IDENTIFICATION OF MIMOTOPES OF HUMAN ENTEROVIRUS 71

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DEDICATION

To my family and friends for their constant support.
ACKNOWLEDGEMENTS

My deepest gratitude and thanks to my supervisor, Professor Dr. Mary Jane Cardose, whose guidance, support and encouragement has helped me to complete my MSc. Thank you Phaik Hooi, Dr. Sim and Dr. David for all the valuable assistance and advice. To all my friends at IHCM, thank you for your help, useful discussion and friendship.
ABSTRACT

A random phage-displayed peptide library was used to screen a raised rabbit serum antibodies against complete recombinant viral protein (VP)1 of B4 strain of human enterovirus (HEV)71 (R410 serum) for mimotopes of VP1 protein of HEV71. One hundred and fifteen different peptide sequences were identified from sequencing 133 phage plaques isolated from four rounds of R410 panning. Of the 115 different peptide sequences identified, 74 different peptide sequences were classified into eight different motif groups (I to VIII). The largest group, group I consisted of peptide sequences with the D/ERPA/D/E motif.

Two peptide sequences from two different motif groups were then chosen for synthesis. The chosen peptide sequences were KLERPAD of group I with the D/ERPA/D/E motif and PQFLSKH of group VII with the QFXSXH motif. Both were synthesised in linear and constrained forms. However, only constrained-KLERPAD peptide was reactive with raised rabbit sera antibodies against complete VP1 recombinant protein of different strains of HEV71. It was also found to mimic an epitope of VP1 protein of B4 strain of HEV71 when superimposed on constructed predictive VP1 structures of B4 strain of HEV71 B4.

These findings show the potential of phage technology in identifying important mimotopes of HEV71 to facilitate the development of diagnostic assays and drugs- or vaccines-based HEV71 mimotopes.
ABSTRAK

Kajian ini telah dijalankan untuk mengenalpasti mimotop yang menyamari epitop pada protein kapsid (VP)1 daripada human enterovirus (HEV)71 dengan menyaring anti-serum daripada arnab terhadap protein rekombinan VP1 daripada strain B4 HEV71 (serum R410) dengan perpustakaan faj yang mempersembahkan peptida rawak. Seratus lima belas jenis jujuan peptida telah diperolehi daripada penjujuan 133 plak faj yang telah dipencilkkan daripada empat pusingan penyaringan. Tujuh puluh empat jenis jujuan peptida daripada jumlah jujuan peptida yang diperolehi telah dikelaskan ke dalam lapan kumpulan (I ke VIII). Kumpulan I dengan jujuan peptida yang bermotif D<sub>E</sub>RPA<sub>D/E</sub> merupakan kumpulan dengan paling banyak jenis jujuan peptida di dalamnya.

Dua jenis jujuan peptida, KLERPAD dan PQFLSKH dalam bentuk linear dan terangkai juga telah disintesiskan. KLERPAD telah dipilih daripada kumpulan I dengan jujuan peptida yang bermotif D<sub>E</sub>RPA<sub>D/E</sub> manakala PQFLSKH telah dipilih daripada kumpulan VII dengan jujuan peptida yang bermotif QF<sub>X</sub>SX<sub>H</sub>. Namun demikian, hanya KLERPAD yang terangkai yang bertindak balas dengan anti-serum daripada arnab terhadap protein rekombinan VP1 daripada strain HEV71 yang berlainan. Selain daripada itu, peptida sintetik ini juga didapati menyamari salah satu epitop pada protein VP1 daripada strain B4 HEV71 apabila dilokasikan pada model struktur protein VP1 daripada strain B4 HEV71.

