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Sperm-CMV interactions: Implications for sperm donor recruitment

By:

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Declaration

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

Katrina J Williams

“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”

Albert Einstein

(1879-1955)

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Presentations arising from this thesis

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Williams, K., Griffiths, P.D., and Pacey, A.A. (2016) Exposure to Human Cytomegalovirus (CMV) *in vitro* has no obvious effect on human sperm function. *Human Fertility*, **19**, 63-69. Proceedings for the British Fertility Society Annual Meeting (Oral).

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Williams, K., Milne, R., Griffiths, P.D., and Pacey, A.A. (2014) Developing a plaque assay to investigate the Cytomegalovirus (CMV) infectivity of human sperm samples. *Human Fertility,* **17**, 145-149**.** Proceedings for the British Fertility Society annual meeting. **Awarded 'Best Scientist' Prize.**

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Abbreviations

ABA	Association of Biomedical Andrologists
AIDS	Aquired Immune Deficiency Syndrome
ALH	Amplitude of Lateral Head Displacement
ART	Assisted Reproduction Technologies
ASRM	American Society for Reproductive Medicine
AZF	Azoospermia Factor
BAS	British Andrology Society
BCF	Beat Cross Frequency
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CTLs	Cytotoxic T Lymphocyte
CLTs	Cytomegalovirys Latency Transcripts
DEAFF	Detection of Early Antigen Flourescent Foci
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EB	Elementary Bodies
EBV	Epstein-Barr Virus
EGFR	Epidermal Growth Factor Receptor
EMEM	Eagles Minimum Essential Media
ES	Equatorial Segment
FCS	Fetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
FSH	Follice Stimulating Hormone
gB	glycoprotein B
GnRH	Gonadtrophin Releasing Hormone
HFEA	Human Fertilisation and Embryology Authority

HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HOST	Hypo-Osmotic Swelling Test
HPV	Human Papilloma Virus
HSPG	Heparin Sulphate Proteoglycans
HSV	Herpes Simplex Virus
ICSI	Intracytoplasmic Sperm Injection
IFN	Interferon
IUI	Intrauterine Insemination
IVF	*In Vitro* Fertilisation
LAT	Latency Associated Transcripts
LB	Luria-Bertani Growth Medium
LH	Lutenising Hormone
LIN	Linearity
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MRC-5	Human Lung Fibroblast Cells
NHS	National Health Service
NICE	National Institute for Clinical Excellence
NIEP	Non-Infectious Enveloped Particle
NK	Natural Killer
NTC	No Template Control
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PT	Perinuclear Theca
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

SCSA	Sperm Chromatin Structure Assay
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
STR	Straightness
TBS	Tris-Buffered Saline
TdT	Terminal deoxyterminonucelotidyl Transferase
TLR	Toll-Like Receptor
UCL	University College London
UL	Unique Long
UV	Ultraviolet
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-Line Velocity
VZV	Varicella Zoster Virus
WOB	Wobble

Abstract

Human Cytomegalovirus (CMV) is a common herpesvirus found in 60% of the population. Normally, it poses no risk, however it can have consequences for unborn babies. This is of concern when donor sperm is used in assisted conception, as CMV is present in semen. The risk of transmission from a positive donor is unclear, as it is not known if sperm can act as a vector for transmission. Additionally, this raises questions about whether CMV might affect sperm function. The hypothesis for this study is that CMV will interact with human sperm and alter sperm function and that sperm will act as a vector for viral transmission.

A survey was conducted to examine how fertility clinics were screening for CMV in sperm donors. This survey found that the majority of UK clinics are screening for CMV in sperm donors in the manner recommended by current guidelines but that the requirement to screen for CMV is causing problems in clinics with regards to sperm donor supply. Fortunately, this thesis has shown that sperm washing by density gradient centrifugation is mostly effective at removing CMV from semen samples infected *in vitro*, with CMV (AD169) grown in the laboratory, and in naturally infected samples. This presents a possible approach for alleviating some of the problems relating to CMV infection in sperm donors in UK fertility clinics. However, co-incubation with CMV has no effect on any of the sperm function parameters tested in this thesis, including, motility, viability, acrosome reaction, tyrosine phosphorylation and levels of DNA damage.

In conclusion, this thesis has highlighted problems with the current approach to screening and managing CMV infection in sperm donors but has provided evidence to show that there could be a simple solution to the problem. No effect on sperm function was observed, but this does not rule out a direct interaction between CMV and sperm. Overall, this thesis shows that fertility clinics should be concerned about CMV infection in sperm donors, but that simple steps could be taken to alleviate the current problems clinics are experiencing.

Chapter 1

Introduction & Literature Review

1.1 Introduction

It is estimated that 1 in 7 heterosexual couples in the UK will experience problems conceiving a child (National Institute for Clinical Excellence, 2013). For these couples, the advent of innovative assisted conception technologies (Edwards and Steptoe, 1983) has provided many new options to achieve this goal. Fertility treatment is now a central part of medical technology and in 2013, 64,600 cycles of *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) were performed in the UK (HFEA, 2013a). In heterosexual couples where male factor fertility issues are unresolved, a sperm donor might be used. In 2013, 4,611 cycles of donor insemination were carried out on 2,379 women (HFEA, 2013a). The number of cycles requiring donor sperm is increasing, with the number of donor insemination cycles in 2013 rising by 3.6% from 2012 and the number of cycles of IVF using donor sperm rising by 6.5% (HFEA, 2013a). Part of the rise in numbers of women undergoing donor insemination is a shift towards single women and same sex couples choosing this treatment (Baetens and Brewaeys, 2001; Leiblum *et al*., 1995), a trend that continues to increase year on year (HFEA, 2014). The increasing number of cycles using donor sperm creates problems due to the lack of availability of sperm donors. In 2013, there were 586 new sperm donor registrations, with a third being imported from overseas (HFEA, 2013b). This is a decrease from the previous year, when there were 631 new registrants (HFEA, 2013b) and falls short of the current demand for the UK (Hamilton *et al*., 2008).

A further complication to the availability of donors is the strict criteria under which a sperm donor is able to donate sperm for treatment (Association of Biomedical Andrologists *et al*., 2008). A study from 2003 showed that of the 1,101 men that applied to be a sperm donor over a 9-year period, only 40 were released as sperm donors, a rate of 3.63% (Paul *et al*., 2003). One limiting factor in the release of sperm donors for treatment is the requirement to go through a strict screening process for genetic and infectious diseases. The list of diseases screened for is not exhaustive and there are often debates surrounding what should and should not be included in this

screening process. One such argument is the requirement to screen for human Cytomegalovirus (CMV), a highly prevalent *Herpesvirus* infection.

CMV is present in 60% of the population and usually presents as an asymptomatic infection in healthy individuals. However, for those with compromised immune systems, such as transplant patients or those suffering from Acquired Immune Deficiency Syndrome (AIDS), infection with CMV can have severe health implications (Griffiths, 2002a). Neonates can also be severely affected by CMV infection, if acquired *in utero*, due to an immature immune system (Griffiths, 2002a). Known as congenital CMV, this type of infection can lead to the development of severe illnesses, including hearing loss and a range of neurological disorders (Griffiths, 2002b).

Given the potential severe health consequences to the neonate, and the evidence that CMV is present in semen cryopreserved for donor insemination (Mansat *et al*., 1997), the requirement to screen for CMV is apparent. However, the risk of transmission of CMV from a semen sample to a female recipient and onto a child is a highly debated subject. Part of the reason behind this is the little scientific evidence surrounding the relationship between CMV and sperm. A better understanding of this relationship and how CMV is transmitted through semen would allow the risk of transmission from sperm donors to be better assessed. Little is known about this aspect of CMV transmission, compared to other pathogens such as Human Immunodeficiency Virus (HIV) and *Chlamydia trachomatis.* For these pathogens, developments have been made in understanding how they interact with sperm, leading to increased knowledge and changes to clinical practice, making assisted conception both available and safer for patients. The same is not true of CMV, which by comparison has been poorly studied. There is a need to address this deficit and better understand if and how CMV might interact with sperm. This is the primary focus of this thesis.

1.2 Literature Review

This literature review will encompass the background to sperm, male fertility and CMV before considering the implications of CMV infection within the assisted conception field. Subsequently, evidence surrounding the interactions between other pathogens, such as bacteria and viruses, and sperm will be detailed as a basis for understanding the relationship between CMV and sperm.

1.2.1 The spermatozoon

Human spermatozoa are highly specialised haploid cells (Fawcett, 1975) evolved for the purpose of delivering the male genome to the oocyte (Suarez and Pacey, 2006). In order to do this, sperm have a highly specialised structure, which is created through the complex process of spermatogenesis. All features of the sperm are specialised for the journey of traversing the female reproductive tract (Suarez and Pacey, 2006) and penetrating the oocyte, in order to pass the male genetic information onto the next generation. Spermatogenesis occurs in the testicles and is responsible for producing millions of fully differentiated sperm every day (Bronson, 2011).

1.2.1.1 The spermatozoon structure

A spermatozoon consists of three major parts, the sperm head, mid-piece and the tail. The major components of the sperm head are the nucleus and the acrosome. The sperm tail can be further divided into four sections, which are connected by the same internal structure. First of all, the connecting piece containing the sperm centriole, the mid-piece containing the mitochondria, the source of ATP required for sperm motility, the principal piece and the end piece (Figure 1.1a).

The nucleus contains the male DNA in a highly condensed and quiescent form (Brewer *et al*., 2002; Dadoune, 2003). During spermiogenesis, the histones bound to DNA are replaced by protamines, making the DNA

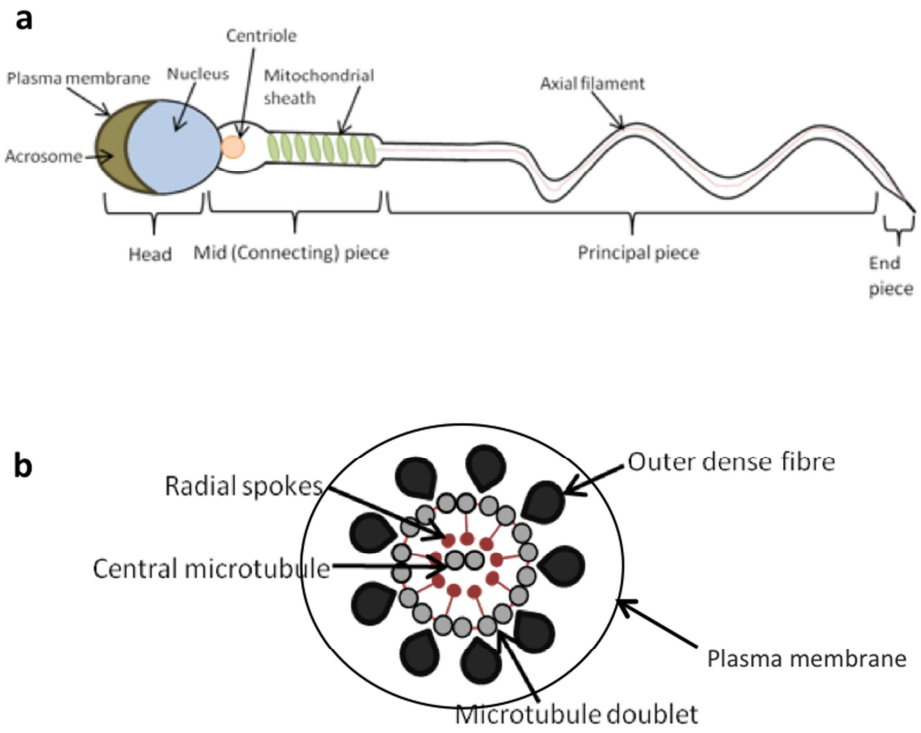

Figure 1.1: The overall structure of the human spermatozoa is represented in diagram (a). The head contains the acrosome and the condensed male DNA in the nucleus. The head is connected to the tail via the mid (connecting) piece containing the centriole and the mitochondria, wrapped around the axial filament which runs throughout the entire sperm tail (Fawcett, 1975). Diagram (b) shows the axial filament is comprised of the microtubule axoneme, which is essential for motility of the sperm tail. Nine outer doublets, connected by radial spokes, surround a central doublet of microtubules. Nine outer dense fibres surround the outer doublets and provide rigidity for the sperm tail (Fawcett, 1975; Porter and Sale, 2000).

inaccessible to enzymes, serving to protect the male genetic information. It is thought that sperm are unable to repair their own DNA (Matsuda *et al*., 1985); therefore once spermatogenesis is complete the DNA needs to be protected from any damage, which might compromise its integrity. The condensation of the male DNA is also thought to serve in aiding the transit through the female reproductive tract and penetration of the oocyte outer layers (Dadoune, 2003). The condensed nature of sperm DNA makes it inaccessible to enzymes and therefore it is thought that transcriptional activity and *de novo* gene expression is unlikely to occur (Kierszenbaum and Tres, 1975). However, evidence pertaining to sperm genomics and proteomics questions this accepted theory, which will be discussed in detail in a Section 1.2.1.2.

