INVESTIGATION OF THE INFECTIOUS CYCLE OF MOLLUSCUM CONTAGIOSUM VIRUS IN HUMAN SKIN AND THE NATURE OF MCV INDUCED IMMUNITY

A thesis submitted in candidature for the degree of

DOCTOR OF PHILOSOPHY

BY

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September 2013

Acknowledgements

There are several people to whom I remain indebted to for their support, inspiration and advice during my time as a PhD student. Firstly, I would like to thank my primary supervisor Dr Joachim Bugert his invaluable guidance, patience and support throughout. I would also like to thank my secondary supervisor, Professor Bernhard Moser for his encouragement and valuable input into my research. I am grateful to all my group members for their continual counsel and enthusiasm.

Many thanks to all my colleagues and staff at the Department of Medical Microbiology, Institute of Infection & Immunity and Henry Wellcome Building for all their help during me time at Cardiff University. I am particularly grateful to: Laura Farleigh, Niamh Blythe and Asif Nizam for help and input with the development of the reporter assays; Timothy Hughes and Lana Hakobayan for all their help with reagents, animal work and the monoclonal screenings; Christopher Holland and Andrew Thomas for their assistance in protein purification; Paul Schnitzler for providing German sera and arranging MC tissue collections; Nidhi Agarwal for providing Indian molluscum positive sera samples; Sam Loveless and Neil Robertson for providing UK sera samples; Eva Hadaschik for providing MC tissue samples; Arwyn T Jones and Edd Sayers for assistance with the confocal microscope; Bernard Moss of the NIH, NIAID LVD, Bethesda, Maryland, U.S.A. for the gift of the vRB12/pRB21 recombination system and Norbert Fusenig of the DKFZ, Heidelberg, Germany for HaCaT cells.

Many thanks family and friends for all their encouragement. A special thanks to my parents for their unreserved support and encouragement throughout my life. Finally, my husband Wajid, for his unconditional support through all the ups and downs of this thesis.

Summary

Molluscum contagiosum virus (MCV) is a significant human pathogen causing benign tumours in the human skin. Molluscum contagiosum (MC) infection is most common in children, young adults and immunodeficient individuals. MCV replicates well in the human skin *in vivo*, but conditions that support MCV replication *in vitro* are unknown. The lack of *in vitro* cell culture system has significantly limited progress in MCV research. The aims of this study were, (i) To develop a reporter assay for the sensitive detection and quantitation of MCV infections *in vitro*, (ii) To express suitable MCV virion surface antigens to develop a sensitive MCV ELISA assay, and (iii) To raise and characterize monoclonal antibodies (mAbs) against these antigens for the detection of MCV in infected human skin. All goals were achieved. A reporter assay based on simultaneous transfection of luciferase/EGFP reporter plasmids and infection with live MCV worked well and was used to test the infectivity of MCV in several human and animal cells. A novel C-terminal MC084 ELISA was established and used to determine MCV seroprevalence in two European populations. mAbs against MC084 and MC133 were raised and partially characterized. Interesting findings were that MCV not only infects human keratinocytes, but also a wide range of animal and human cells *in vitro*, which, however, do not support replication. Our MCV ELISA gives seroprevalences in UK and German general populations comparable with previous Australian and Japanese studies (<40 years of age). Surprisingly, in older age groups (vaccinated against smallpox), a significantly higher MCV seroprevalence was observed. This was not due to crossreactivity with VACV. We propose this increase may be due to childhood MCV antibodies being boosted by subsequent vaccination or vice versa. Finally, the mAbs raised are unique reagents and will be used in future work to test a hypothesis that MCV replicates in human epidermal stem cells.

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Abbreviations

aa	Amino acid
Ab	Antibody
AIDS	Acquired Immune Deficiency Syndrome
A+T	Adenosine + Thymine
ATCC	American Tissue Culture Collection
BHK-21	Baby Hamster Kidney epithelial cell
BJ-1	Human Foreskin Fibroblast Cell
bp	Base- pairs
CAM	Chorioallantoic Membrane
CCV	Clathrin- Coated Vesicles
CD	Cluster of Differentiation
CEV	Cell-Associated Enveloped Virus
CPE	Cytopathogenic Effects
CPXV	Cowpox Virus
CTL	Cytotoxic T Lymphocytes
CV	Coefficient of Variation
CV-1	Male African Green Monkey Kidney Fibroblast Cell
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl-sulfoxide
DED	Death Effector Domains
DNA	Deoxyribonucleic Acid
DOCK8	Dedicator of Cytokinesis 8
ds	Double-stranded
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EEV	Extracellular Enveloped Virus
EM	Electron Microscopy

ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
EV	Extracellular Virion
FADD	Fas-Associated Death Domain Protein
FBS	Foetal Bovine Serum
FF	Firefly
FLIP	FLICE-Like Inhibitory Protein
FPLC	Fast Performance Liquid Chromatography
GAGs	Glycosaminglycans
G+C	Guanine + Cytosine
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
HAART	Highly Active Antiretroviral Therapy
HaCaT	Immortalized Human Skin Keratinocyte Cell
HCMV	Human Cytomegalovirus
HeLa	Human Cervical Epithelial Cell
HFFF	Human Foetal Foreskin Fibroblast Cell
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
HEK 293	Human Kidney Epithelial Cell Line
IEV	Intracellular Enveloped Virion
IFNs	Interferons
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL	Interleukin
ILR #	Internal Laboratory Reference Number
ITR	Inverted Terminal Repeats

IV	Intracellular Virion
kbp	Kilobase pairs
kDa	Kilo Dalton
LB	Luria Bertani (broth)
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
mAb	Monoclonal Antibody
MC	Molluscum contagiosum
MCV	Molluscum contagiosum Virus
MHC	Major Histocompatibility Complex
MOI	Multiplicity Of Infection
MPXV	Monkeypox Virus
mRNA	Messenger RNA
MS	Multiple Sclerosis
MT	Microtubules
MV	Mature Virion
NAb	Neutralising antibody
OD	Optical density
OPV	Orthopox virus
PAMP	Pathogen Associated Molecular Pattern
PBS	Dulbecco's Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pE/L	Synthetic Early/Late poxviral Promoter
pfu	Plaque Forming Units
PhRL-TK	Herpes Simplex Virus Thymidine Kinase Promoter
pi	Post Infection
PPV	Parapox Virus
PPMS	Primary Progressive Multiple Sclerosis
PRNT	Plaque Reduction Neutralization Test
PS	1% Penicillin/Streptomycin
PS	Phosphatidylserine

qPCR	Quantitative Polymerase Chain Reaction
RK13	Rabbit Kidney Epithelial Cell
RLU	Relative Light Units
Ren	Renilla Luciferase
rpm	Rotations per minute
RRMS	Relapsing Remitting Multiple Sclerosis
RT	Room Temperature
RT-PCR	Real Time Polymerase Chain Reaction
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SLAM	Signalling Lymphocyte Activation Molecule
SV40	Simian Vacuolating Virus 40
TNF	Tumour Necrosis Factor
TLR	Toll Like Receptor
T75	$75cm^3$ Cell Culture Flask
UC	Universal Container
UV	Ultra Violet
VAC	Vaccinia
VACV	Vaccinia Virus
VAR	Variola
VARV	Variola Virus
vRNA	Viral RNA
vDNA	Viral DNA
V3	Recombinant form of vaccinia virus expressing firefly luciferase with an early and late poxvirus promoter
WHO	World Health Organisation
vWR RK13	Western reserve preparation of vaccinia virus prepared in RK13 cells
WR	Western Reserve
24wp	24 well cell culture plate
293	Human Kidney Epithelial Cell
3T3	Swiss Mouse Embryo Fibroblast Cell

Chapter 1 - Introduction

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1.1 General Introduction

Molluscum contagiosum virus (MCV) is a significant benign but underreported skin pathogen for children and adults. The typical poxvirus particle morphology and genome organisation of MCV led to its classification as a member of the family *Poxviridae* where it is the sole member of the genus *Molluscipoxvirus*. Compared to other human skin viruses it has failed to garner sustained interest from a research perspective due to the lack of a conventional cell culture system or animal model as well as the benign nature of the clinical disease. The inability to grow the virus *in vitro* has hampered biological studies and rendered the development of specific therapeutics difficult. Also, virions can only be obtained from limited patient material. The resulting lack of reagents has proven a major obstacle in further investigation and research. In the absence of reliable clinical data the true prevalence of molluscum contagiosum (MC) is unknown. Inspite of the common occurrence of the disease, till now only a very limited number of seroprevalence studies have been conducted. No studies have been reported on how common MC is in Europe. Age dependent seroprevalence studies can help establish burden of disease. My doctoral research project focuses on aspects relating to the lifecycle of Molluscum contagiosum virus and the human immune response to it. In light of the fact that any research in this field is affected by a complete absence of reagents enabling its further investigation, one of aims of this PhD project was to develop and design MCV gene specific reagents to enable study of MCV lifecycle and immunity.

1.2 Poxviruses

Poxviruses are large, double-stranded DNA viruses that infect vertebrates and insects (Moss, 2007b). Members of the *Poxviridae* family replicate exclusively in the cytoplasm of vertebrate and invertebrate cells (Fields, 2013). The best known members of the family belong to the orthopoxvirus (OPV) genus, namely variola virus (Moratilla *et al*., 1997), the causative agent of smallpox, and the closely related vaccinia virus

(VAC) which has successfully served as the smallpox vaccine. Vaccinia virus was also the first animal virus to be visualised microscopically, grown in tissue culture, accurately tittered, physically purified and chemically analysed (Fields, 2013). The complete genome sequences of VAC (192 kbp) and VAR (186 kbp) have been determined (Goebel *et al.,* 1990; Shchelkunov *et al.,* 1993). The genome of one other poxvirus, molluscum contagiosum virus has also been sequenced (Senkevich *et al.,* 1996). VAR and MCV are unique among the poxviruses in their specific adaptation to humans as and with the eradication of smallpox in 1977 spearheaded by the World Health Organisation, MCV is the only member of the family that has a strictly human host range. MCV produces small, benign, cutaneous tumours primarily in children and young adults that persist for many months with only a weak immune response and almost no inflammation (Postlethwaite, 1970; Epstein, 1992; Porter *et al.*, 1992; Gottlieb and Myskowski, 1994). In immunodeficient individuals, however, the skin lesions can become extensive, and MCV is a common and untreatable opportunistic infection of AIDS patients (Cotton *et al.*, 1987; Schwartz and Myskowski, 1992 a and b).

Poxviruses are large double-stranded DNA viruses with genomes ranging from 130 to 380 kbp (Fields, 2013) with a preference for the epidermis. They are highly successful pathogens known to infect a wide variety of animals, including insects, reptiles, birds and mammals. They can be spread by aerosol, direct contact, and insects. The most infamous member of the family *Poxviridae* is Variola virus, the causative agent of smallpox, which caused millions of deaths before its eradication from the natural environment (Esposito and Fenner**,** 2001). The threat from multiple poxviruses to humans remains, including the use of smallpox as a bioterrorism weapon in a now largely unvaccinated population and infection from other existing animal poxviruses such as including monkeypox virus, tanapoxvirus, Yaba-like disease virus, and cowpox virus, which can infect humans and cause morbidity (Downie, 1972; Espana, 1971; Espana *et al*., 1971; McNulty *et al*., 1968). Monkeypoxvirus is of particular concern because it causes high human mortality and can spread from human to human (Hutin *et al*., 2001). There are also reports of an emerging poxvirus infection, cantagalo, in Brazil, which has apparently evolved from the locally used smallpox vaccine strain

(Damaso *et al*., 2000). Another member of the poxvirus family, molluscum contagiosum virus (MCV) occurs commonly in humans (39% of a population over 50 year olds tested seropositive) but causes significant disease only in immunocompromised individuals and rarely in children (Konya and Thompson, 1999). MCV may cause long-standing disfiguring infections, mostly on the skin of the face (Xiang and Moss, 2001). The existence of multiple poxviruses that can infect humans raises the possibility of the evolution of a new smallpox-like virus through host gene acquisitions or intervirus recombination events (Upton *et al*., 2003). If the new virus retained the ability to infect animals, however, then its eradication would be unlikely due to the natural animal reservoir of infection. In addition to the importance of poxvirus pathogens, multiple attenuated poxviruses are being used as vectors for clinical purposes, including cancer treatment, vaccines for human immunodeficiency virus, cytomegalovirus, and measles virus, and a successful rabies virus vaccine for feral animals (Artois *et al*., 1990; Hu *et al*., 2001)

Currently, three poxviruses have been established as human pathogens: variola virus (VARV), the etiologic agent of smallpox, monkeypox virus (MPXV), which induces morbidity similar to smallpox, and molluscum contagiosum virus, which causes benign skin lesions. The best understood poxvirus is vaccinia virus (VACV), which is closely related to VARV and was used as a live vaccine for the eradication of smallpox (Goebel *et al*., 1990; Perkus *et al*., 1990; Lun *et al*., 2005; McFadden, 2005).

1.2.1 Classification

The characteristic properties of members of the family *Poxviridae* include cytoplasmic site of replication, a large complex virion containing mRNA synthesizing enzymes and genome consisting of a single linear double-stranded DNA molecule of 130-300 kilobase pairs (kbp) with a hairpin loop at each end (Fields, 2013). Based on vertebrate or insect host range, the family *Poxviridae* is divided into two subfamilies Chordopoxvirinae and Entomopoxvirinae. The subfamily Chordopoxvirinae consists of eight genera: Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus. The members of a

genus are related genetically, morphologically and with respect to host range. Orthopoxviruses have been the subject of intense investigation and DNA sequencing studies reveal that genes common to VACV, VARV, ectromelia virus, camelpoxvirus and cowpoxvirus show greater than 90% identity whereas other poxviruses such as racoonpoxvirus and volepoxvirus indicate genetic divergence (Knight *et al*., 1992). The prototypic virus of the orthopoxvirus genera is the vaccinia virus which has been used as the smallpox vaccine for over 200 years and may no longer exist in the natural host (Slifka and Hanifin, 2004).

Table 1.1. Family *Poxviridae*

Subfamily	Genus	Type
Chordopoxvirinae	Orthopoxvirus	Variolavirus / Vacciniavirus Monkeypoxvirus
	Parapoxvirus	Orfvirus
	Avipoxvirus	Fowlpoxvirus
	Capripoxvirus	Sheeppoxvirus
	Leporipoxvirus	Myxomavirus
	Suipoxvirus	Swinepoxvirus
	Molluscipoxvirus	Molluscumcontagiosum
	Yatapoxvirus	Tanapoxvirus
Entomopoxvirinae	A (Coleoptera)	Melolonthamelolontha
	B (Lipidoptera)	Amsactamoorei
	C (Diptera)	Chironomusluridus

1.2.2 Morphology

The virions of poxviruses (Figure 1.1) are large and barrel shaped particles measuring 400 × 230 nm. The thickness of the outer layer varies between 5-6 nm which along with the density is consistent with the one lipid membrane bilayer (Fields, 2013). The outer membrane has irregular protrusions extending 3-5 nm. Inner structure of the virion consists of a dumbbell shaped core lateral bodies which are aggregates of heterogenous material. The core wall is two layered with an overall thickness of 18-19 nm whereas the inner layer is continuous except for a few lipid channels (Fields, 2013).

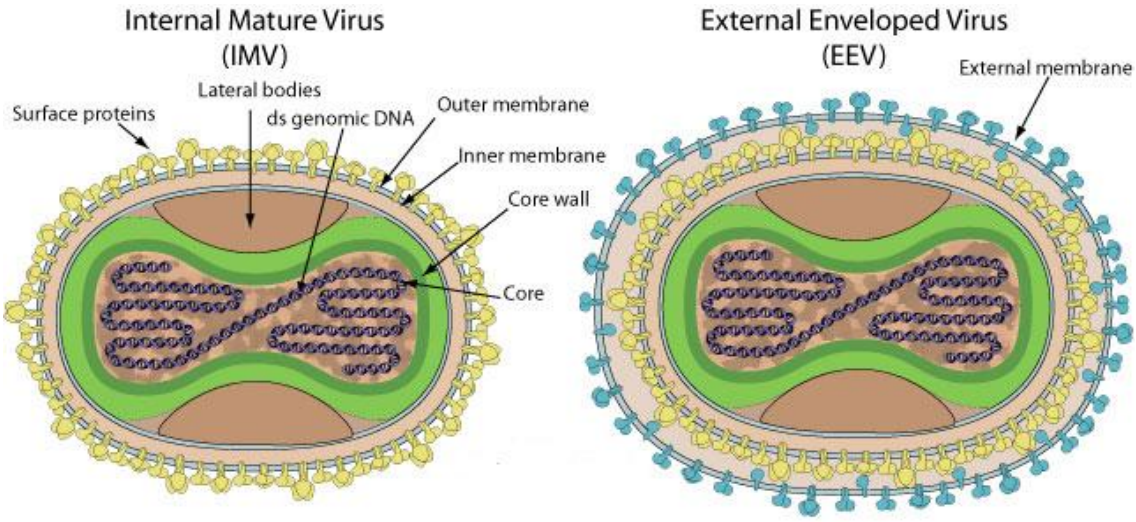


Figure 1.1. Poxvirus virion. *Poxvirus virions are enveloped, brick-shaped or ovoid, 220-450 nm long and 140-260 nm wide. The surface membrane displays surface tubules or surface filaments. Two distinct infectious virus particles exist: the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). (Adapted fromviralzone.expasy.org)*

1.2.3 Genome

The linear double stranded genomes vary from 134 kbp in parapoxviruses to more than 300 kbp in some avipoxviruses (Garon *et al*., 1978). All poxviruses have inverted terminal repeats (ITRs) which contain identical, but oppositely oriented sequences at the two ends of the genome. These are A+T rich, incompletely base paired hair-pin loops which connect the two DNA strands (Baroudy and Moss, 1980).

1.2.4 Poxvirus Tropism

Poxviruses have a narrow animal host range capable of supporting their survival in the wild (McFadden, 2005). The successful eradication of smallpox could not have been possible if there were a non-human reservoir. Genes responsible for tropism and pathogenicity are species specific rather than essential genes which are common to most poxviruses (Fields, 2013). Different aspects of host-virus interactions define the three levels of tropism specific to poxviruses. The first level of tropism which is cellular tropism, refers to the observation that virus replication can be permissive, semi-permissive or abortive in cultured cells of different lineages or species (McFadden,

2005). The second level refers to the frequently observed increased levels of virus replication in specific host organs or tissues, which can be influenced by factors that mediate cellular tropism as well as by tissue-specific antiviral responses. Finally, the third level, manifests with overt pathogenesis and symptoms of disease in the infected organism, is influenced by the first two levels of tropism as well as by the overall host immune and inflammatory responses (McFadden, 2005). At the cellular level poxvirus tropism seems to be regulated by intracellular events downstream of virus binding and entry, rather than at the level of specific host receptors.

Entry of enveloped viruses into the host can be divided into six general steps: binding, cell surface movement, signalling, internalization, intracellular transport, and membrane penetration (Marsh and Helenius, 2006). In many viruses the host tropism is determined by the attachment and entry receptors used, however poxvirus tropism is usually determined by post entry steps (McFadden, 2005) . Poxviruses produce two types of infectious particles namely the mature virions (MVs) and extracellular virions (EVs) (Moss, 2007) (Figure 1.1). Both particles differ in the epitopes which they exhibit on their surfaces and thus use different attachment factors for entry. Fusion of enveloped viruses with cellular membranes can occur either at the plasma membrane or after endocytic uptake of viral particles. While fusion at the plasma membrane would depend less on cellular machinery, fusion after endocytosis would have several striking advantages for both MVs and EVs (Marsh and Helenius, 2006). First, cellular transport mechanisms ferry virus particles to the point of fusion, avoiding cytoskeleton barriers such as cortical actin. Second, spatially/temporally controlled endosomal cues, including acidic pH and proteases, among others, may activate viral fusion at the appropriate cellular location. Third, no virus components that could be recognized by the immune system are left at the plasma membrane. Fourth, endocytic vesicles may protect activated viral fusion proteins, often sensitive to neutralizing antibodies, from immune recognition (Shedlock *et al*., 2010).

VACV MVs consist of the viral core containing the dsDNA genome, two proteinaceous lateral bodies, and one lipid bilayer containing at least 25 viral proteins. EVs consist of

an MV-like particle surrounded by a second viral membrane containing cellular and at least six unique viral proteins. MVs are the more abundantly produced infectious form and are thought to mediate host-to-host transmission, while EVs mediate virus spread within an infected host (Smith *et al*., 2003). The majority of EVs remain associated with the producer cell. These particles can induce the formation of actin tails that push them away from the producer cell mediating local virus dissemination. EVs released into body fluids are thought to mediate virus transmission from tissue to tissue. Thus two forms of infectious particles, each wrapped with a unique number of membranes of varying lipid and protein compositions offer different modes of entry of virus.

MVs use several, partly redundant binding mechanisms that involve glycosaminglycans (GAGs) in addition to other surface molecules. The dependence on GAGs for binding and infection varies with cell type, virus strain, and experimental conditions (Bengali *et al*., 2009; Carter *et al*., 2005; Whitbeck *et al*., 2009). Electron microscopy used to study MV entry point towards either fusion or endocytosis as the major infectious entry pathway for most poxviruses (Granados, 1973; Chang and Metz, 1976; Townsley and Moss, 2007). Both virus particles utilise the host cell machinery of endocytosis for internalisation and cell fusion (Schmidt *et al*., 2012). The size limitations imposed by the large size of the particles make endocytosis the only possible strategy for large particle uptake by non-phagocytic cells and preventing early immune recognition of viral factors at the plasma membrane level especially in the case of MVs.

The mechanisms used by both for macropinocytosis differ (Schmidt *et al*., 2011). MV fusion is facilitated by the inactivation of fusion inhibitory proteins. EVs lack fusion inhibitory proteins and have to shed an additional membrane so as to expose the fusion complex. VAC MVs employ apoptotic mimicry dependent on phosphatidylserine (PS)-binding serum protein in the viral membrane and epidermal growth factor receptor (Butala *et al*., 2013) signalling leading to micropinosome formation (Mercer and Helenius, 2009). It has been demonstrated that macropinocytosis is a productive route of intact EV entry into HeLa cells (Schmidt *et al*., 2011). Unlike MVs, intact EVs do not rely on exposed phosphatidyl serine to trigger macropinocytosis, although both share the need for EGFR signaling. Endocytic vesicles provide the virus with a means

of cytoplasmic transport and promote virus escape from endocytic organelles by membrane fusion. This entry program is typically followed by delivery of incoming viral genomes to the site of virus replication.

An important intracellular event that regulates the efficiency of poxvirus replication is the lineage and differentiation state of the infected cell (Weedon *et al*., 2010). For example, some poxviruses are dependent on the precise differentiation state of the host cell, such as the restriction of productive replication of molluscum contagiosum virus to keratinocytes that arise from the basal epidermal layer of the skin. The cellular factors that are required for the virus to complete its replication cycle beyond the stage of early gene expression are unknown, but it is noteworthy that the molluscum contagiosum virus encodes fewer immunomodulatory proteins than any other poxvirus that can infect humans (Senkevich *et al*., 1996).

1.3 Poxvirus Entry Mechanisms

1.3.1 Mature virions

The mechanisms by which poxviruses enter cells remain the focus of much research, most of which is conducted with the prototypic virus, vaccinia virus (VACV) (Moss, 2007). The morphogenesis of vaccinia virions is summarised in figure 1.2. VACV exists in multiple infectious forms, the most abundant being the mature virion (MV). These can enter cells by a low pH endosomal pathway or through direct fusion with the plasma membrane (Townsley and Moss, 2007; Bengali *et al*., 2009) To achieve entry, enveloped viruses first bind to the cell, activate surface proteins and then fuse with the membrane (Earp *et al*., 2005; Townsley *et al*., 2006). The initial binding to cellular attachment factors is aided by certain membrane proteins. It has been found that A27 and H3L proteins bind to heparan sulphate (Chung *et al*., 1998; Lin *et al*., 2000) and D8 to chondroitin sulphate (Hsiao *et al*., 1999). Experiments conducted by Senkevich and Moss demonstrated that deletion of A27 protein still allowed for entry, illustrating such proteins are not essential for fusion (Senkevich and Moss, 2005). An entry and fusion complex of at lease nine MV transmembrane proteins has been identified as necessary for successful fusion (Roberts and Smith, 2008).

H3L is an integral membrane protein predominantly found in the envelope of IMVs of vaccinia virus. Soluble H3L binds to cell surface heparan sulphate and blocks the adsorption of IMVs thereby indicating that it is a VV receptor involved in target cell binding (da Fonseca *et al*., 2000; Lin *et al*., 2000). VV H3L deletion mutants exhibited a small plaque phenotype and decreased viral yields further substantiated by microscopic examination revealing defective virion morphogenesis (da Fonseca *et al*., 2000; Lin *et al*., 2000). The H3L gene is strongly conserved amongst orthopoxviruses with the H3L protein from VVWR and variola sharing 96% amino acid identity (Davies *et al*., 2005).

It has been extensively investigated that H3L, the vaccinia intracellular mature virion envelope protein, which is a homolog of the molluscum contagiosum virus surface protein MC084 (29% identity), is an antigen capable of eliciting a human humoral immune response recognised by high titre antibodies in human donors particularly after a secondary smallpox vaccination (Davies *et al*., 2005). Experiments by Davies and co-workers concluded that anti-H3L antibody responses provided partial protection *in vivo* in mice from lethal challenges with VV.

1.3.2 Extracellular virions

The MV may become an 'Intracellular Enveloped Virion' (Shchelkunov *et al*., 1993) if subjected to additional membrane wrapping by the trans-golgi or endosomal cisternae (Tooze *et al*., 1993). The IEV subsequently moves to the cell surface and is exocytosed. If retained on the cell surface the virion is referred to as Cell-Associated Enveloped Virus (CEV), but if released it is known as Extracellular Enveloped Virus (Smith *et al*., 2002). CEVs lead to the formation of actin tails which allow propulsion of virions from the cell surface, facilitating the infection of neighbouring cells (Smith *et al*., 2002). The extracellular virion (EV) can be considered an MV surrounded by an additional membrane, which contains at least four glycoproteins and one non-glycosylated protein, none of which exist on the inner, MV membrane (Moss, 2007). Owing to the location of entry proteins on the MV membrane, removal of the additional external membrane is

required for spread of VACV infection (Senkevich *et al*., 2004a; Senkevich *et al*., 2004b).

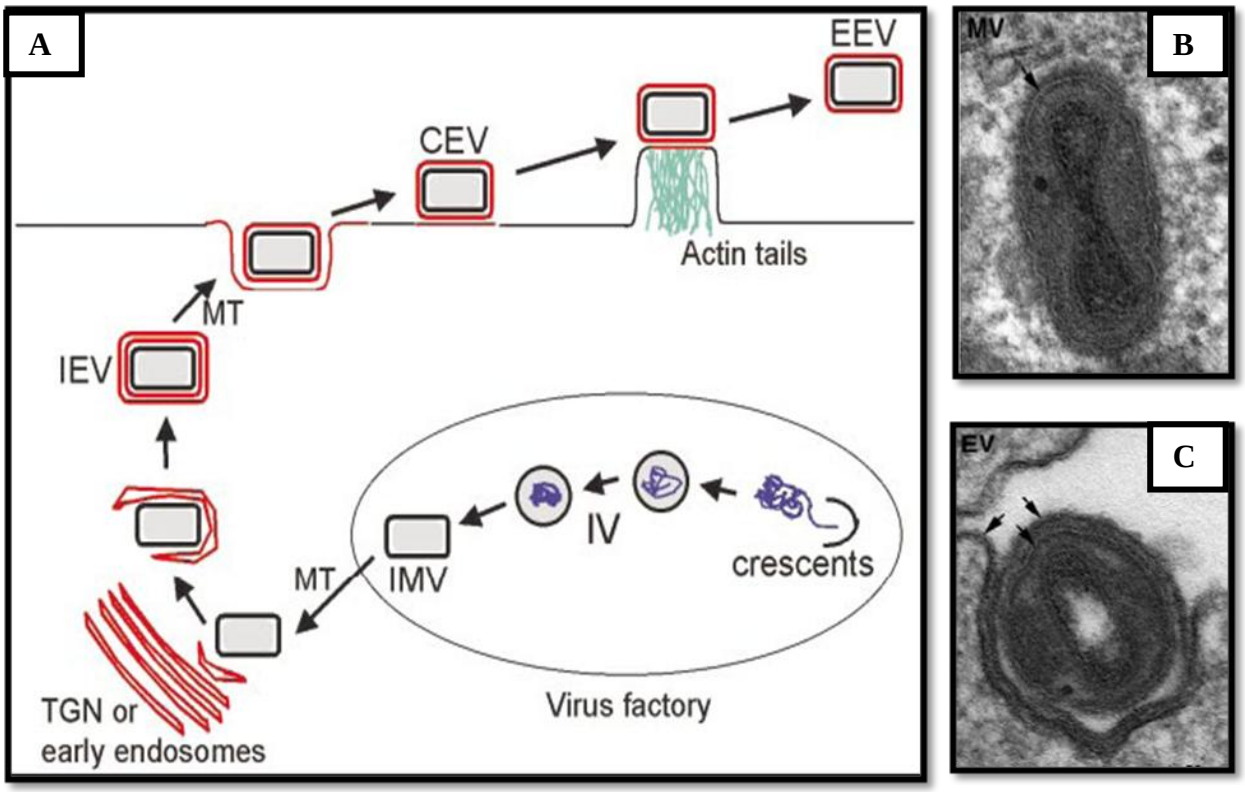


Figure 1.2. Overview of VACV morphogenesis. *(A) IMV made in virus factory and relocated via microtubules (MT) to wrapping membranes. Here IEV is formed which moves to cell surface via MT. At cell surface outermost IEV membrane fuses with plasma membrane to form CEV that induce actin tail formation to propel virion from cell. CEV may also be released to form EEV (From Janeczko et al., 1987). (B) Transmission electron micrograph of MV. Arrow points to the single membrane of MV (Townsley et al., 2006). (C) Transmission micrograph of EV. One arrow points to the outer wrapping membrane that has fused with the plasma membrane and the others to the MV and remaining EV wrapper (Moss, 2007).*

1.3.3 Virus entry

The number of membranes surrounding a VACV virion is fundamental to virus entry. One of the earliest studies used electron microscopy (EM) and demonstrated the presence of virions within intracellular cytoplasmic vesicles (Janeczko *et al*., 1987). Later investigators suggested viral core entry was a result of fusion between the plasma membrane and virus particle envelopes (Sieczkarski and Whittaker, 2002). Such findings, in conjunction with other studies gave rise to the current models of poxvirus

entry. Direct fusion involves the fusion of the MV membrane to the cellular plasma membrane, followed by membrane flattening and release of the naked viral core (Carter *et al*., 2005).

A low-pH endosomal entry pathway was proposed due to evidence of a tenfold increase in MV entry following treatment at a pH <6. In addition, inhibitors of endosomal acidification (bafilomycin A1, concanamycin A, and monensin) reduced entry by more than 70% (Townsley *et al*, 2006). Clathrin, a mediator of the primary route of internalization, is necessary for successful endocytic entry. This forms a clathrin- coated pits on the inner surface of the plasma membrane. Interactions of clathrin with other molecules, e.g. dynamin GTPase, are essential for vesicle formation (Abazeed *et al*., 2005). Recent studies have reported the existence of alternative pathways which do not depend on clathrin (Figure 1.3) (Laliberte and Moss, 2009).

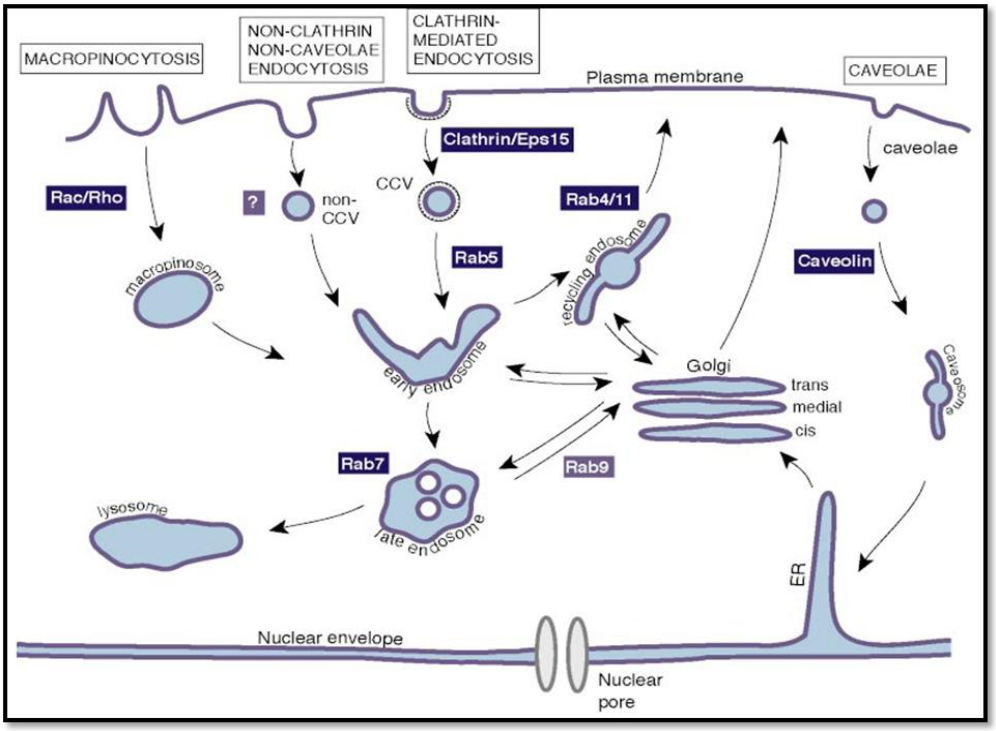


Figure 1.3. Summary of the major routes of endocytosis used by viruses. *The most established is via clathrin-coated pits. Clathrin-coated vesicles (CCVs) progress to early endosomes, which is dependent upon Rab5. Endosomes recycle back to cell surface which is dependent on Rab4 and/or 11 or progress to late endosome/lysosome, controlled by Rab7.*

Other pathways include non-coated pits (non-CCV), macropinocytosis or caveolae (Laliberte and Moss, 2009).

The model proposed by Laliberte and Moss (Laliberte and Moss, 2009) describes a possible apoptotic mimicry entry mechanism whereby phosphatidylserine (PS), located on the VACV membrane, flags virions as apoptotic debris. This triggers a common cellular uptake pathway to gain cell entry, possibly due to an interaction with a PS-specific receptor on the eukaryotic cell surface.

1.4 Zoonoses

Whereas the molecular mechanisms involved in host cell infection, viral replication and virulence are better understood after the genome sequence analysis of many poxviruses, the understanding of poxvirus maintenance in nature and transmission to humans is still less clear. Information on the epidemiology of zoonotic poxviruses is still too poor to enable us to clearly understand and estimate the chances of zoonoses in humans. A recent review has shed light on aspects of the host range of some poxviruses being broader than initially thought (Essbauer *et al*., 2010).

Poxviruses are some of the oldest known viruses known to mankind with respect to infection of humans and are well documented viruses of livestock as well. Only members of the subfamily Chordopoxvirinae are capable of infecting vertebrate hosts, with four genera containing species that induce disease in humans. Of these, three harbour zoonotic potential; namely the genera Orthopoxvirus, Parapoxvirus and Yatapoxvirus.

Despite the general consensus that MCV is highly specific to humans in the skin, MCV lesions have been reported in various animals. As early as 1967, molluscum lesions were reported in chimpanzees by Douglas and colleagues (Douglas *et al*., 1967). Post this observation others were reported in red kangaroos and horses (Bagnall and Wilson, 1974; Lange *et al*., 1991). In spite of these observations no *in vitro* model exists which would allow molecular biological analysis of MCV.

From published literature it seems that the duration of immunity conferred by the smallpox vaccine is uncertain and people vaccinated during the WHO campaign could be susceptible to OPV infections (de Souza Trindade *et al*., 2007) although studies propose a half-life of 92 years for variola virus antibodies (Amanna *et al*., 2007). However information on the epidemiology of zoonotic poxviruses is still too poor to enable us to clearly understand and estimate the chances of zoonotic infections in humans. The end of the vaccination campaign against smallpox in the late seventies to early eighties led to the development of a generation unprotected against OPVs and in turn created a niche for these infections in humans (Essbauer *et al*., 2010). Although studies indicate that the host range of some poxviruses is broader than previously thought (Essbauer *et al*., 2010), it is understood that most cases of poxviral zoonotic infections are the result of occupational risk e.g. for cow workers (PPV) and their families, health care workers (VACV), animal traders (MPXV) and animal keepers (MPXV, CPXV) (Reynolds *et al*., 2006; Croft *et al*., 2007; Trindade *et al*., 2006; Kurth *et al*., 2008).

1.4.1 Orthopoxvirus zoonoses

Orthopoxviruses are immunologically cross-reactive and cross-protective, so that after infection with any member of this genus a protection against an infection with any other member of the genus is obtained. Monkeypox was first described as a rash causing illness in captive monkeys in the zoo of Copenhagen in 1957. The following years saw reports of several outbreaks in European and American zoos. Based on its distinct phenotype monkeypox virus (MPXV) is regarded as a discrete species within the genus orthopoxvirus. Human monkeypox was reported for the first time in the 1970s in countries of Western and Central Africa (Jezek *et al*., 1986). Investigations by the WHO pointed towards a fatality rate of up to 10–17%. Large human monkeypox outbreaks were reported in the Democratic Republic of Congo (DRC) in 1996/1997 and 2001–2004 (Rimoin *et al*., 2007). Whereas in 1996/1997 the mortality was low (1.5%), the secondary attack rate was high (up to 78%).

Monkeypox can be described as an ‘emerging disease’ as cases have not only been reported in Africa but also from USA after its accidental introduction through MPXV-infected rodents imported from Ghana which had been kept with native prairie dogs that were later distributed as pets (Croft *et al*., 2007). Seventy-one human cases were reported of which 26% of the patients had to be hospitalized. However, the MPXV from the USA had a very low virulence with no fatalities (Chen *et al*., 2005). Comparison of different MPXV strains showed that there were two distinct clades i.e. Western Africa/US and a Central Africa clade, the clinical and pathological characteristics of which demonstrated that the Western Africa/US strains are less virulent than the Central Africa strains (Tesh *et al*., 2004; Xiao *et al*., 2005; Sbrana *et al*., 2007).

Various vaccinia virus strains have historically been used for the vaccination of various populations during the worldwide smallpox prevention campaign which eventually led to the eradication of the variola virus prior to 1980. Protective function of antibodies after smallpox infection in humans is displayed by both by neutralising the initial virus inoculums and also by limiting viral particle spread in the host hours after initiation of infection (Fenner *et al*., 1988). Memory B cells contribute to smallpox immunity via their response to infection with an anamnestic antibody response and their potential for maintenance of long term serum antibody levels by replenishment of long lived plasma cells (Crotty *et al*., 2003). Also various T cell responses including the VV-specific memory T-cells are important components of the vaccine mediated protection against smallpox virus (Slifka, 2004).

Numerous strains of vaccinia virus (VACV), differing in their biological properties and virulence in man and in animal models (Mercer *et al*., 2007) exist. Vaccine escape has been hypothesized to account for some VACV strains isolated from domesticated animals and endemic to buffalopox in India. Phylogenetic analysis based on three genes also confirmed that buffalopox virus (BPXV) is closely related to VACV (Singh *et al*., 2006) and is often seen in milkers, who accidently get pox-like local lesions on their hands, forearms and forehead accompanied with fever, axillary lymphadenopathy and general malaise (Singh *et al*., 2007). Reports in 2004–2005 suggested a nosocomial

outbreak of BPXV in humans in five major burn units of the city Karachi in Pakistan (Zafar *et al*., 2007).

Starting in 1999 several VACV strains were shown to be responsible for zoonotic disease affecting more than 1100 dairy cattle and up to 80% of their handlers in rural tropical rainforest and woodland Savanna areas in southeast Brazil (Trindade *et al*., 2006, Trindade *et a*l., 2007). Comparable to BPXV infections, milkers presented pleiomorphic lesions on their hands (mainly papules and painful ulcers), fever, and lymphadenopathy. Initial hypotheses on the reasons for the occurrence of VACV in these areas of South America involved a long-term survival of VACV vaccine strains in nature. Orthopoxviruses are known to have a high tenacity (Essbauer *et al*., 2007), which indeed could account for such a phenomenon. Studies have shown the presence of viral DNA in blood samples of OPV-infected patients. Other works involving VACV-vaccinated patients demonstrated viral DNA in blood until 20 days post-vaccination (Savona *et al*., 2006).

Cowpox virus (CPXV) is known to naturally infect a very broad range of host species in Western Eurasia such as cats, various zoo animals and humans. More than 400 cases of CPXV infections have been described for domestic cats (Essbauer *et al*., 2010; Bennett *et al*., 1986, 1989; Hinrichs *et al*., 1999; Brown *et al*., 1989; Baxby, 1994; Pfeffer *et al*., 2002), but it is estimated that many infections are not recognized by veterinarians and/or owners. The real incidence of CPXV in cats may be reflected by serological data. One study gives a variation of 0-16% in cats with antibodies against CPXV (OPV, respectively) reported from England, Norway, Austria, Germany and Finland (Juncker-Voss *et al*., 2004). The variation is seasonal, with an accumulation of CPXV infections in late summer and autumn (Bennett *et al*., 1986; Pfeffer *et al*., 2002).

Cats as predators are exposed to CPXV while hunting rodents which serve as a reservoir for CPXV (Pfeffer *et al*., 2002. Human CPXV cases are mainly caused by direct contact to ‘‘cuddly’’ cats (Baxby, 1994). Transmission of infection has been described to other close contacts (Coras *et al*., 2005) with reports of systemic involvement and/or fatal outcome of immune-compromised individuals with CPXV infections (Czerny *et al*.,

1991; Pelkonen *et al*., 2003). Cowpox virus infections in humans may become more numerous due to the lack of an adequate immune status of the population because of the abrogated but cross-protective smallpox vaccination in the 1980s (Essbauer *et al*., 2010). Nitsche *et al*. showed the long lasting viremia of the Cowpox virusin humans, and the presence of IgG and IgM were not sufficient to resolve infection (Nitsche *et al*., 2007).

1.4.2 Parapoxvirus zoonoses

Parapoxviruses (PPV) form a divergent group of viruses with zoonotic potential. Infections are mainly reported in persons with close contact to sheep and cattle. The majority of human cases are caused by Orf virus. In the last decade infections of different seal species (e.g. Callorhinus ursinus, Phoca virulina, Halichoerus grypus, Leptonychotes weddellii), sea lions (e.g. Zaophus californianus, Otaria bryonia), camels (Camelus dromedaries), Japanese serow (Capricornis crispus) and deer species (e.g. Cervus nippon centralis, Rangifer tarandus tarandus) with parapoxviruses have been described with an unclear zoonotic potential (Mercer *et al*., 2007).

1.4.3 Yatapoxvirus zoonoses

Yatapoxvirus infection is a rare exotic and mild disease mostly characterized by vesicular skin lesions in humans (Tanapox virus) or monkeys (Yaba-like disease virus). Most reported cases of infection have been from equatorial Africa (Mercer *et al*., 2007). Approximately a dozen case reports from monkeys and humans have been published (e.g. Stich *et al*., 2002; Dhar *et al*., 2004). Tanapox is postulated to be transmitted to humans by mosquitos that have fed on infected monkeys

1.5 Molluscum Contagiosum Virus

Viral skin infections are the most common cause of GP visits in the UK, with over a million cases per year constituting a high burden of diseases and incurring considerable cost to the NHS (Schofield *et al*., 2011). After the eradication of variola virus, the only

remaining poxvirus with a significant prevalence in the human population is molluscum contagiosum virus (MCV), the type member of the genus *Molluscipoxvirus*. MCV causes benign and self-limited tumours of the skin mainly in children and immunodeficient individuals. Meta-analysis suggests a point prevalence of MC in children aged 0 to 16 years of between 5.1% and 11.5% (Olsen *et al*., 2013) in the UK. The lesions are limited to the epidermis, can persist for years and show only weak signs of inflammation (Heng *et al*., 1989). MCV lesions have been histopathologically classified as acanthomas: benign hyperproliferative processes confined to the epidermal layers of the skin. Occurence is worldwide and the infection is regarded as specific for humans (Fields, 2013). There is no evidence of transmission between humans and other animals. MCV has no close relatives and is the only member of the *Molluscipoxvirus* genus (Porter *et al.*, 1992; Murphy *et al.*, 1995).

Four subtypes have been recognized by restriction endonuclease analyses and disease presentation appears to be similar (Parr *et al.*, 1977; Scholz *et al.*, 1989; Porter and Archard, 1992). Attempts to grow MCV in cell culture have been unsuccessful, though limited replication in human foreskin grafted to mice has been reported (Buller *et al.*, 1995; Fife *et al.*, 1996). The lack of an *in vitro* replication system has precluded detailed molecular biological characterization of MCV. The genome of MCV is similar to that of VAC in length and structure but has a much higher GC content (Parr *et al.*, 1977; Porter and Archard, 1987; Darai *et al.*, 1986) similar to parapoxviruses. Early studies focused on small segments of the MCV genome that had been sequenced (Porter and Archard, 1987; Bugert *et al.*, 1993; Douglass *et al.*, 1996; Hadasch *et al.*, 1993; Sonntag *et al.*, 1995; Sonntag and Darai, 1996). However, the complete sequence of the MCV type 1 genome (190 kbp), with the exception of the terminal nucleotides, is available (Senkevich *et al.*, 1996). An initial comparison of the genomes of MCV and OPV indicated that their central regions are conserved, whereas the ends are unique and contain genus-specific host response evasion genes.

1.5.1 MCV replication and pathogenesis

For a virus to produce infection it must gain entry into an appropriate host with susceptible cells. The skin may act as a portal of entry although this may involve breach of the barrier function of the integument or by direct inoculation (Rook, 2012). Host specificity and tissue tropism are the hallmarks of viral infection. Replicative ability in epidermal cells is mainly a feature of DNA viruses; hence the viruses of this group are capable of replication after direct inoculation of the skin. Epithelial cells are permissive for the molluscum contagiosum virus. Unlike variola virus, Molluscum contagiosum does not cause a systemic disease and remains localized at the site of inoculation. Cell proliferation caused by molluscum results in localised tumours (Acanthoma). The replication cycle is summarised in Figure 1.4.

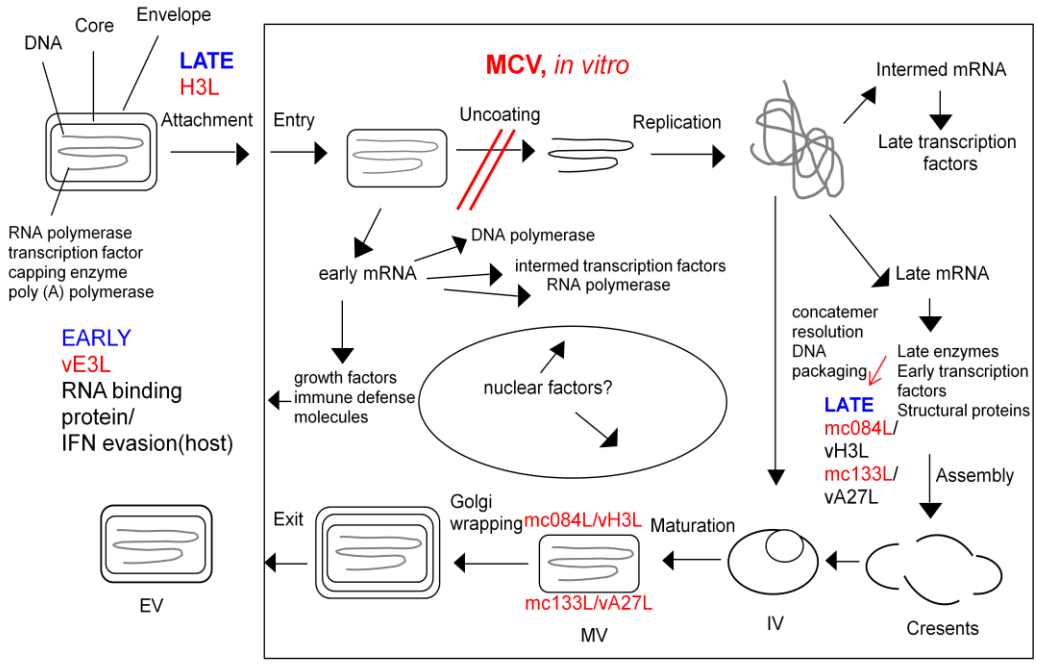


Figure 1.4. Summary of the replication cycle of Molluscum contagiosum virus. *Replication occurs entirely in the cytoplasm. A virion containing the double stranded DNA genome, enzymes and transcription factors attaches to the cells and facilitates fusion with the cell membrane in turn releasing a core into the cytoplasm. The core synthesizes early mRNA which is translated into many different proteins including growth factors, immune defense molecules, factors for DNA replication and intermediate transcription and enzymes. Uncoating takes place and DNA replication takes place to form concatemeric molecules. Intermediate genes are*

transcribed and the mRNA is translated to form late transcription factors. Late genes are then transcribed and the mRNA translated to form virion structural proteins, enzymes and early transcription factors.The assembly process is initiated and the concatemeric DNA intermediates are packaged into immature virions (IV), followed by maturation leading to formation of infectious intracellular mature virions (MV). The genes MC084 and mc133 are associated with the surface of IMVs. These MVs are wrapped by the trans-Golgi and endosomal cistrnae to give wrapped virions which are transported to the periphery of the cell along microtubules. Fusion with plasma membrane leads to release of extracellular enveloped virions (EV). Adapted from B. Moss in Field's Virology.

1.5.2 History and classification

The typical molluscum lesion, a smooth, dome-shaped, flesh-colored protrusion of the skin with a typical central indentation, was first described by Edward Jenner (1749–1823) as a 'tubercle of the skin' common in children. Thomas Bateman (1778-1821) first used the name 'molluscum contagiosum' to describe dome shaped flesh coloured papules with a central indentation and a diameter of between 2 and 5 mm (Bateman 1814). In 1841 W. Henderson and Robert Paterson independently observed intracytoplasmatic inclusions in the epidermal tissues of molluscum contagiosum (MC) lesions; hence Henderson-Paterson bodies (Henderson and Paterson 1841). In 1911 Lipschütz observed 'elementary bodies'-essentially a particular matter, inside of Henderson-Paterson bodies (Lipschütz, 1911). Goodpasture, King and Woodruff recognized similarities of MC-elementary bodies with Borrel-particles of fowlpoxvirus in 1927, and concluded, that the etiological agent of MC must be a poxvirus (Goodpasture and King, 1927; Goodpasture and Woodruff, 1931). At the beginning of the last century, Juliusberg (1905) demonstrated that the etiological agent of MC cannot be removed by filtration through Chamberland filters, implicating a viral etiology (Juliusberg, 1905). Filtrates were used to infect human volunteers, who developed MC between 25 and 50 days after inoculation. An animal model for MC has not been established to this day. Smaller elementary bodies inside the molluscum bodies were observed by Lipschutz in 1911 (Lipschütz, 1911). Electron microscopy revealed these elementary bodies to be poxvirus particles with dimensions of 360 nm to 210 nm. Electron microscopy revealed the dimension of MCV particles with 360 x 210 nm (Pirie *et al.*, 1971, Fenner and Burnet, 1957).

Table 1.2. Characteristic features of Molluscum contagiosum.

Members of the genus	Molluscum contagiosum virus type 1 – 4
Host range	Human
Virion morphology (shape, size)	Brick 210 x 360 nm
Genome size (% GC content)	190215 bp + ~100 (63 % G+C)
Isolate designation and GenBank accession	MCV type 1; U60315
Major biological features	1. Human host [at present no cell culture system] 2. Epidermal tissue tropism [cellular gene acquisition] 3. Immuneevasion [weak inflammatory response] 4. Induces epidermal dedifferentiation 5. Induces epidermal hyperproliferation 6. Induces keratinocyte surface receptor upregulation (EGFR and transferrinR) 7. Analogies to human papillomaviruses

1.5.3 Taxonomy and host range

Based on its poxvirus-like particle morphology, MCV was first reported as an unclassified member of the family *Poxviridae* (Fenner, 1976; Matthews, 1981). It was reclassified as the type member in a separate genus *Molluscipoxvirus* by the ICTV in 1991 (Francki *et al*. 1992), recognizing its poxvirus-like genome structure, in particular the terminal inverted repeats, in the absence of significant cross hybridization to other poxvirus genomes (Bugert *et al*., 1989; Bugert and Darai, 1991). MCV particle analysis in transmission electron microscopy shows spherical, ellipsoidal, brick-shaped, miniature and incomplete forms of MCV (Mihara, 1991).

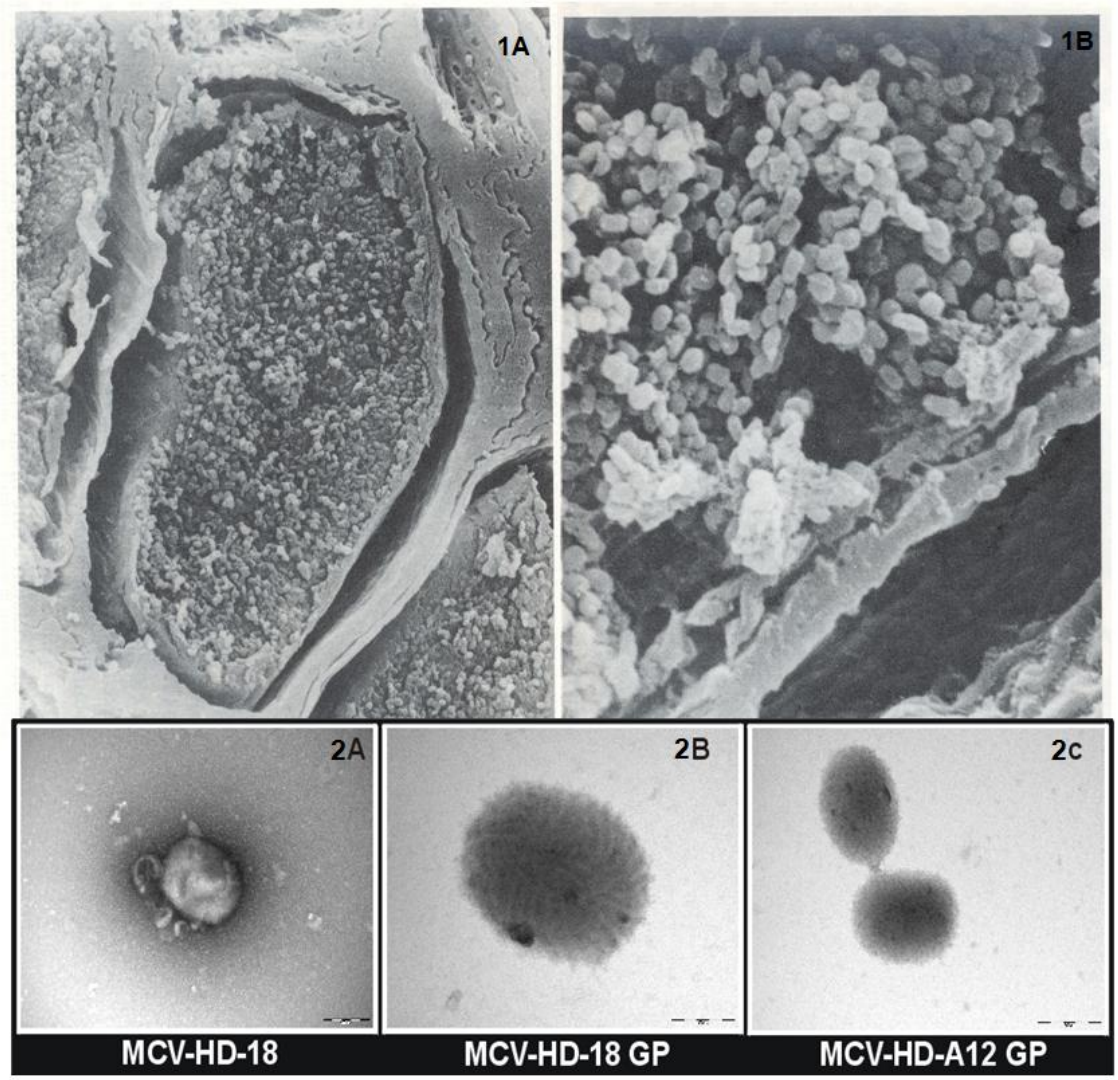


Figure 1.5. MCV infected keratinocytes. *1A: A keratinocyte witb an amorphous shell of compressed cytoplasm and the colony of molluscum virions within a separate retracted membranous sac. SEM (original <11 000) (Shelley and Burmeister, 1986). 1B: Discrete virions within a keratinocyte; the surface of adjacent keratinocytes can be seen at tbe upper right. SEM original > 38000. (Shelley and Burmeister, 1986). 2 Negative stain of MCV particles, ammonium-molybdate technique. A: loose membrane fragments in virions (Isolate MCV HD 18) filtered through 0.45 um Millipore filter (bar 200nm), B: MC virions (isolate MCV HD 18) after Optiprep® gradient purification (bar 100 nm), C: MC virions (isolate MCV HD A12) after Optiprep® gradient purification (bar 100 nm). Electronmicroscopy: Bugert and Hobot, Cardiff University School of Medicine, 2005.*

1.5.4 Disease: clinical aspects and treatment

1.5.4.1 Clinical aspects of the MC infection

The commonest skin infection in humans due to a poxvirus is molluscum contagiosum, an infection usually acquired in childhood (Buxton and Morris-Jones 2009). It is a superficial skin infection which occurs most commonly on exposed skin of children and some in the genital area of some sexually active adults (Brown *et al.*, 2006). It is spread through direct contact, often amongst family members or in schools. The incubation period is variable, between 14 days and 6 months (Buxton and Morris-Jones 2009). In adults florid molluscum may be an indicator of underlying immunodeficiency such as HIV (Schwartz and Myskowski, 1992a). Lesions are often itchy, particularly in patients with atopy which may lead to secondary infection. Transmission is by direct contact.

MC is a common infection that can become a public health issue, especially in areas with low sanitary and hygienic standards. Despite the benign and self-limited nature of the infection, one-third of children have symptoms including pruritus, erythema and, occasionally, bacterial super infection with inflammation and pain.

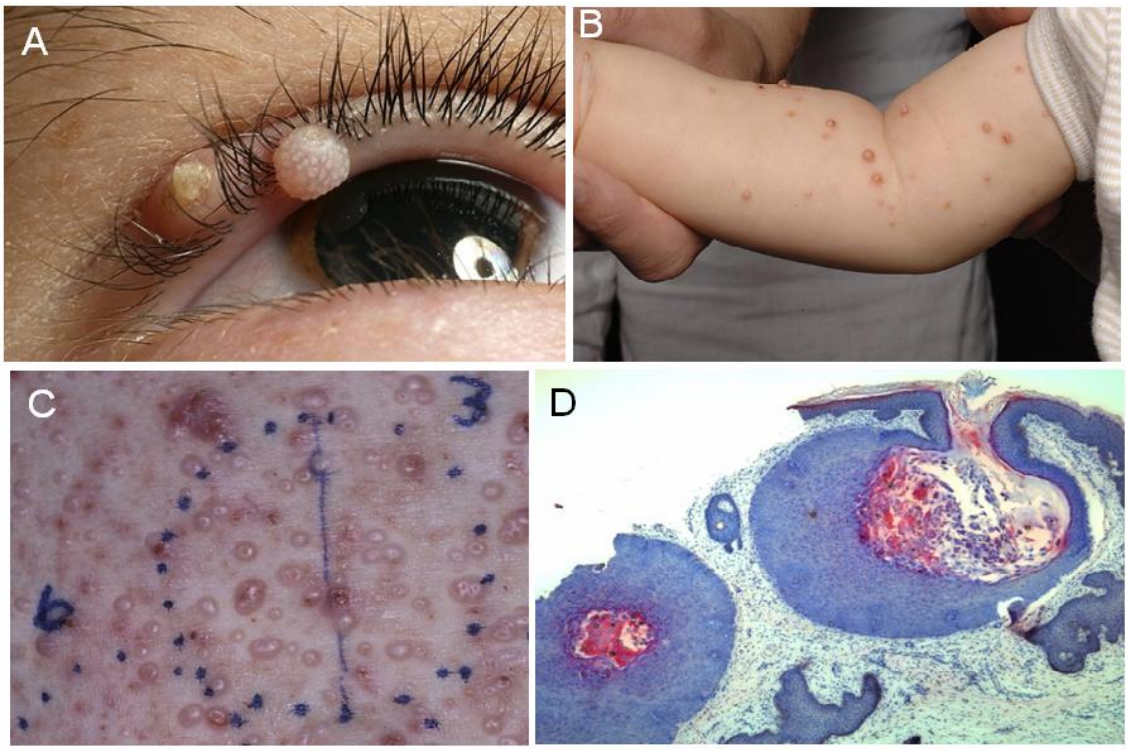


Figure 1.6. **MC clinical presentation and histology**. *(A) MC on the eyelid margin which may result in viral protein spilling into the conjunctiva and causing follicular conjunctivitis. (B)*

Child with typical MCV distribution on upper extremeties. (C) MCV lesions from an immuno-compromised patient with DOCK8, showing varying degrees of acute inflammation. NIH Clinical Center: Bugert and Turner, 1993. (D) MCV histopathology showing lobular hyperplasia of epidermis into dermis forming cup-shaped lesions. Keratinocytes of stratum malpighii have acquired Henderson- Patterson bodies. (From biopsy: University of Heidelberg; stained Sherwani, 2013).

The characteristic lesion begins as a small papule and, when mature, is a discrete, waxy, smooth, dome-shaped pearly or flesh-coloured nodule, often umbilicated (Brown *et al*., 1981). There are usually 1–20 lesions but occasionally there may be hundreds. They may become confluent along the line of a scratch and satellite lesions are occasionally seen. In children, lesions occur mainly on the trunk and proximal extremities. In adults they tend to occur on the trunk, pubic area and thighs, but in all cases infection may be transmitted to other parts by auto-inoculation (Brown *et al*., 1981). Individual lesions last for about two months but the disease usually lasts six to nine months (Steffan and Markman, 1989). More severe and prolonged infection tends to occur in individuals with impaired cell-mediated immunity, including HIV infection (Birthistle and Carrington, 1997; Schwartz and Myskowski, 1992b).

Lesions of MCV occur almost exclusively in the skin, and only rare reports have referred to mucous membrane lesions (Gottlieb and Myskowski, 1994; Lewis *et al*., 1997; Epstein, 1992). Lesions of MCV are most commonly seen in young children, sexually active adults, and in some immune suppressed patient populations (Gottlieb and Myskowski, 1994). Although MCV infections are highest in warm moist climates and in populations where personal hygiene is difficult to maintain, they have a worldwide distribution (Chen, 2013).

Patients with pruritis spread the virus through scratching (Silverberg, 2003). MC frequently occurs around the eyelid, where it is difficult to treat and occasionally causes MC conjunctivitis (Ueyama *et al*., 1985; Khaskhely *et al*., 2000). Unusual cases of MC include an infant with extensive eruptive MC scattered over the back and buttocks that became inflamed with blackening, possible haemorrhaging, and then healed spontaneously (Ogino and Ishida, 1984) or MCV associated with papillomatosis -

common warts (Payne *et al*., 1997). MC presents with an extensive clinical picture in atopic dermatitis (Wetzel and Wollenberg, 2004) and other skin specific and systemic immune dysfunctions. Spontaneous regression of MC may occur by non-inflammatory and traumatic-inflammatory processes (Pierard-Franchimont *et al*., 1983). MC in human immunodeficiency virus (HIV) infections used to be very common (up to 30% of HIV infected individuals in stage 4 clinical disease) before the onset of HAART and cidofovir HCMV prophylaxis (Meadows *et al*., 1997).

In young adults, sexual contact is probably the most common mode of transmission, and genital lesions are common (Gottlieb and Myskowski, 1994) In human immunodeficiency virus type 1-positive (HIV-11) patients, widespread lesions do occur, but head and neck lesions are most common, followed by genital involvement (Smith *et al*., 1994; Schwartz and Myskowski, 1992b). The immune response to the viral infection causes 'molluscum contagiosum' dermatitis which is an erythematous halo with desquamation around the lesion (Brown *et al*., 1981). Large solitary lesions (Giant Molluscum) or infected lesions may have an atypical presentation. Resolving lesions may be surrounded by a patch of inflammation. In most affected patients/individuals the lesions resolve leaving no marks on the skin. HIV patients exhibit bigger lesions which may even be higher in number, as many as 1000 (Cotton *et al*., 1987). In HIV infected individuals large lesions are common on the face (Schwartz and Myskowski, 1992b). Spontaneous resolution is rare.

Lesions are characterised by whitish or flesh coloured umbilicated papules (1-2 mm) or nodules (5-10 mm) (Elston, 2009). Although the typical umbilicated papules occur in all patient populations, in HIV-1 patients, verrucous, warty papules, as well as giant molluscum greater than 1 cm in diameter, are also seen (Buckley and Smith, 1999; Smith *et al*., 1994). In patients without severe immune suppression, lesions produced by MCV typically regress spontaneously usually within months, rarely years (Schwartz and Myskowski, 1992a). MCV is only distantly related to VAR, and lacks DNA cross-hybridization or immunologic cross-reactivity (Senkevich *et al*., 1996).

Four genetically distinct but clinically indistinguishable viral subtypes of MCV have been defined including three MCV-1 variants and MCV-2, MCV-3, and MCV-4 subtypes (Buller *et al*., 1995). MCV-1 subtypes dominate worldwide and, in one report, MCV-1 subtypes occurred exclusively in children under the age of 15 years (Lewis *et al*., 1997; Porter *et al*., 1989) however, there is evidence that other MCV subtypes are more common in the HIV-11 patient population (Nakamura *et al*., 1995; Lewis *et al*., 1997). There is a predilection for the head and neck, trunk, flexural areas, or the genitalia of children and adolescents (Rook, 2009).

Type 1 causes the majority of infections (76-97%) (Porter *et al*., 1989). The virus is has worldwide occurrence although its incidence in areas is not reliably known/documented (Fields, 2013). The disease is rare under the age of one perhaps due to maternally transmitted immunity and a long incubation period (Postlethwaite, 1970). Its incidence reflects an exposure to other humans as well as hygiene conditions. The age of peak incidence is reported to be 2-3years in Fiji (Postlethwaite *et al*., 1967) and 1-4 years in Zaire (Torfs, 1959). The annual infection rate for children under the age of ten in New Guinea has been reported to be 6% (Sturt *et al*., 1971). In cooler climates infection is rare and correlated with a higher age. Use of swimming pools has been correlated with childhood infections in Scotland between the ages of 10-12 years (Postlethwaite *et al*., 1967) and in Japan at the age of 8 (Niizeki *et al*., 1984). In these studies boys were affected more than girls perhaps due to frequent use of communal bathing facilities and contact sports. A peak associated with a later incidence in adults is attributable to sexual transmission. Infection of children through sexual abuse is possible (Bargman, 1986a; Bargman, 1986b).

The clinical association of frequency of molluscum contagiosum in patients with atopic eczema may be a result of impaired immunity (Dohil *et al*., 2006; Solomon and Telner, 1966). Unusually large lesions have been reported in individuals taking immunosuppressive therapy (Cotton *et al*., 1987) and those with HIV. Such clinical manifestations suggest the significance of cell mediated immunity in control and elimination of infection.

The pathogenesis of lesions although unclear suggests the entry of the virus through basal keratinocytes causing an apparent increase in cell turnover extending all the way to the suprabasal layer. As viral DNA synthesis increases, mitosis in the prickle cell layer declines. Cell proliferation produces lobulated epidermal growth that compress the papillae until fibrous septa are formed between the lobules giving rise to a pear shaped structure with an upward apex. Whereas the basal layer remains intact, cells at the core of the lesion exhibit maximum distortion and eventual destruction. These appear as large hyaline bodies (up to 25 µm) called molluscum bodies which contain cytoplasmic masses of viral materials (Shelley and Burmeister, 1986). These bodies are concentrated at the centre of the fully developed lesion and near the surface. Inflammatory changes in the dermis are either absent or very slight. However chronic lesions may show granulomatous infiltrate. It has been suspected that the discharge of the contents of a papule in to the dermis may trigger/induce an inflammatory reaction (Henao and Freeman, 1964). Specific antibodies have been recognised in 60-80% of patients with molluscum contagiosum. Also 5-15% controls exhibit these perhaps due to an unrecognised infection (Shirodaria *et al*, 1979; Watanabe *et al*, 2000). However the role of humoral immunity in regression of lesions is as yet unclear.

The incubation period has been estimated as varying from 14 days to six months. An individual lesion is shiny, pearly-white, hemispherical umbilicated papule which may show a central pore. If less than 1 mm in diameter, the papule may be identified with a hand lens. Enlarging slowly it may reach a diameter of 5-10 mm in 6-12 weeks. Plaques composed of many lesions (agminate form) occur rarely (Lynch and Minkin, 1968). Lesions frequently spread and sometimes the number may be large. After trauma or following many months, inflammatory changes result in suppuration; crusting and eventual destruction of the lesion.Healing is usually without scarring but occasionally in sites with more subcutaneous fat depressed scars may remain (Ghura and Camp, 2001).

There is great variability in the duration of an individual lesion and the attack. Most cases are self-limiting in 6-9 months. Some may persist for 3-4 years. These figures have been confirmed by follow-up studies in children in Fiji (Hawley, 1970). Distribution of lesions is determined by the mode of infection and the type of clothing

worn dictated by the climate. Lesions are commonly seen around the neck or trunk, particularly around the axillae except for sexually transmitted cases where the anogenital area is involved. In tropical regions lesions in children are more common on limbs. Widespread and refractory mollusca associated with HIV patients are seen on the face (Katzman *et al*, 1987). In healthy subjects occasional facial lesions are seen, especially on the eye lids. Molluscum has also been reported in scars (Isaac, 1980) and in tattoos with transfer through pigment (Foulds, 1982). Erythema annulare centrifugum has also been reported (Vasily and Bhatia, 1978). Chronic conjunctivitis and superficial punctuate keratitis may similarly complicate lesions on or near the eyelid (Haellmigk, 1966). Conjunctivitis and eczema subside spontaneously with the removal of lesions.

1.5.4.2 Diagnosis, Treatment, and Prevention

1.5.4.2.1 Diagnosis

After the eradication of smallpox, MCV is the most commonly diagnosed poxviral infection. Ortho and parapoxviral zoonoses are rare by comparison. MCV is readily diagnosed by its clinical appearance and by the typical histopathology found in found in sections of lesion biopsies (Chan *et al*., 2004). Molecular diagnostics by *in situ* hybridization and PCR have been described for unclear cases (Forghani *et al*., 1992; Thompson *et al*., 1990a; Thompson *et al*., 1990b). MCV ELISAs have been described for serological surveys (Konya *et al*., 1992; Thompson *et al*, 1998; Konya and Thompson, 1999). Virions can usually be seen in large numbers if material expressed from the lesion is examined by electron microscopy. The lack of a marked inflammatory response and failure to isolate an agent in cell culture or chorioalantoic membranes (CAM) should eliminate other poxvirus infections.

Diagnosis is mostly based on clinical appearance although this may be challenging in some cases. Biopsy is performed on HIV patients to rule out the invasive fungal infections such as cryptococcosis, histoplasmosis, coccidioidomycosis etc. Microscopy confirms the typical display pattern of numerous intracytoplasmic inclusion bodies called molluscum bodies (Brown *et al*., 2006).

In the differential diagnosis of MC, smallpox must be considered along with other ortho- and parapoxviruses. The anamnesis must cover contacts with pets, especially gerbils and chipmunks of African origin (monkeypox) as well as local rodents and cats (cowpox).

1.5.4.2.2 Prevention and treatment of MCV infection

Infection is benign and recovery usually spontaneous, but treatment may be sought for cosmetic reasons, particularly for facial or multiple lesions. To avoid spread of infection patients must be advised to avoid swimming pools, communal baths, contact sports, shared towels etc. In many cases a natural resolution is awaited and no therapy is required. If resolution is slow, lesions symptomatic or associated eczema is troublesome treatment may be desirable. The treatment of molluscum contagiosum is varied, with no one modality favoured in any country.

Various treatments have been tried (Birthistle and Carrington, 1997). Treatment depends on the age of the patient as well as number of lesions and may range from simple reassurance, topical application to a procedure.The induction of an inflammatory response by mild trauma or destruction of lesions can hasten clearance, which may be due to release of virus-infected cells accessible to the immune system. Prevention is based on attention to personal hygiene and, in developing countries, to general improvements in living conditions. Although relatively unimportant *per se*, the possibility that molluscum may act as a marker for more serious conditions has been raised (Oriel, 1987).

