Development of a reverse genetic system for Human enterovirus 71 (HEV71) and the molecular basis of its growth phenotype and adaptation to mice

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DVM, MVSc

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University. 2009

DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

………………………………..

Patchara Phuektes

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／# ABSTRACT

Human enterovirus 71 (HEV71) is a member of the Human Enterovirus A species within the Family *Picornaviridae*. Since 1997, HEV71 has emerged as a major cause of epidemics of hand, foot and mouth disease (HFMD) associated with severe neurological disease in the Asia-Pacific region. At the present time, little is known about the pathogenesis of acute neurological disease caused by HEV71. The major aim of this study was to generate infectious cDNA clones of HEV71 and use them as tools for investigating the biology of HEV71 and molecular genetics of HEV71 virulence and pathogenesis.

Two infectious cDNA clones of HEV71 clinical isolates, 26M (genotype B3) and 6F (genotype C2) were successfully constructed using a low copy number plasmid vector and an appropriate bacterial host. Transfection of cDNA clones or RNA transcripts derived from these clones produced infectious viruses. Phenotypic characterisation of clone-derived viruses (CDV-26M and CDV-6F) was performed, and CDV-26M and CDV-6F were found to have indistinguishable phenotypes compared to their wild type viruses.

Strains HEV71-26M and HEV71-6F were found to have distinct cell culture growth phenotypes. To identify the genome regions responsible for the growth phenotypes of the two strains a series of chimeric viruses were constructed by exchanging the 5′ untranslated region (5′ UTR), structural protein (P1), and nonstructural protein (P2 and P3) gene regions using infectious cDNA clones of both virus strains. Analysis of reciprocal virus chimeras revealed that the 5′ UTR of both strains were compatible but not responsible for the observed phenotypes. Both the P1 and P2-P3 genome

regions influence the HEV71 growth phenotype in cell culture, phenotype expression is dependent on specific P1/P2-P3 combinations and is not reciprocal.

In the previous study, in order to investigate the pathogenesis of HEV71 infection, a mouse HEV71 model was developed using a mouse-adapted variant of HEV71-26M. Mouse-adapted strain MP-26M caused fore- and/or hindlimb paralysis in mice, whereas HEV71-26M-infected mice did not develop clinical signs of infection at any virus dose or route of inoculation tested. In this study, the molecular basis of mouse adaptation by HEV71 was identified. Nucleotide sequence analysis of HEV71-26M and MP-26M revealed three point mutations in the open reading frame, each resulting in an amino acid substitution in the VP1, VP2 and 2C proteins; no mutations were identified in the untranslated regions of the genome. To determine which of the three amino acid mutations were responsible for the adaptation and virulence of HEV71-26M in mice, recombinant cDNA clones containing one, or a combination of two or three mutations, were constructed. Mouse virulence assays of the mutated viruses clearly demonstrated that a non-conservative amino acid substitution ($G^{710} \rightarrow E$) in the capsid protein VP1 alone was sufficient to confer the mouse virulence phenotype on HEV71.

In addition, a mouse oral infection model was established in this study. Oral inoculation with the mouse-adapted HEV71 virus, MP-26M, induced fore-or hindlimb paralysis in newborn mice in an age- and dose-dependent manner. As oral transmission is the natural route of HEV71 infection, this murine HEV71 oral infection model will provide a suitable tool for studying HEV71 pathogenesis, for

defining neurological determinants, and for testing vaccine efficacy and immunogenicity in the future.

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ABBREVIATIONS

β-gal	betagalactosidase
μF	microfarad
μg	microgram
μL	microlitre
μM	micromolar
μm	micrometre
Ω	ohm
%	percent
ºC	degree Celsius
3′ UTR	3′ untranslated region
5′ UTR	5′ untranslated region
xg	acceleration due to gravity
aa	amino acid
A	adenine
AFP	acute flaccid virus
AMV	avian myeloblastosis virus
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pairs
BEV	bovine enterovirus
BGS	bovine growth serum
BSA	bovine serum albumin
C	cytosine
CAV	Coxsackievirus A
CBV	Coxsackievirus B
cDNA	complementary deoxyribonucleic acid
CDV	clone-derived virus
CHO	Chinese hamster ovarian cells
cm	centimetre
CNS	central nervous system
CMV	cytomegalovirus
COS-7	SV40 transformed African green monkey kidney cells

CPE	cytopathic effects
CSF	cerebrospinal fluid
dH_2O	distilled water
ddH_2O	double deionised water
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	Dithiothreitol
E	echovirus
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
eIF	eukaryotic initiation factor
EMCV	encephalomyocarditis virus
et al.	and others
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMDV	foot-and-mouth disease virus
g	gram
G	guanine
h	hour
HBSS	Hank's balance salt solution
HD_{50}	median humane end point
HEV	human enterovirus
HEV71	human enterovirus 71
HEV-A	human enterovirus group A
HEV-B	human enterovirus group B
HEV-C	human enterovirus group C
HEV-D	human enterovirus group D
HFMD	hand, foot and mouth disease
hnRNP C	heterogeneous nuclear ribonucleoprotein C
HRV	human rhinovirus
i.c.	intracerebral
IFA	immunofluorescence assay

IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
IRES	internal ribosome entry site
i.s.	intraspinal
i.v.	intravenous
IVT	*in vitro* transcription
kb	kilobase pairs
kDa	kiloDalton
kg	kilogram
kV	kilovolt
L	litre
LD_{50}	median lethal dose
M	molar
mAb	monoclonal antibody
mg	milligram
min	minute
mL	millilitre
mM	millimolar
MOI	multiplicity of infection
MOPS	3-N-morpholinopropane sulfonate
mRNA	messenger ribonucleic acid
MVE	Murray Valley encephalitis virus
NB41A3	mouse neuroblastoma cells
ND	not determined
ng	nanogram
NK	natural killer cells
nm	nanometre
nt	nucleotide
NTPase	nucleoside triphosphate hydrolase
OD	optical density
ORF	open reading frame

pAb	polyclonal antibody
PABP	poly (A) binding protein
PBS	phosphate buffered saline
PCBP	poly (C) binding protein
PCR	polymerase chain reaction
PDB	protein databank file
PE	pulmonary edema
PEG	polyethylene glycol
PFU	plaque forming units
pmol	picomole
PTB	polypyrimidine tract-binding protein
PV	poliovirus
PVR	poliovirus receptor
Poly (A)	polyadenylated
RD	rhabdomyosarcoma cells
RE	restriction endonuclease
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
RT	room temperature
SAM	S-adenosylmethionine
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
s	second
T	Thymine
TAE	Tris acetate EDTA buffer
$TCID_{50}$	median tissue culture infectious dose
ts	temperature-sensitive
tr	temperature-resistant
U	Uracil
U	unit
USA	United States of America
UV	ultraviolet

Vero	African green monkey kidney cells
VP	virus protein
VPg	viral protein genome-linked
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside