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Cross-reactive neutralizing antibody epitopes against Enterovirus 71 identified by an *in silico* approach

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ABSTRACT

Currently, infections of hand, foot and mouth disease (HFMD) due to Human Enterovirus 71 (EV71) cannot be prevented or treated, as there are no suitable vaccines or antiviral drugs. This study aimed to identify potential vaccine candidates for EV71 using *in silico* analysis of its viral capsid proteins. A combined *in silico* approach utilizing computational hidden Markov model (HMM), propensity scale algorithm, and artificial learning, identified three 15-mer structurally conserved B-cell epitope candidates lying within the EV71 capsid proteins. Peptide vaccine candidates incorporating a target B-cell epitope and a promiscuous T-cell epitope from the related polio virus were synthesized using solid-phase Fmoc chemistry. Inbred BALB/C mice which were inoculated with two 10 μg doses of the synthetic peptide, generated anti-peptide antibodies. Purified IgG isolated from pooled sera of the inoculated mice neutralized EV71 infections *in vitro*. Furthermore, these neutralizing antibodies were cross-reactive against other members of the *Picornaviridae* family, demonstrating greater than 50% virus neutralization. This indicates that the current approach is promising for the development of synthetic peptide-based vaccine candidates against *Picornaviridae*. Development of effective vaccines is of paramount importance in managing the disease in the Asia Pacific regions where this virus is endemic and has significant social, economic and public health ramifications.

1. Introduction

Five genera within the Picornavirus family, *Apthovirus*, *Cardiovirus*, *Enterovirus*, *Hepatovirus*, and *Parechovirus* can infect humans [1]. *Picornaviridae*, with their subtypes, cause diverse disease manifestations in humans (Table 1).

As the number of polio cases decrease, epidemics of Enteroviruses (EV's) including EV71 have increased in number and severity, and can lead to neurological complications such as acute flaccid paralysis and brain stem encephalitis. Infection with several members of the enteroviral family have been linked to the onset of Type 1 diabetes [2,3] which is speculated to be due to the induction of deleterious antibodies or T cells that cross-react with human islet cells. Treatment for severe cases of EV71 infection is limited to supportive or palliative care.

The majority of licensed antiviral vaccines contain inactivated or live-attenuated virus, which (i) requires maintenance of the cold chain, (ii) protecting workers during the manufacturing process, (iii) carries the risk of reversion to wild-type virus. Vaccination with whole viruses can also lead to autoimmune diseases [4]. It is therefore imperative that any anti-enteroviral vaccine designed does not induce an autoimmune state. Epitope-based vaccines can induce antibodies capable of neutralizing virus without these issues. This study aimed to establish whether computationally derived peptide constructs would be immunogenic and serve as potential vaccine candidates against EV71 [5–9].

Human enteroviruses are encapsulated by four virus capsid proteins (VP1-VP4), with VP1 being the immunodominant surface protein. Detecting B-cell epitopes in a protein is a fundamental step in many immunological applications, this is most efficiently and rapidly achieved by a computational tool that predicts immunological regions. The identification of linear B-cell epitopes has been traditionally based on the physiochemical properties of the amino acids and their relative special spatial to each other; or from hydrophobicity [10,11] or solvent accessibility scales [12,13]. However, false positive rates using sequence scales have usually been quite high as not many predicted epitopes have been able to elicit *in vitro* neutralization. This observation is often due to the degree of ambiguity and error prone alignments in databases and small number of experimentally derived datasets that are available for

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Table 1
The clinical manifestations of multiple Enterovirus subtypes and contribution to disease as adapted from [40].

Clinical manifestation	Enterovirus subtype
Acute flaccid paralysis	Polio virus 1–3; Coxsackievirus A7, A9, B2–5; Echovirus 4, 6, 9, 11, 30; Enterovirus 70, 71
Aseptic meningitis	Polio virus 1–3; Coxsackievirus A2, A4, A7, A9, A10, B1–6; Echovirus 1–11, 13–23, 25, 27, 28, 30, 31; Enterovirus 70, 71
Hand Foot and Mouth Disease	Coxsackievirus A5, A10, A16; Enterovirus 71
Herpangina	Coxsackievirus A2-6, A8, A10; Enterovirus 71
Acute hemorrhagic conjunctivitis	Coxsackievirus A24; Enterovirus 70
Encephalitis	Echovirus 2, 6, 9, 19
Meningoencephalitis	Coxsackievirus B1–5; Enterovirus 70, 71
Pericarditis, myocarditis	Coxsackievirus B1–5
Diabetes Type I	Coxsackievirus B4

training the algorithms. For example, Blyth and Flower [14] found that most propensity scale methods fail to perform better than random predictions.