The sperm head also contains the acrosome, a Golgi-derived vesicle, containing hydrolytic enzymes and receptors (Yoshinaga and Toshimori, 2003) required for interaction and penetration of the oocyte zona pellucida (ZP) (Osman *et al*., 1989). Interaction with a ZP glycoprotein, ZP3, initiates an exocytotic reaction, resulting in the release of the acrosomal components and digestion of the ZP, allowing the sperm to penetrate this layer (Brewis *et al*., 1996). After penetration of the ZP, sperm enter the perivitelline space and are able to bind to the oolemma (Figure 1.2). After the acrosome reaction occurs, receptors present on the inner acrosomal membrane and at the equatorial segment (ES) are unveiled. Receptors located at the ES, such as Fertilin-β were thought to be involved in the fusion with the oolemma (Cho *et al*., 1998). However, it is now thought that a member of a major immunoglobulin family, Izumo1, is the main receptor (Inoue *et al*., 2005) involved with fusion to the putative egg receptor, Juno (Bianchi *et al*., 2014).

The remainder of the sperm head is composed of the perinuclear theca (PT), a matrix of structural proteins that provides support and confers head shape. PT proteins located in the posterior part of the sperm head, the post-acrosomal segment, are thought to function in signalling during early embryogenesis once the PT is dissolved in the oocyte cytoplasm (Sutovsky *et al*., 1997).

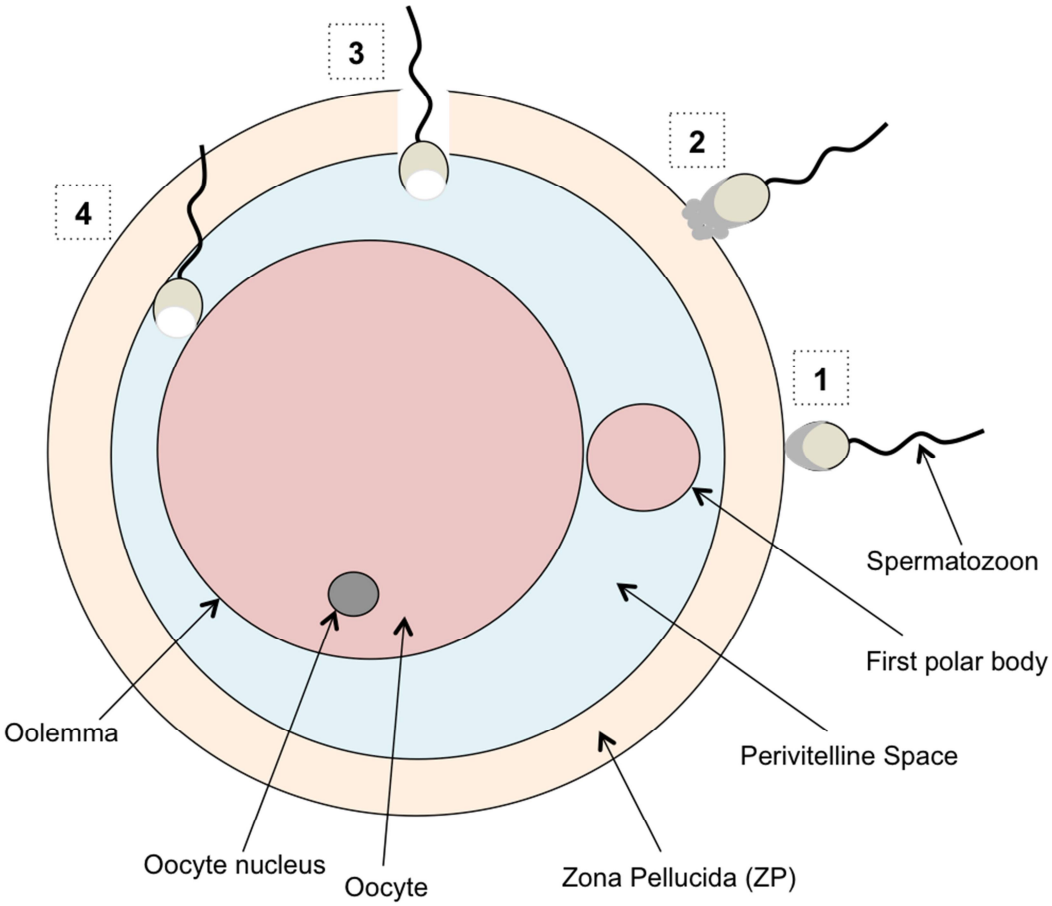

Figure 1.2: Diagrammatic representation of four key steps of fertilisation: (1) sperm bind to the zona pellucida (ZP) initiating an exocytotic reaction, which (2) subsequently releases the contents of the acrosome. Step (3) shows digestive enzymes from the acrosome penetrating the ZP and (4) shows the entry of the sperm to the perivitelline space. Once in this space, the sperm binds to the egg membrane (oolemma), and the sperm nucleus containing the paternal genome is delivered to the oocyte.

The sperm tail provides the motile force for sperm to travel through the female reproductive tract. At the centre of the sperm tail is the microtubule axoneme (Figure 1.1b). This is composed of a 9+2 arrangement of microtubule doublets, with 9 symmetrically arranged outer doublets connected to the two central doublets by radial spokes (Fawcett, 1975). The outer doublets are connected by dynein arms, which are the motor proteins responsible for the creation of mechanical energy from ATP (Turner, 2003). Coordinated asynchronous movement of dynein arms at each microtubule doublet allows for bending of the axoneme and subsequent flagella movement (Burgess *et al.,* 2003; Turner, 2003). Surrounding the outer doublets are 9 outer dense fibres that provide flexibility and support during movement of the flagellum (Figure 1.1b). The sperm tail can be divided into three major sections in addition to the end piece (Figure 1.1a). The connecting piece contains the remaining proximal centriole, leftover from spermatogenesis (Sutovsky and Manandhar, 2006). The mid-piece contains approximately 75-100 mitochondria, arranged helically around the central axoneme (Sutovsky and Manandhar, 2006). The mitochondria supply ATP to the axoneme for conversion into mechanical energy, required for the movement of the flagellum (Piomboni *et al*., 2012). The principal piece has the addition of a fibrous sheath, which is thought to provide support. It is thought that the fibrous sheath is involved in particular steps during capacitation and hyperactivation (Eddy *et al*., 2003).

1.2.1.2 Sperm transcriptome and proteome

It was traditionally thought that the spermatozoon only contributed the male DNA to the resulting zygote, however research into the sperm transcriptome is highlighting that sperm probably contribute more than just the paternal genome. It is now known that sperm contain a population of RNAs (Miller *et al*., 1999), including microRNAs (Ostermeier *et al*., 2005a). It is thought that this RNA is leftover from spermatogenesis, however there is an apparent selection process regarding which RNAs to keep, as the mature spermatozoon RNA population is significantly different from the testis-specific cell transcripts (Miller *et al*., 1999). This suggests that the presence of a

population of RNA is important to the function of the spermatozoon. One potential function is the *de novo* synthesis of proteins. Despite typically being thought of as a translationally inert cell, there is evidence to suggest that sperm are able to translate these mRNAs into protein using mitochondrial ribosomes (Gur and Breitbart, 2006). Other roles for the presence of spermatozoal RNA include a role in early embryonic development (Herrada and Wolgemouth, 1997; Ostermeier *et al*., 2004), epigenetic regulation of genes (Gapp *et al*., 2014), or a structural role within the sperm itself (Miller and Ostermeier, 2006).

The presence of an RNA population within a spermatozoon is also believed to be of importance to male factor infertility. Variation of the RNA present in individual men was reported (Ostermeier *et al*., 2002) and is thought to contribute to infertility, as when certain elements are missing, the ability to achieve a natural pregnancy is compromised (Jodar *et al*., 2015). Interestingly, when assisted reproductive techniques (ART) were used, the absence of certain sperm RNA elements did not affect the outcome of these procedures. This suggests a role for these RNAs in the potential of the spermatozoon to reach and fertilise the egg, rather than an inability to initiate embryonic development. Investigation into whether differences in the RNA carriage of fertile and infertile men can be used as markers for infertility is now being explored (Ostermeier *et al*., 2005b).

Another interesting observation in relation to the sperm genome is the ability to modify histone-bound DNA. Around 15% of human sperm DNA remains associated with histones, rather than protamines (Tanphaichitr *et al*., 1978). This histone bound DNA is therefore still vulnerable to modification by enzymes and studies have shown that sperm are able to digest a portion of their histone bound DNA when challenged with exogenous DNA (Maione *et al*., 1997; Sotolongo *et al*., 2003). Also, sperm are able to uptake exogenous DNA into their nucleus (Francolini *et al*., 1993). It is clear from this evidence that sperm are not as silent and inert as previously thought and are able to respond to environmental triggers.

What is also clear from this evidence is that sperm contain a host of proteins, which regulate these processes. The ability to digest DNA upon exposure to exogenous DNA requires the function of endogenous nucleases, which have been shown to be present in sperm (Maione *et al*., 1997). In addition, whilst the ability for mature sperm to undergo apoptosis is a controversial point, sperm do possess proteins involved in the apoptotic pathway, including caspase-3 (Weng *et al*., 2002). In addition to these unexpected proteomic findings, sperm also contain proteins involved in the fundamental processes of sperm hyperactivation and capacitation, such as AKAP4 (Miki and Eddy, 1998) and the CatSper channels (Ren *et al*., 2001). Sperm also contain numerous receptors involved in egg recognition and penetration, including Izumo1 (Inoue *et al*., 2005) and Fertilin-β (Cho *et al*., 1998). Other receptors have shown to be present on the spermatozoon, including epidermal growth factor (EGFR) (Jaldety *et al*., 2012), fibroblast growth factors (FGFR) (Saucedo *et al*., 2015), heparin sulphate proteoglycans (Foresta *et al*., 2011a), Toll-like receptor (TLR)-2 (Saeidi *et al*., 2014), lactoferrin receptor (Wang *et al*., 2011a), as well as receptors for binding progesterone (Tantibhedhyangkul *et al*., 2014) and oestrogen (Rago *et al*., 2014). These receptors serve different functions to the spermatozoon, from acquisition of motility, ability to undergo the acrosome reaction and defence against exposure of pathogens. It is clear that we are only just beginning to realise the true complexity of the sperm, both inside and out.

1.2.1.3 Spermatogenesis

Functional mature spermatozoa are made through the process of spermatogenesis, occurring in the seminiferous tubules, situated in the testes (Figure 1.3). This process produces millions of fully differentiated sperm everyday (Sutovsky & Manandhar, 2006). The first stage of spermatogenesis involves the mitotic division of non-proliferative type A spermatogonia into type B spermatogonia, which are ready to enter meiosis (Phillips *et al*., 2010). Type A spermatogonia can either commit to differentiate, or self-renew, a step required in order to maintain a population of progenitor cells.

