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Original Article

# Application of a Molecular Method for the Classification of Human Enteroviruses and its Correlation with Clinical Manifestations

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**BACKGROUND/PURPOSE:** A new molecular classification scheme has recently been adopted that groups all enteroviruses into four species, designated human enterovirus A (HEV-A) through D. In this study, we tried to demonstrate the correlation between this molecular classification scheme and clinical manifestations in patients.

**METHODS:** We retrospectively reclassified the clinical isolates of enteroviruses from the preceding 4.5 years in our virology laboratory using reverse transcription-polymerase chain reaction, and reviewed the clinical manifestations of 138 pediatric patients.

**RESULTS:** We reclassified 23 isolates of the five serotypes into the HEV-A group, 110 isolates of 16 serotypes into the HEV-B group, five isolates into the HEV-C group, and no isolate of the HEV-D group. HEV-A species caused significantly more hand-foot-and-mouth disease ($p<0.001$), herpangina ($p=0.029$), and myoclonic jerks ($p<0.001$) compared with HEV-B species. However, HEV-B species caused significantly more pharyngitis ($p=0.043$), respiratory tract infections ($p=0.046$), nausea and vomiting ($p=0.007$), and aseptic meningitis ($p=0.001$). The only death in our report was caused by coxsackievirus A16, which belonged to the HEV-A group.

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**CONCLUSION:** The association between the molecular classification of enteroviruses and related disease patterns is an important finding. We suggest that this molecular classification could be applied in a clinical laboratory as an alternative method under certain circumstances, such as limited availability of antisera or questionable serotyping results, to identify the untypeable isolates.

**KEYWORDS:** aseptic meningitis, clinical manifestations, enteroviruses, molecular classification, RT-PCR

## Introduction

Enteroviruses are common viruses associated with diverse clinical manifestations ranging from mild febrile illness, respiratory tract infection, acute gastroenteritis and aseptic meningitis, to severe and potentially fatal conditions including encephalitis, neonatal sepsis-like syndrome, and pulmonary edema after infection with enterovirus serotype 71 (EV71).[1] Many authors suggest that the disease severity of enterovirus infection is also associated with host factors such as patient age, gender,[2] host cell membrane receptors,[3] and the environment, thus accounting for the greater prevalence of infections in children of lower socioeconomic class. The neutralization assay is usually used for enterovirus serotyping, but it is labor-intensive and time-consuming. Standardized antisera, which are in limited supply, have to be prepared for each serotype. Molecular typing based on reverse transcription-polymerase chain reaction (RT-PCR) has been recently developed to reduce the time required to type an enterovirus isolate and those isolates that are difficult or impossible to serotype.[4,5] More than 80 enterovirus serotypes can now be divided into four human enterovirus (HEV) species, designated HEV-A through D, based on homology within the RNA coding region for the capsid protein, VP1, which contains the major neutralization target (Table 1).[6] The close relationship between polioviruses and HEV-C in the non-capsid coding region indicates that they belong to the same species.[7] In addition, rapid genomic evolution via recombination, deletion, and mutation among enterovirus species may result in an inaccurate serotyping result because most antisera are produced using old prototypes of enteroviruses dating back to the 1950s or 1960s.[8]

None of the published studies have clearly demonstrated a correlation between the virulence of each HEV species and clinical illness. In this study, we reviewed the clinical manifestations of hospitalized patients with enterovirus infections and compared the relationships between species-specific virulence and disease patterns. By establishing a clinical correlation, this new molecular classification may be an alternative method to serotyping for use in a clinical laboratory in certain circumstances

