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Next generation sequencing of human enterovirus strains from an outbreak of enterovirus A71 shows applicability to outbreak investigations

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Abstract

Background—The most recent documented Australian outbreak of enterovirus A71 (EV-A71) occurred in Sydney from 2012 to 2013. Over a four-month period more than 100 children presented to four paediatric hospitals with encephalitic presentations including fever and myoclonic jerks. The heterogeneous presentations included typical encephalomyelitis, and cardiopulmonary complications.

Objectives—To characterise the genomes of enterovirus strains circulating during the 2013 Sydney EV-A71 outbreak and determine their phylogeny, phylogeography and association between genome and clinical phenotype.

Study design—We performed an analysis of enterovirus (EV) positive specimens from children presenting to hospitals in the greater Sydney region of Australia during the 2013 outbreak. We amplified near full-length genomes of EV, and used next generation sequencing technology to sequence the virus. We used phylogenetic/phylogeographic analysis to characterize the outbreak viruses.

Results—We amplified and sequenced 23/63 (37%) genomes, and identified the majority (61%) as EV-A71. The EV-A71 sequences showed high level sequence homology to C4a genogroups of EV-A71 circulating in China and Vietnam during 2012-13. Phylogenetic analysis showed EV-A71 strains associated with more severe symptoms, including encephalitis or cardiopulmonary failure, grouped together more closely than those from patients with hand, foot and mouth disease. Amongst the non-EV-A71 sequences were five other EV subtypes (representing enterovirus subtypes A and B), reflecting the diversity of EV co-circulation within the community.

Conclusions—This is the first Australian study investigating the near full-length genome of EV strains identified during a known outbreak of EV-A71. EV-A71 sequences were very similar to strains circulating in Asia during the same time period. Whole genome sequencing offers additional information over routine diagnostic testing such as characterisation of emerging recombinant strains and inform vaccine design.

Keywords

Enterovirus; EV-A71; Phylogeny; Hand, foot and mouth disease; Whole Genome Sequencing; Australia

1. Background

The World Health Organisation (WHO) has declared much of the world free of polio, however non-polio enteroviruses cause substantial diseases burden and can cause large outbreaks of serious disease such as acute flaccid paralysis and meningitis [1][2]. While most enterovirus infections cause self-limiting hand, foot and mouth disease, a small proportion of infections with enterovirus subtype A71 (EV-A71) can result in severe illness including aseptic meningitis, encephalitis, or cardiorespiratory failure and occasionally death [3-5]. The most recent Australian outbreak of EV-A71 occurred in Sydney from December 2012 until June 2013 with 119 children admitted to tertiary children's hospitals [6]. Of 61 children admitted to the Sydney Children's Hospital Network between January and June 2013, 38% had encephalomyelitis, 33% had brainstem encephalitis, 10% had encephalitis

and 7% had acute flaccid paralysis [5]. Four children (4/61; 7%) died from cardiopulmonary failure [5].

Enterovirus infection is usually detected using real-time reverse transcription polymerase chain reaction (RT-qPCR) targeting the 5'-untranslated region (5'UTR) of the enterovirus genome [7, 8]. Genotyping is most often performed by sequencing the major capsid protein VP1 coding region because the significant genetic diversity between enterovirus genotypes in this region improves identification of genotypes such as EV-A71 and CV-A16 compared with sequencing the 5'UTR region [9]. More recently, next generation sequencing (NGS) has become a useful tool for enterovirus subtyping, particularly in outbreak investigations [10] or where the infectious agent is not easily identified using other methods [11]. Whole genome sequencing (WGS) of enteroviruses has several advantages over single gene analysis, including i) detection of recombination events, ii) ability to identify severity markers across the genome, iii) identification of potential new biomarkers throughout the genome, and iv) allowing for more sensitive and specific tracking of viral evolution.

2. Objectives

There are limited studies using NGS of full-length genomes of EV-A71 outbreaks to define the epidemiology of outbreaks [10, 12], and none performed in Australia. With increasing numbers of outbreaks caused by enteroviruses including EV-A71 and D-68, an investigation of the viral genetic basis for severe illness is warranted. We aimed to characterise the genomes of enterovirus strains circulating during the 2013 Sydney EV-A71 outbreak and determine their phylogeny, phylogeography and association between genome and clinical phenotype.