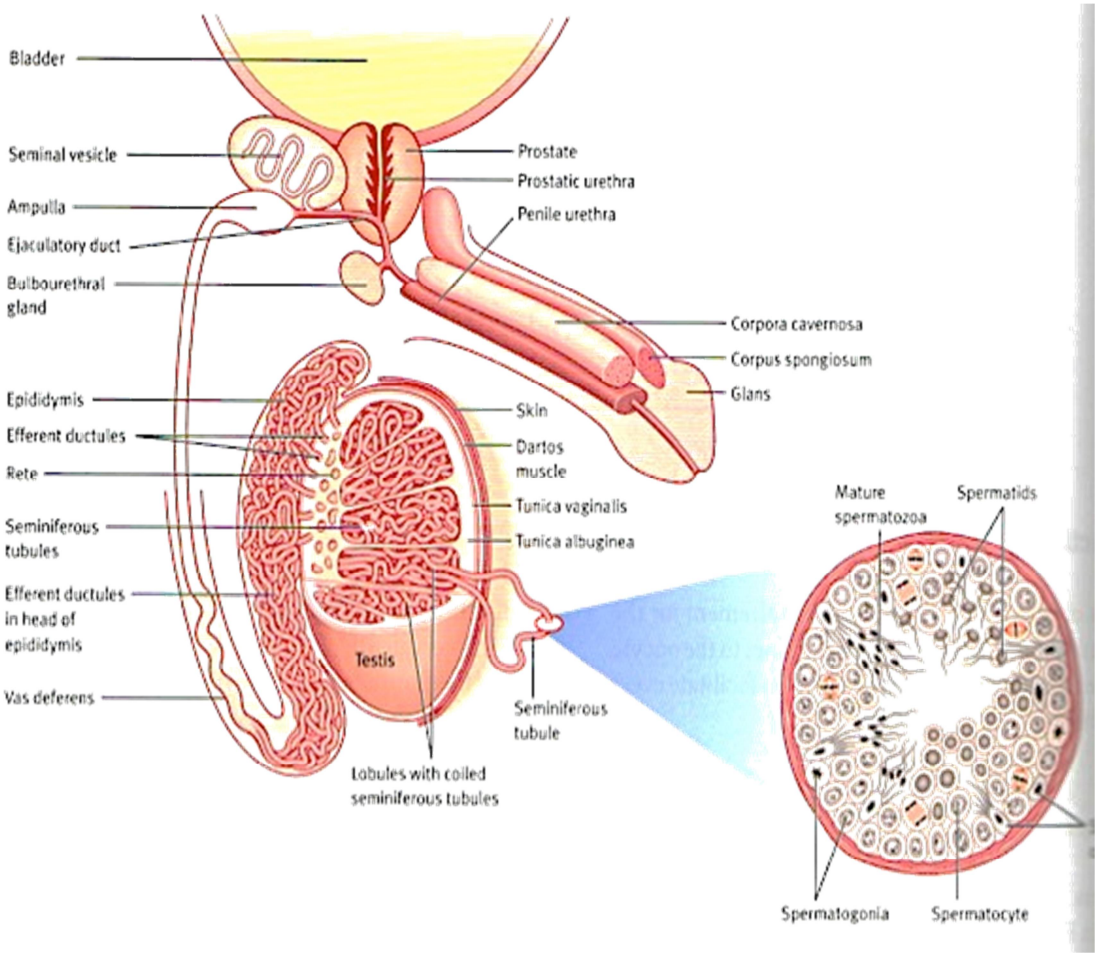

Figure 1.3: Anatomical diagram of the male reproductive tract. Human spermatozoa are produced through the process of spermatogenesis, which occurs in the lining of the seminiferous tubule, located in the testis. Fully differentiated spermatozoa travel through the lumen of the seminiferous tubule to the rete testis. From the rete testis, the spermatozoa travel through the epididymis, undergoing further maturation steps before being stored in the tail of the epididymis. During ejaculation, spermatozoa travel up the vas deferens, joined by seminal plasma from the seminal vesicle before exiting through the urethra. *Reprinted with permission from Cambridge University Press and taken from (Mason, 2010).*

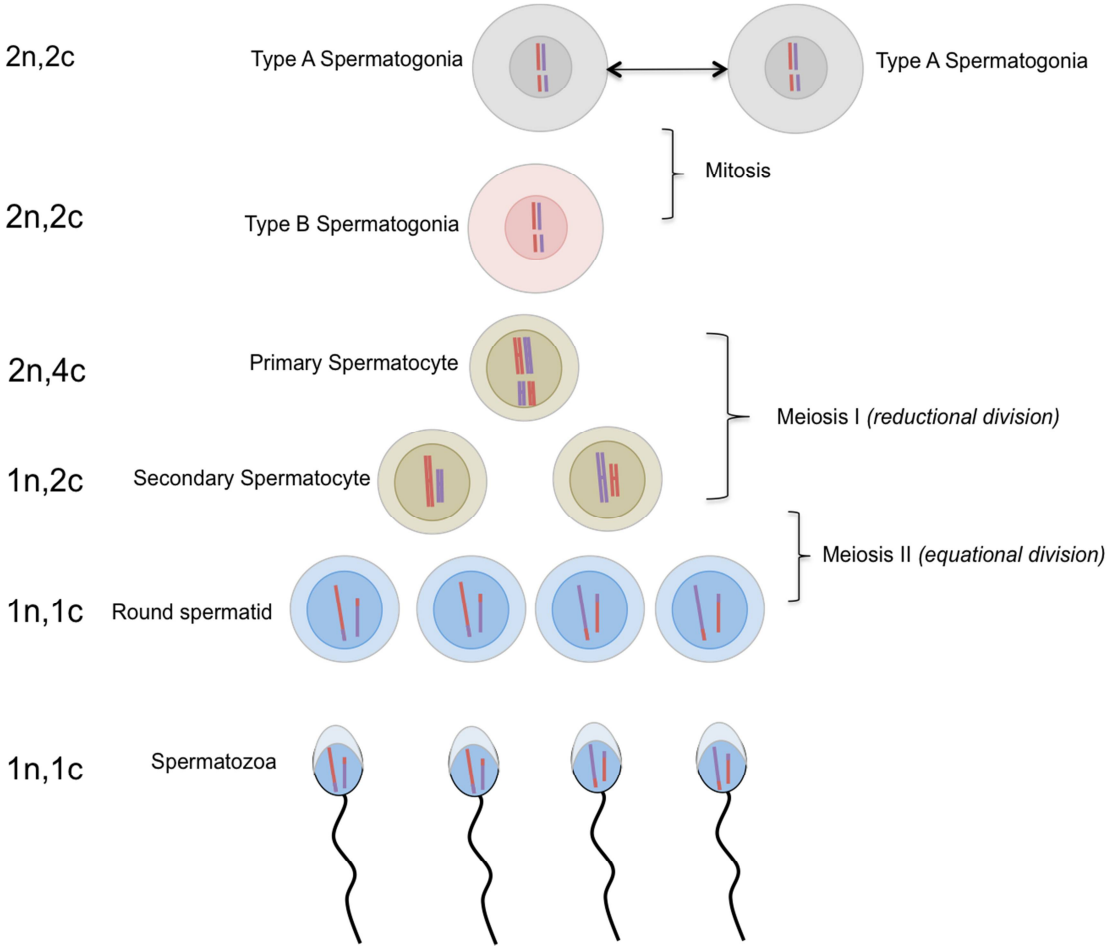

Figure 1.4: Cellular differentiation during spermatogenesis begins with Type A spermatogonia either committing to differentiate into Type B spermatogonia or self-renewal. Type B spermatogonia then further differentiate into primary spermatocytes which are in prophase of meiosis I, consisting of a duplicated complement of DNA, 2n, 4c. In primary spermatocytes, homologous chromosomes line up along the metaphase plate, which allows for homologous recombination, before entering meiosis I and dividing into secondary spermatocytes, which have a haploid complement of chromosomes with sisters chromatids still bound together (1n, 2c). Meiosis I is known as a reductional division as the chromosomal complement has halved to haploid. Secondary spermatocytes then progress through meiosis II to form four round spermatids with one set of chromosomes (1n, 1c). During the final step, spermiogenesis, the round spermatids further differentiate into the specialised form that is required for a functional sperm.

Type B cells then differentiate into primary spermatocytes, which then progress through meiosis I to half their chromosomal complement and form haploid secondary spermatocytes. The final step of spermatogenesis involves a meiosis II division forming haploid round spermatids (Figure 1.4) (reviewed in Wistuba *et al*., 2007). Round spermatids then go through a series of morphological changes during the second stage of this process, known as spermiogenesis. During this stage, many spermatid organelles are remodelled or degraded by ubiquitin-dependent proteolysis (Bedard *et al*., 2011), in order to form a functional sperm with the correct accessory structures. The Golgi apparatus is remodelled to form the acrosomal cap (Moreno *et al*., 2000) and the cytosol becomes the perinuclear theca (Oko, 1995). It is during this step of spermatogenesis that the sperm DNA is remodelled into a more condensed structure more suitable to the function of the spermatozoon (Meistrich *et al*., 2003). Other features of the spermatid are removed, including half of the mitochondrial load and the nuclear pore complexes, involved in mRNA transport (Sutovsky & Manandhar, 2006). Once the round spermatid is remodelled into an elongated form, the process of sperm production is concluded by the release of the sperm cell from the tight associations with Sertoli cells. This last step is known as spermiation. The elongated spermatid is released into the lumen of the seminiferous tubule where the sperm travel to the rete testis and continue their developmental journey through the male reproductive tract (Bronson, 2011).

The architecture of the testis is a complex of looped seminiferous tubules, which end in the rete testis. Spermatogenesis occurs in the epithelium of the seminiferous tubules, which is solely populated by spermatogonial and Sertoli cells (Griswold, 1995). The Sertoli cells surround the germ cells, providing nutrients and are also involved in the hormonal regulation of spermatogenesis (Griswold, 1998). Sertoli cells form tight junctional complexes between each cell, creating a blood-testis barrier which divides the seminiferous epithelium into two compartments: the basal and adluminal compartments (Griswold, 1995). Spermatogenesis and spermiogenesis occur whilst the spermatogonial cells are in close contact with the Sertoli

cells in the basal compartment of the tubule (Griswold, 1995). The final step of the process, spermiation, involves the release of the differentiated spermatid from the close connections with the Sertoli cells into the immune-privileged lumen of the seminiferous tubule.

The process of spermatogenesis is regulated by a complex endocrine feedback loop (reviewed in Holdcraft & Braun, 2004). Gonadotropin releasing hormones (GnRH) secreted from the hypothalamus act on the pituitary gland. Subsequently, Follicle-Stimulating Hormone (FSH) is released, which acts upon Sertoli cells. The pituitary gland also releases Lutenising Hormone (LH), which acts upon Leydig cells. Leydig cells are located in the interstitial space between seminiferous tubules, and upon activation with LH these cells release testosterone. Testosterone then acts upon Sertoli cells, which are involved in the differentiation of spermatogonial stem cells into the spermatozoon.