Recently, B-cell epitope prediction algorithms have included experimentally derived reference datasets and have combined single scale predictions with nearest neighbor or decision tree approaches such as the Hidden Markov model [15], Artificial Neural Network (ANN) models [16], crystallographic structural data from the PDB (Protein data bank) if known, or investigator generated theoretical homology models. Here we follow a multi-step algorithmic approach that utilizes both a sequence (ABCPred, BepiPred) and a structure based (epitopia) servers [17] in an attempt to improve the probability of defining a functional peptide epitope for vaccine use.

In this study, an *in silico* computational analysis model of sequence and structure identified three novel and one previously identified candidate B-cell epitopes from the VP1 and VP3 capsid proteins. The candidates had to be conserved across all strains of EV71 and other Enterovirus family members. These candidates were constructed as linear peptides, specifically incorporating the antibody (Ab) epitope and a promiscuous T-Helper cell epitope from the related polio virus. These constructs were used to inoculate BALB/c mice to determine whether anti-peptide Ab's could be raised that would neutralize EV71 and other viruses from the *Picornaviridae* family.

2. Materials and methods

2.1. Bioinformatics sequence approach

The *Picornavirus* capsid polyprotein (VP1–VP4) regions were computationally analyzed for hydrophobicity, solvent accessibility, surface accessibility of residues, polarity, and the spatial distance orientation relationships [11,12,18,19]. The sequences were obtained *via* NCBI Genbank and scored for the aforementioned key antigenic attributes against the BLASTP [20] query algorithm. Alignment of protein regions was compiled against multiple publicly available database sets and sorted *via* the clustalW alignment program [21]. Conserved sequences demonstrating homology within the protein data bank listings PDB ID: 3VBS Human Enterovirus 71; PDB ID:1BEV Bovine Enterovirus; and PDB ID:1HXS Mahoney Poliovirus were used to construct and verify the model.

2.2. Bioinformatics structure approach

Sequence alignment models demonstrating more than >40% structural conservation with the PDB were used to generate a three-dimensional structural model for the EV71 (VP1 and VP3) assemblies, using the Chimera [22] interface to modeller [23]. The crystallographic atomic coordinates were reconstructed and uploaded to the epitopia server to estimate the rate of amino acid substitutions at each position in the alignment of homologous proteins [17].

2.3. Synthesis of peptide candidates

Four epitopes, including one identified by Foo et al. [24], were synthesized using Fmoc-solid phase peptide synthesis [25]. Each antibody epitope was synthesized as a linear construct containing a universal T helper epitope from Polio virus (KLFAVWKITYKDT) [26]. The constructs were termed K1 (KLFAVWKITYKDTLM-RMKHVRAWIPRPMR), K2 (KLFAVWKITYKDTLFHPTPCIHIPGEVRN), K3 (KLFAVWKITYKDTL GFPTELKPGTNQFLT) and D1 (KLFAVWKI-TYKDTLYPTFGEHKQEKDLEYC) and were commercially synthesized (Auspep Pty. Ltd, Melbourne, Australia).

2.4. Cell growth and maintenance

Vero cells (African green monkey kidney cells; the American Type Culture Collection (ATCC): CCL-81) were maintained in Dulbecco's minimal essential medium with glutamine (DMEM, Gibco Inc., Australia) supplemented with 10% heat-inactivated fetal calf serum and 1% of penicillin streptomycin (50 μg/mL, Gibco Inc., Australia) at 37 °C with 5% CO_2 in air per ATCC recommendations.