MC is best prevented by exposure prophylaxis. Once acquired, molluscum contagiosum lesions are generally self-limiting; it may take between 6 months to 5 years for lesions to disappear. Patients with immune dysfunction or atopic skin conditions have difficulties clearing lesions. There has been a continued debate about whether molluscum contagiosum lesions should be treated or allowed to resolve spontaneously (Tyring, 2003). Therapy is recommended for genital MC to avoid sexual transmission and should begin with gentle skin care and antipruritics to prevent symptoms (Silverberg, 2003). Available treatment options are:

1. In-office-Curettage (surgical): classical removal of the lesion with a sharp spoon. Occasional scarring: While adults cope well with unanesthetized curettage of lesions, children require local anaesthetics or less painful therapeutic options.

2. In-office-Cryosurgery: current technique combines lidocaine/prilocaine topical anesthesia with hyperfocal cryotherapy. Most lesions treated regress without leaving scars (Bardenstein and Elmets, 1995).

3. Novel-at-home painless MCV treatment options in extensive cases are ointments containing retinoids, alpha-hydroxy acids and the topical immunomodulators tacrolimus (e.g. 0.1% ointment), pimecrolimus, imiquimod or podophyllotoxin (0.5% ointment). However, topical immunomodulators may predispose patients to skin infections (Markos, 2001; Berman, 2002; Wetzel and Wollenberg, 2004). Although a variety of such at-home therapies are available, no at-home treatment is as effective as surgical - in-office therapy. Also a recent study published by Katz and Swetman, documents the results of two randomised controlled trials of imiquimod for treatment of MC in children and shows its ineffectiveness (Katz and Swetman, 2013).

4. Antivirals specifically active against MCV DNA polymerase, like acyclic nucleoside phosphonates (HPMPC: cidofovir, Vistide; PMEA: adefovir dipivoxil, Hepsera; and PMPA: tenofovir, Viread). These agents are effective *in vivo* against a wide variety of DNA virus infections (De Clercq, 2003). There is anecdotal evidence that i.v. and topical cidofovir works against MCV, however, comprehensive clinical studies are lacking (Meadows *et al*., 1997). Possible alternative MCV specific antivirals that are presently not pharmaceutically refined target the viral topoisomerase: lamellarin H, coumermycin A1 (50% inhibitory concentration, 32 µM) and cyclic depsipeptide sansalvamide-A (Hwang *et al*., 1998; Hwang *et al*., 1999; Bailly, 2004).

All surgical treatments are painful to some degree and carry the risk of leaving scars. Immunemodulators may predispose to bacterial and fungal infections. Specific antivirals are the most expensive treatment option. Further research in large clinical trials is required to increase current knowledge on prevention, optimal treatment, and long-term outcome with this disease.

1.5.4.3 Epidemiology

1.5.4.3.1 MCV molecular epidemiology

First available in the early seventies was the method known as DNA fingerprinting, which uses bacterial DNA restriction enzymes, to assess genetic variation of viral genome nucleic acids. This method requires access to the viral genome in sufficient quantities to be visualized with ethidium bromide stain on agarose gels. The first purification of MCV DNA suitable for this purpose was reported by Pierie and co-workers (Pirie *et al*., 1971) who found, that MCV can be isolated in large quantities from clinical lesion material.

Parr and co-workers first observed in 1977 genetic variations between MCV isolates based on differences in restriction enzyme patterns (Parr *et al*., 1977). Later work in the labs of G. Darai and L.C. Archard (Darai *et al*., 1986; Scholz *et al*., 1988; Scholz *et al*., 1989; Porter *et al*., 1989; Porter and Archard, 1992) established between 2 and 3 main genetic types, respectively, and a number of subtypes, based on their viral genomic DNA restriction pattern.

Porter and co-workers reported in two studies of patients seen in London hospitals an MCV type 1: type 2 ratio of about 3:1 (Porter *et al*., 1987; Porter and Archard, 1992). Darai and co-workers differentiated MCV types 1, 1 variant (1v), 2, and 2 variant (2v) and went on to characterize 222 MCV isolates from the Grampian region in Scotland. They found that MCV type 1/1v was about 40 fold more common than type 2/2v in this population and that MCV genotypes did not change over time (up to 3 years) in the same patient, or when passed on in a contact group, e.g. family (Scholz *et al*., 1988).

In a study of MCV in Australia for the first time HIV infections were indicated in the study group. MCV 1 or 1v were found in 59% of lesions obtained from 75 Australian patients, 29% of whom were HIV positive, 32% contained MCV 2 or 2v, and 4% contained multiple MCV types, whereas 5% of lesions submitted contained no detectable MCV DNA. The overall ratio of MCV 1/1v to MCV 2/2v was determined to be approximately 1.75:1. MCV type 2 was more frequently detected in general and

specifically in lesions from anogenital areas and immunosuppressed (HIV-positive) patients (Thompson *et al*., 1990).

A Japanese study looked at genomes of 477 Japanese strains of MCV and classified four *BamHI* restriction types, including a newly detected type (MCV type 4). The common markers of the variants of MCV 1 were 24 kbp fusion fragments generated by the loss of a *BamHI* site between the D2 and F fragments of MCV 1p. The variants of MCV 1 were classified into three groups (MCV 1va, MCV 1vb, MCV 1vc), with the variability among them being due to additions and losses of *BamHI* sites located in the right terminus and around the E and I fragments of MCV 1va. Considerable numbers of *BamHI* restriction sites were conserved between MCV 2 and 4, indicating a close analogy between them. The prevalence ratios of MCV types (MCV 1 (MCV 1p): MCV-2: MCV 3: MCV 4), was determined to be 436(0):13:24:4. Thus, the molecular epidemiology of MCV in Japan is characterized by the absence of the European prototype of MCV 1, the exclusive occurrence and abundance of variants of MCV 1, a greater prevalence of MCV 3 over MCV 2, and the presence of MCV 4 (Nakamura *et al*., 1995).

An independent Japanese study of 171 Japanese patients examined whether there were geographic differences in the incidence of MCV types and whether a correlation existed between MCV types and the age, sex, and clinical status of the patients (Yamashita *et al*., 1996). The ratio of MCV 1 to MCV 2 was 13:1. MCV 1 was commonly detected in children (98%) and adult women (92%). MCV 2 was more frequently isolated from adult men (44%) and from patients with HIV infection (75%) (Yamashita *et al*., 1996). In a Spanish study of 147 patients 97 (66%) were children under 10 years, of whom 49% had atopic dermatitis. Atopic patients presented with larger lesions. The MCV 1/MCV 2 ratio was 146:1 (Agromayor *et al*., 2002).

Table 1.3. Prevalence of MCV sub-types (Bugert, 2007).

Prevalence of MCV genetic types							
MCV type	England [Porter *et al*., 1987] N=46	Scotland [Scholz *et al*., 1988] N=222	England [Porter *et al*., 1989] N=93	Australia [Thompson *et al*.,1990] N=75	Japan [Nakamura *et al*., 1995] N=477	Japan [Yamashita *et al*., 1996] N=171	Spain [Agromayor *et al*., 2002] N=147
Type 1	74 %	96% 1p	76%	59% 1p	91% 1v+	92% 1p	99.4% 1p
Type 2	26 %	4%	24%	32%	3%	7%	0.6%
Type 3					5% [1v]		
Type 4					1% [2v]		

1.5.4.3.2 MCV seroepidemiology

Using protein preparations of MC lesion biopsies an early study looked for molluscum contagiosum virus-specific antibodies (Shirodaria *et al*., 1979). The first comprehensive study comparing 35 HIV positive MC cases and a random group of 357 persons (ages, 1 week-69 years) was done 1992 in Australia using a virus coated MCV ELISA format (Thompson *et al*., 1990). MCV antibody was identified in 77% of persons with molluscum lesions: in 17 of 24 HIV-1-negative persons and in 10 of 11 who were HIV-1-positive. No relationship was evident between the serologic responses and the number of lesions or the duration of infection. The population survey revealed an overall seropositivity rate of 23%. The lowest antibody prevalence was in children aged 6 months to 2 years (3%), and seropositivity increased with age to reach 39% in persons >50 years old, indicating that MC is a very common viral infection (Thompson *et al*., 1992).

In order to optimize ELISAs using recombinant MCV antigens, a library of MCV genome fragments was transferred into a cowpox virus expression system and screened with 12 sera from MC patients. Two major antigenic proteins of 70 and 34 kDa were detected by immunoblotting and mapped to the open-reading frames mc133L (70 kD protein: MC133) and mc084L (34 kD protein: MC084), respectively (Vreeswijk *et al*., 1976; Vreeswijk *et al*., 1977). This was confirmed in an independent study using

protein preparations of the virus-induced lesions, where three immunoreactive proteins of 74/80, 60 and 35 kD were detected. The 35 and 74/80 kD proteins turned out to be virus specific, whereas the 60 kD protein band was composed of a mix of human keratins (Agromayor *et al*., 2002). MC133L and MC084L were found to be predominantly expressed on the surface of recombinant virus-infected HeLa cells. MC084R is also detectable on the surface of MCV virion particles (Watanabe *et al.*, 1998). The same group assessed the seroprevalence of antibodies against MCV in 508 Japanese subjects with or without clinical MCV infection using a recombinant truncated MC133L ELISA. Antibodies to MCV were present in 7 (58%) of 12 patients with molluscum contagiosum, 7 (6%) of 108 healthy controls, 7 (9%) of 76 with atopic dermatitis, and 7 (18%) of 39 patients with systemic lupus erythematosus, although no clinical MCV infection was observed in the latter 3 groups. Of 7 human immunodeficiency virus (HIV)-positive patients with molluscum contagiosum, 1 (14%) was antibody positive, compared with 5 (2%) of 266 HIV positive patients without molluscum contagiosum (Watanabe *et al*., 2000).

1.5.4.4 Pathology

1.5.4.4.1 MCV pathogenesis

MCV probably enters the epidermis through microlesions. After a variable, sometimes lengthy, incubation period, papules develop, formed by epidermal hypertrophy. This produces a nodule and also extends the dermis downwards, but the basement membrane usually remains intact. The typical MCV lesion contains conglomerates of hyperplastic epithelial cells organised in follicles and lobes, which all develop into a central indentation towards the surface of the skin. A lesion consists of several inverted lobules of hyperplastic squamous epithelium which expand into the underlying dermis. The lobules are separated by fine septa of compressed dermis. The central indentation is filled with cellular debris, fatty acids and is extremely rich in elementary viral particles, creating a waxy plug-like structure. This plug gets mobilized and spreads the infection to other areas of surrounding skin or contaminates objects in a process similar to holocrine secretion.

The periphery of the MCV lesion is characterized by basaloid epithelial cells with prominent nuclei, large amounts of heterochromatin, slightly basophilic cytoplasm, and increased visibility of membraneous structues, which are larger than normal basal keratinocytes. These hyperplastic cells are dividing faster than normal basal cells, the cytoplasm contains a large number of vacuoles and they are sitting on top of an intact basal membrane (Vreeswijk *et al*., 1976; Vreeswijk *et al*., 1977). The lesion is therefore a strictly intraepidermal hyperplastic process i.e. acanthoma.

Distinct poxviral factories or inclusion bodies appear about 4 cell layers away from the basal membrane in the stratum spinosum (Shelley and Burmeister, 1986). The inclusion bodies grow and push cellular organelles including the cell nucleus to the side. Cells with inclusion bodies do not show mitoses anymore. The cytoplasm of MCV producing cells shows keratinization, which is not expected at that stage of keratinocyte differentiation and indicates dyskeratinization in the sense of abnormal differentiation (Manabe *et a*l., 1996). Characteristic inclusions (Henderson–Patterson bodies) are formed in the prickle cell layer and gradually enlarge as the cells age and migrate to the surface. These cells are replaced by hyperplasia of the basal cell layer. The inclusion is a well-defined sac packed with virions (Shelly and Burmeister, 1986).

The lesion is circumscribed by a connective tissue capsule and the dermis, apart from distortion, remains essentially normal. Occasionally an inflammatory infiltration of the dermis may occur (Brown *et al*., 1981). MCV lesions are conspicuous for the absence of immune effector cells. There are actually fewer immune cells, than in surrounding uninfected skin, e.g. no circulating tissue macrophages, suggesting a local immune evasive effect. MCV infected cells show increased EGFR and transferrin receptor surface density (Viac and Chardonnet, 1990).

1.5.4.4.2 MCV pathology and clinical features

The mean incidence of MC in the general population is 0.1–5%. Seroepidemiological studies have shown that antibody prevalence in persons over 50 years of age is 39% (Konya *et al*., 1999). This indicates that MC is a very common viral skin infection, which is supported by its common occurrence in dermatological practice. MCV

outbreaks occur in crowded populations with reduced hygienic standards. Outbreaks with more than 100 cases have been described in kindergartens, military barracks, and public swimming pools. MC was found to be very common in the Fiji Islands (Hawley, 1970).

MC is most often seen as a benign wart-like condition with light pruritus in preadolescent children, but can occur in immuncompetent adults. MC is more severe in immunocompromised people or individuals with atopic dermatitis, where it can lead to giant molluscum and eczema molluscum. The lesions generally occur on all body surfaces, but not on the palm of the hand or on mucous membranes. They may be associated with hair follicles. MC lesions can grow close to mucous membranes on the lips and eyelids. When situated near the eye, they can lead to conjunctivitis. MC is transmitted by smear-infection with the infectious fluids discharged from lesions and by direct contact with contaminated objects.

MC is not very contagious and therefore infection depends on a high inoculating dose. It is a sexually transmitted disease when lesions are located on or in the vicinity of sexual organs. MC lesions are generally globular, sometimes ovate, 2–5 mm in diameter and sit on a contracted base. Cellular semi liquid debris can be expressed from the central indentation at the top of the lesion. The infection spreads via contact with this fluid. Histologically, the tumours are strictly limited to the epidermal layer of the skin and have a resemblance to hair follicles. They are therefore classified as acanthomas. If not mechanically disturbed, MC lesions will persist for months and even years in immune-competent hosts, but can disappear spontaneously, probably when virus infected tissue is exposed to the immune system. To expose the infection by limited (sterile) trauma is a way of treatment. Scratching or disturbing the lesions leads to a quicker resolution but can complicate the condition through bacterial super infection. This must be avoided in severely immunocompromised hosts, who develop widespread MC with hundreds of lesions of larger size and can succumb to sepsis following bacterial superinfection.

MCV is a marker of late-stage disease in human immunodeficiency virus (HIV)-infected individuals, and in HIV-infected populations the incidence of MC was 30%

before the onset of human cytomegalovirus (HCMV) prophylaxis with cidofovir. MCV probably enters the epidermis through microlesions.

The typical MCV lesion contains conglomerates of hyperplastic epithelial cells organized in follicles and lobes, which all develop into a central indentation toward the surface of the skin in a process similar to holocrine secretion. The whole lesion has the appearance of a hair follicle where the hair is replaced by the virus containing plug. The central indentation is filled with cellular debris and is rich in elementary viral particles in a waxy plug-like structure. This plug becomes mobilized and spreads the infection to other areas of surrounding skin or contaminates objects.

The periphery of the MCV lesion is characterized by basaloid epithelial cells with prominent nuclei, large amounts of heterochromatin, slightly basophilic cytoplasm, and increased visibility of membranous structures. These cells are larger than normal basal keratinocytes, they divide faster than normal basal cells, their cytoplasm contains a large number of vacuoles, and they are sitting on top of an intact basal membrane. The lesion is a strictly intraepidermal hyperplastic process (acanthoma). Distinct poxviral factories (molluscum bodies or Henderson-Patterson bodies) appear about four cell layers away from the basal membrane in the stratum spinosum. The inclusion bodies grow and obliterate cellular organelles. Cells with inclusion bodies do not divide further. The cytoplasm of MCV-producing cells shows keratinization, which is not expected at that stage of keratinocyte differentiation and indicates dyskeratinization in the sense of abnormal differentiation.

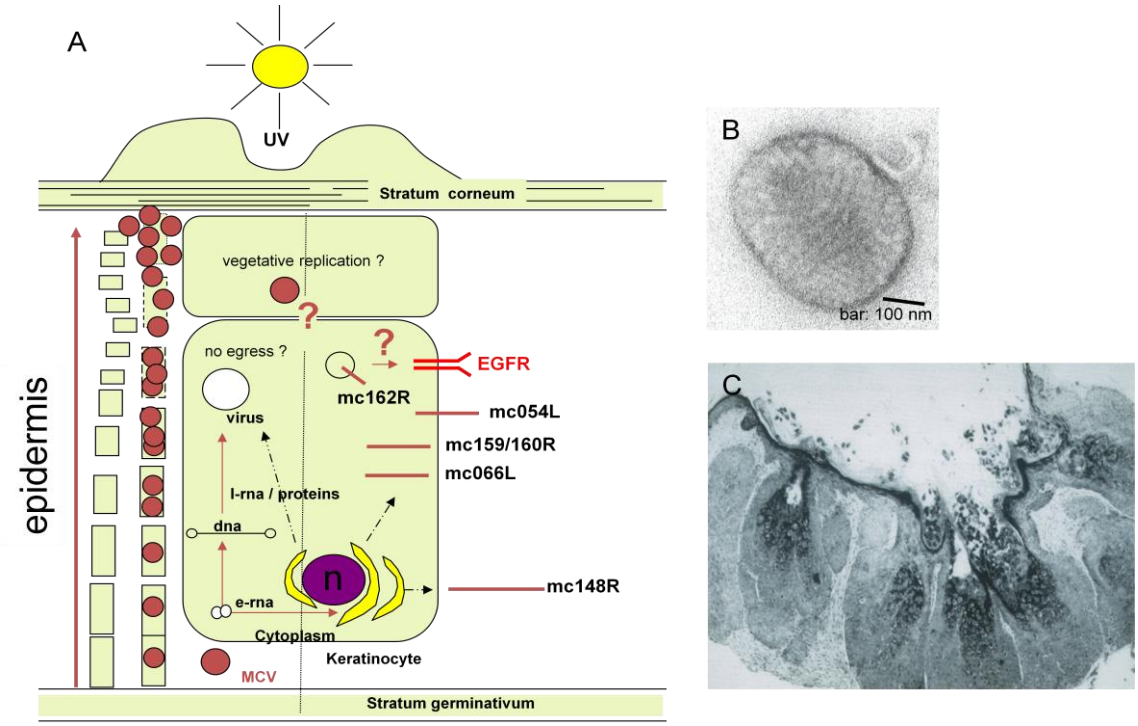


Figure 1.7. MCV life cycle and clinical picture. *(A) MCV replication in the epidermis. Examples of MCV proteins with known functions: a. membrane proteins: MC054L-IL18 binding protein, MC162: Hrs binding protein (possible effect on cell surface receptor regulation). cytoplasmic proteins: MC159/160-FLICE inhibitors, MC066-glutathione peroxidase, c. secreted proteins: MC148R-chemokine antagonist. Virions are shown as blue circles, cell surface receptors as triplet of red lines (example EGFR). (B) MCV virion(C)Histopathology of MC lesion.*

1.5.4.5 MCV tissue and cell tropism

Classical EM studies (Vreeswijk *et al*., 1976; Vreeswijk *et al*., 1977) indicate that MCV is a virus infecting keratinocytes. Granular cells of MCV infected epidermis contain filaggrin, a skin-type keratin pair (K1/K10), and trichohyalin, a hyperproliferation-related keratin pair (K6/K16) (Manabe *et al*., 1996). More recently, eosinophilic intranuclear inclusion bodies, resembling poxviral factories, were described in a melanocytic nevus and confirmed as MCV specific with reverse transcriptase *in situ* polymerase chain reaction (RT *in situ* PCR) (Hahm *et al*., 2002). The latter finding has so far not been independently confirmed.

1.5.4.6 MCV host range

While there is a general consensus that MCV is restricted to the human host, two reports in the more recent literature describe a MCV-like infection in the equine host: Three horses in the Chingola district of Zambia were found to be suffering from a slow progressive skin disease with lesions varying from 4 to 20 mm in diameter in various areas of the body. Microscopically, cytoplasmic inclusions containing many pox virions were found. Attempts at culturing the virus were unsuccessful (Lange *et al*., 1991). There is one report in the literature of 'Equine MCV' being closely related to human MCV by *in situ* hybridization using human MCV hybridization probes (Thompson, 1998).

MCV, like smallpox virus, is considered to be an exclusive pathogen of man. Reports of MCV in a number of animals, including horses, chimpanzees, and kangaroos, have not been supported by DNA sequence confirmation. Many pox-like infections of vertebrates, most of them caused by orthopoxviruses, can be confused with MCV by their clinical appearance. Conventional immune-competent laboratory animals, including mice, rats, guinea pigs, and tree shrews, do not support MCV replication in their skin. MCV infected human keratinocytes have been transplanted into mice with severe combined immune deficiency (SCID) and typical MCV lesions have subsequently developed in these non-natural hosts. Attempts to passage the virus in SCID mice were unsuccessful. However, despite the absence of molecular evidence, an animal reservoir of MCV cannot be excluded.

MCV has so far not been grown in conventional human cell lines, including immortalized tumorigenic/virus-transformed and non-tumorigenic skin keratinocytes (HaCaT, NIKS). Experiments with *ex vivo* cultures of human skin cells (raft cultures) are ongoing. MCV may use a vegetative mechanism for replicating in differentiating keratinocytes. In the absence of culturable virus, classical virological research on MCV is severely restricted. All progress so far has been made by studying MCV genes in isolation, based on the complete MCV genome sequence gained from an overlapping redundant MCV genome fragment library. This reagent has been made available to the ATCC. The entire MCV gene complement is covered by 18 recombinant bacterial

plasmid clones harbouring viral sequences from the *EcoRI*, *BamHI*, and *HindIII* restriction fragment libraries of MCV type 1/80. Further research is being carried out using abortive cell culture systems.

MCV induces a remarkable cytopathogenic effect (CPE) in human fibroblasts, both in primary cells (MRC5) and in telomerase-transduced immortal cell lines (hTERT-BJ-1). The CPE starts 4 h post infection (p.i.) and reaches a maximum at 24 h p.i., with the cells looking as if they have been trypsinized, partially detaching from the monolayer, rounding, and clumping. Cells settle down at 48–72 h p.i., but show a morphological transformation from an oblong fibroblast to a more square epithelial-looking cell type. MCV transcribes early mRNA in these cells. The CPE is not induced by UV-inactivated virions or in the presence of cycloheximide, indicating that expression of viral proteins is required. The mRNA transcription can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR) for months in serially passaged infected cells. A productive MCV infection cannot be rescued nongenetically by co-infection with other chordopoxviruses in these cells.

MCV can infect human HaCaT keratinocytes and transcribes mRNA in these cells, but does not induce a CPE. MCV cannot infect nonhuman cells. It induces type 1 interferons in mouse and human embryo fibroblasts and IL8 in human lung epithelial cells, which it cannot infect, suggesting involvement of surface pathogen-associated molecular pattern (PAMP) receptors like TLR2 or TLR4. Removal of interferon pathways from cell lines susceptible for MCV infection would be worth investigating, in order to exclude interference causing abortive MCV infections. MCV is currently isolated from human-infected skin biopsies. MCV purified from biopsy material can be used for infection studies, electron microscopy, viral DNA extraction, and analyses of early mRNA synthesized by in vitro transcription of permeabilized virions.

1.5.4.7 Cell culture and animal systems

1.5.4.7.1 Abortive cell culture systems

Over many years a large number of primary cells and cell lines were tried for replication of MCV. MCV does not produce infectious progeny in these cells. Non-genetic

reactivation, demonstrated for other chordopoxviruses of different genera does not work with MCV (Postlethwaite, 1970; Pirie *et al*., 1971; Shand *et al*., 1976; Bugert *et al*., 2001). Only human fibroblasts and keratinocytes turned out to be susceptible for MCV infection and MCV early mRNAs can be isolated from abortively infected cells (McFadden *et al*., 1979, Bugert *et al*., 1999, Melquiot and Bugert, 2004). As early as 1967 Postlethwaite at the University of Aberdeen reported cell culture studies with MCV, initially using mouse embryo fibroblasts (Postlethwaite *et al*., 1967). At the same time biological assays for interferon activity were developed using the same mouse embryo fibroblasts and encephalomyocarditis virus as a readout system (Postlethwaite *et al*., 1967). It was found that prior infection of mouse embryo fibroblasts with MCV interfered with the development of EMCV CPE in a dose-dependent manner. Furthermore it was observed, that MCV does not seem to shut down host protein synthesis and does in contrast to vaccinia virus not seem to inhibit the cellular type 1 IFN response. These observations were confirmed recently for human cell lines. MCV induces IFN beta, but not IFN alpha or IL8, in human MRC5 and HaCaT cells.

1.5.4.8 MCV lesion core and biopsy material

Currently MCV is isolated from patient specimens. MCV purified from then debris core of MCV lesions or from biopsy material can be used for infection studies, electron microscopy, viral DNA extraction, and analyses of early mRNA synthesized by in vitro transcription of permeabilized virions (Melquiot and Bugert, 2004).

1.5.4.9 Foreskin xenograft models

MCV replication was observed in human foreskin grafts to the skin of athymic mice. MCV infected xenografts developed morphological changes indistinguishable from patient biopsies (Buller *et al*., 1995). In an independent approach infection with MCV type 1 and 2 virions induced similar histological changes in human foreskin fragments transferred to the renal capsule of athymic mice. Cytoplasmic inclusions containing typical poxvirus particles were seen within 2-3 weeks of implantation. Attempts to pass virus from one infected implant to another were not successful. These findings were confirmed by Paslin and co-workers (Fife *et al*., 1996; Paslin *et al*., 1997). Even though the xenograft models seem to work *in vivo*, the ‘Buller system’ is hampered by low

efficiency of MCV infected graft ‘take’ and a long (146 days) delay before the development of MCV inclusion bodies. Because only one mouse took the infected foreskin graft, there was no attempt to passage the infection. For the ‘Fife system’, though lesions developed faster, it was found that infectious progeny is not produced.

1.5.4.10 Virion, genome and evolution

Molluscum contagiosum virus (MCV) particles have typical poxviral morphology (Figure 1.8). The virions are enveloped, pleomorphic, but generally ovoid to brick shaped, with a dumbbell-shaped central core and lateral bodies similar to those in orthopoxvirus virions. MCV cores show complex structural patterns. Virions are often found to have membrane fragments loosely attached to them, indicating a non-continuous lipid envelope wrapping the core. The genome of MCV is a double-stranded DNA molecule of 190289 bp (GenBank accession U60315: MCV type 1/80) with covalently closed termini (hairpins) and about 4.2 kbp of terminally inverted repeats (Figure 1.8). This excludes 50–100 bp of terminal hairpin sequences that could not be cloned or sequenced because replicative intermediates are not apparent in DNA from MCV biopsy specimens. The genomes of MCV (genus *Molluscipoxvirus*, subfamily Chordopoxvirinae), crocodilepox virus, and parapoxviruses stand out in the family *Poxviridae* because they have GC contents of over 60%. The MCV genome encodes 182 non-overlapping open reading frames of more than 45 codons (Figure 5), almost half of which have no similarities to known proteins. Hypothetical MCV structural proteins and proteins encoding enzymes of the replication and transcription apparatus share obvious homologies to other poxvirus proteins.

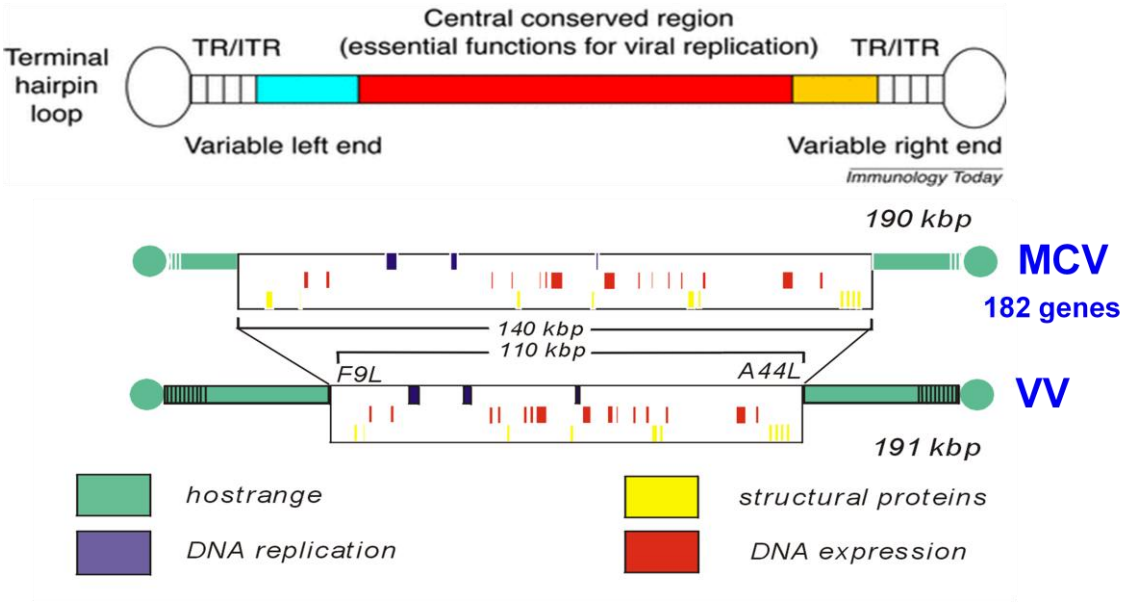


Figure 1.8. MCV genome alignment. *Schematic alignment of the genomes of MCV (190 kbp; top) and vaccinia virus (191 kbp; bottom). The central conserved portion of the MCV genome corresponds to the vaccinia genome between the genes encoding for vaccinia proteins F9L and A44L. Genes encoding amino acid homologs of structural proteins (yellow), proteins of the DNA expression (red) and replication machinery (blue) are positionally conserved between the two poxvirus genomes, genes without significant amino acid homology are interspersed. These include host range genes which are unique to both genomes.*

Less obvious homologies exist between MCV and avipoxviruses (MC130, MC133, and MC131 A-type inclusion body-like proteins) and notably between MCV, parapoxviruses, and crocodilepox virus (MC026 modified RING protein and a number of proteins shared between only two of the above poxviruses) (Chen *et al*., 2013).

Unique MCV non-structural proteins that are not involved in replication or transcription can be divided into two functional classes: (1) proteins dealing with the host immune system (host-response-evasion factors), such as the MCV chemokine antagonist (MC148) and the interleukin-18 (IL18)-binding protein (MC054) and (2) proteins supporting MCV replication in the host cell or the host tissue (host cell/tissue-modulating factors), such as the anti-apoptotic selenoprotein MC066 and the Hrs binding protein MC162 (Chen *et al*, 2013).

An epidermal growth factor (Butala *et al*., 2013) homolog similar to the ones expressed by other poxviruses was not found in the genome of MCV. The only other poxvirus that does not encode this factor is swinepox virus. However, MCV-infected basal keratinocytes seem to increase EGF receptor and transferrin receptor expression, in comparison to uninfected epidermis. Inducing EGF receptor expression may be an indirect mechanism causing epidermal hyperproliferation.

In a phylogenetic analysis of 26 poxvirus genomes, MCV (representing the molluscipoxviruses) formed a group by itself among the subfamily of chordopoxviruses, separate from avipoxviruses (fowlpox virus), orthopoxviruses (vaccinia and variola viruses), and all other genera. Four main genetic subtypes of MCV have been identified by DNA fingerprinting. MCV type 1 prototype is the most common genetic type (98%) in immune-competent hosts in Western Europe (Chen *et al*., 2013). MCV type 1 (including variants) is the most common genetic type worldwide. MCV types 2–4 are relatively more commonly seen in immunocompromised individuals. MCV genotypes discernable by DNA fingerprinting do not change when the viruses are transmitted between family members or in larger contact groups, indicating a low overall mutation rate.

1.5.4.10.1 MCV plasmid clone library and previous limited MCV genome sequencing projects

Using DNA fingerprinting with selected enzymes and denaturation/rehybridization studies, the genome of Molluscum contagiosum virus was found to have a very high G+C content of 63%, which differed from vaccinia virus (G+C content of 30%) and resembled more viruses of the herpesvirus family and parapoxviruses (Parr *et al.*, 1977, Darai *et al*., 1986). Purified DNA restriction fragments as well as recombinant plasmid clones derived by either single or double-digestion of genomic DNA from the subtype I of MCV and DNA hybridization were subsequently used for the establishment of the viral genetic map (Bugert *et al*., 1989; Bugert and Darai, 1991; Scholz *et al*., 1989) and for limited genome sequencing using Klenow enzyme, ss phage DNA and the radioactive Sanger-ddNTP protocols. Early sequencing studies already revealed that there was a centrally conserved poxviral gene arrangement (Blake *et al*., 1991) unlike the terminal regions, where neither significant nucleic acid homologies with the

vaccinia standard genome (Copenhagen), nor in fact any known gene sequences were found (Bugert *et al*., 1993). Limited sequencing was carried on until 1996 (Bugert *et al*., 1989; Hadasch *et al*., 1993; Bugert *et al*., 1993; Moratilla *et al*., 1997) when the complete genome DNA sequence of MCV type 1/80 was determined by fluorescent label sequencing at the LVD, NIAID, NIH in Bethesda, MD using the overlapping MCV genome fragment library established in the Darai lab in Heidelberg. This library has been made availabe to the ATCC in 2004.

1.5.4.10.2 Complete MCV genome sequence and phylogeny

The genome of MCV is a linear, double-stranded DNA molecule with a high GC (63%) content in comparison to vaccinia virus (30%), featuring about 4.2 kbp of terminally inverted repeats (ITR: Figure 1.8), typical of poxviral genomes. The genome of MCV type 1was completely sequenced in 1996 and found to comprise 190,289 bp (+ ~ 50 bp) (GenBank accession U60315: MCV type 1/80) of double stranded DNA with covalently closed ends encoding at least 182 hypothetical genes of 45 amino acids and longer (Senkevich *et al*., 1996; Senkevich *et al*., 1997). Most predicted proteins in the central part of the MCV genome show strong homology to structural proteins of other poxviruses, whereas all the proteins encoded at both ends and numerous proteins interspersed in the centre of the MCV genome are unique.

Initially, the MCV genome was described to encode 164 open reading frames (ORF), likely encoding hypothetical proteins: genes mc001R to mc164L, starting at ITRs (Senkevich *et al.*, 1996) However, a more detailed analysis subsequently revealed 182 genes, with 154 that were more likely to be coding genes (Senkevich *et al*., 1997), 105 hypothetical proteins have homologues to smallpox virus and other poxviruses. The remaining ORFs are unique to MCV and may be involved in the suppression of the host response to infection, nucleotide biosynthesis, and cell proliferation (Bugert and Darai, 1997; Senkevich *et al*., 1997). Most MCV genes conserved in other poxviruses are in the central part of the genome (Figure 1.8) whereas unique MCV genes with cellular homologs or no identified homologs are located on the flanks of the genome and dispersed between conserved genes (Figure 1.8) (Chen *et al*., 2013).

Only a few MCV genes have been analyzed for function (Figure 1.8) and these have been expressed in isolation in bacterial or viral expression systems. In large DNA viruses, gene functions are usually provided by several gene units working in a functional complex (e.g., the vaccinia entry-fusion complex) (Moss, 2006). Conventially poxviral hypothetical proteins with a likely function start with capital letters (e.g., MC007) while the genetic unit uses lower case letters (e.g. mc007). As an essential reference tool for any work with MCV genes, the Heidelberg MCV type 1/80 redundant genome fragment library was deposited in the Molecular Genomics Section of ATCC in 2004 (Chen *et al*., 2013). According to their function, these MCV genes can be divided into two groups: proteins supporting MCV replication in the host cells and genes encoding proteins interacting with the host immune system

1.5.4.10.3 MCV genes supporting replication and survival in the host cell

Several MCV genes with homologues in the vaccinia virus involved in virus replication are especially as targets for antiviral therapy through a variety of different mechanisms:

mc039L is a homologue of the vaccinia virus DNA polymerase E9L gene and is presumed to be the target of cidofovir (Magee *et al*., 2005). **MC142R**, a membrane protein predicted transmembrane helix homologue of vaccinia virus A33R, which is expressed on vaccinia virus infected cells and on EV, may be a possible anti-MCV drug target (Roper *et al*., 1996) To test the anti-MCV antivirals, a reporter system based on EGFP and firefly luciferase reporter plasmids under the control of a poxvirus consensus promoter has recently been established (Sherwani *et al*., 2012).

The mc007L displays no homology with other poxvirus genes. However, an E7-like LxCxE motif was observed and allows MC007L protein to induce a cytosolic sequestration of retinoblastoma protein (pRb) at mitochondrial membranes, leading to the inactivation of the protein by mislocalization. The deregulation of the pRb pathway plays a central role in virus interference with cell cycle and tumor pathogenesis and is notably used by human papilloma virus gene E7 (Mohr *et al*., 2008)

mc66L encodes a homologue of selenocysteine-containing glutathionine peroxidase. This protects infected cells from peroxide radicals and ultraviolet-mediated apoptosis. It may also explain how MCV replicates exclusively in the epidermis: MC66L mediated anti-apoptotic activity increases survival time of MCV infected cells in the UV exposed epidermis, allowing MCV a longer productive cycle (Shisler *et al*., 1998).

mc002L, mc161R, and mc162R were identified by Senkevich and coworkers in the 1996 genome sequencing project and designated a gene family (Senkevich *et al*., 1996). They encode homologues of the human signaling lymphocytic activation molecule (SLAM) protein CD150; thus named the SLAM gene family (Bugert *et al*., 2000) The proteins are expressed early as type I membrane proteins and MC002 and MC162 contain sites of PY motifs in their cytoplasmic domains, capable of interaction with cellular ubiquitin ligases AIP4 and NEDD4 and the Hrs endosomal switching protein. Overexpression of MC162 causes vesicle anomalies in human and mouse fibroblasts. Hypotheses currently investigated include MCV induced over-recycling of surface receptors (MC162), including EGFR, as observed *in situ*, competitive binding of cellular SLAM through an extracellular SLAM homology domain (MC002), and T lymphocyte activation (Bugert, 2007). This viral gene family may hence have a dual role in both the virus life cycle and virus directed immune evasion.

1.5.4.10.4 MCV genes implicated in immune evasion

MCV immunology will be discussed in terms of what the virus does to evade detection and the details of the eventual clearing immune reaction of the host. In MCV biopsy material, there is little or no inflammatory cell infiltration of the epidermis or surrounding dermis in undisturbed lesions. This changes, once the MCV lesion is exposed to the immune system and a full immune response is mounted (Vermi *et al*., 2011). It has been proposed that the initial lack of immune responses to MCV *in situ* is due to the activity of a number of host-response evasion genes present in the MCV genome and the absence of others found in other poxviruses (Figure 1.9) (Moss *et al*., 2000). Analysis of the MCV genome has revealed several candidate genes that could contribute to protection against the immune system (Senkevich *et al*., 1997).

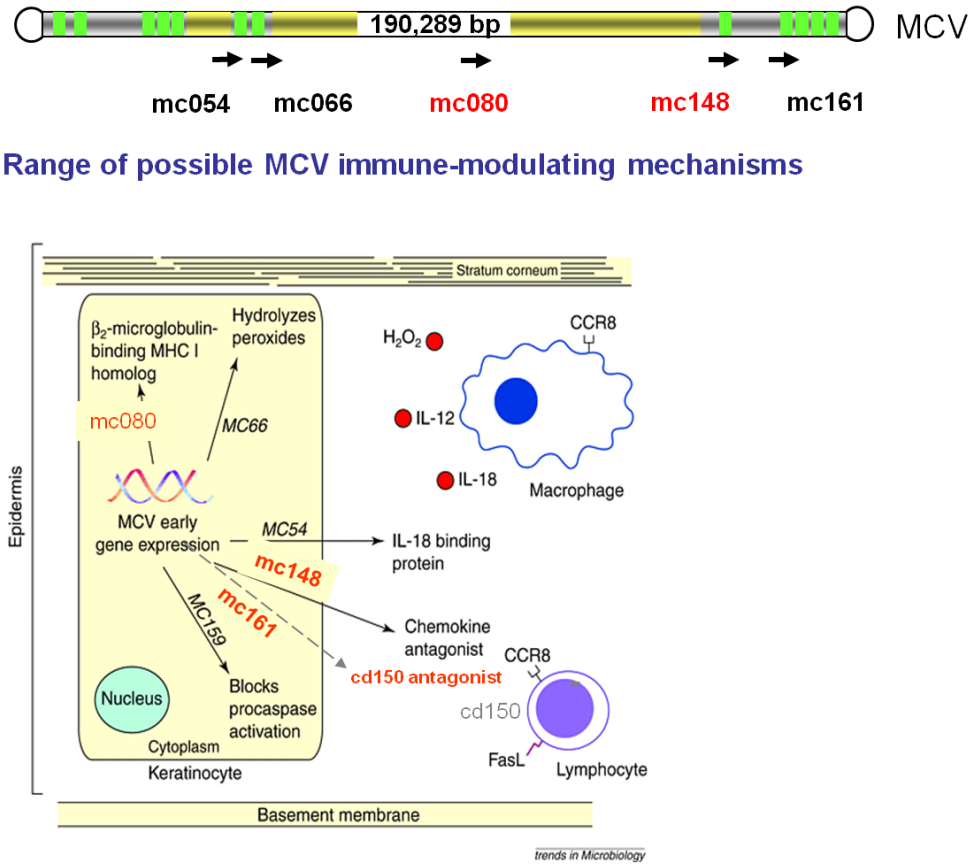


Figure 1.9. MCV Immune defense molecules. *Schematic of immune-defense molecules encoded by Molluscum contagiosum virus. MCV replicates exclusively in keratinocytes within the human epidermis. An MCV-infected keratinocyte that is expressing virus-encoded immune-defense molecules is depicted. The expression of selected antiviral molecules by a macrophage and a lymphocyte that have entered the epidermis in response to tissue injury is also shown. UV rays from the sun, as well as inflammatory cells, can generate peroxides. Abbreviation; MHC, major histocompatibility complex (Adapted from Moss et al. 2000).*

MC54L exhibits sequence similarity to human interleukin 18 binding protein (IL-18BP) and plays as a decoy receptor for IL18, which decreases the IL18 mediated synthesis of interferon gamma, activation of NK cells and Th1 response. Three MCV hypothetical proteins, MC051L, MC053L, and MC054L, have 20 to 35% amino acid sequence identities with human interleukin-18 (hIL-18)-binding protein (hIL-18BP), a naturally occurring antagonist of the proinflammatory cytokine IL18. MC54L is considered to participate in the virus evasion of the immune system (Xiang and Moss, 1999). The same group subsequently found the functional epitope of MC54L which enable its ability and affinity to IL18 (Xiang and Moss, 2001). Full length MC054 might

neutralize locally produced IL18, whereas the N-terminal fragment is soluble and free to systemically neutralize IL18 (Xiang and Moss, 1999a; Xiang and Moss, 1999b; Xiang and Moss, 2001; Xiang and Moss, 2003). This data has been confirmed and compared to the characteristics of other poxviral IL18 binding proteins, including those encoded by vaccinia, ectromelia and smallpoxvirus (Smith *et al*., 2000, Esteban *et al*., 2004).

MC80R, which has a 24.5% amino acid homology to human major histocompatibility complex (MHC) class I was detected in stable endoplasmic reticulum (ER) and Golgi compartments with β2-microglobulin. MHC-I molecule on cell surface may affect the Cytotoxic T lymphocyte (CTL)-mediated cytolysis and NK cell's killing ability (Lopez-Botet *et al*., 1996). However, direct experimental work with the intracellular MC80R has not yet been performed.

MC148R encodes a competitive chemokine receptor antagonist, CCR8, without agonistic activity (Chen *et al*., 2013; Luttichau *et al*., 2001). This MCV protein is probably the best characterized biochemically and in further applications. Initial studies suggested that the protein is secreted (Bugert *et al*., 1998) and demonstrated a broad range inhibitory activity against diverse beta chemokine receptors (Damon *et al*., 1998) as well as inhibitory effects on human hematopoietic progenitor cells (Krathwohl *et al.*, 1997). This, locus chemokine (ILC) is strongly and selectively expressed in the human skin (Ishikawa-Mochizuki *et al*., 1999). ILC has the highest homology to MC148R among the known human CC chemokines and is strongly and selectively expressed in the human skin (Ishikawa-Mochizuki *et al*., 1999). However, the observation that MC148R can inhibit allograft rejection in transgenic mice supported the theory it may possess anti-inflammatory properties (DeBruyne *et al*., 2000). MC148 is expressed early in the MCV lifecycle (Bugert *et al*., 1998), and may provide an anti-inflammatory activity gradient in the transition zone between epidermal and dermal tissue as a form of stand-off defensive device for the virus before other mechanisms engage. The fact that MCV produces a CCR8-specific antagonist at the early stage of infection underscores the importance of $CCR8^+$ peripheral immune surveillance T cells as sentinels in skin immunity (Luttichau *et al*., 2001). In healthy skin $CCR8^+$ T cells make up 50% of all

T_{PS} cells whereas in blood $CCR8^{+}$ T cells are scarce (<5%) (Schaerli *et al*., 2004; McCully *et al*., 2012).

Interestingly, some of the conserved genes that are important for immune evasion in other poxviruses and missing in MCV are homologs of the vaccinia virus E3L and K1L gene which are critical in the type I interferon induction (Meng *et al*, 2009) It is unclear if any and which MCV genes confer protection against type I interferon to MCV. Initial studies by Postlethwaite indicate MCV induces a strong type I interferon response in mouse embryo cells (Postlethwaite *et al*., 1967). However, MCV gene expression is abortive in all current *in vitro* models, and relevant genes may only be expressed in MCV lesions *in situ* and possibly as part of a multi protein complex.

1.5.4.10.5 Immune Response

In undisturbed MC lesions, histological studies have shown a conspicuous absence of effectors of the cellular immune system, in particular skin-specific tissue macrophages (Langerhan cells). The absence of macrophages has been attributed to the activity of various MCV genes that are suspected to make the MC lesion immunologically 'invisible'. This includes a biologically inactive IL8 receptorbinding beta-chemokine homolog (MC148), which may suppress the immigration of neutrophils; a major histocompatibility complex (MHC) class I homolog that may upset MHC class I antigen presentation on the surface of infected cells, or natural killer cell recognition; and an IL18-binding protein, which underlines the importance of this cytokine for the local immune response in human skin. As for the humoral response, MCV-specific antibodies have been detected in several studies, showing a seroprevalence of MCV of up to 40% in the general population, much higher than previously expected. However, these antibodies do not seem to confer a neutralizing immunity. MCV genes were expressed in a cowpox virus expression system and two antigenically prominent MCV proteins identified: mc133L (70 kD protein: MC133) and mc084L (34 kD protein: MC084) (Watanabe *et al*., 1998). These proteins are presumably glycosylated and present on the surface of MCV virions, where they allow binding of antibodies and detection by immune electron microscopy

Typical examples of MCV proteins with homologs in other poxvirus genera (avipoxvirus, parapoxvirus) are the p37k major capsid protein homolog (Blake *et al*., 1991; Sullivan *et al*., 1994) and the MCV DNA polymerase (Sonntag *et al*., 1995; Sonntag *et al*., 1996). Unique MCV genes can be divided into two functional classes: 1. genes encoding proteins dealing with the host immune system (host-response-evasion-factor) and 2. genes encoding proteins supporting MCV replication in the host cell or the host tissue (host-cell/tissue modulating-factors). MCV-specific *host-response-evasion-factors* have been extensively reviewed by several investigators (McFadden, 1998; Moss *et al*., 2000). For review of poxviral homologs of cellular genes see (Bugert *et al*., 2000). Typical MCV host cell/tissue modulating factorsare MC066, a selenocysteine-containing glutathione peroxidase that inhibits peroxide and UV mediated apoptosis and MC159, a FLICE inhibitor presumably inhibiting apoptosis in MCV infected keratinocytes. An epidermal growth factor homolog similar to the ones expressed by other poxviruses was not found in the genome of MCV-1. However, Nanney and coworkers observed that MCV infected basal keratinocytes show an increased density of EGF receptor and transferrin receptor expression, in comparison to uninfected skin (Nanney *et al*., 1992). In a phylogenetic analysis of 26 poxvirus genomes, MCV turned out to be the second-most divergent poxvirus genome in the subfamily Chordopoxviruses, after Avipoxvirues (Gubser *et al*., 2004). MCV seems to have a high degree of homology to crocodile poxvirus, a phylogenetically very old virus (G. Smith, personal communication).

1.5.4.10.6 MCV local immune response in the skin

In addition to the MCV proteins mentioned above which may mediate some degree of MCV immune evasion, the MCV viral colony sac (Shelley and Burmeister, 1986) inside infected cells in the epidermis also provides a layer of invisibility to the immune system, by physically separating MCV antigen from all pathways of presentation to the immune system.

Innate immune responses in MCV infection have not been extensively studied. Ku *et al.* revealed that in MC lesions, Toll-like receptor (TLR) 3 and TLR9 were strongly expressed on the epidermal keratinocytes; IFN-γ and TNF-α were predominately

localized adjacent to the MC bodies (Ku *et al*., 2008) In a hyper-IgE syndrome patient, deficiency of Tyrosine kinase-2 leading to reduced downstream cytokine signals involved in innate and acquired immunity explained the susceptibility to infection by various microorganisms including MCV (Minegishi *et al*., 2006).

Extensive MC has been described in patients with autosomal recessive DOCK8 combined immunodeficiency (Chu *et al*., 2012). DOCK8-deficient patients suffer from bacterial and viral skin infections and an increased incidence of malignancies (Zhang *et al*., 2009). DOCK8 regulates interstitial DC migration (Harada *et al*, 2012). Immunologically, there are two major patterns of response to MCV infection: lesions with immune cells absent and lesions with strong immunological activity. Initially, dense polymorphic lymphocytes infiltration was described in MC skin biopsies (Guitart and Hurt, 1999). However, subsequent studies showed that dendritic cells (DC) were absent in MC lesions but were normal or increasing in the perilesional normal skin (Bhawan *et al*., 1986). Lesions in the process of clearance by strong immune responses may be typical for the immunocompetent host where MC is self-limiting and benign. Patients with immune dysregulation disorders, especially those affecting the cellular immune response, are likely to have more extensive manifestations of MC. Atopic dermatitis, characterized by a T helper 2 (Th2) cytokine switching pattern, leads to more extensive MC lesions, that remain undetected for longer and may be resistant to immune therapy.

The above suggests that MCV in an uninflamed lesion is essentially invisible to the immune system through these multiple layers of viral defence mechanisms. This state of 'Virus suppression of immune responses' is summarized on the left of Figure 1.9. A recent study by Vermi and coworkers has shed light on the mechanism for spontaneous regression of MC, once the virus has been exposed to the immune system (Vermi *et al*., 2011). The extent of a full immune reaction against MCV followed by 'Clearance' is shown on the right of Figure 1.9. This response is overwhelming due to the high immunogenicity of the MCV virions, and comprises almost all elements of immune defense in the human skin.

Using histology and immunohistochemistry, Vermi *et al.* revealed a robust presence of CTLs, plasmacytoid dendritic cells (PDC), and a new class of interferon induced dendritic cells (IFN-DC) in inflammatory MC lesions, as compared to sparse leukocytes in non-inflamed MC lesions (Vermi *et al*., 2011). Kupper *et al.* revealed that skin resident memory T cells (TRM) are potent effector cells in the mice model after localized vaccinia virus (VACV) skin infection (Liu *et al*., 2010). They are superior to circulating central memory T cells at providing rapid long-term protection against cutaneous reinfection. But it is unclear if this mechanism applies to human skin. MCV induced skin immunity emerges in the study by Vermi *et al.* as a valuable model to study skin specific immune defences.

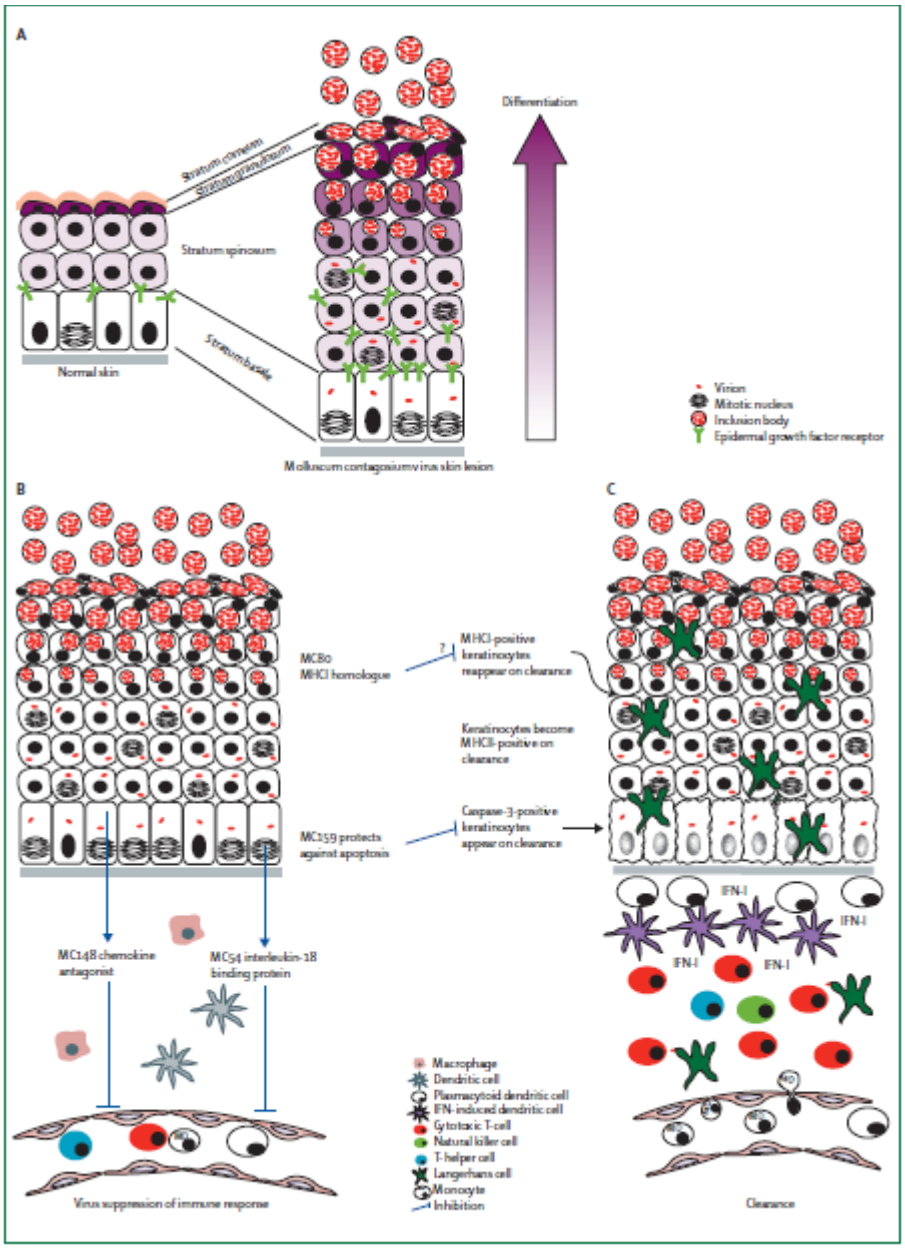


Figure 1.10. Lifecycle of molluscum contagiosum and local immune responses in infected skin. *MC lifecycle in human epidermis. (A) Arrow indicates direction of differentiation from the basement membrane (grey line at bottom) to thetop epidermal layers in the stratum corneum. Virions and inclusion bodies are shown in red, epidermal growth factor receptors in green; mitotic activity is indicated by chromosomes in metaphase. Viral suppression of immune response (B) Lesions are largely devoid of immune effectors in the undetected, non-inflamed state. Clearance (C) Lesions show high immunological activity. Immune effectors are indicated. IFN=interferon (Chen et al., 2013).*

Due to the lack of reagents to detect the virus proteins involved in MCV immune defence and replication in infected skin, or a suitable animal model, to date, no study

has clearly characterized the interaction between immune cells, keratinocytes, and MCV. It is also unclear why spontaneous regression may occur in some clinically inflamed lesions but not in others. MCV could be considered as a new poxviral vaccine vector due to the minimal and non-neutralizing preexisting immunity in the population. Once detected by the immune system it is a powerful immunogen, with the potential to enhance the immune reaction against an expressed transgene.

1.6 Research Aims

As highlighted in the introduction, Molluscum contagiosum virus (MCV) is a significant benign but underreported skin pathogen for children and adults. The inability to grow the virus *in vitro* has hampered biological studies and rendered the development of specific therapeutics difficult. The resulting lack of reagents has proven a major obstacle in further investigation and research. My doctoral research project focuses on aspects relating to the lifecycle of Molluscum contagiosum virus and the human immune response to it. In light of the fact that MCV research has suffered from the absence of reagents enabling its further investigation, one of aim of this PhD project was also to develop and design MCV gene specific reagents to study MCV lifecycle and immunity. The specific aims for each investigative chapter of this thesis are as follows:

Chapter 3 - The MCV lifecycle starts upon entry of the virus particle into human epidermal cells. Although it is not entirely clear where this point is exactly but popular belief is that it may be epidermal stem cells. Infections of epithelial type cells in vitro are non-productive, but produce early gene products. First step of any viral lifecycle is entry but due to the lack of a cell culture system MCV entry has till now not been quantitated so in order to determine whether MCV has entered cells. I have developed a GFP and luciferase based reporter assay and also a quantitative assay to calculate MCV multiplicity of infection (moi) as MCV is characterised by lack of cpe which are detailed in this chapter and have been published as a 'Methods Chapter' (Sherwani *et al*., 2012).

Chapter 4 - To enter cells Molluscum contagiosum virus uses homologs of a cell fusion complex identified in vaccinia virus to gain entry into cells; these are the MCV genes mc084 and mc133 both of which I have mostly worked with. Because of their role in entry these proteins are located on the surface of the virion particle, so most human antibodies versus MCV are raised against mc084 and mc133. I constructed truncated recombinant MCV genes (mc084 and mc133) for overexpression in *E. coli* and purified them. The resulting MC084 antigen was used to develop a sensitive recombinant MCV ELISA using water soluble and highly antigenic truncations of MC084L expressed in *E. coli,* which are more suitable for large scale production. MCV ELISAs based on *E. coli* expressed MCV antigen testing have so far not been reported. Using mc084 antigen in an ELISA format, we have conducted a study to determine MCV seroprevalence for serum collections from Heidelberg and Cardiff. We have used the reporter assay developed in chapter 3 to determine whether these antibodies can neutralize a MCV infection in vitro and whether there is cross reactivity with orthopoxviruses. This is the first such study in Europe (In press; PLOS ONE - PONE-D-13-44356R1).

Chapter 5 – A primary reason for the slow paced research in MCV has been the non-availability of antibody reagents as till now, no MCV specific antibodies exist. My final aim was the production and screening of novel monoclonal antibodies (mAbs) against specific MCV virion surface proteins MC084 and MC133. Selected monoclonal will be used in immuneohistology of MCV disease tissue/biopsies in an effort to better understand the distribution of MCV surface proteins in infected human tissue. These valuable reagents will further aid investigation of the virus.

Chapter 2 -

Materials and Methods

Chapter 2 - Materials and Methods

General Methods

2.1 Cell Culture Technique

2.1.1 Cell lines

All cell lines, aside from HaCaT, were obtained from the American Type Culture Collection (ATCC). A panel of both animal and human cells were used. The human cervical epithelial cell line, HeLa, was established from the cervical adenocarcinoma of a black, 31 year old female and contains Human Papilloma Virus 18 oncogenes. HEK 293T human kidney epithelial cells are highly transfectable derivatives of the 293 cell line, into which the temperature sensitive gene for SV40 T-antigen has been inserted. The human foreskin fibroblast cell line, BJ-1, has proved useful in transfection studies, and has a long lifespan in comparison with other human fibroblast cell lines. Cells of the immortalized human skin keratinocyte cell line, HaCaT, were provided by the German Cancer Research Centre (DKFZ). The animal cell lines tested included the rabbit kidney epithelial cell line, RK13, established from a 5 week old rabbit. It has demonstrated susceptibility to several poxviruses, including vaccinia and is positive for bovine viral diarrhoea virus (BVDV). The African green monkey kidney fibroblast cell line, CV-1, is a suitable transfection host, particularly by SV40 vectors. The 3T3 fibroblast cell line was established from Swiss mouse embryo culturesand BHK-21 Syrian Golden Hamster fibroblasts from the kidneys of one day old hamsters.

2.1.2 Preparation of supplemented media

Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose and L-glutamine without sodium pyruvate 1X (Gibco, UK), supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco, UK) was used for the culture of all cells.

2.1.3 Sub-culturing of cells

All cell lines were sub-cultured before reaching confluency (70% confluency), on average every 2 to 3 days. In order to propagate adherent cells culture medium was

removed and discarded and the cell monolayer was briefly rinsed with trypsin solution TrypLE™ Express (Gibco, UK) (1 ml per T75 flask) to remove all traces of serum that contains trypsin inhibitors. Fresh trypsin solution was added to flask (1 ml per T75 flask) and the cells were incubated at 37˚C until the cell monolayer was dispersed (usually within 5 minutes - inverted microscope observation). The cells were aspirated by gentle pipetting. Upon dispersion of the cells, 5 ml Dulbecco's Phosphate Buffered Saline (PBS) without $CaCl_2$ or $MgCl_2$ 1X (Gibco, UK) was added as a wash and removed. The remaining cells were re- suspended in 10 ml of supplemented media. Harvested cells were centrifuged at room temperature for 5 minutes at 500 g. Appropriate aliquots of the cell suspension were added to culture vessels in supplemented media.

2.1.4 Cell freezing

Cells were frequently frozen for storage and maintenance of laboratory stocks. Trypsinised cells, having reached 80% confluency, were transferred to 10 ml PBS and centrifuged at 1200 rpm for 5 minutes. Before placing the cells in liquid nitrogen, the cells are exposed to a gradual freezing process; an optimum cooling rate is an important factor during this process. When the freezing is slow extracellular ice is produced before intracellular ice crystal start to form leading to osmotic imbalance. As a result water migrates out of the cell preventing intracellular organelles damage due to ice crystal formation and recrystallization during the thawing process. During fast freezing less osmotic imbalance is observed however more intracellular ice is produced. Eukaryotic cells are therefore frozen at a slow rate with an addition of cryoprotectants like dimethyl sulfoxide (DMSO) or glycerol. DMSO acts at two levels; it interferes with the lattice structure of the ice reducing the formation of ice crystals and partially solubilises the membrane so that it is more resistant to damage. Freezing medium contains also high percentage of FBS to dilute DMSO and to reduce its toxic effect (Mazur, 1970). The cells (at 50-80% confluency) were suspended in freezing solution containing: 10% dimethyl sulfoxide (DMSO) (v/v) (Sigma-Aldrich, UK), 40% FBS (v/v) and 50% medium (v/v). The cells were transferred to a cryogenic vial (Nunc, UK). Cells were initially frozen at –70˚C for 24 hours in isopropanol containing vessel (for a

gradual freezing) before transfer to the vapour phase of liquid nitrogen at -196˚C for long-term storage.

2.1.5 Cell thawing

Cryogenic vial containing cells were removed from storage at -70˚C and snap- thawed at 37˚C. The 1 ml suspension was transferred to 10 ml pre-warmed supplemented media with FBS in a sterile T75 flask. After 4 hours incubation at 37˚C the cells underwent media change as to avoid potential toxic effects of DMSO.

2.1.6 Determination of cell number

Cell density was determined using a counting chamber (also known as haemocytometer). The entire counting grid of this chamber consists of 9 large squares (Figure 2.1). Each square has a surface area of 1 mm^2, and the depth of the chamber is 0.1 mm. To determine the final cell density in cells/ml, 15 µl of an aliquot of evenly suspended cells was applied to chamber and the cells in one large square were counted (using a light microscope). Then the total cell count was divided by 0.1 (chamber depth) and divided by 1 mm^2 (the total surface area counted). The count obtained in cells/mm^3 was multiplied by 1000 because there are 1000 mm^3 per ml.

Cell count calculation example

Each large square has surface area of 1 mm^2 and a depth of 0.1 mm, giving it a volume of 0.1 mm^3. If the total cell count in one large square is 80 (number of cells should be statistically significant, about 100 cells), then the cell count is 80 cells per 0.1 mm^3, which equals 800 cells per mm^3. This result needs to be multiplied by 1000 to determine cell count per ml. In this example, the final count is 800000 cells per ml.

After trypsinisation, dispersed cells were aseptically removed and transferred to a 25 ml universal container. A trypan blue solution was prepared by diluting stock 0.4% trypan blue (Sigma, UK) with PBS in a 1:5 ratio. 10 µl of the cell suspension was added to 10 µl of the solution and left to stand for one minute to ensure adequate staining. A

haemocytometer, washed with 70% ethanol, was loaded with 10 µl of the cell/trypan mix.

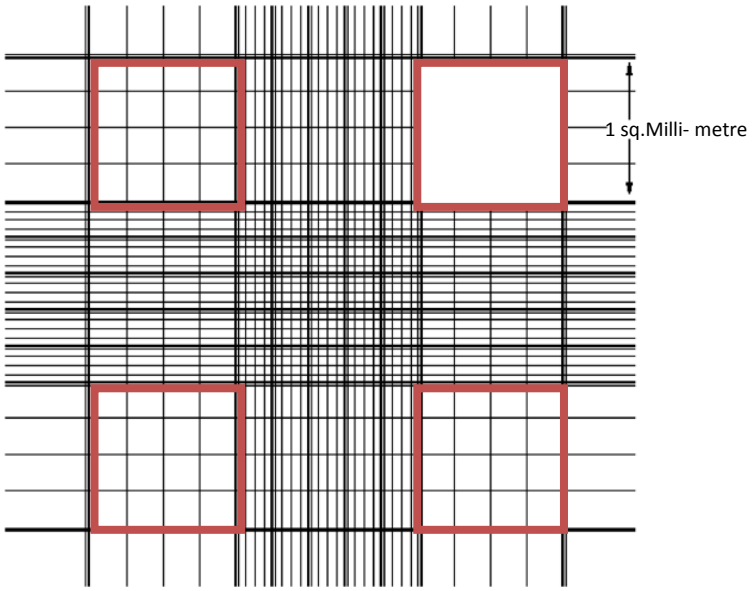


Figure 2.1. Haemocytometer counting grid. *The number of cells in each of four large squares was counted with use of light microscopy and an average taken. Allowing for the PBS dilution, the cell concentration per ml is the average cell count per large square x* 10^5.

2.2 Virus Preparation

2.2.1 VACV

Western Reserve (WR) strain VACV, kindly donated by Bernard Moss, was prepared for stock in different eukaryotic cells following adherent cell protocol. The virus yield was separated into cellular attached virus and supernatant (sn) two days p.i. For the purpose of experiments sn VACV preparations were used. It was decided this was sufficient as VACV was only used as a positive control. The various preparations were subsequently used in infection experiments to deduce the form that yielded optimal signal.

2.2.2 MCV

MCV preparations were made by Dounce homogenising clinical specimen material, taken from a collection of molluscum lesions from Hieldelberg, Germany, collected from 1999 to 2003 and available in the Bugert Lab. For the purposes of the reporter

assay, it was necessary to establish the quantity of virus required to elicit a robust signal. Initially, 4 lesions were used, and then 21. The signal from the latter quantity forms the basis of the results.

2.3 Specific Methods

2.3.1 Preparation of reporter plasmids

2.3.1.1 Preparation of agar plates and liquid bacterial growth medium

The 7.8 g of Columbia Agar Base (Oxoid, UK) was dissolved in 200 ml of distilled water. Agar solution was sterilized by autoclaving for 20 minutes at 121˚C. In order to prepare plates the agar solution was transferred from autoclave to water bath at 45˚C for at least 20 minutes and then 20 ml poured into each 8.25 cm diameter plastic disposable plate (Sterilin Ltd., UK) (20 ml creates a layer of agar 3.5 mm thick) and plates were allowed to cool following overnight incubation at 37˚C. When selective ampicillin plates were prepared 1 µl of 100 mg/ml antibiotic stock solution was added for each 1 ml of media. Ampicillin was added at 42˚C in water bath.

Liquid bacterial growth broth – LB broth (Luria Bertani broth) was prepared by adding 10 g of Tryptone Soy Broth (Oxoid, UK), 5 g of yeast extract (Bio Gene, UK) and 10 g of NaCl to 800 ml of distilled water. pH was then adjusted with NaOH to 7.5 and the volume was adjusted to 1000 ml with distilled water. LB broth was sterilized by autoclaving for 20 minutes at 121˚C and stored at room temperature. When selective ampicillin liquid bacterial growth broth was prepared 1 µl of 100 mg/ml antibiotic stock solution was added for each 1 ml of media.

2.3.1.2 Bacterial growth and expansion

A single bead of transformed *E. coli* from frozen stock (-70˚C) was smeared repeatedly across an agar plate, and incubated overnight at 37˚C. The following day a colony was used to inoculate 4 ml growth broth, Luria Broth (LB), containing 4 µl of 100 mg/ml ampicillin. The culture was incubated for 8 hours at 37˚C then expanded to 100 ml LB with 100 µl ampicillin. Further incubation proceeded at 37˚C for 16 hours in a shaking incubator at 80 rpm (Gallenkamp, UK).

2.3.1.3 Transformation of bacteria

The starter culture was grown in 10 ml of LB medium overnight in 37˚C shaker. The following day 1 ml of Top 10 bacteria was washed 3 times with endotoxin-free water by centrifugation for 1 minute at 500 x g and the pellet was resuspended with 40 µl of endotoxin-free water. 10 µl of plasmid DNA in TE buffer were added to bacteria. DNA suspended in bacteria was transferred gently to an electroporation cuvette and electroporated with a Gene Pulser apparatus (Bio-Rad, UK) that was set at 2.5 kV, 25 µF, and 400 Ω. 400 µl of LB broth was added immediately to the cuvette and cells were incubated in an eppendorf tube for 30 minutes at 37˚C to recover. The electric pulse causes the formation of temporary aqueous pores in cell membrane by disruption of phospholipid bilayers. This damage results in increased electric potential across the membrane so that charged molecules like DNA move through the pores. As this movement takes place the cell membrane discharges and the pores quickly close. Transformed bacteria were then plated onto ampicillin selective agar plates (ampicillin corresponds with the resistance offered by plasmids used in these studies) and the plates were incubated overnight at 37˚C.

After incubation a single colony was selected and grown in 10 ml of selective ampicillin liquid bacterial broth overnight in 37˚C shaker. 100 µl of this culture were transferred in 100 ml of selective LB medium and grown again overnight in 37˚C shaker before plasmid purification.

2.3.1.4 Purification of reporter plasmids

All plasmids were purified using the Qiagen® Plasmid Midi Kit (100) (Qiagen, Germany). The bacteria were centrifuged at 3000 rpm for 30 minutes, the supernatant decanted and the pellet re-suspended in 4 ml of Buffer P1, to which RNase had been added. 4 ml of Buffer P2 was added, then 4 ml of Buffer P3. The lysates were cooled on ice for 15 minutes then filtered (Whatman, UK). Filtrates were applied to pre-equilibrated midi columns and washed twice with 5 ml Buffer QC. The cleared lysates were applied to pre-equilibrated anion exchange midi columns. DNA interacts with the positive charges on the surface of the resin. Under moderate salt condition, plasmid DNA binds to the resin while proteins, carbohydrates and other impurities are washed

away with wash buffer. The washing was performed twice with 10 ml of wash buffer with high salt concentrations. The plasmid DNA was eluted under low salt condition with 5 ml of elution buffer QF. The columns were allowed to drain by gravity flow. 3.5 ml of isopropanol were added to precipitate the plasmid DNA. The DNA preparation was centrifuged in Beckman centrifuge tubes at 13500 rpm for 15 minutes at 4˚C using JA21 rotor and Sorval high speed centrifuge. The pellet was washed with 3 ml 70% ethanol and re-centrifuged. The tube was dried horizontally at room temperature and the plasmid DNA resuspended in 100 µl TE buffer.

2.3.1.5 Determination of DNA concentration

Plasmid DNA concentration was measured using an ND1000 V3.3.0 Nanodrop Spectrophotometer and the corresponding ND1000 V3.3 computer program. An example of the readout obtained can be seen in Figure 2.2.