1.2.1.4 Sperm transit through male reproductive tract

After release into the lumen of the seminiferous tubule and passing through the rete testis, sperm enter the epididymis. At this point, they are incapable of fertilising an egg, as they are biologically immature. Further maturation occurs during transport through the epididymis where spermatozoa acquire fertilisation capability (Moore, 1998). Under the influence of epididymal secretory proteins (Brown *et al*., 1983), spermatozoa acquire the ability to recognise and bind to the oocyte (Hinrichsen and Blaquier, 1980). They also acquire progressive motility (Dacheux *et al*., 1987), through activation of tyrosine phosphorylation signalling pathways (Lin *et al*., 2006). Upon reaching the tail (cauda) of the epididymis, the final storage place before ejaculation (Robaire and Viger, 1995), spermatozoa have acquired the ability to fertilise an egg. In comparison, samples taken from the head (caput) of the epididymis are still biologically immature (Dacheux *et al.*, 1987; Hinrichsen and Blaquier, 1980). During ejaculation, mature spermatozoa are transported through the vas deferens to the urethra, accompanied by secretions from the seminal vesicles and prostate gland, which constitute the seminal fluid

(Nojimoto *et al*., 2009). The accessory proteins present in the seminal fluid contribute to the protection of sperm throughout its transport through the female reproductive tract. An alkaline pH serves to neutralise the acidic pH of vaginal secretions (TeviBenissan *et al*., 1997) and protection from the female immune system through the presence of immune evasion factors such as TGF-β (Lokeshwar and Block, 1992; Robertson *et al*., 2002).

1.2.1.5 Further changes in preparation for fertilisation

Whilst travelling through the female reproductive tract, sperm undergo two further changes in preparation for fertilisation: capacitation and hyperactivation (De Jonge, 2005). During this process, sperm undergo multiple membrane changes, such as cholesterol removal (Zarintash and Cross, 1996) and binding of a calcium binding glycoprotein, SABP (Banerjee and Chowdhury, 1995), to the sperm head (Banerjee and Chowdhury, 1994). These changes constitute the molecular processes of capacitation and cause the sperm head membrane to be more fluid (De Jonge, 2005) and more permeable to Ca^{2+} (Banerjee and Chowdhury, 1995). Binding to ZP3, induces further calcium influxes within the sperm, resulting in initiation of the acrosome reaction (O'Toole *et al*., 2000). This process is enabled by the changes to the plasma membrane during capacitation. Hyperactivation is also induced by changes in membrane permeability and subsequent calcium influxes. This is a change in the beating of the sperm tail, imparting thrust upon the sperm, necessary for penetration of the ZP (Stauss *et al*., 1995) and release from storage in the tubual isthmus (Pacey *et al*., 1995). This is thought to occur as a result of increased intracellular calcium (Suarez *et al*., 1993), which has been linked (Harayama *et al*., 2012) to the additional increase in levels of cAMP (Calogero *et al*., 1998) involved in increased tyrosine phosphorylation, known to be responsible for acquisition of motility (Lin *et al*., 2006).

Before considering how CMV could affect sperm function and assisted conception, it is necessary to discuss the biology and pathogenesis of CMV.

1.2.2 “The stealth virus’” human Cytomegalovirus (CMV)

Cytomegalovirus (CMV) is a member of the *Herpesviridae* family, a group of double-stranded DNA viruses capable of establishing a latent infection in the host, resulting in a lifelong infection with periods of reactivation (Slonczewski and Foster, 2008). There are different types of CMV, specific to different species. There are eight different strains known to infect humans, with over 150 known to infect many other species (Lou & Zhou, 2007).

The prototypical herpesvirus structure (Figure 1.5) consists of a lipid bilayer envelope, derived from cellular internal membranous structures, containing multiple different types of glycoprotein, essential for entry into the host cell. Inside the virion, the double stranded DNA genome is enclosed in a protective protein capsid, known as the nucleocapsid. This is surrounded by the tegument, which contains proteins needed for survival of the virus, including those that support viral gene expression and mechanisms for evading the host response (Lou & Zhou, 2007).

1.2.2.1 Lytic life cycle in susceptible cells

CMV has a broad cell tropism, enabling the infection of multiple different cell types *in vivo*. Among the types of cells CMV can infect are fibroblasts, endothelial, epithelial, monocytes and hepatocytes (Sinzger *et al*., 1995). The ability to infect multiple cell types is thought to be due to a complex mechanism of binding to broadly expressed cellular receptors in conjunction with cell-specific receptors (Compton & Feire, 2007). Upon infection of a cell, a characteristic cytopathic effect is observed. Cells become enlarged and undergo rounding, as a direct result of the formation of intracellular and intranuclear exclusion bodies (Rowe *et al*., 1956).

Binding and entry of CMV to a cell is a complex pathway of multiple binding events via the three different glycoprotein complexes, gCI, gCII and gCIII. Complex gCI, containing a homodimer of glycoprotein B (Cranage *et al*., 1986), is thought to be responsible for the initial binding step (Figure 1.6), involving interaction with a broadly expressed type of receptor, heparin

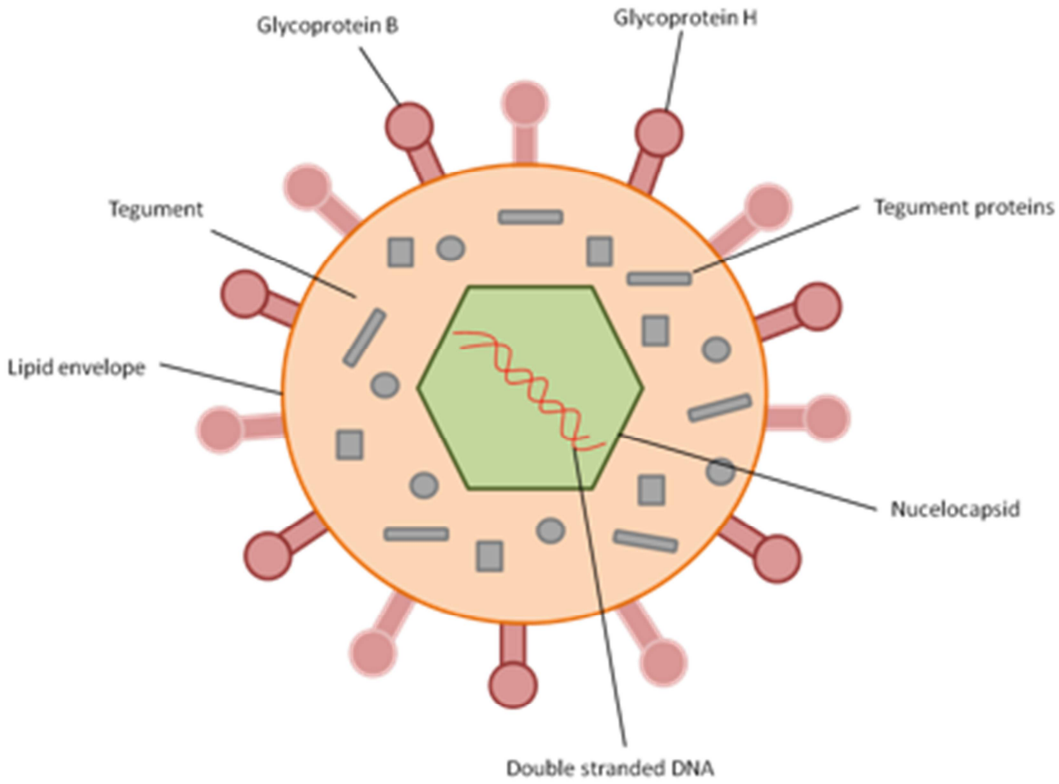

Figure 1.5: The prototypical structure of a herpesvirus virion. The lipid bilayer, derived from the host cell inner membranous structures, contains multiple types of glycoprotein (Emery and Griffiths, 2000), including glycoprotein B and H. Viral glycoproteins are essential for entry into a host cell by binding with putative receptors. The ~250kB double stranded DNA genome is enclosed inside the nucleocapsid which delivers the DNA to the host cell nucleus (Slonczewski and Foster, 2008). The nucleocapsid is surrounded by the tegument, which contains proteins essential for viral survival inside the host cell (Griffiths and Grundy, 1987).

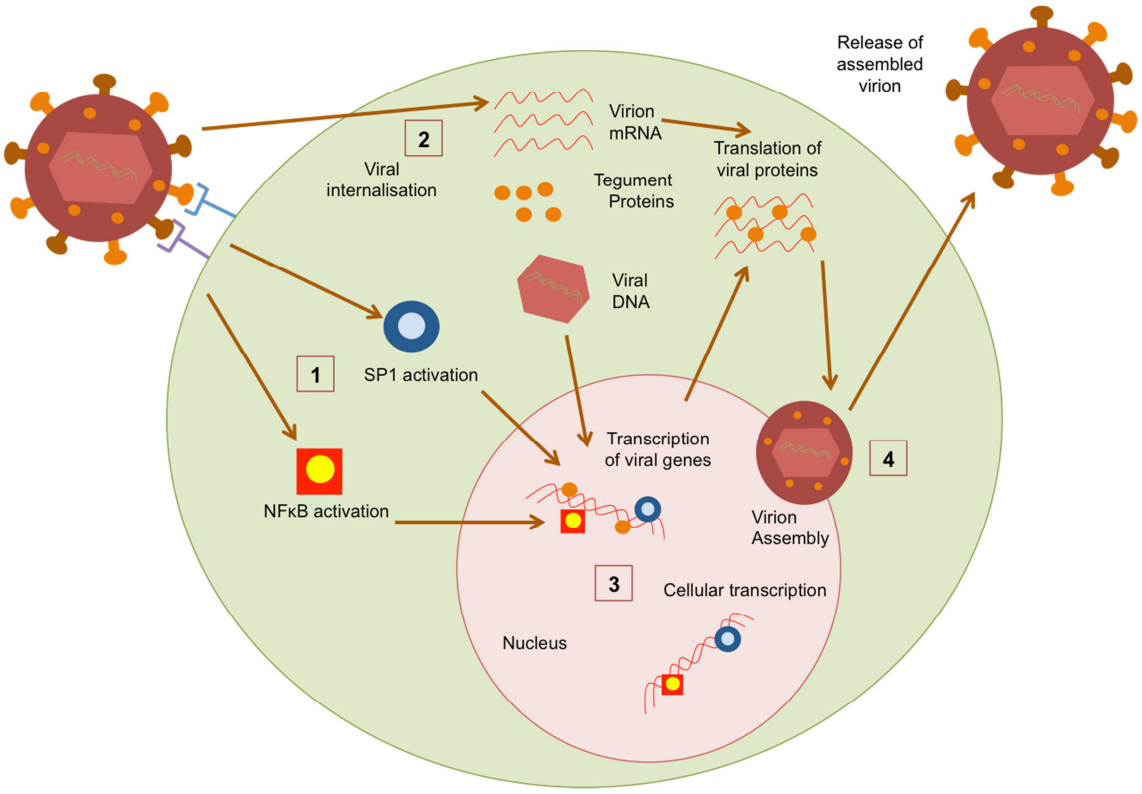

Figure 1.6: Diagrammatic representation of the CMV lytic life cycle. CMV binds to a susceptible host cell via initial interaction between gB and HSPGs. Further interactions with other glycoproteins are also involved which serve to stabilise the interaction and initiate viral and cell membrane fusion. (1) Binding of CMV activates cellular transcription factors necessary for expression of the IE genes. (2) Upon entry to the cell, the nucleocapsid is transported to the nucleus where the viral DNA is delivered. It is thought that CMV also contains mRNAs, which function to translate proteins in the absence of translation of viral DNA (Bresnahan and Shenk, 2000). (3) The replicated viral DNA and translated tegument proteins are packaged into the virion, assembled from newly translated structural proteins. (4) The virus is released from the nucleus where multiple steps of envelopment and de-envelopment result in the virus obtaining its lipid bilayer. During transit through the cytoplasm and the various trafficking vehicles the cell employs, the virion acquires the remaining elements of the viral envelope and is ultimately released from the cell. *Adapted with permission from Nature Publishing Group from (Huang and Johnson, 2000).*

sulphate proteoglycan (HSPG) (Carlson *et al*., 1997; Compton *et al*., 1993). The second complex, gCII, containing glycoproteins gM and gN, is also involved in this interaction, by binding to HSPGs (Kari & Gehrz, 1992). gCII potentially also aids gB in binding to a secondary receptor and stabilising the interaction (Boyle & Compton, 1998). The third glycoprotein complex, gCIII, is a heterodimer containing gH and gL, complexed with either gO or an unknown product of a particular region of the CMV genome, the UL128 locus (Huber & Compton, 1997; Ryckman *et al*., 2008) This complex is thought to be involved with the fusion of virus and host membranes, allowing entry of the virus to the host cell (Topilko and Michelson, 1994; Compton *et al*., 1992). Whilst fusion of the virus and host cell membranes is thought to be the main route of entry to a cell, pathways that employ endocytosis-mediated entry are also thought to play a role in the entry of CMV to certain cell types (Ryckman *et al*., 2006; Wang *et al*., 2007).

