in treatment and shorter hospital stays. Methods should be selected according to the goals, function and resources of each individual laboratory.

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Biography

Anna Lau is a final year PhD student working at the Centre for Infectious Diseases and Microbiology, Westmead Hospital, University of Sydney, NSW. Her project involves the development of new diagnostic platforms for the rapid detection and identification of fungal pathogens and their resistance mechanisms. After completing her degree, Anna will take up her postdoctoral position as a Clinical Microbiology Fellow at the National Institutes of Health Clinical Centre, USA.

New approaches to enterovirus identification

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The human enteroviruses (HEVs) are members of the Picornaviridae family and cause a diverse range of diseases from respiratory illness to paralysis. Historically, HEVs were isolated in cell culture and subtype identification was by neutralisation using specific antisera. Currently, diagnostic virology laboratories use nucleic-acid-based tests to detect and identify HEVs in clinical specimens.

Human enteroviruses

The human enteroviruses (HEVs) are single-stranded, positive-sense RNA viruses in the *Picornaviridae* family. The International Committee for the Taxonomy of Viruses (ICTV) has classified the HEVs into four species A–D (Figure 1). HEVs are highly infectious, typically transmitted via the faecal-oral route, and cause a wide range of clinical symptoms that can be fatal, including respiratory illness, fever, diarrhoea, meningitis, acute haemorrhagic conjunctivitis, hand-foot-and-mouth disease, poliomyelitis, rash and paralysis. HEVs are also associated with chronic disease and there is mounting evidence for a causal role in the onset of type 1 diabetes [1]. As the symptoms and signs of enteroviral infections are not pathognomonic, prompt laboratory

diagnosis is important for patient management and control of possible outbreaks [2].

Virus culture – limitations

The historical classification of HEVs into coxsackievirus types A and B, echovirus and poliovirus was based on biological activity and disease presentation in patients and laboratory-infected mice[3]. During the early period of coxsackievirus characterisation, respiratory and faecal specimens were inoculated into suckling mice. HEVs may produce a cytopathic effect when clinical specimens are inoculated onto mammalian cell lines, which can be confirmed by immunofluorescent-labelled anti-pan-HEV antibodies and the specific serotype identified by antisera neutralisation [3]. However, this approach has not provided consistent results and includes the following limitations: (i) it requires the judicious use of a number of cell lines susceptible to infection by a range of HEVs; (ii) some HEVs do not grow in cell culture; (iii) many HEVs cannot be typed by the antisera commonly available; (iv) the supply of antisera is limited; (vi) it can take up to two weeks to issue a negative report; (vi) it is expensive to maintain routine passages of cell lines and (vii) the interpretation of the cytopathic effect is subjective (Figure 2).

The use of eight intersecting specific antisera pools (Lim Benyesh-Melnick; LBM) has been the mainstay for enterovirus serotyping prior to nucleic acid sequence analysis. The limited availability of the LBM antisera pools and requirement for virus neutralisation and cell culture facilities have restricted enteroviral subtyping to reference laboratories.

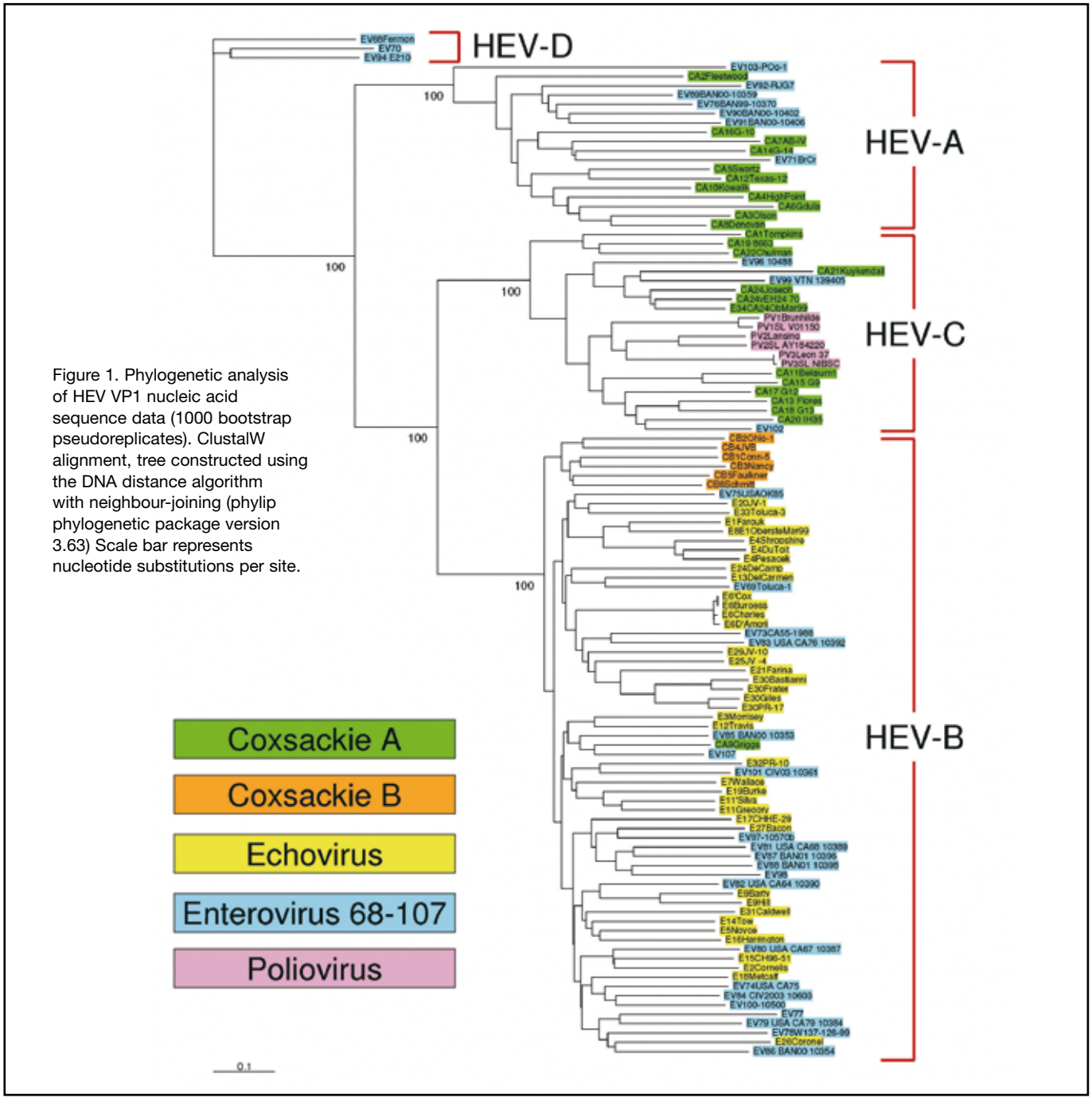

Figure 1. Phylogenetic analysis of HEV VP1 nucleic acid sequence data (1000 bootstrap pseudoreplicates). ClustalW alignment, tree constructed using the DNA distance algorithm with neighbour-joining (phylip phylogenetic package version 3.63) Scale bar represents nucleotide substitutions per site.

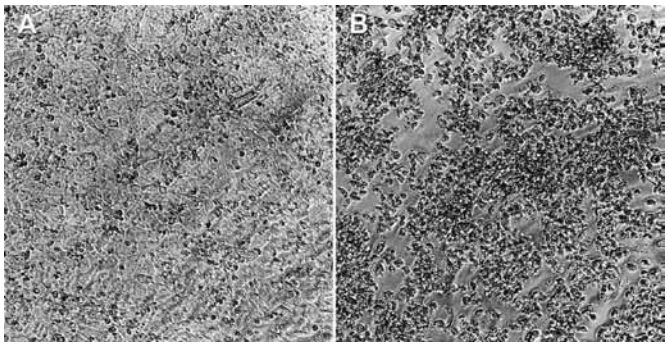

Figure 2. Photomicrograph showing typical enterovirus cytopathic effect (A) uninfected Buffalo Green Monkey Kidney cells (BGMK) and (B) infected BGMK cells. Image courtesy of TW Kok and A Gaeguta, Institute of Medical and Veterinary Science.

Human enterovirus identification by PCR

Since the early 1990s, many diagnostic laboratories have replaced cell culture with reverse-transcription PCR (RT-PCR) as the principal means of detecting HEVs in clinical specimens [4,5]. An indication of the shift from conventional culture methods to PCR is the availability of an Enterovirus Molecular Diagnostic quality assurance panel from the Royal College of Pathologists of Australasia. The relatively low cost of RT-PCR screening for HEVs compared to the routine maintenance of numerous cell lines is an attractive option, with a faster turnaround time (6–48 hours) and the capability of high-throughput analysis. Syndrome-based screening based on PCR multiplex assays have been designed for respiratory, central nervous system and congenital infections[6]. More recently, diagnostic laboratories have introduced real-time RT-PCR (qRT-PCR) detection systems [7], which have further reduced reporting times to less than six hours when combined with automated nucleic acid extraction. The rapid detection of HEVs in clinical specimens is important for patient care [8].