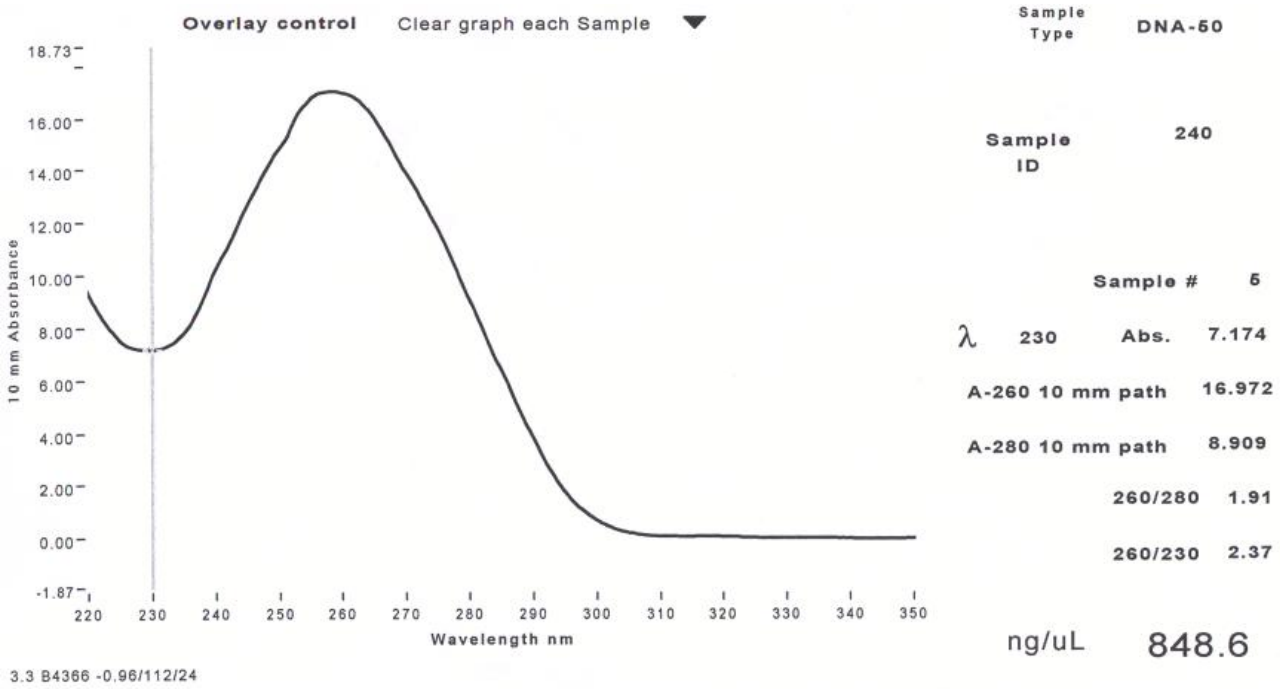


Figure 2.2. Measurement of DNA concentration. *Report obtained for measurement of DNA concentration of plasmid #240 using Nanodrop 1000 Spectrophotometer.*

2.3.1.6 Restriction digest analysis and agarose gel electrophoresis

20 units of *XhoI* and *BamHI* restriction enzymes plus 10x Surecut buffer B (Roche, Germany) were used to digest the 5 µL of DNA in TE Buffer, in a total volume of 50 µl. This was incubated at 37˚C for 2 hours. The digested DNA was combined with 10 µl loading buffer and inserted into a corresponding well of a vertical gel electrophoresis instrument (Hoeffer-Pharmacia SE280, UK). 1X electrophoresis buffer (EPB) was used as a running buffer. Two molecular weight ladders (1 kbp and 100 bp) were used as size markers (Fermentas, UK). Electrophoresis on 2% agarose gel took place for 90 minutes at 100V and was stained with ethidium bromide solution (200 µg/ml). A BIORAD UV transiluminator was used to view the DNA.

2.3.2 Dual luciferase reporter assay

The Dual-Luciferase® Reporter Assay System (Promega, UK) allows for simultaneous measurement of two reporter enzyme activities (luciferases) within a single sample. One enzyme is derived from the light-emitting organ of the firefly (*Photinus pyralis*) another from sea pansy (*Renilla reniformis*). The reporter gene is under the control of the target promoter. The luciferase activity correlates with the target promoter activation. The *Renilla* reporter gene is under the control of the herpes simplex virus (HSV) thymidine kinase (TK) promoter. The *Renilla* luciferase activity provides an internal control to normalise against transfection efficiency. Firefly protein is a 62 kD molecular weight oxidase. This enzyme requires ATP, molecular oxygen, Mg^{2+} and firefly luciferin as a substrate to generate light. *Renilla* luciferase is 36 kD protein which catalyses coelenterazine oxidation by oxygen to produce light (Figure 2.3).

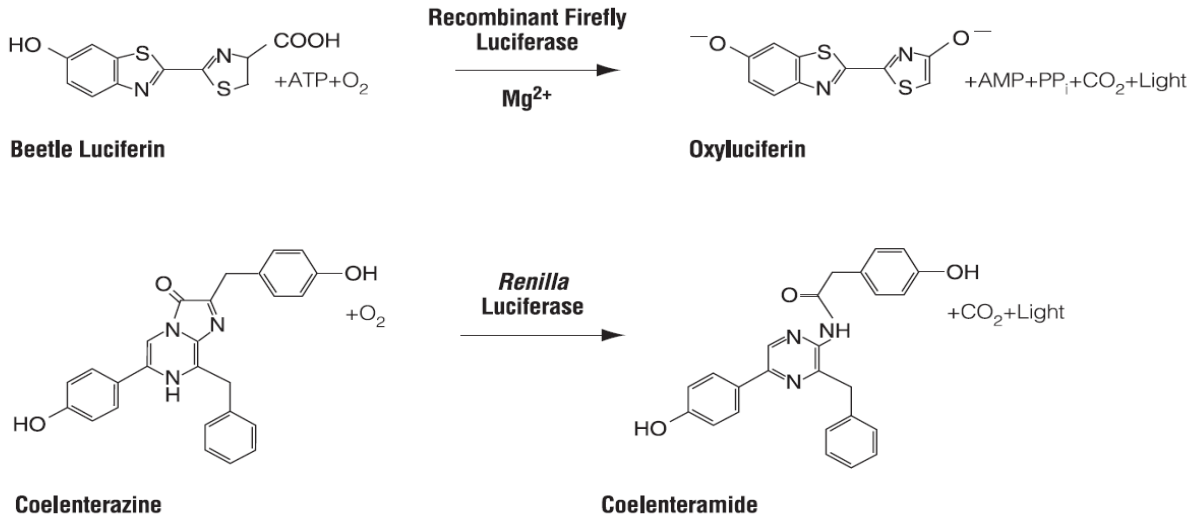


Fig. 2.3. Bioluminescent reactions catalysed by firefly and *Renilla* luciferases *(Adapted from Promega).*

2.4 Assessment of Molluscum contagiosum virus infectivity

2.4.1 MCV Luciferase Reporter Assay

Molluscum contagiosum virus isolated from human tissue specimen was integrity checked, quantitated by PCR and used to infect a series of epithelial and fibroblast type cell lines of human and animal origin. The cells were subsequently transfected with a reporter plasmid encoding firefly luciferase gene under the control of a synthetic early/late poxviral promoter and a transfection control plasmid. After 16 hours cells were harvested and tested for MCV infection, interferon production and luciferase expression. If eukaryotic cells are transfected with reporter plasmid followed by virus entry into cells, the MCV early transcription complex should recognize the E/L synthetic poxviral promoter (pE/L) of the reporter plasmid resulting in firefly luciferase expression, which can also be quantified. Reaction of firefly luciferase present in the cell lysate with firefly luciferase substrate (Beetle Luciferin) produces luminescence, which can be quantified as an indirect measurement of virus uptake.

2.4.1.1 Plasmids

All experiments involving the use of genetically modified materials were performed in the Bugert lab (L2) under the GM permissions GM 130/53 and GM 130/07.5. All plasmid DNA was purified using 100 µg capacity midiprep-columns (HPVNA kit - Roche) and then stored in elution buffer at −20˚C until used.

1. *PCR control plasmid*. The complete MCV-1 genome was cloned (Bugert and Darai, 1991) and sequenced (Senkevich *et al*., 1996; Senkevich *et al*., 1997) and the redundant MCV genome fragment library of MCV type 1 was submitted to ATCC for safekeeping in 2003 and 2008. For the quantitative PCR assay, the genomic MCV-1 *EcoRI* fragment C (25,516 bp) cloned into bacterial plasmid vector pACYC184 was used as a MCV target control (pyMCV1-E-C, Figure. 2.4a).

2. *Transfection control plasmid*. Plasmid phRG-TK (Promega GenBank accession number AF362545: 4,045 bp), expressing renilla luciferase under the control of the herpes simplex virus TK gene promoter. In this protocol, this plasmid is called p238 and used as plasmid transfection control (p238 Promega; Figure. 2.4b).

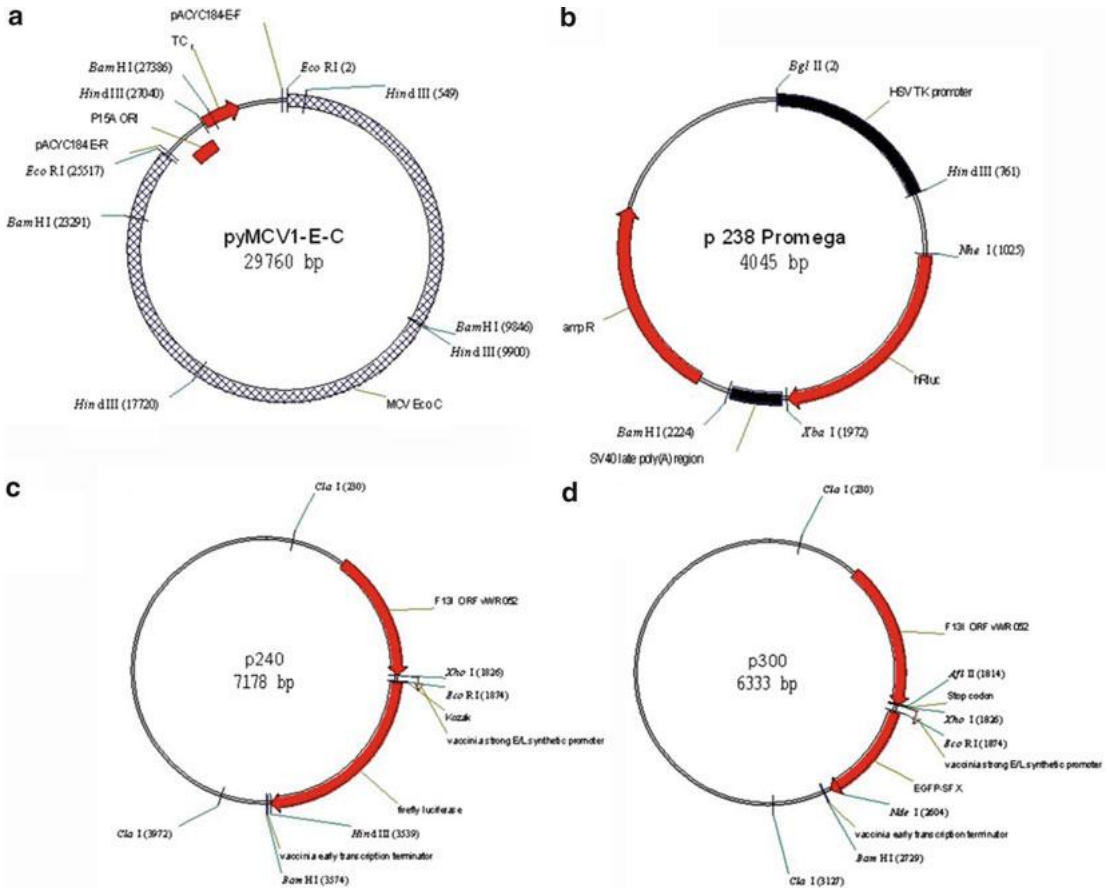


Figure 2.4. Plasmid constructs. *VectorNTI drawings for the recombinant plasmids (a) pyMCV1-EcoRI-fragment C (pyMCV1-E-C; available from ATCC molecular section), (b) phRG-TK (Promega; Internal lab reference number p238), (c) pRB21-pE/L-FF luciferase (p240), and (d) pRB21-pE/L-EGFP-SFX (p300) (Sherwani et al., 2012).*

3. *Poxviral luciferase reporter plasmid.* The coding sequence of firefly luciferase (*Photinus pyralis* GenBank accession number M15077) was amplified with a modified Kozak sequence by PCR and ligated into the pRB21 donor plasmid (kind gift of B. Moss) (Blasco and Moss, 1991) using the *EcoRI* and *HindIII* restriction sites in the donor plasmid multiple cloning site, resulting in the pRB21-E-Koz-Firefly luciferase-H (also called pRB21-pE/L-FF luciferase) construct of 7,178 bp with the internal lab designation p240 (p240, Figure. 2.4c).

4. *Poxviral EGFP reporter plasmid*. The coding sequence of EGFP was amplified from a commercially available plasmid with a modified Kozak sequence by PCR and ligated into the pRB21 donor plasmid using the *EcoRI* and *NheI* restriction sites in the donor

plasmid multiple cloning site, resulting in the pRB21-E-Koz-EGFP-X-flag-strepII-N construct (also called pRB21-pE/L-EGFP-SFX) of 6,333 bp with the internal lab designation p300 (p300, Figure. 2.4d).

5. *IFN-β firefly reporter plasmid*. This plasmid was referred to by us as IFN-β firefly reporter plasmid (ILR#231). The pGL3-IFN-β-Luc plasmid was generated by Lin and co-workers (Lin *et al*. 2000) by cloning the *Eco*RI-*Taq*I fragment (−280 to +20; filled in with the Klenow enzyme) from pUCβ26 into the *Nhe*I site (filled in with the Klenow enzyme) of the pGL3 basic vector (Promega) (Figure 2.5).

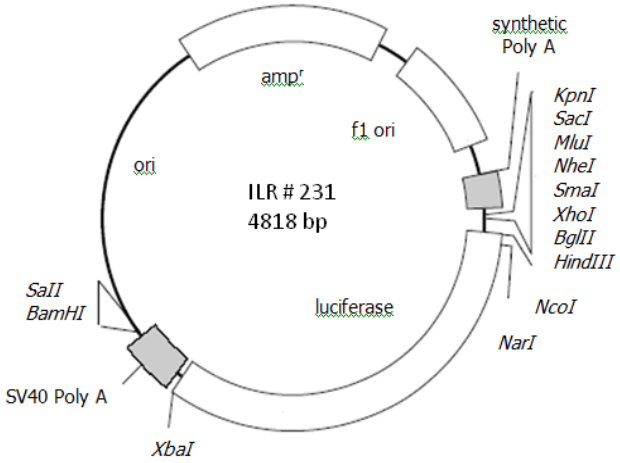


Figure 2.5 IFN-β firefly reporter plasmid *(ILR#231) Schematic showing firefly luciferase reporter gene.*

2.4.2. Infection – Transfection: Luciferase assay

2.4.2.1 Infection/ Transfection

Enough 12-well plates were prepared containing human HEK293 (ATCC CRL1573) cells in growth media (DMEM, high glucose with glutamine –Life Technologies, 10% FCS - Gibco) to allow for infection/transfection in triplicate for each experimental condition (including a mock that will be transfected but not infected, as well as wells that will be harvested at 16 h and wells that will be continued to be incubated for days). Transfection efficiency can vary considerably from cell batch to cell batch. Passage number (best low), cell confluence (best below 60%), and time of culture prior to

experiment (best no longer than 24 h) are determinant factors. Virus aliquots are thawed, sonicated, and kept on ice. Plasmid DNA is thawed and and kept on ice. Transfection reagents OptiMEM (Life technologies) and Lipofectamine 2000 (Life technologies) at room temperature (RT) are used to prepare transfection mixes by adding a dilution of 2 µl of Lipofectamine 2000 in 50 µl of OptiMEM to a dilution of 0.3 mg of each plasmid DNA (p240 FF reporter and p238 transfection control plasmid, p300 EGFP reporter) in 50 µl of OptiMEM. These are mixed gently for 15 min at RT in the dark to allow formation of transfection complexes. The growth medium is removed from HEK293 cells and 100 µl of transfection mix is added in each well. 100 µl each of ice-cold virus in PBS and 100 µl of transfection mix at RT are combined and the mixture is transferred into appropriate wells of HEK293 cells. While this protocol has included transfection of adherent cells, we have found that for some harder-to-transfect cells (e.g., human fibroblasts) we can get higher transfection efficiency when cells are in suspension. The assay depends to a significant degree on the transfectability of the cell cultures involved. Human keratinocytes and fibroblast cell lines are most interesting as possible natural hosts for MCV, but they are also hard to transfect. The cells are incubated for 16 h at 37˚C in 5% CO_2 atmosphere. The incubation time of 16 h allows for a robust signal from the transfection control plasmid p238, so firefly signal readings can be adjusted to renilla transfection efficiency readings between experiments.

2.4.2.2. Microscopy and collection of cells for luciferase assay

At 16 h post infection (p.i.), cells transfected with the GFP reporter plasmid are inspected using live cell microscopy and GFP-positive cells are documented noting that MCV does not show GFP positive cells after 16 h, whereas WR shows multiple GFP positive cells. Upon further incubation for another 4 days (5 days p.i.), some individual cells in the MCVinfected wells will show medium to strong GFP signals.For luciferase assay, at 16 h p.i., adherent cells in wells are washed once with PBS and 100 ml of 1× passive lysis buffer (part of the Dual-Luciferase® Reporter Assay System from Promega) is added to each well. The samples for the luciferase assay are collected at 16 h post infection. This allows the control plasmid ILR#238 to get to the nucleus and be expressed to yield a robust control signal. In HEK 293 cells, the Renilla luciferase signal can be seen in vaccinia infected cells after 2 h, and is seen in the MCV infected

cells after 8 h. The 12-well plate is covered with clear film plate protectors to stop evaporation and incubated with agitation on a belly-shaker at RT for 15 min. Plates are then frozen at −20˚C for at least 15 min or stored overnight or for up to 2 weeks before assayed. Cell lysates are tested for luciferase activity by adding 100 µl Dual Luciferase Assay Substrate (part of The Dual-Luciferase ® Reporter Assay System from Promega**)** to each well. If one does not have instrumentation that can add 100 µl of PROMEGA Dual luciferase firefly substrate and pipette series of four samples in a row, then load the plate, and read. The reading time, including the initial shake for four samples, is 20s. This results in a signal loss per reading of <1%, which is less than the sample-to-sample variation in triplicate samples, when compared to machine pipetting sample per sample. Luciferase activity is then measured in a FLUOROSTAR Luminometer. Data is compiled in a Microsoft EXCEL file and evaluated using standard statistical protocols (average, standard deviation, Student's *P* test). It is worth noting that the incubation time of 16 h allows for a robust signal from the transfection control plasmid p238, so firefly signal readings can be adjusted to renilla transfection efficiency readings between experiments. The signal from early poxviral promoters can be used as a surrogate parameter of viral infectivity. The signal can be further dissected and used to look at early and late transcription activity using transfected plasmids with a reporter gene behind the respective promoters in isolation.

2.4.3. Quantitative PCR assay

2.4.3.1 PCR design

To design a MCV quantitative PCR we needed to correlate MCV genome units with genome units of a sister virus which could be titrated i.e. vaccinia virus. MCV and VACV genomes shown below were assessed for homology using BLAST2N (Figure 2.6).

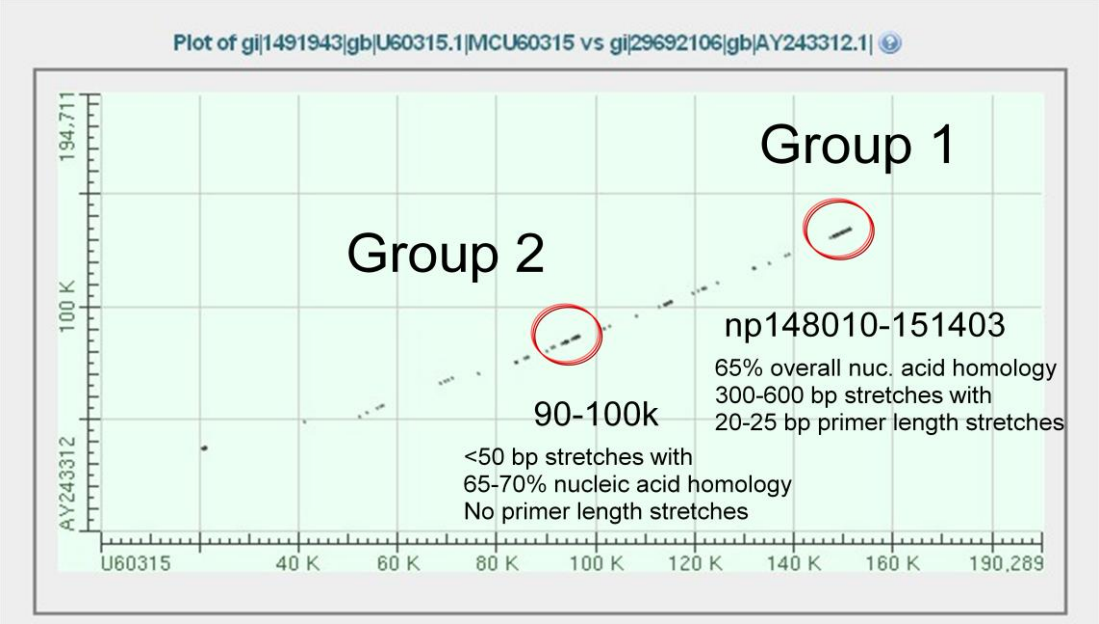


Figure 2.6. BLAST 2N – MCV vs VACV.

Group 1 consisted of genes mc128 and mc129. Of these the hypothetical ORF mc129R (Figure 2.7), which is the putative second largest subunit of DNA-dependent RNA polymerase, was selected for the design of primers (Figure 2.8) which could be used to detect identical reaction rates in MCV as well as VAVC

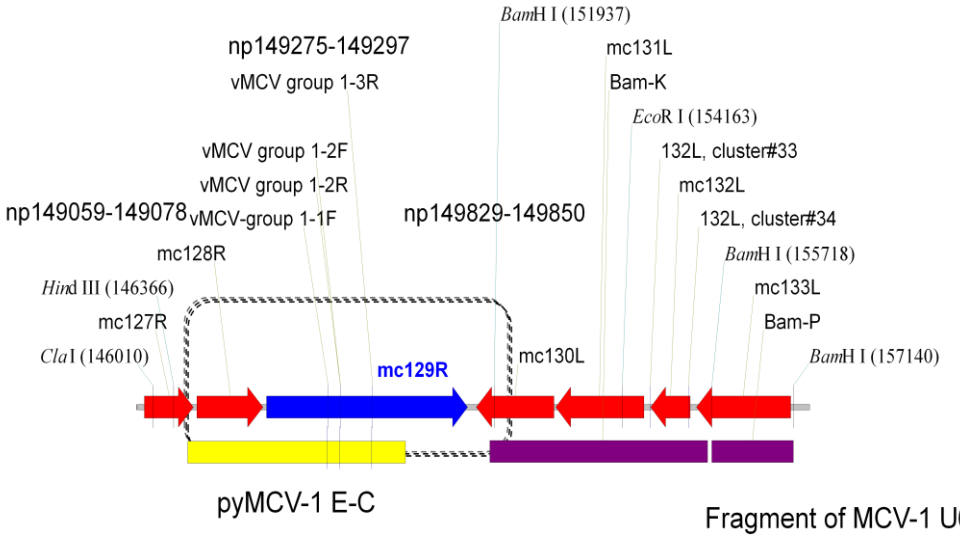


Figure 2.7. MC129R. *Putative second largest subunit of DNA-dependent RNA polymerase.*

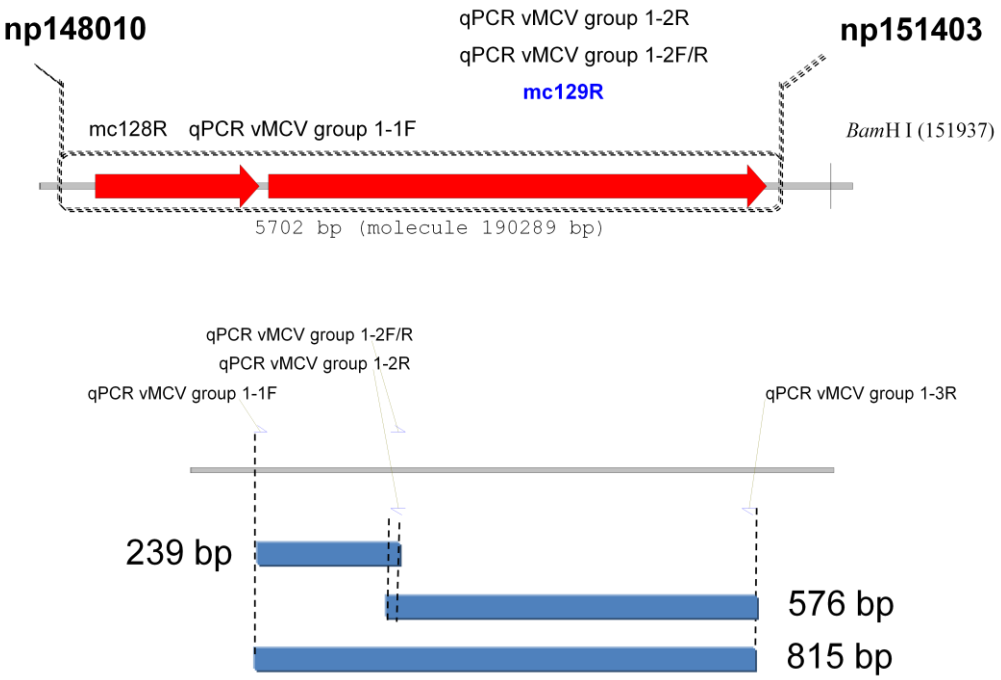


Figure 2.8. qPCR primer design

2.4.3.2. Virus and DNA preparation

Equal volumes of freshly thawed virions (100 µl aliquot) are incubated in 100 µl DNAse/*BamHI* buffer for 30 min at 37˚C. Viral genomic DNA is extracted using a HPVNA kit (Roche) following the manufacturer's instructions. The control plasmid pyMCV1-EC (Figure. 2.1a) was prepared using the same procedure. The DNA concentration of the control plasmid was determined using a Nanodrop Spectrophotometer or a similar device. Molecule numbers were calculated using the average molecular weight of DNA molecules and Avogadro's number (6.02×10^{23} per mole). The molecular weight of a plasmid (in Daltons) can be estimated as MW of a double-stranded DNA molecule (http://www.epibio.com/techapp.asp) = (# of base pairs) × (650 Da/base pair). The plasmid pyMCV1-E-C has 29,760 bp. Thus, the molecular weight is calculated as 19.344 MDa and thus 19.344 ng of plasmid would be 6.02×10^{8} mol. The actual plasmid concentration was 21 ng/µl (±1.7) and, thus, represented 6.5×10^{8} mol/µl. From this value, the molecule numbers for the pyMCV1-E-C twofold dilution series are calculated. The molecule numbers are then correlated to the pixel numbers of bands on a gel quantitated by the software ImageJ.

2.4.3.3. PCR reaction

MCV was isolated from human skin biopsy material as described previously (Melquiot and Bugert, 2004) and kept in 100 µl aliquots frozen at −70˚C in PBS. VACV-WR, vaccinia virus, strain WR (gift of B. Moss) was prepared and purified from infected HeLa cells, titrated in BSC-1 cells, and kept in 100 µl aliquots frozen at −70˚C in PBS. We prepared vaccinia virus and MCV preparations in 1 ml PBS and then immediately made ten 100 µl aliquots which were frozen. The vaccinia virus stock was generated by infecting one T150 flask containing adherent HeLa cells. Harvesting of the infected cells and resuspending them in 1 ml PBS yielded a titer of 2×10^7 pfu/ml. Thus, each of the ten 100 µl aliquots of vaccinia virus used here contained 2×10^6 pfu. The 100 µl aliquot of MCV contained an unknown number of MCV particles, but is quantified using the described PCR method. Primers outlined in Table 2.1 were suspended in injection-grade water to a final concentration of 100 pmol/µl and stored at −20˚C.

Table 2.1.MCV–VACV quantitative PCR assay primers (Sherwani *et al*., 2012).

Primer ID	Primer sequence (nhb)	Primer length	Product size (nucleotide position), GenBank Acc. #
Mcv129 1-2F149275	5′-CCGCACTAC TCCTGGATGCAGAA-3′	23	576 bp (149,275–149,850), U60315
Mcv129 1-3R149850	5′-CTGGATGTC GGAGAAGGTCATG-3′	22	
VACV-WR 1-2F132482	5′-CCTCACTAT TCATGGATGCAGAA-3′ (3)	23	573 bp (132,482–122,054), AY243312
VACV-WR 1-3R133054	5′-CTGAATGTC AGAGAATGTCATG-3′ (3)	22	

nhb nonhomologous bases underlined (number of mismatches)
Primers were designed using BLAST2 (NCBI: http://blast.ncbi.nlm.nih.gov/) alignment of MCV (GenBank accession # U60315) and VACV-WR (GenBank accession # AY243312) genome sequences and Vector NTI vs. 4.0, 1994–1996 InforMax Inc.

Two-fold dilutions of viral genomic DNA and plasmid control were prepared in injection-grade water and store at −20˚C. PCR assays were prepared as outlined in Table 2.2. PCR reaction conditions are included in the table.

Table 2.2 PCR Reaction (Sherwani *et al*., 2012)

	Volume (µl)
Primer 1-2F[a] (100 pmol/µl)	0.5
Primer 1-3R[a] (100 pmol/µl)	0.5
Injection-grade water	36.8
10× PCR buffer	5.0
TaKaRa dNTP (0.2 mM)	2.0
Template (series of twofold dilutions)	5.0
AmpliTaq 360 polymerase (1 unit of 5 U/µl)	0.2
Total	50.0

PCR reaction: 2 min of denaturation at 96˚C; and then 45 cycles of 1 min at 96˚C, 2 min at 55˚C, and 3 min at 72˚C. Block and then cool to 10˚C. For MCV Primer 1-2F and Primer 1-3R, use MCV129 1-2F149275 and MCV129 1-3R149850, respectively. For WR Primer 1-2F and Primer 1-3R, use VACV-WR 1-2F132482 and VACV-WR 1-3R133054, respectively (Sherwani *et al*., 2012).

PCR bands were visualised by loading a 2% agarose gel with 10 µl from each PCR reaction and running gel for 1 h at 100 V (constant voltage). This was stained with ethidium bromide, photographed with a digital unit and export into a jpeg file. The source template of the PCR products produced can also be determined by *XhoI* digest, which cleaves the MCV product into 227- and 349 bp sub-fragments but does not cleave the VACV-WR PCR product. To quantitate the PCR product, one can use the captured bands on the jpeg photograph with a series of identical gates using the Image J software (Wayne Rasband - wayne@codon.nih.gov) Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). The conventional PCR assay described has the problem ofassay-to-assay variation due to agarose gel and staining artefacts.Future advances may be the development of a real-timePCR assay using molecular Taqman probes (Life technologies) specific to eitherVAVC-WR or MCV and binding in the internal section of therather large PCR product (550 bp). Quantitative results of digital imaging (quantified pixel output) of a two-fold dilution

series of plasmid pyMCV1-E-C are plotted against molecule numbers using Microsoft EXCEL software. The molecule/genome equivalent numbers are taken from the calibration plot and compared to the pixel readings obtained for VACV-WR. Results are tabulated and evaluate using standard statistical protocols (average, standard deviation, Student's *P* test).

2.5 MCV gene specific constructs

2.5.1 Bioinformatics

NCBI Blast was used to establish amino acid homologies between mc084 and vaccinia H3L (VACV097). Vector NTI (vNTI) was used to produce virtual molecules and schematic diagrams of constructs prior to molecular cloning (InforMax, Inc), TMHMM was used to determine hydrophobic and transmembrane regions whereas the Kyte Doolittle plot was used to identify hydrophilic regions with predicted high antigenicity.

2.5.2 MCV expression constructs

The MCV genes of interest (mc084 and mc133) were cloned with different promoters and different combinations of four epitope tags to allow a rational optimization of overexpressed protein purification and protein detection. These recombinant plasmid constructs would be used to generate recombinant vaccinia viruses (vWR-delta F13L-RB12) and infect mammalian cells (BSC-1, CV-1) and express specific MCV genes. Expression has been achieved in vaccinia virus infected cells (v/pRB system) and in *E. coli* (pGEX2TK). These are being compared with an aim to optimize protein production for ELISA, immunization of animals and selection of T cell clones. All plasmid constructs have been confirmed by DNA nucleotide sequencing. The constructs made are listed in Table 2.3.

The two expression systems used were

1. Vaccinia virus vRB12/ pRB21 plaqueless mutant system (cell infection/ infection controls). Construction of recombinant vaccinia WR (vRB12 system)/MVA and overexpression of protein in a poxviral background (vWR) to

express full length proteins with posttranslational modifications similar to *in vivo* MCV infection. To be used as infected cell controls, neutralisation assays and hybridoma screening.

2. pGEX-2TK GST fusion system (antigen for ELISA and mAb) in *E. coli* (RIL^{+})

Table 2.3. MCV Expression constructs

MCV gene	ATCC plasmid	pRB21 – rec VACV ILR# (promoter/tag)	pGEX 2TK ILR# (tag)	Seq control vs U60315 (GenBank)	Expression
Mc084L	pyMCV1-E-D	316 (pe/l- XV5) 319 (pe/l- XSV5)	332 (S)	as in GenBank as in GenBank as in GenBank	v316 IF + v319 IF + Ec WB pos (34Kd)
Mc133L	pMCV1-B-KP	343 (pe/l-SV5)	334 (S)	2 aa subs (same in both)	v343 IF + Ec WB pos (66 Kd)
Promoters: **Tags:** pVGF = vaccinia growth factor (early) S = strep II; V5 = V5 tag; F= Flag tag pe/l = vaccinia synthetic opt. (early/ late)			mc080 subs: chem. cons. – pos >>>>> mc084 subs: chem. cons.- pos >>>>> mc133 subs: chem. cons – pos >>>>>		

2.5.2.1 Construction of recombinant vaccinia virus WR expressing mc084S

The full length surface protein gene mc084S of MCV was cloned intothe donorplasmid pRB21 plasmid under the control of the synthetic optimized early/late poxviral promoter, to complement the plaqueless phenotype virus vRB12 delta F13L and rescue recombinant virus producing normal sized plaques (Blasco and Moss, 1991). MC084 was amplified by PCR using specific primers tailed with restriction enzyme sites and C-terminal StrepII epitope tag (Forward primer: 5'-CTTCGTgaattcgccaccatggccgagagcgaaagcacg and reverse primers: 5'-CTTCGTgctagcaagcttTTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCAGATCCACGCGGAACCAGCTGGAGCAGCCCCGACGC and 5'CTTCGTgctagcaagcttTTATTTTTCGAACTGCGGGTGGCTCCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCAGATCCACGCGGAACCAGCTgGAGCAGCCCCGA respectively. The donor plasmids p316 and p319 were sequenced

using the ABI Prism 3.1 reagent kit (Applied Biogenes), and the recombinant virus v316 and v319 (Figure 2.9) rescued by infection/transfection of BSC-1 cells and three subsequent plaque purifications (1-1-1) using the LMP agarose overlay method. Virus stocks (IMV) were produced in adherent HeLa cells following the procedure outlined by Townsley and coworkers. v319 expression characterised in figures 2.10 and 2.11.

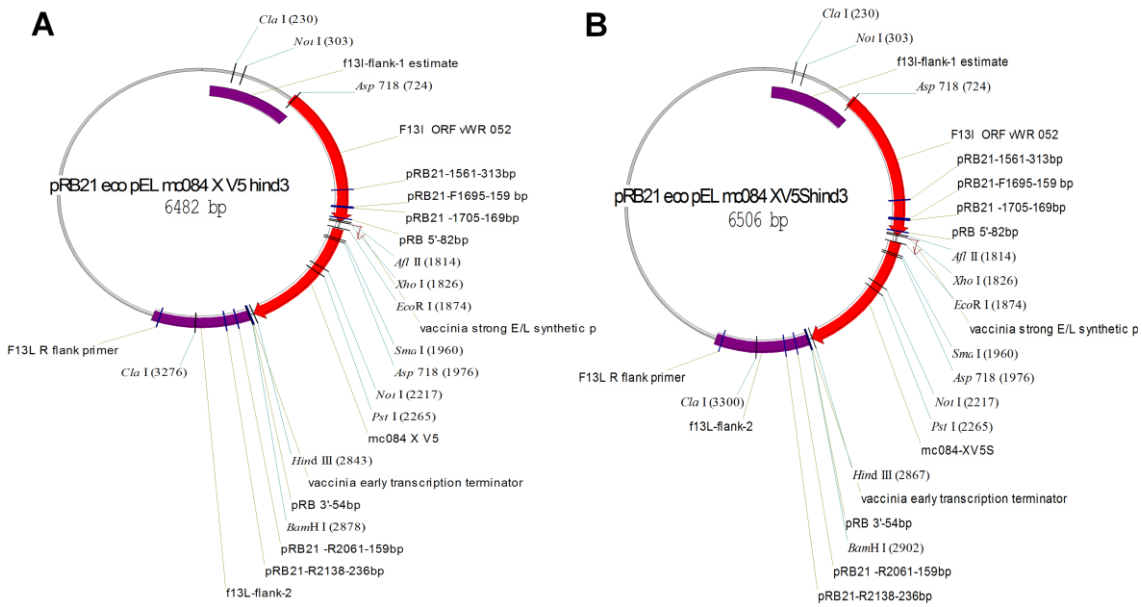


Figure 2.9. vNTI virtual mc084 constructs of the donor plasmids pRB21. *Each construct was cloned with strong Early/Late synthetic promoter and inserted MCV gene mc084 used to construct v316 (pE/Lmc084XV5) and v319 (pE/Lmc084XSV5).*

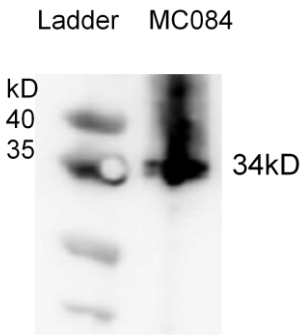


Figure 2.10. Western Blot detection of viral MC084S. *vMC084S(34 kD) detected using Strep-Tactin HRP conjugate antibody (1:100).*

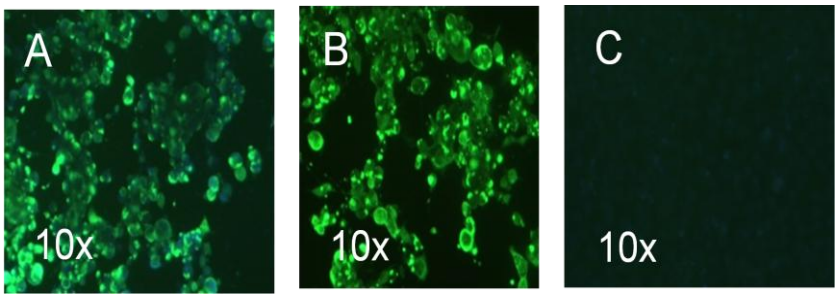


Figure 2.11. Immunofluorescent detection of expression of vmc084. *Detection of expression of v319 using (A) anti V5–anti mouse 488 (1:100) (10x), (B) Classic strep MAB (1:100) (10x) and (C) Mock+/- DAPI in CV-1 cells.*

2.5.2.2 Construction of recombinant vaccinia virus WR expressing mc133FS

The full length surface protein gene mc133FS of MCV was cloned intothe donorplasmid pRB21 plasmidunder the control of the synthetic optimized early/late poxviral promoter, to complement the plaqueless phenotype virus vRB12 delta F13L and rescue recombinant virus producing normal sized plaques (Blasco and Moss, 1991). Mc133 was amplified by PCR using specific primers tailed with restriction enzyme sites and C-terminal Flag StrepII epitope tag (Forward primer:5'-CTTCGTgaattcgccaccATGGCTGATAGCGAGGCTGTCGAT and reverse primer: 5'CTTCGTaagcttgaattcTTATTTTTCGAACTGCGGGTGGCTCCACTTATCGTCGT CATCCTTGTAAGATCCACGCGGAACCAG*CTCGTATACCATGTCGTCTCTGTAG CCT).*The donor plasmid p343 was sequenced using the ABI Prism 3.1 reagent kit (Applied Biogenes), and the recombinant virus v343 (Figure 2.12) rescued by infection/transfection of BSC-1 cells and three subsequent plaque purifications (1-1-1) using the LMP agarose overlay method. Virus stocks (IMV) were produced in adherent HeLa cells following the procedure outlined by Townsley and coworkers. Expression of MC084S in CV-1 cells infected with v319 is characterised in figures 2.13 and 2.14.

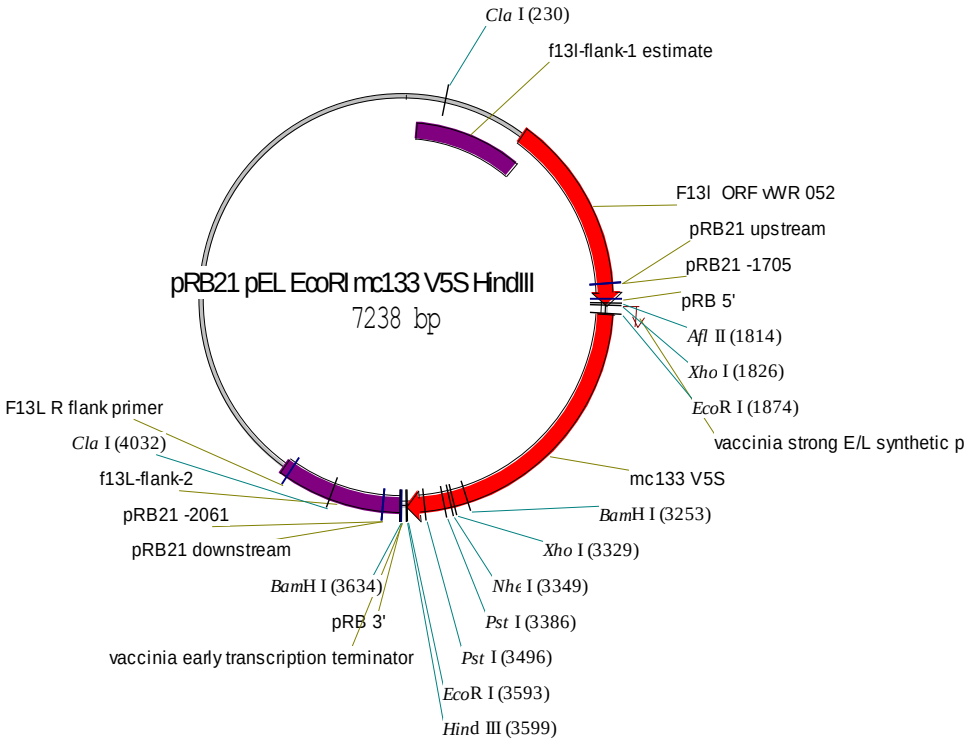


Figure 2.12. **vNTI virtual mc133 construct of the donor plasmid pRB21**. *Constructs with strong Early/Late synthetic promoter and cloned MCV gene mc133-V5S.*

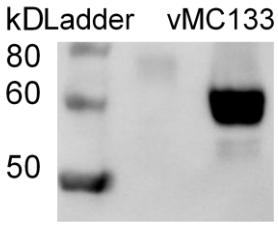


Figure 2.13. Western Blot detection of viral MC133. *vMC133S (61 kD) detected using Strep-Tactin HRP conjugate antibody.*

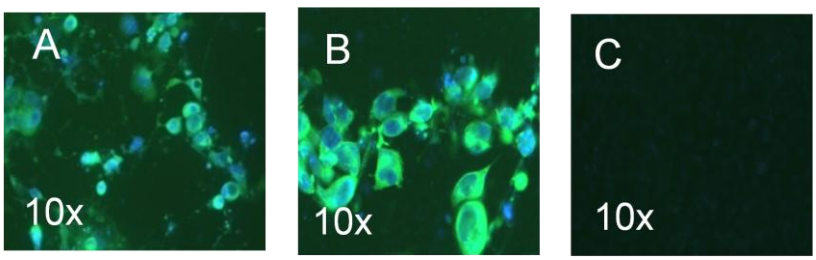


Figure 2.14. Immunofluorescent detection of expression of vMC133. *Immunofluorescent detection of vMC133S (aa1-546) using (A) Classic Strep MAB (10x), (B) Anti flag mouse 488 antibodies (10x) and (C) mock (10x) in BSC-1 cells +/- DAPI.*

2.5.2.3 Expression of recombinant vaccinia viruses in cell culture

BSC-1 cells (ATCC CCL-26) were used to rescue recombinant vaccinia virus. HeLa cells (ATCC CCL-2) (gift of Norbert Fusenig, DKFZ, Heidelberg) were used for vaccinia virus stock production. HaCaT cells were used to demonstrate mc084 expression in immortalized human keratinocytes infected with recombinant vaccinia virus v319. Similarily HaCaT cells were also used to demonstrate mc133 expression in cells infected with recombinant vaccinia virus v343. All cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Life technologies, Paisley, UK) with 4.5 g/L glucose and L-glutamine without sodium pyruvate 1X (Gibco, Life technologies, Paisley, UK), supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS; Gibco) was used for the culture of all cells. Cells were cultured in the absence of antibiotics until used for assay, when penicillin/streptomycin was added (Gibco). Visibly contaminated cultures were discarded, mycoplasma contamination was ruled out following lab standard procedure (Mycoplasma PCR kit, Geneflow, UK).

2.5.2.4 Constructon of GST-MC084S (V123-R230) fusion vector

The mc084S (V123-R230) (originally cloned in the vector pRB21) was digested with *EcoRI* and *BamHI* to generate a 1049 bp (14 kD) fragment. The same restriction enzymes were also used to cut the expression vector pGEX-2TK to obtain a 4965 bp fragment. Both fragments were purified after electrophoresis on 2% agarose gel using Qiagen gel extraction kit and ligated overnight at 4˚C using T_4 DNA Ligase (Promega). The ligation product was used to transform competent BL21 RIL^+ *E. coli* cells. Five independent transformants were selected for plasmid DNA analysis by restriction enzyme digestion, confirmed by sequence analysis and tested for protein expression.

2.5.2.5 Constructon of GST-MC084v5 (V33-G117) fusion vector

The mc084S (V33-G117) (originally cloned in the vector pRB21) was digested with *EcoRI* and *BamHI* to generate a 998 bp (10 kD) fragment. The same restriction enzymes were also used to cut the expression vector pGEX-2TK to obtain a 4965 bp fragment. Both fragments were purified after electrophoresis on 2% agarose gel using Qiagen gel extraction kit and ligated overnight at 4˚C using T_4 DNA Ligase (Promega). The ligation product was used to transform competent BL21 RIL^+ *E.coli* cells. Five

independent transformants were selected for plasmid DNA analysis by restriction enzyme digestion, confirmed by sequence analysis and tested for protein expression.

2.5.2.6 Constructon of GST-MC133S (M1-N370) fusion vector

The mc084S (M1-N370) (originally cloned in the vector pRB21) was digested with *EcoRI* and *BamHI* to generate an 1835 bp (40 kD) fragment. The same restriction enzymes were also used to cut the expression vector pGEX-2TK to obtain a 4956 bp fragment. Both fragments were purified after electrophoresis on 2% agarose gel using Qiagen gel extraction kit and ligated overnight at 4˚C using T_4 DNA Ligase (Promega). The ligation product was used to transform competent BL21 RIL^+ *E.coli* cells. Five independent transformants were selected for plasmid DNA analysis by restriction enzyme digestion, confirmed by sequence analysis and tested for protein expression.

2.6 Protein expression and purification

2.6.1 Recombinant protein expression

Single colonies of *E. coli* strain BL21 RIL^+ transformed with the truncated and C-terminal epitope tagged GST-MC084S (V123-R230)/GST-MC084v5 (V33-G117)/ GST-MC133S (M1-N370) fusion vector were inoculated into 500 ml LB medium containing 500 µl Ampicillin (100 µg/ml) as the selection marker. Bacterial cultures were grown overnight at 30˚C (approx. 8 h) in a shaking incubator set at 70 rpm. The following morning these cultures were checked and when the bacteria attained log phase (A_{550} of 0.5-0.7) expression of recombinant protein was induced by addition of IPTG to a final concentration of 1.5 mM. The cultures remained at 37˚C and 70 rpm and after 3 h bacteria were harvested by centrifugation at 10000 rpm for 20 min. The supernatants were discarded and pellets resuspended in 20 ml Lysis buffer (PBS, Lysozyme, triton-X and protease inhibitor cocktail), sonicated on ice for five to eight cycles of 15 sec using a probe sonicator followed by 30 second cooling on ice and centrifuged at 5000 rpm for 15 min. The supernatant was used as a source of soluble protein. Cultures grown under identical conditions but without IPTG addition served as uninduced controls. Presence of the recombinant fusion protein was confirmed by SDS-

PAGE analysis on 4-12% Bis-Tris NuPAGE gels followed by staining with Coomassie Brilliant Blue.

2.6.2 Purification of GST-MC084S (V123-R230), GST-MC084v5 (V33-G117) and GST-MC133S (M1-N370) on glutathione sepharose beads

Appropriate volumes of glutathione sepharose 4B beads (GE Healthcare) washed with PBS-Triton X and equilibrated with 1xPBS were mixed with the solubilised protein in a batch reaction and incubated with gentle agitation at 4˚C for 24 hrs. The mixture was centrifuged at 3000 rpm for 5 min to sediment the matrix. Unbound protein was removed by washing the gel pellet three times with GST Bead wash (PBS-Triton X) and three times with 1xPBS.

2.6.3 Purification of GST-MC084S (V123-R230)

The fusion protein GST-MC084S (V123-R230), bound matrix was equilibrated by washing with 10 bed volumes of Thrombin cleavage buffer consisting of 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT and 0.5 mM EDTA at 4˚C. The matrix bound fusion protein was suspended in one bed volume of fresh cleavage buffer containing Thrombin (Pharmacia) and incubated with gentle agitation at 4˚C. After 4 h of incubation, the supernatant was collected by centrifugation at 500 g for 5min and analysed by SDS-PAGE. The protease cleaved 14 kD MC084S was further purified via AKTA-FPLC using Superdex S200 column (GE Healthcare). Final protein concentrations were estimated spectrophotometrically using NanoDrop (Nanodrop Limited). To compare the reactivity of components in fusion protein with tested sera, pure GST was obtained by expressing pGEX-6P-1 without an insert in BL21 (RIL^{+}) chemically competent *E. coli.*

2.7 Protein visualisation and detection

2.7.1 SDS-PAGE

Protein preparations were separated using denaturing sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) in NuPAGE Novex 4-12% Bis tris Gels (Life technologies) and MOPS SDS running buffer (Invitrogen). Protein bands were

visualised by staining with 0.01% Coomassie Brilliant Blue R-250 (Sigma-Aldrich) followed by destaining in Coomassie destaining solution (40% Methanol and 10% glacial acetic acid).

2.7.2 Western transfer and immunodetection

For immunodetection proteins prepared by SDS-PAGE were electrotransferred onto nitrocellulose and probed with Strep tactin-HRP conjugate (1:100) (IBA Lifesciences) or Strep MAB Classic-HRP conjugate (1:100) (IBA Lifesciences). Detection by chemiluminescence was performed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's recommendations.

2.8 Recombinant MC084S – ELISA screening methods

2.8.1 Human serum/ tissue samples

522 serum samples (aged 2 months -101 years) were collected for routine screening and medical diagnosis at University Hospital Heidelberg, Germany as well as 44 serum samples (aged 8-62 years) from patients undergoing treatment for various diseases at the University of Heidelberg, Germany, between 2007-2011. Lesion material from patients with molluscum contagiosum was obtained from the University Hautklinik, Heidelberg, Germany. Sample collection was approved by the Human Ethics Committee of the University **(**Ethikvotum S-091/2011 Hautklinik Heidelberg). 149 sera samples (aged 2-69 years) were from a UK population collected as part of a study on MS at Cardiff University ('An Epidemiological study of Multiple sclerosis and other neuroinflammatory demyelinating disorders in South Wales', 05/WSE03/111). Twelve serum samples were collected from MCV patients (4 Male and 8 Female, aged 2-62 years). Of these 10 were collected at the Dr P N Behl Skin Institute and School of Dermatology, New Delhi, India and two were collected in South-western UK (Bath, Cardiff).

2.8.2 MCV direct binding ELISA

Ninety six well Maxisorp ELISA plates (Nunc) were coated with 3 µg/ml of FPLC purified recombinant truncated MC084S (aa123-230) protein per well in 100 µl of 0.05

M carbonate-bicarbonate buffer (pH 9.6) and incubated at 37˚C for 2 hours and then overnight at 4˚C. Plates were washed three times with PBS containing 0.05% Tween-20 (Fischer Scientific) prior to the addition of 200 µl/well of 5% skim milk (Vitamel Skim Milk). The plates were incubated for 2 h at 37˚C and washed eight times with PBS-T. Test sera, diluted 1:100 in dilution buffer were coated across the plate (100 µl/well). The plate was incubated at 37˚C for 2 h and washed ten times with PBS-T. Secondary anti-human IgG (raised in sheep) conjugated to horseradish peroxidase (GE Healthcare), diluted 1:2000 in dilution buffer was subsequently added (100 µl/well). After incubation at 37˚C for 2 h the plate was washed ten times with PBS-T and 100 µl of BD OptEIA™ substrate reagents (TMB in 0.05 M phosphate citrate buffer containing hydrogen peroxide - BD Biosciences) was added to each well. 50 µl of 1M H_2SO_4 was used to stop the enzyme reaction after 20 min incubation at RT. The OD of the reaction product was read at 450 nm on an FLUROSTAR OPTIMA - ELISA plate reader (BMG Labtech).

2.8.3 Plate description

Forty two sera samples were tested in duplicate on each plate along with a panel of four control sera consisting of two negative and two positive as well as four blanks, all in duplicate. The results were expressed as δODU (δODU =mean of duplicate wells minus mean of the blank wells).

2.8.4 ELISA performance

Plate to plate variation was monitored by comparing the control panel results between the different wells of the same plate; same sera samples run on different plates on the same day as well as on different days.

Intra-assay variation was tested by running eight sera samples (of varying concentration) in replicates of 10 across the microtitre plate and determining the percent coefficient of variation (CV). Acceptable criteria are typically CVs of 80-120%.

Inter-assay variation was again determined by evaluating at least eight sera samples (of varying concentration) in duplicate on at least three different microtitre plates on different days using the same reagent lots. The percent CVs are then calculated.

CV=SD/AVG x 100

2.8.5 Cut-off value determination

We defined the cut-off for ELISA based on the 66 sera samples from infants aged 0-1years which were tested on ELISA plates coated with MC084S (aa 123-230) and the mean of their δODU readings was 0.12043 and the SD was 0.08300. In comparison the mean δODU for 12 MCV infected patients was 0.833 and the S.D. 0.571. The infant group was therefore used to define negativity with the upper limit being the mean δODU plus 3 S.D. (i.e. 0.36). Assuming that these values are indicative of a negative response to the recombinant protein, we defined a positive antibody response as being a value greater than mean plus 3 SDs i.e. 0.36.

2.8.6 Binding Affinity - Direct ELISA

Direct binding ELISA was performed on flat-bottom 96-well polystyrene immunoplates (Maxisorp TM; Nalgene Nunc International, Roskilde, Denmark), as described above. Briefly, plates were coated with 100 µl of antigen (5 µg/ml) for 2 h at room temperature and overnight at 4˚C. Plates were washed three times with PBS containing 0.05% Tween-20 (Fischer Scientific) and unoccupied sites were blocked with 200 µl of 5% bovine serum albumin in PBS-T for 2 h at room temperature. The test serum (diluted 1: 100) in PBS-T (100 µl per well) was adsorbed for 2 h at room temperature and overnight at 4˚C. Bound antibodies were assayed secondary anti-human IgG (raised in sheep) conjugated to horseradish peroxidase (GE Healthcare); diluted 1:2000 in dilution buffer was subsequently added (100 µl/well).After incubation at 37˚C for 2 h, the plate was washed ten times with PBS-T and 100 µl of BD OptEIA™ substrate reagents (TMB in 0.05 M phosphate citrate buffer containing hydrogen peroxide - BD Biosciences) was added to each well. 50 µl of 1M H_2SO_4 was used to stop the enzyme reaction after 20 min incubation at RT. The OD of the reaction product was read at 450 nm on an FLUROSTAR OPTIMA - ELISA plate reader (BMG Labtech).

2.8.7 Specificity – Inhibition ELISA

The antigenic specificity of the antigen was determined by competition ELISA. Varying concentrations of inhibitors such as BSA and human IgG (0–10 µg/ml) were allowed to interact with a constant amount of antiserum for 2 h at room temperature and overnight at 4˚C. The immune complex thus formed was incubated in the wells (instead of the serum taken in direct binding ELISA) and the bound antibody levels were detected as in direct binding ELISA. The percent inhibition was calculated using the formula: Percent inhibition = [1 – (A inhibited /A uninhibited)] x 100 where A inhibited is the absorbance at 1 µg/ml of inhibitor concentration and A uninhibited the absorbance at zero inhibitor concentration.

2.8.8 Comparison of antigen reactivity

Purified GST or GST-tagged fusion proteins (100 µl/well) were applied to the Glutathione Immobilizer plates (Thermo scientific) in concentrations of 5 µg/ml in PBS, without any mixing. The plates were incubated at 4˚C overnight. The plates were washed three times with 300 µl/well PBS-T (0.05% Tween v/v). The use of blocking solution is not recommended. Anti-Strep II (100 µl/well) diluted 1: 100 in PBS-T is applied to the plates and these are incubated at room temperature for one hour. The plates were washed three times with 300 µl/well PBS-T again. The OD of the reaction product was read at 450 nm on a FLUROSTAR OPTIMA - ELISA plate reader (BMG Labtech). 100 µl of BD OptEIA™ substrate reagents (TMB in 0.05 M phosphate citrate buffer containing hydrogen peroxide - BD Biosciences) was added to each well. 50 µl of 1 M H_2SO_4 was used to stop the enzyme reaction after 20 min incubation at RT. The OD of the reaction product was read at 450 nm on an FLUROSTAR OPTIMA - ELISA plate reader (BMG Labtech).

2.8.9 Immunofluorescent detection of MC084S in v319-MC084 infected keratinocytes

HaCaT cells were seeded at low cell density on glass coverslips. Cells growing on coverslips were allowed to adhere for 24 hours and were infected with recombinant vaccinia virus v319 expressing MC084. At 24-48 h post infection, cells were washed

three times with PBS and fixed by immersing coverslips in 100% methanol at -20˚C for 5minutes. These were then blocked with IF block buffer for 45 min at 37˚C. Coverslips were washed three times in PBS and then cells were stained with human high tire antiserum HD V0901071 or mouse anti Strep II tag diluted 1:100 in IF blocking buffer for 45 min at 37˚C, washed extensively in PBS again and incubated for 30 min at 37˚C with a secondary Alexa Fluor 488 goat anti-human IgG (H+L) (1:1000) (Invitrogen). Samples were washed several times with PBS and mounted with Vectashield® mounting medium containing DAPI (Vector Laboratories) on glass slides.

2.8.10 Confocal scanning laser microscopy

Samples were analysed for fluorescence emission properties in green and blue regions at excitation wavelengths of 458, 488 and 543 nm by using confocal scanning laser microscopy (CSLM) (Leica TCS SP2 AOBS) mounted on a Zeiss Axiovert 200M on an inverted-based microscope with a 63x objective.

2.8.11 Immunohistochemistry

Paraffin embedded sections were deparaffinized and rehydrated. Staining was done using the Dako Cytomation Envision®+Dual Link System-HRP (DAB+) kit (Dako) as per manufacturer's instructions. For staining of tissue with high titre or low titre human sera, ECL Anti-human IgG (1:2000) (GE Healthcare) was used in conjunction with the kit. Staining was completed with counterstaining with Mayers haematoxylin and eosin. Slides were fixed with coverslips. All sections were analysed using an Olympus BX51 light microscope. Expression of MC084S specific antibodies were considered positive in the epidermis when the tissue showed golden-brown staining in a focal or diffused pattern.

2.8.12 Statistical Analysis

Serological data was stratified by age or diagnosis. Statistical significance of differences between the ELISA responses of different groups was assessed by one way ANOVA. Tukey post hoc anova was used to identify and compare statistically significant means and differences of different group.

2.8.13 MCV serum neutralisation test

Reporter assays were conducted as per protocol described by Liu and coworkers (Liu *et al*., 2012). Neutralisation was measured using the neutalisation assay developed by us using the poxvirus reporter system v3, where virus was inhibited by neutralizing antibodies present in serum samples. HeLa cells were added as trypsinized single cell suspensions. Viral progeny were allowed to grow in the cells for up to 24 h after infection. Cell densities were determined to be 10^4 cells per well. Varying dilutions of serum as 1:3, 1:9, 1:27, 1:81, 1:243 and 1:729 containing neutralizing antibodies and 400 PFU/50 µl diluted virus. Diluted serum (100 µl) was distributed into the corresponding wells in a 96 well plate and 50 µl of VAC was added to each well. Plates were incubated at 37˚C, 5% CO_2 for 1 h. Following incubation, 100 µl cells were added to each well. Plates were placed on a plate shaker for 1 min and further incubated at 37˚C, 5% CO_2 for 24 h. After incubation cell lysates were tested for luciferase activity by adding 100 µl Dual Luciferase Assay Substrate (part of The Dual-Luciferase® Reporter Assay System from Promega**)** to each well. Luminescence was measured on aFLUOStar Luminometer. The 50% neutralization titre (NT_{50}) for each serum sample was defined as the serum dilution at which the relative light unit (RLU) was reduced by 50% compared to virus containing control wells. Titres greater than 1:9 were considered positive.

2.8.14 MCV plaque assays

The plaque assay remains the gold standard for virus quantification. The plaque itself can be described as an area of dead cells or as area of infected cells in a cell monolayer. The production of plaques representing original titre under liquid medium is impossible. Plaque reduction neutralisation tests were conducted as per protocol described by Liu and coworkers (Liu *et al*., 2012). Sera were serially diluted to five-fold dilutions in the range of 1:40 to 1:25000, depending on the expected titre. Wells containing 550 µl of each dilution and an equal volume of vaccinia virus (400 pfu/ml) were mixed. After incubation at 37˚C, 5% CO_2 for 1 h, 550 µl was added to Vero cells in duplicate and plates were incubated for 2 h at 37˚C, 5% CO_2 with intermittent rocking, followed by the addition of 3 ml of overlay containing 0.5% agarose. On day 4, viral plaques were

visualised by staining with 0.1% crystal violet in PBS containing 0.2% formalin and counted. The NT_{50} was defined as the reciprocal of the serum dilution required for 50% reduction in vaccinia plaques.

2.9 Monoclonal Antibody Production

2.9.1 Antibody Production

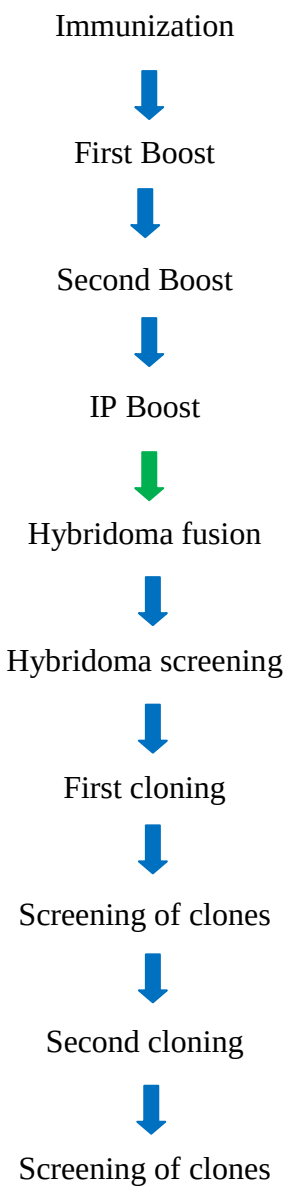


Figure 2.15. Monoclonal antibody production.

- Each immunization/boost with 50 μg purified MC084S (V123-R230) protein or GST-MC133S per BALB/c mouse.
- Each blue arrow represents approximately two weeks
- The green arrow represents 2-3 days.

2.9.2 Immunisations

Adult mice of theBALB/c strainwere used for immunisations and fusions and were either purchased from The Jackson Laboratory (Bar Harbor, Maine) or were produced in the animal colonies by members of the Morgan Lab. The primary immunization was performed by intramuscular injection of 50 μg of antigen. Boosts with 50 μg of the same antigen were given at a variety of intervals as indicated in Figure 2.15. Fusion was performed at 2 days after the final intraperitoneal (IP) boost, also as indicated in Figure 2.15

2.9.3 Hybridoma fusion

A day prior to fusion, SP2/0 myeloma cells, were split to ensure they will be in log phase growth on day of fusion. The immunized mice spleen cells were mixed with myeloma cells (SP2/0) at a 2:1 (spleen:myeloma) ratio in the presence of polyethylene glycol (PEG) 2000 (Molecular weight) (Roche) and a volume of 200 μl is pipetted in to each well of 96-well plates in RPMI + 15% FBS/HAT + 1% 3A(L-glutamine, pen/strep, sodium pyruvate) medium and incubated at 37 °C in an atmosphere of 5% CO_2. These plates are left incubator for hybridoma clones to grow and after 6-7 days fusions are fed by adding 1 ml/well fresh complete RPMI + 1X HAT.

2.9.4 Screening and subcloning of monoclonal hybridomas

Nine or ten days after the fusion process, the individual hybridomas begin to make the media slightly acidic. Hybridomaswere screened by ELISA after 10 days for antibodies against MC084S (aa123-230). Hybridomas showing significant MC084S (aa123-230) specific inhibition, were cloned three times by limiting dilution. The cells from this point on are grown in complete RPMI media with HT. These cells are expanded to T25

10 ml flasks containing RPMI with HT and at least two lines of each subclone are frozen down.One clone was chosen for further study.

Selected positive wells are fed as appropriate with complete RPMI medium+1xHAT (fusion should be fed with HAT containing medium for approximately 2 weeks and then only with HT (Gibco) containing medium. The hybridomas usually become visible colonies at day 7-8, and the supernatants are harvested and tested generally between day 10-15 days following the fusion.

ELISA plates are prepared according to the number of wells showing growth. A stock solution of antigen is prepared for screening by add 5 μg of protein to 10 ml PBS. This is mixed well and 100 μl of antigen is coated per well. A positive (polyclonal sera) and negative (BSA) control are also used. The wells that are ELISA positive are immediately transferred to a 24 well plate with 1 ml of HAT media and the 96 well plates are also refed. The day before subcloning, the SP2/0 cells are counted and plated at a density of 2 X 10^4 cells per well in 100 μl of complete RPMI. Two 96 well plates must be prepared for every hybridoma that will be subcloned and placed at 37˚C in an incubator. Peritoneal macrophages are isolated from two mice in RPMI media, washed and resuspended in 1xHT-complete RPMI out of which 100 μl/well is added in x (# of fusion wells to clone x 2) 96-well plates and incubate at 37˚C.

2.9.5 Crypreservation of cell lines

Before freezing, cells are checked using an inverted microscope and should appear >90% viable. Lower cells counts may not be able to recover from the freezing process. Cells are counted with hemocytometer. If cells are in normal density range these are pelleted in a sterile centrifuge tube by spinning between 1200-1500 rpm for 10 minutes, after which the supernatant was removed with a pipette and discarded. The bottom of tube is tapped to dislodgecells and they are resuspended in an appropriate amount of cold Freezing Media to obtain necessary density.

Freezing Media: for 200 ml

120 ml RPMI 1640 medium (Invitrogen)

60 ml of foetal calf serum (FCS-Gibco) (Final concentration 30%)

20 ml dimethylsulfoxide (DMSO) (Final concentration 10%)

The medium is filter sterilized and then aliquoted to 15 ml conical tubes and stored frozen at -20˚C. Before use the media should be defrosted and placed in the ice bucket covered with ice for at least 10 minutes to ensure it is cold before use. The DMSO should not be added directly to FCS as precipitation may occur. Cells should be resuspended in 1 ml of media per cryovial and should have a density between 3 x 10^6 and 7 x 10^6 cells/ml. As an example, if in 10ml, the cell count is 4 x 10^5 cells/ml, then the cell count is actually 4 x 10^6 cells total in the flask. So after pelleting, the cells should be resuspended in 1 ml of Freezing Media to maintain this density. Cells should be transferred immediately in cell suspension to appropriately labelled cryovial and put on ice until they can be frozen. Also these vials should be transferred to -80˚C freezer and left overnight. The cells should never be stored at -20˚C for any period of time and never transferred directly to Liquid N_2 tank, as this will be too great of a temperature change and cause loss of viability. After 24 hours, the cell line may be transferred to liquid nitrogen storage.

2.9.6 ELISA assay

96 well Maxisorp ELISA plates (Nunc) are coated with appropriate antigen (MC084S/vMC084/MC133S/GST) and incubated overnight at 4˚C. The antigen is removed and plates washed with PBS-T (PBS - 0.1M sodium carbonate-Fisher Scientific, pH 7.5 + Tween 20) once. These are then blocked with 200 µl of blocking solution (5% Vitamel skim milk) for 1-2 hours. The blocking solution is decanted and the plate washed two times with PBS-T using 200 µl volumes per well. After each wash the plate is inverted onto tissue to blot dry. Primary antibody or hybridoma supernatant is transferred undiluted to the ELISA plate, 100 µl/ well, using sterile tips in a hood and the plates are incubated for 1½ -2 hours at room temperature. The plate is washed with 100-200 µl of PBS-T three times and on the third wash let to stand for ten minutes. The secondary antibody i.e. Polyclonal Goat Anti-mouse IgG-HRP (Dako) is prepared in PBS (1:2000) and 100 µl/well of the secondary antibody is plated out using a multichannel pipette and plates incubated for 1½ -2 hours. The plates are washed with

PBS-T five times. After removal of all liquid from the final wash, 100 µl volumes of BD OptEIA™ substrate reagents A+B (BD Biosciences), in a ratio of 1:1, are added to each well and plates incubated at RT for 20 min, avoiding direct light.Colour change from colourless to blue is observed. The reaction is stopped by adding 50 µl volumes of stop reagent (2 N H_2SO_4) to each well allowing yellow colour to develop.Plate is read on FLUROSTAR OPTIMA - BMG Labtech plate reader at 450 nm (Table 1) and 570 nm (Table 2). Values obtained at 570nm are subtracted from readings obtained at 450 nm to give final readout (Table 3) and corrected for optical imperfections in the ELISA plate. Readings with a δODU≥0.6 are considered positive.

2.9.7 Binding affinity - Direct ELISA

Direct binding ELISA was performed in flat-bottom 96-well polystyrene immunoplates (Maxisorp TM; Nalge Nunc International, Roskilde, Denmark). Plates were coated with 100 µl/well MC084S (aa123-230) antigen at a concentration of 5 µg/ml for 2 h at room temperature and overnight at 4˚C. Plates were washed three times with PBS containing 0.05% Tween-20 (Fischer Scientific) and unoccupied sites were blocked with 200 µl of 5% skim milk (Vitamel) in PBS-T for 2 h at room temperature. Plates were washed three times with PBS-T. The serum collected from immunised sacrificed miceas well as from a normal healthy non-immunised mouse serving as control serum was pipetted into the plate wells and adsorbed for 2 h at room temperature and overnight at 4˚C. Plates were washed three times again with PBS-T. Bound antibodies were assayed using secondary anti-mouse IgG (raised in sheep) conjugated to horseradish peroxidase (Dako); diluted 1:2000 in dilution buffer was subsequently added (100µl/well).After incubation at 37˚C for 2 h, the plate was washed ten times with PBS-T and 100 µl of BD OptEIA™ substrate reagents (TMB in 0.05M phosphate citrate buffer containing hydrogen peroxide - BD Biosciences) was added to each well. 50 µl of 1M H_2SO_4 was used to stop the enzyme reaction after 20 min incubation at RT. The OD of the reaction product was read at 450 nm on an FLUROSTAR OPTIMA - ELISA plate reader (BMG Labtech).

2.9.8 Specificity – Inhibition ELISA

The antigenic specificity of the antigen was determined by competition ELISA. Increasing concentrations of inhibitorMC084S (aa123-230) (0.01–10 µg/ml) were allowed to interact with a constant amount of immunogen MC084 (4 µg/ml) for 2 h at room temperature and overnight at 4˚C. The immune complex thus formed was incubated in the wells (instead of the serum taken in direct binding ELISA) and the bound antibody levels were detected as in direct binding ELISA. The percent inhibition was calculated using the formula: Percent inhibition = [1 – (A inhibited /A uninhibited)] x 100 where A inhibited is the absorbance at 1 µg/ml of inhibitor concentration and A uninhibited the absorbance at zero inhibitor concentration.