Keputusan-keputusan yang diperolehi daripada kajian ini menunjukkan potensi teknologi faj dalam mengenalpasti mimotop penting daripada HEV71 dalam usaha untuk mencari kaedah terbaik mendiagnoskan jangkitan HEV71 mahupun penawar ataupun vaksin terhadap HEV71.
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ABBREVIATIONS

BEV  bovine enterovirus
BGM  buffalo green monkey kidney cells
BSA  bovine serum albumin
BEV  bovine enterovirus
CAV  coxsackievirus A
CBV  coxsackievirus B
cDNA complementary DNA
CMC  carboxymethyl-cellulose
CNS  central nervous system
CO₂  carbon dioxide
C-PBS casein-PBS
CPE  cytopathic effect
CSF  cerebrospinal fluid
DMEM Dulbecco’s Modified Eagle Medium
DNA  deoxyribonucleic acid
E. coli  Escherichia coli
EDTA ethylene diamine tetraacetate
ELISA enzyme-linked immunosorbant assay
FBS  foetal bovine serum
FCA  Freund complete adjuvant
FIA  Freund incomplete adjuvant
HCl  hydrochloric acid
HEV  Human enterovirus
HFMD hand, foot and mouth disease
HRP  horseradish peroxidase
HPLC high performance liquid chromatography
H₂SO₄  sulphuric acid
IFA  immunofluorescence assay
IgG  immunoglobulin G
IgM  immunoglobulin M
IMR  Institute for Medical Research
IPTG isopropyl-β-D-thiogalactosidase
kb  kilobase
kDa  kiloDalton
LB  Luria-Bertani
M protein membrane protein
Mab5-D8/1 mouse monoclonal antibody clone 5-D8/1
MK  Rhesus monkey kidney
MNS  mouse negative serum
MPS  mouse anti-HEV71 VP1 serum
MRC-5  human diploid fibroblast cells
ND  not done
NaCl  sodium chloride
NaOH  sodium hydroxide
NPEV  non-poliovirus enterovirus
UTR  untranslated region
OD  optical density
OPD  o-phenylenediamine
PAGE polyacrylamide gel electrophoresis
PBS  phosphate buffer saline
PCR  polymerase chain reaction
PEG  polyethylene glycol
pre-R410  pre-immunisation blood collected from rabbit selected for immunisation with HEV71 B4 recombinant protein
pfu  plaque forming unit
PV  Poliovirus
R410  rabbit anti-HEV71 B4 VP1 recombinant protein serum
R401  rabbit anti-HEV71 B3 VP1 recombinant protein serum
R402  rabbit anti-N-terminal HEV71 B3 VP1 recombinant protein serum
R403  rabbit anti-C-terminal HEV71 B3 VP1 recombinant protein serum
R404  pooled pre-immunisation blood collected from rabbits selected for immunisation with HEV71 B3 recombinant proteins
RD  rhabdomyosarcoma cells
RNA  ribonucleic acid
RO  reverse osmosis
rpm  rotation per minute
RT-PCR  reverse transcription-polymerase chain reaction
S protein  spike glycoprotein
SARS-CoV  Severe Acute Respiratory Syndrome coronavirus
SM  (non-fat) skimmed milk
SDS  sodium dodecyl sulphate
TBS  Tris-buffered saline
TBST  TBS-Tween-20
TMTC  too many to count
UHQ  ultra high quality
Unimas  Universiti Malaysia Sarawak
VP  viral coat protein
v/v  volume/volume
w/v  weight/volume
CHAPTER 1: INTRODUCTION

1.1. Picornaviruses

Picornaviruses are small, non-enveloped viruses with a single positive strand RNA genome. They are divided into nine genera: *Rhinovirus, Enterovirus, Aphthovirus, Cardiovirus, Hepatovirus, Parechovirus, Teschovirus, Erbovirus* and *Kobuvirus* (Mayo & Pringle, 1998; Institute of Animal Health, 2002). Each genus is then divided into species which consist of different virus serotypes. The picornaviruses are originally classified into their respective genera based on their physical and antigenic properties such as particle density, pH sensitivity and by neutralisation with specific anti-sera (Committee on Enteroviruses, 1962; Melnick, 1997). Recently, they are classified based on their molecular properties because some picornaviruses are misidentified or untypable when they were classified based on their antigenic properties (Muir *et al.*, 1998; Oberste *et al.*, 1999a & b; Kubo *et al.*, 2002; Brown *et al.*, 2003; Bendig & Earl, 2005).

1.1.1. Human enteroviruses

Enteroviruses are so-named because they are usually found inhabiting the gastrointestinal tract of their hosts (Rueckert, 1996). They also cause the greatest hazards to human health among all known picornaviruses (Shimizu *et al.*, 1999; Chaves *et al.*, 2001). To date, there are 74 serotypes of human enteroviruses identified and they are classified into five species, namely *Poliovirus* (PV), *Human enterovirus A* (HEV-A), *Human enterovirus B* (HEV-B),
Human enterovirus C (HEV-C) and Human enterovirus D (HEV-D) (King et al., 2000; Norder et al., 2003; Oberste et al. 2005) (Table 1.1).