**Table 1.** Correspondence between human enterovirus species and previous serotypes[a]

| Species | Previous serotypes | | | | |
|---|---|---|---|---|---|
| | Coxsackievirus A | Poliovirus | Coxsackievirus B | Echovirus | Enterovirus |
| HEV-A | CAV2–8, 10, 12, 14, 16 | – | – | – | EV71, 76, 89–92 |
| HEV-B | CAV9 | – | CBV1–6 | E1–7, 9, 11–21, 24–27, 29–33 | EV69, 73–75, 77–88, 93, 97, 98, 100, 101 |
| HEV-C | CAV1, 11(15), 13(18), 17, 19–22, 24 | Poliovirus 1–3 | – | – | EV95, 96, 99, 102 |
| HEV-D | – | – | – | – | EV68, 70, 94 |

[a]Data from the Taiwan Centers for Disease Control.[6] HEV=Human enterovirus; CAV=Coxsackievirus A; CBV=Coxsackievirus B; E=Echovirus; EV=Enterovirus.

such as limited availability of antisera, dubious serotyping results, or where serotyping is difficult or impossible. Furthermore, future antiviral therapies, vaccine development, or other novel treatments could be developed, based on the results of this molecular classification.

## Methods

### *Patient characteristics and virus isolation*

One hundred and forty clinical enterovirus isolates from 140 pediatric patients were identified in the Taipei Veterans General Hospital (VGHTPE) virology laboratory from January 2001 to June 2005. We retrospectively reviewed the medical charts of the 140 pediatric patients, who were treated in either VGHTPE or other rural hospitals. Of the 140 isolates, 17 strains were untypeable and 123 were typeable. To reduce the errors associated with conventional laboratory diagnostic methods, 50 of the 123 typeable strains were randomly selected and reclassified using RT-PCR plus sequencing, revealing 100% compatibility. The 17 untypeable enteroviruses were also reclassified according to the PCR sequencing results.

During the 4.5 years of this study, 243 enterovirus isolates were originally identified by neutralization or immunefluorescence assay (IFA) in this virology laboratory. A total of 9,107 virus samples were sent to our laboratory within the study period, with an enterovirus isolation rate of 2.67%. Among them, only the 140 isolates from pediatric patients (< 18 years of age) were subjected to analysis. The VGHTPE is a national medical center and teaching hospital with approximately 3,000 beds. The virology laboratory receives approximately 2,000 clinical samples per year, including throat and rectal swabs, feces, serum, and cerebrospinal fluids collected from patients in local clinics or hospitals in Taipei City and suburban areas with various infectious diseases.

### *RNA extraction and RT-PCR*

Virus serotypes were identified by sequence analysis of the *VP1* gene. For the initial testing, RNA was extracted using the QIAamp viral RNA mini kit (Qiagen Ltd., Crawley, UK) in accordance with the manufacturer's recommendations.

A 50-μL reaction consisted of 10 μl of 5 × RT-PCR buffer (1 × buffer), 1.75 mM $MgCl_2$, 800 μM dNTP mix, 5 mM DTT, 400 μM each forward (5′-MIGCIGYIGARACNGG-3′) and reverse (5′-CICCIGGIGGIAYRWACAT-3′) serotype-specific primers,[8,9] 25 U MultiScript reverse transcriptase (Roche Ltd., Branchburg, NJ, USA), 2.5 U Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 10 U RNase inhibitor, and 8 μL of template RNA. The reactions were incubated at 42°C for 30 minutes and then 94°C for 3 minutes. Thermocycling was performed in a 9600 model thermocycler (Perkin-Elmer, Norwalk, Connecticut) with 35 cycles at 94°C for 30 seconds, 42°C for 30 seconds, and 60°C for 30 seconds, followed by incubation at 72°C for 5 minutes. PCR products were separated at 120 volts in a 1.5% agarose gel stained with 0.5 μg/mL ethidium bromide for 60 minutes.

The Qiagen gel extraction kit (Qiagen) was used to extract the DNA from the agarose gels in accordance with the manufacturer's recommendations. Both strands of the PCR amplicons were cycle-sequenced with rhodamine-labeled dideoxynucleotide chain terminators (DNA sequencing kit; ABI) and analyzed on an ABI Prism 310 automatic sequencer (Applied Biosystems, USA). PCR primers were used for the sequencing. The sequences were aligned with published enterovirus sequences obtained from GenBank.