2.9.9 Immunohistochemical detection using MC084-6C in MC biopsy paraffin sections and frozen sections

Paraffin embedded sections were deparaffinized and rehydrated. Staining was done using the Dako Cytomation Envision®+Dual Link System-HRP (DAB+) kit (Dako) as per manufacturer's instructions. Staining was completed with counterstaining with Mayers haematoxylin and eosin. Coverslips were fixed over the stained sections. All sections were analysed using an Olympus BX51 light microscope. Expression of MC084S specific antibodies were considered positive in the epidermis when the tissue showed golden-brown staining in a focal or diffused pattern.

2.9.10 Immunofluorescent detection of MC084S in v319-MC084 infected HaCaT/BSC-1 cells

HaCaT/BSC-1 cells were seeded at low cell density on glass coverslips. Cells growing on coverslips were allowed to adhere for 24 hours and were infected with recombinant vaccinia virus v319 expressing MC084. At 24-48 hrs post infection, cells were washed three times with PBS and fixed by immersing coverslips in 100% methanol at -20˚C for 5minutes. These were then blocked with IF block buffer for 45 min at 37˚C. Coverslips were washed three times in PBS and then cells were stained with MC084 monoclonal antibody MC084-6C or Classic Strep MAB (IBA Lifesciences) diluted 1:100 in IF blocking buffer for 45 min at 37˚C, washed extensively in PBS again and incubated for

30 min at 37˚C with a secondary antibody Alexa Fluor 488 (Green) chicken anti-mouse IgG (H+L) (1:1000) (Invitrogen). Samples were washed several times with PBS and mounted with Vectashield® mounting medium containing DAPI (Vector Laboratories) on glass slides.

2.10 IT equipment and software

Immunofluorescence was examined using the fluorescence microscope Olympus BX5I and AxioVision software version 4.4. Confocal scanning laser microscopy was done on the Leica *TCS SP2 AOBS.* Plates with plaque assays were scanned on a flat bed scanner HP Scanjet 5470C, using HP Precisionscan Pro software version 3.1 and examined by using COREL® PHOTO – PAINT® X3 software version 3. Luciferase activity was measured using FLUROstar OPTIMA Luminometer BMG LABTECH and FLUROstar* OPTIMA software. Statistical analysis was performed using SPSS software version 12, assessed by one way ANOVA. Tukey post hoc anova was used to identify and compare statistically significant means and differences of different groups. Bibliography was created using End Note version x7.

Chapter 3 - Development of a new method for the assessment of Molluscum contagiosum virus infectivity

Chapter 3 - Development of a new method for the assessment of Molluscum contagiosum virus infectivity

Introduction

Molluscum contagiosum is a viral infection of differentiating epidermal keratinocytes resulting in cutaneous tumours. Clinical presentation as an acanthoma with large keratohyalin granules and the lack of reactivity of these granules to antiflaggrin monoclonal antibodies suggest that the pathogenesis involves abnormal differentiation of epidermal cells (Takahashi, Izutani *et al*. 1999). MCV replicates well in the human skin *in vivo*, but not *in vitro* in standard monolayer cell culture. It has a large genome which has many genes which may encode function for its survival in human skin.

The MCV lifecycle starts with the entry of virus particles into human epidermal cells. Although it is not entirely clear in which cells the virus replicates, the point of replication is thought to be epidermal stem cells. Infections of epithelial type cells *in vitro* are non-productive, but produce early gene products. Molluscum contagiosum virus (MCV) does not produce a quantifiable cytopathic effect and does not produce viral progeny in infected standard cell cultures. The lack of a cell culture system has further limited MCV research. Consequently, all stages of MCV viral life-cycle have been under-investigated, accounting for the sizeable gaps in the literature. Small amounts of viral mRNA and protein expression can be detected indicating that MCV virions are transcriptionally active (Bugert *et al*., 1999; Bugert *et al.,* 2001, Mohr *et al*., 2008).

The first step of any virus lifecycle is entry. The MCV lifecycle *in vivo* as well as *in vitro* is summarised in Figure 3.1. In order to determine whether MCV has entered cells we have developed a combination of GFP and luciferase based reporter assays. This new method, developed in the Bugert lab, was published in 2012 (Sherwani *et al.,* 2012), and enables measurement of early transcriptional activity of MCV in a luciferase reporter assay, and has allowed us to make an initial contribution to addressing the basic

question of MCV entry into target cells. The present research concerns the first step of the viral life cycle, entry.

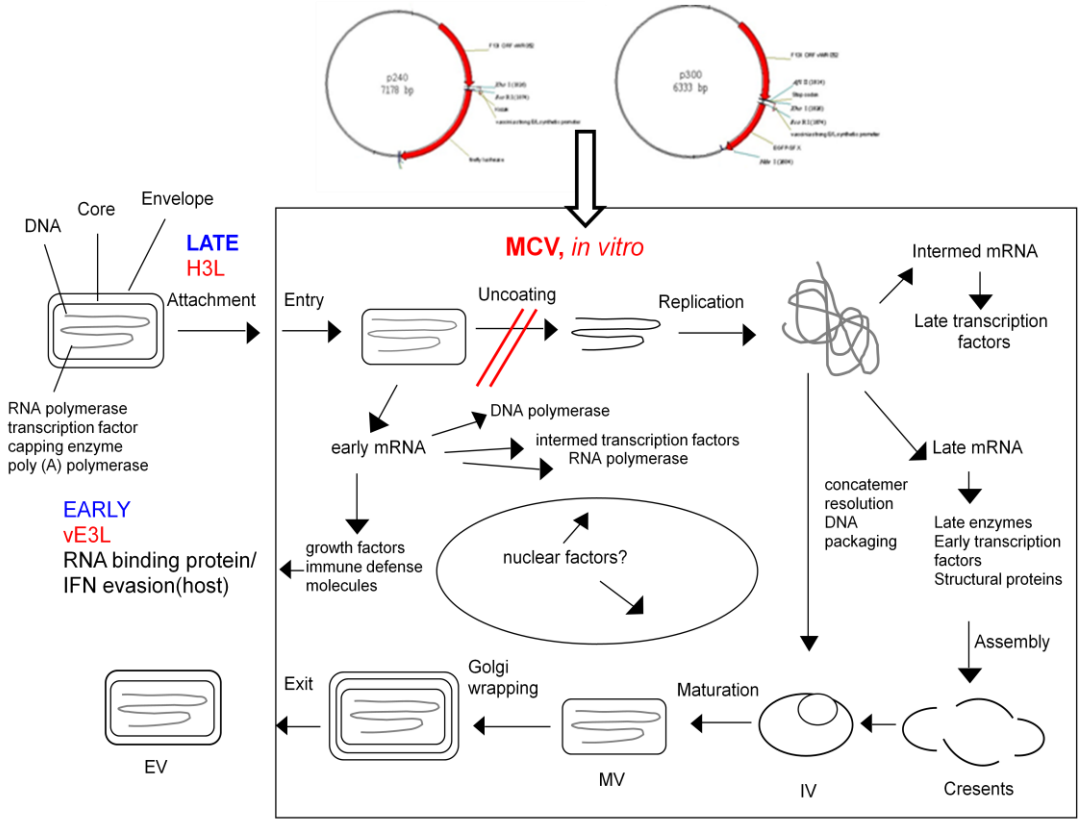


Figure 3.1. Summary of the replication cycle of Molluscum contagiosum virus. *Replication occurs entirely in the cytoplasm. A virion containing the double stranded DNA genome, enzymes and transcription factors attaches to the host GAGs on cells and facilitates fusion with the cell membrane in turn releasing a core into the cytoplasm. In the natural life cycle in vivo the core synthesizes early mRNA which is translated into many different proteins including growth factors, immune defence molecules, factors for DNA replication and intermediate transcription and enzymes. In vitro the life cycle is arrested at this stage with the transcription of early gene products in detectable levels. Uncoating takes place in vivo and DNA replication takes place to form concatemeric molecules. Intermediate genes are transcribed and the mRNA is translated to form late transcription factors. Late genes are then transcribed and the mRNA translated to form virion structural proteins, enzymes and early transcription factors. The assembly of progeny virions is initiated in cytoplasmic viral factories and the concatemeric DNA intermediates are packaged into immature virions (IV), followed by maturation leading to formation of infectious intracellular mature virions (MV). These MVs are wrapped by the trans-Golgi and endosomal cisternae to give wrapped virions which are transported to the periphery of the cell along microtubules. Fusion with plasma membrane leads to release of extracellular enveloped virions (EV). Adapted from B. Moss in Field's Virology.*

In order to determine the nature of the replication deficiency *in vitro,* the MCV infection process in standard cell culture has to be studied step by step. We developed a method which uses luciferase reporter constructs to measure poxviral mRNA transcription activity in MCV infected cells in standard culture.

Many investigators have observed that poxvirus transcription complexes can drive luciferase reporters under the control of poxviral promoters in plasmids in poxvirus infected cells. A recent paper uses this as a method to diagnose orthopoxvirus infections (Levy *et al.,* 2010). We introduce a luciferase reporter expression in trans as a new surrogate marker of infectivity and gene expression for MCV. To compare the infectivity of MCV with other poxviruses (Bengali *et al.*, 2009), the number of virions must be determined. Quantitation by EM or OD300 can be used (Melquiot and Bugert, 2004), but requires relatively large amounts of gradient purified virons. However, currently, MCV can only be isolated from clinical specimens and thus is difficult to obtain amounts sufficient for gradient purification (Melquiot and Bugert, 2004). PCR is an alternative method of quantification of smaller amounts of poxviruses from clinical specimens, which is both reliable and highly specific for individual poxviruses. This method uses a novel PCR target in an area with significant DNA homology (~65%) between MCV and vaccinia virus strain WR (VACV-WR). The MCV gene mc129R, which is homologous to the VAVWR144 (also called A24R gene encoding RPO132, the large subunit of the DNA-dependent RNA polymerase). The method provides a means to quantitate poxviral genome units in the same virus preparations used to compare transcriptional activity and infectivity of MCV and VACV-WR.

Despite the general consensus that MCV is highly specific to both humans and the skin, MCV lesions have been reported in various animals. As early as 1967, molluscum lesions were reported in chimpanzees by Douglas and colleagues (Douglas *et al*., 1967). Post this observation others were reported in red kangaroos and horses (Bagnall and Wilson, 1974; Lange *et al*., 1991). In spite of these observations no *in vitro*or *in vivo*model exists which would allow molecular biological analysis of MCV. The present work also aims to address questions about MCV entry and the possible development of *in vitro* MCV cell culture model (MINUCELL) which may be

developed to facilitate specific therapeutic strategies for MCV infection as well as possible vaccine delivery strategies.

The main aims that this chapter addresses are the development and optimisation of methods to achieve a luciferase reporter entry assay that produces sufficient luminescent signal for MCV entry from which conclusions may be drawn. Also the development of a quantitative assay to calculate MCV multiplicity of infection (moi) as MCV is characterised by lack of cpe and hence difficult to quantitate on entry and using both of the above mentioned, the testing and quantification of MCV entry into a panel of human and animal cells in order to identify the host cell line with maximal virus entry.

Results

3.1 Determination of optimal VACV

In virus titrations RK13 cells (p45 05.03.10) at a quantity of $1x10^4$ cells/well were infected with five different preparations of VACV in 10^{-2} dilution. The VACV supernatant (sn) preparations used and corresponding titres are shown in Table 3.1.

Table 3.1. VACV preparations and titrations.

VACV preparation	Viral titre (pfu/ml)	Multiplicity of infection (MOI)
vWR RK13 sn 8/211-1 (25.1.08)	$4.0x10^6$	4
vWR BSC-1 sn 8/221-1 (25.1.08)	$1.4x10^5$	0.14
vWR HeLa sn 7/1412-11 (7.12.07)	$3.6x10^6$	3.6
vWR CV-1 sn (13.12.06)	$2.8x10^6$	2.8
vWR BJ-1 sn (16.11.06)	$2.0x10^4$	0.02

Viral titres were calculated by using VACV-infected BSC-1 cells seeded in 24 well plates. Infected plates were incubated at 37˚C and 5% CO_2 for 45 mins. 500 µl microcrystalline cellulose 1.2% was added to each well before incubation for an additional 3 days. Wells were washed twice with PBS, stained with 400 µl crystal violet solution and incubated at RT for 3 hours. After rinsing with cold water and passively drying, the plates were examined. The number of plaques was counted for each VACV preparation to achieve a titre in plaque forming units pfu/ml. MOI, the ratio of pfu of virus to the number of cells being infected, was calculated using a viral volume of 10 µl and a cell number of $1x10^4$.

VACV infected cells were collected 16 hours p.i. and measured for luciferase activity. Luminescent firefly signals achieved were corrected to MOI=3 and normalised to renilla values as a well-to-well transfection control. Values varied markedly between the five viral preparations demonstrating that the cell type from which the viral preparation was acquired does appear to have an effect on the entry capacity of the virus. vWR BJ-1 (16.11.06) produced the greatest quantity of luminescence, averaging 35,850 RLU, when corrected for MOI. However, as the MOI for this VACV preparation was very low (MOI=0.02). The MOI for vWR RK13 (MOI=4) was considered more appropriate

for use as a positive control. No differences in CPE between infected cell lystates were evident upon photographic observation.

In order to achieve optimal signal (ideally less than 10,000RLU) due to VACV at 16 p.i., 100 µl vWR RK13 was diluted in a series of 900 µl DMEM P/S pools as to produce viral preparations of different dilutions (10^{-1} to 10^{-6}). These corresponded to MOIs of 40 to 0.0004. RK13 cells (p45, 05.03.10, p32 15.01.10), numbering $1x10^4$/ well, were transfected, infected with VACV and measured for luminescence. Formation of a standard curve allowed for deduction of a suitable dilution for use in subsequent experiments, which would provide a more comparable signal to MCV as well as help to preserve viral stocks. Figure 3.2 illustrates that cells infected with VACV at an MOI of 4 produce a luminescent signal which appears exceptionally large when compared to readings gained from MCV infected cell lysates in preliminary experiments. The figure also shows movement towards a plateau effect in signal from MOI 0.004 onwards. An MOI of 0.04 was chosen for use in subsequent experiments.

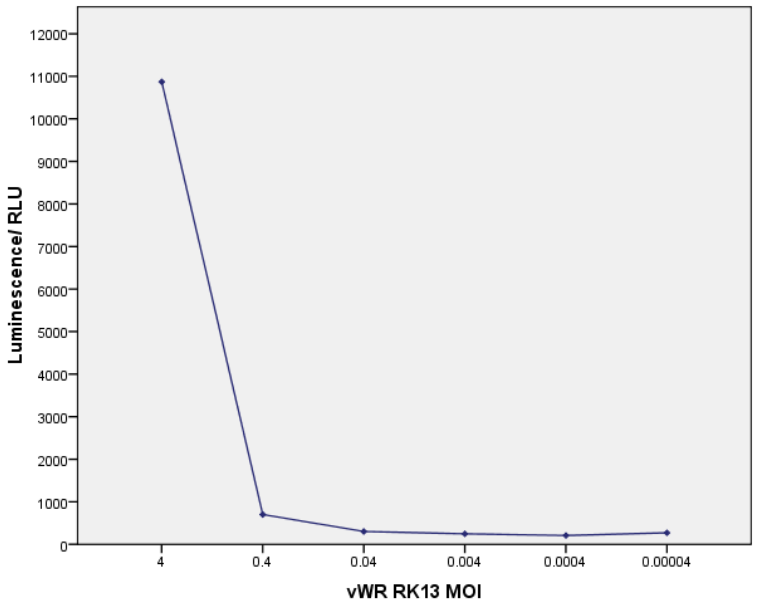


Figure 3.2. vWR RK13 dilution curve. *Luminescent signals achieved from RK13 cell lysates infected with vWR RK13 at $4.0x10^6$ pfu/ml. Cells were tranfected using a dual luciferase® reporter assay with ILR#p238 and ILR#p240 each at 300 ng/well. Values are normalised to renilla as well- to- well transfection control and given as mean luminescence in relative light units of n=2 triplicates. Viral dilutions are given as MOI.*

Subsequent to insufficient signal using 10 µl of a pooled MCV preparation, the experimental method was altered to include the use of a greater volume of virus (50 µl/well). The second preparation of MCV, pooled from a greater number of lesions, was centrifuged at 10,000 rpm in 1.5 ml non-sterile PCR tubes on two occasions. The supernatant was transferred to new tubes after each centrifugation in order to minimise the amount of debris in the sample. Consequently 50 µl of this optimal MCV preparation was used per well in subsequent experiments, which provided both an uncontaminated infection and sufficient signal from which to draw conclusions.

3.2 Quantitative PCR detecting MCV mc129/ vWR A24L homolog

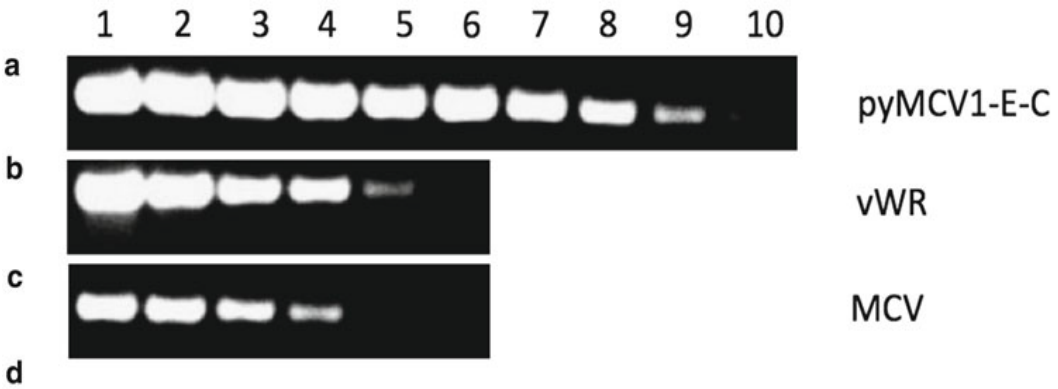
It is clear that vaccinia plaque-forming units cannot be directly compared to MCV virion units because of the different nature of their biological activity. However, the PCR method described in materials and methods allows a relative quantification of MCV genome equivalents to VACV-WR infectious units measured in pfu/ml based on amplifiable genomic DNA units/molecule numbers calculated for a relatively large plasmid containing 25,517 bp of MCV sequence.

As described in the methods section the plasmid pyMCV1-E-C has 29,760 bp. Thus, the molecular weight is calculated as 19.344 MDa and thus 19.344 ng of plasmid would be 6.02×10^8 mol. The actual plasmid concentration was 21 ng/µl (±1.7) and, thus, represented 6.5×10^8 mol/µl. From this value, the molecule numbers for the pyMCV1-E-C two-fold dilution series are calculated (Figure. 3.3d). The molecule numbers are then correlated to the pixel numbers of bands on a gel quantitated by ImageJ.

The pfu-to-molecule ratio for vaccinia virus (mature virions) comes out as 1:31, in keeping with previously published ratios (Payne and Kristensson, 1982). For the same PCR reaction, in the figure 3.3a, lane 9, the signal (27,779 pixels) for the dilution of the MCV control plasmid (pyMCV1-E-C) correlates to 1.586×10^6 plasmid units. The band in gel 3.3c (MCV PCR) with comparable pixel density (i.e., within 10%) is in lane 4 with a pixel value of 28,726 pixels. If the 1.586×10^6 mol are multiplied by the dilution factor (16x), the MCV aliquot of 100 µl used for genomic DNA preparation contained

2.5 × 10^7 mol/genome units. If the pixel values obtained for vWR in gel b, lane 4 (71,008), are used in the same way and related to gel a, lane 8 (67,495 pixels), after multiplying by the dilution factor (16x), a molecule number of 5 × 10^7 is obtained. This can then be used to relate to the pfu. If WR was at 1.6 × 10^6 pfu in 100 ml, the pfu-to-genome unit ratio is 1:31.

The PCR data can be used to calculate an MCV multiplicity of infection equivalents in relation to the control plasmid molecule numbers as well as in the form of pfu equivalents in relation to a titered vaccinia stock for comparison purposes. We havefound this approach to be both more reproducible and more specific than electron microscopy or OD quantifications of virions.



d

lane# (gel a)	1	2	3	4	5	6	7	8	9	data
DNA										
pyMCV	406,250	203,125	101,562	50,781	25,390	12,695	6,347	**3,173**	**1,586**	mol
pyMCV	138,794	159,136	140,851	129,871	107,126	112,165	89,590	67,495	27,779	pixel
lane# (gel b)					1	2	3	4	5	
vWR					156,659	126,644	87,696	71,008	15,102	pixel
lane# (gel c)						1	2	3	4	
MCV						77,875	73,384	55,270	28,726	pixel

Figure 3.3. PCR quantification of purified DNA. *Panels a – c show two fold dilutions of poxviral genomic DNA purified from DNAse-treated virions (b and c) and plasmid DNA repurified using the HPVNA kit (**a**). Lane numbers at the top of the figure refer to log 2 dilutions from 1 to 10. Panel d tabulates the calculated molecular number for the reference plasmid pyMCV1- E-C (pyMC) (1,000 × by nanodrop in 10 ml of original DNA prep) and the ImageJ pixels for each band in gels a to c. (Sherwani et al., 2012).*

3.3 Quantification of MCV entry into host cells and MCV infection of both human and animal cell lines

In order to identify the host cell lines with maximal viral entry a panel of animal and human cells, namely, HEK 293, 3T3, BHK-21, BJ-1, CV-1, HaCaT, HeLa and RK13 were transfected with ILR#p238 and ILR#p240 using the dual luciferase® reporter assay and subsequently infected with either MCV or vWR RK13, the latter at an MOI of 0.04. vWR RK13 was once again used as a control that the system was functioning. All virus infected cell lysates were collected 16 hours p.i. and measured for luciferase activity.

As predicted from previous optimisation experiments, MCV pooled from a greater number of lesions promoted elevation in strength of the FF signal achieved. Figure 3.4a illustrates that MCV-infected 293T cells emit the highest quantity of FF luminescence, more than double the luminescent signal of any other cell line. BHK-21 cells also provided high signal emission, but distribution of values about the mean was widespread. Results show that CV-1 consistently gave the lowest signals by a relatively large margin, even when compared to other animal cell lines of fibroblastic origin. Luminescent emissions from the 3T3 mouse fibroblast line were 2.44 fold greater than those from CV-1.

The phRL-TK reporter plasmid provided optimal renilla luciferase expression by which to normalise FF readings to correct for well to well deviation within triplicates and also to provide a transfection comparison between different cell lines. Figure 3.4b illustrates the mean renilla signals achieved from the assays. There appear to be remarkable differences in transfectability amongst the various cell types. HEK 293T cells proved most easily transfected, with renilla signals averaging 16,209 RLU, whereas BJ-1 appeared most difficult to tranfect with an average signal of 216 RLU. Correcting FF signals for these differences in cell transfectability allows identification of the host cells that underwent maximal virus entry, by calculation of the firefly readings that would be attained if all cell- lines were equally transfected. Figure 3.4c shows the corrected firefly signals. Subsequent to correction by renilla, the 3T3 cell line achieved the highest luminescent signal, undergoing the greatest quantity of MCV entry. The mean

reading from BJ-1 cells was also high, though the standard deviation was wide. Interestingly, cells derived from the human skin keratinocyte cell line, HaCaT, did not produce the greatest quantity of luminescence.

Although VACV was initially used as a positive control, it also became possible to compare the entry capacity of MCV and VACV. A semiquantitive PCR for MCV and VACV infectious particle genome equivalents was conducted using a conserved DNAdRNA pol primer pair as described in the previous result. This allowed calculation of a titre for the MCV pool used in experiments (2×10^8 pfu/mL).Using the calculated VACV titre, it became possible to compare MCV and VACV entry. Table 3.2 shows comparisons made between the entry capacity of the two viruses and demonstrates that VACV possesses a significantly greater ($p<0.05$) capacity to enter all cell lines except for HaCaT and HeLa.

Table 3.2. Comparisons of MCV and VACV entry

Cell line	Origin	MCV (RLU/MOI)	VACV (RLU/MOI)	Fold Difference	T- test for equality of means 95% Confidence Interval of the difference.		P-Value
					Lower	Higher	
3T3	Murine fibroblasts	45.35	188727.00	4161.57	-2.80809E5	-96554.10439	0.005*
BHK-21	Hamster kidney	31.90	530544.00	16631.47	-7.00794E5	-3.60230E5	0.01*
RK13	Rabbit kidney	22.55	261379.50	11591.11	-4.41493E5	-81221.52559	0.016*
HEK293T	Human embryonic kidney	5.27	26340.00	4998.10	-8647.04925	-6186.41475	<0.0001*
BJ-1	Human foreskin fibroblast	42.25	456285.90	10799.67	-6.68191E5	-2.44296E5	0.004*
CV-1	African green monkey kidney epithelial	8.19	325660.67	39763.21	-4.20462E5	-2.30843E5	0.001*
HaCaT	Human keratinocytes	20.27	268456.67	13244.04	-5.68543E5	31670.12735	0.68
HeLa	Human epithelial	3.17	41668.33	13144.58	-65212.1926	-18118.13225	0.68

*Both viruses at an MOI =1, hence values represent Relative Light Units (RLU)/MOI. Values given as mean of triplicates, corrected to renilla. Independent samples t test results are shown with resulting p-value. Results marked * show a statistically significant ($p<0.05$) difference between entry capacity of MCV and VACV.*

MCV isolated from human- infected skin biopsies was pooled and used to infect a panel of human and animal eukaryotic cells. Vaccinia virus has been used as a positive control. Results showed that the 3T3 cell line demonstrated maximum MCV entry. The present study is encouraging and the experimental approach used may prove helpful in the establishment of an *in vitro* cell culture model of MCV infection.

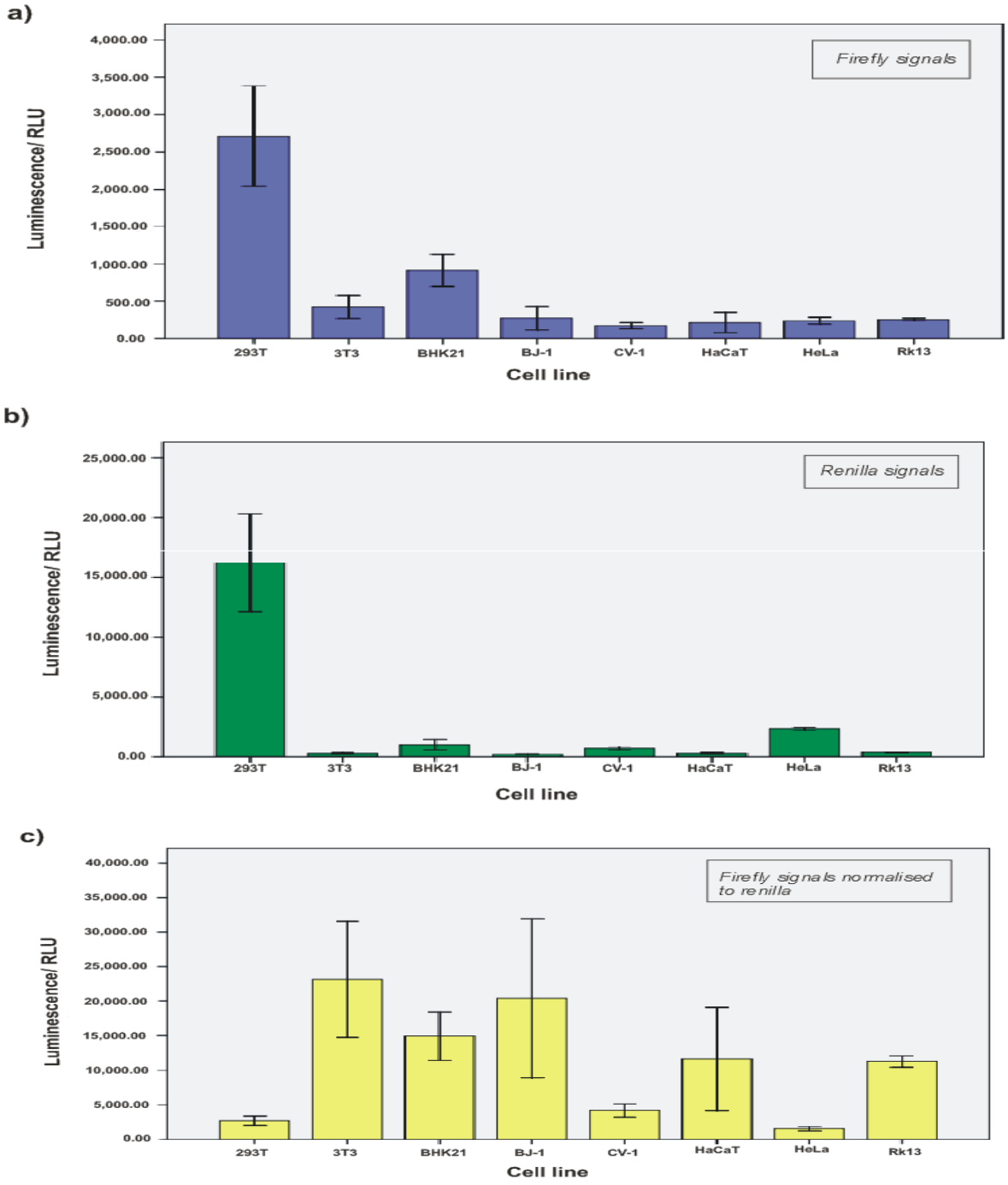


Figure 3.4. Luminescent signals obtained from MCV entry assay. *(a) Firefly (FF) signals achieved from eight MCV-infected cell types. Cells were infected with 50 µL MCV pool diluted*

1:10 in PBS (MOI = 500) and transfected with 300ng/well each of reporter plasmids ILR#p238 and ILR# p240. Values are corrected for well to well deviation in transfectability within cell line triplicates and are given as relative light units (RLU) as mean +/- 1 SD of triplicates. (b) Renilla signals achieved from the same infection/transfection assay. (c) Firefly signals normalized to renilla readings, correcting for differences in transfectability between cell lines.

Preliminary data obtained from the entry reporter assays conducted, show that firefly signals for MCV entry appear substantially greater than mock, i.e. the signals are high in the absence of viral infection. Although accurate comparisons cannot be made between VACV and MCV entry, due to difficulties in titrating MCV, it is possible to compare entry between different cell lines.

3.4 MCV drives expression of EGFP reporter in HEK293 cells

The results illustrated in figure 3.5 show that RK13 cells, derived from rabbit kidney epithelium, emit the greatest quantity of luminescence.This is extremely interesting, due to the fact that RK13 is a rabbit kidney epithelial cell line. MCV shows GFP positive cells after 16 h (Figure 3.5a), whereas WR shows multiple GFP positive cells (Figure 3.5b). Upon further incubation for another 4 days (5 days p.i.), some individual cells in the MCV-infected wells show medium to strong GFP signals (Figure 3.5c). At the same time point, the WR-infected wells show extensive plaques and cell degradation (Figure 3.5d). Figure 3.5e shows chemiluminescence in HEK 293 cells infected with MCV and vWR and collected 16h post infection. The luciferase signal can be seen in vaccinia-infected cells (MOI 0.01 - 1) after 2 h and is seen in the MCV infected cells (MOI of 5) after 8 h confirming MCV entry though to a much reduced degree as compared to vaccinia.

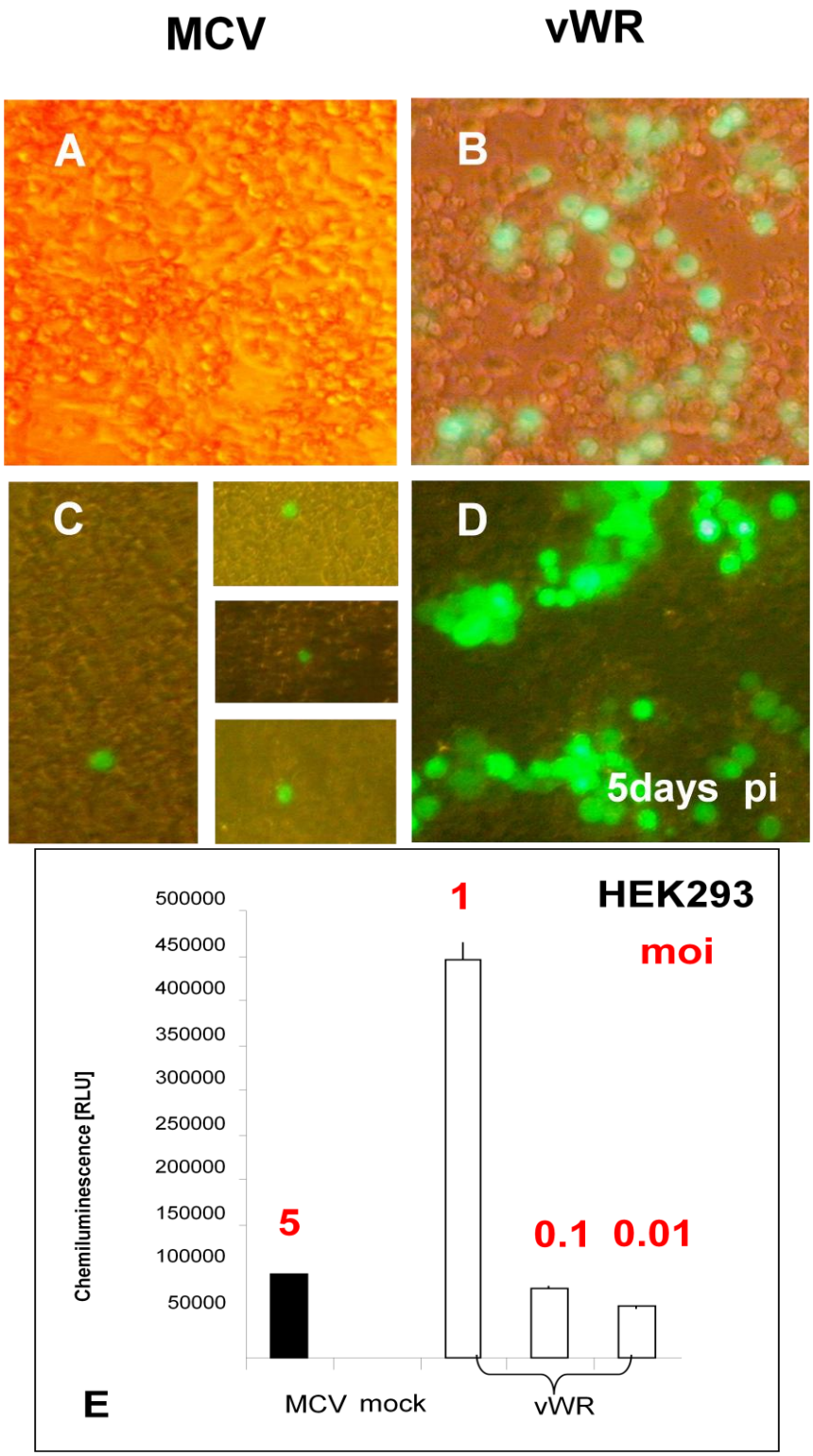


Figure 3.5. Images of luciferase and GFP in infected/transfected cells and quantfication of luciferase output. *Panels (a – d) show HEK 293 cells infected with MCV (a and c) and vWR (b and d). Inserts in c show individual GFP-positive cells. Panel e shows a histogram of luciferase data giving chemiluminescence in RLU. HEK 293 cells were infected with MCV or vWR at the indicated MOI and collected at 16 h p.i.*

Discussion

Determination of the optimal VACV for use as a postive control resulted in the vWR preparation in RK13 cells achieving the most favourable luminescent signal. The titre measured $4.0x10^6$ pfu/ml, giving it an MOI of 4 when $1x10^4$ cells are infected. This was viewed as a more desirable MOI than that of the preparation that emitted the strongest luminescent signal, vWR. (MOI=0.02) Prior to correction by renilla, the FF signal achieved from vWR was extremely low, suggesting it may not be appropriate as a postive control. Upon dilution of the vWR RK13 VACV preparation, MOI=0.04 was chosen as optimal. Dilutions after this tended to plateau in effect, with more concentrated dilutions providing too large a luminescent signal to provide an appropriate postive control. Although VACV was initially used as a control, semiquantitative PCR allowed this poxvirus to act as a comparator to MCV. Difficulties in the titration of MCV have meant that this was not previously possible.

Determination of the optimal Renilla luciferase vector resulted in use of the phRL-TK reporter plasmid (ILR#238) in subsequent experiments as this expressed low to moderate levels of renilla luciferase which appeared to be more consistent. This provided a more appropriate transfection control by which to normalise firefly readings.

Since vaccinia plaque-forming units cannot be directly compared to MCV virion units because of the different nature of their biological activity the PCR method described in allows a relative quantification of MCV genome equivalents to VAVC-WR infectious units measured in pfu/ml based on amplifiable genomic DNA units/molecule numbers calculated for a relatively large plasmid containing 25,517 bp of MCV sequence. The pfu-to molecule ratio for vaccinia virus (mature virions) comes out as 1:31, in keeping with previously published ratios (Postlethwaite *et al*., 1987). The PCR data can be used to calculate an MCV multiplicity of infection equivalents in relation to the control plasmid molecule numbers as well as in the form of pfu equivalents in relation to a titered vaccinia stock for comparison purposes. We have found this approach to be both more reproducible and more specific than electron microscopy or OD quantifications of virions. The biological activity of virions can be assessed using an *in vitro* transcription

reaction (Shand *et al*., 1976; Melquiot and Bugert, 2004). It is unclear to which exten the different GC content of the two virus genomes would affect the PCR product. This was not further investigated.

Through analysis of the renilla signals achieved from different cell lysates it became apparant that not all cell-lines are transfected equally with reporter plasmid. For example, HEK 293 cells were easily transfected resulting in an extremely high mean renilla reading, whereas cell-lines such as 3T3 and BJ-1 were relatively poorly transfected. (Fig 3.4b) These discrepancies would impact on the FF signal emitted and consequently under-estimate viral entry. Such transfectability issues were compensated for by correcting firefly signals to renilla, the transfection control. Analysis of the corrected data (Fig 3.4c) demonstrates that cells of 3T3 lineage experienced maximum MCV entry, indicating that an *in vitro* model using this cell line may be successful. These results corroborate with earlier studies, the first dating back to 1967, when Postlethwaite and colleagues described MCV cultures using mouse embryo fibroblasts (Postlethwaite *et al.*, 1978b) Notably, the host cell line derived from human keratinocytes, cells of the skin to which MCV may be specific, did not produce the strongest signal. Statistical comparisons involving mean corrected firefly signals from both MCV and VACV infected cell lysates demonstrated that in the majority of cases, viral entry is significantly different. In all but two cell lines, HaCaT and HeLa, MCV entry was significantly lower than that of VACV.

Variability in luminescent signal is evident by wide error bar range (Figure 3.4c). This was most noticeable among BJ-1 and 3T3 cell lines. This may be in part due to sub-optimal manual operation of the FLUORObstar OPTIMA. Ideally, luminescence should be measured immediately after the enzymatic reaction to ensure a true reading. However, for practical purposes, signal from four wells were read in sequence per measurement. The use of an injector system would guarantee an immediate measurement of signal, thus increasing accuracy.

Although cells were seeded to number $2x10^4$/well, epithelial cell lines divide at a higher rate than fibroblasts. Consequently, 16 hours incubation may lead to a greater number of

epithelial cells to fibroblasts. This could potentially result in elevated signals from cell lysates of epithelial origin. In addition, routine practice in animal cell culture technique encourages replacement of tissue culture flasks subsequent to each cell passage. This ensures no sub-optimal cells remain in culture. Due to preservation of laboratory stocks, cells were kept in the same culture flask for ten passages. It is therefore plausible that incompetent cells were plated as viable, as cell viability was not taken into account during cell number determination. Ramifications of this may include diminished capacity with reference to host cell infection and transfection, thus causing variation in signal.

In contrast to the current thinking that MCV is highly specific in both host and cell, we have indications that entry host-range is broad. Thus the lack of productivity in cell culture is not due to non-entry- or receptor issues. The virus gets into mouse (3T3), hamster (BHK-21), monkey (Vero) and various human cells (A549, HeLa, HaCaT) and drives early poxviral promoters. High specificity is unusual amongst poxviruses and we therefore hypothesise that MCV entry host-range is not so limited.

Cells of 3T3 lineage demonstrated maximum MCV entry. The inability to grow MCV in cell culture has hampered biological studies using the virus and therefore development of therapeutic strategies. The considerable MCV entry measured in 3T3 cells in this study is encouraging, and demonstrates that an *in vitro* model using this cell line may be successful. However, progression to late gene transcription needs to be investigated. Further repetition of the experiments may also confirm entry into alternative cell lines, e.g. BJ-1. Compared to VACV, it was found that MCV is much less able to enter cells, significantly so in all cell lines aside from HeLa and HaCaT.

The assay depends to a significant degree on the transfectability of the cell cultures involved. Human keratinocytes and fibroblast cell lines are most interesting as possible natural hosts for MCV, but they are also hard to transfect. We found HEK 293 cells to be the best transfected cell line. However, while this cell line shows robust reporter signals, it is clearly not the type of cell MCV naturally infects.

Transfected plasmids with poxviral transcription signals can be transcribed by the poxviral transcription complex produced by transcriptionally active cores after entry. It is not clear whether the transcription complex is accessed inside partially uncoated virions, with plasmid DNA getting inside cores, or by transcription complex that is released into the cytoplasm. MCV infected cells produce a robust luciferase signal after 16 h. However, GFP is only visibly expressed in a small number of individual cells, detectable after 5 days of incubation. Potentially, other cells may express GFP at a level undetectable by microscopy. The process where cores are accessible for the reporter plasmids may be delayed in MCV-infected cells. At the same time point, the WR infected wells will show extensive plaques and cell degradation.

The present study has successfully optimised a reporter assay based on simultaneous transfection of luciferase/EGFP reporter plasmids and infection with live MCV virus. This method can be used to infect a series of epithelial and fibroblast type cell lines of human and animal origin with MCV and determine infectivity. Cells of 3T3 lineage demonstrated maximal virus entry. Though robust luminescent signals can be obtained 16 hrs p.i., microscopic visualisation (using pE/L EGFP reporter) is only possible at 5 days p.i. The use of qPCR has enabled quantification of MCV and MOI calculations documented for the first time and published in 2012 (Sherwani *et al*., 2012).

Chapter 4 -

Seroprevalence of Molluscum contagiosum virus antibodies in German and UK populations

Chapter 4 - Seroprevalence of Molluscum contagiosum virus antibodies in German and UK populations

Introduction

After the eradication of smallpox, Molluscum contagiosum virus remains the principal poxvirus causing human disease (Chen *et al*., 2013). It has unique features that are distinct from otherpoxvirus genera pathogenic for humans such as othropoxviruses (OPV), including smallpox and monkeypox (Buller, 1991). MCV shares the highest level of amino acid similarity and unique proteins with parapoxviruses such as ORF and Milkers nodules (Delhon *et al.* 2004). MCV exclusively infects the human skin (Postlethwaite, 1970). The infection occurs worldwide and in all racial groups (Birthistle and Carrington, 1997). MC is most common in young children and teenagers. MCV infection in immunocompromised patients results in more numerous and extensive lesions, some of which may be giant (Buckley and Smith, 1999).

The true prevalence of MC has probably been underestimated because of the generally benign clinical manifestation and rare complications. Since it is a non-notifiable disease clinical cases are often neglected and underreported. Development of assays which could assist in seroprevalence studies has been hampered by unsuccessful attempts to cultivate MCV *in vitro* and by the lack of standardized serodiagnostic tools (Burnett and Neva, 1966; Burnett and Sutton, 1968; Buller *et al*., 1995; Fife *et al.,* 1996). Few studies have addressed questions of incidence trends or burden of infection.

In the first known MCV antibody study in 1953, Mitchell found three out of 14 MC patients had complement-fixing antibodies to an antigen prepared from human MC lesions (Mitchell, 1953). Mitchell suggested that it was likely that in a larger proportion of patients absorption of small amounts of soluble antigen caused the activation of the antibody-forming mechanism. However, the amount of circulating complement-fixing antibody was too low to be detectable. Incidents which cause vascular congestion, such as irritation or bacterial infection of a lesion, would cause absorption of larger amounts of antigen and the subsequent production of detectable antibody. Mitchell also stated

that the formation of antibody, as a secondary response, would lead to rapid disappearance of lesions as is commonly observed clinically as a sequel to secondary infection.

Shirodaria *et al*. used MCV cryostat sections in an immunofluorescence study of MCV antibodies, reporting IgM only in MCV patients and IgG antibody responses in 16.7% of healthy control subjects (n=30) (Shirodaria *et al*., 1979). They also reported a higher incidence of anticellular IgM antibodies in molluscum sections in comparison to sections with warts or psoriasis.

A study conducted by a Swiss group compared the pattern of dermatoses in different age groups within the pediatric population referred to Aargau Hospital in Switzerland, a hospital associated dermatology department, between 1998 to 2001 (Wenk and Itin, 2003). A prospective analysis was conducted using the data files of patients seen (outpatients and inpatients) in the department of dermatology dusring this period. The patients (n=1105) were grouped by age into four categories: infants (less than 2 years old), preschool children (3–5 years old), school children (6–11 years old), and adolescents (12–15 years old). The prevalence of MC was found to be highest in school children (10.6%) and was also found to be frequent in pre-school children (6.9%) as well. The ratio of females with MC was slightly higher (5.2%) than males (4.5%). The study suggested a lower frequency compared to general prevalence in literature due to an underreporting of cases.

A study by Reynolds *et al*. was unique in the fact that it used population based estimates to describe the trends in incidence and epidemiology of MC in the United States among American Indian and Alaska Native populations reported between 2001-2005 (Reynolds *et a*l., 2009). Based on outpatient visit rates, it was found that the MC-associated outpatient visits for American Indian and Alaska native persons for 2001-2005 was 20.15/10000, which was comparable to the rate for the general US population of 22/10000. American Indian and Alaskan native children in the age groups of 1-4 years had the highest incidence (77.12%) which was more than twice that for children in the

age group of 5-14 year old (30.79%) (Reynolds *et al*., 2009). They also found that the incidence for infants <1 years of age was more than that for adults.

So far only two seroprevalence studies using ELISA, have been reported; one by Konya and Thompson (Konya and Thompson, 1999) in 1999 and one by Watanabe and co - workersin 2000 (Watanabe *et al*., 2000).

Konya and co-workers described in 1992 a virion based enzyme linked immunosorbent assay (Konya *et al*., 1992). MCV virions were isolated from human lesion material. The antigen was extracted from pooled lesions of different genotypes with epidermal protein extract used as a control. MCV virions were isolated from human lesion material and plated for ELISA assay. ELISA plates were batch-coated with purified MCV-1 virion proteins (5 ug/ml), with no further details on how they were purified. Their 1999 serological survey of a healthy Australian population (n=357), MCV infected patients (n=35) out of which 24 were HIV-1 negative and 11 were HIV-1 infected individuals, revealed a overall seroprevalence of 23%, with 77% prevalence among HIV negative MCV patients and, and 91% among HIV positive MCV patients. This was perceived as a high seroprevalance, likely to be an overestimate due to a lack of specificity of the antigen purified from human biopsy material considering variation of MCV antigen from different biopsies and patients and skin protein background.

The basis for the development of recombinant antigen ELISAs was laid down with the sequencing of the viral genome in 1996 (Senkevich *et al*., 1996). Based on MCV sequence information then available, in 1998 Watanabe *et al*. identified two immunodominant proteins of 70 and 34 kD and mapped them to the ORFs mc133L (vaccinia virus homolog A27L) and mc084L (vaccinia virus homolog H3L), respectively using a Cowpox expression system (Watanabe *et al*. 1998). The proteins are homologues of vaccinia virus proteins H3L and A27L, and major antigenic peptides of the virion particle (Senkevich *et al.*, 1996; Watanabe *et al*., 1998).

The first MCV ELISA based on a recombinant protein was reported in 2000 by Watanabe and co-workerswho used the Sendai virus expression system and an N-

terminal truncation of MCV virion protein MC133 (Watanabe *et al*., 2000). They presented seroepidemiological data in a representative Japanese population of 508 subjects with and without clinical MCV infection. MCV specific antibodies were found in 58% (7/12) patients with molluscum contagiosum, 6% (7/108) healthy controls, 9% of patients with atopic dermatitis (7/76) and 18% of patients with systemic lupus erythematosus (7/39). Of the HIV positive patients with MCV only 14% (1/7) were seropositive compared to 2% (5/266) patients who were HIV positive but MCV negative. The low antibody detection rate in patients with clinical disease and the generally low seroprevalence in comparison to the Australian study indicates that the Watanabe ELISA may be underestimating MCV prevalence and that MC133 may not be the best choice of antigen for an MCV ELISA. Their claim of MC133 being a superior antigen to MC084 was not supported by data.

The objective of this study was to use MC084 which is a MCV surface viral protein which is a part of the entry complex of the virion and develop highly sensitive recombinant MCV ELISA using water soluble and highly antigenic truncations of MC084L, expressed in *E. coli,* which are more suitable for large scale production. MCV ELISAs based on *E. coli* expressed MCV antigen testing have so far not been reported. Another aim of this study was also to establish seroprevalence trends in a German and a UK sera collection and to provide a comprehensive view of epidemiology of MC across different age groups in a German population; the first in any European population.

In light of the information on poxviral zoonoses and smallpox vaccination also reviewed extensively in the introduction, we further investigated the specificity of what appear to be human Molluscum contagiosum antibodies, (high titre antibodies as determined by recombinant MC084S (V123-R230) ELISA, versus vaccinia virus in plaque and reporter inhibition assays (v3 recombinant VACV reporters). Using the same sera dilutions used in ELISA (1:100), we wanted to test whether any neutralization was observed over this dilution in reporter and plaque assays. Also if so, was the neutralisation a more common phenomenon in younger or older people?

Results

4.1 Selection of antigen, cloning and purification

4.1.1 Antigen selection

The molluscum gene mc084 was selected as a candidate for expression and purification as an immunogenic peptide based on a previous study by Watanabe and co-workers (Senkevich *et al.*, 1996; Watanabe *et al.*, 1998). Amino acid sequences of the MC084 (298 aa, 34 kD) were analysed using standard software packages to determine overall homology with related proteins in the GenBank and identify transmembrane regions and region of high hydrophilicity and high antigenicity.

Two transmembrane regions predicted in the C-terminal end of the protein (Blasco and Moss, 1991) were excluded to avoid solubility issues in the *E. coli* expression system (Figure 4.1A). Of the remaining 230 amino acids, two subclones were defined; an N-terminal truncation (V33-G117; 30% aa identity with VACV097) and a C-terminal truncation (aaV123-R230; 27% aa identity with CPXV_UK2000_K2984_106), both containing one region of high hydrophilicity in the Kyte–Doolittle plot (Figure 4.1B) (Kyte and Doolittle, 1982), which were further analysed for subcloning.

An open unlimited NCBI BLASTN search was used to compare the truncated amino acid sequences of mc084 to Genbank sequences (nr). MC084V5 (aa33-117) showed the highest overall amino acid identity with vaccinia virus protein VACV097 (H3L) of 30%, with no significant homologies detected using NCBI BLAST 2 algorithm between the MCVs most antigenic motif (GGNIRNDDKYTH) and its corresponding sequence in vaccinia virus.

MC084S (aa123-230) showed the highest overall amino acid identity with the cowpox virus IMV heparin binding protein (CPXV_UK2000_K2984_106) of 27%, with no significant homologies detected using NCBI BLAST 2 algorithm between the MCV most antigenic motif (NELRGREYGASLR) and its corresponding sequence in cowpox virus. A similar homology of 30% was found with the agmatine deiminase enzyme of

Listeria monocytogenes. MC133 and MC084 share an overall level of homology to orthopoxvirus homologs of between 27% (MC084) and 30% (MC133), making MC084 potentially the more MCV specific antigen.

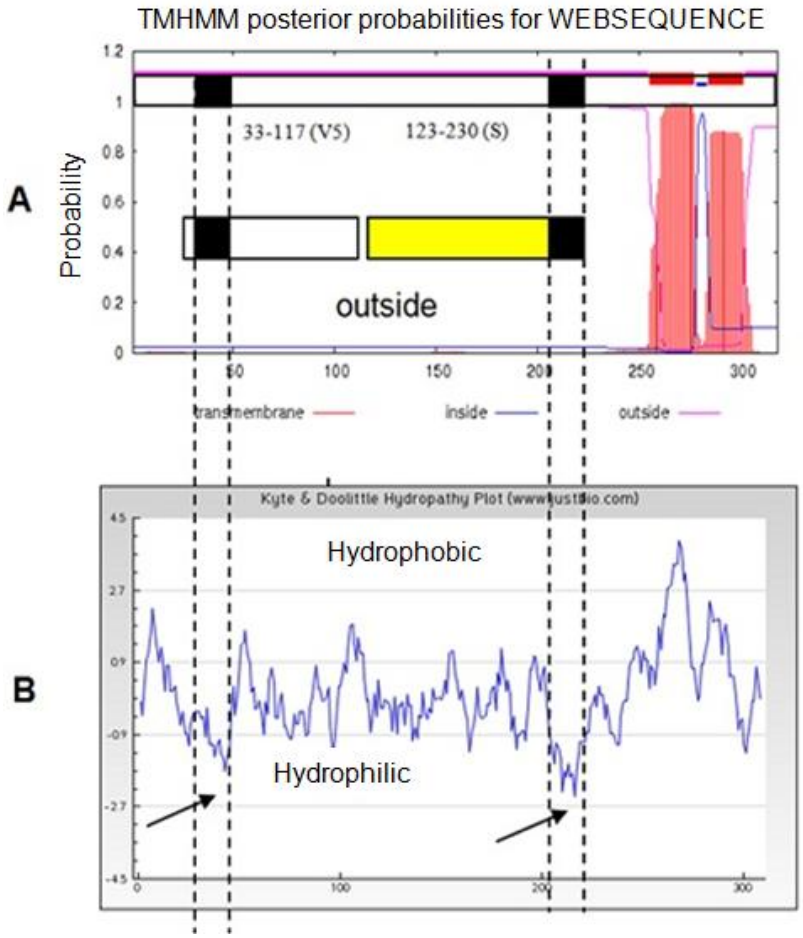


Figure 4.1. Bioinformatics for MC084. *(A) Transmembrane plot (TMHMM Server v. 2.0) of mc084 amino acids 1-318; (B) Hydropathy plot of MC084 protein with predicted high hydrophilic / antigenic regions indicated by black boxes. The full length ORF (MC084 1-318; predicted molecular weight 34.2 kD; shown on top) was cloned into vRB12 using specific primers tailed with restriction enzyme sites BamHI-HindIII and C-terminal StrepII epitope tag. The resulting plasmid p319 was sequenced and the recombinant vaccinia virus v319 isolated on BSC-1 cells using the plaqueless mutant system. N- and C-terminal (in yellow) truncations were subcloned from the original full length MCV gene into pGEX-2TK for overexpression in E. coli BL21 (RIL^{+}). TMHMM was used to determine transmembrane regionswhereas the Kyte Doolittle plotwas used to identify hydrophilic regions with predicted high antigenicity.*

4.1.2 Cloning of mc084

The C-terminal truncation of MC084 (V123-R230, predicted MW 14 kD), comprising 107aa, has the lowest homology to orthopoxvirus proteins and contains a region of high antigenicity (218-NELRGREYGASLR-230) with no significant homology to vaccinia/ cowpox virus. The C-terminal truncation of MC084 (V123-R230) was subcloned into the pGEX-2TK vector (Figure 4.2A) with a Strep II tag and in frame with glutathione S-transferase separated by a thrombin kinase site (Figure 4.2B), and due to the high GC content, overexpressed as a GST fusion protein in codon optimized *E. coli* (BL21 RIL^{+}).

The GST fusion protein was identified with an apparent molecular weight (MW) similar to the predicted MW of 40 kD in IPTG induced cultures (Figure 4.3A).

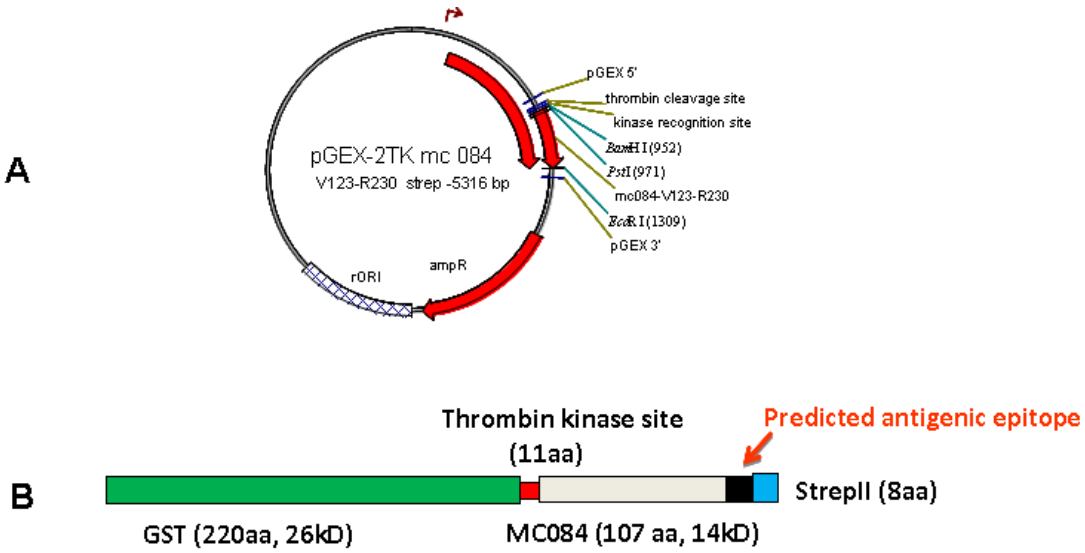


Figure 4.2. MC084-pGEX 2TK construct. *(A) Schematic of recombinant plasmid p332 with a MC084 specific insert of 107 amino acids (V123-R230); predicted molecular weight 14 kD. (B) Schematic of fusion protein of GST (green), followed by Thrombin cleavage site (red), MC084 V123-R230 (grey), and strep II tag (blue); predicted antigenic site (black).*

4.1.3 Purification of recombinant MC084 protein

The protein was protease cleaved and the C-terminal truncation of MC084 with an apparent MW of 14 kD was further purified using a Superdex S200 column via FPLC (Figure 4.3B). The Strep II tag was identified in Western Blot in both the fusion protein and the cleaved MC084 (V123-R230)-Strep II truncation (Figures 4.3C and 4.3D).

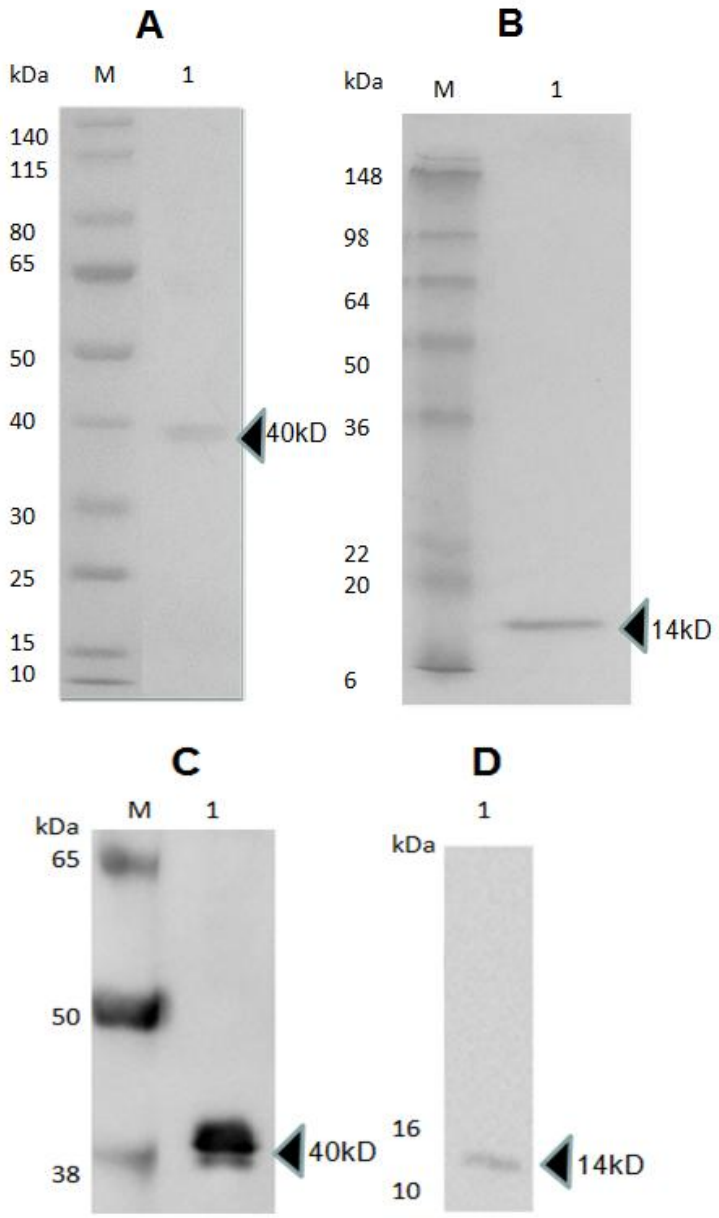


Figure 4.3. Protein purification. *Characterisation of overexpressed recombinant fusion protein GST-MC084S and FPLC purified recombinant MC084S protein by SDS-PAGE and Western Blot. M: Molecular weight markers expressed in kDa. (A) Overexpressed 40kDa recombinant GST-MC084S fusion protein separated in a 4-12% Bis-Tris gel. (B) FPLC purified 14kDa protein separated in a 15% Bis-Arylamide gel. Both gels were stained with Coomassie Brilliant Blue R-250. (C) GST-MC084S fusion protein after transfer to nitrocellulose (D) FPLC purified MC084S. The membranes were probed with Strep MAB-Classic HRP conjugate (IBA-lifesciences). Arrow heads indicate the locations of proteins.*

4.2 Antigen specificity and comparisons

4.2.1 Antigen specificity

The antigenicity of MC084S (aa123-230) was determined by direct binding ELISA.The strong reactivity of C-MC084S towards high titre human serum sample referred to as HDV0901071 antibodies belonging to a seven year old male was determined through C-MC084S ELISA. Antigen antibody immune complex equilibrium was achieved at a concentration of 4 µg/ml (Figure 4.4A). Antigen and antibody (serum HD V0901071) binding specificity was further ascertained by competitive inhibition ELISA using protein inhibitors that are abundantly present in human serum such as bovine serum albumin (BSA) and human immunoglobulin-G (IgG). A maximum of 80% inhibition of anti-serum antibodies with MC084S as inhibitor was observed whereas inhibition observed with BSA and human IgG was negligible (Figure 4.4B).

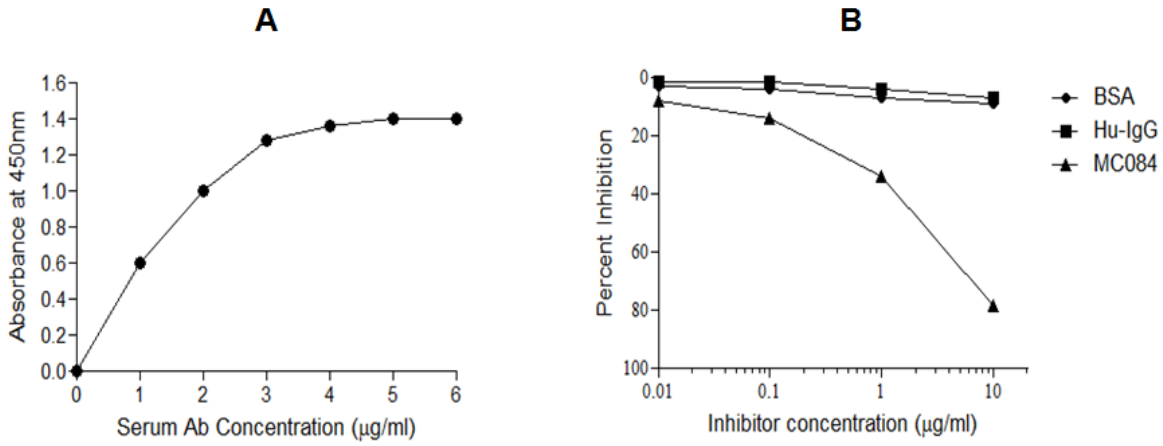


Figure 4.4. Antigen specificity. *High titre antibodies from a representative high titre serum HD V0901071 (1:100) used to check (A) binding affinity to MC084 (V123-R230) protein antigen coated in increasing concentration (0-6 µg/ml) and (B) cross reactivity (% inhibition) with MC084S, BSA or human IgG in a 96 well plate. Each value represents mean of three independent assays.*

4.2.2 Antigen comparisons

The N-terminal truncation of MC084 i.e. MC084v5 (aa33-117), C-terminal truncation of MC084 i.e. MC084S (aa123-230), N-terminal truncation of MC133 i.e. MC133S (aa1-370) and GST were prepared and compared as uncleaved fusion proteins on a GST

affinity plate to compare antigen affinity and seroreactivities (Figure 4.5). MC084 C-terminal has a slight advantage over MC084 N-terminal and performs similar to MC133. The relative absorbances of sixteen individual sera were the same against all antigens tested with only minimal differences in absorbance.

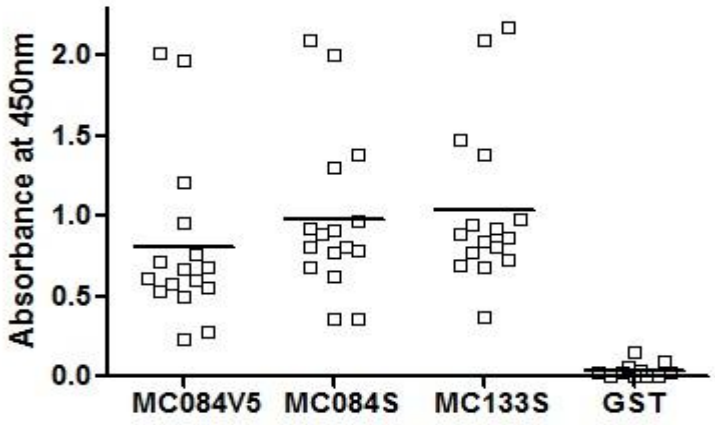


Figure 4.5. Antigen comparisons. *Comparison of relative antigen affinities of MC084v5 (aa1-117), MC084S (aa123-230) and MC133S (aa1-370) expressed as GST fusion proteins and GST coated on an anti GST coated plate against a selection of high and low titre sera (n=16) from a representative healthy German population. The horizontal bar within each group represents the median absorbance measurement.*

On comparing the two truncations of MC084, the N-terminal truncation of MC084 i.e. MC084v5 (aa33-117) showed reduced reactivity against the sixteen sera samples with a lower median absorbance measurement of 0.805 in comparison to the C-terminal truncation of MC084 i.e. MC084S (aa123-230), which had a slightly higher median absorbance measurement 1.00. In direct antigen comparison there was no significant difference between absorbance measurments of truncations of MC084 and MC133 (median absorbance 1.05), and no serum showed prevalent reactivity against one or another of the antigen used. Purified GST antigen showed hardly any reactivity against the sixteen sera samples (median absorbance 0.023).

4.2.3 Strep II tag interference

To rule out strep II epitope tag interference in the binding of serum antibodies against MC084S (aa123-230), we tested high titre human serum antibodies alone as well as after incubation overnight with GST-MC133S fusion protein on glutathione sepharose beads. After overnight incubation at 4˚C, the serum was collected and used in ELISA. Commercially available Strep antibodies (Classic Strep MAB) were used either alone or after overnight incubation under similar conditions as mentioned above and served as positive and negative controls for this assay (Figure 4.6).

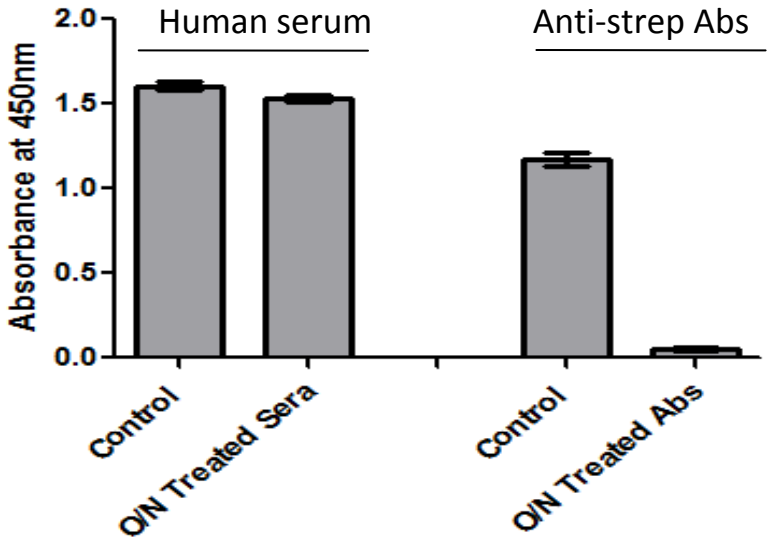


Figure 4.6.**Strep interference**. *ELISA plate was coated with MC084S (aa123-230). Control and overnight treated sera of human (HD V0901071) alone as well as treated overnight with strep tagged Glutathione sepharose bead bound fusion protein GST-MC133S. Anti-strep antibodies alone as well as treated overnight served as positive and negative controls respectively.*

It is clear from the assay that the Strep II tag did not interfere with the binding of MC084S specific serum antibodies with MC084S (aa123-230) epitope.

4.3 ELISA Sensitivity and cut-off

4.3.1 ELISA sensitivity

MC084S (aa123-230) ELISA specificity and sensitivity were established on the basis of twelve sera samples from MCV patients. Ten were collected at the School of Dermatology, Skin Institute New Delhi and two were collected in the South-western UK (Bath and Cardiff). To establish sensitivity a panel of these 12 sera samples from patients with known and clinically active MCV was first screened in comparison to sera from 0-1 year old individuals from the neonatal screening program of the Heidelberg University Clinics. In the group of sera from patients with diagnosed Molluscum contagiosum (n=12), the ELISA gave high absorbance for all (median δODU 1.5), with the most recent sample from Cardiff (CF2012-1) exhibiting the highest binding (Figure 4.7). The control group of seventeen neonates from the Heidelberg University Clinics showed low reactivity with a mean δODU of 0.1 as shown in Figure 4, with one outlier (0.61 δODU). The confidence interval for the difference between positive and negative control groups was highly significant (Figure 4.7). Sensitivity was 100% for MC patients.

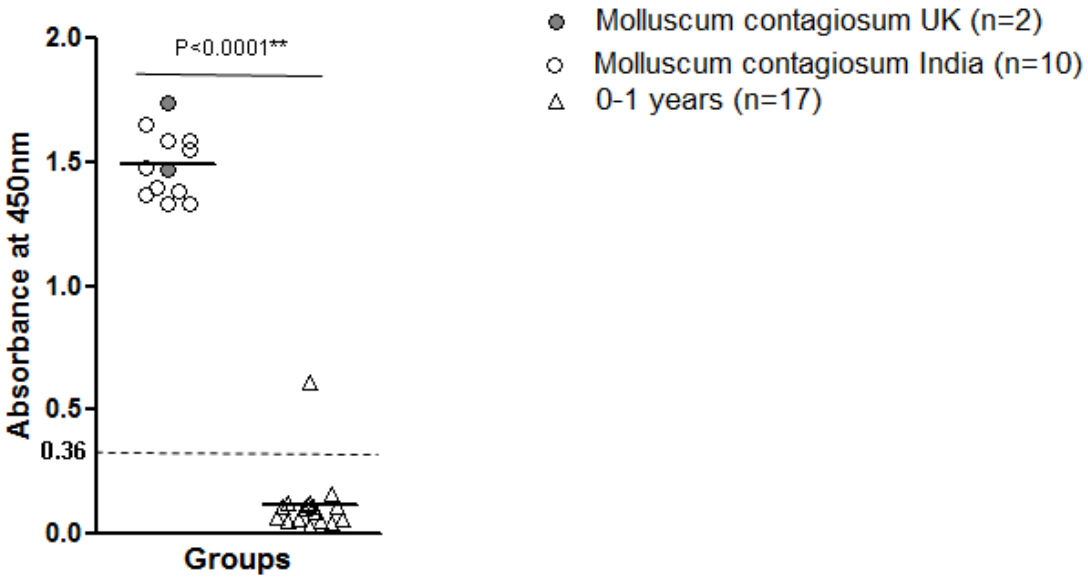


Figure 4.7. ELISA sensitivity. *Absorbance plot of twelve sera from patients clinically diagnosed with MCV (India n=10; UK n=2); control group of 0-1 year old individuals (n=17).*

4.3.2 ELISA cut-off

The cut-off for ELISA was calculated based on 66 sera from infants seen in the neonatal unit aged 0-1 years. The mean of δODU readings was 0.12043 and the SD was 0.08300. In comparison the mean δODU for 12 MCV infected patients was 1.57 and the S.D. 0.571. The infant group was used to define negativity with the upper limit being the mean δODU plus 3 S.D. (i.e. 0.36). Assuming that these values are indicative of a negative response to the recombinant protein, we defined a positive antibody response as being a value greater than mean plus 3 SDs i.e. δODU 0.36. Two more outliers were identified in this group (δODU 0.36 and 0.359) which led to an alternative cut-off calculation based in inclusion or exclusion of the outliers (Table 4.2). The MCV status of these subjects (aged 2 months, 9 months and 11months) could not be determined. Inter-well, intra-assay and inter-assay variability was found to be 3%, 5.25% and 6.72 % respectively.