2.5. Viruses

The stock clinical isolate strains: Human Enterovirus 71 (EV71: isolate number 99018233), Coxsackie A16 (CVA16: isolate number CAIG 9902-2745-4PMEK 9.4.00), Coxsackie B4 (CVB4: isolate number 99039838), Coxsackie B6 (CVB6: isolate number 273370/P BO (14.09.1989)), and Echovirus 13 (Echo13: isolate number 28-606PMEK (07.06.1990)) were supplied by the Victorian Infectious Disease Reference Laboratory (VIDRL, Melbourne, Australia).

2.6. Virus growth and maintenance

Vero cell monolayers at 80–100% confluence were infected with clinical isolate strains of EV71, CVA16, CVB4, CVB6, and Echo13 were used to infect and then incubated at 37 °C with 5% CO_2 in air until virally induced cytopathic effects (CPE) were visible. The supernatant was then harvested and the titre for each virus was determined by the Reed and Muench formula for $TCID_{50}$ based on the CPE produced from the viral infection [27].

2.7. Mice and immunization protocols

Groups of six male BALB/c mice (6–8 weeks old) obtained from the animal house facility, Department of Microbiology and Immunology, The University of Melbourne, were inoculated subcutaneously at the base of the tail with the peptide immunogens. Animals received two doses of either 10 or 20 μg of peptide 4 weeks apart; the primary inoculation was administered with Complete Freund's Adjuvant (CFA) and the subsequent inoculation with Incomplete Freund's Adjuvant (IFA). Sera were obtained from animals 10 days after each vaccination.

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2.8. Enzyme linked immunosorbent assay (ELISA)

The anti-peptide antibodies in the mouse sera were detected by ELISA as described previously [28,29], using Horseradish peroxidase conjugated rabbit anti-mouse antibody (Sigma Aldrich, Castle Hill, Australia). The assays were performed in triplicate. Antibody titres were expressed as the reciprocal of the highest dilution that gave a reading above the endpoint cut-off [30].

2.9. IgG purification

IgG was purified from pooled mouse sera by affinity chromatography using a column (10 mm × 85 mm) of Protein A-Sepharose Fast Flow (Sigma Aldrich, Castle Hill, Australia) following the manufacturer's instructions.

2.10. Positive controls

A monoclonal antibody (MAb) against the VP1 protein of EV71 (MAB979, Millipore, Australia), and monoclonal antibodies for CA16 and CVB4 (VIDRL, Melbourne, Australia) served as positive controls.

2.11. Neutralization assay

Neutralizing activity of IgG purified from pooled sera from the immunized mice was measured using an *in vitro* micro neutralization assay in 96-well plates (Imunoblot HB) [31]. Purified IgG (10 μg/mL) at six 2-fold serial dilutions was pre-incubated with an equal volume of 200$TCID_{50}$ of each virus (EV71, CVB4, CVB6, CA16 and Echo13) and then used to infect 80–100% confluent Vero (CCL-81) cells. The cells were washed and then incubated at 37 °C with 5% CO_2 until the first sign of complete CPE in the virus control wells. At this point, 25 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega Corp., Madison, WI) one-shot solution was added to all wells and an absorbance was recorded at 490 nm. The MAbs were included as positive controls. Results were read as positive if there was more than a 50% CPE reduction as compared to naïve controls.

2.12. Statistical analyses

Statistical analyses were performed using a one way parametric ANOVA test with 95% confidence interval.

3. Results

3.1. Bioinformatics

The combined *in silico* approach identified three 15-mer linear epitope sequences of potential interest across multiple EV subtypes (Table 2). These epitopes were termed K1, K2 and K3. The antibody inducing peptide epitope previously published by Foo et al. [24], here termed D1, which was also highlighted using this computational approach was also included. Epitopes were discounted if their normal position lay buried within the viral coat rather than on a surface. K1 was structurally most conserved with no more than 6 amino acid substitutions as compared to the VP1 sequence of other virus strains. K1 also exhibited more surface exposed residues on the PDB ID: 3VBS, 1HSX and 1BEV entries. K2 and K3 (from the VP3 protein) had less sequence conservation with other viruses and represented less exposed, more buried regions. Other peptide epitopes were identified from VP2 and VP4 but were not as promising as K1, K2 and K3 in terms of their conservation and antigenicity and were not evaluated as potential vaccine targets.