Poorly-amplified strains were re-cultured with rhabdomyosarcoma cells separately at 37°C in 5% $CO_2$, and the cytopathic effect (CPE) observed daily. When about 60% CPE was evident, the tube was frozen at −70°C and then re-amplified as described above.

### *Statistical analysis*

The $\chi^2$ or Fisher's exact test was used for contingency table analysis of the symptoms and signs associated with the different virus species. SPSS version 11 (SPSS Inc., Chicago, IL, USA) was used for the analysis and $p < 0.05$ was considered statistically significant.

## Results

All 140 isolates were initially serotyped using neutralization or IFA for the presence of a characteristic CPE. After RT-PCR reconfirmation using sequences alignment and after excluding two rhinoviruses, we were able to reclassify 138 isolates into the categories HEV-A, -B, -C, or -D. RT-PCR and serotyping results were discordant for two isolates. Echovirus 31 (E31) was typed as E30 and E28

**Table 2.** Comparisons of the clinical manifestations of human enterovirus species[a,f]

| Variable | HEV-A[d] ($n=23$) | HEV-B[e] ($n=110$) | $p^b$ | HEV-C[c] ($n=5$) |
|---|---|---|---|---|
| Sex, male | 12 (52.2) | 64 (58.2) | 0.766 | 4 (80.0) |
| Age (mo) | 47.6 | 35.7 | – | 10.8 |
| Hand-foot-and-mouth disease | 11 (47.8) | 2 (1.8) | <0.001 | 0 (0) |
| Herpangina | 9 (39.1) | 18 (16.4) | 0.029 | 0 (0) |
| Respiratory tract infection | 2 (8.7) | 35 (31.8) | 0.046 | 2 (40.0) |
| Acute pharyngitis | 1 (4.3) | 29 (26.4) | 0.043 | 0 (0) |
| Vomiting/nausea | 2 (8.7) | 45 (40.9) | 0.007 | 3 (60.0) |
| Diarrhea | 0 (0) | 11 (10.0) | 0.243 | 3 (60.0) |
| Aseptic meningitis | 1 (4.3) | 47 (42.7) | 0.001 | 1 (20.0) |
| Myoclonic jerk | 4 (17.4) | 0 (0) | <0.001 | 0 (0) |
| Convulsion | 1 (4.3) | 1 (0.9) | 0.772 | 0 (0) |
| Encephalitis | 2 (8.7) | 3 (2.7) | 0.444 | 0 (0) |
| Newborn sepsis-like syndrome | 0 (0) | 15 (13.6) | 0.129 | 0 (0) |
| Death | 1 (4.3) | 0 (0) | 0.385 | 0 (0) |