Upon entry to the cell, the nucleocapsid is delivered to the nucleus (Figure 1.6), facilitated by the capsid and tegument components, in a process known as uncoating (Compton & Feire, 2007). Once the viral DNA is delivered to the nucleus, the viral genes are expressed in a temporal cascade (Sinclair & Sissons, 2006). Gene expression begins with the immediate early (IE) genes, which does not require *de novo* protein synthesis to occur prior to expression. These genes are responsible for controlling viral and cellular gene expression in order to optimise the cellular environment for production of daughter progeny (Sinclair and Sissons, 2006). The expression of IE genes is essential for the production of viral progeny as they are involved in activating the expression of the early (E) genes which function in the replication of the viral DNA (Emery & Griffiths, 1990). Finally, the late (L) genes are expressed once DNA replication is complete (Emery & Griffiths, 1990). The L genes are mainly structural proteins, such as those required for the nucleocapsid. Upon entry to a host cell, expression of IE genes also serves to promote survival in the cell by hijacking the host cell DNA synthesis and cell cycle pathways, as well as encoding mechanisms for avoiding cellular defence mechanisms (reviewed in Fortunato *et al*., 2000).

Viral assembly begins in the nucleus, where the newly replicated DNA is packaged into the nucleocapsid (Griffiths and Grundy, 1987). The capsid then acquires its tegument proteins and through a process of repeated steps of envelopment and de-envelopment, the virion acquires its lipid bilayer (Skepper *et al*., 2001). The mature virion is then transported to the host cell membrane where it is released into the extracellular environment via exocytosis (Das *et al*., 2014) (Figure 1.6).

The activation of the IE genes is thought to be the event that commits CMV to a productive (lytic) life cycle. However, in addition to undergoing a productive life cycle, CMV is also able to undergo a latent cycle, enabling the virus to remain hidden from the cellular immune response (Sinclair and Sissons, 2006).

1.2.2.2 Sites of CMV latency and persistence

All members of the *Herpesviridae* family are able to establish sites of latency within the host. This serves as a mechanism to ensure the continued survival of the virus through multiple opportunities to infect other hosts during repeated bouts of reactivation. Sites of latency are regions of specific cellular sub-types where the viral genome is present, but there is no production of infectious virus (Sinclair, 2008). Unlike other viruses which establish persistent or latent infections, such as HIV or EBV, the CMV genome is not thought to integrate into the genome of the host cell, rather it remains in an episomal form, similar to a circular plasmid, in the nucleus of the cell (Bolovan-Fritts *et al*., 1999).

Different herpesviruses use different cell types as sites of latency and CMV utilises myeloid progenitor cells (Mendelson *et al*., 1996). When these progenitor cells undergo differentiation into monocytes and ultimately macrophages/dendritic cells, CMV is reactivated (Taylor-Wiedeman *et al*., 1994), allowing the virus to re-enter the lytic life cycle, resulting in secretion of infectious virus by the host (Figure 1.7). Due to this unique method of reactivation, CMV has been described as a persistent infection in the cell populations where it remains latent (Seckert *et al*., 2012).

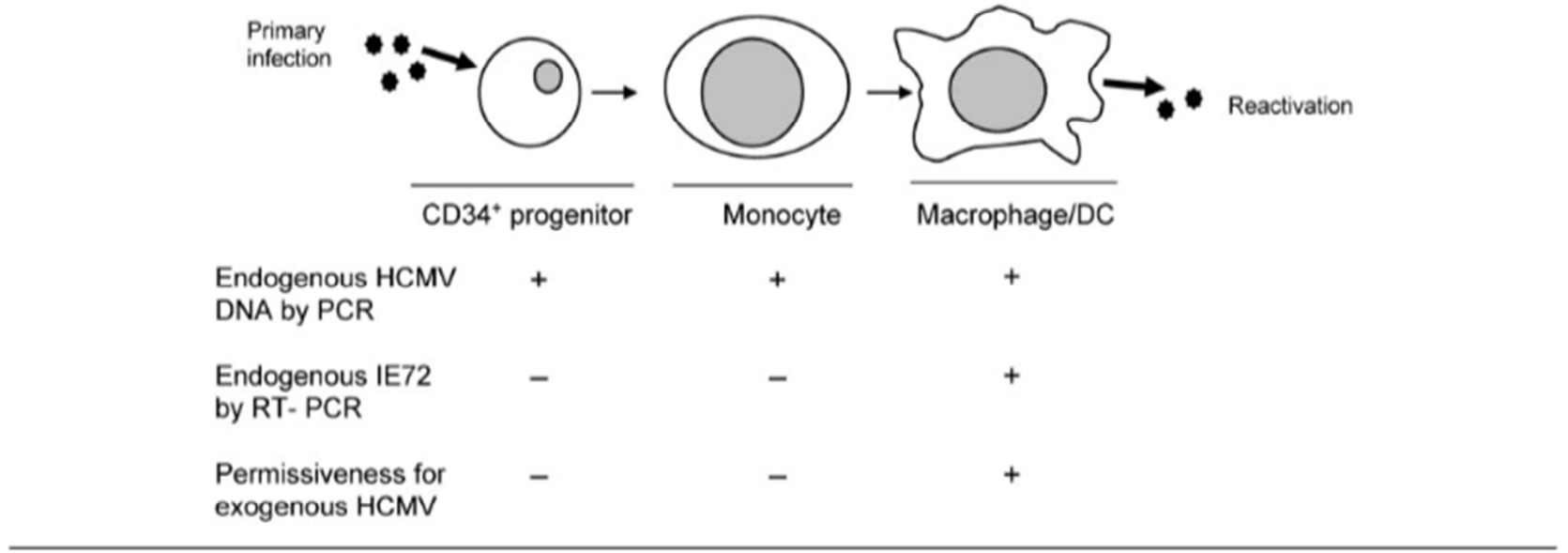

Figure 1.7: A model of reactivation from latency upon progenitor cell differentiation into macrophages. One of the sub-types of cell CMV is able to establish sites of latency in are the CD34+ progenitor cell type, which gives rise to monocytes and macrophages/dendritic cells upon differentiation. In the progenitor cells, CMV can be detected by PCR but no expression of CMV IE genes is detected, showing no viral gene expression is occurring, supporting the presence of CMV in these cells in a latent form. The same situation is found upon differentiation into monocytes but upon terminal differentiation into macrophages or dendritic cells, expression of CMV IE genes is found, supporting the ability for CMV to reactivate and enter a lytic life cycle in this cell type. Also, prior to this point, the progenitor cells are not permissive to exogenous CMV infection, further supporting the presence of CMV in these cells in a latent form only. *Reprinted from (Sinclair and Sissons, 2006) with permission from the Microbiology Society.*

Constant reactivation of the virus due to monocyte differentiation modulates the immune system as more cytotoxic T lymphocytes (CTL) become dedicated to stopping dissemination of the virus during reactivation episodes (White *et al.*, 2012). This can leave the host vulnerable to other infections in a process called immunosenescence. Normally, the immune system is able to control the reactivation of CMV, but in those with compromised immune systems, reactivation of CMV from latency can result in a systemic infection that can be life threatening (Smyth *et al*., 1991). Reactivation from latency is dependent upon the expression of the IE genes (Sinclair and Sissons, 2006), which are repressed during latency. This is achieved by modulation of the chromatin surrounding the promotor regions of these genes, making the DNA inaccessible to transcription factors, conferring transcriptional silence and a state of latency (Reeves *et al*., 2005a). During differentiation of the progenitor cells into macrophages and dendritic cells, this repressive chromatin is remodelled again and transcription of viral IE genes is initiated (Reeves *et al*., 2005b). The mechanism that modulates this chromatin remodelling is not fully understood in CMV, however it is known that HSV utilises latency associated transcripts (LATs) to achieve this. These genes are expressed during latency to maintain a state of repressed chromatin surrounding the promotor regions of IE genes (Wang *et al*., 2005). CMV is known to express similar transcripts, known as cytomegalovirus latency transcripts (CLTs) (Lunetta and Wiedeman, 2000), but their function in maintaining latency in CMV is not clear.

1.2.2.3 CMV virulence vs host immune response

A productive life cycle for CMV is a delicate balance between promoting viral replication and growth whilst down regulating cellular growth and promoting cell survival (Sinclair and Sissons, 2006). In addition, the ability to establish a latent infection and remain persistent within a population of cells relies on the ability to effectively evade the host immune response. CMV has developed many intelligent mechanisms for continued survival within a host (reviewed in Miller-Kittrell and Sparer, 2009). The majority of CMV gene products are thought to be involved in modulation of the host response to CMV, as they

are not essential for viral growth in culture (Dunn *et al*., 2003; Yu *et al*., 2003). This section will cover a few of these mechanisms in order to understand how the pathogenesis of CMV has evolved.

Initial interaction of the virus with the host cell initiates an innate immune response, thought to be through the Toll-Like Receptor 2 (TLR2) (Compton *et al*., 2003). This response results in the activation of several transcription factors, including NF-κB and SP1, which has an effect on cell gene expression (Yurochko *et al*., 1995). Initial activation of these transcription factors is thought to serve to enable the transcription of IE genes. However, in addition to initiating viral gene transcription, virally encoded proteins act to shut down cellular gene expression, allowing for all resources to be diverted to the transcription of viral genes only (Castillo & Kowalik, 2002). CMV also acts to stop the cell cycle progressing, further acting to focus cellular resources on viral reproduction, as opposed to cellular growth and division (reviewed in Fortunato *et al*., 2000).

After infection with CMV, a peak in the activation of p53, a gene responsible for growth arrest and activation of apoptosis, is observed (Muganda *et al*., 1994). Whilst activation of cell cycle arrest is beneficial to CMV, induction of apoptosis is not. Despite employing these mechanisms to promote viral replication over cellular replication, the host immune response deployed upon activation of such mechanisms is not beneficial for the continued replication and dissemination of CMV. As such, CMV has developed mechanisms to evade these innate responses. Whilst p53 is activated, CMV is able to repress apoptosis through the action of two viral genes, which prevents the cell from dying. One viral gene inhibits caspase-8 (Skaletskaya *et al*., 2001), a key component in the initiation of apoptosis. The second gene prevents cytochrome C release from mitochondria, which is an intermediary step in the pathway of mitochondrion mediated apoptosis (Goldmacher *et al*., 1999; Roberedo *et al*., 2004).

Similarly, infection with CMV results in the activation of NF-κB, which is required to induce viral replication (Yurochko *et al*., 1995), but this also

results in the activation of cytokines and interferon (Boehme *et al*., 2004). Activation of this signalling pathway acts to induce an inflammatory response, resulting in the recruitment of cells involved in the innate immune reaction to the site of infection. Whilst this might at first appear disadvantageous for CMV, it actually serves as an opportunity for further dissemination. The cells of the innate immune response, such as macrophages, are permissive to CMV infection and therefore present as additional sources for its continued productive infection (Castillo & Kowalik, 2002).