1.2. Properties of enteroviruses

Like all piconarviruses, enteroviruses are non-enveloped spherical ribonucleotide acid (RNA) viruses with sizes ranging from 22 to 30 nm in diameter. PVs, the most studied enteroviruses, were the first in having their atomic structures characterised by x-ray crystallography. Atomic structures of coxsackievirus A (CAV) 9 (Hendry et al., 1999), CAV21 (Xiao et al., 2001), coxsackievirus B (CBV) 3 (Muckelbauer et al., 1995), bovine enterovirus (BEV) (Smyth et al., 1995) were also characterised using the same method. Each of the enteroviruses has a buoyant density of 1.34 g/cm³ in cesium chloride and carries a single-stranded, positive sense RNA genome (Melnick, 1997). Their icosahedral capsids are constructed from 60 identical blocks or protomers (Figure 1.1). A protomer consists of four types of structural proteins, namely viral protein 1 (VP1), viral protein 2 (VP2), viral protein 3 (VP3) and viral protein 4 (VP4) (Rueckert, 1996).

The viruses can be inactivated by exposure to ultraviolet light, formalin, chlorine solution and temperature above 50°C (Rueckert, 1996; Nuanualsuwan & Cliver, 2003). However, they are stable against treatments with detergents and organic solvents. Enteroviruses are also able to withstand extreme acidic environments. This acid stability property enables enteroviruses to replicate in their native habitat, the gastrointestinal tract (Rueckert, 1996). The viruses remained viable when left for days at room temperature in moist conditions but
they become highly viable at any temperature once stabilised by magnesium chloride (Melnick, 1997).

1.2.1. Genome organisation

The single-stranded positive sense RNA genome of enteroviruses consists of approximately 7.5 kilobases (kb) as shown in Figure 1.2. The genome has a single open reading frame and can directly be translated once it enters a host cell.

The 3' and 5' ends of the viral RNA contain untranslated regions (UTRs). The two UTRs are conserved in all enteroviruses and contain elements important for RNA replication and translation. The 3' end of the genome carries a poly-A sequence while a small viral protein called VPg protein is attached to the 5' end of the genome (Andino et al., 1999).

The protein-coding region is organised into P1, P2 and P3 regions. The P1 region encodes four types of viral structural proteins (VP1 to VP4), which make up an enterovirus capsid. The nucleotide sequences in P1 coding region are highly variable because the viral capsid is constantly subjected to pressure caused by host antibodies. Meanwhile, the P2 and P3 regions contain genes that encode seven types of non-structural proteins and several forms of intermediate cleavage products. The products formed from these regions act as important viral enzymes required in the propagation of enteroviruses. Therefore, the nucleotide sequences in P2 and P3 coding regions are more conserved than those in P1 coding region (Rueckert, 1996).
Table 1.1. Classification of 74 serotypes of human enteroviruses.

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</tr>
<tr>
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<td>Coxsackievirus A9, Coxsackievirus B1-6, Echovirus 1-9, 11-21, 24-27, 29-33, Enterovirus 69, 73*, 74*, 75*, 77*, 78*</td>
</tr>
<tr>
<td>HEV-C (11 serotypes)</td>
<td>Coxsackievirus A1, 11, 13, 15, 17-22, 24</td>
</tr>
<tr>
<td>HEV-D (2 serotypes)</td>
<td>Enterovirus 68, 70</td>
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# Recently identified HEV-A (Oberste et al., 2005).
*Recently identified HEV-B (Norder et al., 2003).
This classification was modified according to King et al. (2000), Norder et al. (2003) and Oberste et al. (2005).
Figure 1.1. The structural organisation of an enterovirus. (A) A protomer is formed by the proteolytic cleavage products of the P1 polyprotein to capsid proteins VP1 to VP4. The surface of the virion is formed by VP1, VP2 and VP3, while VP4 is found internally. (B) Five protomers assemble into a pentamer. The fivefold axis of the pentamer is shown. (C) An icosahedral capsid is formed by twelve pentamers. One of the twenty threefold axes is marked. (Reproduced from Rueckert, 1996).
Figure 1.2. Organisation and expression of an enterovirus genome. The top box represents the enterovirus genome organisation: VPg protein, 5'-UTR, coding region for structural and non-structural proteins, 3'-UTR and a poly-A tail. The box below indicates the end products obtained from cleavage of P1, P2 and P3 precursor proteins by viral-coded proteinases. (Adapted from Reuckert, 1996 and Andino et al., 1999).
1.2.2. Viral proteins

