

MOLECULAR IDENTIFICATION AND TYPING OF HUMAN ENTEROVIRUSES



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Human enteroviruses (HEVs) are common human pathogens associated with a wide spectrum of symptoms. These range from asymptomatic infection to serious illness, especially in infants and the immunocompromised. Clinical syndromes include aseptic meningitis, encephalitis, fever and rash, acute flaccid paralysis, and gastrointestinal and respiratory illness. Enteroviruses (e.g., EV71) have been associated with large outbreaks in Southeast Asia. Polioviruses (including vaccine strains) have re-emerged recently, and have been associated with clinical disease. Although Australia is “polio-free”, there have been recent outbreaks of polio in Indonesia, and a recent importation of a case from Pakistan.

The original classification of enteroviruses consists of polioviruses, coxsackie A or B viruses, echoviruses and other enteroviruses, based on biological activity and disease. They are often difficult to culture *in vitro*, and serological testing for diagnosis has poor sensitivity and specificity. Nucleic acid amplification testing (NAAT) methods provide more rapid diagnosis and increased sensitivity. They usually use oligonucleotide primers complementary to conserved HEV sequences, such as those in the 5' untranslated region (UTR).

Enteroviral isolates can be typed serologically, but this is relatively slow and insensitive. Some enteroviruses cannot be serotyped ('untypeable'). Currently CIDMLS is one of two laboratories in Australia that perform HEV serotyping as a reference service. Since 1979, approximately 7,000 enteroviruses have been serotyped, including 1052

isolates from cerebrospinal fluid collected from meningitis and encephalitis cases. Approximately 200 enteroviruses were untypeable. The data on enteroviruses isolated at CIDMLS over the last 27 years demonstrate that all serotypes were common throughout the year, but were most frequent during the summer and autumn, especially for echoviruses 11, 9, 30, 6, 13 and enterovirus 71. Isolation of enteroviruses also varied annually - for example, echoviruses 11 appeared to cause periodic epidemics lasting 2-3 years, and occurring every 3-4 years.

With sequence analysis, the over ninety HEV serotypes can be classified into four species, HEV-A to D, based largely on phylogenetic relationships across multiple genome regions. The 3'UTR of enteroviruses is highly conserved within a species but highly divergent between species. Sequences derived from the VP1 gene correlate best with serotype, and therefore provide the opportunity for the development of molecular typing methods consistent with present serological methods. These molecular methods may also prove their utility through the detection of enteroviruses that fail to grow in cell culture (i.e. directly on clinical samples), and allow the characterization of strains that are untypeable by serotyping using neutralization assays. Currently between 5-10% of enteroviruses are untypeable - it is unknown whether there are 'novel' enteroviruses, or previously 'typeable' strains that have drifted genetically. Molecular analysis of these viruses will also assist with developing more reliable diagnostic assays.

We have developed species-specific reverse-transcriptase (RT)-PCR amplification and sequencing of the complete VP1 region to identify HEV isolates that could not be typed serologically, and PCR/RLB to rapidly characterise the most common serotypes isolated at CIDMLS.

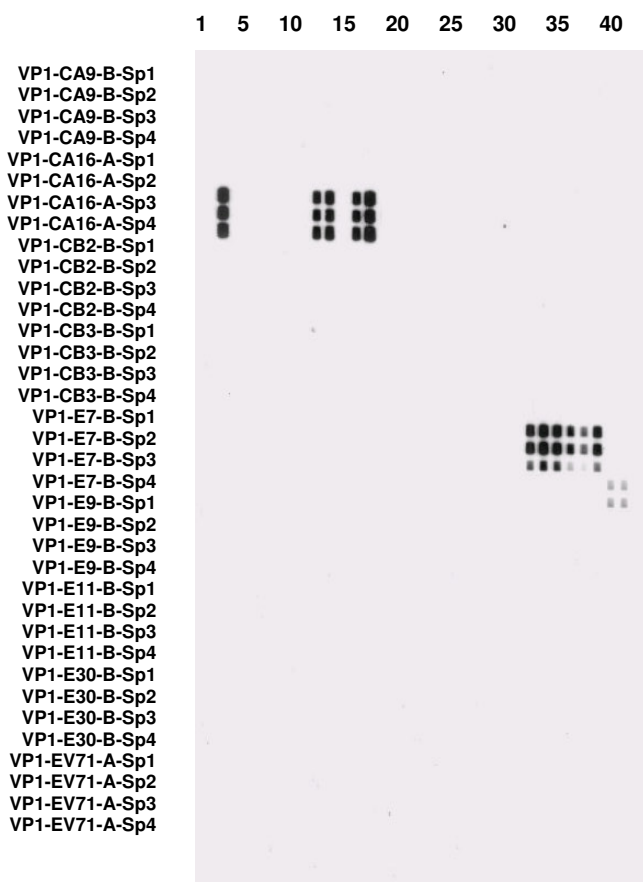
We have detected forty-five untypeable HEV isolates using pan-enterovirus primers. They were initially screened using species-specific RT-PCR primers to confirm their species group. Their complete VP1 sequences were generated using VP1 sequencing primers, and the HEV serotype was confirmed by pairwise comparison of the complete VP1 sequence to a database of complete VP1 sequences of all known enterovirus serotypes. Strains which were at least 75% identical (85% amino acid identity) in VP1 nucleotide