It is clear that during the initial stages of CMV replication, there is a fine balance between activating pathways that allow CMV to replicate whilst also down regulating cell death pathways. However, the immune system is generally able to eventually control the productive infection, but not before CMV is able to establish sites of latency within some cells. During periods of reactivation, the cell-mediated immunity, primarily CD8+ lymphocytes, will continue to fight the productive infection and keep the infection below a level at which disease is manifested (Griffiths and Grundy, 1987). However, CMV has developed ways to evade these mechanisms, in order to promote its spread from one person to another. In individuals experiencing a reactivation, symptoms of CMV might not be present, but CMV will be shed in the bodily fluids of the individual, making them infectious to others (Ling *et al*., 2003; Cannon *et al*., 2010).

One major mechanism CMV has evolved to avoid detection by cytotoxic T-lymphocytes (CTL) is to downregulate the presentation of viral antigens on the surface of the cell. Major histocompatability complex (MHC) class I molecules are responsible for presenting viral antigens to CTLs, which act to destroy the cell and subsequently destroy the virus (Slonczewski and Foster, 2008). CMV prevents this response from occurring by initiating the degradation of MHC class I molecules (Jones *et al*., 1995). CMV also produces a MHC class I homologue which inhibits destruction of the infected cell by Natural Killer (NK) cells (Reyburn *et al*., 1997). Similarly, CMV controls cellular production of cytokines in response to infection, by

sequestering a certain type of cytokine and removing it from the extracellular environment, preventing the recruitment of NK and CTL cells to the site of the infection (Bodaghi *et al*., 1998).

These are only a few of many mechanisms in place to evade the human immune response. Over many years of co-evolving with the mammalian immune system, CMV has developed many more mechanisms to evade this response. Effective evasion of attack from the immune system allows promotion of virus survival, explaining why CMV is so successful at persisting in the human population. If these immune evasion mechanisms were not in place, the virus would be destroyed before it was able to establish sites of latency within the host. Conversely, in the immunocompromised individual, these mechanisms of immune evasion are redundant as there is no immune system for CMV to fight against, allowing the virus to become widely disseminated in the body and cause serious damage to the infected organs. At this point, the symptoms of CMV pathogenesis become apparent and the virus becomes life threatening to the host.

1.2.2.4 CMV pathogenesis

Infection with CMV can be grouped into three different types: (i) primary infection, where CMV is contracted for the first time; (ii) re-infection, with a different strain of CMV; and (iii) reactivation of the individual's own latent virus. Each type of infection can result in the same disease manifestation, dependent on the integrity of the immune system of the individual.

Infection with CMV can occur at any age and can be contracted through contact with any bodily fluid from an infected individual, including saliva, urine, breast milk (Hamprecht *et al*., 2001) and through sexual secretions (Chandler *et al*., 1985; Handsfield *et al*., 1985; Staras *et al*., 2008). Infected children are thought to be a large contributor to the transmission of CMV (Cannon *et al*., 2011), and are a particular risk factor to pregnant women (Pass *et al*., 1986), due to the continued secretion of CMV during the first few years of life (Stagno *et al*., 1975) and the frequent transmission between children (Adler *et al*., 1985). Infection with CMV is normally asymptomatic in a

healthy individual, although occasionally it can result in CMV mononucleosis (Manfredi *et al*., 2006), similar to the symptoms of Epstein-Barr virus (EBV). Infection can also result in CMV hepatitis in both healthy individuals, and as an added complication in immunocompromised patients (Castiglione *et al*., 2000).

Primary infection or reactivation of latent virus in immunocompromised individuals can lead to a host of diseases through direct and indirect effects (Freeman, 2009). In AIDS patients, CMV is a common opportunistic pathogen resulting in progressive blindness due to inflammation of the retina (Sugar *et al.*, 2012). However, with the advent of effective antiretroviral therapy against HIV, the incidence of CMV-induced retinitis has decreased (Sugar *et al*., 2012). CMV is also thought to drive the pathogenicity of HIV by acting as a co-factor to the retrovirus (Griffiths, 2006).

Prior to the advent of antiviral drugs, CMV was a major concern to organ transplant patients due to the risk of transmission of CMV via an organ from a seropositive donor (Grundy *et al*., 1988). This is of most risk to seronegative recipients, but is also of concern to seropositive recipients due to the risk of reactivation and re-infection (Smyth *et al*., 1991). A further complication in this scenario is the immunosuppressed nature of the patient, necessary during transplant surgery to prevent rejection of the organ. Systemic CMV infection in a transplant patient can result in a range of direct and indirect effects, including pneumonitis (de Maar *et al*., 1998), hepatitis, retinitis and death (Rubin, 1989).

The elderly are also vulnerable to the effects of CMV due to the biological phenomena of immunosenescence. As the host experiences continued bouts of CMV reactivation, an increasing number of cytotoxic T lymphocytes (CTL) become dedicated to killing CMV infected cells. This leaves the host vulnerable to new infections, such as the seasonal influenza virus, due to a lack of naive T cells available to fight the new infection (Khan *et al*., 2002).

If acquired *in utero*, CMV can have severe health consequences for the neonate, ranging from progressive bilateral sensorineural hearing loss

(Grosse *et al.*, 2008) to severe neurological disorders (Preece *et al*., 1983). Clinically recognised as congenital CMV, the effect CMV has on neonates is of primary concern for the objectives of this thesis and will be discussed in more detail in Section 1.2.2.6.

Pathogenesis associated with CMV infection correlates with the viral load of CMV in the blood of a patient in a non-linear relationship (reviewed in Griffiths *et al*., 2012). This is known as the 'threshold effect', in which the chance of developing symptoms and the severity of those symptoms increases once the viral load passes a threshold value (Emery *et al*., 2000). In transplant patients, the chance of developing viraemia, and subsequent end-organ disease increases greatly after passing this threshold value (Figure 1.8a) (Cope *et al*., 1997a,b). A similar relationship between viral load and severity of sensorineural hearing loss is observed in congenital CMV infection (Figure 1.8b) (Walter *et al*., 2008). In transplant patients, pre-emptive treatment given before the level of CMV reaches this critical threshold value has proven effective at reducing the incidence of CMV related disease in these patients (Mattes *et al*., 2004). This type of treatment relies on the ability to detect CMV accurately at low levels, in order to monitor the progression of the viral load. This is now possible due to the development of DNA technologies. Prior to this, different methods of diagnosis and treatment were employed to try and control the development and progression of CMV related disease in infected individuals.

1.2.2.5 Diagnosis and treatment

Diagnosis of CMV infection can only be determined by detecting the virus in a biological specimen from the infected individual. Prior to the advent of DNA technologies, using cell culture methods to detect CMV was the only method of diagnosis. This involved exposing permissive cells to patient swabs and waiting until the typical CPE developed. The time taken for CPE to develop is typically two to three weeks, therefore this method was hindered by the long lead time on test results (Griffiths *et al.*, 1984). In the early 1980's scientists searched for a faster method of diagnosis, and a technique was developed

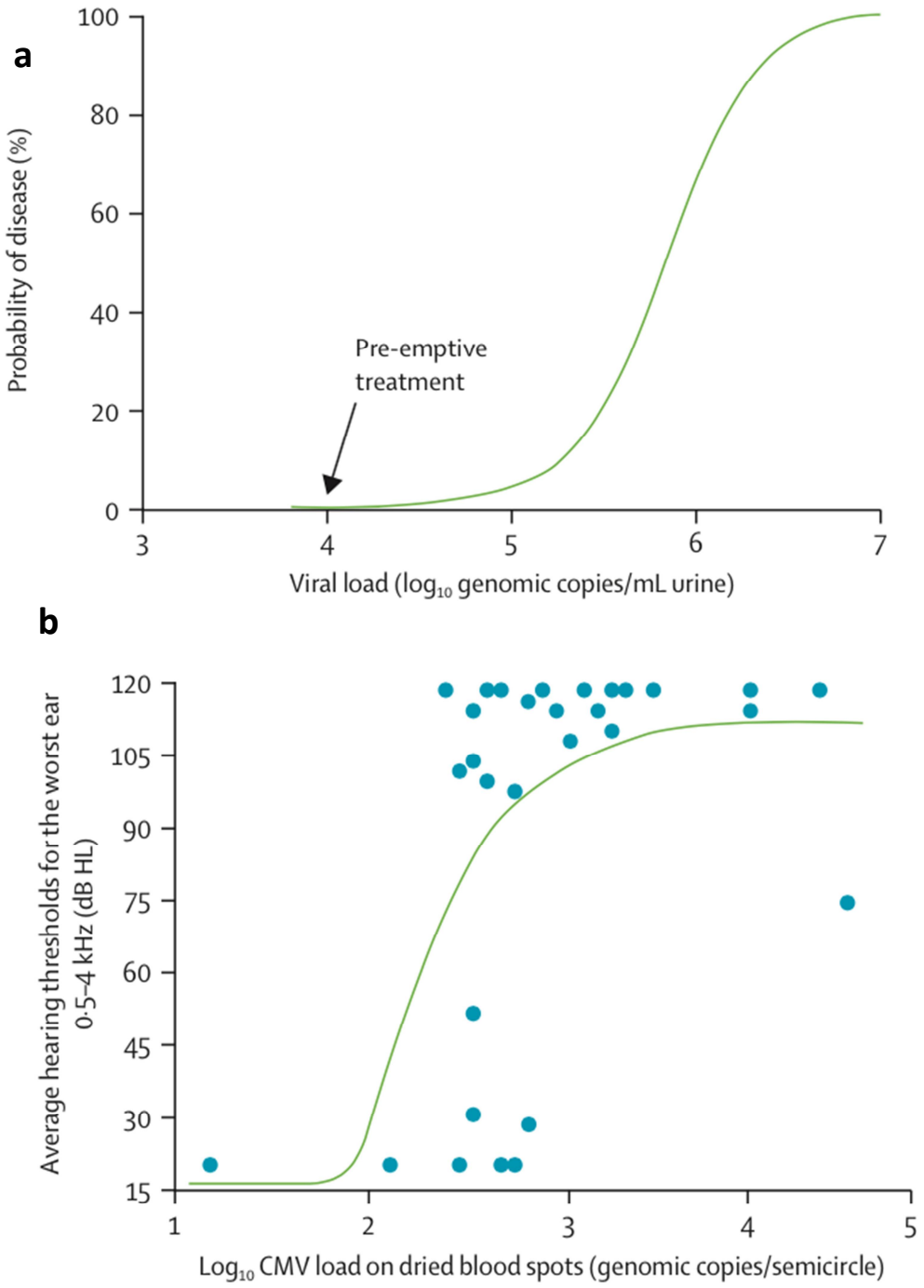

Figure 1.8: Graphs depicting the 'threshold model' of infection for CMV-related disease. Graph (a) depicts the increase of disease risk in transplant patients once the viral load passes a certain 'threshold' level. Pre-emptive treatment prior to this point helps to protect the patient from systemic disease (Cope *et al*., 1997a,b). Graph (b) displays a similar relationship between viral load and the severity of hearing loss in neonates (Walter *et al*., 2008). *Reprinted from (Griffiths et al., 2012) with permission from Elsevier. Originally published in (Cope et al., 1997b) and (Walter et al., 2008) and permission was granted for re-use from the Journal of Medical Virology and BMI Publishing Group Ltd, respectively.*

utilising a monoclonal antibody against proteins found to be present in cultures within hours of inoculation with CMV (Stinski *et al*., 1982). By using indirect immunofluorescence, the detection of early antigen fluorescent foci (DEAFF) test was proven effective at rapidly detecting the presence of CMV, however it was ineffective at detecting low viral titres, which were often found in blood (Griffiths *et al*., 1984). Another early test for determining CMV infection was the pp65 antigenemia test. This method utilises a monoclonal antibody to detect a viral structural late protein, pp65, which is expressed in blood leukocytes (Van der Bij *et al*., 1988).

The methods described above are able to provide information on whether infectious CMV is present, but they are both labour intensive and insensitive (Ross *et al*., 2011). Another method of diagnosing CMV that has been used for many years is the detection of CMV specific IgG and IgM antibodies via serology testing. Whilst this technique is retrospective and is not able to give an accurate picture on the presence of infectious virus in an individual, it is able to show if a person has previously been infected with CMV and therefore harbouring a latent infection.