Table 2
B-cell epitope sequences identified using bioinformatics with sequence alignment across multiple *Picornaviridae*. This layout shows representative sampling from across multiple viruses.

Virus Strain	GenBank Accession Number	Peptide			
		D1:VP1 (YPTFGEHKQEKDLEYG)	K1:VP1 (MRMKHVRAWIPRPMR)	K2:VP3 (FHPTPCIHIPGEVRN)	K3:VP3 (GFPTELKPGTNQFLT)
Enterovirus 71 (EV71)	Genbank ID: AAK13008.1	YPTFGEHKQEKDLEYG	MRMKHVRAWIPRPMR	FHPTPCIHIPGEVRN	GFPTELKPGTNQFLT
Coxsackie A16 (CVA16)	Genbank ID: Abx55895.1	YPTFGEHIQANDLDYG	MRIKHVRAWIPRPLR	FHPTPPIHIPGEVRN	GIPTELKPGTNQFLT
Coxsackie B4 (CVB4)	Genbank ID: AAL37156.1	WSNF-----SRDGIYG	FKPKHVKAVVPRPER	FDVTPEMNIPGQVRN	GLPTMLTPGSTQFLT
Coxsackie B6 (CVB6)	Genbank ID: AAD02132.1	WSNF-----SNKGIYG	FKPKHVKAWVPRPER	FDVTPEMNIPGQVNN	GLPVMTTPGSTQFLT
Echo virus13 (Echo13)	Genbank ID: AAQ73076.1	WSNF-----TQNGVYG	MKPKHVKAWIPRPPR	FDVTPEMHIPGEVRN	GLPTMNTPGSTQFLT

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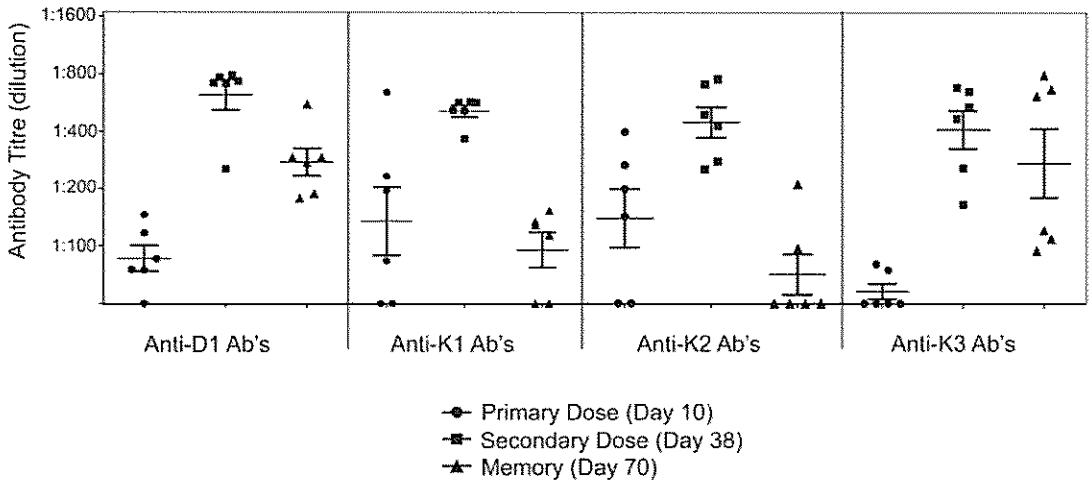

Fig. 1. Induction of anti-peptide specific Abs. Mice were vaccinated sub-cutaneously with the peptide constructs, D1, K1, K2 and K3 containing a helper epitope from Polio virus, on days 0 and 28. Mice were bled 10 days after both inoculations and again 6 weeks after the second dose. The specific Ab titres of individual mice are expressed as the reciprocal of the highest serum dilution that gave a reading above endpoint cut-off [29].

In order to prevent induction of deleterious auto-antibodies, the peptides were checked for sequence similarities with any known human proteins. We found that the four peptides did not share significant sequence similarities with human islet cell antigens, insulin, glutamic acid decarboxylase 65, tyrosine phosphatases, heat shock protein 60/65 or any human proteins for which the sequence is known.