4.3.3 Sera specificity confirmation by microscopy

4.3.3.1 Typical staining pattern with MCV positive serum (CF2012-1)

An example for specific reactivity human in MCV infected tissue section obtained from the Heidelberg University Dermatology Unit was tested with high titre serum from a MCV positive UK patient CF2012-1 is demonstrated in Figure 4.8. The section shows a dome-shaped contour with cup shaped lesions with central invagination, representing a typical MCV lesion consisting of two inverted lobules of hyperplastic squamous epithelium (red arrows) with several sub-lobules. The MC lesion shows acanthosis with the appearance of intraepidermal lobules with enlarged basophilic nuclei filled with cellular as well as MC Henderson Patterson bodies (black arrows). Intraepidermal lobules are separated by septa consisting of compressed dermis (dotted arrow). MCV inclusion bodies stain strongly golden-brown with the human polyclonal serum CF2012-1 taken from a patient with clinical MCV infection. The stain is confined to areas where MCV cores, mature and released virus particles would be expected. In a number of tissue sections stained, the pattern was repeatable and sensitive to tissue preparation. Interestingly, the debris areas filled with mature MCV particles and lipid

debris are also sensitive to removal by xylene/ethanol treatment of paraffin sections. The areas most consistently stained are the suprabasal and spinous layers.

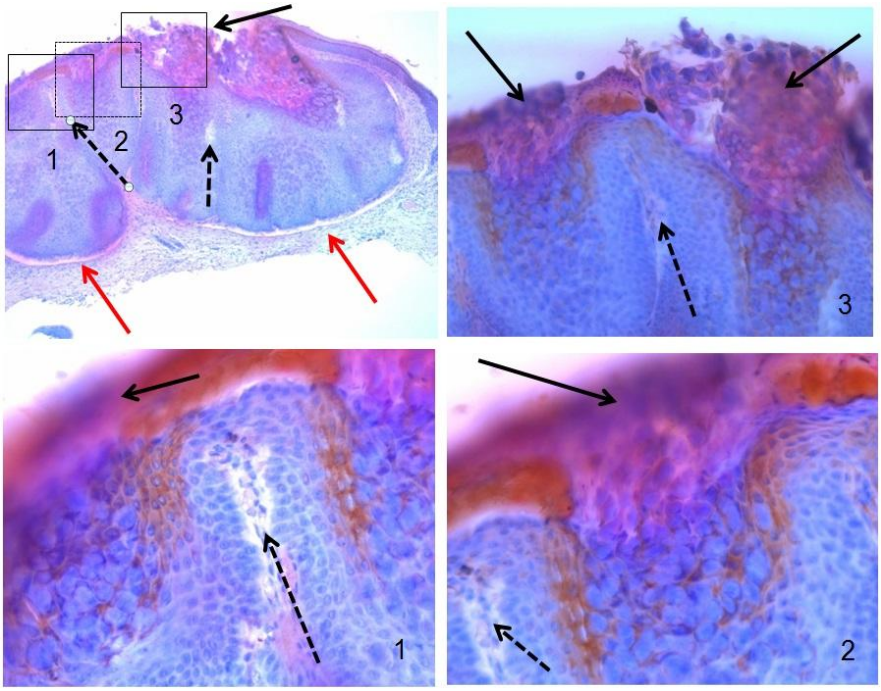


Figure 4.8. MCV infected tissue stained with MC positive human serum. *Microscopy (4x) of a Molluscum contagiosum lesion section (17315/11) stained with MC patient positive serum (CF2012-1) and haematoxylin-eosin counterstain (upper left hand corner). Three insets showing details at various magnifications [inset 1-(10x), inset 2-(20x) and inset 3-(20x)].*

4.3.3.2 Typical staining pattern with MCV negative serum

As an example for specific reactivity of serum from an unifected individual in infected skin, a MCV infected human tissue section obtained from the Heidelberg University Dermatology Unit was tested with control serum from a 32 year old female with no prior history of molluscum contagiosum as demonstrated in Figure 4.9. The section shows a multi-lobular section of an MCV lesion consisting of lobules of hyperplastic squamous epithelium. Staining patterns consistent with typical haematoxylin-eosin staining show blue staining of nuclei of cells and pink-red of eosinophilic structures over the entire lesion. There is a clear absence of golden-brown staining pattern in the lesion.

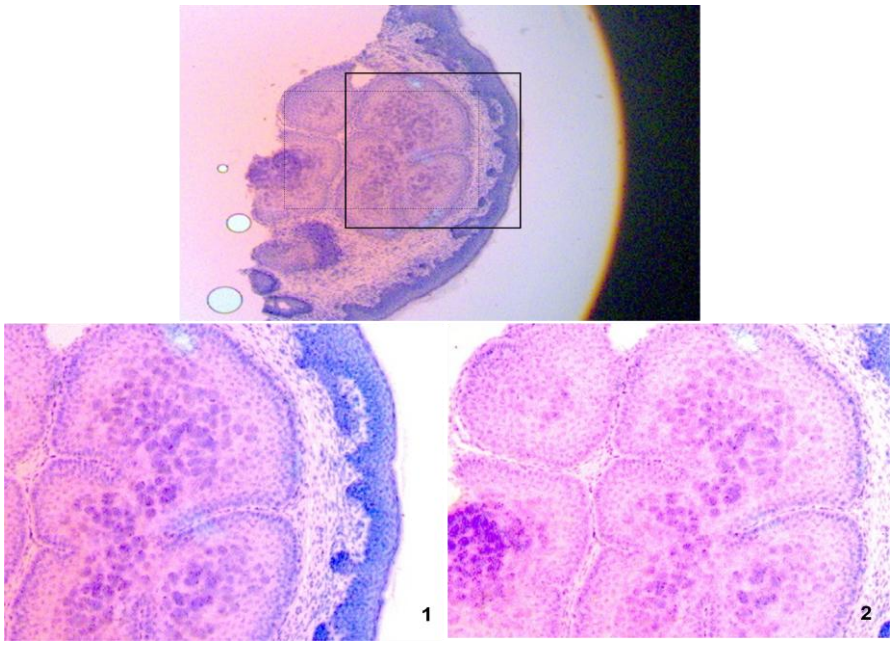


Figure 4.9. MC infected tissue stained with control human serum. *Microscopy (4x) of a Molluscum contagiosum lesion section (12710/12) stained with control human serum from an individual with no history of MC infection and haematoxylin-eosin counterstain. Two insets showing details at a similar magnification [inset 1-(10x) and inset 2-(10x)].*

4.3.3.3 Staining patterns with high and low titre sera in MCV biopsy sections

ELISA specificity was established by using human MCV infected tissue sections obtained from the Heidelberg University Dermatology Unit which were tested with high and low titre sera as determined from the MC084S (V123-R230) ELISA. A panel of high and low titre sera samples used to stain tissue samples are tabulated in Table 4.1.

Table 4.1. High tire and low titre serum samples used in MC biopsy section staining.

High Titre Sera (Positive)				Low Titre Sera (Negative)			
Serial No.	**Serum No.**	**Age (years)**	**Sex**	**Serial No.**	**Serum No.**	**Age (years)**	**Sex**
1.	HD V0901071	7	M	1.	HD V0905374	1	M
2.	HD V0905794	5	F	2.	HD V000040	1	M
3.	HD V0903005	6	F	3.	HD V901798	1	F
4.	HD AZ004482	47	F	4.	HD V0905511	1	M
5.	HD AZ005203	51	M	5.	HD AZ005860	50	M

In figure 4.10 panels 1-5 show molluscum contagiosum lesions stained with high titre human sera samples at various magnifications as determined by recombinant MC084S (V123-R230) ELISA and indicated in Table 4.1, together with a haematoxylin eosin

counterstain. The sections show epidermal stratification as basal, suprabasal and spinous layers. The molluscum lesions typically consist of hyperplastic squamous epithelium with several sub-lobules bearing molluscum bodies. The lesion(s) lie adjacent to normal appearing epidermis in panel 1, 3-5. Intraepidermal lobules are separated by septa consisting of compressed dermis. All sections show strong golden brown staining of the lesional area indicating specificity of the serum antibodies established through the MC084 (V123-R230) ELISA. The staining is especially strong in the the acanthotic intraepidermal lobules exhibiting central cellular MC bodies' debris and released viral material. The skin adjacent to the MC lesions showed either no histological alterations or mild epidermal acanthosis.

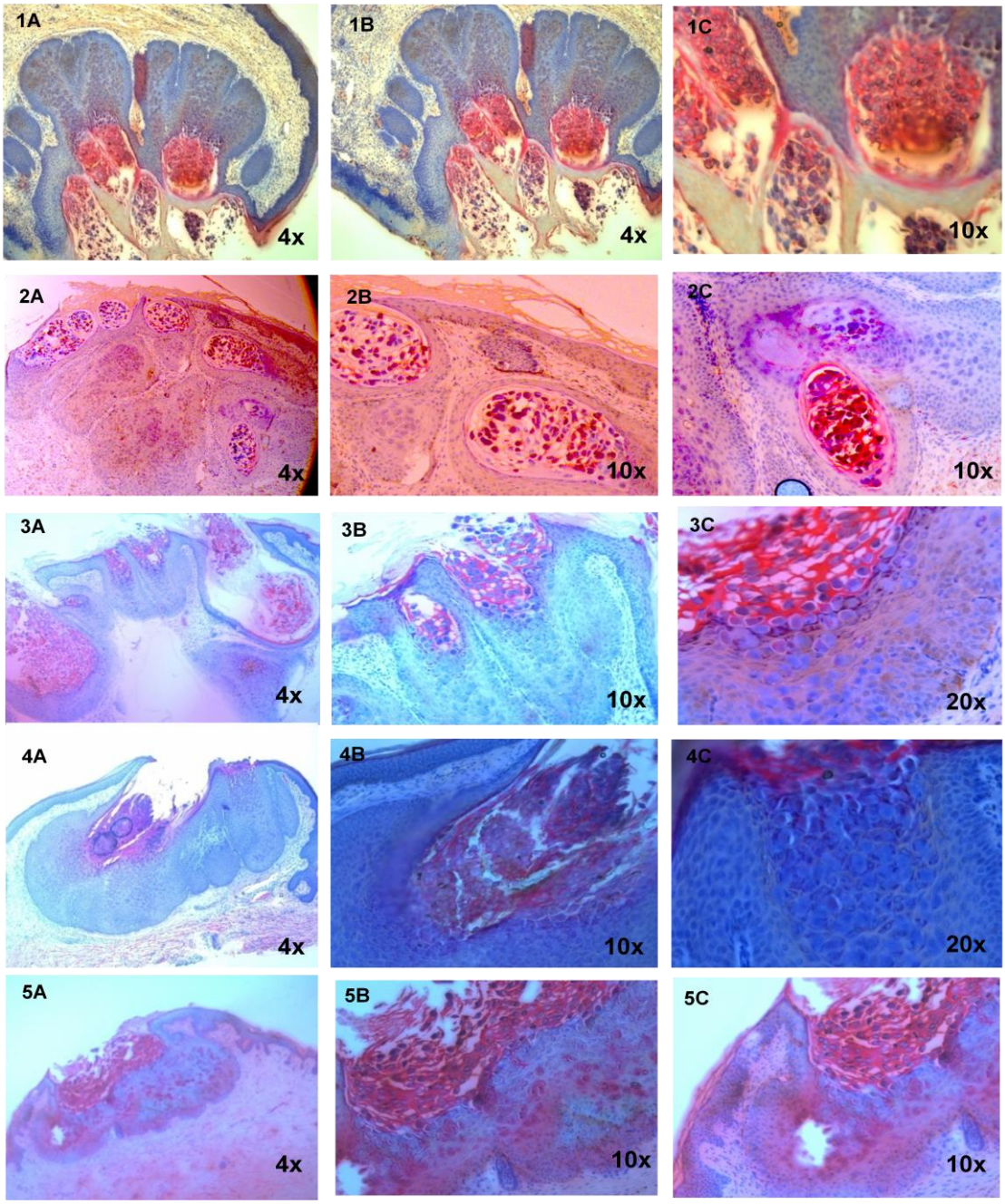


Figure 4.10. MC tissue stains with high titre sera confirming ELISA specificity. *Tissue sections from MCV positive patient biopsies stained with high titre human sera samples as determined by recombinant MC084S(V123-R230) ELISA. Panel 1 shows section 5911/12 stained with HD V0901071-1A (4x), 1B (4x) and 1C (10x). Panel 2 shows section 7633/11 stained with HD V0905794-2A (4x), 2B (10x) and 2C (10x). Panel 3 shows section 19858/11 stained with HD V0903005-3A (4x), 3B (10x) and 3C (20x). Panel 4 shows section 17314/11 stained with HD AZ005203-4A (4x), 4B (10x) and 4C (20x). Panel 5 shows section 12877/11 stained with HD AZ004482-5A (4x), 5B (10x) and 5C (20x). (Haematoxylin-eosin counterstain).*

In contrast, Figure 4.11 panels 1-5 show various magnifications of molluscum contagiosum lesions stained with low titre human sera samples as indicated in table 4.1 together with a haematoxylin eosin counterstain. Again, the molluscum lesions typically consist of hyperplastic squamous epithelium with several sub-lobules separated by compressed dermis and bearing molluscum bodies. The lesion(s) lie adjacent to normal appearing epidermis in panel 1-2 and 4-5. All sections show either no staining or mild golden brown staining of the lesional area. It can be inferred that the sera used for staining these sections have either no MC084S (V123-R230) specific antibodies or very low titres, as already established by the MC084S (V123-R230) ELISA. The skin adjacent to the MC lesions showed either no histological alterations or mild epidermal acanthosis.

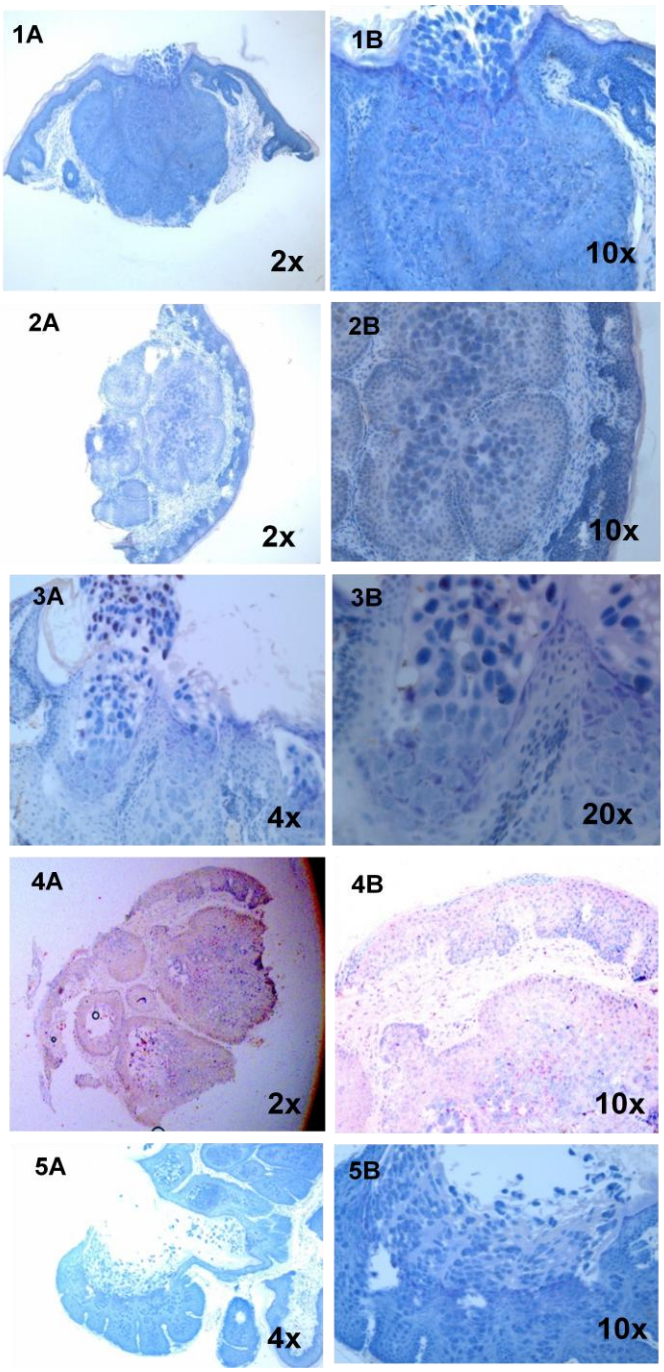


Figure 4.11. Molluscum contagiosum tissue stains with low titre sera confirming ELISA specificity. *Tissue sections from MCV positive patient biopsies stained with low titre human sera samples as determined by recombinant MC084S(V123-R230) ELISA. Panel 1 shows section 10624/12 stained with HD V0905374-1A (2x) and 1B (10x). Panel 2 shows section 12712/12 stained with HDV000040-2A (2x) and 2B (10x). Panel 3 shows section 12720/12 stained with AZ005860-3A (4x) and 3B (20x). Panel 4 shows section 63541/12 stained with HD V901798-4A (2x) and 4B (10x). Panel 5 shows section 10195/12 stained with HD V0905511-5A (4x) and 5B (10x). (Haematoxylin-eosin counterstain).*

4.3.3.4 Staining patterns with high titre versus low titre sera in biopsy sections from same patient series

To further demonstrate the difference in tissue staining with high and low titre sera, tissue sections obtained from the same patient series, were stained with a panel of five sera each. In figure 4.12 panel 1A, 1B and 2A,2B showtwo tissue sections stained with a high (HD V0901071, HD V0903005) and in figure 4.12 panel 1C, 1D and 2C, 2D show two tissue sections stained with low titre sera (HDV0900471, HDV0900040) at two magnifications (4x and 10x). Sections in panel 1 belong to a single patient series whereas sections in panel 2 belong to another patient series.

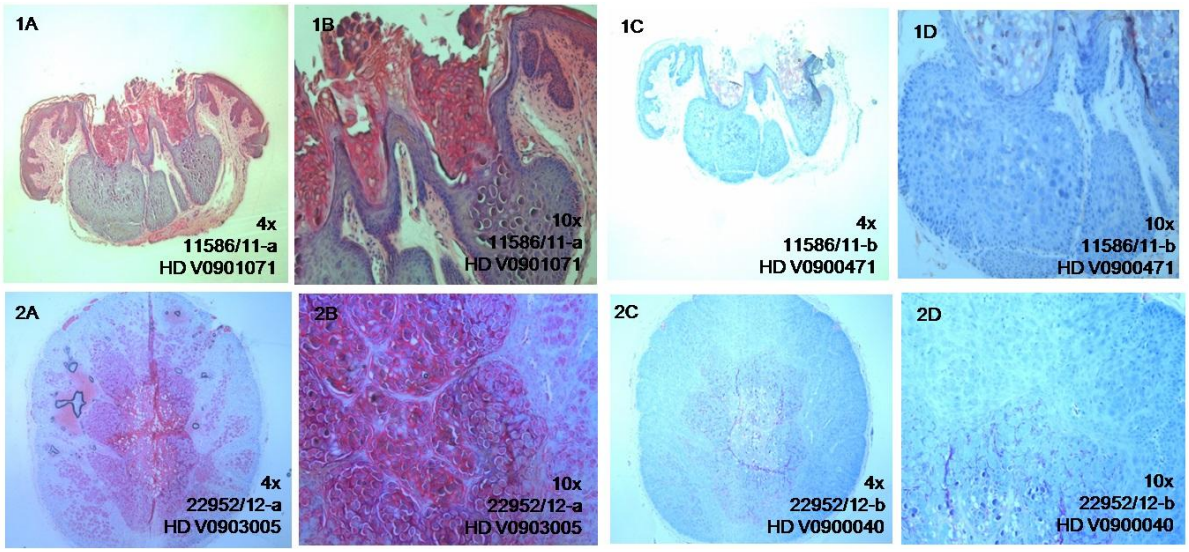


Figure 4.12. Serum specificities in tissue stains. *Tissue sections from MCV positive patient biopsies stained with high titre human sera HD V0901071-1A (4x) and 1B (10x), HD V0903005-2A (4x) and 2B (10x) and low titre human sera HD V0900471-1C (4x), 1D (10x) and HD V0900040-2C (4x) and 2D (10x). (Haematoxylin-eosin counterstain).*

Reactivity of high titre sera are shown on the left (Figure 4.12 panel 1A, B/ transverse section-perpendicular to level skin and 2A, B/ plane section-parallel to level skin). High titre sera show strong MC084 specific staining of cellular debris and MC bodies extruded from and in the centre of lesions, as well as infected cells in lobules extruding infectious virus into the centre of the lesion and upwards. Molluscum rich lipid debris areas are well preserved in these lesions. There is much weaker staining with low titre sera (same lesions in transverse and plane sections; figure 4.12 panel 1C, D and 2C, D).

4.3.3.5 Confirmation of antigen specificity in HaCaTs expressing vMC084 by immunofluorescence

To further establish antigen specificity we also infected human HaCaT keratinocytes with a vaccinia virus expressing full length mc084 (v319; aa 1 to 318) as shown in Figure 4.13. Infected keratinocytes were immune stained with the high titre serum HD V0901071 (Figure 4.13 A – 60x). Virus infected cells show a vesicular stain similar to an endosomal/lysosomal pattern. Infected keratinocytes were similarly immune stained with the low titre serum HD V0900040 and showed no reactivity (Figure 4.13 B – 60x). Uninfected cells (Figure 4.13 C – 60x) show no background signal, indicating the human polyclonal serum does not recognize keratinocyte antigens in cultured HaCaT cells.

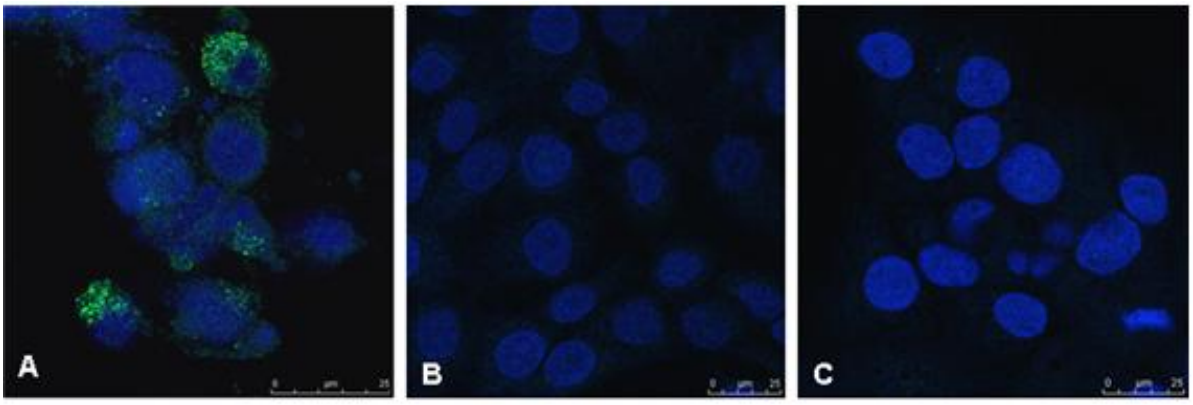


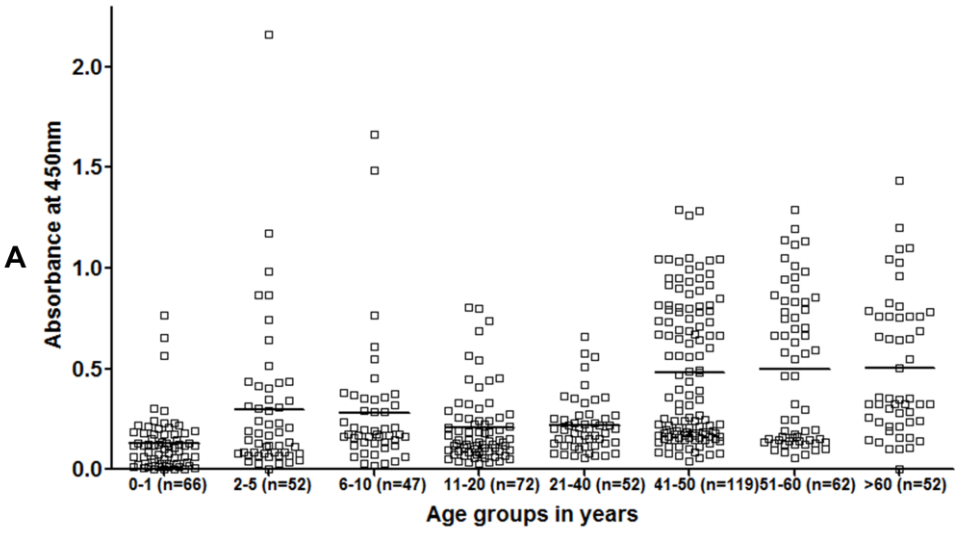
Figure 4.13. HaCaT Immunofluorescence. *(A) HaCaT cell culture infected with recombinant vaccinia virus expressing MC084S (v319). Reactivity of high titre human serum HDV0901071 and secondary antibody Alexafluor 488 (Green) goat anti-human IgG (H+L). (B) HaCaT cell culture infected with recombinant vaccinia virus expressing MC084S (v319). Reactivity of low titre human serum HDV0900040 and secondary antibody Alexafluor 488 (Green) goat anti-human IgG (H+L). (C) Mock infected cells. Reactivity of high titre human serum HDV0901071 and secondary antibody Alexafluor 488 (Green) goat anti-human IgG (H+L). Nuclei are stained with DAPI (Hoechst) and shown in blue. Samples were analysed for fluorescence emission properties by using confocal scanning laser microscopy Leica TCS SP2 AOBS.*

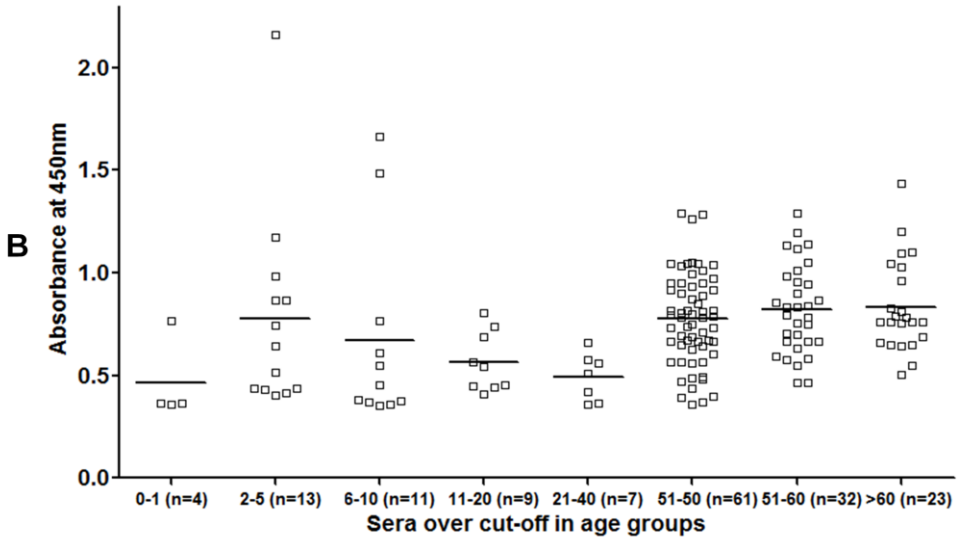
4.4 ELISA population studies

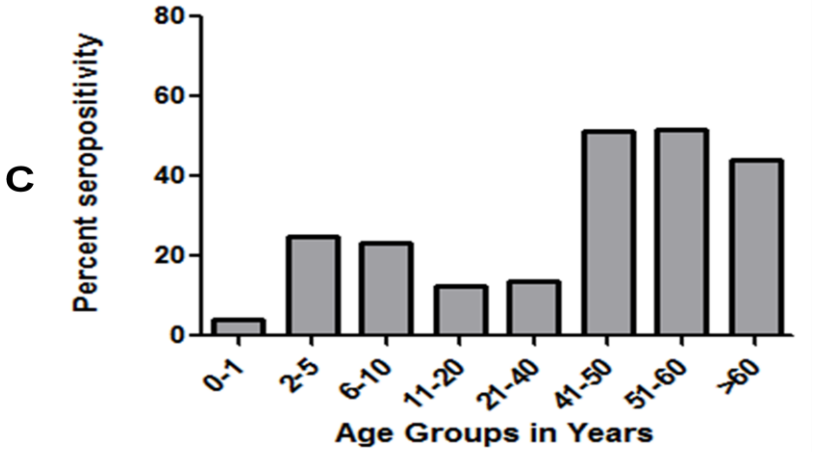
4.4.1 German population studies

Sera from 522 individuals aged 2 months to 101 years were collected at University Hospital Heidelberg, Germany, between 2007-2011. The study has ethical approval for the use of German tissues and sera (Ethikvotum S-091/2011 Hautklinik Heidelberg). They were tested for the presence of anti-MC084S (aa123-230) antibodies (Figure 4.14). Subjects are divided into groups on the basis of age: 0-1 years (n=66), 2-5 years (n=52), 6-10 years (n=47), 11-20 years (n=72), 21-40 years (n=52), 41-50 years (n=119), 51-60 years (n=62) and >60 years (n=52) (Figure 4.14A). The reactivity in infants was significantly lower than in other groups. Based on the minimum cut-off value of δODU 0.36, 159 (30.5%) sera of the 522 sera from a representative healthy German population tested positive in the MC084S (123-230) ELISA. Positive antibody responses in the age groups were as follows: 4.5% (n=3) 0-1 year olds, 25% (n=13) in 2-5 year olds, 23.4% (n=11) in 6-10 year olds, 12.5% (n=9) in 11-20 year olds, 13.5% (n=7) in 21-40 year olds, 51.26% (n=61) in 41-50 year olds, 51.61% (n=32) in 51-60 year olds and 44.2% (n=23) in >60 year olds (Figure 4.14B and 4.14C). Results are tabulated in Table 4.2.

Also based on the cut-off value of δODU 0.28, 199 (38.12%) sera of the 522 sera from a representative healthy German population tested positive in the MC084S (123-230) ELISA. Positive antibody responses in the age groups (Table 4.2) were as follows: 4.5% (n=3) 0-1 year olds, 36.5% (n=19) in 2-5year olds, 34% (n=16) in 6-10 year olds, 22.2% (n=16) in 11-20 year olds, 19.2% (n=10) in 21-40 year olds, 54.6% (n=65) in 41-50 year olds, 54.83% (n=34) in 51-60 year olds and 69.23% (n=36) in >60 year olds (Figure 4.14D and 4.14E).


A
Absorbance at 450nm
2.0
1.5
1.0
0.5
0.0
0-1 (n=66)
2-5 (n=52)
6-10 (n=47)
11-20 (n=72)
21-40 (n=52)
41-50 (n=119)
51-60 (n=62)
>60 (n=52)
Age groups in years


B
Absorbance at 450nm
2.0
1.5
1.0
0.5
0.0
0-1 (n=4)
2-5 (n=13)
6-10 (n=11)
11-20 (n=9)
21-40 (n=7)
51-50 (n=61)
51-60 (n=32)
>60 (n=23)
Sera over cut-off in age groups


C
Percent seropositivity
80
60
40
20
0
0-1
2-5
6-10
11-20
21-40
41-50
51-60
>60
Age Groups in Years

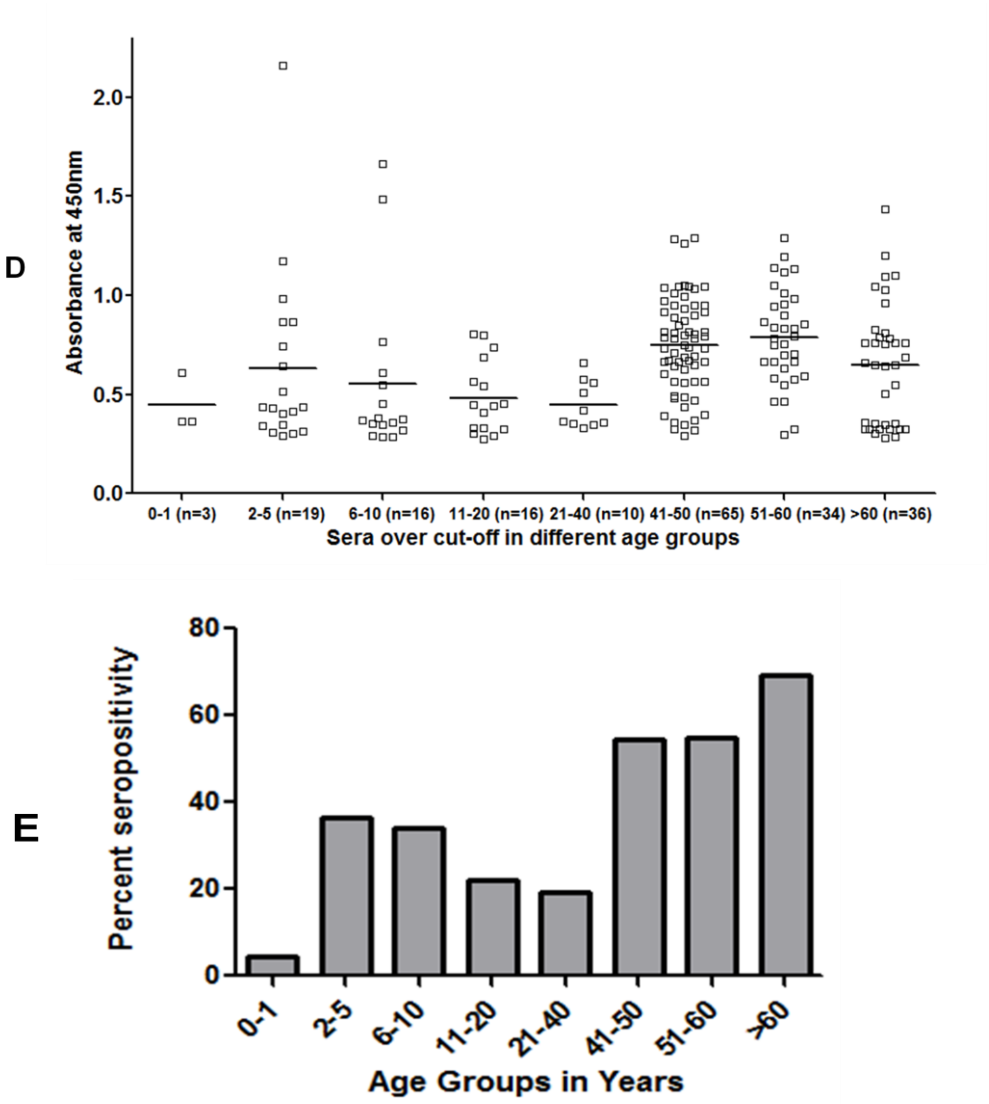


Figure 4.14. Seroprevalence in a German population. *Distribution of anti-MC084S antibodies in a German population tested by direct binding ELISA (A) Serological responses to MCV antigen MC084 in a German population (n=522; ages 0-101 years) expressed as the δODU value of an individual serum sample. The horizontal bar within each group represents the median absorbance measurement. (B) Serological responses to MCV antigen MC084 in a German population (n=522; ages 0-101 years) expressed as the δODU value of an individual serum sample after a cut-off of 0.36. The horizontal bar within each group represents the median absorbance measurement. (C) Percent seropositivities in different age groups after cut–off of 0.36 (i) 0-1years (4.5%), (ii) 2-5 years (25%), (iii) 6-10 years (23.4%), 11-20 years (12.5%), 21-40 years (13.5%), 41-50 years (51.26 years), 51-60 years (51.61%) and >60 years (44.2%). (D) Serological responses to MCV antigen MC084 in a German population (n=522; ages 0-101 years) expressed as the δODU value of an individual serum sample after a cut-off of 0.28. The horizontal bar within each group represents the median absorbance measurement. (E) Percent seropositivities in different age groups after cut–off of 0.36 (i) 0-1years (4.5%), (ii) 2-5 years (36.5%), (iii) 6-10 years (34%), 11-20 years (22.2%), 21-40 years (19.2%), 41-50 years (54.6%), 51-60 years (54.83%) and >60 years (69.23%).*

Table 4.2. Summary of seroprevalences in German and UK populations

Age Group	Total sera	Cut-off= Mean+3*SD (0-1yr+outlier) 0.36	Cut-off= Mean+2*SD (0-1yr+outlier) 0.28
		Positive Sera	**Positive Sera**
German Sera			
0-1	66	3 (4.5%)	3 (4.5%)
2-5	52	13 (25%)	19 (36.5%)
6-10	47	11 (23.4%)	16 (34)%
11-20	72	9 (12.5%)	16 (22.2%)
21-40	52	7 (13.5%)	10 (19.2%)
41-50	119	61 (51.26%)	65 (54.6%)
50-60	62	32 (51.61%)	34 (54.83%)
>60	52	23 (44.2%)	36 (69.23%)
	522	**Seropositivity in healthy subjects** 159 (30.45 %)	**Seropositivity in healthy subjects** 199 (38.12%)
Psoriasis	5	1 (20%)	1 (20%)
SLE*	19	2 (10.5%)	4 (21.05%)
Autoimm†	20	2 (10%)	2 (10%)
UK Sera			
Healthy Controls	50	12 (24%)	14 (28%)
PPMS#	49	10 (20.4%)	13 (26.53%)
RRMS‡	50	6 (10%)	11 (22%)
Total	149	28 (18.7%)	38 (25.5%)
20-40 years	79	16 (20.2%)	21 (26.58%)
>40 years	70	11 (15.71%)	16 (22.85%)
Total	149	27 (18.1%)	37 (24.83%)

Values reflect the prevalence of MCV seropositivity in members of a German and UK population comprising of different groups based on age and disease diagnosis.
* SLE – Systemic Lupus Erythematosus
† Autoimm. – General autoimmune conditions
# PPMS – Primary progressive multiple sclerosis
‡ RRMS – Relapsing remitting multiple sclerosis

Analysis of data was also done after only considering sera from 289 individuals aged 2 months to 40 years (median age 21 years) from the same sera collection tested for the presence of anti-MC084S (aa123-230) antibodies. The reactivity in infants was significantly lower than in other groups. Based on the minimum cut-off value of δODU 0.36, 43 (14.87%) sera of the 289 sera from a representative healthy German population tested positive in the MC084S (123-230) ELISA.

4.4.1.1 Statistical analysis using Anova in German population before cut-off:

A one way anova was used for detection of variances and differences between the different groups divided on the basis of age (Figure 4.14A) (Table 4.3). From the Anova results for the German sera before cut-off the test statistic (F value) for anti-MC084S antibodies amongst the eight different groups divided on the basis of age differed significantly F (1.431, 0.086) = 16.676, p=0.000 (Table 4.4). Using the Tukey test for further post hoc analysis, it was possible to determine the most statistically relevant differences between age groups before a cut-off as shown in Appendix I.

Table 4.3. One way Anova for German population before cut-off

	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
0 - 1 year	66	.131553	.1045174	.105859	.157247
2 - 5 year	52	.299317	.3736997	.195279	.403356
6 - 10 years	47	.278372	.3171693	.185248	.371497
11 - 20 years	72	.206757	.1807952	.164272	.249242
21 - 40 years	52	.218635	.1364325	.180652	.256618
41 - 50 years	119	.482466	.3435129	.420108	.544825
51 - 60 years	62	.498734	.3720875	.404241	.593226
>61 years	52	.556632	.4199750	.354210	.759053
Total	522	.332305	.3241976	.303499	.361111

Table 4.4. Anova analysis of German titres before cut-off.

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	10.017	7	1.431	16.676	.000
Within Groups	41.274	481	.086		

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	10.017	7	1.431	16.676	.000
Within Groups	41.274	481	.086		
Total	51.291	488			

4.4.1.2 Statistical analysis using Anova in German population after cut-off 0.36

After cut-off the mean difference for the 0-1 year age group was 0.80 with 95% confidence interval of 0.10-0.80 (Figure 4.14C) (Table 4.5). The mean difference for the 2-5 year age group was 0.30 with 95% confidence interval of 0.50-1.10. The mean difference for the 6-10 year age group was 0.70 with 95% confidence interval of 0.40 - 1.000. The mean difference for the 11-20 year age group was 0.60 with 95% confidence interval of 0.50-0.70. The mean difference for the 21-40 year age group was 0.50 with 95% confidence interval of 0.40-0.60. The mean difference for the 41-50 year age group was 0.80 with 95% confidence interval of 0.72-0.831. The mean difference for the 51-60 year age group was 0.821 with 95% confidence interval of 0.743 -0.90. Finally, the mean difference for the group comprising of >61 year olds was 0.834 with 95% confidence interval of 0.711-0.80 (Table 4.5).

A one way anova was used for detection of variances and differences between the different groups divided on the basis of age stratification. After a cut-off of 0.36, the variance or mean square deviation between groups was 0.212 whereas the variance within groups was 0.072. The test statistic (F value) was 2.950 and the significance was found to be is 0.006 (Table 4.6). Further post hoc analysis was done using Tukey test to identify and measure statistically significant difference between groups of data as pairs shown in Appendix 3.

In conclusion the one way anova for the detection of variances and differences between the different groups of a German population divided on the basis of age and a cut-off value of 0.36 yielded a test statistic (F value) for anti-MC084S antibodies amongst the eight different groups as F (2.950), p=0.006 which was a significant result but less than that calculated before cut-off.

Table 4.5. One way Anova for German population after cut-off 0.36

	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
0 - 1 year	3	.446833	.1417395	.094733	.798934
2 - 5 year	13	.774962	.4873230	.480475	1.069448
6 - 10 years	11	.670000	.4680104	.355586	.984414
11 - 20 years	9	.565222	.1447487	.453958	.676486
21 - 40 years	7	.493000	.1154913	.386188	.599812
41 - 50 years	61	.774779	.2225712	.717776	.831782
51 - 60 years	32	.821469	.2163133	.743479	.899458
>61 years	23	.834678	.2249314	.737411	.931946
Total	159	.755152	.2795715	.711361	.798942

Table 4.6. Anova analysis of German titres after cut-off 0.36

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.485	7	.212	2.950	.006
Within Groups	10.864	151	.072		
Total	12.349	158			

4.4.1.3 Statistical analysis using Anova in German population after cut-off 0.28:

A one way anova was used for detection of variances and differences between the different groups divided on the basis of age stratification (Table 4.7). After a cut-off of 0.28 (Figure 4.14D), the variance or mean square deviation between groups was 0.333 whereas the variance within groups was 0.084. The test statistic (F value) was 3.973 and the significance was found to be is 0.000 (Table 4.8). Further post hoc analysis was done using Tukey test to identify and measure statistically significant difference between groups of data as pairs (Appendix 2).

Similarly when a one way anova was used for detection of variances and differences between the different groups in a German population divided on the basis of age and with a cut-off of 0.28, the test statistic (F value) for anti-MC084S antibodies amongst the eight different groups was found to be highly significant F (3.973), p=0.000. This significance was found to be more than that obtained using a cut-off of 0.36.

Table 4.7. One way Anova for German population after cut-off 0.28

	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
0 - 1 year	3	.446833	.1417395	.094733	.798934
2 - 5 year	19	.630447	.4541187	.411569	.849326
6 - 10 years	16	.556406	.4200983	.332552	.780261
11 - 20 years	16	.483750	.1850200	.385160	.582340
21 - 40 years	10	.448050	.1189933	.362927	.533173
41 - 50 years	65	.746854	.2419522	.686901	.806807
51 - 60 years	34	.791294	.2428502	.706560	.876029
>61 years	36	.650778	.3058595	.547290	.754266
Total	199	.669947	.3043301	.627404	.712490

Table 4.8. Anova analysis of German titres at cut-off 0.28

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.331	7	.333	3.973	.000
Within Groups	16.007	191	.084		
Total	18.338	198			

The findings summarized in Table 4.2, include alternative results based on different cut-off calculations. Our main reference is a cut-off calculated as three standard deviations of an average from postnatal screening of 0 to 1 year old German newborns and babies. In this group three outliers were observed. As it was not possible to determine the MCV status of these subjects (aged 2 months, 9 months and 11months) they could not be excluded. The calculation of cut-off as Mean + 2×SD or Mean + 3×SD has a significant effect on the cut-off values which are 0.28 or 0.36 respectively. 0.36 has been taken as the most accurate cut-off for data analysis.

4.4.2 German patient population

Sera samples from 44 German patients with dermatological conditions such as such as Systemic lupus erythematosus (n=19), Psoriasis (n=5) and general autoimmune conditions (n=20) including patients with Autoimmune haemolytic anaemia, Autoimmune cerebilitis and Autoimmune hepatitis were collected at University Hospital Heidelberg, Germany, between 2007-2011 from frozen ‘patient sera’ on the

basis of clinical examinations and tested for the presence of anti-MC084S (aa123-230) antibodies (Figure 4.15).

The sera were divided into groups on the basis of diagnosis as: Psoriasis (n=5), Systemic lupus erythematosus (SLE) (n=19) and Autoimmune conditions (n=20) (Figure 14.15). Based on the minimum cut-off value of δODU 0.36, positive antibody responses in MC084S (123-230) ELISA were as follows; 20% (n=1) in patients with Psoriasis, 10.5% (n=2) in patients with SLE and 10% (n=2) in patients with various autoimmune conditions, with an overall seroprevalence of 11.36% (Table 4.2). In addition, using a cut-off value of δODU 0.28, seroprevalences in the same patient groups were found to be 20% (n=1) in patients with Psoriasis, 21.05% (n=4) in patients with SLE and 10% (n=2) in patients with various autoimmune conditions with an overall seroprevalence of 16% (Table 4.2).

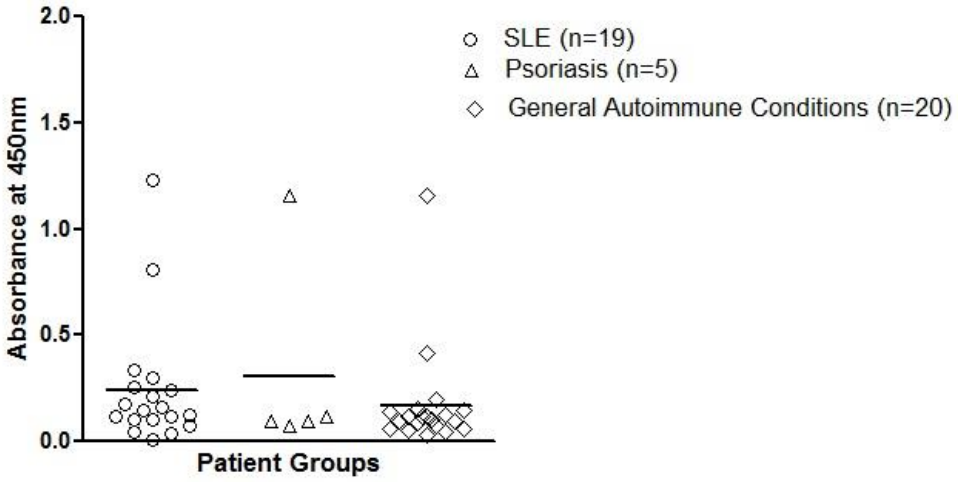


Figure 4.15. Serorectivity amongst different patient groups. *(A) Patients with Systemic lupus erythematosus (n=19), (B) Patients with Psoriasis (n=5) and (C) Patients with General autoimmune conditions including Autoimmune haemolytic anaemia, Autoimmune cerebilitis and Autoimmune hepatitis (n=20).*

Thus the patients with Psoriasis and SLE had similar rates of MC antibody seroprevalence whereas patients suffering from autoimmune diseases had a seroprevalence rate which was half of the above rates. MCV seroprevalence is above

the average rate in skin specific autoimmune conditions, but similar in general autoimmune conditions.

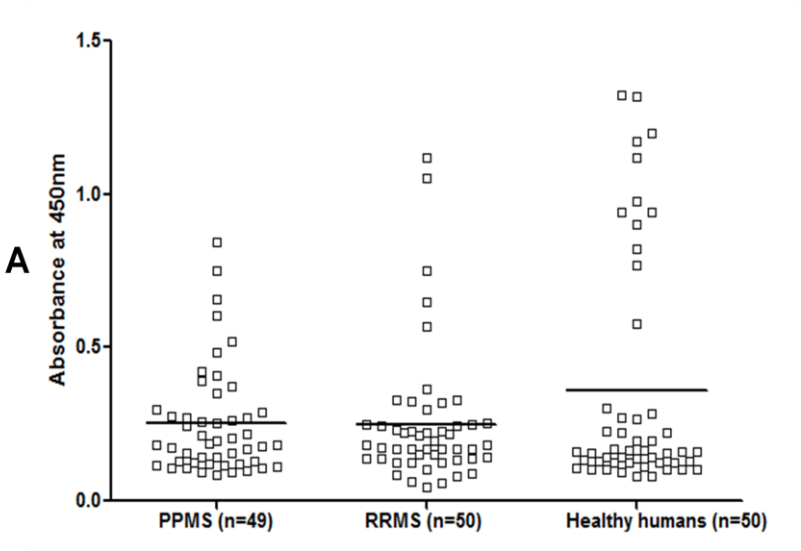
4.4.3 UK population studies

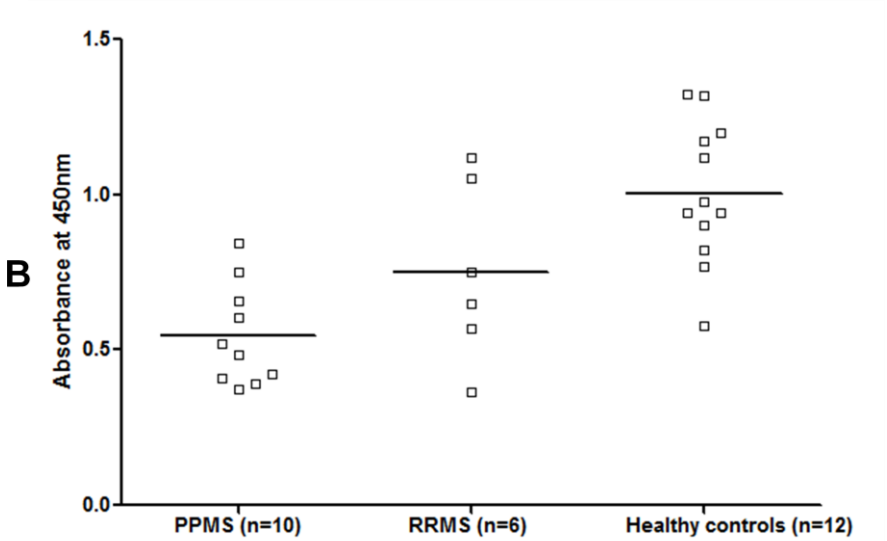
4.4.3.1 UK population studies on basis of disease

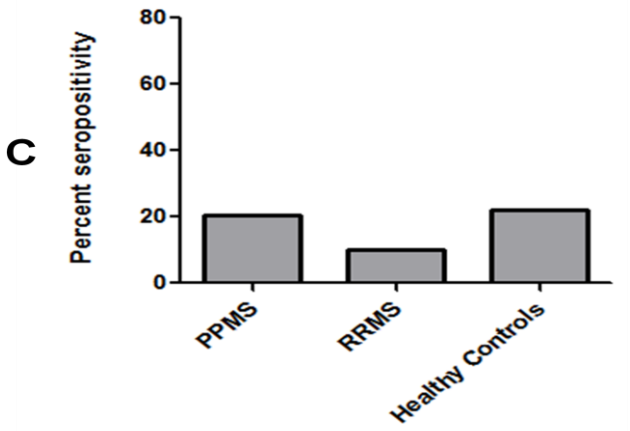
Sera samples (n=149) from a UK population (aged 21-69 years) which had been collected as part of a study on Multiple sclerosis (MS) at Cardiff University entitled 'An Epidemiological study of Multiple sclerosis and other neuroinflammatory demyelinating disorders in South Wales', 05/WSE03/111 were also analysed. These subjects were grouped as Primary progressive multiple sclerosis (n=49), relapsing remitting multiple sclerosis (n=50) and healthy human/controls (n=50) (Figure 14.6A). Using the same calculated cut-off of δODU 0.36, the overall UK seroprevalence was found to be 18.7% and MCV antibodies were detected in 12 of 50 UK sera samples (24%) from healthy individuals. In patients with Primary progressive multiple sclerosis seroprevalence was 20.4% (n=10/49), as compared to 10% (n=6/50) in patients with relapsing remitting multiple sclerosis (Figure 14.6B and 14.6C). Using the calculated cut-off of δODU 0.28, the overall UK seroprevalence was found to be 25.5% and MCV antibodies were detected in 14 of 50 UK sera samples (28%) from healthy individuals. In patients with Primary progressive multiple sclerosis seroprevalence was 26.53% (n=113/49), as compared to 22% (n=11/50) in patients with relapsing remitting multiple sclerosis (Figure 14.6D and 14.6E).

Before establishing a cut-off the mean difference for the PPMS group was 0.3 with 95% confidence interval of 0.10-0.80. The mean difference for the age group comprising of 2-5 year olds was 0.30 with 95% confidence interval of 0.10-0.422. The mean difference for the RRMS age group was also 0.30 with 95% confidence interval of 0.20-0.40. The mean difference for the healthy controls group was 0.414 with 95% confidence interval of 0.30-0.60. A one way anova was used for detection of variances and differences between the different groups divided on the basis of disease or lack of it.

As tabulated in the Table given in Appendix 7 for the statistical analysis of UK population before cut-off usingAnova, the test statistic (F value) for anti-MC084S antibodies amongst the eight different groups divided on the basis of age differed as F (0.197, .112) = 1.756, p=0.180. Further post-hoc analysis was done using 'Tukey' test to identify and measure statistically significant difference between pairs of data. From the multiple comparisons table (Appendix 7) the following conclusions can be drawn: Between groups PPMS and RRMS, the observed mean difference was -0.033 with 95% confidence interval of (-0.330-0.30) with a p value =0.963. Similarly between the groups PPMS and healthy controls, the observed mean difference was -0.20 with 95% confidence interval of (-0.50- 0.133) with a p value =0.381. Between groups RRMS and PPMS, the observed mean difference was 0.033 with 95% confidence interval of (-0.30-0.330) and a p value =0.963. Similarly between groups RRMS and healthy controls the observed mean difference was -0.14 with 95% confidence interval of (-0.33- 0.1) with a p value =0.216. Finally, between the groups healthy controls and PPMS, the observed mean difference was 0.20 with 95% confidence interval of (-0.133- 0.50) with a p value =0.381. Whereas, between the groups healthy controls and RRMS, the observed mean difference was 0.14 with 95% confidence interval of (-0.1- 0.33) with a p value =0.216.


A
Absorbance at 450nm
1.5
1.0
0.5
0.0
PPMS (n=49)
RRMS (n=50)
Healthy humans (n=50)


B
Absorbance at 450nm
1.5
1.0
0.5
0.0
PPMS (n=10)
RRMS (n=6)
Healthy controls (n=12)


C
Percent seropositivity
80
60
40
20
0
PPMS
RRMS
Healthy Controls

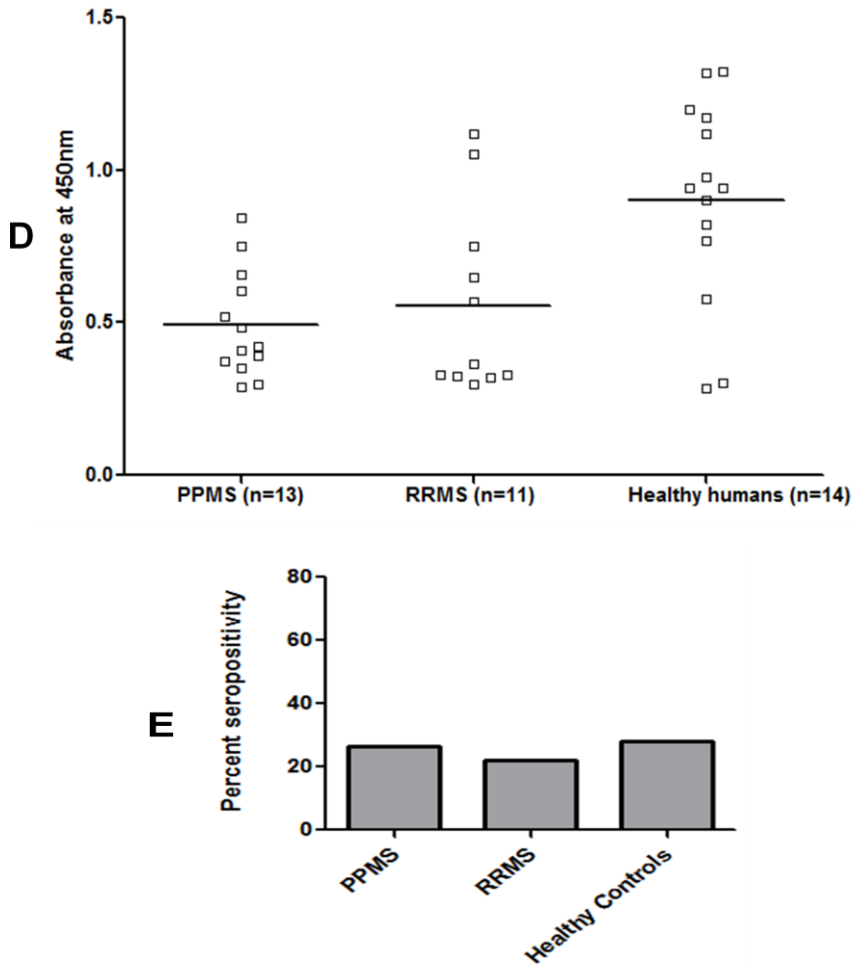
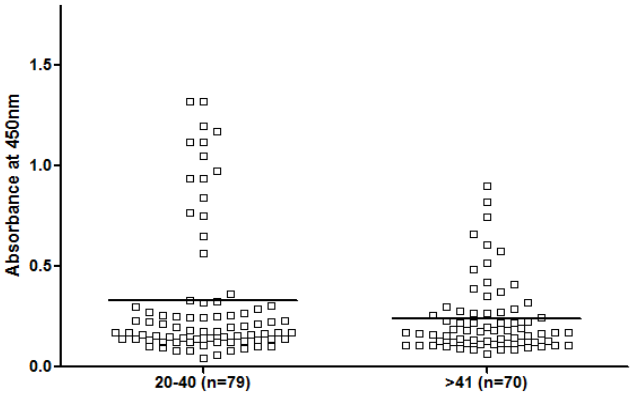
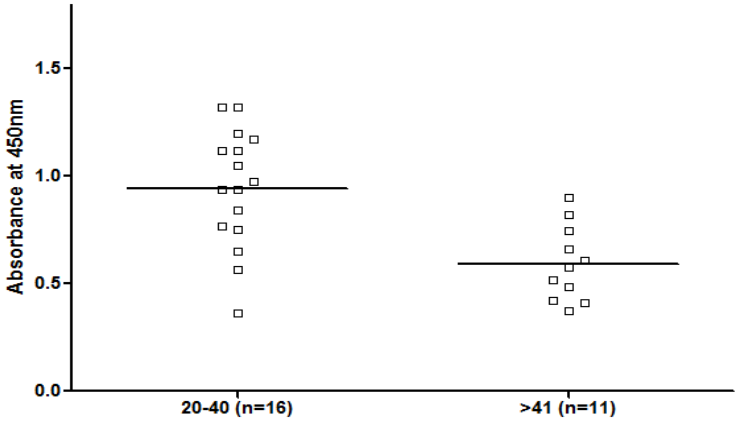


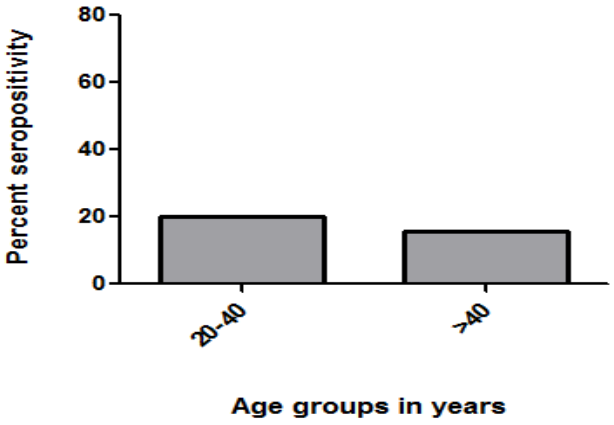
Figure 4.16. Seroprevalence in UK population classified on the basis of disease. *Distribution of anti-MC084S antibodies in a UK population tested by direct binding ELISA. (A) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=149) expressed as the δODU value of an individual serum sample in different groups before cut-off (i) Primary progressive multiple sclerosis (PPMS; n=49), (ii) Relapsing remitting multiple sclerosis (RRMS; n=50) and (iii) Healthy humans (n=50). The horizontal bar within each group represents the median absorbance measurement. (B) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=149) expressed as the δODU value of an individual serum sample in different groups after cut-off 0.36 (i) Primary progressive multiple sclerosis (PPMS; n=10), (ii) Relapsing remitting multiple sclerosis (RRMS; n=6) and (iii) Healthy humans (n=12). (C) Percent positivity in individual groups for MC084S after cut-off 0.36 (i) PPMS (20.4%), (ii) RRMS (10%) and (iii) Healthy humans (24%). (D) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=149) expressed as the δODU value of an individual serum sample in different groups after cut-off 0.28 (i) Primary progressive multiple sclerosis (PPMS; n=13), (ii) Relapsing remitting multiple sclerosis (RRMS; n=11) and (iii) Healthy humans (n=14). (C) Percent positivity in individual groups for MC084S after cut-off 0.28 (i) PPMS (28%), (ii) RRMS (26.53%) and (iii) Healthy humans (28%).*

4.4.3.2 UK population studies on basis of age

The 149 sera samples were also redistributed into two groups based on age i.e. 20-40 years (n=78) and ≥41 years (n=70) (Figure 14.7A). Using the cut-off of 0.36, anti-MC084S antibodies were detected in 20.2% (16/79) of UK sera samples from individuals aged 20-40 years and 15.71% (n=11/70) of UK sera samples from individuals aged >41 years (Figure 14.7B and 14.7C). Using a cut-off of 0.28 the seroprevalnce was found to be 26.58% (n=21/79) and 22.85% (n=16/70) respectively (Figure 14.7D and 14.7E). Since these are only two groups an independent t-test was performed for the two samples with unequal variance. The computed test statistic or t value = 2.1076 at 147 degrees of freedom, p value = 0.03 which is less than level of significance of 0.05. Hence a statistically significant difference exists in the age groups 20 – 40 years and ≥ 41 years of age group. After a cut-off of 0.36 the T value = 4.069 at 24 degrees of freedom and p value = 0.0004. Similarly for the UK population at cut off 0.28 the T value = 3.03 at 34 degrees of freedom and p value = 0.004 (Appendix 8).


Absorbance at 450nm
1.5
1.0
0.5
0.0
20-40 (n=79)
>41 (n=70)


Absorbance at 450nm
1.5
1.0
0.5
0.0
20-40 (n=16)
>41 (n=11)


Percent seropositivity
80
60
40
20
0
20-40
>40
Age groups in years

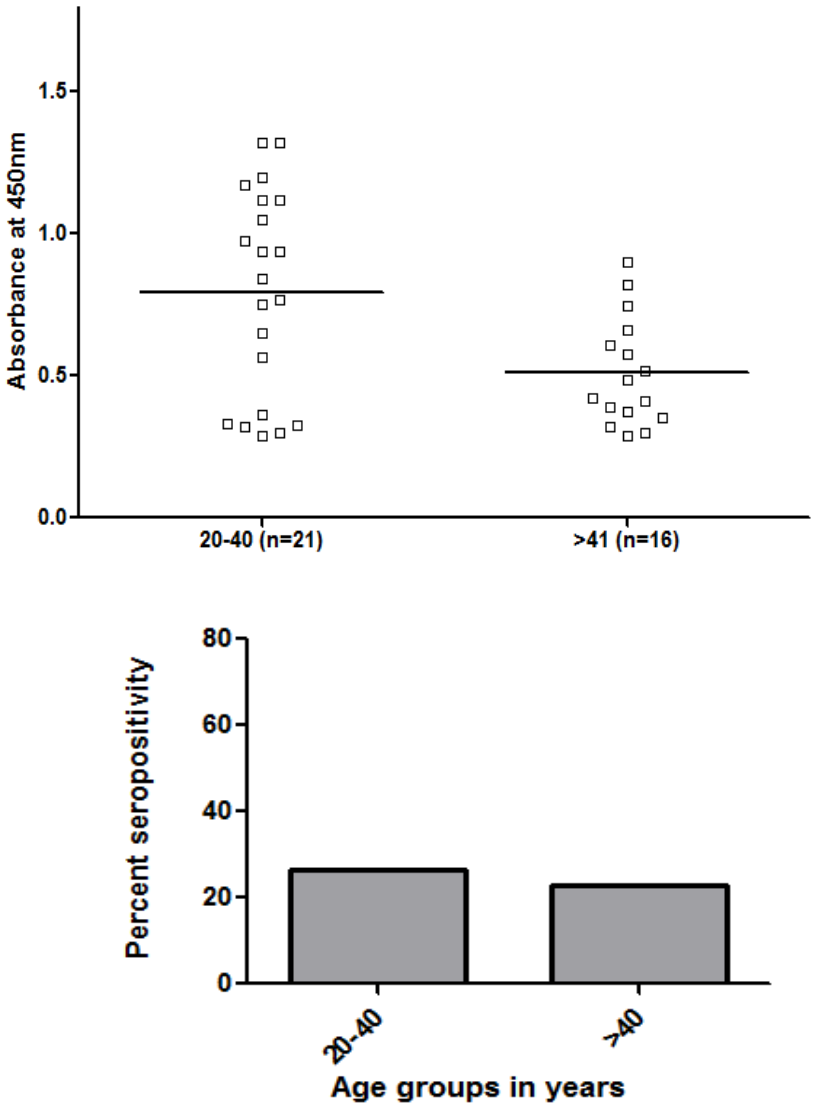


Figure 4.17. Seroprevalence in UK population grouped on the basis of age. *Distribution of anti-MC084S antibodies in a UK population tested by direct binding ELISA. (A) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=149) expressed as the δODU value of an individual serum sample in different groups classified on the basis of age before cut-off (i) 20-40 years (n=79) and (ii) >40 years (n=70). The horizontal bar within each group represents the median absorbance measurement. (B) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=149) expressed as the δODU value of an individual serum sample in different age groups after cut-off 0.36 (i) 20-40 years (n=16) and (ii) >40 years (n=11). (C) Percent positivity in individual age groups for MC084S after cut-off of 0.36 (i) 20-40 years (20.2%), (ii) >40 years (15.7%). (D) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=149) expressed as the δODU value of an individual serum sample in different age groups after cut-off 0.28 (i) 20-40 years (n=21) and (ii) >40 years (n=16).(E) Percent positivity in individual age groups for MC084S after cut-off of 0.28 (i) 20-40 years (20.6%), (ii) >40 years (23%).*

4.5 Neutralising antibodies in sera samples

4.5.1 Detection of neutralising antibodies in different sera samples using reporter assay

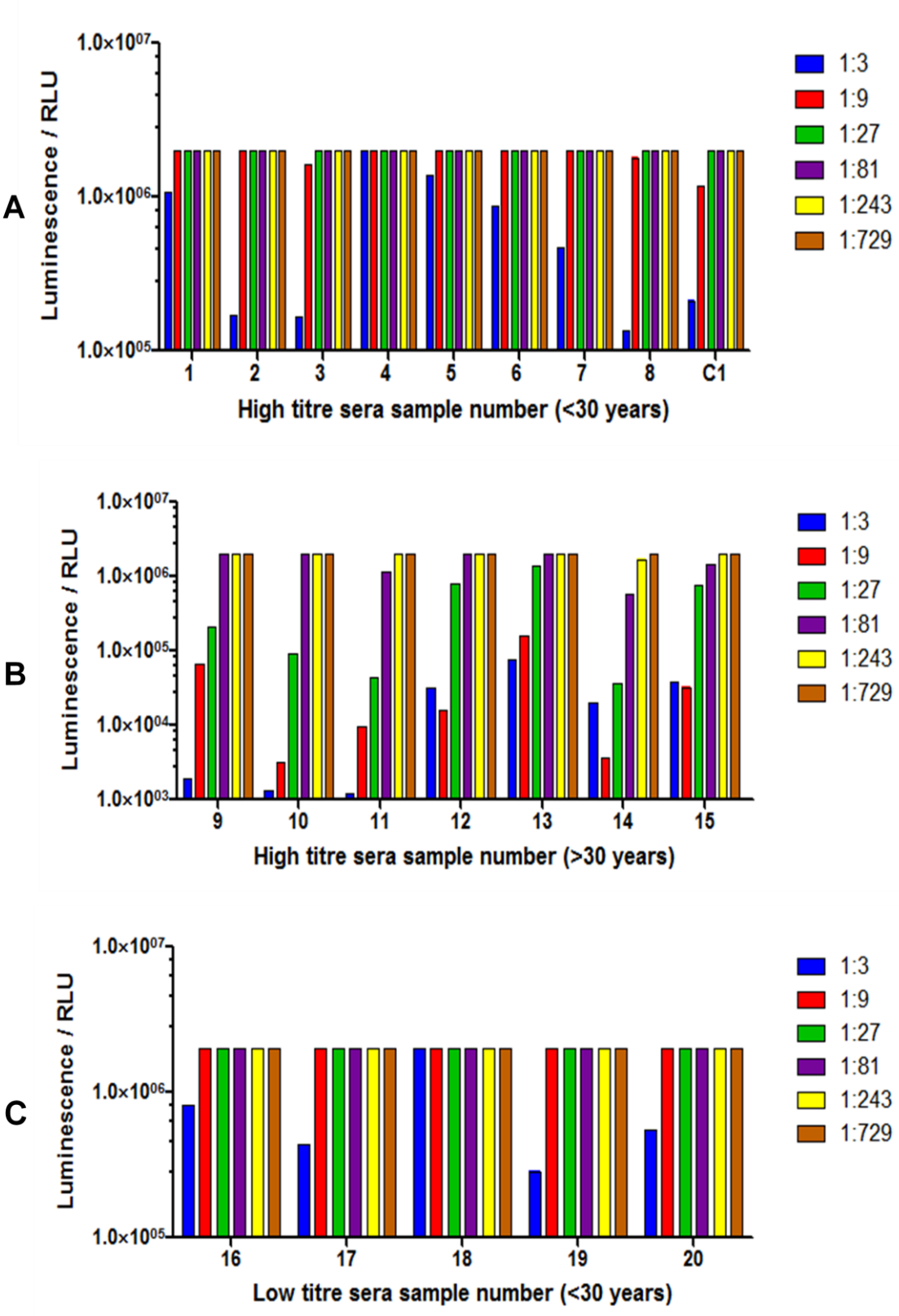
Using the neutralisation assay developed by us in 96 well format, that uses poxviral reporter system v3, we conducted a preliminary study evaluating the neutralizing antibody (NAb) activity 24 hours post infection of HeLa cells, as a function of reduction of luminescent signal and hence transgene expression inhibition, in the presence of specific anti-MC antibodies in the sera samples tested in our MC084S (V123-230) ELISA (Table 4.9).

Table .4.9. Sera samples used in neutralisation assays.