3. Study Design

3.1 Samples

We collected samples testing positive for enterovirus (EV) through Virology Reference Laboratories at tertiary paediatric hospitals in the greater Sydney and Newcastle regions of New South Wales (NSW), Australia between March 1 and June 30 2013 (4 months). These included laboratories servicing patients in eastern, northern, near western and far northern NSW.

Samples were tested by nested multiplex PCR testing for EV [13]. The 5'UTR was amplified and sequenced in a subset of samples by the diagnostic laboratory at the time of the outbreak, as performed previously [14]. All samples were stored at -20°C from 2013 in the diagnostic laboratory until use in the present study. Sample types included swabs (throat, rectal or nose), nasopharyngeal aspirates, cerebrospinal fluid, faeces or cell culture from children aged 2 months to 14yrs old. We categorised clinical symptoms presented by patients into one of four stages by clinical severity [15-17]: uncomplicated HFMD or fever (Stage 1), myoclonic jerks or meningitis (Stage 2), with encephalitis with or without cardiopulmonary failure (Stage 3A/3B).

The Sydney Children's Hospital Network and South Eastern Sydney Local Health District Human Research Ethics Committees approved the study (LNR/13/SCHN/443, LNR/16/POWH/172).

3.2 Near full length genome PCR and sequencing

Viral nucleic acid was extracted using the QIAamp Viral RNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that linear polyacrylamide (Sigma Aldrich, Missouri, USA) was used instead of carrier RNA.

Near full-length enterovirus genomes (approximately 7.4 kb; EV genome is between 7.2 kb and 8.5 kb in length) were amplified as described previously [18], except KlenTaq polymerase was used for amplification instead of Takara LA DNA polymerase (Clontech). Library preparation for NGS was performed as described previously [18].

3.3 Sequence Analysis

Sequencing was performed at the Centre for Infectious Diseases and Microbiology Laboratory Service within the Institute of Clinical Pathology and Medical Research at Westmead Hospital, using a NextSeq500 generating 2 x 150 bp paired-end reads (Nextera XT). We constructed EV contigs as previously described [18]. We annotated the sequences against a recent complete genome of EV-A71 (GenBank accession [JX244186](#)), and trimmed them to the complete coding sequence (CDS) for comparative analysis.

Genotyping was performed using the Basic Local Alignment Search Tool (BLAST, available through the National Center for Biotechnology Information (NCBI) website [19]) to genotype using the final consensus as the query sequence. Consensus sequences were submitted to GenBank (Accession numbers [MK697678-MK697695](#)). Multiple sequence alignment was performed in Geneious v11.0.4 (Biomatters)[20] using the ClustalW [21] plug-in. Phylogenetic analyses of the aligned sequences for the CDS and VP1 gene was performed using the neighbour joining method with 1,000 bootstraps (Geneious, Biomatters) [20].

Separately, we compared our EV-A sequences to those published from Sydney in 2016 [15]. Here, we used PhyML [22] in Geneious 11.1.4 and specified a generalised time-reversible substitution model with an NNI and SPR topology search and a bootstrap of 1,000.

We used BEAST v.1.10.4 [23] to examine the phylogeography of EVA-71 sequences. We implemented a Bayesian Stochastic Search Variable Selection (BSSVS) procedure [24] using discrete trait analysis of the geographic areas (North, East, South, and Southwest Sydney) and the patient's severity. We used Bayesian Tip Significance (BaTS) [25] to study the individual hypotheses that the EV-A71 phylogeny was structured by geography as well as clinical outcome (i.e. severe outcomes were genetically grouped together).

We used HYPHY to examine selection and calculate the non-synonymous synonymous (dN/dS) rate ratio using both the single likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) approaches. We analysed recombination using the Recombination Detection Program (RDP) [26] on our EV-A71 sequences. We used the methods: RDP,

GENECONV, MaxChi, Chimaera, SiScan and 3Seq within the program. We only considered recombination events if they were detected as a significant event by at least three different detection methods.

