White rabbits intravenously with 5 ml of UV-inactivated virus stock (typically 10⁸ 50% cell culture infective doses/ml) and boosted them four times at 2-day intervals. We checked the antibody titer at day 36, inoculated the rabbits with 10 ml of virus stock at the final boosting on day 42, and then tested their sera for neutralizing antibodies (7) the following week. The elicited titers calculated using the Reed-Muench method (15) for each serotype were 1:5,620 (CVA10), 1:22,387 (CVA2), 1:25,176 (CVA5), 1:53,826 (CVA6), and 1:79,250 (CVA4), and no cross-reactivity (titer of less than 1:8) was observed with the...
neutralization titration among the prepared antisera. In checkerboard dilution studies, the optimal concentration of antiserum was obtained to make sure that no cross-reaction could be generated in the IFA. Each antiserum was evaluated individually for its efficiency and sensitivity by the IFA before a blend was prepared. The formulated CVA IFA typing kit set 1 (an additional kit targeting CVA3, -8, -12, and -21 is in progress) contains a reagent blend for the detection of CVA2, -4, -5, -6, or -10 either alone or in a mixture of serotypes in the IFA. Each individual antiserum allows identification of the specified virus.

The laboratory procedure for IFA staining is adapted from Chemicon’s monoclonal antibody IFA kit. In brief, on a glass slide which is warmed to room temperature, the coxsackievirus antiserum blend is pipetted to adequately cover each testing well. Then the slide is placed for 30 min at 37°C in a humidified chamber. After incubation, the slide is washed with phosphate-buffered saline (pH 7.2 to 7.4, with 0.05% Tween 20) and dried. Later, a volume of 10 μL of anti-rabbit immunoglobulin G fluorescein isothiocyanate reagent (Chemicon catalog no. AP132F or equivalent) is added to each well. The wells are incubated for 30 min at 37°C and washed with phosphate-buffered saline, and the slide is dried. Finally, a drop of mounting oil is added to each well, a cover glass is placed on the slide, and observa-

![FIG. 1. Results of retest of untypeable NPEV by use of the coxsackievirus IFA typing kit set 1. (Upper panel) Pie charts display the percentages of enteroviruses taken from patient specimens of throat swabs, rectal swabs, nasopharyngeal swabs, stool, and CSF by the virology laboratory network and reported to CDC Taiwan from 2002 to 2006. The specific serotype is indicated if the rate was higher than 10%. "Others" include all other serotypes that were reported at rates lower than 10%. Untypeable NPEV are indicated by the black portions of the charts. (Lower panel) Pie charts indicating the changes in identification rates for untypeables after the specimens were retested with the designed kit.]

# TABLE 1. Evaluation of sensitivity and specificity of the IFA CVA kit set 1 by testing clinical isolates

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of isolates with indicated test results</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVA2</td>
<td>87/87 (7*/179, 0/87)</td>
<td>100.0</td>
<td>96.1</td>
</tr>
<tr>
<td>CVA4</td>
<td>209/218 (8*/213, 9/218)</td>
<td>95.9</td>
<td>96.7</td>
</tr>
<tr>
<td>CVA5</td>
<td>46/46 (7*/165, 0/46)</td>
<td>100.0</td>
<td>95.8</td>
</tr>
<tr>
<td>CVA6</td>
<td>73/73 (4*/144, 0/73)</td>
<td>100.0</td>
<td>97.2</td>
</tr>
<tr>
<td>CVA10</td>
<td>96/99 (6*/153, 3/99)</td>
<td>97.0</td>
<td>96.1</td>
</tr>
</tbody>
</table>

* These clinical isolates were stored at CDC Taiwan from 2000 and confirmed by determining the partial VP1 gene sequences (11).
* Test +, the tested isolates were confirmed to be the specified serotype by partial VP1 gene sequences and used for the specificity test; Test -, the tested isolates were confirmed to not be the specified serotypes and were used for the specificity test. Randomly selected isolates, HSV and adenovirus included, were used for the specificity test. All other enteroviruses and adenoviruses were negative. IFA + and IFA -, the tested isolates were positive and negative, respectively, for the specified serotype by the coxsackievirus IFA kit set 1. HSV isolates showing cross-reactivity with the IFA kit. For example, in the CVA2 specificity test (IFA +/Test −), the numbers of isolates used for the different virus serotypes were as follows: for CVA4, 14; CVA5, 47; CVA6, 6; CVA8, 1; CVA10, 5; CVA12, 3; CVA16, 22; CVA21, 3; echovirus 3 (E3), 1; E6, 3; E11, 4; E18, 4; E25, 1; CVB1, 1; CVB2, 3; CVB3, 3; CVB4, 4; CVB5, 2; EV71, 45; and HSV, 7.
tions of the fluorescence are made. A positive IFA staining result is indicated by a bright greenish color in the cytoplasm and nuclei of the infected cells (Fig. 2A1 to A5). A negative reaction is indicated by the absence of fluorescence and by a dull red color displayed by the cell due to the Evans blue counterstain (Fig. 2C1 to C5, D1, and D2). Each positive specimen should be further tested with monospecific antiserum, i.e., CVA2 antiserum and others, for serotype identification. To further accomplish the serotyping by using the monospecific antiserum, another glass slide is prepared as before. The serotype-specific antiserum (i.e., CVA2, -4, -5, -6, or -10) is added individually to each well (the cell control and known positive control are also included). The procedure as previously described is repeated, and the specific serotype of the specimen is determined (Fig. 2B1 to B5).

Viral identification tests with the IFA kit set 1 were performed on 523 known, previously sequenced viruses of CVA2, -4, -5, -6, and -10 (Table 1), and the tests demonstrated that the kit provided 95.9 to 100% sensitivity. Also, identification tests were performed on virus panels (Table 1), not only on other randomly selected enterovirus types but also on herpes simplex virus (HSV) and adenovirus. This evaluation demonstrated specificity values of 95.8 to 97.2%, with only HSV showing cross-reactivity. We recognize that it is possible for HSV to be present in certain clinical specimens (e.g., cerebrospinal fluid and stool specimens), and we would alert investigators to this fact. Although the cross-reactivity between HSV and this IFA kit remains to be elucidated, it is our opinion that this will not be an issue, since in a real scenario the scientist would be able to distinguish the cytopathic effect caused by either an enterovirus (cytolytic and degenerated cells) or an HSV (swollen, rounded, and syncytial cells).

To evaluate the efficiency of this newly designed IFA kit,
we analyzed the untypeable NPEV isolates that were reported to CDC Taiwan from 2002 to 2006 by 13 reference virology laboratories. As shown in Fig. 1 (upper panel), the annual untypeable NPEV isolates initially ranged between 19 and 78%. When these untypeables were retested using the coxsackievirus IFA kit set 1, the rate of untypeable NPEV for all years dropped to below 3% with the exception of 17% in 2006. The strains identified by the IFA were confirmed by determining partial VP1 gene sequences (11). More identification tests of the untypeables in 2006 are planned, since they may represent strains not frequently occurring in previous years.

We introduced this IFA blend kit to 13 local reference laboratories in 2007, and they have found the trial kit to be simple, reliable, and effective when attempting to identify the above-mentioned CVA serotypes. To strengthen Taiwan’s enterovirus surveillance system, we plan to distribute this IFA kit to local reference laboratories semiannually, since the shelf life of the reagents is determined to be at least 6 months (there is no loss in the fluorescence intensity after 12 months of storage at 4°C; we will continue to follow up). We will offer this reagent kit globally to interested laboratories and institutions at no cost while supplies last.

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REFERENCES