S. No.	Serum sample ID	Serum type	Age (years)	Sex
1.	HDV0905794	High titre	5	F
2.	HDV0901772	High titre	5	F
3.	HDV0905796	High titre	2	M
4.	HDV0903090	High titre	2	M
5.	HD V0903005	High titre	6	F
6.	HD V0905374	High titre	1	M
7.	HD V0904696	High titre	4	M
8.	HDV0901071	High titre	7	M
9.	HD AZ003970	High titre	47	F
10.	HD AZ003735	High titre	56	M
11.	HD AZ005203	High titre	51	M
12.	HD AZ004482	High titre	47	F
13.	HD AZ005347	High titre	56	M
14.	HD AZ003746	High titre	48	F
15.	HD AZ003749	High titre	45	F
17.	HD V0900040	Low titre	1	M
18.	HD V0900471	Low titre	16	M
19.	HD V0901798	Low titre	1	F
20.	HD V0900223	Low titre	2	M
21.	HD AZ005860	Low titre	50	M
22.	HD AZ005914	Low titre	47	F
23.	HD AZ006064	Low titre	45	M
24.	HD AZ007182	Low titre	57	M
25.	HD AZ007363	Low titre	51	F
C1	CF2012-1	MCV positive	27	M
C2	VV8191 Anti-vaccinia	Rabbit serum		

High titre sera samples from individuals aged <30 years showed little to no inhibition and neutralising antibody responses at sera dilutions varying from 1:3 to 1:9 (Figure 14.18A). High titre sera samples from individuals aged >30 years showed neutalising antibody responses at sera dilutions varying from 1:3 to 1:243 (Figure 14.18B) with sera numbers 11, 14 and 15 showing reporter inhibition (Figure 14.18B). Low titre sera samples fromindividuals aged <30 years showed neglegible inhibition with measurable neutalising antibody responses at sera dilutions of 1:3 (Figure 14.18C). Finally, low titre sera samples from individuals aged >30 years showed neutalising antibody responses at sera dilutions varying from 1:3 to 1:81 with reporter onserved in sera numbers 22-24 (Figure 14.18D). On comparing the neutralisation titres of sera in the reporter assay it was found that a low prevalence of neutralising antibodies with no reporter inhibition was observed in the groups consisting of individuals <30 years of age ranging from a dilution of 1:3 to a maximum of 1:9 which may be considered non-specific

However, in the groups consisting of individuals >30 years of age, a high reporter inhibition is observed due to neutralising antibodies, ranging from a low dilution of 1:3 (considered non-specific) to a maximum of 1:243. This group consists of individuals who would have definitely received the smallpox vaccine and may therefore exhibit a basal titre of VACV specific antibodies as is exhibited by sera samples 11, 14 and 15.


A
Luminescence / RLU
1.0×10^07
1.0×10^06
1.0×10^05
1 2 3 4 5 6 7 8 C1
High titre sera sample number (<30 years)
1:3
1:9
1:27
1:81
1:243
1:729
B
Luminescence / RLU
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
9 10 11 12 13 14 15
High titre sera sample number (>30 years)
1:3
1:9
1:27
1:81
1:243
1:729
C
Luminescence / RLU
1.0×10^07
1.0×10^06
1.0×10^05
16 17 18 19 20
Low titre sera sample number (<30 years)
1:3
1:9
1:27
1:81
1:243
1:729

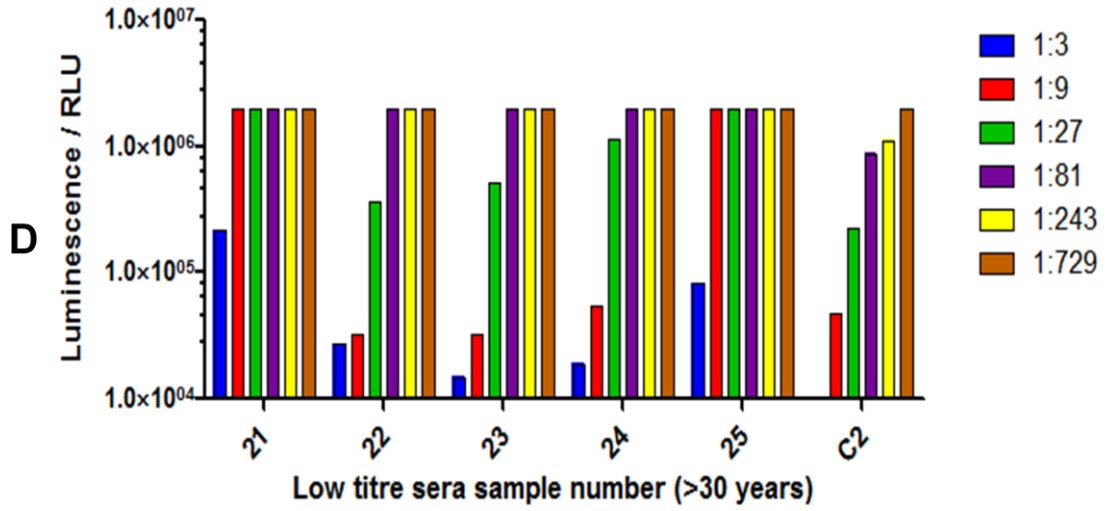


Figure 4.18. Neutralising antibodies as determined by reporter assay. *Neutralising antibody titres determined as luminescence using reporter assay for high and low titre sera samples differentiated on the basis of age. (A) High titre sera samples (1-8 and C1) from individuals <30 years of age. (B) High titre sera samples (9-15) from individuals 30 years of age. (C) Low titre sera samples (16-20) from individuals <30 years of age. (D) Low titre sera samples (21-25) and C2 from individuals >30 years of age. Each value is the arithmetic mean of three independent assays.*

4.5.2 Detection of neutralising antibodies in different sera samples using plaque reduction neutralisation tests

Plaque reduction neutralisation assays were conducted for all of the above mentioned sera samples to obtain MCV neutralising antibody titres required for replication inhibition.Plaque reduction was assessed three days post infection. Figure 14.19 shows the results from the PRNT using ELISA defined low and high titre sera samples. Wells from the plaque reduction neutralisation test corresponding to neutralising serum titre in reporter assay displayed by the bars in histogram (Figure 14.19). Maximum virus infection was observed in control wells without serum and the anti-vaccinia rabbit polyclonal was included as a positive control. Comparison of the NAb titres between the high titre and sera samples from individuals aged <30 years show that sera samples 3,4,5,7 and 8 show low levels of neutralising Abs at a dilution of 1:3 whereas sera samples 1,2 and 6 show slightly higher Nab levels at a dilution of 1:9. Compared to the previous group, NAb levels in the group consisting of high titre sera samples from

individuals aged >30 years are slightly elevated, with sera samples 9-13 showing antibodies in a dilution range of 1:27 and samples 14-15 showing Nabs in a dilution range of 1:81. Results with low titre sera samples as determined by recombinant MC084 ELISA were also analysed after division on the basis of age. For the sera samples from individuals aged <30 years it was found that samples 16, 17, 19 and 21 showed detectable levels of neutralising antibodies in wells with serum dilutions of 1:3. No neutralising activity was evident in samples 18 and 19 with plaque formation even at the lowest dilution levels. In the group consisting of samples from individuals aged >30 years the neutralising antibody activity was evident for samples 22-24 in a dilution of 1:3 and at a higher dilution of 1:9 in sera sample 25.

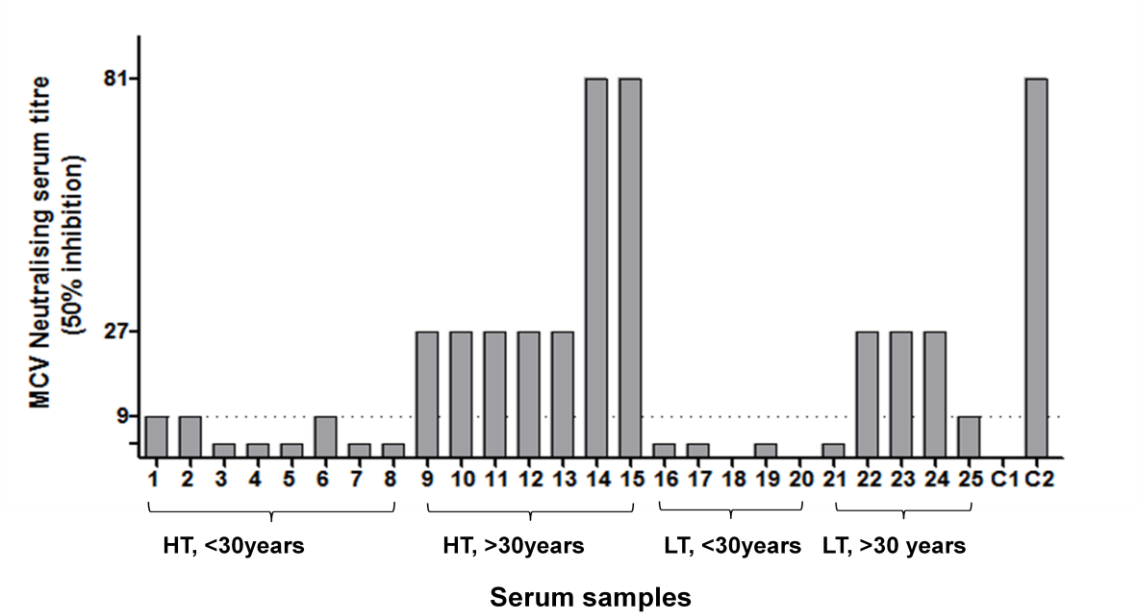


Figure 4.19. PRNT-Neutralising antibody titres. *NAb titres(A) determined using plaque reduction neutralisation test three days post infection for high and low titre sera samples differentiated on the basis of age as high titre sera samples (1-8) and <30 years of age, high titre sera samples (9-15) and individuals >30 years of age, low titre sera samples (16-20) and <30 years of age, low titre sera samples (21-25) and >30 years of age and controls C1 and C2. Each value is the arithmetic mean of three independent assays.*

Discussion

We describe here for the first time a seroepidemiological study of MCV in Europe, the largest survey reported so far (n=715) and the first MCV ELISA based on viral antigen expressed in *E. coli*.

The selection of antigen was both based on a previous study by Watanabe *et al*. and independent analysis of the amino acid sequences of candidate MCV proteins. Watanabe had previously identified MC084 and MC133 as two major antigenic virion particle proteins of MCV (Watanabe *et al*., 1998).They offered no data for their decision to establish an ELISA on mc133 antigen, other than that it performed better than mc084 in their assays.

Previously reported MCV ELISAs used antigen from human lesion material (Konya and Thompson, 1999) or Sendai virus (Watanabe *et al*., 1998) expressed full length MC133, raising issues with background skin antigens and posttranslational antigen processing. To improve water solubility and provide an expression platform more suitable for commercial production of a MCV ELISA we decided to compare antigen prepared from truncated MC084 and MC133 and expressed in *E. coli*.

On the basis of previous work by Watanabe *et al.* and our own homology analyses we chose a C-terminal truncation of MC084 (V123-R230), upstream of A238-Q298 previously found non-reactive in ELISA by Watanabe, as our candidate ELISA (Watanabe *et al*,. 2000) antigen. Our choice of antigen minimizes the possibility of cross reactivity with vaccinia virus specific antibodies, exclude the membrane spanning domains of mc084, but include a possible major antigenic site, identified by hydrophilicity plotting (MC084 N218-R230).

Using a C-terminal truncation of MCV ORF mc084 as an antigen, we were able to establish an ELISA both sensitive (100%, n=12) and specific, with low inter- and intra-assay variability.This is in comparison to the lower sensitivities of 77% and 58%, in the ELISAs reported by Konya *et al.* (Konya and Thompson, 1999) and Watanabe

(Watanabe *et al.*, 2000), respectively. Our experiments determining specificity in MCV tissue sections also compare well to data shown in Konya *et al.* (Konya and Thompson, 1999), while Watanabe only showed specificity in an immunoblot using recombinant protein (Watanabe *et al*., 2000).

Our homology studies indicate that both mc084 and mc133 share amino acid homologies with vaccinia virus proteins H3L and A27L. We studied truncated versions of the proteins, N-and C-terminal halves separately, and found that homologies vary between 27% - 30% amino acid identities, with the C-terminal part of mc084 sharing the lowest homology with vaccinia H3L of 27% amino acid identity. We consequently chose MC084 (aa123-230) as our candidate ELISA antigen to minimize possible cross reactivity with vaccinia virus specific antibodies. Interestingly, Watanabe had tested a peptide (aa238-298) further downstream of the C-terminal of our truncation, well into the transmembrane domain (TMHH prediction), which showed no reactivity with human sera at all.

The C-terminal truncation of mc084 chosen by us (aa123-230) exclude the membrane spanning domains of mc084, but include a possible major antigenic site, identified by hydrophilicity plotting (Kyte–Doolittle): NELRGREYGASLR (mc084 amino acids 218-230).

Also, while Watanabe chose mc133 expressed using a Sendai virus expression system in hen eggs using the infected allantoic fluid (Watanabe *et al.*, 2000), we decided to express mc084 and mc133 protein truncation excluding membrane spanning domains in *E. coli* to provide an expression platform more suitable for commercial production of a MCV ELISA.

Both N- and C-truncations of mc084 and the N-terminal truncation of mc133 were expressed as fusion proteins with GST and purified using a glutathione sepharose beads. All truncations tested were water soluble. On the relative merits of mc084 and mc133 as suitable antigens for detection of MCV antibodies, we made a direct comparison, using a high titre serum from a patient with active MCV infection. In this assay the mc084 C-

terminus performed slightly but not significantly better than mc084 N-terminus and was similar to mc133.

The C-terminal MC084S (aa123-230) truncation without GST was further purified using FPLC on a Sephadex 200 column, resulting in a sufficiently pure protein for ELISA studies. The antigen load for plating was optimized and *in vitro* specificity shown in an inhibitor titration assay. A strep tag was used for detection of recombinant antigen in western blots. The tag did not interfere with ELISA results in a serum study of 149 serum samples.

A stringent cut-off was determined as mean ODU plus three standard deviations, using a serum sample collection of Heidelberg university clinics neonates up to 1 year of age, assuming a minimal exposure to MCV in this age group. We have specifically looked at sera from newborns and babies from 0-1 years after birth, where under hygienic conditions, the exposure and incidence to MC should be the lowest. Surprisingly despite this, serum samples from a 2 month, a 7 month and a 9 month old subject showed significant reactivity in ELISA, indicating a possible exposure and active infections. This would be considered rare. However a study has reported a case of vertically transmitted molluscum contagiosum viral infection in which the maternal infection was clinically documented before vaginal delivery (Luke and Silverberg, 2010). Also a clinical case of a female patient with DOCK8 deficiency presenting extensive clinical MC from birth and eventually dying in her early teens was evidenced by Dr. Bugert. Also the greatest likelihood of infection with MC in babies is from transmission from immediate family members. However a confirmation of diagnosed clinical Molluscum contagiosum in these three cases could not be made as this was a study with an anonymised sera collection for which no clinical data was available apart from age and sex. For the purposes of the cut-off calculations we have presented cut-offs calculated with and without these outlying sera results. The respective cut-offs of 0.36 and 0.28, result in a significant difference of overall seroprevalence of 30.45 to 38.12 percent in the German population or 18.7 to 25.5% in the UK population.

In direct antigen comparison there was no significant difference between truncations of mc084 and mc133 and no serum showed prevalent reactivity against one or another of the antigen used. Also the difference in reactivity between the MC084S (aa123-230) or MC084S-GST with sera samples was negligible. A strep tag was used for detection of recombinant antigen in western blots. The tag did not interfere with ELISA results as established by our strep interference assay confirming no cross-reactivity between MC084S (aa123-230) specific serum antibodies and strep tag.

To determine sensitivity of our ELISA, we were able to collect 12 sera from patients with ongoing clinical MCV. All sera showed significant reactivity in comparison to a control group of healthy individuals. The serum with the highest absorbance value was collected form a British patient (CF2012-1) with acute molluscum contagiosum and frozen immediately with little handling. This was higher than the Watanabe study, where only 7 of 12 young children with clinical MC showed substantial reactivity in the mc133 ELISA, indicating that their ELISA may be underestimating the true seroprevalnce (Watanabe *et al*., 2000).

The same serum (CF2012-1) was used to determine specificity of MC antibodies using MCV infected human skin tissue samples. This and a series of other high titre sera reacted positively with the expected tissue distribution in clinical lesions: stratum spinosum up to egress in debrisomes. There was a significant difference between high and low titre sera in tissue reactivity. With the ELISA thus validated as highly sensitive and specific we proceeded to look at larger population to determine seroprevalnece. We also infected human HaCaT keratinocytes with a vaccinia virus expressing full length mc084 (v319). Infected keratinocytes were immune stained with the same high titre serum HDV0901071. Virus infected cells show a vesicular stain similar to an endosomal/lysosomal pattern. Uninfected cells show no background signal, indicating the human polyclonal does not recognize keratinocyte antigens in cultured HaCaT cells.

Antibody cross reactivity between MCV and VACV was never reported and is in fact not observed as documented in publications on MCV serology (Agromayor *et al.*,2002).

Also amino acid homology studies do not indicate a potential for cross reactivity using C-terminal mc084, as mentioned before.

Information on the epidemiology of zoonotic poxviruses especially those belonging to the genera OPV is still too poor to enable us to clearly understand and estimate the chances of zoonoses in humans. A recent review sheds light on aspects of the host range of some poxviruses being broader than initially thought (Essbauer *et al*., 2010). Presently the two most studied OPV infections responsible for zoonoses are monkeypox virus and cowpoxvirus (CPXV).

Of these monkeypox infection is rare with no case reports outside of Africa and America. Cowpoxvirus is the most likely natural source of zoonoses amongst European populations with cats being the main mode of transmission. The literature points to more than 400 cases of CPXV infections described for domestic cats (Essbauer *et al*., 2010; Bennett *et al.*, 1986; Bennett *et al.*1989; Hinrichs *et al*., 1999; Brown *et al*., 1989; Baxby, 1994; Pfeffer *et al.*, 2002), but it is estimated that many infections are not recognized by veterinarians and/or owners. The real incidence of CPXV in cats may be reflected by serological data. One study gives a variation of 0 up to 16% in cats with antibodies against CPXV (OPV, respectively) reported from England, Norway, Austria, Germany and Finland (Juncker-Voss *et al*., 2004). The variation is seasonal, with an accumulation of CPXV infections in late summer and autumn (Bennett *et al*., 1986; Pfeffer *et al*., 2002). Cats as predators are exposed to CPXV while hunting rodents which serve as a reservoir for CPXV (Pfeffer *et al.,* 2002).

Human CPXV cases are mainly caused by direct contact to ‘cuddly’ cats (Baxby, 1994). It is suspected that the cowpox virus infections in humans maybe more common than thought due to the lack of an adequate immune status of the population because of the abrogated but cross-protective smallpox vaccination in the 1980s (Essbauer *et al*., 2010). However currently there is a lack of published data giving true incidence and serology in human populations rather than most studies which are reports of cases in isolated populations. Hence it would be difficult to estimate the contribution of neutralizing antibodies in our study due to zoonotic poxviral infections.

Looking at an age stratified grouping of 522 sera from healthy German individuals, we observed lowest δODU in the youngest age group, increasing to the highest mean in 1-5 year olds, then falling to lower values in higher age groups of 20-40 year olds. This is perfectly reflected in the literature with respect to these various age groups. It is also in agreement with data published by Konya and Thompson, using a virion particle ELISA, the lowest mean δODU in their youngest subjects (<6 years), where they consider it to be representative of maternally acquired antibodies (Konya and Thompson, 1999). Another study from the Netherlands found that the childhood from of MC was common, with a cumulative incidence of 17% by age 15 (Koning *et al*., 1994). However after cut-off of 0.36 the seroprevalences in the age groups of 41-50 years and over are significantly higher; with rates in the 41-50 years and 50 to 60 years strictly comparable (51.26% and 51.61% respectively) and then showing a slight decrease in >60 year olds (44.2%). These results may also be somewhat affected by sera numbers within each individual group however it would not have been possible to increase or reduce sera samples tested without creating bias in the final result. The study by Konya and Thompson also reported an increase in seropositivity rate with age to a final high rate of 39% in those ≥50 years old.

From the statistical analysis using Anova, results for the German sera before cut-off, the test statistic (F value) for anti-MC084S antibodies amongst the eight different groups divided on the basis of age differed significantly F (1.431, .086) = 16.676, p=0.000. However from the statistical analysis using Anova calculations for the German sera after cut-off 0.36, the test statistic (F value) for anti-MC084S antibodies amongst the eight different groups divided on the basis of age, differed statistically as F (0.212, 0.072) = 2.950, p=0.006. The F values in both cases were significant. The Tukey test was used for further post hoc analysis before and after cut-off was established (Appendix) however the statistical significances between groups before cut-off are much greater than after cut-off. These differences may be attributed to difference in group sizes.

In a serum collection from a UK MS study, the sera from healthy individuals revealed a lower overall seroprevalence of 18.7%, as compared to 30.45% in the German

population using the cut-off calculated with outliers. This may be explained by the fact that the UK sera collection (n=149) consisted largely of patients with multiple sclerosis (n=99) who may exhibit altered antibody profiles due to disease.

For the UK population divided on the basis of disease, post-hoc analysis done by using Tukey test, found no statistically significant differences between Healthy controls, PPMS and RRMS before or after cut-off calculations. After redistribution of the UK data set on the basis of age and performing an independent t-test with unequal variance a *p* value was calculated as 0.03 which is less than level of significance of 0.05. Hence a statistically significant difference exists in the titres in the age groups 20-40 years and ≥41 years of age group. After a cut-off of 0.36 the p value was found to be highly significant as 0 .0004. Similarly after a cut off of 0.28 the calculated p value = 0.004 was highly significant.

This is the largest seroprevalences study conducted in European populations. The findings of our serological survey are higher than those reported for an immunofluorescence study of MCV antibodies in Northern Ireland and two previous ELISA studies in Australia and Japan even though different antigen and expression systems were used (Shirodaria *et al*., 1979; Konya and Thompson, 1999; Watanabe *et al.*, 2000). Importantly, considering the MC seroprevalence of 14.8% in 0-40 year olds in our German population is a threefold increase over the reported incidence of MC in a comparable Swiss population of 4.9% (Wenk and Itin, 2003) supporting the notion, that MC is a underreported infection.

We find an overall seropositivity in a general German population of 30.45%, depending on cut-off 0.36 and 18.7% in the UK as compared to 16.7% in Ireland (n=30; IgG responses) (Mitchell, 1953), 23% in an Australian population (Konya and Thompson, 1999) and 6% in a Japanese survey (Watanabe *et al.*, 2000). Whereas the German seroprevalence is higher than previously published seroprevalences, the UK seroprevalence is and slightly lower than the Australian seroprevalences. The calculated seroprevalence of anti-MC084S (123-230) antibodies in 0-40 year olds in the German population is found to be much lower i.e. 14.8% with a cut-off of 0.36. However with

the same stringent cut-off, the overall German seroprevalence rate was calculated to be higher, because of the clear trend of higher antibody titres in a large group of people in the age groups consisting of >40 years; a population which has definitely received the smallpox vaccine and is most likely to exhibit some neutralising antibody titres as previously published (Liu *et al*., 2012).

The age profile determined using the MC084 ELISA correspond well with our understanding of the natural history of MCV infections, with low exposure of very young children and a high prevalence among toddlers and kindergarten aged children, where MCV smear infections is most likely to be transmitted among larger numbers of children. Our data confirm previously reported findings of stronger antibody responses, mostly in the 2-10 age group, with waning immunity as the population reaches adulthood. This would suggest very little reexposures boosting immunity up to 40 year of age.

An MC incidence study based on outpatient visits in American Indian and Alaska Native populations also notes a relatively high incidence amongst 1-4 year olds (Reynolds *et al*., 2009). Other studies measuring age-related health care utilization for MC also suggest that the overall numbers of MC associated visits were highest among 2-5 year olds (Molino *et al.*, 2004; Dohil *et al*., 2006). A Swiss study (Wenk and Itin, 2003) also found high prevalence of MC in pre-school children (6.9%) and school children (10.6%). Increased utilization of childcare leading to a lowering in age of socialization outside the immediate household may be contributory factors this increased MC incidence in pre-school children.

In contrast to Konya *et al*. (Konya and Thompson, 1999), who report a very high seropositivity rate in their 0-6 year old population of 31%, explaining this with maternal antibodies, our data do not indicate a high seropositivity rate in very young children. Seroprevalence with the mc084 (V123-R230) ELISA is below 5% in 0-1 year olds and only increases in the age group of 2-5 year olds, not exceeding 25%.

Watanabe *et al*. explained their low overall seropositivity (n=108, 6%) in healthy subjects (Watanabe *et al*., 2000) in comparison to the prior Australian study (n=357; 23%) (Konya and Thompson, 1999)with their mc133 ELISA failing to pick up sera with mc084 antibodies as shown in immunoblots, indicating that mc133 may not be the best choice of antigen underestimating seroprevalence.

The objective of this study was to develop a convenient and specific ELISA based on recombinant viral antigen by expression of selected molluscum contagiosum open reading frame in *E. coli* as a strep tagged protein. The virion surface protein, with serodiagnostic utility was identified, expressed and incorporated into an ELISA that offers the prospect of examination of seroprevalence of antibodies against MCV in subjects with and without clinical Molluscum contagiosum infection.

This solves the problem of finding sufficient MC antigen for ELISAs as established by Konya *et al.*, and circumvents the problem of standardization in eukaryotic expression systems as established by Watanabe *et al*. Due to outliers in our cut-off control group, we propose a possible seroprevalence in Europe of about 30% in healthy populations with lower seroprevalences of 14.7% in the age groups of 0-40 years with a clear increase in those aged >40 years.

On comparison of data comprising of healthy 0-40 year olds the seroprevalences are lower (14.8% in Germans) than the Australian survey (n=357; 23%) (Konya and Thompson, 1999) based on a MCV virion particle ELISA which may have overestimated seroprevalence due to lack of antigen specificity. Our data indicates a seroprevalence higher than found in the Japanese survey (n=108, 6%) (Watanabe *et al*., 2000). However they used MC133 which may be an inferior antigen for maximum sensitivity, and they expressed their antigen in embryonated chicken eggs and monkey cells, where host specific posttranslational modifications may be an issue.

We have only looked at very small groups of patients with specific clinical diagnoses other than MC. Of interest may be the fact that all patient groups comprising of general autoimme conditions and skin conditions, psoriasis and SLE seem to show a lower than

average seroprevealence of MC. The seroprevalence in German patients with SLE is lower (10.9%) than that reported by Watanabe and group (57%) in their study (Watanabe *et al*., 2000) which was described to be a result of hyperimmunity or hypergammaglobulinemia. In MS patients, who are mostly under therapeutic immune suppression we observe a lower than average seroprevalence, which may be due to suppression of immune cells involved in antibody production. This data is necessarily weak due to lack of numbers. Also the seropositivity in patients with primary progressive seroprevalence is almost twice that in patients with relapsing remitting multiple sclerosis.

This is the first seroepidemiological study of MCV in Europe, the largest survey reported so far (n=715) and the first MCV ELISA based on surface viral antigen involved in the viral entry complex expressed in *E. coli.*In summary, we propose MC084 (V123-R230) is a suitable antigen for MCV serological surveys when expresse in *E. coli*. It includes a probable highly antigenic site at amino acid position N219-R230. Our study suggests that Molluscum contagiosum is strikingly common in the UK and Germany, especially in children under 5 years of age as is well documented in literature but also, as was apparent in the German population, in the older community comprising of individuals >40 years of age. The assay allows further investigations into the seroprevalence of MCV in other geographical areas, including the US, China, Japan and Australia.

The reporter assay described in chapter 3 is based on the inhibition of infection introduced in cells by a recombinant v3 virus that carries a firefly luciferase gene with the resulting inhibition of transgene expression. Quantitative analysis of the assay allows measurement of a luminescent signal in relative light units which is directly proportional to transgene expression. We conducted neutralisation assays based on a working range of 22-1800 pfu/30,000 cells and an optimum pfu of 400 for reproducibility and sensitivity established by a group using a similar vaccinia virus neutralisation assay (Liu *et al*., 2012). It was found that sera from individuals of different age groups had different levels of non-specific reporter inhibition at different dilutions. Comparison of the neutralisation titres obtained by the two assays, i.e.

reporter assay and plaque reduction neutralisation test, indicated a high correlation between the two (near identical results for 15 sera samples), with a slight enhanced sensitivity of the reporter assay in results for 7 seven sera samples (samples 3, 8, 11, 14, 20 and C1), compared to PRNT for 5 sera samples (samples1, 2, 4, 6 and 25). These differences may be due to the differences in protocols followed. A low prevalence of neutralising antibodies with no reporter inhibition was observed in the groups consisting of individuals <30 years of age ranging from a dilution of 1:3 to a maximum of 1:9 which may be considered non-specific

In the groups consisting of individuals >30 years of age, a high reporter inhibition is observed due to neutralising antibodies, ranging from a low dilution of 1:3 (considered non-specific) to a maximum of 1:243. This group consists of individuals who would have definitely received the smallpox vaccine and may therefore exhibit a basal titre of VACV specific antibodies as is exhibited by sera samples 11, 14 and 15. The levels of neutralising antibodies amongst the two different groups sorted on the basis of high/low titres established by ELISA are somewhat different. Within the group consisting of low titre, sera samples antibody titres are in the range of 1:3 (sample 21) or 1: 27 (samples 22-24) and one serum sample (25) shows a higher prevalence of NAbs at a dilution of 1:81. In the group consisting of high titre sera samples, the neutralising antibody titres are found to me much higher with detectable titres at dilutions ranging from 1:27 (samples 9, 10, 12 and 13), 1:81 (samples 11 and 15) to as high as 1:243 (sample 14). The levels of neutralising antibodies amongst the two different groups sorted on the bases of high/low titres established by ELISA are almost comparable, with the exception of two samples (sample 3 and 8) which show greater neutralising activity.

We consider readings at dilutions of 1:3 and 1:9 to be non-specific in reporter assay and PRNT as they are not observed to the same levels. A likely explanation for higher neutralising antibody titres in sera samples of individuals >30 years may be that since these individuals would have received the small pox vaccine they would retain some residual immunity to vaccinia. It is well known that serological tests such as ELISA and PRNT for orthopoxvirus species may not be virus species specific due to high levels of cross-antigenicity amongst species of the genus (Liu *et al.*, 2012).However, from the

reporter assay we found no neutralization in serum dilutions over 1:100 i.e. the same dilution used in our recombinant ELISA in those aged <30 years. This clearly indicates no cross-reactivity between MCV and VACV neutralising antibodies. There is a possibility that some sera from individuals aged >30 years may have neutralising antibodies against MCV as well as exhibiting a low affinity for vaccinia. Although not clearly understood, the higher MCV seroprevalence we observed in the over 30, vaccinated populations, could be a booster affect on the immunity of these individuals either through exposure to MCV, VACV or other poxviruses. Also, if this is the case then the booster effect may function in both ways i.e. MCV exposure boosts anti-VACV antibodies or vice versa.This may have implications for a role of MC when smallpox is endemic.

Chapter 5 -

MC084 and MC133 Monoclonal Antibodies

Chapter 5 - MC084 and MC133 Monoclonal Antibodies

Introduction

Vaccinia virus which is the representative virus of the genus orthopoxvirus is a well studied virus for which a number of valuable antigen (ELISA) and antibody reagents are available. Apart from interest in the virus on account of its use in the eradication of smallpox and various vaccine delivery systems, the ease of reagent generation and availability has been one of the major reasons for the thorough investigation of this virus and its mechanisms of action. On the other hand compared to other well known poxviruses, Molluscum contagiosum has failed to garner sustained interest from a clinical perspective due to the benign nature of the disease but has also suffered from dearth of interest from a research perspective due to the lack of a conventional cell culture system or animal models.The severe lack of reagents has greatly impacted our understanding of the virus in general and also the true nature and extent of the disease in particular. One of the main aims that I hoped to address with this study was to develop MCV gene specific reagents which would contribute to further investigation of the MCV.

It would be extremely interesting to be able to detect and visualise Molluscum contagiosum at the site of the infection. Molecular detection assays could possibly provide more specific and rapid detection which could be a useful supplement to diagnosis on basis of physical examination alone, especially in patients showing complicated and unclear presentation. Nucleic acid-based techniques rely on detection of specific DNA sequences in the pathogen genome. The methods typically consist of sample extraction, a PCR amplification step and a product analysis step. The techniques are highly specific but generally time consuming and confined to a dedicated research laboratory environment. Pathogen detection using specific antibodies offers the potential for more rapid analysis than nucleic acid-based techniques due to limited sample preparation. Also an antibody based system would allow other disease related investigations such as a more specific sandwich ELISA. Currently no monoclonal antibodies specific for Molluscum contagiosum virus exist.

Monoclonal antibodies (mAb) specific to cell-surface antigens have systematically provided a means of identification and characterisation of the structure and function of many integral membrane proteins. The fast advances in molecular biology cloning have allowed the isolation of novel genes encoding specific membrane proteins. However, in the case of molluscum contagiosum research, the initial characterization of these novel gene products has been greatly hampered by the lack of specific antibodies. Currently most poxviral antibodies which are available recognize mostly VACV proteins that are involved in virion assembly or/and are major antigens in smallpox vaccine. These antibodies are useful for tracking distinct steps in virion assembly and for studying the B cell epitopes in smallpox vaccine.

Thus the development of monoclonal antibodies raised against specific MCV surface proteins would greatly contribute to the investigation and understanding of the disease. It would help us to identify the distribution of MC proteins MC084 and MC133 in infected skin biopsy material as well as potentially contribute to the development of a more accurate and specific assays such as a sandwich ELISA which would enable more thorough and accurate screening of sera samples.

The aim of the work presented in this chapter was the production and screening of novel monoclonal antibodies (mAbs) against specific MCV virion surface proteins MC084 and MC133 by immunizing mice with purified MC084S (aa123-230) characterised in Chapter 4 and due to time constraints against the fusion protein MC133S-GST (aa1-370), as possible reagents for future implementation in skin biopsy staining, development of routine serological reagents and development of a possible sandwich ELISA.

Results

5.1 Selection of antigen, cloning and purification

The two candidate immunogenic peptides selected for the purpose of production of MCV specific monoclonal reagents were MC084 and MC133, both of which are homologues of proteins which are part of the virus particle entry complex in VACV.

5.1.1 Antigen selection – MC084

The selection, purification and characterisation of the recombinant C-terminal truncation of molluscum surface protein MC084, used for monoclonal antibody production i.e. MC084S (V123-230), has been extensively discussed in Chapter 4 and to avoid repetition will not be discussed here.

5.1.2 Antigen selection – MC133

The molluscum gene mc133 was selected as a candidate for expression and purification as an immunogenic peptide based on a previous study by Watanabe and co-workers where they identified MCV proteins capable of recognition by the humoral immune system of molluscum patients (Senkevich *et al*., 1996; Watanabe *et al.,* 1998). This was done by screening 12 sera from molluscum contagiosum patients with a library of molluscum contagiosum transferred into cowpox expression system (Watanabe *et al.,* 1998). A 70 kD recombinant protein was detected by immunoblotting and mapped to the open reading frame MC133L. Consensus sites were also determined between MC133 and homologs in vaccinia and variola (Watanabe *et al.,* 1998). MC133 is an IMV membrane associated type A inclusion protein. Amino acid sequences of the MC133 (aa1-546), calculated molecular weight 61.5 kD) were analysed using standard software packages to determine overall homology with related proteins in the GenBank and identify transmembrane regions and region of high hydrophilicity and high antigenicity.

Of the 546 amino acids in total, two subclones were defined; an N-terminal truncation (aa1-370; calculated molecular weight 40.5 kD) and a C-terminal truncation (aa 371-

546; calculated molecular weight 20.6 kD) (Figure 5.1A), both containing one region of high hydrophilicity in the Kyte–Doolittle plot (Figure 5.1B) (Kyte and Doolittle, 1982), which were further analysed for subcloning.

An open unlimited NCBI BLASTN search was used to compare the truncated amino acid sequences of mc133 to Genbank sequences (nr). Compared to the N-terminal mc133S (aa1-370), the C-terminal mc133v5 (aa371-546) showed the highest overall amino acid identity with vaccinia virus protein A27L and variola virus protein A30L.

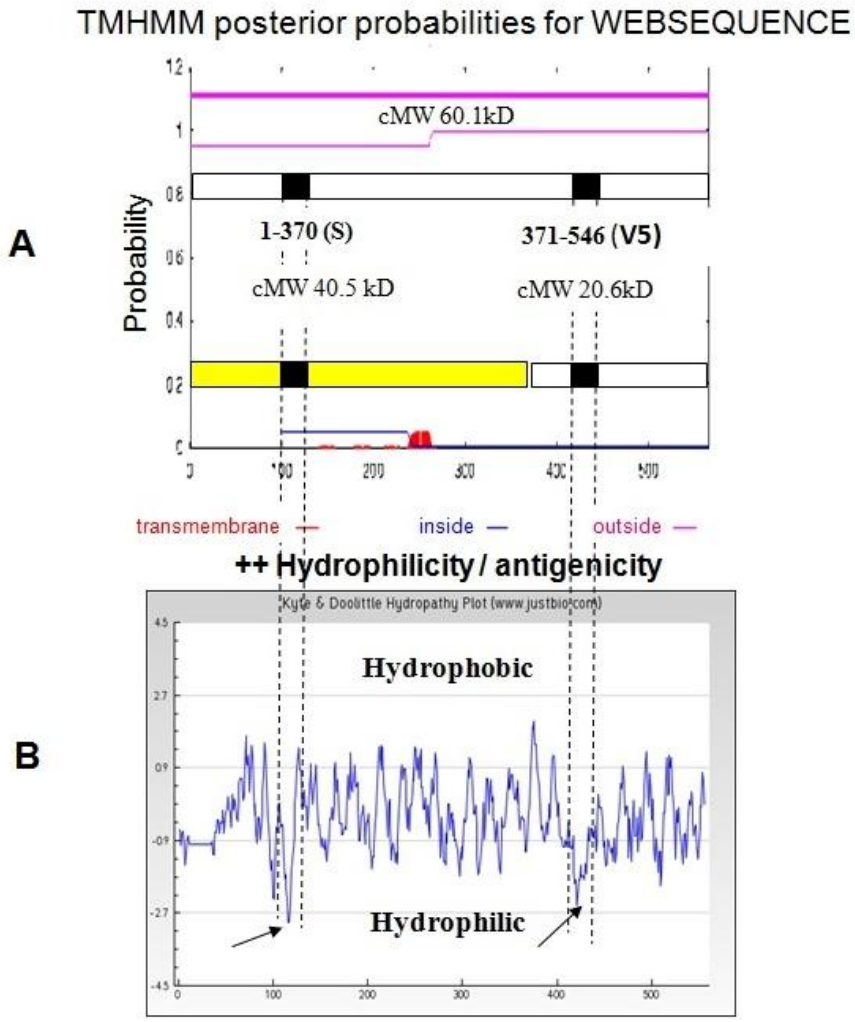


Figure 5.1. Bioinformatics of MC133. *(A) Transmembrane plot (TMHMM Server v. 2.0) of mc133 amino acids 1-546 with the two most antigenic regions indicated by black boxes. (B) Hydropathy plot of MC133 protein with predicted high hydrophilic / antigenic regions indicated by black arrows in the corresponding transmembrane plot. The full length ORF (MC133; aa1-546; predicted molecular weight 61.5 kD; shown on top) was cloned into vRB12 using specific primers tailed with restriction enzyme sites EcoRI-HindIII and C-terminal Flag StrepII epitope tag. The resulting plasmid p343 was sequenced and the recombinant vaccinia virus v343 isolated on BSC-1 cells using the plaqueless mutant system. N-terminal (in yellow) and C-terminal (in white) truncations were subcloned from the original full length MCV gene into pGEX-2TK for overexpression in E. coli BL21 (RIL^{+}). TMHMM was used to determine transmembrane regionswhereas the Kyte Doolittle plotwas used to identify hydrophilic regions with predicted high antigenicity.*

5.1.2.1 Cloning of mc133

The N-terminal truncation of mc133 (M1-N370, predicted MW 40.5 kD), comprising 370 aa, has the lowest homology to orthopoxvirus proteins and contains a region of high antigenicity with no significant homology to vaccinia/cowpox virus. The N-terminal truncation MC133 (M1-N370) was then subcloned into the pGEX-2TK vector (Figure 5.2A) with a Strep II tag and in frame with glutathione S-transferase separated by a thrombin kinase site (Figure 5.2B) and overexpressed as a GST fusion protein in codon optimized *E. coli* (BL21 RIL^{+}).

The GST fusion protein was identified with an apparent molecular weight (MW) similar to the predicted MW of 66.5 kD in (40.5+26 kD) IPTG induced cultures (Figure 5.2B).

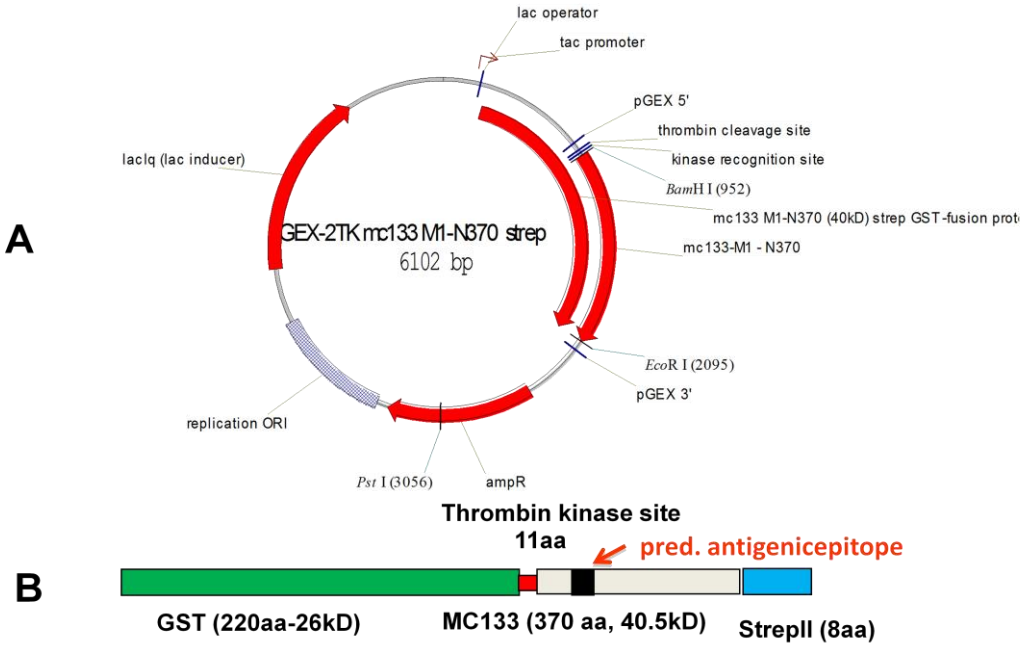


Figure 5.2. pGEX 2TK-mc133 (M1-N370) construct and fusion protein. *(A) Schematic of recombinant plasmid p334 with a MC133 specific insert of 370 amino acids (M123-N370); predicted molecular weight approx. 40.5 kD. Vector NTI (vNTI) was used to produce virtual molecules and schematic diagrams of constructs prior to molecular cloning (InforMax, Inc). (B) Schematic of fusion protein of GST (green) followed by Thrombin cleavage site (red), MC133 (M123-N370) (grey), and strep II tag (blue); predicted antigenic site (black).*

5.1.2.2 Purification of antigen

The recombinant GST fusion N-terminal truncation of MC133 was obtained in overexpressed bacterial lysate and purified over glutathione sepharose beads using a batch purification method. The eluted fusion protein was visualised using the Strep II tag for detection in Western Blot of the fusion protein GST-MC133 (M1-N370)-StrepII using a an anti-strep antibody i.e Strep MAB-Classic HRP conjugate (Figure 5.3).

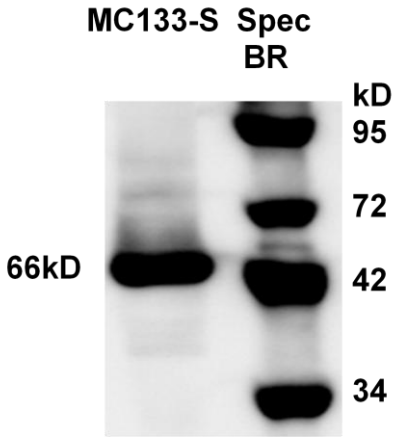


Figure 5.3. Characterisation of GST-MC133S(M1-N370). *Characterisation of overexpressed recombinant fusion protein GST-MC133S (M1-N370) in Western Blot. Proteins were separated on a 4-12% Bis-Tris gel and after transfer to nitrocellulose overexpressed 66 kD recombinant GST-MC133S fusion protein is seen. Spectra BR: Molecular weight marker expressed in kD. The membrane was probed with Strep MAB-Classic HRP conjugate (IBA-lifesciences).*

5.2 Hybridoma production

5.2.1 Production of MC084S (123-230) antibody producing hybridomas and hybridoma yield following fusion

In this study we used the previously characterised MC084S (123-230) (Chapter4) as an immunogen for the production of specific mouse mAbs. The amino acid sequence of MC084S, was evaluated for regions of high hydrophilicity/ high antigenicity, making it an ideal candidate for immunising mice. All animal immunisations and hybridoma fusions were carried out with the help of individuals in the Morgan Lab. Initially three BALB/c mice (1R, 1L and 1B) were immunised with MC084S (123-230) according to the protocol in materials and methods. Unfortunately each splenocyte fusion from the three mice immunised with MC084S (123-230), suffered from fungal and mycoplasma contamination within the first week of fusion, which was the result of large scale contamination of shared CO_2 incubators and hoods in the cell culture lab used for the

procedures. This was confirmed visually and by PCR testing for mycoplasma. Further to this two other BALB/c mice (2R and 2L) were immunised with MC084S following the same protocol. These mice were sacrificed sequentially based on the highest peritoneal Ab titre of the mouse sera, assessed by ELISA experiments described previously and from the splenocyte fusions fully cloned antibody-producing cell lines were obtained. The hybridomas were selected on the basis of a positive reaction with MC084S coated microtitre ELISA wells. All cell lines were found to produce IgG-isotype antibodies. Mouse 2R generated 9 clones while Mouse 2LR produced 6 clones, respectively (Table 5.1).

Table 5.1. Summary of hybridoma yield following fusion for mice immunised with MC084S

Mouse	Total hybridomas	Single clones	% single clones	Original positive hybridomas	Subcloned
2R	9	3	33.33%	2	1
2L	6	2	30%	1	1

5.2.2 Production of MC133 (M1-N370) antibody producing hybridomas and hybridoma yield following fusion

Similarily we used the fusion protein MC133S-GST (M1-N370) characterised in previous result (Figure 5.3), as an immunogen for the production of specific monoclonal antibodies mAbs. The amino acid sequence of MC133S was evaluated for regions of high hydrophilicity/high antigenicity. A region of high hydrophilicity was confirmed and thus this was considered an ideal immunogen. Three BALB/c mice (1R, 1L and 1B) were immunised with MC133S (M1-N370) according to the protocol in materials and methods. These mice were sacrificed sequentially based on the highest peritoneal Ab titre of the mouse sera, assessed by ELISA experiments described previously and from the splenocyte fusions of fully cloned antibody-producing cell lines were obtained. Antibody-containing culture supernatants from the mAb producing clones were tested and hybridomas were selected on the basis of negative screening by comparing reactions with MC133S-GST, vMC133 (v343) and GST coated microtitre ELISA wells. In the end clones showing strongest reactivity against MC133S-GST and vMC133 and no reactivity with GST were selected and found to produce IgG-isotype antibodies.

Mouse 1L generated 6 clones, while Mouse 1R and 1B produced 2 and 3 clones, respectively (Table 5.2).

Table 5.2. Summary of hybridoma yield following fusion for mice immunised with MC133S

Mouse	Total hybridomas	Single clones	% single clones	Original positive hybridomas	Subcloned
1R	6	2	33.33%	2	2
1L	12	6	50%	3	1
1B	7	3	43%	2	1

5.2.3 Specificity of hybridoma supernatants for mouse and human

Hybridomas from MC084 fusions were initially screened using ELISA as described in 2.9.5. However, Mouse 2L produced six hybridomas that responded to the the ELISA and mouse 2R produced nine. These further screened for positive single clones and the positive hybridomas were subcloned by limiting dilution as described in materials and methods. The specificity of one of these clones i.e. MC084-(6-2, 11-A-2, 11-D-2, 6C) or MC084-6C has been investigated in 5.3. The yield from MC133 immunised mice spleens were higher (1R-6, 1L-12, 1B-7) perhaps due to the fusion with a larger GST fusion protein for immunization in comparison to the peptide alone. To rule out hybridomas against GST, the hybridomas from MC133 fusions were also screened using ELISA with three proteins GST, MC133S and v343. Mouse 2L produced six hybridomas that responded to the ELISA screening and mouse 2R produced nine. These further screened for positive single clones and the positive hybridomas were subcloned by limiting dilution as described in materials and methods. Due to time constraints the positive single clones for the producing MC133S (M1-N370) hybridomas have been screened using ELISA and are yet to be fully characterised.

5.3 Characterisation of MC084 (V123-R230) monoclonal (6-2, 11-A-2, 11-D-2, 6C) or MC084-6C

5.3.1 Specificity of MC084 (V123-R230) monoclonal MC084-6C for antigen

TheMC084 (V123-R230) monoclonal (6-2, 11-A-2, 11-D-2, 6C) which shall hence forth be referred to as MC084-6Cfor simplicity, was characterised for specificity for

antigen used in immunisation protocol. A 4-12% Bis-Tris gel was run with lanes 1 and 2 loaded with the 14 kD purified MC084S (V123-R230) peptide; lanes 3 and 4 loaded with the 40 kD fusion protein GST-MC084S (V123-R230); lane 5 with 26 kD GST alone and lane 6 with 66 kD fusion protein GST-MC133S (M1-N370). The gel was transferred to nitrocellulose and the western blot stained with MC084S-6C and an ImmunoPure® secondary goat anti-mouse IgG antibody and visualised using the Supersignal WestPico chemiluminescent kit (Figure 5.4).

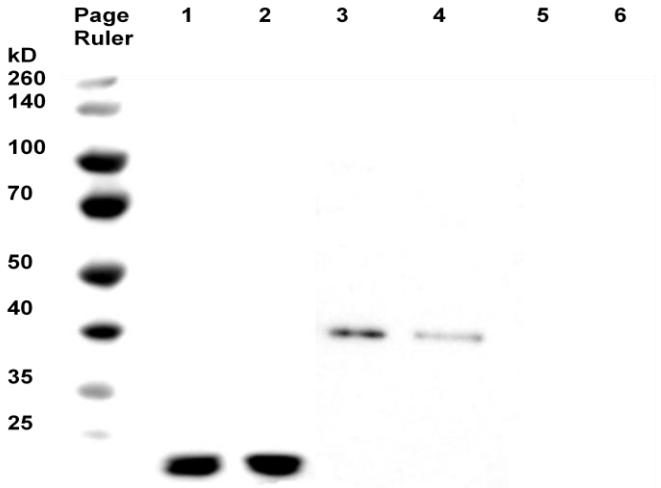


Figure 5.4. Characterizationof MC084 (V123-R230) monoclonal MC084S-6C. *Page Ruler: Molecular weight marker expressed in kD. Lanes 1&2: Purified 14kD recombinant MC084S (V123-R230), Lanes 3&4: MC084S-GST fusion protein (60.5 kD), Lane 5 GST protein (26kD) andLane 6:MC133S-GST fusion protein by SDS-PAGE and Western Blot. The membrane was probed with ImmunoPure® antibody 2º goat anti-mouse IgG (Pierce). Detection using SuperSignal™ WestPico Chemiluminescent Substrate (Pierce).*

5.3.2 Antigenicity and specificity of MC084 (V123-R230) monoclonal MC084-6C

Mice were sacrificed 2-3 days after final intraperitoneal immunisation and bled to collect sera by cardiac puncture. Serum from a normal healthy non-immunised mouse was also collected that served as control. Immune serum against MC084 antigen showed a high titer of at least >12400 when tested by direct binding ELISA (Figure 5.5A). Serum from healthy mouse (control) exhibited no binding with MC084 antigen.

Antigen-antibody binding in direct binding ELISA was confirmed by inhibition ELISA. In this assay serum was incubated with increasing amounts of inhibitor (MC084) for four hours at 37ºC or overnight at 4ºC. Immune complexe(s) formation increased with

increase in inhibitor concentration (0.1-10 μg/ml). Instead of directly using sera as in direct binding ELISA, these sera was treated overnight with inhibitors and coated on ELISA plates. The remaining steps were identical to direct binding ELISA. Maximum inhibition (83.4%) was achieved with highest the concentration (10 μg/ml) of inhibitor i.e. MC084S (V123-R230) (Figure 5.5B). Fifty percent inhibition was achieved at 2 μg/ml of inhibitor (Figure 5.5B). This shows that immunised mouse serum antibodies exhibited high specificity and affinity towards immunogen (MC084S (V123-R230)).

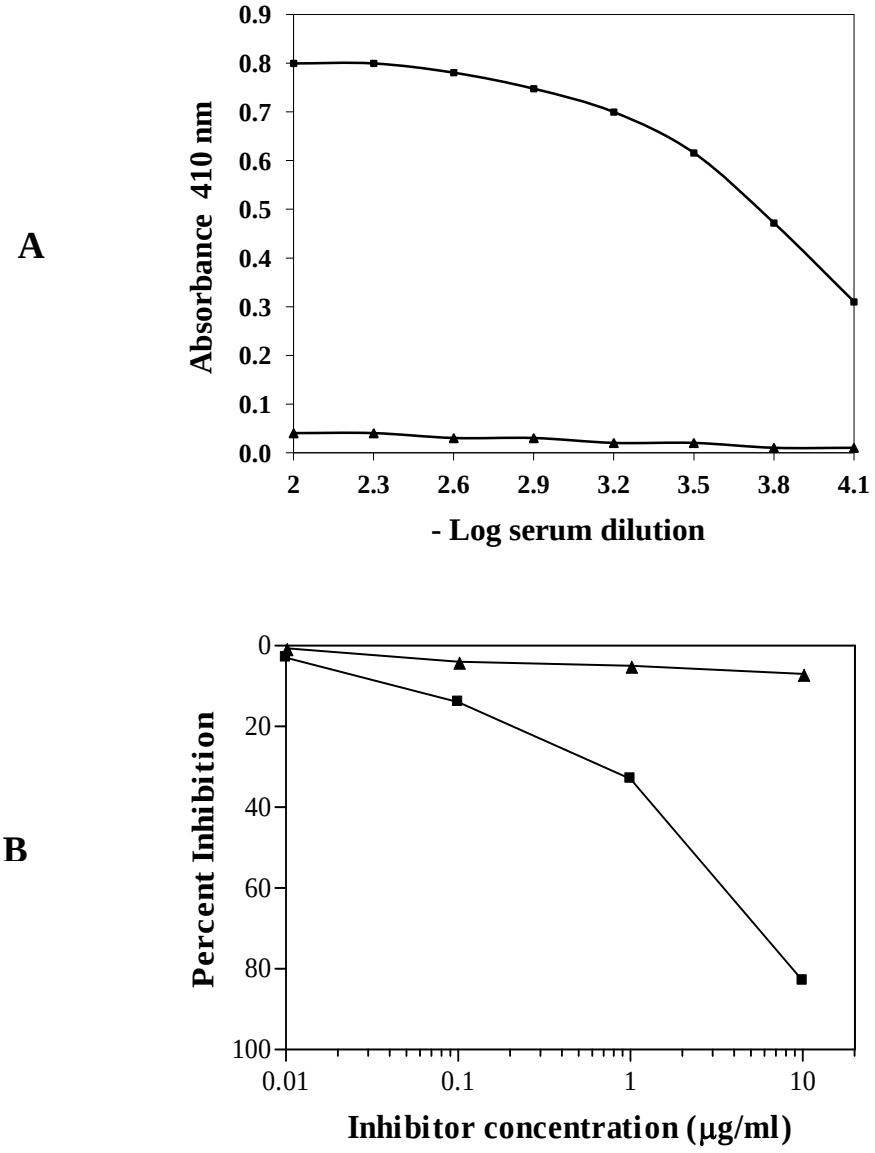


Figure. 5.5. Immune serum antigenicity and specificity of MC084 (mouse-2R). *(A) Direct binding ELISAs were performed with serial dilution of immune sera (■) and sera from non-*

immunised healthy BALB/c mice (control) (▲) against immunogen MC084 (V123-R230). (B) Inhibition ELISAs were performed with immune (■) and control (▲) sera against MC084. MC084S (V123-R230) was used as an inhibitor with increasing concentrations (0.01-10 μg/ml) Microtitre plates were coated with immunogen MC084S (V123-R230) (4 μg/ml).

Characterisation of monoclonal antibody MC084-6C was done using ELISA assays. Increasing concentrations (0-20 μg/ml) of antigen MC084S (V123-R230) was coated on ELISA plates and MC084 monoclonal antibody supernatant was used to perform direct binding ELISA. Antigen-antibody binding is found to increase with increase in concentration of antigen (Figure 5.6A). Antigen MC084S (V123-R230) and antibody (MC084-6C mAb) binding equilibrium was achieved at an antigen concentration of 3 μg/ml. Antigen antibody antigenicity was confirmed by a more sensitive inhibition ELISA (Figure 5.6B). A high inhibition of 93% was detected with 10 μg/ml of inhibitor (MC084). Fifty percent inhibition was achieved at 1 μg/ml.

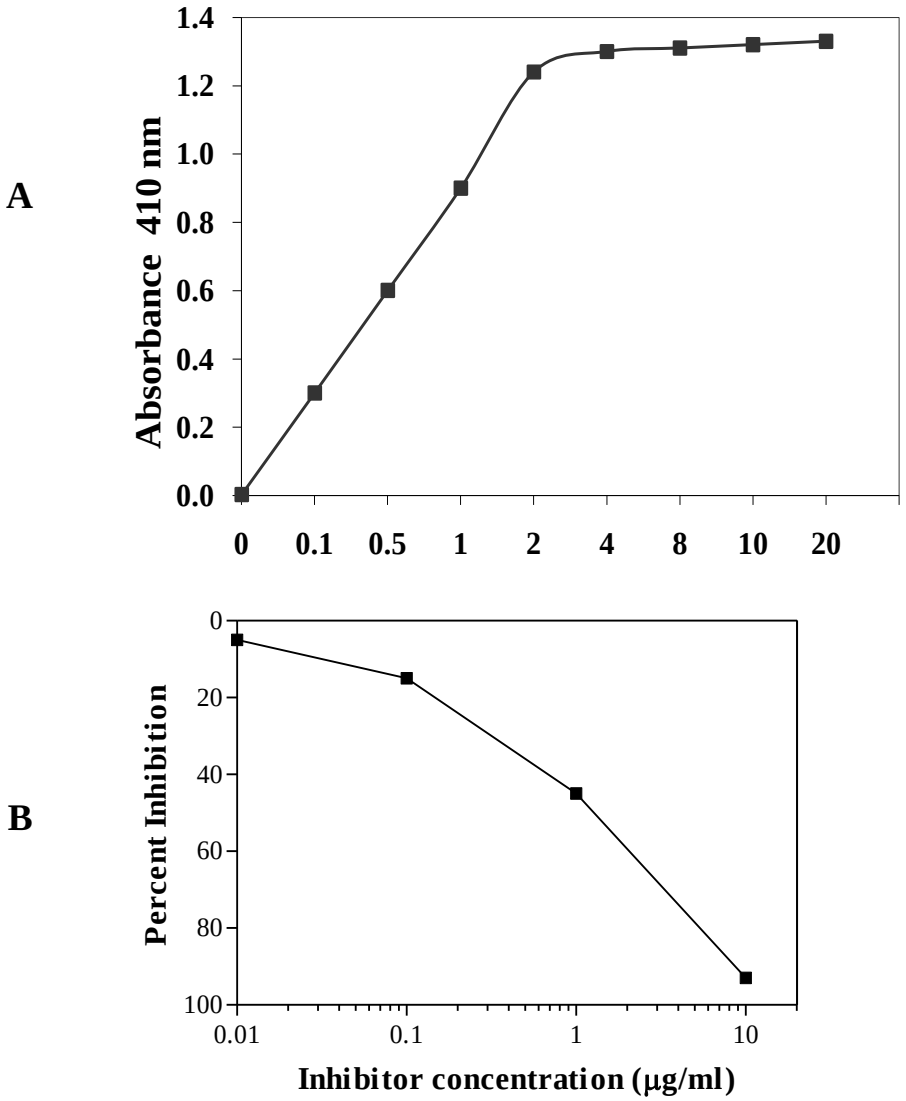


Figure 5.6. Antigenicity and specificity of MC084 (V123-R230) monoclonal antibody MC084-6C by direct binding and inhibition ELISA. *(A) Direct binding ELISAs were performed for MC084 monoclonal antibodies (MC084-6C) against increasing concentration (0-20 µg/ml) of immunogen MC084. (B) Inhibition ELISAs were performed for monoclonal MC084-6C. MC084 was used as an inhibitor with increasing concentrations (0.01-10 µg/ml) Microtitre plates were coated with immunogen MC084 (4 µg/ml).*

5.3.3 Specificity determined by Molluscum contagiosum tissue staining with MC084-6C

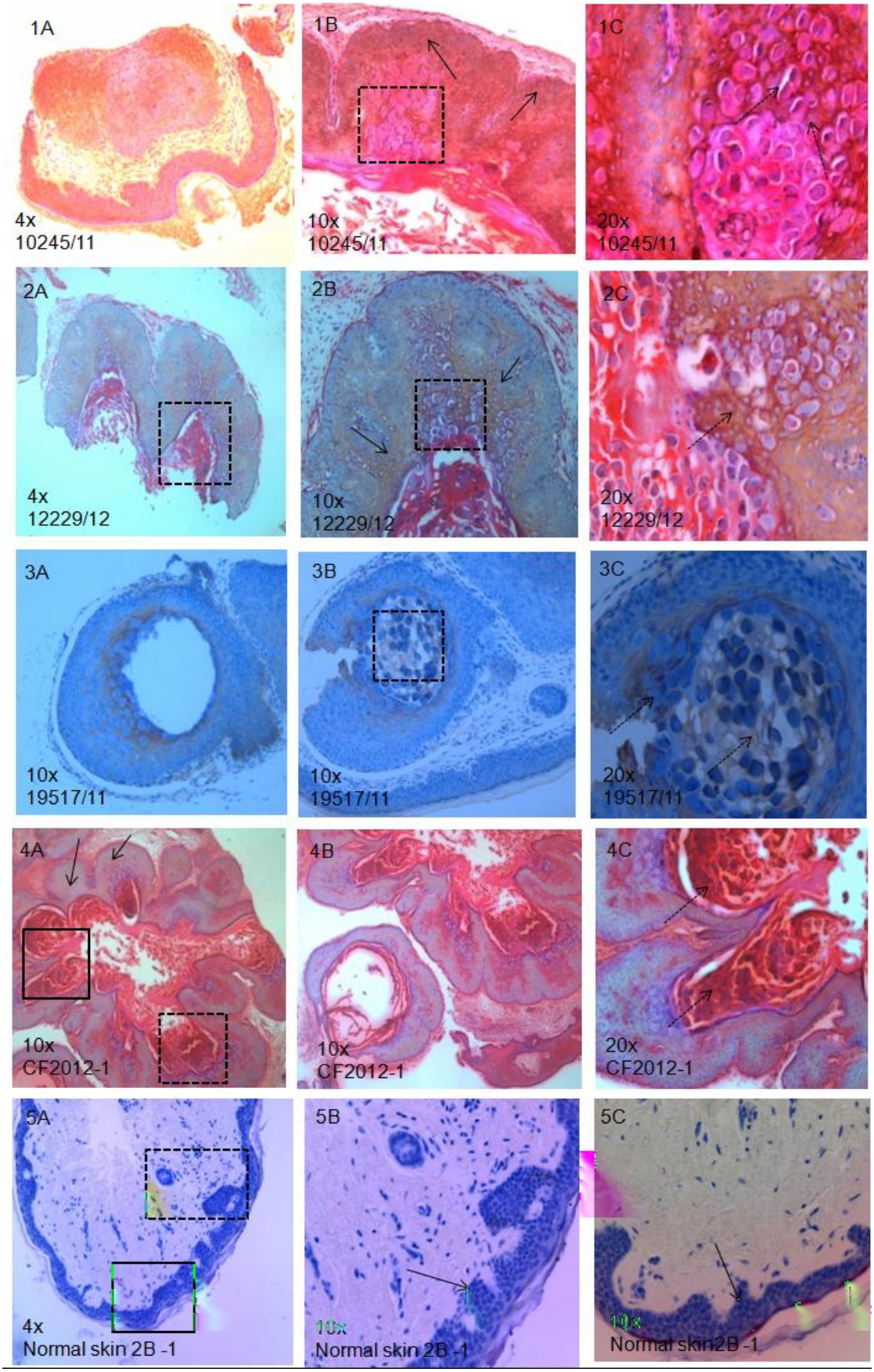
5.3.3.1 Specificity determined by Molluscum contagiosumparaffin section staining with MC084-6C

The MC084 (V123-R230) clone designated MC084-6C, was further characterised for specificity by tissue staining. MC biopsy tissue sections consisting of infected skin were stained with the MC084 monoclonal i.e.MC084-6C (Figure 5.7 panels 1-3 and 5). MC positive patient serum CF2012-1 (Figure 5.7 Panel 4) was used as positive control and normal human epidermal skin (Figure 5.7 panel 5) as a negative control.

Panels 1-4 show molluscum contagiosum lesions with typical pattern of staining at various magnifications counterstained with haematoxylin eosin. Figure 5.7-1A shows a MC section (4x) with concentrated golden brown stain in areas. Figure 5.7-1B (10x) shows a small area of the same section (not pictured in Figure 5.7-1A) with concentrated golden brown stain in basal layers of the cup shaped lesion as show by solid arrows. Figure 5.7-1C inset higher magnification (20x) of central crater containing cell and viral debris and showing strong golden brown staining (dotted arrows). Figure 5.7-2A shows a lesion with typical molluscum contagiosum histology (4x). Figure 5.7-2B shows an inverted lesion with clear epidermal stratification as basal, suprabasal and spinous layers (10x). The majority of brown stain is observed in the spinous layers (dotted arrows) surrounding the central crater. Figure 5.7-2C inset; cell and viral debris filled crater with staining shown by arrows (20x). Figure 5.7-3A shows a molluscum lesion with staining around central waxy plug where viral material has been washed away (10x). Figure 5.7-3B shows the same lesion at the same magnification (10x) with the viral plug intact and stained golden brown. Figure 5.7-3C shows the same follicular infundibulum at a higher magnification (20x).

Figure 5.7-4A shows MC section with intraepidermal lobules stained with MCV positive patient serum CF2012-1 (4x). Figure 5.7-4B is an area in the same lesion shown by dotted box in previous panel (10x). Figure 5.7-4C is a magnification (20x) of the area shown in panel A in solid box showing the cell debris and MC bodies filled crater. Figure 5.7-5A shows normal skin section showing no staining with MC084-6C

(4x). Figures 5.7-5B and 5C show different areas of normal epidermis (arrows) with no apparent staining with the monoclonal antibody (both 10x).


1A
4x
10245/11
1B
10x
10245/11
1C
20x
10245/11
2A
4x
12229/12
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12229/12
2C
20x
12229/12
3A
10x
19517/11
3B
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19517/11
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4C
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CF2012-1
5A
4x
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Normal skin 2B -1
5C
10x
Normal skin2B -1

Figure 5.7. MC084S (V123-R230) monoclonal antibody MC084S-6C specificity in MC tissue stains. *Tissue sections from MCV positive patient biopsies stained with MC084S* (V123-R230) *monoclonal MC084S-6C showing specific golden brown stain in lesions (Panel 1-4). Panel 1 shows section 10245/11-1A (4x), 1B (10x) and 1C (20x). Panel 2 shows section 12229/12-2A (4x), 2B (10x) and 2C (20x).Panel 3 shows section 19517/11-3A (10x), 3B (10x) and 3C (20x). Panel 4 shows section 18894/11 stained with positive control MC positive serum CF2012-1-4A (10x), 4B (10x) and 4C (20x) and showing positive golden brown staining. Panel 5 shows negative control normal human epidermal skin stained with MC084-6C- 5A (4x), 5B (10x) and 5C (10x) and showing no golden brown staining. (Haematoxylin-eosin counterstain).*

5.3.3.2 Specificity determined by Molluscum contagiosum frozen biopsy section (HD91) staining with MC084-6C

The MC084 (V123-R230) monoclonal antibody MC084-6C, was further characterised for specificity by immune histology. MC frozen sections from an old collection belonging to Dr. Bugert from 1991 were stained with the MC084 monoclonal i.e. MC084-6C (Figure 5.8). Due to the age of these frozen biopsy sections and fluctuation in storage temperature the sections did not show clear histology. However when stained with MC084-6C and a secondary Alexa Fluor 488 (Green) chicken anti-mouse IgG (H+L) antibody (1:1000) the section showed clear distribution of an apple green stain in the cup shaped lesion. It was possible to distinguish the lesion into a central crater filled with viral debris containing cells (Figure 5.8A, solid arrow) and the surrounding epidermal basal membrane (Figure 5.8A, dotted white arrow). Figure 5.8B shows immunofluorescent (blue filter) staining of the nuclei concentrated in and around the central lesional crater (solid white arrows). Some blue staining is also visible in the basal layer surrounding the central crater (dotted white arrow).

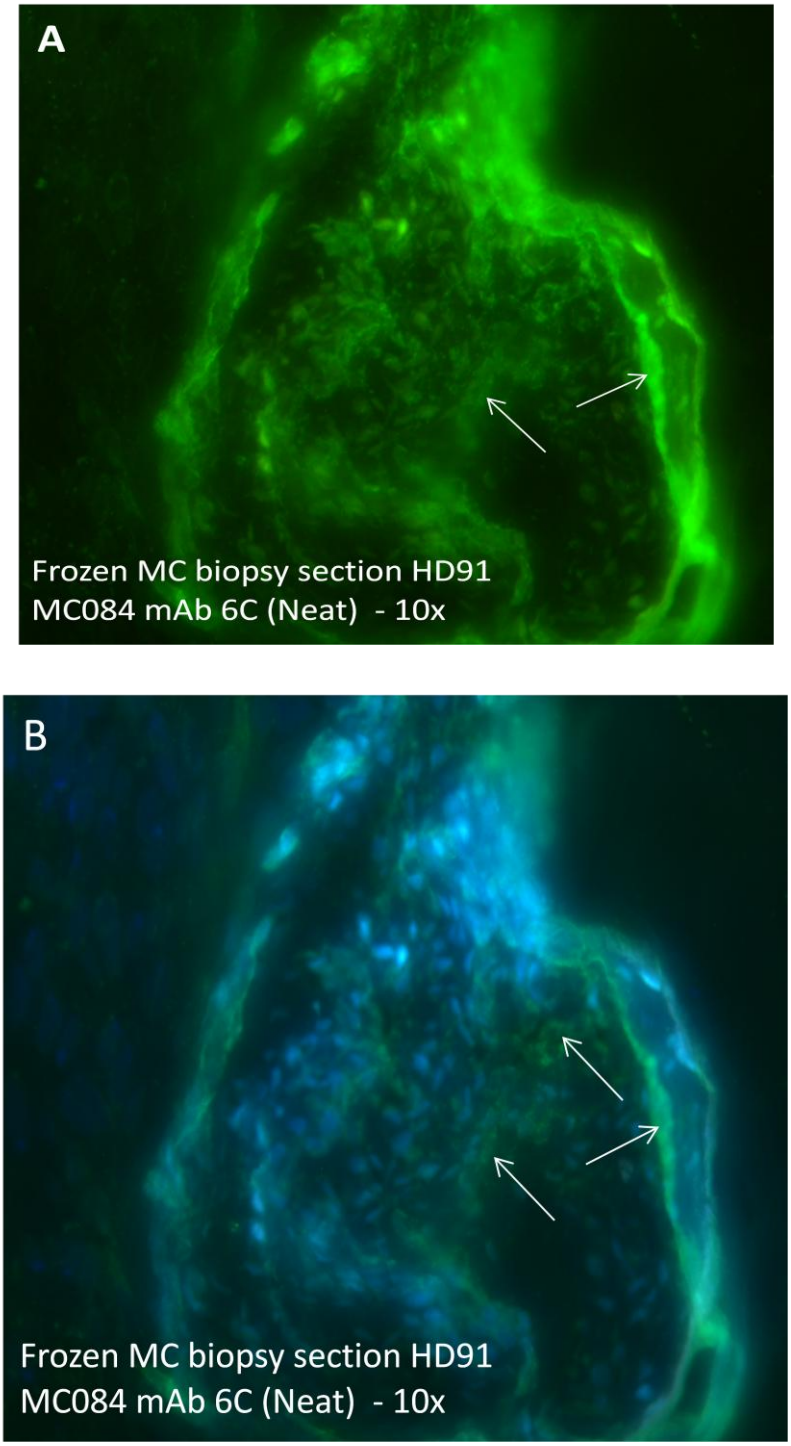

Figure 5.8. Immunofluorescent specificity of MC084S-6C monoclonals in MC frozen tissue sections. *(A) Reactivity ofMolluscum contagiosum biopsy freeze cuts (HD 91) withMC084S (V123-R230) monoclonal MC084-6 (neat) in and around central virus plug and the surrounding epidermis. (B) Blue staining of nuclei with DAPI mostly in central crater of MC lesion. Secondary antibody Alexa Fluor 488 (Green) chicken anti-mouse IgG (H+L) (1:1000). Nuclei are stained with DAPI and shown in blue. Sample was analysed for fluorescence emission properties by using an Olympia BX51 (A) green filter and (B) blue filter.*

5.3.4 Specificity determined by immunofluorescent staining of viral mc084 with MC084-6C

5.3.4.1 Binding specificity of MC084-6C for viral mc084 expressed in BSC-1 cells

To establish monoclonal MC084-6C specificity for antigen we infected African green monkey kidney epithelial cells with a vaccinia virus expressing full length mc084 (v319; aa 1 to 318). These were fixed and stained with MC084-6C (1:50). Most cells on the coverslip (Figure 5.9A) showed binding of expressed viral MC084 with the monoclonal antibody MC084-6C. Figure 5.9B shows some uninfected cells with blue DAPI stained nuclei (solid white arrows) but no apple green indicating no expression of MC084. Figure 5.9C shows strep control.