[a]Data presented as $n$ (%); [b]comparison of HEV-A group and HEV-B group; [c]only 5 isolates of HEV-C; [d]HEV-A included CAV2 ($n=1$), CAV4 ($n=2$), CAV6 ($n=1$), CAV16 ($n=18$) and EV71 ($n=1$); CAV16 ($n=4$) contributed to all myoclonic jerks; CAV16 ($n=11$) and EV71 ($n=1$) caused hand-foot-and-mouth disease; CAV2 ($n=1$), CAV4 ($n=1$), CAV6 ($n=1$), and CAV16 ($n=6$) are associated with herpangina; CAV16 ($n=2$) were responsible for encephalitis; [e]HEV-B included 16 serotypes in our study: CAV9 ($n=2$), CBV2 ($n=2$), CBV3 ($n=51$), CBV4 ($n=6$), CBV5 ($n=8$), E4 ($n=1$), E5 ($n=1$), E6 ($n=5$), E7 ($n=3$), E9 ($n=1$), E11 ($n=6$), E14 ($n=1$), E19 ($n=2$), E24 ($n=5$), E30 ($n=14$), and E31($n=2$); aseptic meningitis caused by the serotype CBV3 ($n=12$), CBV4 ($n=1$), CBV5 ($n=5$), E6 ($n=4$), E7 ($n=2$), E11 ($n=2$), E24 ($n=4$), E30 ($n=14$), and E31 ($n=2$); sepsis-like syndrome caused by the serotype CBV3 ($n=10$), CBV5 ($n=2$), E6 ($n=1$), E11 ($n=1$), E14 ($n=1$); the serotype CAV9 ($n=1$), CBV3 ($n=13$), CBV4 ($n=1$), CBV5 ($n=4$), E5 ($n=1$), E6 ($n=3$), E7 ($n=2$), E11 ($n=2$), E24 ($n=4$), E30 ($n=11$), E31 ($n=2$) were responsible for nausea and vomiting; the serotype CBV3 ($n=4$), CBV4 ($n=1$), CBV5 ($n=2$), E7 ($n=1$), E11 ($n=1$), E30 ($n=1$), and E31 ($n=1$) were responsible for diarrhea; the serotype CBV2 ($n=1$), CBV3 ($n=10$), CBV4 ($n=2$), CBV5 ($n=4$), E5 ($n=1$), E6 ($n=2$), E11 ($n=3$), E24 ($n=2$), E30 ($n=5$), and E31 ($n=1$) were responsible for respiratory tract infections; [f]two rhinoviruses were excluded from 140 previously isolated strains. HEV=Human enterovirus.

as E30. This may have been due to antigenic variation or viral aggregation during the cross-reaction.[11]

Samples from the 138 pediatric patients were analyzed. Seventy-three were sourced from our hospital (VGHTPE) and 65 from other medical facilities. We reclassified 23 isolates of five serotypes into the HEV-A group, 110 isolates of 16 serotypes into the HEV-B group, and five isolates into the HEV-C group (Table 2). All five HEV-C isolates were polioviruses and were most likely due to oral polio vaccinations. No isolate was classified into the HEV-D group in this study. Since the number of isolates in the HEV-C group was small, we only compared the clinical manifestations of patients suffering from HEV-A or HEV-B infections to avoid inaccurate interpretations.

Enteroviruses in the HEV-B group accounted for the majority of infections in our study (79.7%), while enteroviruses in the HEV-A group accounted for 16.7%. HEV-A caused significantly more hand-foot-and-mouth disease ($p<0.001$), herpangina ($p=0.029$) and myoclonic jerks ($p<0.001$) compared with HEV-B. However, HEV-B caused significantly more pharyngitis ($p=0.043$), respiratory tract infections ($p=0.046$), nausea/vomiting ($p=0.007$) and aseptic meningitis ($p=0.001$). The two cases of encephalitis in the HEV-A group were both caused by serotype coxsackievirus A16 (CAV16) and one patient eventually died. This was the only mortality.

## Discussion

To the best of our knowledge, this is the first formal study comparing the biological behavior of enteroviruses in human disease based upon this new molecular classification. It is not surprising that two of the enteroviruses previously classified using the former system were reclassified

as rhinoviruses. Along with enteroviruses and several other virus genera, human rhinoviruses belong to the family *Picornaviridae*, which share many common features including a non-enveloped icosahedral capsid, a messenger-sense RNA genome, and partial nucleotide and amino acid sequence homology.[12] Also, two isolates were reclassified because their serotyping was discordant with the conventional diagnostic method, possibly due to antigenic variations or viral aggregation during cross-reaction.[11]