1.2.2.1. Non-structural proteins

Non-structural proteins and their intermediates coded from P2 and P3 regions of enterovirus RNA genome take part in enterovirus RNA replication and protein processing. The P2 region encodes proteins 2A, 2B and 2C while the P3 region encodes proteins 3A, 3B, 3C and 3D. Uncompleted cleavage of both regions forms functional intermediates known as proteins 2BC, 3AB and 3CD (Rueckert, 1996; Andino et al., 1999).

Protein 2A cleaves translated long polypeptide between VP1 and itself to release capsid protein precursor P1 from the rest of the polyproteins (Toyoda et al., 1986). Proteins 2B, 3A and their respective precursor proteins (intermediates 2BC and 3AB) increase host cell plasma membrane and organelles membranes permeability to create the required environment for viral RNA replication (Lama & Carrasco, 1992; Aldabe et al., 1996; Towner et al., 1996; Dodd et al., 2001; de Jong et al., 2002 & 2004). Meanwhile, protein 2C is a helicase (Klein et al., 2000) involved in the encapsidation of viral RNA (Vance et al., 1997). Protein 3B or Vpg protein initiates RNA synthesis by protein 3D which is also known as viral RNA-dependent RNA polymerase (Paul et al., 2003). Two important proteinases coded from P3 region are protein 3C and the intermediate 3CD. They are mostly involved in the cleavage of viral proteins (Ansardi & Morrow, 1995; Parsley et al., 1999; Patick et al., 1999).
1.2.2.2. Structural proteins

The capsid protein precursor P1 is cleaved into individual capsid proteins, VP1 to VP4 by enzymes coded from P2 and P3 regions. VP1, VP2 and VP3 capsid proteins are the main structural components of an enterovirus and each of them consists of approximately 240 to 290 amino acids residues folded into eight-stranded anti-parallel β-sheets with a ‘jelly-roll’ topology to form the outer surface capsid. Meanwhile, VP4, which is smaller in size, about 70 amino acids residues, is located in the inner surface of the VP1, VP2 and VP3 (Symth & Martin, 2002). Of the four types of viral structural proteins, VP1 plays the major role in determining the antigenicity of enteroviruses.

An enterovirus infection may become severe or enteroviruses may become more virulent when changes occurred to certain antigenic sites. This is because changes in the antigenic sites alter the mechanisms of immune system in hosts towards enteroviruses (Ramsingh et al., 1997; Halim & Ramsingh, 2000). Variation of amino acids in the VP1 protein within or close to one of the important β-sheets, the BC loop is observed in different enterovirus serotypes (Norder et al., 2003). This may explained why the gene that expresses VP1 has the most variable nucleotide sequences compared to the other viral structural proteins (Mulder et al., 2000; Oberste et al., 1999a,b & 2000). Monoclonal antibodies against enterovirus capsid proteins (Emini et al., 1982; Blondel et al., 1983) or synthetic peptides based on certain regions of VP1 (Hovi & Roivanen, 1993; Airaksinen et al., 2001; Shin et al., 2003) have been generated to identify the epitopes of VP1. Some of these epitopes are conserved among enteroviruses and can be used for diagnosing enterovirus infections. Mouse monoclonal antibody clone 5-D8/1 (Mab5-D8/1) from DAKO A/S, Copenhagen, Denmark is an
example of a commercially available monoclonal antibody derived from a conserved epitope (mapped from residues 40 to 48) of the VP1 of a majority of enterovirus serotypes (Samuelson et al., 1995). Mab5-D8/1 cross-reacts with most of enterovirus serotypes and is useful for rapid diagnosis of enterovirus infections (Trabelsi et al., 1995; Zhang et al., 2000).