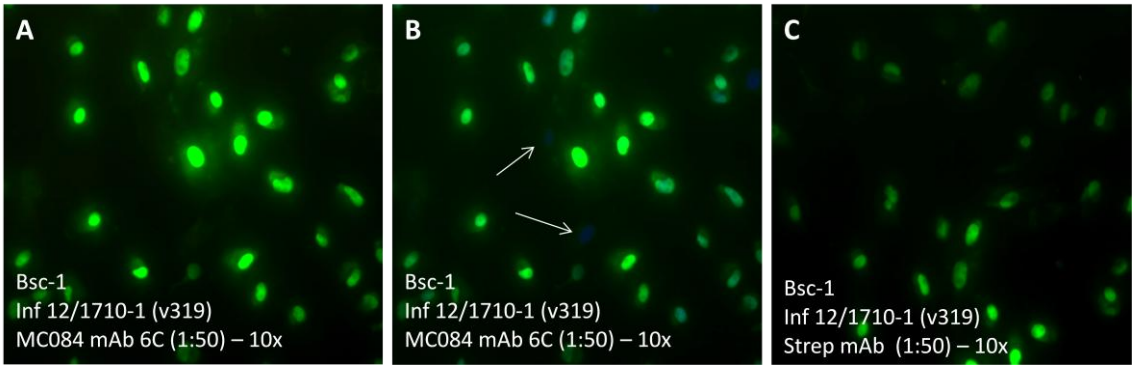

Figure 5.9. Determination of specificity of MC084S-6C for vMC084 in BSC-1s. *Immunofluorescent detection of recombinant vaccinia virus expressed MC084S (v319) in BSC-1 cell culture with MC084-6C. (A&B) Reactivity of MC084S (V123-R230) monoclonal MC084-6 expressed in hybridoma supernatant (1:50) with MC084 (10x) and secondary antibody Alexa Fluor 488 (Green) chicken anti-mouse IgG (H+L) (1:1000). (C) Reactivity of Classic Strep MAB (1:100) with MC084S. Nuclei are stained with DAPI and shown in blue (solid white arrows). Coverslips were analysed for fluorescence emission properties by using an Olympia BX51.*

5.3.4.2 Binding specificity of MC084-6C for vmc084 expressed in HaCaT cells

To further establish monoclonal MC084-6C specificity for antigen we infected human keratinocytes with a vaccinia virus expressing full length mc084 (v319; aa 1 to 318) and stained with MC084-6C (1:50). Fewer keratinocytes were infected compared to uninfected cells as evidenced by an apple green stain. Single infected cells shown by solid white arrows indicating binding of monoclonal MC084-6C with expressed viral MC084 shown in Figure 5.10, Panels A and B (10x). Figure 5.10C shows a higher

magnification (20x) of two keratinocytes with large DAPI stained nuclei surrounded by viral MC084 stained an apple green (solid white arrows). It is also apparent that the MC084 is produced outside of the nucleus in an endosomal pattern. Figure 5.10D shows the strep antibody control (1:100) (10x) in cells indicated by solid white arrows.

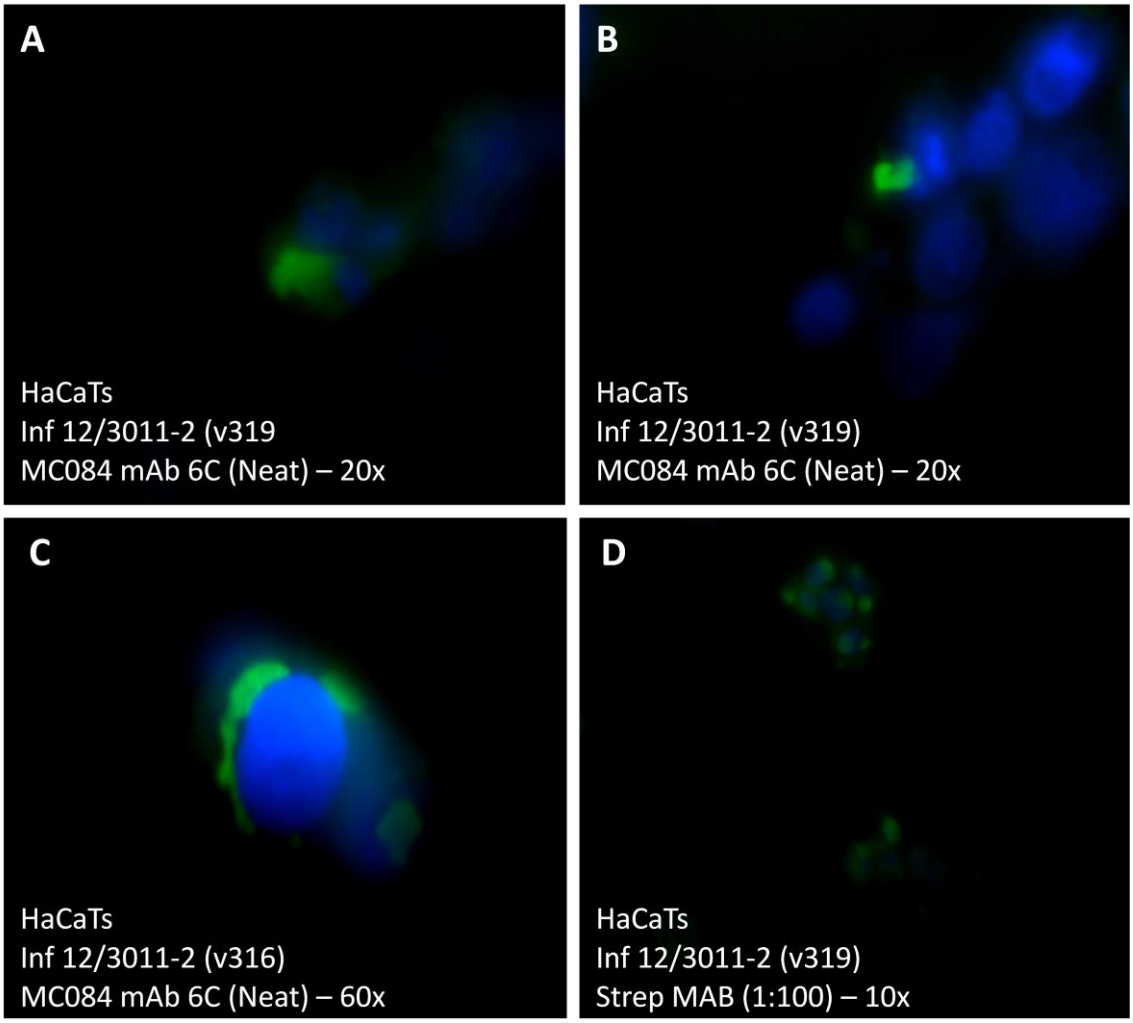

Figure 5.10. Determination of specificity of MC084S-6C for vMC084in HaCaTs. *Immunofluorescent detection of recombinant vaccinia virus expressed MC084S (v319) in HaCaT cell culture with MC084-6C. (A and B) Reactivity of MC084S (V123-R230) monoclonal MC084-6 expressed in hybridoma supernatant (1:50) with MC084 (10x) and secondary antibody Alexa Fluor 488 (Green) chicken anti-mouse IgG (H+L) (1:1000). (C) Higher magnification (20x) of two cells showing staining of nuclei and reactivity with MC084-6C in endosomes. (D) Reactivity of Classic Strep MAB (1:100) with MC084S. Nuclei are stained with DAPI and shown in blue. Samples were analysed for fluorescence emission properties by using an Olympia BX51.*

Discussion

We developed monoclonal antibodies against MCV recombinant surface proteins MC084 and MC133 truncations i.e. MC084S (V123-R230) and MC133S (M1-N370). BALB/c mice were immunised with the purified MC084S peptide and GST-MC133 fusion protein according to immunization protocol as discussed in materials and methods. The mice were sacrificed sequentially and the splenocytes were fused with SP2/0 cells to achieve hybridoma fusion. These hybridomas were ELISA screened and subcloned as described in materials and methods. The percentage of hybridomas obtained from MC133 fusions was greater than that for MC084 fusions. This may be the result of the nature (fusion protein) and size of protein (40 kD) used for MC133 immunisation. Of the two original positive MC084 hybridomas which were subcloned, the clone (6-2, 11-A-2, 11-D-2, 6C) or MC084-6C was further investigated.

Polyclonal antibodies isolated from immune sera of experimental animal after final boost showed MC084 recombinant viral protein as highly immunogenic and induced high titre anti-MC084 antibodies. After several routine screens of clones, we successfully selected MC084-6C clone that was further characterised by western blot for specificity for immunogen. The antibodies showed reactivity with 14 kD peptide used in immunisation as well as the 40 kD GST-MC084S fusion protein but no binding with GST (26 kD) alone or with GST-MC133 fusion protein. The specificity and immune-reactivity were also ascertained by direct binding and inhibition ELISAs. The results revealed high affinity of MC084-6C for the immunogen, MC084S (V123-230), suggesting that the monoclonal antibodies are immunogen specific and possibly recognise the most antigenic motif (GGNIRNDDKYTH) of MC084 protein.

Specificity of MC084-6C was determined in Molluscum contagiosum infected tissue biopsies by clear golden brown staining confirming reactivity of the monoclonal with the MC084 in sections (Figure 5.7 panels 1-3). Thee staining pattern of tissues stained with MC084-6C, show the concentration of golden brown stain in the basal, suprabasal layers as well as the central crater indicating MC084-6C specificity and possible abundance and aggregation of MC084 in these layers. Immunofluorescent staining of

frozen MC biopsy sections with the monoclonal MC084-6C showed clear staining of the MC lesion with concentration of green signal in the central crater probably filled withviral and cell debris as well as the surrounding basal epidermal layer. Blue coloured nuclei stained with DAPI were visible in the lesion, particularly concentrated in the centre of the lesion.

Monoclonal MC084-6C specificity for antigen was further investigated by immunofluorescent staining of different cells infected by a vaccinia virus expressing full size mc084 (v319; aa 1 to 318) constructed earlier as a part of my project and described in the materials and methods. The African green monkey kidney epithelial cells infected with the vaccinia virus v319 stained positive and showed strong apple green signal in a pattern resembling virus factories. Infected cells showed a blue DAPI stained nucleus. Similarly infected keratinocyes (HaCaTs) showed an apple green stain which was found to surround the DAPI stained nucleus in and endosomal pattern.

In summary, we have developed and screened hybridomas producing monoclonal antibodies against MC084S (V123-R230 and MC133S (M1-N370). From these the monoclonal antibodies produced by hybridoma designated as MC084-6C producing mAbs against MC084S have been characterised and these seem to work very well in recombinant vaccinia controls and in human MCV infected tissues. These monoclonal antibodies are unique reagents and will be used in future work to test a hypothesis that MCV replicates in human epidermal stem cells.

Chapter 6 -
General Discussion

Chapter 6 - General Discussion

Molluscum contagiosum virus (MCV) is a significant benign, but underreported pathogen for children and adults, which causes a skin infection similar to warts. It causes molluscum contagiosum (MC) a stigmatising skin infection which severely impedes quality of life and may develop into chronic infections lasting 12 months and longer.

Viral skin infections are the most common cause of GP visits in the UK, with over a million cases per year constituting a high burden of diseases and incurring considerable cost to the NHS (Schofield *et al*., 2011). Meta-analysis suggests a point prevalence of MC in children aged 0 to 16 years of between 5.1% and 11.5% (Olsen *et al*., 2013). The lack of effective therapies is largely due to a poor understanding of the virus specific immune responses in the human skin.

Compared to other human skin viruses it has failed to garner sustained interest from a research perspective due to the lack of a conventional cell culture system or animal model. The resulting lack of reagents has proven a major obstacle in further investigation of this unique virus. My doctoral research project focuses on aspects relating to the lifecycle of Molluscum contagiosum virus and the human immune response to it. In light of the fact that MCV research has suffered from the absence of reagents enabling its further investigation, the major aim of this PhD project was to develop and design valuable MCV gene specific reagents to enable this work.

As outlined in the introduction and chapter 3, the MCV lifecycle starts upon entry of the virus particle into human epidermal cells or more specifically differentiating keratinocytes. Although the seat of replication is still to be investigated and substantiated, popular belief is that it may be epidermal stem cells. Another challenge to any investigations is that infections of epithelial type cells *in vitro* are non-productive but do produce early gene products. The lack of a cell culture system also contributes to this dilemma. Since the first step of any viral lifecycle is entry, so in order to determine whether MCV has entered cells, we have developed a couple of a GFP and luciferase

based reporter assays detailed in chapter 3 of my work. Also due to the fact that MCV cannot be titrated, we developed a qPCR correlating MCV genome units with genome units of its surrogate virus which is titratable i.e. vaccinia virus. Although these are different viruses but this has enabled quantification of MCV and MOI calculations which can be used together with a reporter assay.Some of the findings during the course of this study were quite unexpected. When we infected a range of cells with MCV using our reporter assay (Sherwani *et al.*, 2012) we found rather unexpectedly that MCV is not limited to infecting keratinocytes (HaCaT) or even to human cells. So the lack of productivity in cell culture is not due to non-entry- or receptor issues as the live virus gets into mouse (3T3), hamster (BHK-21), monkey (Vero) and various human cells (A549, HeLa, HaCaT) and drives early poxviral promoters.

To enter cells,the Molluscum contagiosum virus uses homologs of a cell fusion complex identified in vaccinia virus; these are the MCV genes mc084 and mc133 both of which I have mostly worked with. Because of their role in entry these proteins are located on the surface of the virion particle, so human antibodies versus MCV are raised against mc084 and mc133. We have expressed mc084 and mc133 in prokaryotic and eukaryotic expression systems and then purified the antigens to test human patient serum samples for the presence of polyclonal antibodies in Chapter 4. We have used the reporter assay to determine whether these antibodies can neutralize a MCV infection *in vitro* and whether there is cross reactivity with orthopoxviruses. We have compared parts of MC084 and MC133 for suitability as antigens and identified why the C-terminal domain of a MC084 peptide selected by Watanabe *et al.*, failed to show reactivity with sera samples of Molluscum positive patients in an ELISA. This was because the peptide used was a part of the transmembrane region of that protein.

In a serosurvey using mc084 antigen in an ELISA format versus serum collections from Heidelberg and Cardiff, we found that MC is very common in Germany, especially in children under 5 years of age. This is the first such study in Europe (In print; PLOS ONE PONE-D-13-44356R1). Our numbers are higher than previous Australian and Japanese studies, but this may be due to the use of different antigens.

We have used the most stringent cut-off conditions as we have specifically looked at sera from new-borns and toddlers from 0-1 years after birth, where due to the least exposure to MC due to hygienic conditions, the incidence should be the lowest. But Molluscum contagiosum (MC) seems to be strikingly common in general populations and we have despite this, found evidence for MCV infections in very young children, basically new-borns, and a few months after birth. Because our serum sample collections were anonymized, we could not follow up on this clinically with more sample information. However though rare the occurrence of such infections in new-borns is not unheard of as proved by a case of a DOCK8 deficient patient as previously observed by my supervisor Dr. Bugert at the NIH, who had extensive MC (Figure 1.5C) from birth, and eventually died from the condition as a teenager.

Another interesting case was a patient in our collection that we could follow up clinically during the course of this PhD in collaboration with a Bath dermatologist; the patient suffers from a haematological cancer and has a documented chronic severe and incurable case of MCsince 2008. It is known that MC thrives in immunesuppressed hosts, but is well controlled in immune competent individuals (Bugert and Darai, 1997). A recent study has shown MCV actually induces a massive immune response in immune competent hosts (Vermi *et al.*, 2011).

We have further investigated the specificity of what appear to be human MC antibodies versus vaccinia virus in plaque and reporter inhibition assays using the recombinant VACV reporters and found no neutralization in serum dilutions over 1:100 i.e. the same dilution used in our recombinant ELISA in those aged <30 years. This clearly indicates no cross-reactivity between MCV and VACV antibodies.Three sera samples from individuals aged >30 years whose ages in fact range from 45-51 years, showed neutralisation which can only be explained as the result of vaccinia antibodies acquired through small pox vaccination.Although not clearly understood, the higher MCV seroprevalence we observed in the over 40, vaccinated populations, could be a booster effect on the immunity of these individuals either through exposure to MCV, VACV or other poxviruses. Also, if this is the case then the booster effect may function in both

ways i.e. MCV exposure boosts anti-VACV neutralising antibodies or vice versa.This may have implications for a role of MC when smallpox is endemic.

As discussed in Chapter 5, we have also raised monoclonal antibodies against mc084 and 133. Whereas the monoclonal raised against mc133 have been screened and are yet to be properly characterised, monoclonal antibodies specific for the antigen MC084S (V123-R230) have been characterised. We have tested MC084-6C and theseseem to work very well in recombinant vaccinia controls and in human MCV infected tissues. They will be used in helping to determine the MCV host cell in the epidermis.

Future work emanating from this research project includes the possible development of a capture ELISA using our characterised mAbs and immunogenic peptide for more seroprevalence studies focussing on different geographical areas. Also, the possible identification of the host cell, by monoclonal detection of the virus in infected skinand testing the stem cell hypothesis using our reporter assays and in skin models.The peptides characterised in this study could also lead to the development of novel reagents to detect and characterize T cells in the blood of healthy and MCV infected patients, and investigate the virus specific immune responses by immunehistocytochemistry in sections of MCV skin lesions.

As part of Dr. Bugerts poxvirus group I have also been involved in some of the other work of the group that could be used to address MCV issues. We used the reporter assay to test antiviral compounds and found that Laura Farleighs (another PhD student in the group) ddBCNAs, active against vaccinia virus, also inhibit MCV (part of IBSc Niamh Blythe). I was also involved in a single gene study on mc162, specifically a recombinant of vaccinia virus v354 made by Asif Nizam, which induces class e endosomes in a range of eukaryotic cells, through binding of ESCRT pathway proteins, and possibly inducing EGFR overexpression in tissue studies. This could explain in part the proliferative nature of MC. We have a new collaboration with a HPV group in Novara, Italy, with a raft system who will help by testing the monoclonals in a MCV raft infection model. We will provide lentiviral expression vectors for MCV single gene studies on proliferation and they will provide more virus and sera with clinical data,as

well as test candidate antiviral compounds in clinical studies (ethical permissions have recently been obtained).

In summary as part of my PhD project, I carried out the largest survey yet of serum antibodies directed against the surface virion protein MC084 in European populations. I was able to show for the first time an unexpectedly high seroprevalence of MCV in Europe, and a very early onset in children. I have also produced a number of novel reagents, including monoclonal antibodies specific for MCV virion proteins MC084 and MC133and established a luciferase reporter assay to efficiently evaluate MCV in panels of potential host cells *in vitro*.

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Appendices

Appendix-1- Before cut off

Statistics analysis of German Population

	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
0 - 1 year	66	.131553	.1045174	.105859	.157247
2 - 5 year	52	.299317	.3736997	.195279	.403356
6 - 10 years	47	.278372	.3171693	.185248	.371497
11 - 20 years	72	.206757	.1807952	.164272	.249242
21 - 40 years	52	.218635	.1364325	.180652	.256618
41 - 50 years	119	.482466	.3435129	.420108	.544825
51 - 60 years	62	.498734	.3720875	.404241	.593226
>61 years	52	.556632	.4199750	.354210	.759053
Total	489	.332305	.3241976	.303499	.361111

ANOVA

German titres before cut off

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	10.017	7	1.431	16.676	.000
Within Groups	41.274	481	.086		
Total	51.291	488			

Multiple Comparisons

German titres before cut off
Tukey HSD

(I) German age groups	(J) German age groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 - 1 year	2 - 5 year	-.1677643*	.0543168	.044	-.333122	-.002407
	6 - 10 years	-.1468193	.0559094	.149	-.317025	.023387
	11 - 20 years	-.0752039	.0499192	.804	-.227174	.076766
	21 - 40 years	-.0870816	.0543168	.748	-.252439	.078276
	41 - 50 years	-.3509134*	.0449580	.000	-.487780	-.214047
	51 - 60 years	-.3671808*	.0518088	.000	-.524903	-.209458
	>61 years	-.4250785*	.0762653	.000	-.657255	-.192902

*. The mean difference is significant at the 0.05 level.

Appendix-2- After 0.28 cut off

	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
0 - 1 year	3	.446833	.1417395	.094733	.798934
2 - 5 year	19	.630447	.4541187	.411569	.849326
6 - 10 years	16	.556406	.4200983	.332552	.780261
11 - 20 years	16	.483750	.1850200	.385160	.582340
21 - 40 years	10	.448050	.1189933	.362927	.533173
41 - 50 years	65	.746854	.2419522	.686901	.806807
51 - 60 years	34	.791294	.2428502	.706560	.876029
>61 years	36	.650778	.3058595	.547290	.754266
Total	199	.669947	.3043301	.627404	.712490

ANOVA

German titres at 0.28 cut off

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	2.331	7	.333	3.973	.000
Within Groups	16.007	191	.084		
Total	18.338	198			

Multiple Comparisons

German titres at 0.28 cut off
Tukey HSD

(I) German age groups	(J) German age groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 - 1 year	2 - 5 year	-.1836140	.1798533	.971	-.734836	.367608
	6 - 10 years	-.1095729	.1821380	.999	-.667797	.448652
	11 - 20 years	-.0369167	.1821380	1.000	-.595141	.521308
	21 - 40 years	-.0012167	.1905704	1.000	-.585285	.582852
	41 - 50 years	-.3000205	.1709549	.651	-.823970	.223929
	51 - 60 years	-.3444608	.1743593	.501	-.878845	.189923
	>61 years	-.2039444	.1739662	.939	-.737124	.329235

*. The mean difference is significant at the 0.05 level.

Appendix-3- After 3.6 cut off

	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
0 - 1 year	3	.446833	.1417395	.094733	.798934
2 - 5 year	13	.774962	.4873230	.480475	1.069448
6 - 10 years	11	.670000	.4680104	.355586	.984414
11 - 20 years	9	.565222	.1447487	.453958	.676486
21 - 40 years	7	.493000	.1154913	.386188	.599812
41 - 50 years	61	.774779	.2225712	.717776	.831782
51 - 60 years	32	.821469	.2163133	.743479	.899458
>61 years	23	.834678	.2249314	.737411	.931946
Total	159	.755152	.2795715	.711361	.798942

ANOVA

German titres at 0.36 cut off

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1.485	7	.212	2.950	.006
Within Groups	10.864	151	.072		
Total	12.349	158			

Multiple Comparisons

German titres at 0.36 cut off
Tukey HSD

(I) German age groups	(J) German age groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 - 1 year	2 - 5 year	-.3281282	.1718031	.546	-.856235	.199978
	6 - 10 years	-.2231667	.1747071	.906	-.760199	.313866
	11 - 20 years	-.1183889	.1788184	.998	-.668059	.431282
	21 - 40 years	-.0461667	.1850946	1.000	-.615130	.522796
	41 - 50 years	-.3279454	.1586236	.441	-.815539	.159648
	51 - 60 years	-.3746354	.1619578	.293	-.872478	.123207
	>61 years	-.3878449	.1646514	.271	-.893968	.118278

Appendix-4- Multiple comparisons – Before cut off

Multiple Comparisons						
German titres before cut off Tukey HSD						
(I) German age groups	(J) German age groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 - 1 year	2 - 5 year	-.1677643*	.0543168	.044	-.333122	-.002407
	6 - 10 years	-.1468193	.0559094	.149	-.317025	.023387
	11 - 20 years	-.0752039	.0499192	.804	-.227174	.076766
	21 - 40 years	-.0870816	.0543168	.748	-.252439	.078276
	41 - 50 years	-.3509134*	.0449580	.000	-.487780	-.214047
	51 - 60 years	-.3671808*	.0518088	.000	-.524903	-.209458
	>61 years	-.4250785*	.0762653	.000	-.657255	-.192902
2 - 5 year	0 - 1 year	.1677643*	.0543168	.044	.002407	.333122
	6 - 10 years	.0209450	.0589567	1.000	-.158538	.200428
	11 - 20 years	.0925604	.0533101	.663	-.069733	.254853
	21 - 40 years	.0806827	.0574486	.855	-.094209	.255575
	41 - 50 years	-.1831491*	.0486956	.005	-.331394	-.034904
	51 - 60 years	-.1994166*	.0550835	.008	-.367108	-.031725
	>61 years	-.2573143*	.0785267	.025	-.496375	-.018254
6 - 10 years	0 - 1 year	.1468193	.0559094	.149	-.023387	.317025
	2 - 5 year	-.0209450	.0589567	1.000	-.200428	.158538
	11 - 20 years	.0716154	.0549319	.897	-.095615	.238846
	21 - 40 years	.0597377	.0589567	.972	-.119745	.239221
	41 - 50 years	-.2040940*	.0504659	.002	-.357728	-.050460
	51 - 60 years	-.2203615*	.0566546	.003	-.392836	-.047887
	>61 years	-.2782592*	.0796366	.012	-.520699	-.035820
11 - 20 years	0 - 1 year	.0752039	.0499192	.804	-.076766	.227174
	2 - 5 year	-.0925604	.0533101	.663	-.254853	.069733
	6 - 10 years	-.0716154	.0549319	.897	-.238846	.095615
	21 - 40 years	-.0118777	.0533101	1.000	-.174171	.150415
	41 - 50 years	-.2757094*	.0437364	.000	-.408857	-.142562
	51 - 60 years	-.2919769*	.0507524	.000	-.446484	-.137470
	>61 years	-.3498746*	.0755517	.000	-.579878	-.119871
21 - 40 years	0 - 1 year	.0870816	.0543168	.748	-.078276	.252439
	2 - 5 year	-.0806827	.0574486	.855	-.255575	.094209
	6 - 10 years	-.0597377	.0589567	.972	-.239221	.119745
	11 - 20 years	.0118777	.0533101	1.000	-.150415	.174171
	41 - 50 years	-.2638318*	.0486956	.000	-.412077	-.115587
	51 - 60 years	-.2800993*	.0550835	.000	-.447791	-.112407
	>61 years	-.3379970*	.0785267	.001	-.577057	-.098937
41 - 50 years	0 - 1 year	.3509134*	.0449580	.000	.214047	.48778
	2 - 5 year	.1831491*	.0486956	.005	.034904	.331394
	6 - 10 years	.2040940*	.0504659	.002	.050460	.357728
	11 - 20 years	.2757094*	.0437364	.000	.142562	.408857
	21 - 40 years	.2638318*	.0486956	.000	.115587	.412077
	51 - 60 years	-.0162675	.0458814	1.000	-.155945	.123410
	>61 years	-.0741652	.0723695	.971	-.294481	.146151

51 - 60 years	0 - 1 year	.3671808*	.0518088	.000	.209458	.524903
	2 - 5 year	.1994166*	.0550835	.008	.031725	.367108
	6 - 10 years	.2203615*	.0566546	.003	.047887	.392836
	11 - 20 years	.2919769*	.0507524	.000	.137470	.446484
	21 - 40 years	.2800993*	.0550835	.000	.112407	.447791
	41 - 50 years	.0162675	.0458814	1.000	-.123410	.155945
	>61 years	-.0578977	.0768133	.995	-.291742	.175947
>61 years	0 - 1 year	.4250785*	.0762653	.000	.192902	.657255
	2 - 5 year	.2573143*	.0785267	.025	.018254	.496375
	6 - 10 years	.2782592*	.0796366	.012	.035820	.520699
	11 - 20 years	.3498746*	.0755517	.000	.119871	.579878
	21 - 40 years	.3379970*	.0785267	.001	.098937	.577057
	41 - 50 years	.0741652	.0723695	.971	-.146151	.294481
	51 - 60 years	.0578977	.0768133	.995	-.175947	.291742

*. The mean difference is significant at the 0.05 level.

Appendix-5- Multiple comparisons after 0.28 cut off

Multiple Comparisons						
German titres at 0.28 cut off Tukey HSD						
(I) German age groups	(J) German age groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 - 1 year	2 - 5 year	-.1836140	.1798533	.971	-.734836	.367608
	6 - 10 years	-.1095729	.1821380	.999	-.667797	.448652
	11 - 20 years	-.0369167	.1821380	1.000	-.595141	.521308
	21 - 40 years	-.0012167	.1905704	1.000	-.585285	.582852
	41 - 50 years	-.3000205	.1709549	.651	-.823970	.223929
	51 - 60 years	-.3444608	.1743593	.501	-.878845	.189923
	>61 years	-.2039444	.1739662	.939	-.737124	.329235
2 - 5 year	0 - 1 year	.1836140	.1798533	.971	-.367608	.734836
	6 - 10 years	.0740411	.0982294	.995	-.227017	.375099
	11 - 20 years	.1466974	.0982294	.810	-.154360	.447755
	21 - 40 years	.1823974	.1131010	.742	-.164240	.529034
	41 - 50 years	-.1164065	.0755006	.784	-.347804	.114991
	51 - 60 years	-.1608467	.0829213	.525	-.414988	.093294
	>61 years	-.0203304	.0820914	1.000	-.271928	.231267
6 - 10 years	0 - 1 year	.1095729	.1821380	.999	-.448652	.667797
	2 - 5 year	-.0740411	.0982294	.995	-.375099	.227017
	11 - 20 years	.0726563	.1023527	.997	-.241039	.386351
	21 - 40 years	.1083563	.1167001	.983	-.249311	.466024
	41 - 50 years	-.1904476	.0807924	.269	-.438063	.057168
	51 - 60 years	-.2348879	.0877667	.136	-.503879	.034103
	>61 years	-.0943715	.0869831	.959	-.360961	.172218
11 - 20 years	0 - 1 year	.0369167	.1821380	1.000	-.521308	.595141
	2 - 5 year	-.1466974	.0982294	.810	-.447755	.154360
	6 - 10 years	-.0726563	.1023527	.997	-.386351	.241039
	21 - 40 years	.0357000	.1167001	1.000	-.321967	.393367
	41 - 50 years	-.2631038*	.0807924	.029	-.510720	-.015488
	51 - 60 years	-.3075441*	.0877667	.013	-.576535	-.038553
	>61 years	-.1670278	.0869831	.539	-.433617	.099562
21 - 40 years	0 - 1 year	.0012167	.1905704	1.000	-.582852	.585285
	2 - 5 year	-.1823974	.1131010	.742	-.529034	.164240
	6 - 10 years	-.1083563	.1167001	.983	-.466024	.249311
	11 - 20 years	-.0357000	.1167001	1.000	-.393367	.321967
	41 - 50 years	-.2988038	.0983373	.054	-.600192	.002585
	51 - 60 years	-.3432441*	.1041433	.025	-.662427	-.024061
	>61 years	-.2027278	.1034837	.512	-.519889	.114434
41 - 50 years	0 - 1 year	.3000205	.1709549	.651	-.223929	.823970
	2 - 5 year	.1164065	.0755006	.784	-.114991	.347804
	6 - 10 years	.1904476	.0807924	.269	-.057168	.438063
	11 - 20 years	.2631038*	.0807924	.029	.015488	.510720
	21 - 40 years	.2988038	.0983373	.054	-.002585	.600192
	51 - 60 years	-.0444403	.0612725	.996	-.232231	.143350
	>61 years	.0960761	.0601447	.751	-.088258	.280410
51 - 60 years	0 - 1 year	.3444608	.1743593	.501	-.189923	.878845

	2 - 5 year	.1608467	.0829213	.525	-.093294	.414988
	6 - 10 years	.2348879	.0877667	.136	-.034103	.503879
	11 - 20 years	.3075441*	.0877667	.013	.038553	.576535
	21 - 40 years	.3432441*	.1041433	.025	.024061	.662427
	41 - 50 years	.0444403	.0612725	.996	-.143350	.232231
	>61 years	.1405163	.0692313	.465	-.071667	.352700
>61 years	0 - 1 year	.2039444	.1739662	.939	-.329235	.737124
	2 - 5 year	.0203304	.0820914	1.000	-.231267	.271928
	6 - 10 years	.0943715	.0869831	.959	-.172218	.360961
	11 - 20 years	.1670278	.0869831	.539	-.099562	.433617
	21 - 40 years	.2027278	.1034837	.512	-.114434	.519889
	41 - 50 years	-.0960761	.0601447	.751	-.280410	.088258
	51 - 60 years	-.1405163	.0692313	.465	-.352700	.071667
*. The mean difference is significant at the 0.05 level.						

Appendix-6- Multiple comparisons – After 0.36 cut off

Multiple Comparisons						
German titres at 0.36 cut off Tukey HSD						
(I) German age groups	(J) German age groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 - 1 year	2 - 5 year	-.3281282	.1718031	.546	-.856235	.199978
	6 - 10 years	-.2231667	.1747071	.906	-.760199	.313866
	11 - 20 years	-.1183889	.1788184	.998	-.668059	.431282
	21 - 40 years	-.0461667	.1850946	1.000	-.615130	.522796
	41 - 50 years	-.3279454	.1586236	.441	-.815539	.159648
	51 - 60 years	-.3746354	.1619578	.293	-.872478	.123207
	>61 years	-.3878449	.1646514	.271	-.893968	.118278
2 - 5 year	0 - 1 year	.3281282	.1718031	.546	-.199978	.856235
	6 - 10 years	.1049615	.1098857	.980	-.232816	.442739
	11 - 20 years	.2097393	.1163113	.619	-.147790	.567269
	21 - 40 years	.2819615	.1257470	.333	-.104573	.668496
	41 - 50 years	.0001828	.0819375	1.000	-.251685	.252051
	51 - 60 years	-.0465072	.0882192	.999	-.317684	.224670
	>61 years	-.0597167	.0930720	.998	-.345811	.226378
6 - 10 years	0 - 1 year	.2231667	.1747071	.906	-.313866	.760199
	2 - 5 year	-.1049615	.1098857	.980	-.442739	.232816
	11 - 20 years	.1047778	.1205593	.988	-.265810	.475366
	21 - 40 years	.1770000	.1296864	.872	-.221643	.575643
	41 - 50 years	-.1047787	.0878635	.933	-.374863	.165305
	51 - 60 years	-.1514688	.0937489	.740	-.439644	.136706
	>61 years	-.1646783	.0983292	.703	-.466933	.137576
11 - 20 years	0 - 1 year	.1183889	.1788184	.998	-.431282	.668059
	2 - 5 year	-.2097393	.1163113	.619	-.567269	.147790
	6 - 10 years	-.1047778	.1205593	.988	-.475366	.265810
	21 - 40 years	.0722222	.1351740	.999	-.343290	.487734
	41 - 50 years	-.2095565	.0957781	.365	-.503969	.084856
	51 - 60 years	-.2562465	.1012043	.190	-.567339	.054846
	>61 years	-.2694560	.1054613	.181	-.593634	.054722
21 - 40 years	0 - 1 year	.0461667	.1850946	1.000	-.522796	.615130
	2 - 5 year	-.2819615	.1257470	.333	-.668496	.104573
	6 - 10 years	-.1770000	.1296864	.872	-.575643	.221643
	11 - 20 years	-.0722222	.1351740	.999	-.487734	.343290
	41 - 50 years	-.2817787	.1070395	.153	-.610808	.047250
	51 - 60 years	-.3284687	.1119210	.073	-.672503	.015566
	>61 years	-.3416783	.1157847	.070	-.697589	.014233
41 - 50 years	0 - 1 year	.3279454	.1586236	.441	-.159648	.815539
	2 - 5 year	-.0001828	.0819375	1.000	-.252051	.251685
	6 - 10 years	.1047787	.0878635	.933	-.165305	.374863
	11 - 20 years	.2095565	.0957781	.365	-.084856	.503969
	21 - 40 years	.2817787	.1070395	.153	-.047250	.610808
	51 - 60 years	-.0466901	.0585470	.993	-.226658	.133278
	>61 years	-.0598996	.0656318	.984	-.261645	.141846

51 - 60 years	0 - 1 year	.3746354	.1619578	.293	-.123207	.872478
	2 - 5 year	.0465072	.0882192	.999	-.224670	.317684
	6 - 10 years	.1514688	.0937489	.740	-.136706	.439644
	11 - 20 years	.2562465	.1012043	.190	-.054846	.567339
	21 - 40 years	.3284687	.1119210	.073	-.015566	.672503
	41 - 50 years	.0466901	.0585470	.993	-.133278	.226658
	>61 years	-.0132095	.0733240	1.000	-.238600	.212181
>61 years	0 - 1 year	.3878449	.1646514	.271	-.118278	.893968
	2 - 5 year	.0597167	.0930720	.998	-.226378	.345811
	6 - 10 years	.1646783	.0983292	.703	-.137576	.466933
	11 - 20 years	.2694560	.1054613	.181	-.054722	.593634
	21 - 40 years	.3416783	.1157847	.070	-.014233	.697589
	41 - 50 years	.0598996	.0656318	.984	-.141846	.261645
	51 - 60 years	.0132095	.0733240	1.000	-.212181	.238600

Appendix-7- Statistical analysis of UK population grouped on basis of disease

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
PPMS	49	.246167	.2292621	.0764207	.069940	.422393
RRMS	50	.278703	.2466215	.0405443	.196475	.360930
Healthy	50	.414303	.4307495	.0749839	.261566	.567040
Total	149	.331639	.3385956	.0380950	.255798	.407481

ANOVA

UK Population

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.395	2	.197	1.756	.180
Within Groups	8.548	76	.112		
Total	8.942	78			

Multiple Comparisons

UK Population

Tukey HSD

(I) Age Group	(J) Age Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PPMS	RRMS	-.0325360	.1246437	.963	-.330494	.265422
	Healthy	-.1681364	.1261130	.381	-.469607	.133334
RRMS	PPMS	.0325360	.1246437	.963	-.265422	.330494
	Healthy	-.1356003	.0802980	.216	-.327551	.056350
Healthy	PPMS	.1681364	.1261130	.381	-.133334	.469607
	RRMS	.1356003	.0802980	.216	-.056350	.327551

Appendix-8 - Statistical analysis of UK population grouped on basis of age – Independent t-test

	UK age groups before cut off	N	Mean	Std. Deviation
UK titre before cut off	20 - 40 years	79	.331639	.3385956
	>40 years	70	.239207	.1803878

Since these are only two groups we performed independent t test for two samples with unequal variance. The compute test statistic or t value = 2.1076 at 147 degrees of freedom, p value = 0.03 which is less than level of significance of 0.05. Hence statistically significant difference exists in the titres in the age groups 20 – 40 and more than 40 years of age group.

Similarly for the UK population at cut off 0.28

T value = 3.03 at 34 degrees of freedom and p value = 0.004

And for UK population at cut off 0.36

T value = 4.069 at 24 degrees of freedom and p value = .0004

List of publications and presentations

Publication

1. **Subuhi Sherwani**, Laura Farleigh, Nidhi Agarwal, Sam Loveless, Neil Robertson, Paul Schnitzler and Joachim Jakob Bugert (**2014**). Seroprevalence of Molluscum contagiosum virus in German and UK populations. *PLOS ONE* (In print; PONE-D-13-44356).

2. **Subuhi Sherwani,** Niamh Blythe, Laura Farleigh and Joachim J Bugert (**2012**). New method for the assessment of Molluscum contagiosum virus infectivity. In: Stuart N. Isaacs, (ed. 2nd): Vaccinia Virus and Poxvirology: Methods and Protocols, Methods in Molecular Biology, Springer**,** Vol. 890: 135-146.

Conferences-Oral Presentation

1. **Subuhi Sherwani,** Nidhi Agarwal, Laura Farleigh, Sam Loveless, Neil Robertson, Paul Schnitzler and Joachim J. Bugert (25-28th March **2013**) Seroepidemiology of Molluscum contagiosum virus in representative German and UK populations, SGM Spring Conference 2013, Manchester, UK.

2. **Subuhi Sherwani,** Nidhi Agarwal, Laura Farleigh, Sam Loveless, Neil Robertson, Paul Schnitzler and Joachim J Bugert (26th February **2013**) Molluscum contagiosum virus (MCV) lifecycle and immunity: Developing tools for MCV research. Infection and Immunity Seminar, Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK.

Conferences- Poster Presentation

1. **Subuhi Sherwani,** Nidhi Agarwal, Laura Farleigh, Paul Schnitzler and Joachim J Bugert (6-9th March **2013**). Seroepidemiology of Molluscum contagiosum virus in a representative German population, 23rd Annual Meeting of the Society of Virology-Kiel, Germany.

2. **Subuhi Sherwani**, Niamh Blythe, Laura Farleigh, Amanda Tonks and Joachim Bugert (21th Sept. **2012**) Novel method for assessment of entry of Molluscum contagiosum virus into host cells and subsequent innate immune responses, MITReG: Interdisciplinary Postgraduate Research Day, Viriamu Jones Gallery, Cardiff University, UK.

3. **Subuhi Sherwani**, Niamh Blythe, Laura Farleigh, Amanda Tonks and Joachim Bugert (12th Sept. **2012**). Novel method for assessment of entry of Molluscum contagiosum virus into host cells and subsequent innate immune responses, Cardiff Institute of Infection and Immunity Annual Meeting 2012, Cardiff University, UK.

4. **Subuhi Sherwani**, Asif Nizam, Laura Farleigh and Joachim J Bugert (11th Nov. **2011**). Overexpression of Molluscum contagiosum virus proteins for functional studies and to establish burden of disease, 26th Annual Postgraduate Research Day, Graduate School of Biomedical and Life Sciences 2011, Cardiff University, UK.

5. Niamh Blythe, **Subuhi Sherwani**, Laura Farleigh, Asif Nizam, Amanda Tonks and Joachim J Bugert (11-14th April **2011**). Novel method for assessment of entry of Molluscum Contagiosum Virus into host cells and subsequent innate immune responses, SGM Spring Conference 2011, Harrogate, UK.

Chapter 8

New Method for the Assessment of Molluscum Contagiosum Virus Infectivity

Subuhi Sherwani, Niamh Blythe, Laura Farleigh, and Joachim J. Bugert

Abstract

Molluscum contagiosum virus (MCV), a poxvirus pathogenic for humans, replicates well in human skin in vivo, but not in vitro in standard monolayer cell cultures. In order to determine the nature of the replication deficiency in vitro, the MCV infection process in standard culture has to be studied step by step. The method described in this chapter uses luciferase and GFP reporter constructs to measure poxviral mRNA transcription activity in cells in standard culture infected with known quantities of MCV or vaccinia virus. Briefly, MCV isolated from human tissue specimen is quantitated by PCR and used to infect human HEK293 cells, selected for ease of transfection. The cells are subsequently transfected with a reporter plasmid encoding firefly luciferase gene under the control of a synthetic early/late poxviral promoter and a control plasmid encoding a renilla luciferase reporter under the control of a eukaryotic promoter. After 16 h, cells are harvested and tested for expression of luciferase. MCV genome units are quantitated by PCR targeting a genome area conserved between MCV and vaccinia virus. Using a GFP reporter plasmid, this method can be further used to infect a series of epithelial and fibroblast-type cell lines of human and animal origin to microscopically visualize MCV-infected cells, to assess late promoter activation, and, using these parameters, to optimize MCV infectivity and gene expression in more complex eukaryotic cell culture models.

Key words: Molluscum contagiosum virus, Luciferase reporter construct, Eukaryotic cells, Infection, Transfection, Quantitative PCR

1. Introduction

Molluscum contagiosum virus (MCV) does not produce a quantifiable cytopathic effect and does not produce viral progeny in infected standard cell cultures. But small amounts of viral mRNA and protein expression can be detected indicating that MCV virions are transcriptionally active (1–3). Many investigators have observed

Stuart N. Isaacs (ed.), *Vaccinia Virus and Poxvirology: Methods and Protocols*, Methods in Molecular Biology, vol. 890,
DOI 10.1007/978-1-61779-876-4_8,

that poxvirus transcription complexes can drive luciferase reporters under the control of poxviral promoters in plasmids in poxvirus-infected cells. A recent paper uses this as a method to diagnose orthopoxvirus infections (4).

In the assay described in this chapter, the same principle is used. We introduce a luciferase reporter expression in trans as a new surrogate marker of infectivity and gene expression for MCV. To compare the infectivity of MCV with other poxviruses (11), the number of virions must be determined. Quantitation by EM or OD300 can be used (5), but requires relatively large amounts of gradient purified virons. However, currently, MCV can only be isolated from clinical specimens and thus is difficult to obtain amounts sufficient for gradient purification (5). PCR is an alternative method of quantitation of smaller amounts of poxviruses from clinical specimens, which is both reliable and highly specific for individual poxviruses. The method described in this chapter uses a novel PCR target in an area with significant DNA homology (~65%) between MCV and vaccinia virus strain WR (VACV-WR). The MCV gene is mc129R, which is homologous to the VAVWR144 (also called A24R gene encoding RPO132, the large subunit of the DNA-dependent RNA polymerase). The method provides a means to quantitate poxviral genome units in the same virus preparations used to compare transcriptional activity and infectivity of MCV and VACV-WR.

2. Materials

2.1. MCV Luciferase Reporter Assay

2.1.1. Infection–Transfection of Cell Cultures

1. OPTIMEM, stored at 4°C.
2. Plasmids described in Subheading 2.3 (see Note 1).
3. Lipofectamine 2000, stored at 4°C until used.
4. Human HEK 293 cells (ATCC CRL1573) (see Note 2).
5. Dulbecco's modified Eagle medium: DMEM, high glucose with glutamine, stored at 4°C until used.
6. Fetal calf serum: FCS, stored in aliquots at –70°C until used.
7. Cell growth medium: DMEM with 10% FCS.

2.1.2. Luciferase Assay (The Dual-Luciferase® Reporter Assay System from Promega)

1. Dual Luciferase Assay Substrate (lyophilized) stored at –20°C for up to 6 months reconstituted.
2. 10 ml Luciferase Assay Buffer II, stored in a 1-ml aliquots at –20°C for up to 6 months until used.
3. Stop and Glo Substrate (50×) stored at –20°C.

4. 10 ml of Stop & Glo Buffer, stored in a 1-ml aliquots at –20°C for up to 6 months until used.
5. 30 ml of Passive Lysis Buffer (5×) stored at –20°C, then diluted to 1× using sterile water, and kept at 4°C until used.
6. Clear film plate protectors (to prevent evaporation from wells).
7. FLUOStar Luminometer.

2.2. MCV and VACV Quantitative PCR Assay

1. Primers outlined in Table 1 suspended in injection-grade water to a final concentration of 100 pmol/μl and stored at –20°C.
2. MCV isolated from human skin biopsy material as described previously (5) and kept in 100-μl aliquots frozen at –70°C in PBS (see Note 3).
3. VACV-WR, vaccinia virus, strain WR (kind gift of B. Moss) was prepared and purified from infected HeLa cells, titrated in BSC-1 cells, and kept in 100-μl aliquots frozen at –70°C in PBS (see Note 3).
4. DNAse at 1 mg/ml.
5. DNAse/BamHI buffer: 78 μl water, 2 μl DNAse, 20 μl 10× BamHI buffer from New England Biolabs.
6. High Pure viral nucleic acid (HPVNA) kit (e.g., Roche).
7. Nanodrop-Spectrophotometer.

Table 1
MCV–VACV quantitative PCR assay primers

Primer ID	Primer sequence (nhb)	Primer length	Product size (nucleotide position), GenBank Acc. #
Mcv129 1-2F149275	5′-CCGCACTACTCCTGGATGCAGAA-3′	23	576 bp (149,275–149,850), U60315
Mcv129 1-3R149850	5′-CTGGATGTCGGAGAAGGTCATG-3′	22	
VACV-WR 1-2F132482	5′-CCTCACTATTCATGGATGCAGAA-3′ (3)	23	573 bp (132,482–122,054), AY243312
VACV-WR 1-3R133054	5′-CTGAATGTCAGAGAATGTCATG-3′ (3)	22	

nhb nonhomologous bases underlined (number of mismatches)
Primers were designed using BLAST2 (NCBI: http://blast.ncbi.nlm.nih.gov/) alignment of MCV (GenBank accession # U60315) and VACV-WR (GenBank accession # AY243312) genome sequences and Vector NTI vs. 4.0, 1994–1996 InforMax Inc.

8. ImageJ (Wayne Rasband (wayne@codon.nih.gov) Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA).
9. Injection-grade water.
10. AmpliTaq 360 polymerase (5 U/μl).
11. dNTP (0.2 mM).
12. 10× PCR buffer.
13. 2% Agarose gel.
14. Ethidium bromide solution: 10 mg/ml stock solution in demineralized water and used at 20 μl per 200 ml.

2.3. Plasmids (See Fig. 1)

1. *PCR control plasmid*. The complete MCV-1 genome was cloned (6) and sequenced (7, 8) and the redundant MCV genome fragment library of MCV type 1 was submitted to

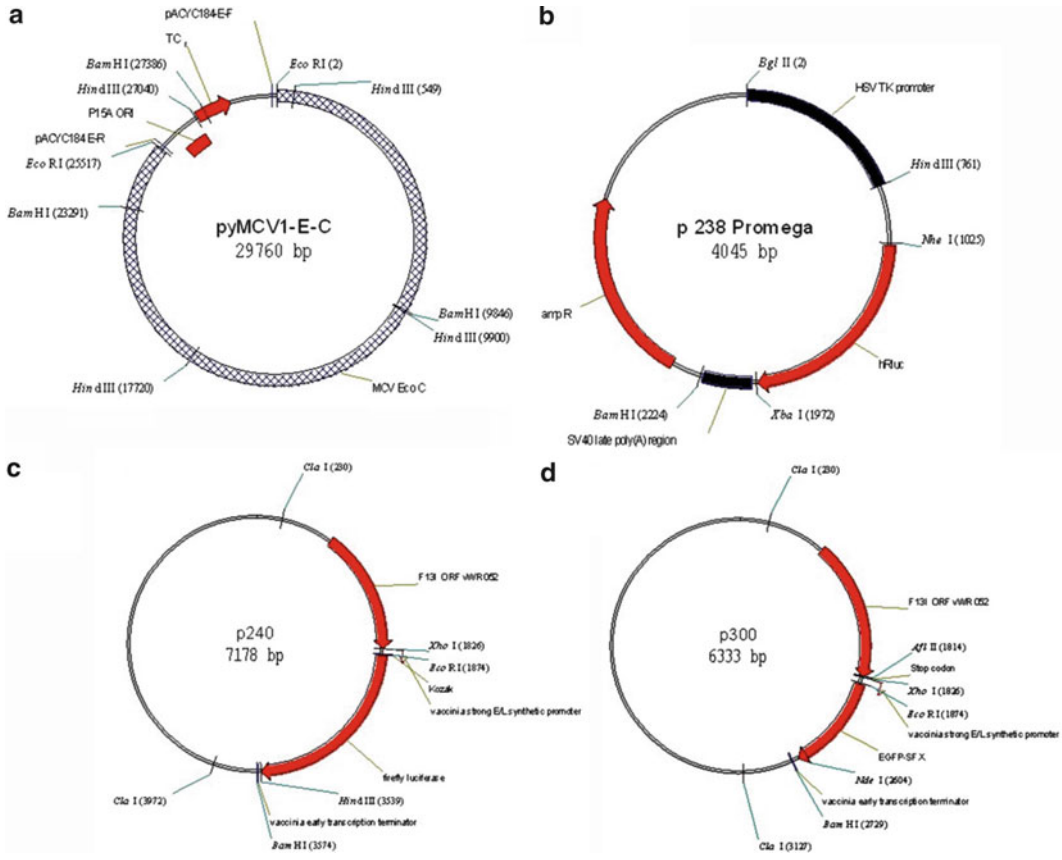


Fig. 1. Plasmid constructs. VectorNTI drawings for the recombinant plasmids (**a**) pyMCV1-EcoRI-fragment C (pyMCV1-E-C; available from ATCC molecular section), (**b**) phRG-TK (Promega; Internal lab reference number p238), (**c**) pRB21-pE/L-FF luciferase (p240), and (**d**) pRB21-pE/L-EGFP-SFX (p300).

ATCC for safekeeping in 2003 and 2008. For the quantitative PCR assay, the genomic MCV-1 *Eco*RI fragment C (25,516 bp) cloned into bacterial plasmid vectorp pACYC184 was used as a MCV target control (pyMCV1-E-C, see Fig. 1a).

2. *Transfection control plasmid*. Plasmid phRG-TK (Promega GenBank accession number AF362545: 4,045 bp), expressing renilla luciferase under the control of the herpes simplex virus TK gene promoter. In this protocol, this plasmid is called p238 and used as plasmid transfection control (p238 Promega; see Fig. 1b).
3. *Poxviral luciferase reporter plasmid*. The coding sequence of firefly luciferase (*Photinus pyralis* GenBank accession number M15077) was amplified with a modified Kozak sequence by PCR and ligated into the pRB21 donor plasmid (kind gift of B. Moss (9, 10)) using the EcoRI and HindIII restriction sites in the donor plasmid multiple cloning site, resulting in the pRB21-E-Koz-Fireflyluciferase-H(alsocalledpRB21-pE/L-FF luciferase) construct of 7,178 bp with the internal lab designation p240 (p240, see Fig. 1c).
4. *Poxviral EGFP reporter plasmid*. The coding sequence of EGFP was amplified from a commercially available plasmid with a modified Kozak sequence by PCR and ligated into the pRB21 donor plasmid using the EcoRI and NheI restriction sites in the donor plasmid multiple cloning site, resulting in the pRB21-E-Koz-EGFP-X-flag-strepII-N construct (also called pRB21-pE/L-EGFP-SFX) of 6,333 bp with the internal lab designation p300 (p300, see Fig. 1d).

3. Methods

3.1. Infection–Transfection: Luciferase Assay

3.1.1. Infection/Transfection

1. Prepare enough 12-well plates containing HEK 293 cells in growth media to allow for infection/transfection in triplicate for each experimental condition (including a mock that will be transfected but not infected, as well as wells that will be harvested at 16 h and wells that will be continued to be incubated for days) (see Note 4).
2. Thaw virus aliquots, sonicate, and keep on ice.
3. Thaw plasmid DNA and keep on ice.
4. Bring OptiMEM and Lipofectamine 2000 to room temperature (RT).
5. Prepare transfection mixes by adding a dilution of 2 µl of Lipofectamine 2000 in 50 µl of OptiMEM to a dilution of 0.3 µg of each plasmid DNA (p240 FF reporter and p238

transfection control plasmid, p300 EGFP reporter) in 50 µl of OptiMEM. Mix gently for 15 min at RT in the dark to allow formation of transfection complexes.

6. Remove growth media from HEK293 cells and put 100 µl of transfection mix in each well.
7. Combine 100 µl each of ice-cold virus in PBS and 100 µl of transfection mix at RT and transfer the mixture into appropriate wells of HEK293 cells (see Note 5).
8. Incubate for 16 h at 37°C in 5% CO_2 atmosphere (see Note 6).

3.1.2. Microscopy and Collection of Cells for Luciferase Assay

1. At 16 h post infection (p.i.), inspect cells transfected with the GFP reporter plasmid using live cell microscopy. Document GFP-positive cells noting that MCV does not show GFP-positive cells after 16 h, whereas WR shows multiple GFP-positive cells.
2. Upon further incubation for another 4 days (5 days p.i.), some individual cells in the MCV-infected wells will show medium to strong GFP signals (see Note 7). At the same time point, the WR-infected wells will show extensive plaques and cell degradation (see Fig. 2a–d).
3. For luciferase assay, at 16 h p.i., wash adherent cells in wells once with PBS and add 100 µl of 1× passive lysis buffer to each well (see Note 8).

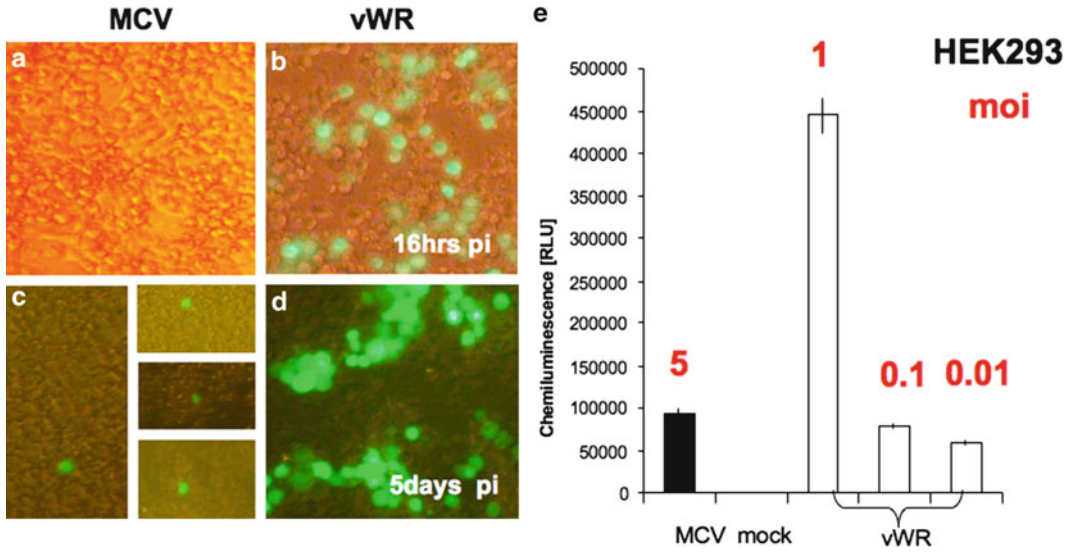


Fig. 2. Images of luciferase and GFP in infected/transfected cells and quantification of luciferase output. *Panels* (**a**–**d**) show HEK 293 cells infected with MCV (**a** and **c**), and vWR (**b** and **d**). Inserts in **c** show individual GFP-positive cells. *Panel* **e** shows a histogram of luciferase data giving chemiluminescence in RLU. HEK 293 cells were infected with MCV or vWR at the indicated moi, and collected at 16 h p.i.

4. Cover the 12-well plate with clear film plate protectors to stop evaporation and incubate with agitation on a belly-dancer at RT for 15 min. Plates are then frozen at −20°C for at least 15 min or stored overnight or for up to 2 weeks before assayed.
5. Cell lysates are tested for luciferase activity by adding 100 µl Dual Luciferase Assay Substrate to each well (see Note 9).
6. Luciferase activity is then measured in a FLUOStar Luminometer.
7. Data is compiled in a Microsoft EXCEL file and evaluated using standard statistical protocols (average, standard deviation, Student's *P* test). A typical result is shown in Fig. 2e (see Notes 6, 10, and 11).

3.2. Quantitative PCR Assay

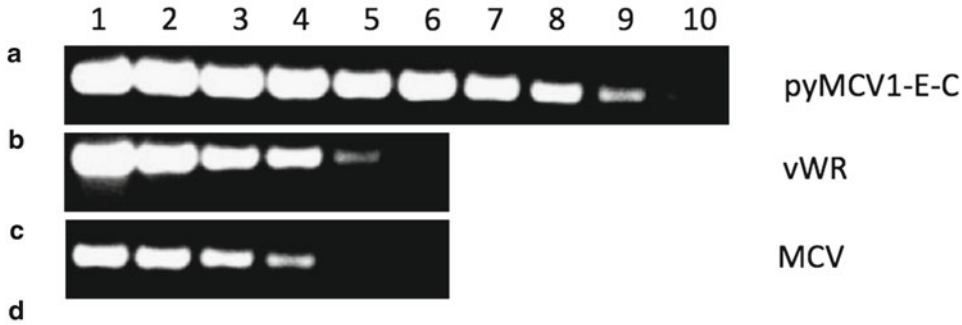
1. Incubate equal volumes of freshly thawed virions (100-µl aliquot) in 100 µl DNAse/BamHI buffer for 30 min at 37°C.

3.2.1. Virus and DNA Preparation

2. Extract viral genomic DNA using a HPVNA kit following the manufacturer's instructions. The control plasmid pyMCV1-E-C (see Fig. 1a) is prepared using the same procedure.
3. Determine the DNA concentration of the control plasmid using a Nanodrop-Spectrophotometer or a similar device.
4. Calculate molecule numbers using the average molecular weight of DNA molecules and Avogadro's number (6.02×10^{23} per mole). The molecular weight of a plasmid (in Daltons) can be estimated as MW of a double-stranded DNA molecule (http://www.epibio.com/techapp.asp) = (# of base pairs) × (650 Da/base pair). The plasmid pyMCV1-E-C has 29,760 bp. Thus, the molecular weight is calculated as 19.344 MDa and thus 19.344 ng of plasmid would be 6.02×10^8 mol. The actual plasmid concentration was 21 ng/µl (±1.7) and, thus, represented 6.5×10^8 mol/µl. From this value, the molecule numbers for the pyMCV1-E-C twofold dilution series are calculated (see Fig. 3d). The molecule numbers are then correlated to the pixel numbers of bands on a gel quantitated by ImageJ (see Fig. 3d).

3.2.2. PCR Reaction (see Note 12)

1. Prepare twofold dilutions of viral genomic DNA and plasmid control in injection-grade water and store at −20°C.
2. Prepare PCR assays as outlined in Table 2. PCR reaction conditions are included in the table.
3. Visualize PCR bands by loading a 2% agarose gel with 10 µl from each PCR reaction and run for 1 h at 100 V (constant voltage). Stain with ethidium bromide, photograph with a digital unit, and export into a jpeg file (see Note 13).
4. To quantitate the PCR product, one can use the captured bands on the jpeg photograph with a series of identical gates



d

lane# (gel a)	1	2	3	4	5	6	7	8	9	data
DNA										
pyMCV	406,250	203,125	101,562	50,781	25,390	12,695	6,347	**3,173**	**1,586**	mol
pyMCV	138,794	159,136	140,851	129,871	107,126	112,165	89,590	67,495	27,779	pixel
lane# (gel b)					1	2	3	4	5	
vWR					156,659	126,644	87,696	71,008	15,102	pixel
lane# (gel c)						1	2	3	4	
MCV						77,875	73,384	55,270	28,726	pixel

Fig. 3. PCR quantification of purified DNA. *Panels* **a**–**c** show twofold dilutions of poxviral genomic DNA purified from DNAse-treated virions (**b** and **c**) and plasmid DNA repurified using the HPVNA kit (**a**). Lane numbers at the top of the figure refer to $\log_2$ dilutions from 1 to 10. *Panel* **d** tabulates the calculated molecular number for the reference plasmid pyMCV1-E-C (pyMC) (1,000× by nanodrop in 10 μl of original DNA prep) and the ImageJ pixels for each band in gels **a** to **c**. ImageJ (Wayne Rasband (wayne@codon.nih.gov) Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) was used to quantitate pixel densities in boxes of 212 × 42 pixels.

Table 2
PCR reaction

	Volume (μl)
Primer 1-2F[a] (100 pmol/μl)	0.5
Primer 1-3R[a] (100 pmol/μl)	0.5
Injection-grade water	36.8
10× PCR buffer	5.0
TaKaRa dNTP (0.2 mM)	2.0
Template (series of twofold dilutions)	5.0
AmpliTaq 360 polymerase (1 unit of 5 U/μl)	0.2
Total	50.0

PCR reaction: 2 min of denaturation at 96°C; and then 45 cycles of 1 min at 96°C, 2 min at 55°C, and 3 min at 72°C. Block and then cool to 10°C

[a]For MCV Primer 1-2F and Primer 1-3R, use Mcv129 1-2F149275 and Mcv129 1-3R149850, respectively. For WR Primer 1-2F and Primer 1-3R, use VACV-WR 1-2F132482 and VACV-WR 1-3R133054, respectively

using IMAGEJ software to produce a quantified pixel output that can be imported into a Microsoft EXCEL file.

5. Plot quantitative results of digital imaging (quantified pixel output) of a twofold dilution series of plasmid pyMCV1-E-C against molecule numbers using Microsoft EXCEL software.
6. Take molecule/genome equivalent numbers from the calibration plot and compare to the pixel readings obtained for VACV-WR. Tabulate results and evaluate using standard statistical protocols (average, standard deviation, Student's *P* test).
7. Results from a PCR reaction and the corresponding mole numbers and pixels are shown in Fig. 3d. In that figure, for gel a, lane 9, the signal (27,779 pixels) for the dilution of the MCV control plasmid (pyMCV1-E-C) correlates to 1.586×10^6 plasmid units. The band in gel c (MCV PCR) with comparable pixel density (i.e., within 10%) is in lane 4 with a pixel value of 28,726 pixels. If the 1.586×10^6 mol are multiplied by the dilution factor (16×), the MCV aliquot of 100 µl used for genomic DNA preparation contained 2.5×10^7 mol/genome units. If the pixel values obtained for vWR in gel b, lane 4 (71,008), are used in the same way and related to gel a, lane 8 (67,495 pixels), after multiplying by the dilution factor (16×), a molecule number of 5×10^7 is obtained. This can then be used to relate to the pfu. If WR was at 1.6×10^6 pfu in 100 µl, the pfu-to-genome unit ratio is 1:31 (see Notes 14–16).

4. Notes

1. All plasmid DNA should be purified using 100-µg capacity midiprep-columns (HPVNA) and then stored in elution buffer at –20°C until used.
2. The assay depends to a significant degree on the transfectability of the cell cultures involved. Human keratinocytes and fibroblast cell lines are most interesting as possible natural hosts for MCV, but they are also hard to transfect. We found HEK 293 cells to be the best transfected cell line. However, while this cell line shows robust reporter signals, it is clearly not the type of cell MCV naturally infects.
3. We prepare vaccinia virus and MCV preparations in 1 ml PBS and then immediately make ten 100-µl aliquots and freeze. The vaccinia virus stock was generated by infecting one T150 flask containing adherent HeLa cells. Harvest of the infected cells and resuspending them in 1 ml PBS yielded a titer of 2×10^7 pfu/ml. Thus, each of the ten 100-µl aliquots of vaccinia virus used here contained 2×10^6 pfu. The 100-µl aliquot MCV

contained an unknown number of MCV particles, but is quantified using the described PCR method.

4. Transfection efficiency can vary considerably from cell batch to cell batch. Passage number (best low), cell confluence (best below 60%), and time of culture prior to experiment (best no longer than 24 h) are determinant factors.
5. While in this protocol we transfect adherent cells, we have found that for some harder-to-transfect cells (e.g., human fibroblasts) we can get higher transfection efficiency when cells are in suspension.
6. The incubation time of 16 h allows for a robust signal from the transfection control plasmid p238, so firefly signal readings can be adjusted to renilla transfection efficiency readings between experiments.
7. Transfected plasmids with poxviral transcription signals can be transcribed by the poxviral transcription complex produced by transcriptionally active cores after entry. It is not clear whether the transcription complex is accessed inside partially uncoated virions, with plasmid DNA getting inside cores, or by transcription complex that is released into the cytoplasm. MCV-infected cells produce a robust luciferase signal after 16 h. However, GFP is only visibly expressed in a small number of individual cells, detectable after 5 days of incubation. Potentially, other cells may express GFP at a level undetectable by microscopy. The process where cores are accessible for the reporter plasmids may be delayed in MCV-infected cells.
8. The samples for the luciferase assay are collected at 16 h post infection. This allows the control plasmid ILR#238 to get to the nucleus and be expressed to yield a robust control signal. In 293 cells, the Renilla luciferase signal can be seen in vaccinia-infected cells after 2 h, and is seen in the MCV signal after 8 h.
9. If one does not have instrumentation that can add 100 μl of PROMEGA Dual luciferase firefly substrate one can hand, pipet series of four samples in a row, then load the plate, and read. The reading time, including the initial shake for four samples, is 20 s. Doing this results in a signal loss per reading of <1%, which is less than the sample-to-sample variation in triplicate samples, when compared to machine pipetting sample per sample.
10. The signal from early poxviral promoters can be used as a surrogate parameter of viral infectivity.
11. The signal can be further dissected and used to look at early and late transcription activity using transfected plasmids with a reporter gene behind the respective promoters in isolation.

12. The conventional PCR assay described has the problem of assay-to-assay variation due to agarose gel and staining artifacts. Future advances may be the development of a real-time PCR assay using molecular Taqman probes specific to either VAVC-WR or MCV and binding in the internal section of the rather large PCR product (550 bp).
13. The source template of the PCR products produced can also be determined by XhoI digest, which cleaves the MCV product into 227- and 349-bp subfragments but does not cleave the VAVC-WR PCR product.
14. It is unclear to which extent the different GC content of the two virus genomes would affect the PCR product. This was not further investigated.
15. It is clear that vaccinia plaque-forming units cannot be directly compared to MCV virion units because of the different nature of their biological activity. However, the PCR method described in Subheading 3.2 allows a relative quantification of MCV genome equivalents to VAVC-WR infectious units measured in pfu/ml based on amplifiable genomic DNA units/molecule numbers calculated for a relatively large plasmid containing 25,517 bp of MCV sequence. As described in Subheading 3.2.2, **step 7**, the pfu-to-molecule ratio for vaccinia virus (mature virions) comes out as 1:31, in keeping with previously published ratios (12). The PCR data can be used to calculate an MCV multiplicity of infection equivalents in relation to the control plasmid molecule numbers as well as in the form of pfu equivalents in relation to a titered vaccinia stock for comparison purposes. We have found this approach to be both more reproducible and more specific than electron microscopy or OD quantifications of virions.
16. The biological activity of virions can be assessed using an in vitro transcription reaction (1, 5, 13).

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PLOS ONE

Seroprevalence of Molluscum contagiosum virus in German and UK populations

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Seroprevalence of Molluscum contagiosum virus in German and UK populations
Short Title:	Molluscum contagiosum virus burden of disease
Corresponding Author:	Joachim Bugert Cardiff University School of Medicine UNITED STATES
Keywords:	Molluscum contagiosum; viral skin infection; paediatric infection; seroprevalence study; general population; immunesuppression; ELISA; MC084 antigen
Abstract:	Molluscum contagiosum virus (MCV) is a significant but underreported skin pathogen for children and adults. Seroprevalence studies can help establish burden of disease. Enzyme linked immunosorbent assay (ELISA) based studies have been published for Australian and Japanese populations [1-2], and the results indicate seroprevalences between 6 and 22 percent in healthy individuals, respectively. To investigate seroprevalence in Europe, we have developed a recombinant ELISA using a truncated MCV virion surface protein MC084 (V123-R230) expressed in E. coli. The ELISA was found to be sensitive and specific, with low inter- and intra-assay variability. Sera from 289 German adults and children aged 0-40 years (median age 21years) were analysed for antibodies against MC084 by direct binding ELISA. The overall seropositivity rate was found to be 14*8%. The seropositivity rate was low in children below the age of one (4*5%), peaked in children aged 2-10 years (25%), and fell again in older populations (11-40 years; 12*5%). Ten out of 33 healthy UK individuals (22%; median age 27 years) had detectable MC084 antibodies. MCV seroconversion was more common in dermatological and autoimmune disorders, than in immunocompromised patients or in patients with multiple sclerosis. Overall MCV seroprevalence is 2.1 fold higher in females than in males in a UK serum collection. German seroprevalences determined in the MC084 ELISA (14*8%) are at least three times higher than incidence of MC in a comparable Swiss population (4.9%; [3]). While results are not strictly comparable, this is lower than Australian seroprevalence in a virion based ELISA (n=357; 23%; 1999) [1], but higher than the seroprevalence reported in a Japanese study using an N-terminal truncation of MC133 (n=108, 6%; 2000) [2]. We report the first large scale serological survey of MC in Europe (n=393) and the first MCV ELISA based on viral antigen expressed in E. coli.
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Ethics Statement All research involving human participants must have been approved by the authors' institutional review board or equivalent committee(s) and that board must be named by the authors in the manuscript. For research involving human participants, informed consent must have been obtained (or the reason for lack of consent explained, e.g. the data were analyzed anonymously) and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should submit a statement from their ethics committee or institutional review board indicating the approval of the	Ethics statementThe study has ethical approval for the use of German tissues and sera (Ethikvotum S-091/2011 Hautklinik Heidelberg. Ethical approval was given by the Heidelberg University board in charge of ethical approvals, the ‘Ethikkommission’. Ethical approval for UK samples was part of 'An Epidemiological study of Multiple sclerosis and other neuroinflammatory demyelinating disorders in South Wales', 05/WSE03/111. Ethical approval was given by the Cardiff University 'Biobank Ethics Board'. All patients provided prospective informed consent in writing upon admission. All children’s' parents/guardians provided informed consent in writing. Class 2 GM work was notified to HSE with the project number GM 130/10.3.

research. We also encourage authors to submit a sample of a patient consent form and may require submission of completed forms on particular occasions. All animal work must have been conducted according to relevant national and international guidelines. In accordance with the recommendations of the Weatherall report, "The use of non-human primates in research" we specifically require authors to include details of animal welfare and steps taken to ameliorate suffering in all work involving non-human primates. The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. Please enter your ethics statement below and place the same text at the beginning of the Methods section of your manuscript (with the subheading Ethics Statement). Enter "N/A" if you do not require an ethics statement.	

