INVESTIGATIONS INTO THE UTILITY OF REAL-TIME PCR FOR THE DETECTION, QUANTITATION AND CHARACTERISATION OF CLINICALLY RELEVANT VIRUSES.

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DECLARATION

Except where due reference is cited in the text, this thesis is the original work of the author. No part of the original studies from this thesis has been submitted, in any form, to this or any other University.

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ABSTRACT

The use of PCR as a tool for the diagnostic virology and viral research laboratories has greatly increased in recent years, however the use of conventional PCR and amplicon detection systems can be a complex and relatively slow process that increases the risk of amplicon carry-over contamination. Many conventional PCR systems are unsuited for, or unable to perform as accurate diagnostic and quantitative tools because viruses are present in such a diverse variety of patient tissues and in a broad range of concentrations. Traditional viral culture, while still the gold standard for the detection of many viruses, is lengthy, expensive and often subjective. In addition, successful isolation of infectious virus is variable and dependent upon appropriate cell lines, lengthy incubations and careful transport and storage of clinical specimens. Many of the disadvantages arising from the use of traditional assays for the detection of viruses have been overcome by the development of real-time PCR. The technology has continued to develop due to the introduction of several commercial thermal cycling platforms and the appearance of numerous specific and non-specific fluorogenic chemistries.

For the purpose of this thesis, human virology was sectioned into three diagnostic divisions containing the synthetic viruses, the well characterised viruses and the new or emerging viruses. This thesis

proposes the hypothesis that real-time PCR could greatly improve upon traditional techniques for the detection, quantitation and characterisation of the members of these three divisions in both research and diagnostic environments.

Conventional competitive quantitative PCR assays and a non-oligoprobe real-time PCR assay were constructed to detect novel synthetic gene therapy vectors developed from retroviruses. When compared to oligoprobe-based real-time PCR, it was clear that conventional molecular assays, whilst improving upon traditional methods of viral culture and immunofluorescence, were slower, more complex, less versatile and were hindered by a limited dynamic range. Synthetic control templates were developed and an improved method of assaying these template preparations was devised. The controls were used to precisely optimise each assay, create quality assurance reagents and to construct external standard curves permitting the absolute quantitation of viral templates.

Real-time PCR achieved several significant goals during the studies performed for this thesis. The new assays detected human enterovirus (HEV) and the emerging pathogen, human metapneumovirus (hMPV) which were both responsible for seasonal outbreaks of serious disease that would otherwise have gone undiagnosed. These data led to the first description of hMPV outside

of the Netherlands, as well as the first description of two validated rapid diagnostic RT-PCR assays which permitted the definitive classification of hMPV as a global pathogen of children and adults. Building upon its detection, an extensive molecular epidemiological study permitted the description of subtle differences between Australian and the more recently described international hMPV strains resulting in the classification of two distinct types of hMPV (A and B) and within these, four subtypes (A1, A2, B1 and B2).

Real-time PCR rapidly detected, quantitated and genotyped herpes simplex viruses in a single reaction and determined the successful delivery of human and non-human genes by novel retroviral vectors in less time than any other phenotype detection assay. Additionally, these studies produced quantitative data which permitted the rapid calculation of transduction efficiency. Real-time PCR was able to quickly assess the efficiency of the PCR either in response to the titration of individual reaction components or as a result of amplification modifiers present within specimen extracts. The use of nucleotide sequencing studies ideally complemented earlier diagnostic studies of HEV and permitted the discrimination of pathogenic enterovirus 71.

This thesis demonstrated that real-time PCR is more able to accommodate the demanding aspects of viral research and

diagnostics than any other single method, and is now in a position to replace many of the traditional techniques still used by laboratories unfamiliar with the benefits of real-time PCR. The assays, techniques, reagents and publications resulting from these studies have benefited several areas of viral research and diagnostics and have improved the understanding of the role of real-time PCR in virology and of the technique in general, among the greater scientific community whilst successfully addressing the proposed hypothesis.

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ABBREVIATIONS

aa	Amino acid
APV	Avian pneumovirus
AR	Analytical reagent grade
BHQ	Black hole quencher
bRSV	Bovine respiratory syncytial virus
CCD	Charge-coupled device
Cftr (CFTR)	Cystic fibrosis transmembrane conductance regulator gene (protein)
C_T	Threshold cycle
C_P	Crossing point
CVRU	Clinical Virology Research Unit
DABCYL	4-[4'-dimethylamino-phenylazo]-benzene
DIG	Digoxigenin
dNTP	deoxyribonucleotides
dsDNA	Double-stranded DNA
ELAHA	Enzyme linked amplicon hybridisation assay
ERV	Endogenous retrovirus
EV71	Enterovirus 71
FAM	6-carboxy-fluorescein
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FMCA	Fluorescence melting curve analysis.
FRET	Fluorescent resonance energy transfer
Gfp (GFP)	Green fluorescent protein gene (protein)
HEV	Human enteroviruses
hMPV	Human metapneumovirus
hRSV	Human respiratory syncytial virus
IC	Internal control
LB	Luria Bertani medium
LTR	Long terminal repeat
LUX	Light-up upon extension
MGB	Minor groove binding molecule
MHC	Major histocompatibility complex
MOI	Moiety of infection.
MoMLV	Moloney murine leukaemia virus

MQAE	*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
mRNA	Messenger RNA
MSV	Murine Sarcoma Virus
NDV	Newcastle disease virus
NFQ	Non-fluorescent quencher
NTR	Non-translated region
ORF	Open reading frame
bRSV	Bovine respiratory syncytial virus
oRSV	Ovine respiratory syncytial virus
PCR	Polymerase chain reaction
PIV	Parainfluenza virus
PNA	Peptide nucleic acid
Poly(A)	Polyadenylated
PVM	Pneumonia virus of mice
qcPCR	Quantitative competitive PCR
QHPS	Queensland Health Pathology Service
RCH	Royal Children's Hospital
RCR	Replication competent retrovirus
rRNA	Ribosomal RNA
ROX	6-carboxy-N,N,N',N'-tetramethylrhodamine
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
SASVRC	Sir Albert Sakzewski Virus Research Centre
SNP	Single nucleotide polymorphisms
SV	Simian virus
TAMRA	6-carboxy-tetramethyl-rhodamine
Taq	*Thermus aquaticus*
$TCID_{50}$	50% Tissue culture infective dose
T_D	Denaturation temperature
T_M	Melting temperature
TMB	Tetramethylbenzidine
TRTV	Turkey rhinotracheitis virus
TV	Trypsin-versene solution
UTR	Untranslated region
VSV	Vesicular stomatitis virus

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