One of the limitations of PCR technology is the requirement that the oligonucleotide primers have a high degree of target specificity. Pan-HEV RT-PCR primers are directed to highly conserved sequence within the 5′ non-translated region (5′NTR) (Figure 3). This assay can detect all known HEVs but may also cross-react with closely related members of the picornavirus family, specifically rhinoviruses (Jason Roberts, Bruce Thorley unpublished observation), resulting in the detection of false positives. This can be resolved by sequencing the RT-PCR amplicon but may not be feasible in a diagnostic laboratory. Another consideration is to ensure the absence of PCR inhibition, which is a particular problem with faecal and CSF specimens [9]. PCR inhibition can be detected by the inclusion of an internal PCR amplification system, a process recommended by the National Pathology Accreditation Advisory Council (NPAAC) [10].

Enteroviral genomic sequence characterisation

The non-enveloped capsid of HEVs consists of four proteins, VP1-4, with major antigenic determinants located within VP1-3 while VP4 is not exposed on the exterior of the virion. An electron micrograph and visual representations illustrating the molecular topography of virus capsid proteins are available as Supplementary Figures 1–6. The use of genetic sequencing to characterise the HEVs led to their reclassification into species A–D and the identification of new types numbered sequentially from enterovirus 73 to, currently, 109 [11]. Molecular characterisation methods also enabled the reclassification of a number of HEVs such as echovirus 22 and 23 as parechovirus, enterovirus 72 as hepatovirus and echovirus 10 as a reovirus (Refer to the ICTV website, www.ictvdb.org).

The identification of HEVs based on the sequence of VP1, VP2 and VP4 capsid regions is possible [12,13] but the adoption of a standard target region enables phylogenetic comparison of HEVs detected in clinical specimens. The identification of HEVs based on the VP1 sequence was found to correlate with the traditional antisera serotype classification enabling the continuation of the existing HEV prototype nomenclature, although the usage of the term genotype rather than serotype has not been resolved [14]. If difficulties are encountered identifying the HEV type from capsid sequence, primers directed to the 3′NTR can classify the virus to the level of HEV species [15].

The dominant region of interest in VP1 is the BC loop, illustrated in Figure 4, which is a neutralising antibody binding site and a major antigenic determinant [14]. A fragment of the VP1 nucleic acid sequence, including the BC loop, can be amplified directly from clinical specimens using highly degenerate primers containing deoxy-inosine, a method termed consensus degenerate hybrid oligonucleotide primer (CODEHOP) [16]. HEV sequence and subsequent HEV serotype identification can be reported within 48 hours of receiving a specimen using the CODEHOP method. The availability of cognate HEV sequences via internet-based repository systems such as GenBank provides the laboratory with sequence data to infer phylogeny. The provision of sequence data is useful in outbreak investigations, such as a wild poliovirus importation [17], the identification of specific genogroups of public health significance, such as enterovirus 71 genogroup C4 that has been associated with serious neurological sequelae and mortality[18,19] and establishing the epidemiology of HEV infections [20].

Derivation of DNA sequence based on Sanger dideoxynucleotide chain termination sequencing may be limited in a diagnostic laboratory by time constraints and cost-effectiveness. While the development of sequence-by-synthesis methods are currently cost-prohibitive for routine diagnostic testing, the ability to screen clinical specimens for microbes by sequence-

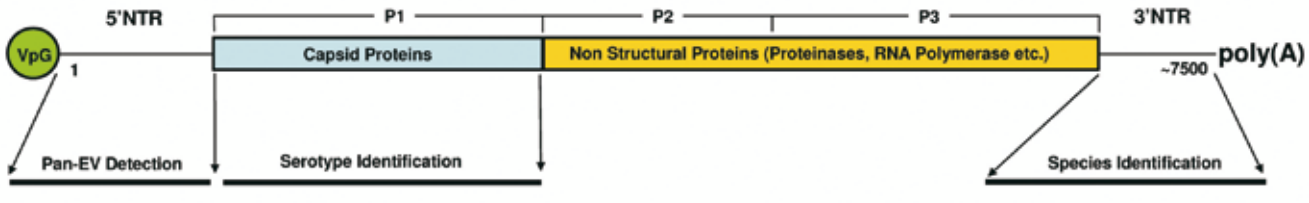

Figure 3. Simplified schematic diagram of a typical enterovirus genome, identifying targets for RT-PCR amplification.