Our findings confirm previous study results, with the serotype CAV16 accounting for the majority of infections involving HEV-A (18/23 HEV-A isolates were serotype CAV16; 78.3%). Furthermore, the strain is known to be the major cause of hand-foot-and-mouth disease, herpangina and, rarely, meningitis. The only death in our study was caused by CAV16. In the literature, however, CAV16 is rarely associated with a fatal outcome, whereas another HEV-A member, EV71, caused many deaths in the Malaysian and Taiwanese outbreaks in 1997–2000.[13,14] The two serotypes usually co-circulate within a given geographical area and result in similar clinical manifestations, such as hand-foot-and-mouth disease. Further study is required to establish whether the fatality was due to recombination between the two HEV-A members resulting in the exchange of virulent sequences, or just a sporadic event. In our study, four patients had myoclonic jerks, which were all caused by serotype CAV16. HEV-A also included the less common serotypes CAV2, CAV4 and CAV6, all of which are associated with herpangina and respiratory tract diseases.

The HEV-B species accounted for the highest percentage of studied cases (79.7%). A recent, comprehensive survey of enteroviruses conducted in the United States shows that these serotypes belong to HEV-B and account for many human diseases worldwide.[15] In the present study, a marked number of patients suffered from aseptic meningitis caused by HEV-B group viruses (47/110 patients of HEV-B group isolates; 42.7%). The most common serotypes causing aseptic meningitis in our study were E30 (14 patients) and coxsackievirus B3 (CBV3; 12 patients). This result is similar to that reported in previous studies.[16–19]

Gastrointestinal symptoms such as vomiting are significantly more prominent in HEV-B infected patients. As we know, coxsackieviruses and echoviruses replicate in the small bowel, and they are frequently cited as causes of nonbacterial diarrhea or gastroenteritis. Outbreaks of E11, E14 and E18 have occasionally been responsible for epidemic diarrhea in young infants.[20] However, in our report, episodes of diarrhea resulting from enterovirus infection were not frequent (occurring in 9.7% of HEV-B infected patients). In fact, conflicting results regarding the rate of enterovirus isolation from children with acute diarrheal illness and matched healthy control children have been reported.[21]

Our study also showed that HEV-B species tended to be the dominant pathogens in neonatal sepsis-like syndrome, although this was not statistically significant. Early descriptions of coxsackievirus B group viruses in neonatal disease came from outbreaks in nurseries in South Africa, Zimbabwe and the Netherlands.[22] Many nursery outbreaks of neonatal echovirus infection have been recorded, with the severity of neonatal disease varying according to the viral serotype.[23] Neonates are uniquely susceptible to coxsackievirus and echovirus diseases. Although many enterovirus serotypes can still cause self-limiting clinical syndromes in neonates, some serotypes are capable of producing fulminant and frequently fatal diseases in this patient group. CBV2–5 and E11 are most frequently associated with overwhelming systemic neonatal infections.[20,24]

One of the limitations of our study is that none of the HEV-C (except for poliovirus) or HEV-D viruses were isolated in our laboratory. As we know, in most studies the isolation rate for CAVs is probably an underestimation. It has been shown that the growth of CAVs is somewhat problematic in most cell lines (except for CAV9 and CAV16). The efficacy of suckling mice for the isolation of difficult-to-cultivate CAVs has been reported in a number of studies.[25] This may account for the relatively few HEV-A cases and the lack of HEV-C isolates in our virus pool. A new diagnostic method is needed to overcome this problem.

Other major limitations of our study are its retrospective design and the reliance on reviews of medical records from different hospitals. This may increase the likelihood of variation in descriptive medical terminology and classification. In addition, the imperfections that are inherent in conventional cultural diagnostic methods suggest that some serotypes and species may have been underestimated. It appears prudent to suggest that a large prospective survey utilizing the latest diagnostic laboratory methods is required for further elaboration of our findings.

In conclusion, if the correlation of genotyping and clinical manifestations can be confirmed by future studies, we suggest that this method of molecular classification could be applied (under certain circumstances) in a clinical laboratory as an alternative to serotyping. The advantages of this molecular classification method are that it is less labor-intensive and less time-consuming, without the need to prepare all the standardized antisera and the ability to type enteroviruses that are untypeable using neutralization or IFA.

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