In addition, VP1 is also involved in viral attachment to host cell receptor and uncoating during infection (Fricks & Holge, 1990; Couderc et al., 1996; Ward et al., 1999; Airaksinen et al., 2001). Mapping of epitopes of the capsid proteins of enteroviruses especially VP1 is crucial in the development of vaccines against enterovirus infections. A vaccine is considered to be effective when it induces host humoral immune response to produce neutralising antibodies (Herremans et al., 2000). Thus, sequences obtained from the mapping of epitopes of VP1 can be used in developing drugs to treat enterovirus infections. The potential drugs should inhibit viral uncoating and/or attachment to the receptors of host cell (Barnard et al., 2004).

1.3. Enterovirus infections

1.3.1. Transmission and pathogenesis

Enteroviruses enter the human body via the oral and respiratory routes in the form of eye, nose or throat discharges and fluid from blisters or stool of infected persons. Once inside the host, they replicate in the gastrointestinal and upper respiratory tracts. In some cases, viremia may occur on the third day after the infection (Melnick, 1997). Enteroviruses then spread during the viremic phase to secondary target organs such as the central nervous system (CNS),
heart and muscles for further replication. Most complications arise from the infection of these target organs (Alexander et al., 1994; Kandolf et al., 1999; Chaves et al. 2001).

1.3.2. Clinical manifestations of enterovirus infections

Infection with human enteroviruses are usually asymptomatic, but they can cause a wide spectrum of clinical manifestations, which range from conjunctivitis, hand, foot and mouth disease (HFMD), herpangina, encephalitis, myocarditis, neonatal systemic enteroviral disease and paralytic poliomyelitis. Certain clinical manifestations are associated with specific enterovirus serotypes. HFMD, for example is usually caused by infection with CAV16 and HEV71 (Melnick, 1997). Meanwhile, CBV is well recognised as the causative agent of myocarditis (Gauntt et al., 1995; Kandolf et al., 1999). Enterovirus-associated diseases occur throughout the year in the tropics but in countries with temperate climates, enteroviruses are associated with epidemics during the summer.

1.4. Diagnosis of enterovirus infections

There are three main methods to detect an enterovirus infection. Traditional diagnosis of enterovirus infection is based on the detection of viable viruses or anti-enterovirus antibodies. When no result is obtained from these approaches, molecular techniques are used to detect the presence of viral genome.
1.4.1. Detection of viable viruses

The classical laboratory approach to diagnose enterovirus infection is to first isolate the virus and then identify its serotype by neutralisation assay. Enteroviruses can be isolated from cerebrospinal fluid (CSF), vesicular fluid, serum, urine, faeces and throat or rectal specimens taken from patients. The viruses are identified by the cytopathic effect (CPE) shown by cells that they are grown in. Buffalo green monkey kidney (BGM) cells, human diploid fibroblast (MRC-5) cells, primary Rhesus monkey kidney (MK) cells, rhabdomyosarcoma (RD) cells and Vero cells are the commonly used cell lines for the isolation of enteroviruses (Hamparian et al., 1985; Chonmaitree et al., 1988; Ho et al., 1999).

A few cell lines are usually used at the same time to increase isolation speed and sensitivity. This is because the infectivity of enteroviruses varies in different cell lines. The RD cell line is especially sensitive for the isolation of echoviruses (Hamparian et al., 1985) although most enteroviruses can be isolated from the same cell line (Pe'rez-Ruiz et al., 2003). Meanwhile, BGM cell line is more sensitive for isolating CBVs (Menegus & Hollick, 1982). Some enteroviruses such as CAVs do not grow in cell cultures. CAVs are more successfully isolated if they are infected into suckling mice (Lipson et al., 1988). Therefore, the practice of using several cell lines does increase the sensitivity in isolating enteroviruses but it requires massive labour and cost (Chonmaitree et al., 1988). Sometimes, certain virus cultures need to be sub-passaged due to low initial titer of virus inoculated or to rule out false CPE. The condition and quality of collected clinical specimens also affect the efficiency in isolating enteroviruses (Melnick, 1997).

Detection of enteroviruses by cell culture usually takes a longer period of time than other diagnosis methods. Confirmation of enterovirus infections (or