4. Results

4.1 Patients involved in the 2013 outbreak

Of 63 samples available from 45 patients (Supplementary figure 1), we amplified and sequenced genomes from 23 (41%) samples (18 patients; 40%) (Table 1), with a mean age of 24 months (range 28 days-6 years) and 61% male. Clinically, three patients presented with uncomplicated HFMD or fever (Stage 1), 12 with myoclonic jerks or meningitis (Stage 2), and three with encephalitis with or without cardiopulmonary failure (Stage 3A/3B).

An average of 2,916,214 reads per sample were assembled with average coverage of 430 reads per position, resulting in trimmed sequence length of approximately 7 kb. The majority of patients were identified as infected with EV-A71 (11/18, 61%, Table 1), presenting with Stage 2 disease or above. There were 5/18 other subtypes identified as enterovirus A (COX-A6 (n=2), COX-A2 (n=1), and COX-A16 (n=1)) or enterovirus B subtypes ECHO-E6 (n=1) and ECHO-E9 (n=2), with 5/7 of these patients also with neurological disease including myoclonic jerks (Stage 2, Table 1).

Those samples with whole genomes sequenced had 5'UTR sequences generated in only two thirds (12/18) (Table 1), with discordance between 5'UTR and whole genome sequences in half (6/12) where both were available (Table 1).

There were three patients with the EV genomes sequenced from more than one sample, either because samples either taken from different body sites (n=2), or from an original sample and its cell culture isolate (n=1). In Supplementary Figure 3, we show the phylogenetic relationships of the CDS regions of these subtypes which demonstrates that the samples from different body sites were genetically identical. The cell culture isolate of sample Sydney57 resulted in 22 nucleotide changes to the EV-A71 genome, and 11 amino acid missense mutations in the polyprotein from the original nasopharyngeal aspirate sample (data not shown).

4.2 Diversity of HEV genotypes circulating during an outbreak of EV-A71

In Figure 1, we show the genetic diversity of enterovirus strains co-circulating during the 2013 outbreak of EV-A71. Sequence comparison revealed that the Sydney outbreak strains from this study grouped closely with strains from the C4 sub-genotype isolated in China in previous years (Figure 1 & Supplementary Figure 2). Figure 2 shows the comparison of the EV-A sequences with those from a 2016 molecular epidemiology study conducted in Sydney [15]. The results show an overall grouping of our 2013 Sydney strains with the 2016 strains by genotype with high bootstrap support.

4.3 Phylogeographic analysis of strains sequenced in the study

In Figure 4 we show the maximum clade credibility (MCC) of 11 EV-A71 samples. Here, we colour the branches by geographic area North (N), South (S), East (E), and Southwest

(SW) (locations shown in Figure 3). The root state of the phylogeny suggests the origin of the outbreak was within the East (including Randwick City Council and Waverly Council, red lines in Figure 4). The BaTS analysis suggests that the phylogeny of the tree was not structured by geography (association index p-value = 0.86) indicating that specific geographic areas were not harbouring genetically distinct viruses. Figure 5 shows the same tree but with the branches coloured by severity. In contrast, the BaTS analysis here shows the phylogeny was structured by severity (association index p-value = 0.02).

We did not find evidence for positive selection of amino acids from these EV-A71 sequences. The amino acid differences for EVA71 viruses in our study are highlighted in Supplementary figure 4. We detected a single recombination event using four methods (Supplementary Table 1) in one EV-A71 strain, Sydney 57. The analysis detected a breakpoint at nucleotide position 5447.

5. Discussion

This is the first Australian study investigating the near full-length genome of enterovirus strains obtained during an outbreak of EV-A71, which has demonstrated the utility of NGS over single site sequencing of the 5'UTR. While a high proportion of strains were EV-A71 (61%), other enterovirus subtypes also circulating at the same time caused serious disease in children presenting to hospital. These new data add important information about enterovirus diversity and concurrent circulation of strains during an outbreak, one of the principal reasons for conducting enterovirus surveillance as described in the recent framework "Enterovirus Surveillance Guidelines" published by the WHO Europe http://www.euro.who.int/__data/assets/pdf_file/0020/272810/EnterovirusSurveillanceGuidelines.pdf).

The genetic similarity of our Sydney 2013 EV-A71 strains to those isolated in Vietnam and China in the surrounding years indicates a possible introduction of an Asian EV-A71 strain into Australia in 2012. The 2013 outbreak strain was subgenotype C4, which was the most common EV-A71 genotype in China, Hong Kong, Korea, and Vietnam [27]. Liu and colleagues characterised EV-A71 from the C4 genogroup which caused a large outbreak of HFMD in China in 2012 [28] [29]. Serious outbreaks caused largely by the EV-A71 C4 genogroup also occurred in Vietnam [12] and Cambodia [10] during the same time period. Sydney has a large Chinese and Vietnamese migrant population (4% and 2% respectively, according to the Australian Bureau of Statistics [30]), and increasingly frequent air travel to South and East Asia will likely result in future introductions of new enterovirus strains and potential for recombination of subtypes and outbreaks.

Outbreaks of enterovirus, particularly EV-A71, are common in Asia and occur every 2-3 years in Malaysia [31] and other Asian countries [32, 33]. Outbreaks of enterovirus D68 occur approximately every two years in the United States (possibly due to waning enterovirus antibodies in the population years [33], with a large outbreak in 2014 involving 1153 people (mostly children) [34]. In contrast, outbreaks of EV-A71 in Australia do not seem to have followed a cyclical pattern. Prior to the 2013 outbreak in Sydney, other documented outbreaks have occurred in 2000 (Sydney), 1999 (Perth), 1986 (Melbourne and

Sydney), and 1972 (Melbourne) [35-38]. This could be because non-polio enterovirus surveillance in Australia is currently done by a network of 11 laboratories, primarily as a means to detect imported poliovirus cases [39]. Without a sentinel or active national surveillance system for non-polio enteroviruses such as EV-A71, smaller outbreaks may be missed.

The phylogenetic analyses included whether neighbouring taxa in a phylogenetic tree shared either a common geographical location or clinical outcome. These results demonstrated EV-A71 strains causing more severe clinical outcome were grouped together phylogenetically. This has clinical significance as regular collection and sequencing of enteroviruses, combined with clinical data, is important for early detection of viruses with outbreak potential, supporting the Enterovirus surveillance guidelines published by the WHO Europe [41]. The clustering of EV-A71 in the phylogeographical tree showed the estimated origin of the outbreak strain may have been a single area (East). This would be consistent with spread from a single node, resulting from person to person spread. We also looked for recombination, as intra- and inter-typic recombination is common in enterovirus (Volle et al 2019). We found one incidence with a recombination break point in the P3 region of the genome, which encodes non-structural proteins involved in viral replication, consistent with recombinations detected by previous studies (Volle et al., 2019; Cobbin et al., 2018).

This study was limited by the small number of samples, and sample storage at -20°C for approximately four years, which may have resulted in some loss of viral RNA [42]. We were not able to amplify EV from cerebrospinal fluid samples ($n=3$ samples). Other studies have also reported difficulties in amplifying EV from cerebrospinal fluid [43]. In addition, there were no contemporaneous pre- and post-outbreak EV positive samples for comparison, which would have been ameliorated by state-wide enterovirus surveillance systems, (which are not currently in place). The full impact of these near full length genome data will be utilised when effective anti-virals and widespread vaccines are available. Continued surveillance of enteroviruses is important to build an understanding of this ever-changing pathogen.

Vaccination against EV-A71 would likely prevent large scale outbreaks, as seen with the near-eradication of poliovirus [44]. Vaccines have been developed and trialled in China, some are now in commercial production [27] and have demonstrated effectiveness in children 5 years old [45]. These whole, inactivated virus vaccines are useful against the C4a EV-A71 strain which is common in China. In other countries, surveillance and full genome sequencing of enterovirus positive samples is necessary to determine whether the vaccine strain matches strains that are circulating in that particular country. Understanding the molecular epidemiology of EV-associated neurological disease may help detect outbreaks, emerging recombinant strains and allow for early intervention strategies such as infection control. Ultimately vaccination will be one of the best defences against enterovirus outbreaks, and studies such as these can inform vaccine design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Near full-length genome analysis using next generation sequencing identified EV-A71 and other enterovirus A and B subtypes circulating
- The Sydney EV-A71 2013 strain was very similar to EV-A71 circulating in China and Vietnam during the previous year
- Strains causing more severe clinical manifestations grouped together phylogenetically

Figure 1. Phylogenetic analysis of complete coding sequence (CDS) from Sydney outbreak 2013 strains and sequences from GenBank.