The detection of CMV IgM is thought to be indicative of a primary infection, which is known to cause more severe symptoms if contracted in the neonate from the mother (Fowler *et al*., 1992). However, the presence of IgM antibodies has also been shown to be present in re-infection and reactivation (GrangeotKeros *et al*., 1997), which causes less severe symptoms in the neonate (Fowler *et al*., 1992). These discrepancies presented problems when counselling expectant mothers on the risk of transmission to their fetus, as the three different types of infection carry significantly different risks of symptomatic infection in the neonate. Due to this, a different test, which was able to better quantify the risk of symptomatic congenital CMV, was required.

In an attempt to fulfil this diagnostic requirement, the IgG avidity test was developed, aimed at testing the strength of binding between antigen and antibody. A strong avidity was thought to be indicative of a re-infection or reactivation whereas a weak avidity indicated a primary infection

(GrangeotKeros *et al*., 1997). This technique was first used for CMV infection in the early 1990's (Boppana and Britt, 1995) and was able to give mothers more accurate information on the risk of having a baby born with congenital CMV, through a more accurate understanding of the type of infection the mother was experiencing.

In more recent years, more sophisticated techniques for detecting CMV at even low titres were developed upon the advent of DNA technologies, most importantly, the discovery of polymerase chain reaction (PCR) (Saiki *et al*., 1988). PCR detection of CMV DNA in liver transplant patients showed that the presence of viral DNA was associated with the development of CMV related diseases and rejection of the organ (Lao *et al*., 1997). This evidence showed that detection of CMV could predict disease progression in the patient. Subsequently, quantitative PCR (qPCR) assays were developed which allowed virologists to accurately quantify the levels of CMV DNA in the blood of a patient. Through this, it is now possible to use pre-emptive treatment, preventing the viral load reaching the critical threshold value and the onset of symptoms (Mattes *et al*., 2004).

The first antiviral drug targeted to CMV was ganciclovir, which acts by disrupting viral DNA synthesis. This drug was shown to be effective in the prevention of CMV pneumonitis in bone marrow transplant patients (Schmidt *et al*., 1991), also showing that pre-emptive therapy was an effective treatment plan in preventing disease onset after organ transplantation (Rubin, 1991). In addition, ganciclovir was also effective in protecting the hearing of neonates born with symptomatic congenital CMV through an intensive six-week course given after birth (Kimberlin *et al*., 2003). Subsequently, an oral version of the drug, valganciclovir was developed to treat neonates born without symptoms (Kimberlin *et al*., 2008), previously excluded from treatment due to the toxic side effects of ganciclovir (Schmidt *et al*., 1991).

Attempts to develop a vaccine for CMV have been underway for many years and multiple phase I and II clinical trials have been carried out (reviewed in

Reider & Steininger, 2014). Whilst there have been multiple vaccine candidates trialled, the development of a vaccine against CMV was hindered by evidence suggesting that prior immunity to CMV did not confer protection against the virus. Cases of seropositive mothers having babies born with congenital CMV fuelled this argument (Fowler *et al.*, 1992). Fortunately, this discrepancy was explained by further discoveries showing that prior immunity only failed to confer protection against a different strain of CMV (Boppana *et al*., 2001).

Most of the current vaccine candidates are targeted toward CMV gB, and a vaccine containing recombinant gB was shown to reduce the transmission of CMV and decrease death rate in guinea pigs (Schleiss *et al*., 2004). Whilst these findings may not be translatable to humans, a phase II clinical trial using CMV gB in combination with a water emulsion adjuvant, MF59, was found to be safe and effective at reducing the length of viraemia in post transplant patients. Whilst this trial did not demonstrate complete immunity to CMV, the levels of CMV antibodies were increased in those treated with the vaccine over 6 months, in comparison to a placebo (Griffiths *et al*., 2011). Furthermore, in seronegative patients who received an organ from a seropositive donor, the number of days treated with ganciclovir was reduced (Griffiths *et al*., 2011). These findings are promising for the future of CMV vaccines, in particularly for transplant patients in reducing post surgery complications related to CMV. However, the development of a CMV vaccine is also thought to provide hope for preventing the transmission of CMV from mother to fetus (Griffiths, 2012).

1.2.2.6 Congenital CMV infection

Congenital CMV infection is the leading cause of sensorineural hearing loss in neonates along with neurological problems such as cognitive impairment, visual impairment and cerebral palsy (Fowler *et al.*, 2003). A seronegative woman has a 32% chance (Kenneson and Cannon, 2007) of transmitting the virus to the fetus through infection of the placenta (Hemmings *et al*., 1998). It is estimated that the overall birth prevalence of CMV is 0.7% (Dollard *et al*.,

2007). Most babies born with the virus will remain asymptomatic, however a small proportion ~13.5% (Dollard *et al*., 2007) will develop symptoms within the first year of life and ~12% will be born with symptoms at birth (Dollard *et al*., 2007; Preece *et al*., 1983).

It is estimated that between 40-58% of symptomatic infants will develop permanent severe symptoms of CMV infection (Dollard *et* al., 2007). The severity of any symptoms is proportional to the viral load, which was first observed by detecting the amount of excreted virus in the urine of infants (Stagno *et al*., 1975). Those born with symptoms had greater quantities of virus in their urine than those who were born without symptoms (Stagno *et al*., 1975). Progressive hearing loss of babies born with no symptoms during the first year of life was explained due to the continual excretion of virus in the urine some time after birth, indicating a continual productive infection (Stagno *et al*., 1975).

The onset of symptoms due to a high viral load is a direct result of maternal antibody status (Fowler *et al*., 1992). In women with a primary infection, the virus is able to establish viraemia and infect the layers of the placenta and pass onto the fetus (Hemmings *et al*., 1998). In mothers with prior immunity, the virus is managed by the immune system, keeping the viral load low and preventing, or limiting, placental transfer (Fowler *et al*., 1992).

The three types of infection, primary, re-infection and reactivation, can be ranked with regards to the risk of transmission of CMV to a neonate and the severity of any subsequent symptoms. Primary infection during pregnancy poses more risk to the neonate, due to a high viral load in the mother, increasing the chance of transmission and onset/severity of symptoms. In women experiencing a recurrent infection (reactivation or reinfection), the rate of transmission of CMV is ~1.4%, in comparison to 32% in women experiencing a primary infection (Kenneson and Cannon, 2007). Additionally, in mothers experiencing a primary infection, symptoms of congenital CMV are more likely to occur and the severity of the symptoms is likely to be higher (Fowler *et al*., 1992). According to a meta-analysis of the risk of

hearing loss, the risk was slightly greater in babies born to mothers with a primary infection (13%), than a non-primary infection (11%) (de Vries *et al*., 2013).

Despite these statistics that show a higher risk of vertical transmission in seronegative mothers, it is estimated that the majority of babies born with CMV are from seropositive mothers that have experienced a recurrent infection (de Vries *et al*., 2013). Approximately 8% of pregnancies in seropositive women result in a re-infection with an exogenous strain of CMV (Yamamoto *et* al., 2010). This evidence appears to contradict the evidence that a primary infection poses a greater risk of transmission to a fetus and the onset of symptoms. However, it is thought that this epidemiological finding is due to the evidence that "the risk of re-infection among seropositive women outweighs the combined risks of both acquisition and maternal-to-fetal transmission among seronegative women" (de Vries *et al*., 2013). Therefore, whilst the greatest risk of a baby being born with congenital CMV is a woman experiencing a primary infection during pregnancy, the role of seropositive women in contributing to the rates of congenital CMV infection should not be underestimated.

Whilst the main source of infection to both seronegative and seropositive expectant mothers is thought to be infected children (Cannon *et al*., 2011; Pass *et al*., 1986), sexual transmission of CMV does occur, and can also be thought of as a risk factor for pregnant women (Chandler *et al*., 1985; Handsfield *et al*., 1985; Staras *et al*., 2008). Furthermore, the evidence that CMV is able to survive the cryopreservation process of sperm (Mansat *et al*., 1997), presents a dilemma to the assisted conception field when using donor sperm from CMV positive men.

1.2.3 Male infertility and the implications of CMV in assisted reproduction

Before considering the role CMV plays in donor insemination, it is important to consider the wider context of assisted reproduction, including the techniques available and the reasons for requiring intervention. As previously

stated, one in seven heterosexual couples will experience fertility problems; defined as the failure to conceive after 2 years of unprotected sexual intercourse. According to the 2013 National Institute for Clinical Excellence (NICE) fertility assessment guidelines, fertility problems can be divided into 3 categories. Female factors, including tubal damage (20%), ovulatory disorders (25%) or uterine/peritoneal disorders (10%), a further 25% can be attributed to unexplained fertility in either party (male or female) and the final 30% constitutes issues surrounding male factor infertility (NICE, 2013).

1.2.3.1 Male factor infertility

The majority of male infertility is of an unknown cause (idiopathic), however there are some known medical problems associated with fertility issues in the male, including varicocele, accessory gland infection (Irvine, 1998) or the absence of a vas deferens which is often found in cystic fibrosis sufferers (Chillon *et al*., 1995).

Aside from issues that affect the male reproductive tract, male infertility can arise from problems with the spermatozoa themselves. A complete lack of production of any sperm is known as azoospermia, which can be divided into non-obstructive and obstructive causes. Alternatively, sperm may be produced, but in small numbers (oligozoospermia), or defective due to problems with sperm production or maturation, resulting in reduced motility (asthenozoospermia), or have an abnormal morphology (teratozoospermia) (World Health Organisation, 2010). Defective sperm are unable to cope with the female reproductive environment and get filtered out, resulting in only the normal sperm reaching the Fallopian tubes and potentially fertilising the egg (Suarez and Pacey, 2006). Subsequently, this reduces the reproductive potential of an individual. Defects in sperm production or sperm function can be caused by genetic mutations, lifestyle or occupational factors or due to infection.

Non-obstructive azoospermia can be caused by a number of genetic mutations, including microdeletions in the azoospermia factor (AZF) region of the Y chromosome, which deletes genes involved in spermatogenesis (Oates

et al., 2002). Similarly, mutations in genes involved in the endocrine regulation of spermatogenesis can result in an inability to produce sperm, such as hypogonadotropic hypogonadism (Bhagavath *et al*., 2006). Obstructive azoospermia, where sperm are produced but unable to be ejaculated due to blockages in the reproductive tract (Seshagiri, 2001), is often caused by a reproductive tract infection.

An infection in the reproductive tract can also have a direct effect on sperm. Infection leads to inflammation, which results in the production of reactive oxygen species (ROS). ROS can be produced by sperm themselves prior to ejaculation, or by seminal leukocytes, which would affect sperm during ejaculation and after ejaculation, and would result in sperm membrane lipid peroxidation (Aitken *et al*., 1989). This changes the composition of the sperm membrane and can impair sperm function, including the ability to fuse to the oocyte. Infection with certain pathogens, such as *Chlamydia trachomatis* can also result in direct damage to the spermatozoon, either through exposure prior to ejaculation, during storage in the epididymis for example, or after ejaculation either through exposure during the ejaculatory process, or exposure from pathogens present in the female reproductive tract. *In vitro* incubation of sperm with *C. trachomatis* leads to decreased motility and increased sperm death (Hosseinzadeh *et al*., 2003), thought to be due to a receptor mediated interaction between the bacterium and sperm.

Whilst the reasons for male factor infertility are able to be diagnosed in many cases, the underlying cause is often not known. The presence of common pathogens, such as CMV, in the semen of men, might indicate an infection throughout the reproductive tract with a pathogen that has the potential to affect sperm function, and therefore might provide an explanation for infertility in men that present without an obvious cause.