3.2. Immunogenicity of vaccine constructs

Following the *in silico* identification of D1, K1, K2 and K3, we next tested their ability to induce anti-peptide antibodies in mice. Each peptide was synthesized as a linear construct containing a universal T helper epitope by standard F-moc chemistry. These peptide constructs elicited low levels of anti-peptide antibodies after the first inoculation while the second dose generated significant anti-peptide antibody responses from all mice in all groups (Fig. 1). Six weeks after the second vaccine dose (day 70), Ab levels against D1 and K3 were higher than for the K1 and K2 peptides (Fig. 1).

3.3. Functional activity of anti-peptide antibodies

The functional activity of the anti-peptide antibodies was tested using purified IgG from the serum of inoculated mice in an *in vitro* micro neutralization assay. Fig. 2 demonstrates that IgG from serum of naïve mice did not protect cells as no significant neutralization was noted ($p < 0.05$). In contrast, all anti-peptide antibodies were capable of neutralizing EV71 (Fig. 2), resulting in cell viabilities greater than 60% in all samples. With anti-D1, anti-K1 and anti-K2 antibodies, more than 80% of cells were viable. The anti-VP1 specific MAb (MAB979) also neutralized the virus, but to a lesser extent than the anti-peptide Abs, giving approximately 60% cell viability.

Anti-peptide antibodies as shown by neutralization assays against CVA16, CVB4, CVB6 and ECHO13, were found to be cross-reactive (Fig. 3). Assessment of viable cells after three days of incubation with anti-peptide antibodies (10 μg/mL) and 200$TCID_{50}$ of virus showed that CVA16, the virus with the greatest sequence similarity to EV71 (>90%), could be cross-neutralized by all four of the anti-peptide Abs. The anti-D1 and anti-K2 antibodies both resulted in greater than 80% cell viability, with the anti-K1 antibodies showing a similar neutralizing ability. The anti-K3 Ab's also neutralized CVA16, although to a lesser extent, where cell viability was approximately 65%.

For CVB4 and CVB6, with sequence identity of approximately 64% compared to EV71, the degree of neutralization was significantly lower but still evident with multiple anti-peptide Abs. Both the anti-D1 and anti-K1 antibodies cross-neutralized CVB4 virus, where 53% and 60% cell viability was noted respectively. The anti-K2 and anti-K3 Abs did not show any neutralizing activity as less than 50% cell viability was noted in these samples. Similarly, the naïve IgG sample showed no significant neutralizing ability. With CVB6, only the anti-K1 antibodies were capable of cross-neutralizing virus, where almost 70% cell viability was noted after infection with virus. All other anti-peptide antibodies, including the naïve sample demonstrated less than 50% cell viability so were considered non-neutralizing. Samples were considered significant at values of $p < 0.05$ (Fig. 3).

ECHO13 virus has less than 60% protein sequence identity with EV71 and is therefore the most divergent virus included in this study. IgG samples from D1, K2, K3 immunized and the naïve mice did not neutralize ECHO13 virus, with cell viabilities below 50%. Interestingly, the anti-K1 antibodies, generated from the most conserved peptide construct, did cross-neutralize the ECHO13 virus, giving greater than 50% cell viability at the point of analysis.

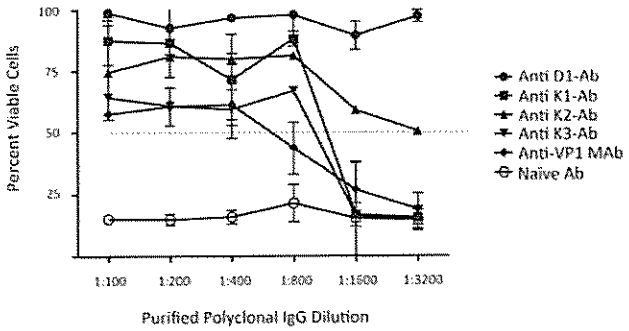

Fig. 2. Ability of anti-peptide antibodies to neutralize EV71 virus *in vitro*. EV71 virus was added to Vero cells after 2 h pre-incubation with serial dilutions (starting concentration: 10 μg/mL) of polyclonal anti-peptide IgG pooled from inoculated mice, or a commercial anti-EV71 MAb directed against VP1. The graph shows the percent of viable cells in wells 3 days after infection. Antibodies that allow more than 50% cell viability after infection were considered to be neutralizing. The experiments were repeated multiple times. Results shown are the mean of triplicates performed in one of these experiments. The results were shown to be statistical significant, $p < 0.05$.