Dear editor,

I am writing in support of our manuscript 'Seroprevalence of molluscum contagiosum virus in German and UK populations' by Subuhi Sherwani, Laura Farleigh, Nidhi Agarwal , Samantha Loveless , Neil Robertson , Eva Hadaschik , Paul Schnitzler, and Joachim J. Bugert.

With this research article we establish for the first time seroprevalence of Molluscum contagiosum virus (MCV), a significant skin pathogen of children and young adults, in a large European population (n= 393) using a novel MC084 based ELISA assay.

MCV is easily recognized clinically, but understudied, and what little is available of seroepidemiological data for European populations dates back to the 1950s. Two previous ELISA studies on MCV seroprevalence were published in 1999 and 2000 but use different and in our opinion less suitable antigens.

Our ELISA assay is based on part of the MC084 antigen expressed in E coli and establishes interesting patterns of humoral immune responses to MCV. Our data provide evidence, that MC is a very common skin disease in Europe and probably underreported by a factor of three or more.

All authors have contributed to, seen, and approved the final, submitted version of the manuscript.

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Sincerely,

J J Bugert

Seroprevalence of Molluscum contagiosum virus in German and UK populations

Short:

Molluscum contagiosum virus burden of disease

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Abstract

Molluscum contagiosum virus (MCV) is a significant but underreported skin pathogen for children and adults. Seroprevalence studies can help establish burden of disease. Enzyme linked immunosorbent assay (ELISA) based studies have been published for Australian and Japanese populations [1-2], and the results indicate seroprevalences between 6 and 22 percent in healthy individuals, respectively. To investigate seroprevalence in Europe, we have developed a recombinant ELISA using a truncated MCV virion surface protein MC084 (V123-R230) expressed in *E. coli.* The ELISA was found to be sensitive and specific, with low inter- and intra-assay variability. Sera from 289 German adults and children aged 0-40 years (median age 21years) were analysed for antibodies against MC084 by direct binding ELISA. The overall seropositivity rate was found to be 14·8%. The seropositivity rate was low in children below the age of one (4·5%), peaked in children aged 2-10 years (25%), and fell again in older populations (11-40 years; 12·5%). Ten out of 33 healthy UK individuals (22%; median age 27 years) had detectable MC084 antibodies. MCV seroconversion was more common in dermatological and autoimmune disorders, than in immunocompromised patients or in patients with multiple sclerosis. Overall MCV seroprevalence is 2.1 fold higher in females than in males in a UK serum collection. German seroprevalences determined in the MC084 ELISA (14·8%) are at least three times higher than incidence of MC in a comparable Swiss population (4.9%; [3]). While results are not strictly comparable, this is lower than Australian seroprevalence in a virion based ELISA (n=357; 23%; 1999) [1], but higher than the seroprevalence reported in a Japanese study using an N-terminal truncation of MC133 (n=108, 6%; 2000) [2]. We report the first large scale serological survey of MC in Europe (n=393) and the first MCV ELISA based on viral antigen expressed in *E. coli.*

Introduction

After the eradication of smallpox, MCV is the principal poxvirus causing human disease [4-7]. MCV is classified as a member of the family *Poxviridae,* in its own genus *Molluscipoxvirus* [8]. It has unique features that are distinct from other poxviruses pathogenic for humans, including smallpox and monkeypox [9]. MCV shares the highest level of amino acid (aa) similarity and unique proteins with parapoxviruses such as Orf viruses [10].

MCV infects the human skin and Molluscum contagiosum (MC) is a sexually transmitted disease, with infections occuring worldwide [4, 11-13].Clinical infection is characterized by a variable number of papules, each forming a central crater filled with a waxy plug of cell debris mixed with a large numbers of virus particles. Histopathologically, MC causes a benign epidermal hyperproliferation, known as an acanthoma [14]. MC is most common in young children and teenagers. MC in immunocompromised patients results in more numerous and extensive lesions [15]. In immune-competent patients, lesion may persist for up to 12 months [14]. Spontaneous regression of MC lesions is commonly preceded by clinical signs of inflammation [16], indicating a vigorous immune response [17].

The true prevalence of MC has probably been underestimated because of the benign clinical manifestation and rare complications. Development of assays which could assist in seroprevalence studies has been hampered by unsuccessful attempts to cultivate MCV efficiently *in vitro* [18-21]. The viral genome was sequenced in 1996 [5].

In the first known MCV antibody study in 1952, Mitchell found three out of 14 MC patients with complement-fixing antibody to an antigen prepared from human MC lesions [22]. Shirodaria *et al*. used MCV cryostat sections in an immunofluorescence study of MCV antibodies, reporting IgM class of antibodies only in MCV patients and IgG antibody responses in 16.7 % of healthy control subjects (n=30) [23]. Only two seroprevalence studies using ELISA, have been reported; one by Konya and Thompson [1] in 1999 and another by Watanabe *et al*. in 2000 [2].

Konya and coworkers described in 1992 a virion based enzyme linked immunosorbent assay [24]. MCV virions were isolated from human lesion material. The antigen was extracted from pooled lesions of

different genotypes with epidermal protein extract used as a control. Their 1999 serological survey of a healthy Australian population (n=357) revealed an overall seroprevalence of 23% and up to 77% in MCV infected HIV negative individuals [1].

Based on MCV sequence information then available, in 1998 Watanabe *et al*. identified two immunodominant proteins of 70 and 34 kDa and mapped them to the ORFs mc133L and mc084L, respectively [25]. The proteins are homologues of vaccinia virus proteins H3L and A27L, and major antigenic peptides of the virion particle [4, 25].

Using this information they developed an ELISA, based on an N-terminal truncation of MCV virion protein MC133produced in a Sendai virus expression system [2]. Their survey of a Japanese population of 508 subjects found mc133 specific antibodies only in 58% of patients with MC, and in only 6% of healthy controls (n=108).

The objective of our current study was to develop a recombinant MCV ELISA using water soluble and highly antigenic truncations of MC084L expressed in *E. coli* and to establish seroprevalence in a German and a UK serum collection.

Materials and Methods

Ethics statement

The study has ethical approval for the use of German tissues and sera (Ethikvotum S-091/2011 Hautklinik Heidelberg. Ethical approval was given by the Heidelberg University board in charge of ethical approvals, the ‘Ethikkommission’. Ethical approval for UK samples was part of 'An Epidemiological study of Multiple sclerosis and other neuroinflammatory demyelinating disorders in South Wales', 05/WSE03/111. Ethical approval was given by the Cardiff University 'Biobank Ethics Board'. All patients provided prospective informed consent in writing upon admission. All children’s' parents/guardians provided informed consent in writing. Class 2 GM work was notified to HSE with the project number GM 130/10.3.

pGEX-2TK expression of truncated MCV –GST fusion proteins

The plasmid pGEX-2TK was used for expression of truncated and epitope tagged MCV ORFs mc084 (V33-G117V5), MC084 (V123-R230 StrepII), and MC133 (M1-N370 StrepII) in *E. coli* with Glutathione S-Transferase (GST) fusion protein at the N terminus. Recombinant plasmids were constructed by PCR using specific primers tailed with restriction enzyme sites (BamHI-EcoRI) and C-terminal epitope tags.

Expression and purification of MC084S (V123-R230) protein

pGEX 2TK GSTmc084S (ILR#332; MC084 specific insert 107 amino acids; 14 kD) was transformed into *E. coli* BL21 (RIL^+).Cultures were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and fractions analysed for fusion protein expression by SDS-PAGE and StrepII tag expression by western blotting. Cultures were incubated at 37ºCfor 4hrs after which the cells were harvested by centrifugation at 10,000 × g for 20 min and lysed by sonification in buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M and Tris-HCl, pH 8.0). Lysate containing the protein of interest was added to glutathione sepharose beads and GST-MC084S was bound to beads using batch purification. The fusion protein was cleaved using Precision protease at RT overnight. AKTA-FPLC of the resulting 14kD sized protein was done using size exclusion Superdex S200 column (GE Healthcare).

SDS-PAGE, western transfer and immunodetection

Protein preparations were separated using denaturing sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) in NuPAGE Novex 4-12% Bis-tris Gels (Life technologies) and MOPS SDS running buffer (Invitrogen). Protein bands were visualised by staining with 0.01% Coomassie Brilliant Blue R-250. For immunodetection proteins prepared by SDS-PAGE were electrotransferred onto nitrocellulose and probed with Strep MAB Classic HRP conjugate (IBA). Detection by chemiluminescence was performed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's recommendations.

Human serum/ tissue samples

314 serum samples and lesion material from patients with molluscum contagiosum were collected at University Hospital Heidelberg, Germany, between 2007-2011. 79 UK sera samples were collected at Cardiff University. Twelve serum samples were collected from MCV patients (10 from Dr P N Behl Skin

Institute and School of Dermatology, New Delhi, India; two from UK; aged 2-62 years) as diagnostic specimens.

MCV direct binding ELISA

Ninety six well Maxisorp ELISA plates (Nunc) were coated with 3 µg/ml of FPLC purified recombinant truncated MC084S (aa123-230) protein per well in 100 µl of 0.05M carbonate-bicarbonate buffer (pH9.6) and incubated at 37ºC for 2 hours and then overnight at 4ºC. Plates were washed with PBS and blocked with 5% skim milk. Test sera, diluted 1:100 in dilution buffer, and were coated across the plate (100µl/well). The plates were incubated at 37ºC for 2h and washed ten times with PBS-T. Secondary anti-human IgG conjugated to horseradish peroxidase (GE Healthcare), diluted 1:2000 in dilution buffer was subsequently added (100µl/well). After incubation at 37ºC for 2h the plate was washed ten times with PBS-T and 100 µl of BD OptEIA™ substrate reagents (BD Biosciences) was added to each well. 50 µl of 1M H_2SO_4 was used to stop the enzyme reaction after 20min incubation at RT. The OD of the reaction product was read at 450nm on an FLUROSTAR OPTIMA - ELISA plate reader (BMG Labtech).

Plate description

42 serum samples were tested in duplicate on each plate along with a panel of four control sera consisting of two negative and two positive as well as four blanks, all in duplicate. The results were expressed as δODU (δODU =mean of duplicate wells minus mean of the blank wells).

ELISA performance

Plate to plate variation was monitored by comparing the control panel results between the different wells of the same plate; same sera samples run on different plates on the same day as well as on different days.

Immunofluorescence and Immunohistochemistry

Paraffin embedded sections were deparaffinized and rehydrated. Dako Cytomation Envision®+Dual Link System-HRP (DAB^+) kit (Dako) was used as per manufacturer's instructions. For staining of tissue with human sera, ECL Anti-human IgG (1:2000) (GE Healthcare) was used. Staining was completed with

Mayers haematoxylin and eosin counterstaining. All sections were analysed using an Olympus BX51 light microscope. Vaccinia virus infected HaCaT cells were grown on glass coverslips and fixed with 3% paraformaldehyde for 10 min, followed by staining with human serum antibodies (1:100) and an anti-human Alexafluor-488 secondary antibody (Invitrogen).

Statistical Analysis

Serological data was stratified by age or diagnosis. Statistical significance of differences between the ELISA responses of different groups was assessed by one way ANOVA. Tukey post hoc anova was used to identify and compare statistically significant means and differences of different groups.
Additional information on material and methods is shown in supporting information.

Results

Selection of MC084 antigen, cloning and purification

Amino acid sequences of MC084 (298aa, 34kD) were analysed to determine overall homology with related proteins in the GenBank and identify transmembrane regions and region of high hydrophilicity / high antigenicity. Two transmembrane regions predicted in the C-terminal end of the protein [26], were excluded to avoid solubility issues in the *E. coli* expression system (Figure 1A). Of the remaining amino acids, a N-terminal region (V33-G117) and a C-terminal region (aaV123-R230), both containing one region of high hydrophilicity in the Kyte–Doolittle plot (Figure 1B) [28] were further analysed for subcloning.

The C-terminal truncation of mc084 (V123-R230, predicted MW 14kD), comprising 107 aa, has the lowest homology to orthopoxvirus proteins and contains a region of high antigenicity (218-NELRGREYGASLR-230) with no significant homology to vaccinia/ cowpox virus. The C-terminal truncation MC084 (V123-R230) was then subcloned into the pGEX-2TK vector (Figure 2A) with a Strep II tag and in frame with glutathione S-transferase separated by a thrombin kinase site (Figure 2B) and overexpressed as a GST fusion protein in codon optimized *E. coli* (BL21 RIL^{+}).

The GST fusion protein was identified with an apparent molecular weight (MW) similar to the predicted MW of 40 kD in IPTG induced cultures (Figure 3A) and was absent in the uninduced cultures. The protein was protease cleaved and the C-terminal truncation of MC084 with an apparent MW of 14kD was further purified via FPLC using a size exclusion Superdex S200 column (Figure 3B). The Strep II tag was identified in Western Blot in both the fusion protein and the cleaved MC084 (V123-R230)-Strep II truncation (Figures 3 C and D). Additional data on antigen selection and optimization can be found in supporting information figures S1 and S2.

ELISA sensitivity, cut-off, and specificity

MC084S (V123-R230) antigen coated ELISA plates were produced as described in material and methods. Serum samples were diluted 1:100.

To establish sensitivity a panel of 12 sera from patients with known and clinically active MCV was first screened in comparison to sera from 0-1 year old individuals from the neonatal screening program of the Heidelberg University Clinics. In the group of sera from patients with diagnosed Molluscum contagiosum (n=12) the ELISA gave high readings for all (median δODU 1.5), with the most recent sample from Cardiff (CF2012-1) giving the highest (Figure 4). The control group of seventeen neonates from the Heidelberg University Clinics showed low readings with a mean of 0.1 δODU as shown in Figure 4, with one outlier (0.61 δODU). The confidence interval for the difference between positive and negative control groups was highly significant (Figure 4). Sensitivity was 100% for MC patients.

The cut-off for ELISA was calculated based on 66 sera from infants seen in the neonatal unit aged 0-1 years. The mean of δODU readings was 0·12043 and the SD was 0·08300. In comparison the mean δODU for 12 MCV infected patients was 0·833 and the S.D. 0·571. The infant group was used to define negativity with the upper limit being the mean δODU plus 3 SD (i.e. 0·36). Assuming that these values are indicative of a negative response to the recombinant protein, we defined a positive antibody response as being a value greater than mean plus 3 SDs i.e. δODU 0·36. Two more outliers were identified in this group (δODU 0·36 and 0·35). The MCV status of these subjects (aged 2 months, 9 months and 11months) could not be determined. Inter-well, intra-assay and inter-assay variability was found to be 3%, 5·2% and 6·7 %, respectively.

In order to establish ELISA specificity, human MCV infected tissue section obtained from the Heidelberg University Dermatology Unit were tested with high and low titre sera form our serum collections (supporting information Figure S3). An example for specific reactivity (MCV positive UK patient CF2012-1) is demonstrated in Figure 5. The section shows a dome-shaped contour with cup shaped lesions with central invagination, representing a typical MCV lesion consisting of two inverted lobules of hyperplastic squamous epithelium (red arrows) with several sub-lobules. The MC lesion shows acanthosis with the appearance of intraepidermal lobules with enlarged basophilic nuclei filled with cellular as well as MC bodies' debris (black arrows). Intraepidermal lobules are separated by septa consisting of compressed dermis (dotted arrow). MCV inclusion bodies stain strongly golden-brown with the human polyclonal serum CF2012-1 taken from a patient with clinical MCV infection. The stain is confined to areas where MCV cores, mature and released virus particles would be expected. In a number of tissue sections stained, the pattern was repeatable and sensitive to tissue preparation. Interestingly, the debris areas filled with mature MCV particles and lipid debris are also sensitive to removal by xylene /ethanol treatment of paraffin sections. The areas most consistently stained are the suprabasal and spinous layers.

To further establish antigen specificity we also infected human HaCaT keratinocytes with a vaccinia virus expressing full length mc084 (v319; aa 1 to 318) as shown in Figure 6. Infected keratinocytes were tested with the high titre serum HD V0901071. Virus infected cells show a vesicular stain similar to an endosomal / lysosomal pattern. Uninfected cells show no background signal, indicating the human polyclonal does not recognize keratinocyte antigens in cultured HaCaT cells.
Antigen optimization and comparisons are described in supporting information.

ELISA population studies

Sera from 289 individuals aged 2 months to 40 years (median age 21 years) were randomly selected from frozen 'normal control sera' collected at the University of Heidelberg, Germany, and tested for the presence of anti-MC084S (aa123-230) antibodies (Figure 7 A). Healthy subjects are divided into groups on the basis of age: 0-1 years (n=66), 2-5 years (n=52), 6-10 years (n=47), 11-20 years (n=72) and 21-40 years (n=52). The reactivity in infants was significantly lower than in other groups. Based on the minimum cut-off value of δODU 0·36, 43 (14·8%) sera of the 289 sera from a representative healthy

German population tested positive in the MC084S (123-230) ELISA. Positive antibody responses in the age groups were as follows: 4·5% (n=3) 0-1 year olds, 25% (n=13) in 2-5year olds, 23·4% (n=11) in 5-10 year olds, 12·5% (n=9) in 10-20 year olds and 13·5% (n=7) in 20-40 year olds (Figure 7 B). A one way anova was used for preference differences between the different age groups. The test statistic (F value) is 4.587 and the p value is 0.001. Further post hoc analysis was done using Tukey test to identify and measure statistically significant difference between groups of data as pairs. From the multiple comparisons it can be concluded that there is a sharp increase in positive sera responses between 0-1 year olds and 2-5 year olds which are statistically significant at p value =0.001. The differences in the sera responses between 0-1 year olds and 6-10 year olds are also statistically significant (p=0.011). Differences in sera responses between all other groups are not statistically significant. The results of the serological survey in members of the German populations are shown in Table 1.

We analysed 25 patients (8-40 years of age) with dermatological conditions such as Systemic lupus erythematosus (n=10), Psoriasis (n=3) and general autoimmune conditions (n=12), including patients with Autoimmune haemolytic anaemia, Autoimmune cerebilitis and Autoimmune hepatitis diagnosed at the University of Heidelberg. The findings are summarized in Table 1. MCV seroprevalence is above the average rate in skin specific autoimmune conditions, but similar in general autoimmune conditions.

79 serum samples from a UK population (aged 21-40 years; median age 27 years) were analysed which had been collected as part of a study on Multiple sclerosis (MS) at Cardiff University. These subjects were grouped as Primary progressive multiple sclerosis (n=9), relapsing remitting multiple sclerosis (n=37) and healthy humans (n=33) (Figure 8A).

Using the same cut-off of 0·36, MCV antibodies were detected in 10 of 33 healthy UK serum samples (22%). In patients with primary progressive multiple sclerosis seroprevalence was 11·1% (n=1/9), as compared to 16·2% (n=6/37) in patients with relapsing remitting multiple sclerosis (Figure 8B). A one way anova was used for preference differences between the different groups. The test statistic (F value) is 1.756 and the p value is 0.180. Further post hoc analysis was done using Tukey test to identify and measure statistically significant difference between groups of data as pairs. From the multiple comparisons it can be concluded that differences in sera responses between groups are not statistically

significant. An overall gender ratio (M: F) of 1.4:1 (183:131) was found in the German serum collection, as compared to 1:2.1 (25: 54) in the UK population. The results of the serological survey in members of all UK populations are shown in Table 1.

Discussion

We describe here for the first time a seroepidemiological study of MCV in Europe, the largest survey reported so far (n=393) and the first MCV ELISA based on viral antigen expressed in *E. coli*.

Previously reported MCV ELISAs used antigen from human lesion material or Sendai virus expressed N-terminal amino acid sequences of MC133, raising issues with background skin antigens and posttranslational antigen processing. To improve water solubility and provide an expression platform more suitable for commercial production of a MCV ELISA, we decided to use hydrophilic antigenic regions of MC084 expressed in *E. coli*. On the basis of previous work by Watanabe *et al.* [2] and our own homology analyses we chose a C-terminal truncation of MC084 (V123-R230), upstream of A238-Q298 previously found non-reactive in ELISA by Watanabe [2], as our candidate ELISA antigen. Our choice of antigen minimizes the possibility of cross reactivity with vaccinia virus specific antibodies, exclude the membrane spanning domains of mc084, but include a possible major antigenic site, identified by hydrophilicity plotting (MC084 N218-R230).

The ELISA is sensitive (100%) and specific, with low inter- and intra-assay variability. This is in comparison to the lower sensitivities of 77 and 58%, in the ELISAs reported by Konya *et al.* [1] and Watanabe [2], respectively. We have determined specificity in MCV tissue sections, similar to Konya *et al.* [1]. To determine specificity quantitatively, a collection of sera would be needed

We have calculated cut-off for our ELISA to include outlier results from our neonatal control group. The MC status of the outliers could not be determined, as the data was anonymised.

Any comparisons of our findings with previous ELISA results must be fundamentally flawed, because different antigen and expression systems were used. However, no other data are available, so with the above reservations, we compared the findings of our serological survey to results reported for Northern

Ireland and two previous ELISA studies in Australia and Japan [1-2, 23]. We find an overall seropositivity in a general German population of 14·7% and 22% in the UK. This correlates well with previous findings of16.7% in Ireland (n=30; IgG responses) [23], 23 % in an Australian population [1] and less so with 6 % reported in a Japanese survey [2].

The age profile determined using the MC084 ELISA correspond well with our understanding of the natural history of MCV infections, with low exposure of very young children and a high prevalence among toddlers and preschool children, where MCV smear infections is most likely to be transmitted among larger numbers of children. Our data confirm previously reported findings of stronger antibody responses in acute MC [1], mostly in the 2-10 age group [3, 29], with waning antibody levels being detectable as the population ages. This would suggest very little re-exposure in older age groups.

In contrast to Konya *et al*. [1], who report a very high seropositivity rate in their 0-6 year old population of 31%, explaining this with maternal antibodies, our data do not indicate a high seropositivity rate in very young children. Seroprevalence with the mc084 (V123-R230) ELISA is below 5% in 0-1 year olds and only increases in the age group of 2-5 year olds, not exceeding 25%. Watanabe *et al*. explained their low overall seropositivity (n=108, 6%) [2] in healthy subjects in comparison to the prior Australian study (n=357; 23%) [1], with their mc133 ELISA failing to pick up sera with mc084 antibodies as shown in immunoblots, indicating that mc133 may not be the best choice of antigen, underestimating seroprevalence.

The findings in immunocompromised patients and patients with skin and other inflammatory disorders indicate an increased seroprevalence in skin disorders, and a decrease in generally or therapeutically immunocompromised populations, but lack statistical power because of low sample numbers. The gender ratios calculated, indicate a higher seroprevalence (2.1 fold) in females than males of in the UK serum collection, but a lower ratio in the German collection.

In summary, we propose MC084 (V123-R230) is a suitable antigen for MCV serological surveys when expressed in *E. coli*. It includes a probable highly antigenic site at amino acid position N219-R230. Importantly, the MC seroprevalence of 14·8% in our German population is a threefold increase over the

reported incidence of MC in a comparable Swiss population of 4·9% [3], supporting the notion, that MC is an underreported infection. The assay will allow further investigations into the seroprevalence of MCV in other geographical areas, including the US, China, Japan and Australia.

Ongoing work includes possible use of a subpeptide of MC084 (N218-R230) comprising only the highly antigenic site for a capture ELISA and T cell studies, and the development of aIgMMC084 (V123-R230) ELISA. We are also in the process of investigating the MC084 (V123-R230) peptide for its potential to compete with MCV/VACV entry in a MCV/VACV reporter assay [30].

Acknowledgements

We thank Bernhard Moser for evaluating this manuscript and offering many helpful suggestions. We thank Arwyn T Jones and Edd Sayers for assistance with the confocal microscope, Bernard Moss of the NIH, NIAID LVD, Bethesda, Maryland, U.S.A. for the gift of the vRB12/pRB21 recombination system, Norbert Fusenig of the DKFZ, Heidelberg, Germany for HaCaT cells, Christopher Holland for assistance with protein purifications, Kamalpreet Banga for help with the statistics analyses and Frau S. Martinache for technical support with MCV section material provided by the Hadaschik group.

Author contributions

Subuhi Sherwani did experimental work, organised funding, contributed to literature research and wrote the manuscript.

Laura Farleigh contributed recombinant vaccinia construction and cell culture.

Nidhi Agarwal contributed Indian sera collection.

Sam Loveless contributed UK sera collection.

Neil Robertson contributed UK sera collection.

Eva Hadaschik contributed German MCV tissue sections.

Paul Schnitzler contributed German sera collection.

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Legends to figures

Figure 1. Bioinformatics

(A) Transmembrane plot (TMHMM Server v.2.0) [26] of mc084 amino acids 1-318; (B) hydropathy plot of MC084 protein with predicted high hydrophilic / antigenic regions indicated by black boxes. The full length ORF (MC084 1-318; predicted molecular weight 34.2 kD; shown on top) was cloned into vRB12 using specific primers tailed with restriction enzyme sites BamHI-HindIII) and C-terminal StrepII epitope tag. The resulting plasmid p319 was sequenced and the recombinant vaccinia virus v319 isolated on BSC-1 cells using the plaqueless mutant system [27]. N- and C-terminal (in yellow) truncations were subcloned from the original full length MCV gene into pGEX-2TK for overexpression in *E. coli* BL21 (RIL^+). TMHMM was used to determine transmembrane regions [26] whereas the Kyte-Doolittle plot was used to identify hydrophilic regions with predicted high antigenicity [28].

Figure 2.pGEX 2TK construct

(A) Schematic of recombinant plasmid p332 with a MC084 specific insert of 107 amino acids (V123-R230); predicted molecular weight 14 kD (B) Schematic of fusion protein of GST (green), followed by Thrombin kinase site (red), MC084 V123-R230 (grey), and strep II tag (blue); predicted antigenic site (black) (C) Western blot giving 40kD GST fusion protein GST-MC084S (V123-R230) detected using Strep MAB-Classic HRP conjugate (IBA-lifesciences). Vector NTI (vNTI) was used to produce virtual molecules and schematic diagrams of constructs prior to molecular cloning (InforMax, Inc)

Figure 3. Protein purification

Characterisation of over expressed recombinant fusion protein GST-MC084S and FPLC purified recombinant MC084S protein by SDS-PAGE and Western Blot. M: Molecular weight markers expressed in kDa. (A) over expressed 40kDa Recombinant GST-MC084S fusion protein separated in a 4-12% Bis-Tris gel. (B) FPLC purified 14kDa protein separated in a 15% Bis-Arylamide gel. Both gels were stained with Coomassie Brilliant Blue R-250. (C) GST-MC084S fusion protein after transfer to nitrocellulose (D) FPLC purified MC084S. The membranes were probed with Strep MAB-Classic HRP conjugate (IBA-lifesciences). Arrow heads indicate the locations of proteins.

Figure 4.Sensitivity

Absorbance plot of twelve sera from patients clinically diagnosed with MCV (India n=10; UK n=2; control group of 0-1 year old individuals n=17).

Figure 5. Tissue stain details

Microscopy (4x) of a Molluscum contagiosum lesion section (17315/11) stained with MC patient positive serum (CF2012-1) and haematoxylin-eosin counterstain (upper left hand corner). Three insets showing details at various magnifications [inset 1-(10x), inset 2-(20x) and inset 3-(20x)].

Figure 6. HaCaT Immunofluorescence

HaCaT cell culture infected with recombinant vaccinia virus expressing MC084S (v319). Reactivity of high titre human serum HDV0901071 and secondary antibody Alexafluor 488 (Green) goat anti-human IgG (H+L). Nuclei are stained with DAPI (Hoechst) and shown in blue. Samples were analysed for fluorescence emission properties by using confocal scanning laser microscopy Leica TCS SP2 AOBS.

Figure 7. German seroprevalence

Distribution of anti-MC084S antibodies in a German population tested by direct binding ELISA (A) Serological responses to MCV antigen MC084 in a German population (n=289; ages 0-40 years) expressed as the δODU value of an individual serum sample. The horizontal bar within each group represents the median absorbance measurement. (B) Percent seropositivities in different age groups

after cut–off of 0.36 (i) 0-1years (4.5%), (ii) 2-5 years (25%), (iii) 6-10 years (23.4%), 11-20 years (12.5%), and 21-40 years (13.5%).

Figure 8. UK seroprevalence

Distribution of anti-MC084S antibodies in a UK population tested by direct binding ELISA. (A) Serological responses to MCV antigen MC084S (V123-R230) in UK population (n=79) expressed as the δODU value of an individual serum sample in different groups (i) Primary progressive multiple sclerosis (PPMS; n=9), (ii) Relapsing remitting multiple sclerosis (RRMS; n=33) and (iii) Healthy humans (n=37). The horizontal bar within each group represents the median absorbance measurement (B) Percent positivity in individual groups for MC084S after cut-off of 0.36 (i) PPMS (22%), (ii) RRMS (11.11%) and (iii) healthy humans (16.21%).

Supporting Information Figure Legends

Figure S1. MC084 antigen optimization.

The figure shows the antigenicity of MC084S (aa123-230) as determined by direct binding ELISA using high titre human serum (HD V0901071). (A) Saturation was achieved at 3µg/ml. (B) A maximum of 80% inhibition of anti-serum antibodies with MC084S as inhibitor was observed whereas negligible inhibition was observed with BSA and human IgG.

Figure S2. Comparison of antigen reactivity.

The N-terminal truncation of MC084 i.e. MC084v5 (33-117), C-terminal truncation of MC084 i.e. MC084S (123-230), N-terminal truncation of MC133 i.e. MC133S (1-370) and GST tested as uncleaved fusion proteins on a GST affinity plate to compare antigen affinity and seroreactivities. The relative absorbance of individual sera was the same against all antigens tested with only minimal differences in absorbance.In direct antigen comparison there was no significant difference between truncations of mc084 and mc133, and no serum showed prevalent reactivity against one or another of the antigen used. A strep tag was used for detection of recombinant antigen in western blots. The tag did not interfere with ELISA results in a serum study of 149 serum samples (supplementary data).

Figure S3. Tissue staining with high and low litre sera

Tissue sections stained with high (**1A-D:** HD V0901071, HD V0903005) and low titre sera (**2A-D:** HDV0900471, HDV0900040) in two magnifications (4x and 10x). High titre sera stained the spinous layers as well as cellular debris and MC bodies in and around the intraepidermal lobules golden-brown. The same section stained with low titre sera as determined in MC084S ELISA showed much reduced or no reactivity in the same tissue areas.

Table 1. Summary of seroprevalences in German and UK populations

Groups	**Total sera**	**Cut-off = Mean+3*SD (0-1yr+outlier) 0·36**
		Positive Sera
German sera		
0-1 years	66	***3 (4·5%)***
1-5 years	52	13 (25%)
6-10 years	47	11 (23·4%)
11-20 years	72	9 (12·5%)
21-40 years	52	7 (13·5%)
	289	**Seropositivity in healthy subjects** 14·87 %
Psoriasis	10	2 (20%)
SLE*	3	1 (33·3%)
Autoimm†	12	2 (16·6%)
UK sera		
Healthy Humans	33	10 (22%)
PPMS#	9	1 (11·1%)
RRMS‡	37	6 (16·2%)
Total	293	65 (22.1%)

* SLE – Systemic Lupus Erythematosus

† Autoimm. – General autoimmune conditions

539 [#]PPMS – Primary progressive multiple sclerosis

540 [ǂ]RRMS – Relapsing remitting multiple sclerosis

Figure 1
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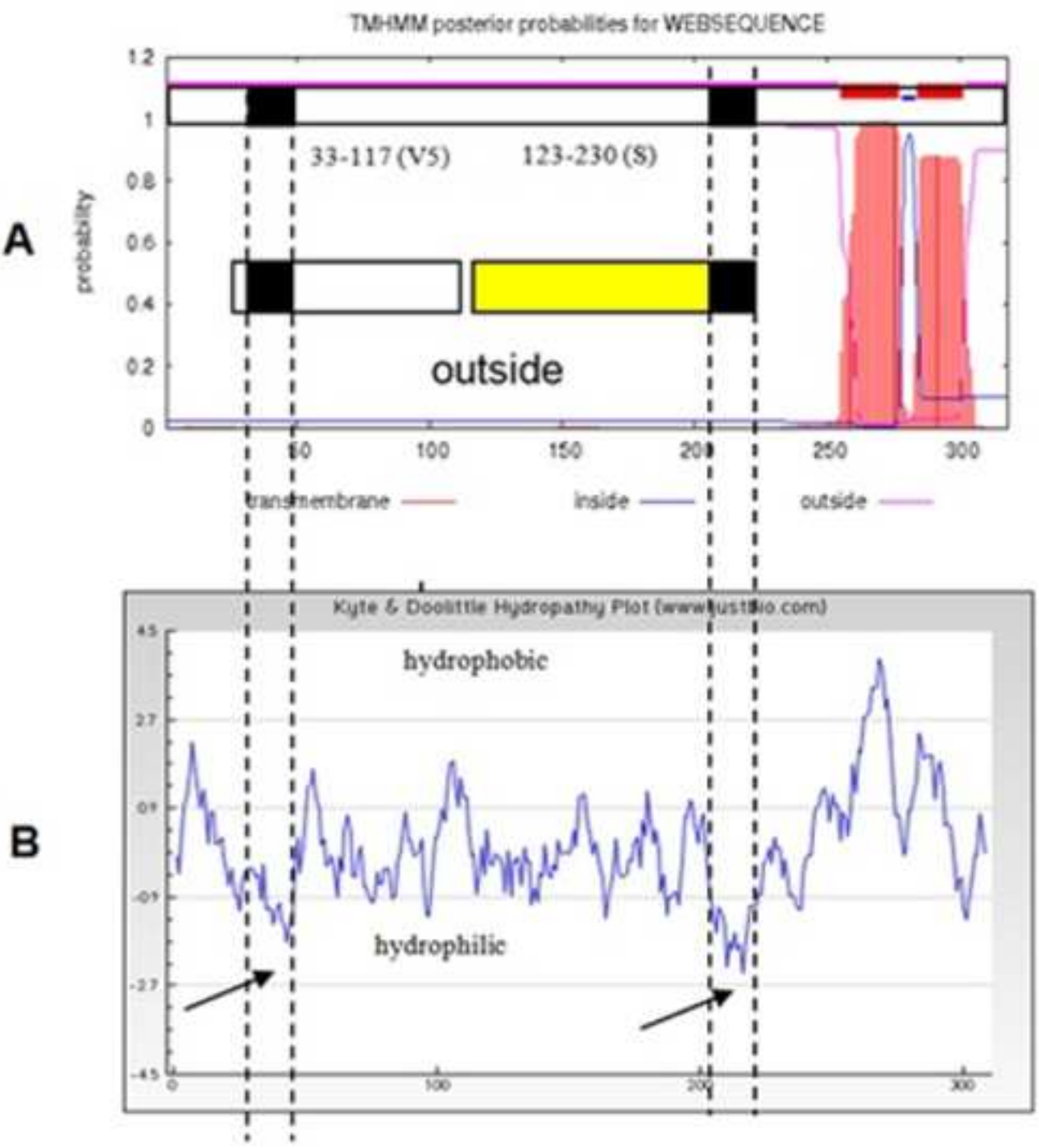

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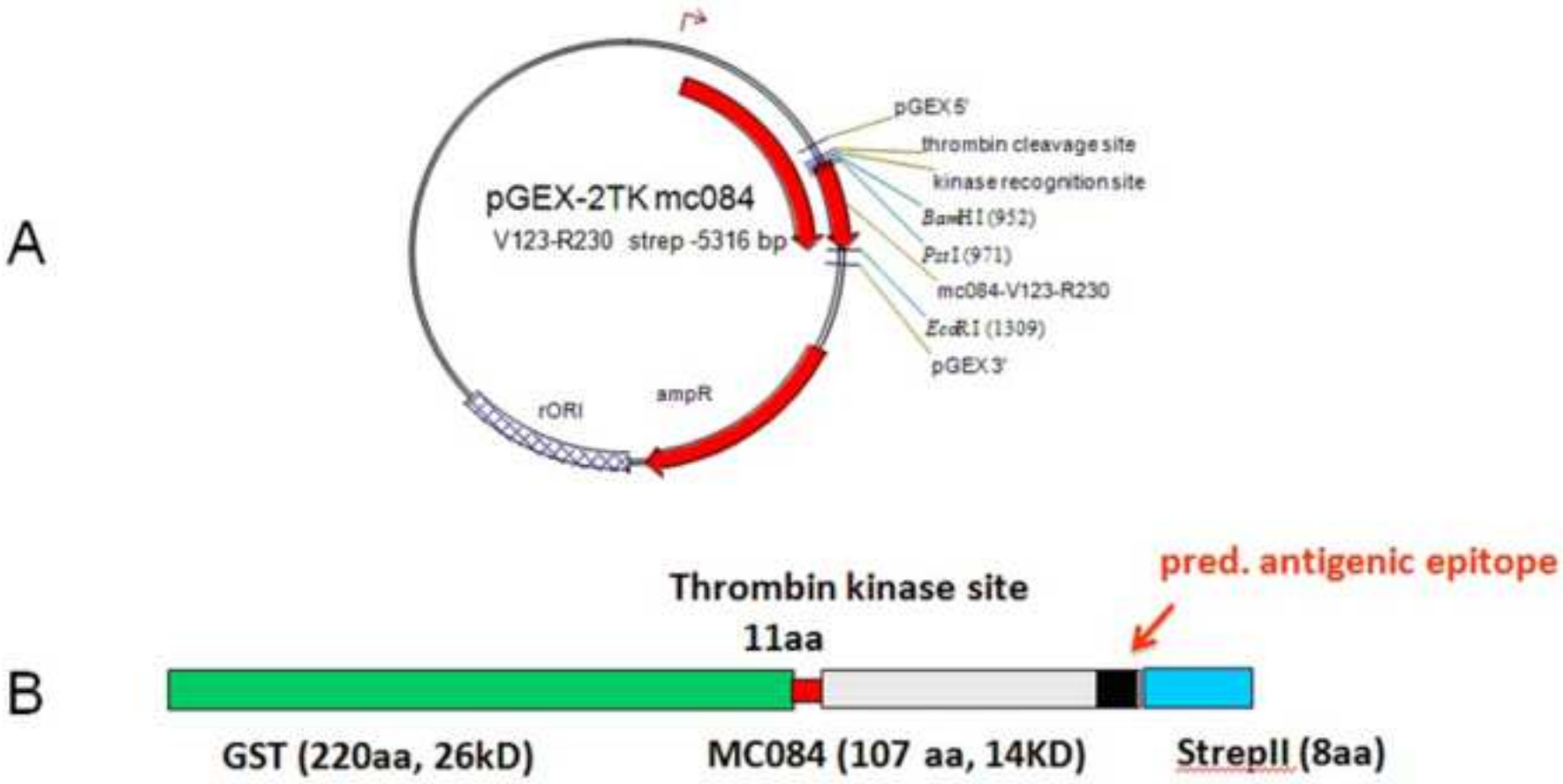

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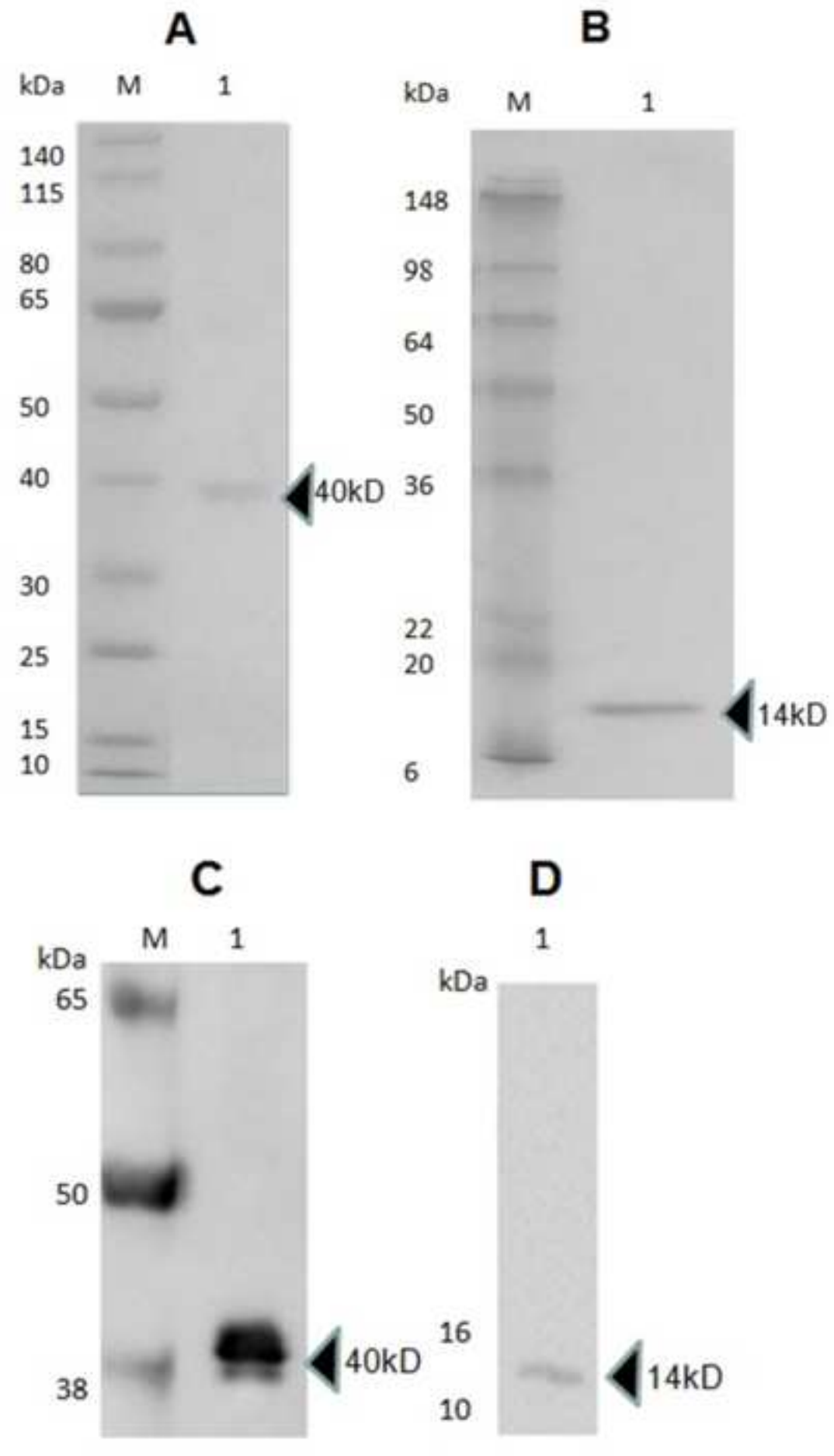

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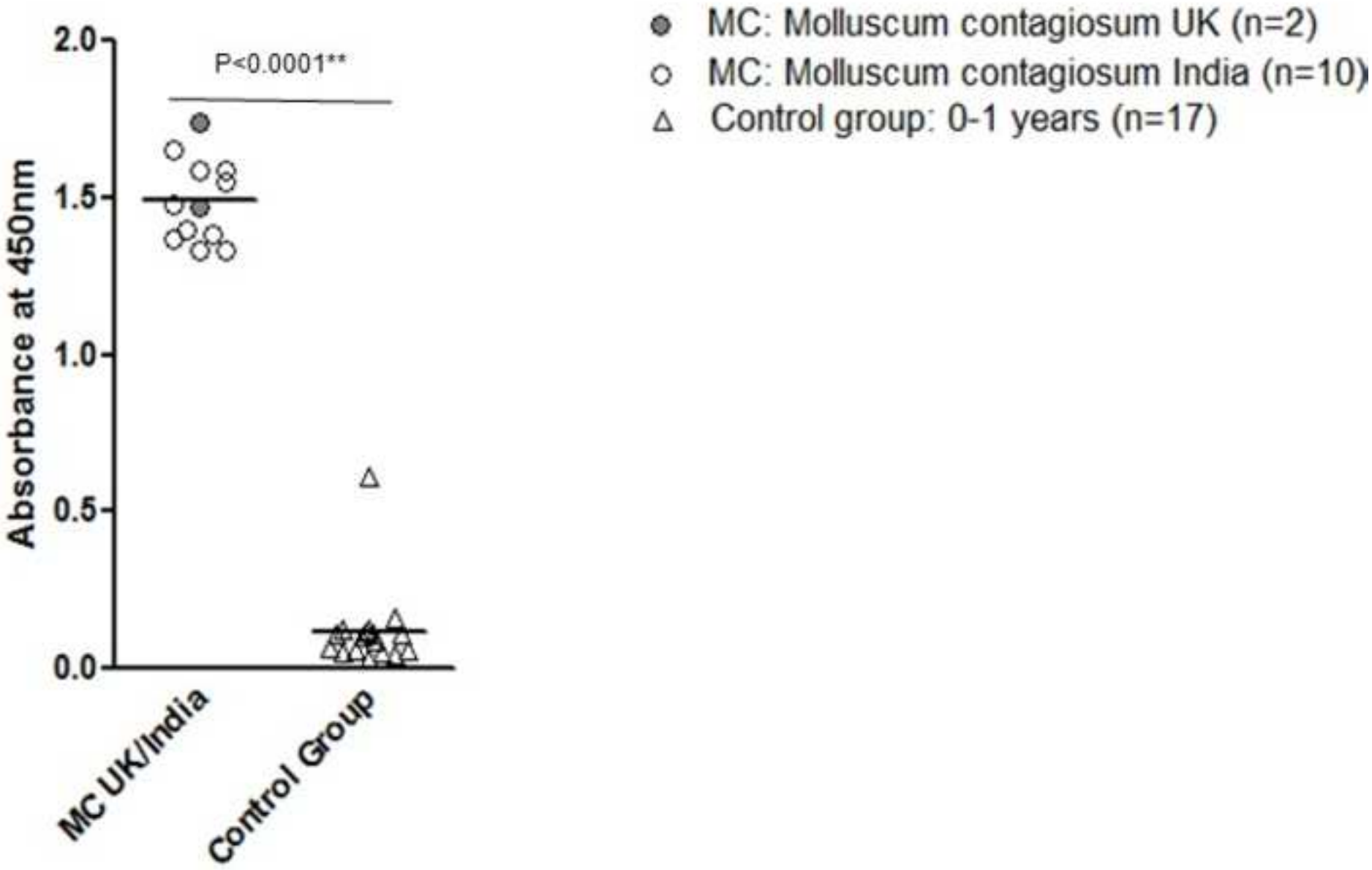

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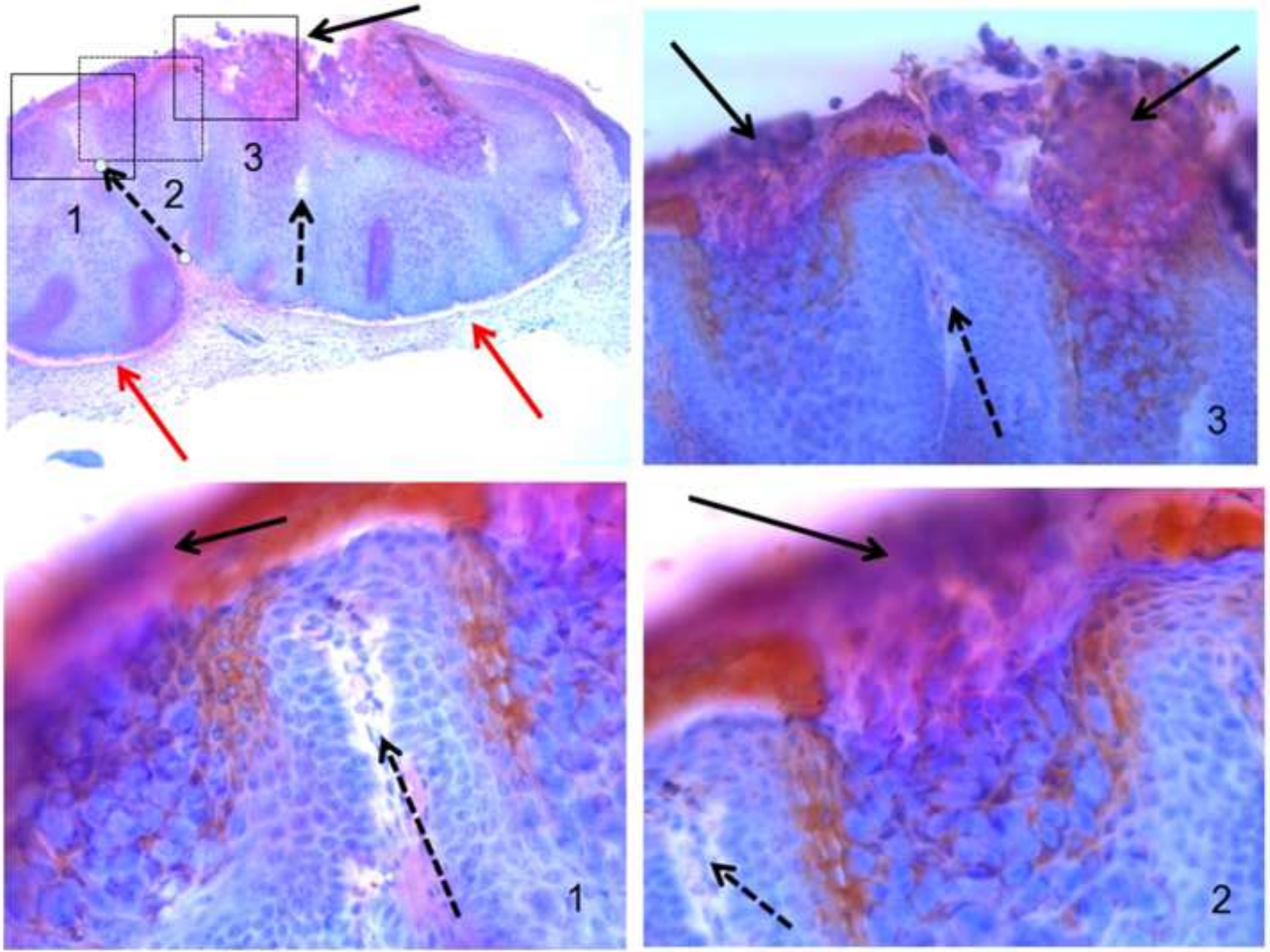

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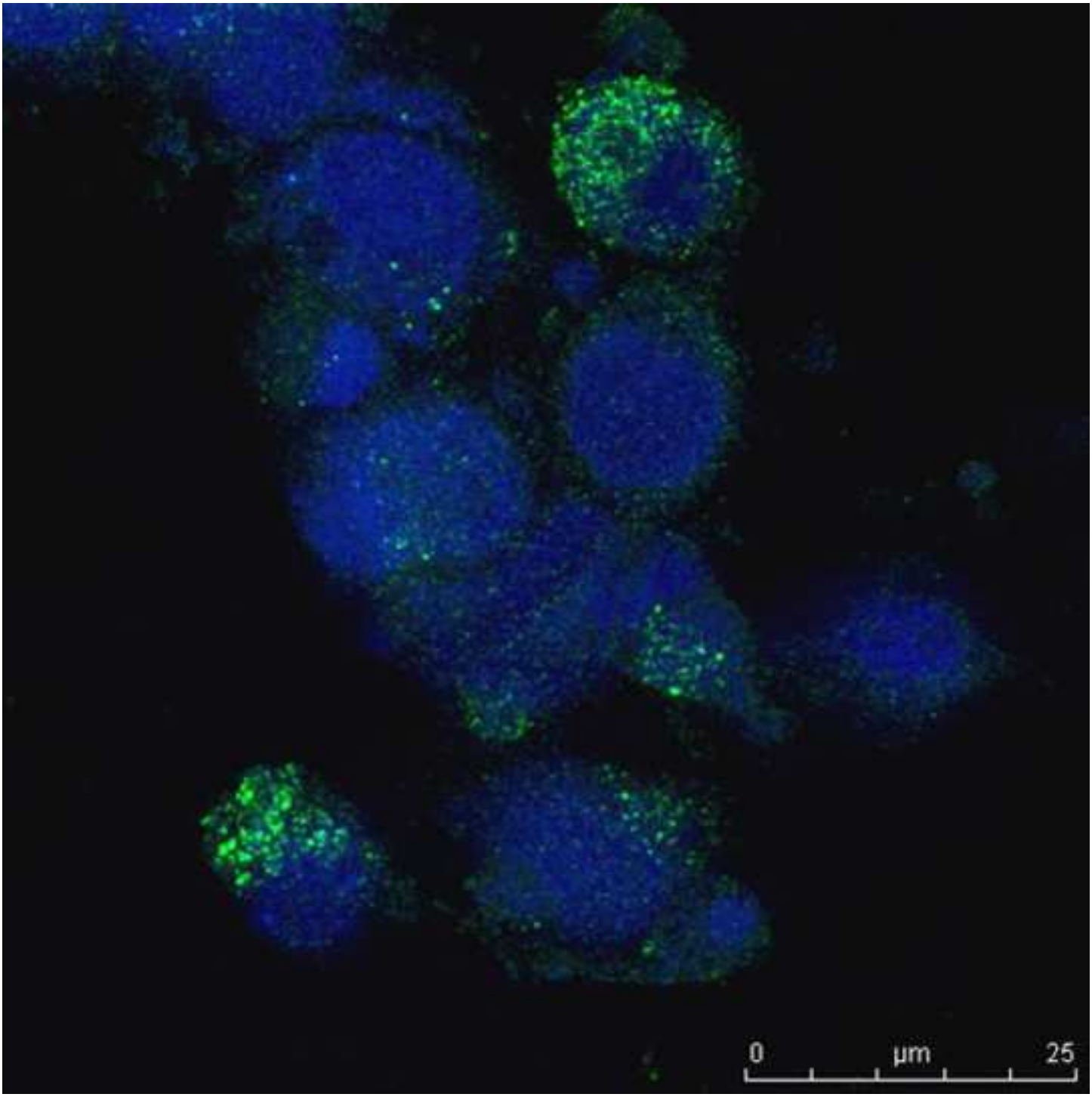

Figure 7

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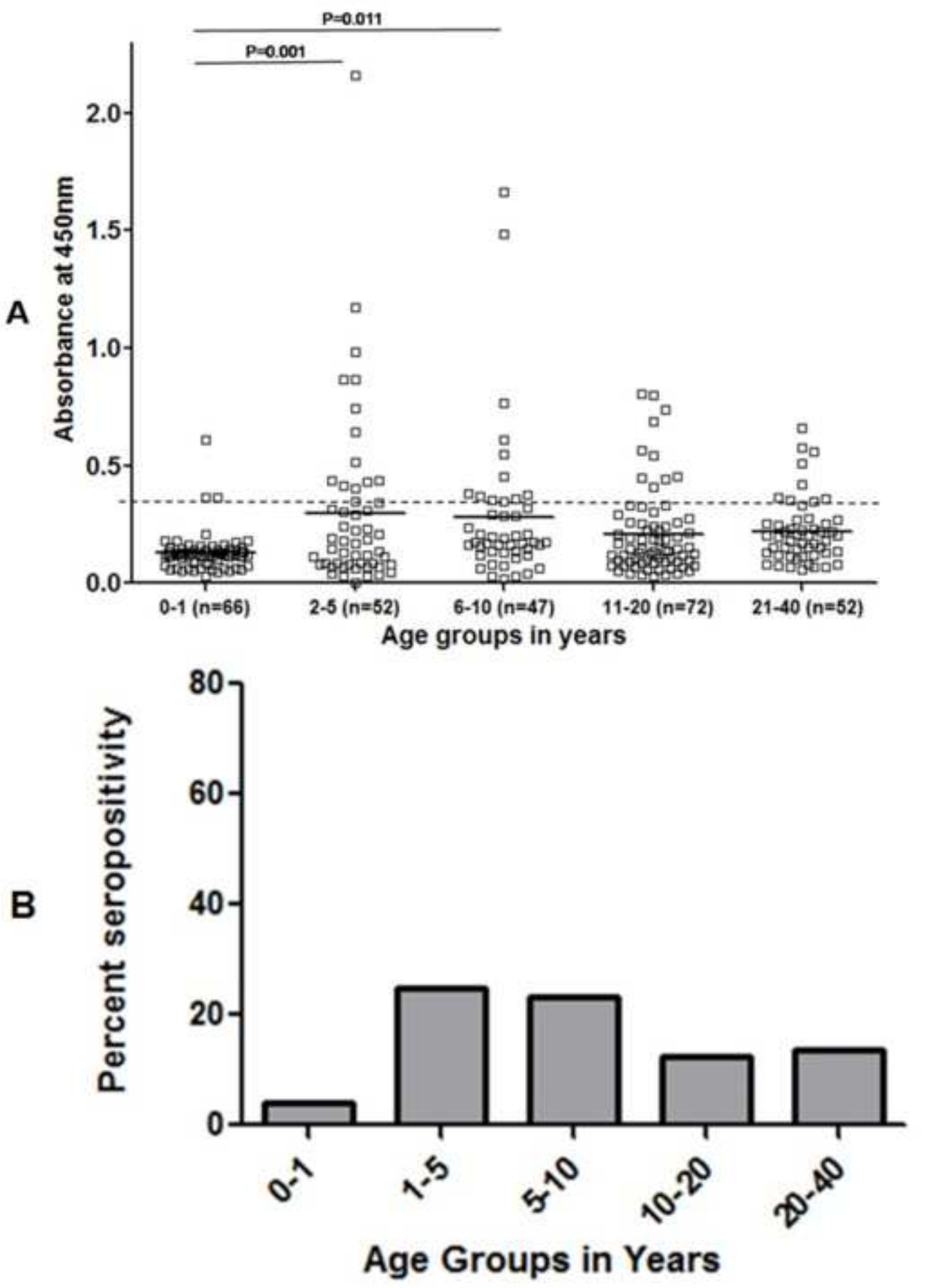

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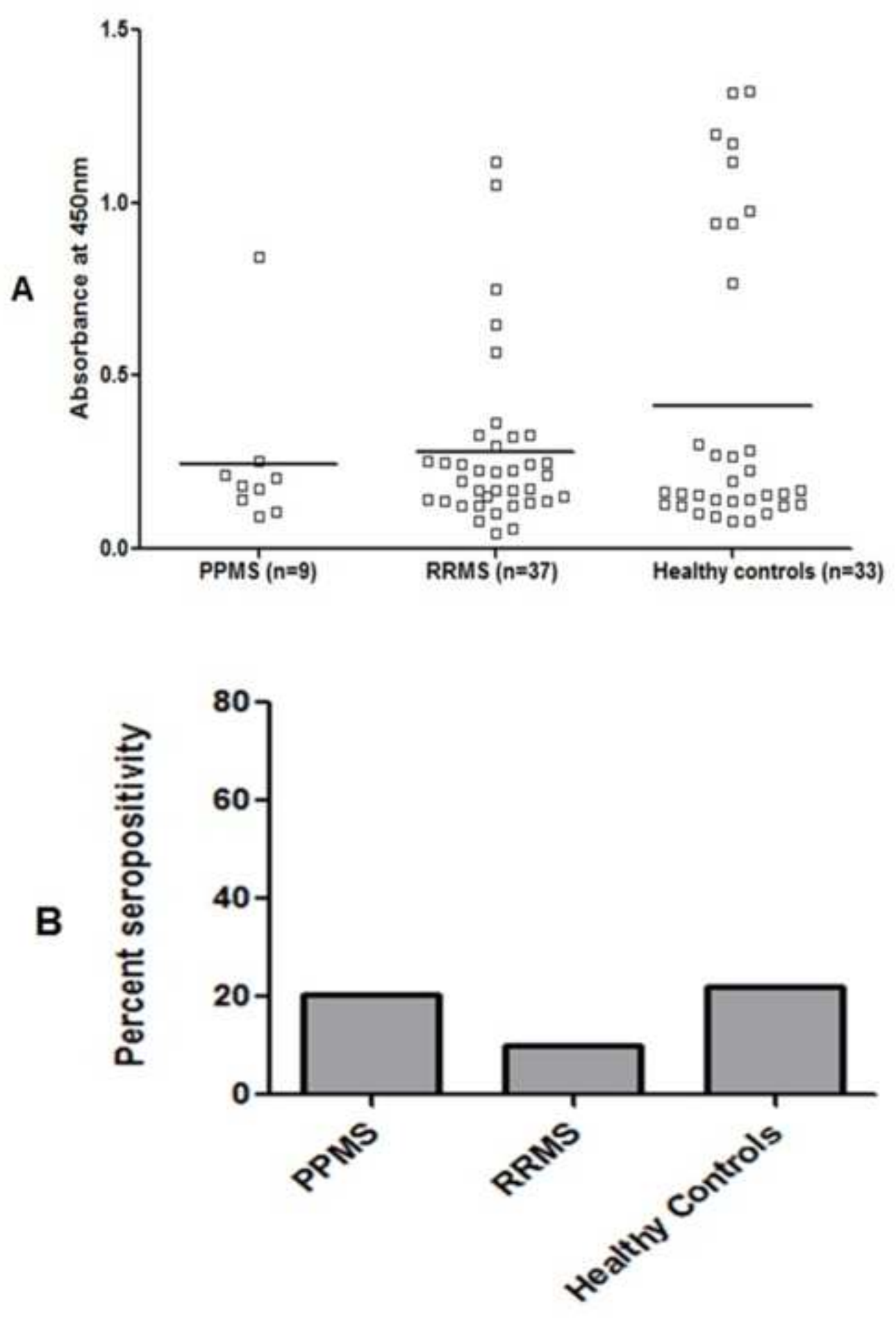

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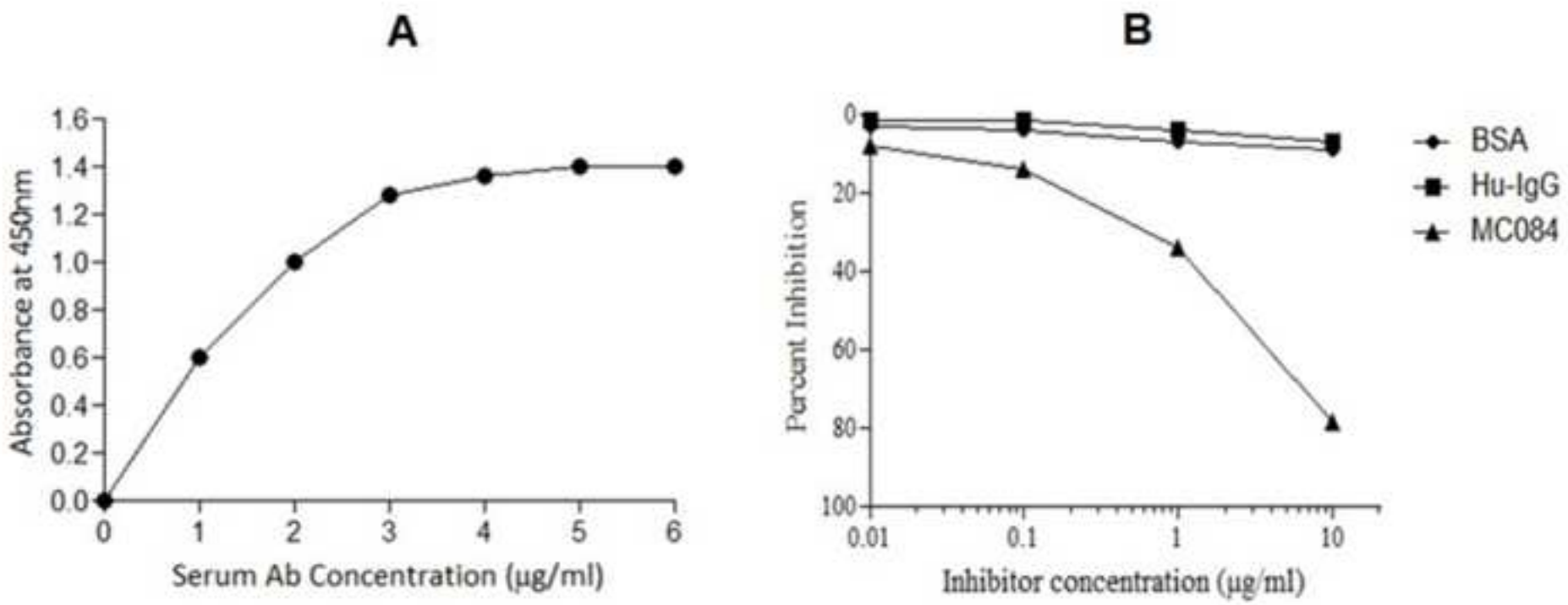

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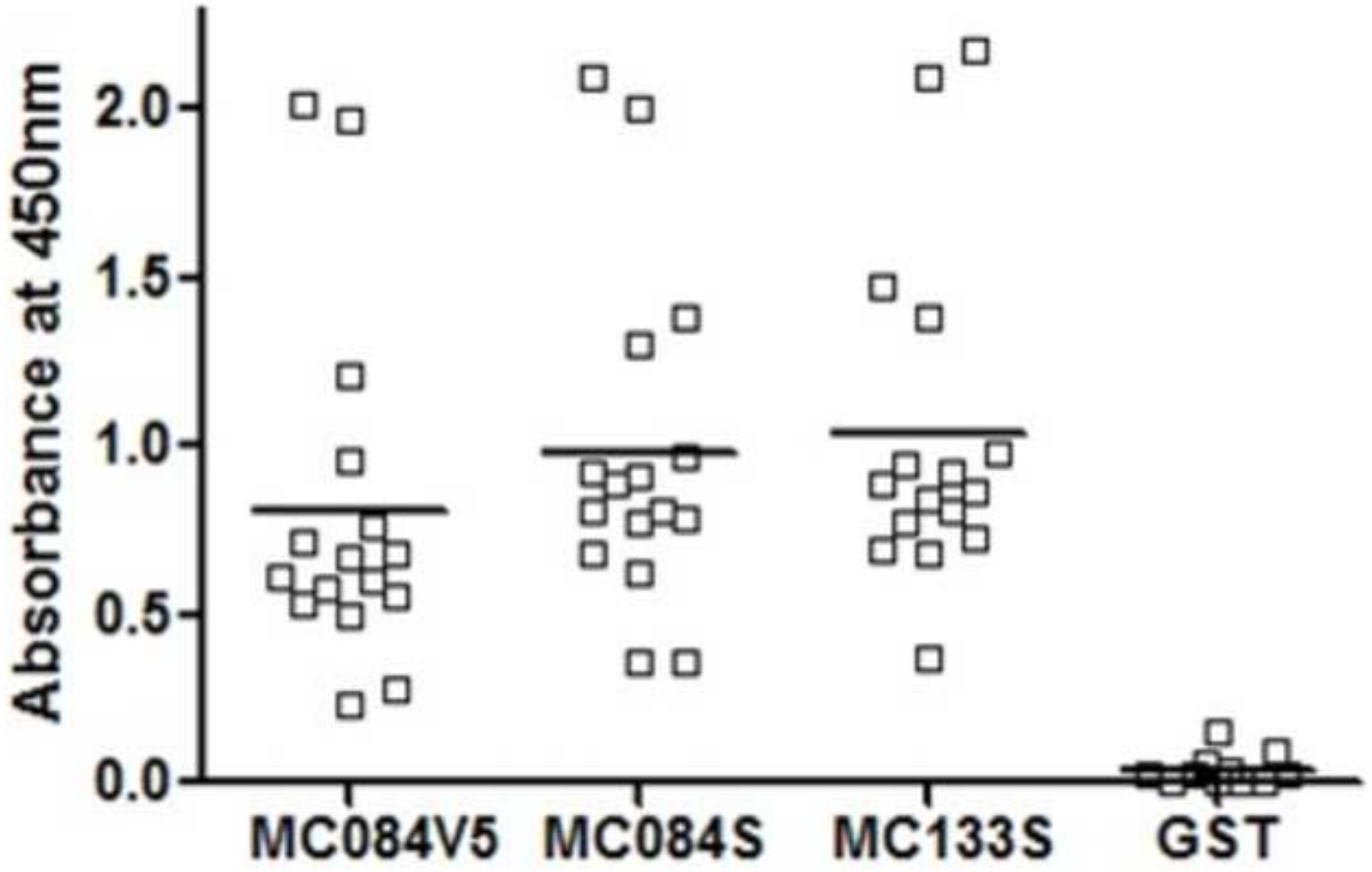

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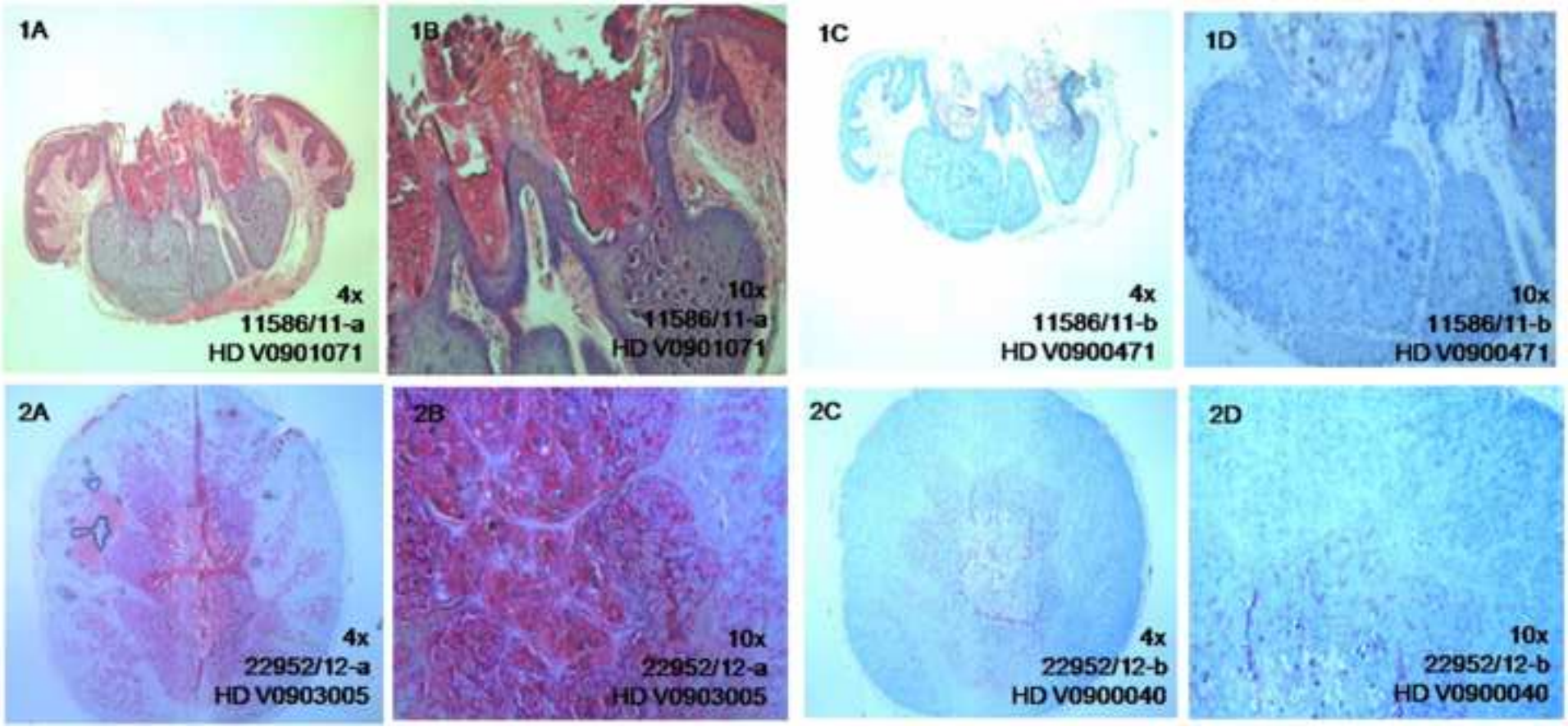

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