sequences were considered to represent strains of the same serotype. We then designed species-specific primers/probes and serotype-specific primers/probes targeting two fragments within VP1 region for the most common ten serotypes isolated over the last 30 years at CIDMLS. These included coxsackie A9, A16, coxsackie B2, B3, echovirus 7, 9, 11, 30, enterovirus 71 and poliovirus 1.

Using this approach, we successfully identified 45 previously untypeable HEV isolates as echovirus 30 (13 isolates), echovirus 18 (8), echovirus 25 (5), echovirus 9 (5), echovirus 14 (3), echovirus 5 (2), echovirus 11 (2), coxsackie A16 (2), and 1 each of echovirus 1, echovirus 7, echovirus 16, coxsackie A24 and coxsackie B2. These are mostly HEV-B. The genotypes corresponded with the conventional serotypes in 91.78% of isolates by PCR/RLB method (Fig.1).

Molecular typing methods of HEVs using complete (or partial) VP1 sequences can be used as an alternative to serotyping by neutralization. Molecular typing also allows examination of genetic drift of enteroviruses associated with HEV outbreaks and significant disease. Our HEV database shows that the most frequent HEV species is HEV-B, mainly including echoviruses and coxsackie B viruses. The appearance of untypeable HEVs may be reflective of the drift or evolution of these viruses, which may be better explored by molecular approaches instead of neutralization assays. Genotyping by PCR/RLB has the feasibility to complement traditional serotyping method to some extent, and has the advantage of convenience, speed and accuracy.

Figure 1. PCR/RLB results for 40 enterovirus isolates.



STAFF PROFILE LOU ORSZULAK



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With over 16 years of experience within the NSW health industry, Lou Orszulak's projects have included implementation of clinical standards in SWAHS Cancer Services, co-ordination and facilitation of quality improvement teams and projects across SWAHS, and facilitation of education forums for clinical staff on the use of quality improvement tools and methodologies.

Most recently, Lou established new multi-disciplinary approaches to patient care in SWAHS Cancer Services, resulting in 16 teams meeting regularly to review patient care. This project positively impacted the NSW Health system with the NSW Cancer Institute providing funds to support the creation of MDT's state-wide.

Lou has presented at several national quality health improvement and oncological conferences and has contributed to the NSW Cancer Plan 2007-2010.

Lou's current Project Officer role involves the management and promotion of the CIDM Public Health education, research and development program. Lou is involved in organising local and international workshops and conferences, website content, newsletters, annual reports and quality improvement of operational procedures.

Upcoming Meetings, Workshops & Conferences

Australasian Society for Antimicrobials Annual Meeting	26-28 February 2009	Sydney
Royal College of Pathologists of Australasia Annual Update Meeting	13-15 March 2009	Sydney
Australian Society for Infectious Diseases Annual Meeting	25-28 March 2009	Sydney
Communicable Disease Control Conference	4-6 May 2009	Canberra
American Society for Microbiology Annual Meeting	17-21 May 2009	Philadelphia USA

To join our e-list to receive updates please email Lou_Orszulak@wsahs.nsw.gov.au