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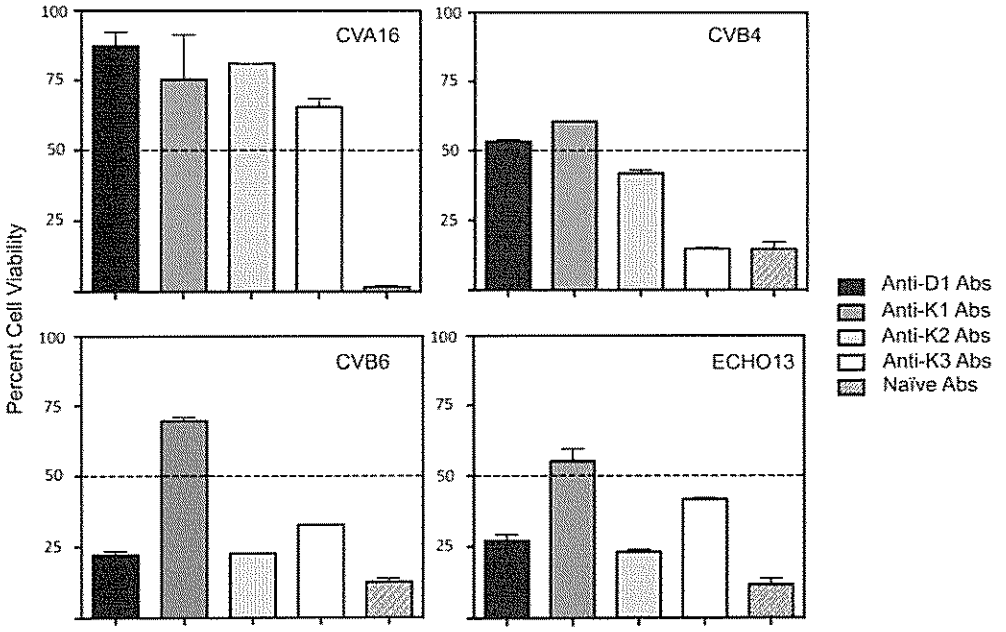

Fig. 3. Ability of anti-peptide antibodies to neutralize other *Picornaviridae in vitro*. 10 μg/mL of polyclonal IgG from naïve and mice inoculated with peptide constructs were added to four different *Picornaviridae*, CVA16, CVB4, CVB6 and ECHO13, these were incubated at 37 °C for 2 h, prior to being adding cell culture wells. Cells were incubated for 3 days at 37 °C with 5% CO_2. Antibodies that allow more than 50% cell viability after infection were considered to be neutralizing. The experiments were performed multiple times. Results shown are the mean of triplicates performed in one of these experiments. The results were shown to be statistical significant, $p < 0.05$.

4. Discussion

Due to the problems associated with traditional vaccines and the fact that some enteroviral infections can induce autoimmune disease, such as Type 1 diabetes, the binding of Enterovirus derived peptides to immune receptors has important biomedical applications in drug discovery and vaccine design. Additionally, the immunogenicity of peptide–protein conjugates (as used by Foo et al. [24] and Liu et al. [32]) contained in traditional vaccines, such as tetanus or diphtheria toxoid, may be poorly immunogenic and cause epitopic suppression; a phenomena due to the cross antigenic competition and/or pre-existing immunity against other protein fragments during previous vaccination or exposure. The construction of our peptide–peptide candidates eliminates such regions that may have the potential to later elicit pathogenic or autoimmune responses [33,34]. Unlike a peptide–protein conjugate, a small peptide only vaccine should incorporate both a B-cell and T-Helper cell epitope in order to facilitate the induction of a strong protective response [35].