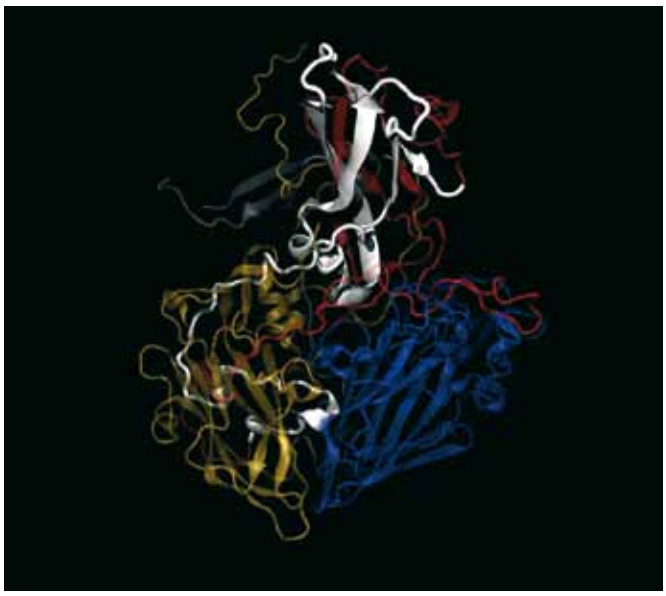

Figure 4. Computer-generated "New Cartoon" representation of a poliovirus type 3 protomer (refer to Supplementary Figure 4). The structural protein subunits are highlighted in color: VP1-Red, VP2-Blue, VP3-Yellow and VP4-Grey (a small discontiguous fragment of VP1 is highlighted in Purple. The translated protein encoded by the VP1 PCR target sequence commonly used for serotype identification is highlighted in white. Image representation created using VMD with the STRIDE function; Frishman, D., and Argos, P. (1995) Knowledge-based protein secondary structure assignment. Proteins-Structure Function and Genetics 23, 566-579. Protein coordinate data obtained from protein data bank file 1PVC.pdb available at http://www.pdb.org/pdb/explore/explore.do?structureId=1PVC. Protomer reconstructed and visualised using the Visual Molecular Dynamic software package (VMD) http://www.ks.uiuc.edu/Research/vmd/.

independent methods is a great advance [21]. The availability of microarray detection and pyrosequencing allows rapid serotype identification of enteroviruses based on the capsid region [22,23]. The PyroMark MD™ pyrosequencing system enables laboratories to sequence biotinylated RT-PCR products within an hour at a significantly reduced cost compared to standard dideoxynucleotide sequencing. Such a system may prove useful for the rapid investigation of HEV outbreaks. The larger 454 Genome Sequencer FLX™ system enabled a metagenomic analysis of patient specimens [24], with the ability to provide full genome sequence data theoretically from all non-host genetic information present in the sample. The storage and analysis of potentially huge amounts of data needs refinement before the next-generation sequencing methods become routine [25]. Caution is also needed before assigning causality to any microbe identified from a clinical specimen by these methods, especially for HEV given that most infections in humans are asymptomatic. Despite these limitations, over the coming years this may be the test of choice for pathogens in general.

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Biographies

Bruce Thorley is the Head of the Australian National Polio Reference Laboratory, which is accredited by the World Health Organization and located at the Victorian Infectious Diseases Reference Laboratory. He is also the Chief Investigator for the national Acute Flaccid Paralysis Surveillance program that investigates polio-like illness in children. In recent years he has focussed on broadening surveillance for poliovirus in Australia by establishing an Enterovirus Reference Laboratory Network and testing environmental samples from sentinel sites.

Jason Roberts is a Senior Medical Scientist at the Victorian Infectious Diseases Reference Laboratory with a background in molecular diagnostic assay development. He is a consultant virologist for the Australian Polio Expert Committee and acts a temporary advisor for the WHO Polio Laboratory Network. His research interests relate to neurotropic RNA viruses, in particular the characterisation and molecular modelling of enteroviruses. He is currently collaborating with RMIT University, the Victorian Partnership for Advanced Computing and the Victorian Life Sciences Computation Initiative to recreate 'in-silico', novel enterovirus and poliovirus proteins to determine the mechanisms of specific mutations associated with neurovirulence.