The Neighbour-Joining method was used with 1000 bootstrap analysis. Tips are named by: year of collection, genotype, country, and GenBank accession number. Sequences in this study are labelled as ‘Sydney (study number)’, reference sequences are included for each genotype with their GenBank accession number. Poliovirus (Brunhilde strain) was used as an outgroup. Country abbreviations: CHN-China, HK-Hong Kong and AUS-Australia.

Figure 2. Maximum likelihood tree of Sydney EVA strains from the 2013 outbreak and a 2016 epidemiology study (Cobbin et al., 2018).

We used PhyML (Guindon et al., 2010) in Geneious 11.1.4 and specified a generalised time-reversible (GTR) substitution model with an NNI and SPR topology search and a bootstrap of 1,000. Viruses not included in our samples are labelled by their GenBank accession number and coloured by EV genotype. Branches are labelled with bootstrap values.

Figure 3. Map of local government areas in Sydney and surrounding area of NSW, with areas where patients in this study were infected with EV highlighted. Highlighted are Bayside Council (blue), Blue Mountains City Council (cyan), Canterbury-Bankstown Council (green), Northern Beaches Council (red), Parramatta City Council (yellow), Randwick City Council (orange) and Waverley Council (pink). Three samples were from Lake Macquarie Council and Port Stephens Council, which are not featured on this map.

Figure 4. Phylogeographic maximum clade credibility (MCC) tree of eleven EV-A71 samples from this study. The branches are coloured by geographical area.

Abbreviations: E-East (includes Randwick City Council and Waverley Council; N-North (includes North Sydney Council, Northern Beaches Council and Port Stephens Council); S-South (includes Bayside Council); SW- South West (includes Canterbury-Bankstown Council).

Figure 5. Maximum Clade Credibility (MCC) tree of 11 EV-A71 Sydney 2013 samples with branch colour indicating clinical severity.

Severity codes were based on: 1 = uncomplicated HFMD or fever; 2 = myoclonic jerks or meningitis; 3A = encephalitis; 3B = CNS involvement with cardiopulmonary failure.

Table 1.

Patient characteristics, symptoms and enterovirus genotypes, with those discordant between 5'UTR and whole genome sequencing shaded.

Study ID	Sex	Age (months)	Specimen type	Disease severity α	ID by 5' UTR sequencing	ID by genome sequencing
Sydney03	M	31	Rectal swab	2	ND	EV-A71
Sydney05	M	60	Rectal swab	2	ND	EV-A71
Sydney09	F	17	Faeces	2	EV-A71	EV-A71
Sydney14	F	14	Throat swab	2	EV-A71	EV-A71
Sydney18	F	31	Nose swab	2	ND	EV-A71
Sydney20	F	6	Faeces	3A	ND	EV-A71
Sydney30	M	38	Throat swab	3B	ND	EV-A71
Sydney11	M	24	Throat swab	2	EV-A71	EV-A71
Sydney56	F	5	Nasopharyngeal aspirate	3A	ND	EV-A71
Sydney57	M	20	Cell culture from Stool	1	EV-A71	EV-A71
Sydney60	M	9	Throat swab	2	EV-A71	EV-A71
Sydney02	M	20	Throat swab	2	EV-A71	CV-A6
Sydney16	M	26	Throat swab	2	EV-A71	CV-A2
Sydney17	M	39	Perianal swab	2	EV-A71	CV-A6
Sydney42	F	77	Faeces	2	EV-A71	E6
Sydney48	F	12	Faeces	1	CV-A16	CV-A16
Sydney43	M	2	Faeces	2	CVB2	E9
Sydney55	M	<1	Faeces	1	E30	E9

α Severity score according to (Huang et al., 2017).

ND, not done.