Some peptide–protein complexes lack specific T-cell epitopes derived from the pathogen, this can prevent the development of cognate help, which would normally ensure long term immunity. In contrast, the peptide–peptide constructs used here may offer cognate help as the T-cell epitope was also from the same family of viruses, *Picornarviridae*. As such, synthetic peptides and peptide–peptide based vaccines allow for a reductionist approach to vaccine design, whereby deleterious sequences can be eliminated and a vaccine can consist of the minimal proportion of a pathogen required to induce an effective and efficient immune response.

In this study we used an *in silico* approach to identify Ab epitope candidates for EV71. These epitopes were all screened to confirm a lack of sequence similarity to human proteins associated with autoimmune disease such as Type I diabetes. Autoantibodies to various human proteins have been linked to the onset of Type I diabetes [36]. Several studies have shown a correlation between Enterovirus infection and the incidence of Type I diabetes [34–38]. When identifying the potential Ab epitopes, we made certain that no peptides were included that share sequence homology with human proteins which could induce a deleterious response.

The epitopes identified were from the VP1 protein (K1 and D1) or the VP3 protein (K2 and K3) of the EV71 capsomere. Previous studies have shown that the VP1 protein is immunogenic and can induce neutralizing antibodies [24]. All of the additional target epitopes, identified using the *in silico* approach induced virus neutralizing anti-peptide antibodies in inoculated mice.

The vaccine candidates all induced high titre specific anti-peptide antibodies after two 10 μg doses. Earlier studies which induced anti-EV71 antibodies in mice [39–42] administered doses upwards of 50 μg. Furthermore, in our study these titres were maintained in the mice 6 weeks after the second dose, indicating that the response was long lived, which is desirable in a vaccine candidate. Antibodies against all four peptide constructs demonstrated high neutralizing activity *in vitro* against EV71, the virus from which the epitopes were found. This further demonstrates the validity of this structure and sequence-based *in silico* identification method.

CVA16 is an Enterovirus A, closely related to EV71 sharing 75% nucleotide and 88% sequence identity [37]. The anti-D1 and K1 antibodies, demonstrated cross-reactive neutralizing activity to other Group-A and Group-B EV's such as CVA16. The anti-K1 antibodies also neutralized ECHO13, another member of the *Picornaviridae* family. These results indicate that the antibodies generated are not only peptide-specific and EV71-specific, but they are also cross-reactively neutralizing. The ability of the K1 and D1 anti-peptide Abs to neutralize multiple viruses may be due to the high degree of sequence similarity between the VP1 protein of most *Picornaviridae* and because VP1 has been reported to be immunodominant in generating neutralizing responses [38]. Additionally, some structural reasons could account for this; the K1 and D1 peptides appear to be in a closer spatial proximity on the capsid protein thus allowing for a greater induction of a conformational fit with the antigen.

The constant evolution of viral serotypes and sub-species is an important reason for investigating conserved synthetic

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peptide based vaccines. The large amount of structural and sequence variation between viruses poses challenges in the development of traditional attenuated, inactivated or sub-unit vaccinations.

This challenge was illustrated with the K2 and K3 peptide constructs from VP3, which has been reported as an antigenic region of the virus [39], although these induced responses which neutralized Group A Enteroviruses, they did not cross-neutralize the Group B viruses. This could be attributed to the VP3 region of EV71 being less conserved and having greater structural differences than VP1. Both VP3 peptides, K2 and K3, vary by more than 7 amino acids when compared with viruses outside of the Enterovirus A group, indicating that these sequences are not conserved across multiple genotypes of virus (Table 2).

In this study we have identified B-cell epitopes from the EV71 capsid protein, that when incorporated into a synthetic peptide-based vaccine construct elicit antibodies with the ability to neutralize EV71. Furthermore, the anti-peptide antibodies elicited can cross neutralize other viruses from the Enterovirus group A. The K1 epitope was the most conserved across viral species and located in the immunodominant VP1. The K1 anti-peptide antibodies could neutralize several of the species of *Picornaviridae*. These peptide epitopes represent potential new vaccine candidates not only for EV71, but multiple members of the *Picornaviridae* family, which are important human pathogens.

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