1.2.3.2 Assisted conception

Different assisted conception treatment options are available for infertile couples based on the medical cause of their fertility issues, including intrauterine insemination (IUI) (Guzick *et al*., 1999), IVF (Edwards and

Steptoe, 1983) and ICSI (Palermo *et al*., 1992). When infertility is attributed to a male factor cause, such as oligozoospermia or asthenozoospermia, ICSI is the most common form of assisted reproduction technology employed in the UK, with over half of fresh IVF cycles in 2013 using ICSI to achieve a pregnancy (HFEA, 2013b). In cases of azoospermia, some couples may consider the use of a sperm donor, and in other instances of unresolved infertility issues, donor eggs or embryos might be used. Treatment using donor gametes is not only available for infertile heterosexual couples but also to same-sex couples (Baetens and Brewaeys, 2001) and single women (Leiblum *et al*., 1995) who are considered to suffer 'social infertility' and need medical intervention to reproduce (Pacey, 2010).

1.2.3.1 Donor conception

Donor conception using donor sperm (Clarke *et al*., 1997a), eggs (Wiggins and Main, 2005) or embryos (Devroey *et al*., 1989) was carried out in 1 in 10 fresh IVF cycles in 2013 (HFEA, 2013a). The number of donor insemination and IVF cycles using donor sperm rose in 2013 from 2012 by 3.6% and 6.5% respectively, highlighting the increasing demand for this type of treatment.

The ability to use donor gametes in assisted conception has been facilitated by the advent of freezing gametes and embryos in liquid nitrogen. The discovery of cryogenic properties of agents such as dimethyl sulphoxide (DMSO) (Chen, 1986) and glycerol (Paz *et al*., 1991) revolutionised this field as it allowed freezing of gametes with limited damage. Cryopreservation of eggs, sperm and embryos is now routinely used in both donor and non-donor assisted conception procedures. Donor conception poses a variety of health and ethical risks to the mother and unborn child. Not only is there a risk of transferring genetically inherited diseases (Gebhardt, 2002), there is also the risk of both horizontal and vertical transmission of infections, such as HIV (Wortley *et al*., 1998).

In the UK, these risks have been identified and screening guidelines for the donation of gametes and embryos have been in place for a number of years. Screening for sperm donors was first suggested in Barton *et al*., (1945) and

the British Andrology Society (BAS) formally recommended screening in 1993 (Barratt *et al*., 1993) and later revised their guidelines in 1999 (British Andrology Society, 1999). A further updated version of the guidelines was published in 2008 as a collaborative effort between all professional bodies in the field. The aim was to update and consolidate all the guidelines across the field for sperm, egg and embryo donation, in order to increase safety and consistency (Association of Biomedical Andrologists, Association of Clinial Embryologists., British Andrology Society, British Fertility Society and Royal College of Obstetricians and Gynaecologists., 2008).

1.2.3.2 General UK screening guidelines

UK screening guidelines for all donors currently recommend an initial clinical assessment of the potential donor to assess age and basic medical history (Association of Biomedical Andrologists *et al*., 2008). Further to this, an extensive genetic history and exhaustive genetic tests, including karyotyping, are carried out to rule out any obvious inheritable diseases.

Potential donors are also screened for the presence of sexually transmitted infections (STIs) such as *Neisseria gonorrhoea* and *C. trachomatis* in addition to viral infections, including HIV, Hepatitis B and C (Association of Biomedical Andrologists *et al*., 2008). The 2008 guidelines also advise on quarantine procedures, which differ between sperm and eggs due to different success rates in cryopreservation procedures. The current guidelines stipulate that donor sperm should be quarantined for >180 days, to allow for detection of any seroconversion events, which may take place in the case of a recent infection. These guidelines are very similar to those published by the American Society for Reproductive Medicine (ASRM, 2013).

In addition to the viral infections mentioned above, CMV infection is also tested for as part of the sperm donor screening process. Although it is not solely transmitted through sexual contact, and the risk of transmission is unknown, it is included in the screening process, as the guidelines state; “it is clear that the risk to the neonate of a maternal CMV infection during

pregnancy can be significant and should be avoided if at all possible" (Association of Biomedical Andrologists *et al*., 2008).

1.2.3.3 CMV specific UK screening guidelines

In 1993, the BAS advised that serum IgG antibody testing for CMV should be carried out on all donors (Barratt *et al*., 1993). Further advice stated that if a donor was seropositive for CMV, that donor must only be used on seropositive recipients. In response to the evidence that CMV is present in cryopreserved semen samples (Mansat *et al*., 1997; Prior *et al*., 1994), the BAS revised their guidelines regarding CMV screening in 1999, controversially recommending that only seronegative donors be recruited (British Andrology Society, 1999). The revised 1999 guidelines also recommended IgM antibody testing. The recommendation to recruit only CMV negative donors was a highly debated subject and in response to this, the guidance for CMV screening was further revised in 2008. A summary flowchart of how the current screening process should occur, as recommended by the current 2008 guidelines can be found in Figure 1.9. These guidelines recommend that IgG and IgM testing be carried out on all sperm donors. If found to be IgM positive before or after the quarantine period, the donor should be deferred from use, as this serology result is indicative of a current infection (Ljungman, 2007). However, these guidelines state that where possible, CMV negative donors should be used, but IgG positive (IgM negative) donors can be used for seropositive recipients, if required, "at the clinicians discretion".

Whilst the current UK guidelines are taking measures to diagnose an active infection in the form of IgM testing, there are other indicators of active CMV infection that are not currently tested for. For example, the standards for screening in the UK fall short of those in the USA. More thorough tests are performed in the US to detect an active infection, including urine or throat cultures, in addition to detecting changes in the antibody titre levels (ASRM, 2013). Both a positive culture and an increase in antibody titre levels (including IgG) are indicators of a current active infection in a sperm donor,

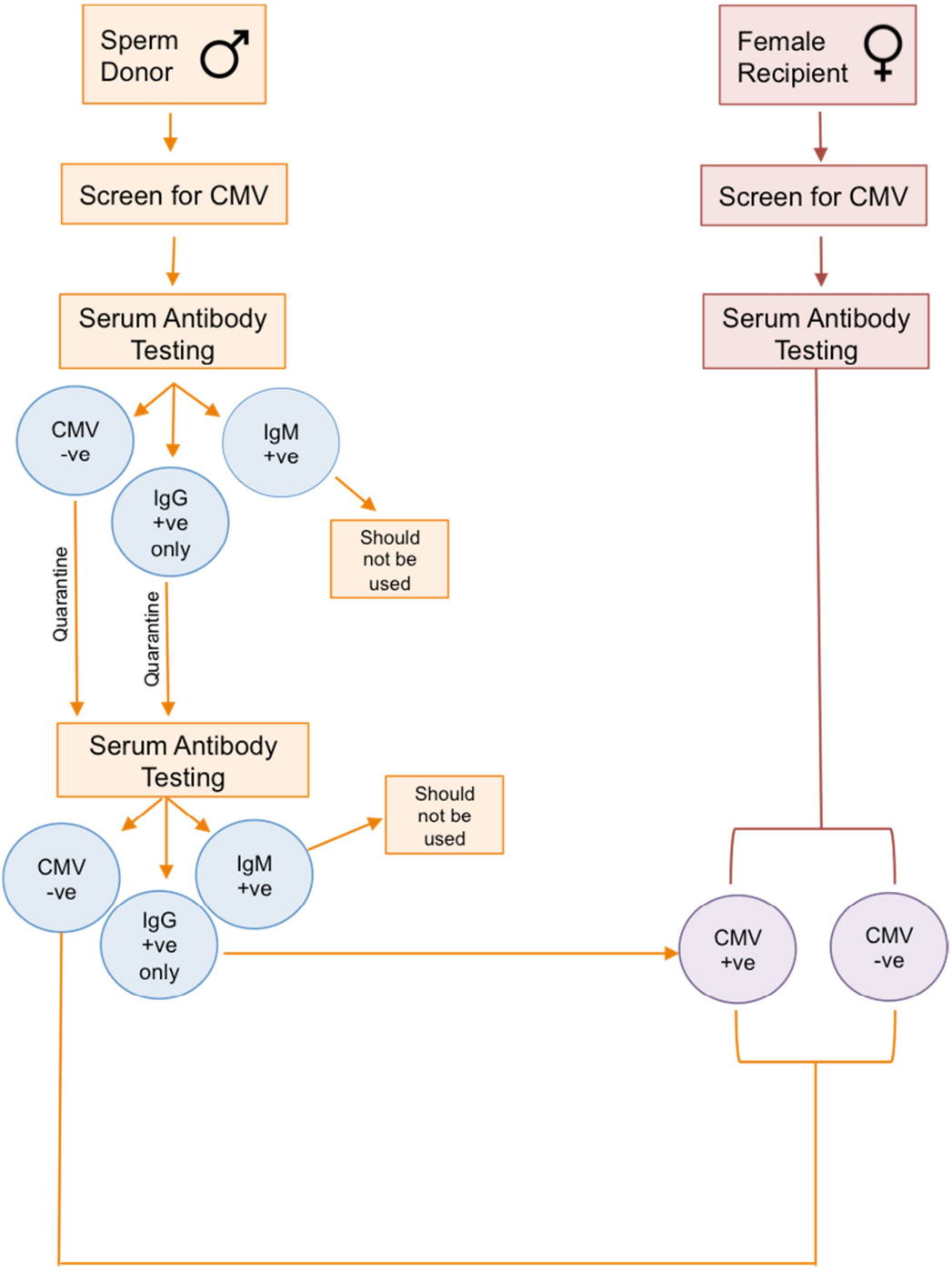

Figure 1.9: A flowchart summarising the screening process for CMV in sperm donors and those undergoing donor insemination. Briefly, current guidelines state all sperm donors should be screened for CMV by serum antibody testing. IgM positive donors should be deferred from donating, but seronegative and IgG positive donors are allowed to be used, dependent upon continued IgM negative results after a quarantine period. Donors and recipients should be matched based on their CMV serostatus and CMV IgG positive donors should only be given to seropositive recipients.

and therefore, the ASRM recommend that a donor with such positive test results should be excluded from use. A primary or reactivation infection poses a greater risk as it has been shown that in these instances the level of viral secretion, detected by PCR, can be up to 100 fold higher than IgG positive patients (Bresson *et al*., 2003). These cases represent an active infection with the potential to infect the recipient, in comparison to IgG positive donors, which represent a past infection and may no longer be infectious.

1.2.3.4 Risk of CMV transmission via donor conception

Although all sperm donors are screened for the presence of CMV, there is no evidence to suggest that there is a risk of CMV transmission via egg or embryo donation (Witz *et al*., 1999) compared to sperm donation. During non-donor assisted conception, the transmission of CMV is not problematic as the female is in regular contact with her partner's bodily secretions, suggesting prior contact with CMV infected secretions and existing immunity to the virus. However, in donor conception, a seronegative female could potentially contract CMV from a positive donor, which could lead to a primary infection. This leaves the neonate vulnerable to congenital CMV, as there is no maternal immunity protecting from placental transfer (Hemmings *et al*., 1998). The risk of transmission through donor conception is not known and any incidences of congenital CMV infection through donor conception are likely to go unreported. These epidemiological factors make it difficult to assess the true risk of CMV transmission through assisted conception.

1.2.3.5 Consequences for donor conception

The recommendation for exclusion of all CMV seropositive donors from the BAS in 1999 sparked huge controversy and debate about the feasibility of such an exclusive recruitment strategy (Liesnard *et al*., 1998; Matson, 2001). Arguments were made that this decision would drastically impact upon the number of available donors, a realistic consequence as approximately 60% of the population will have encountered CMV in their lifetime and would

present as seropositive for the virus. It has been shown that the number of available sperm donors is significantly below what is needed to meet the demand of couples waiting for donor fertility treatment (Hamilton *et al*., 2008). This is potentially exacerbated further by the removal of donor anonymity by the Department of Health in 2005 (Burr, 2010). In turn, this ultimately increases the length of waiting lists and pressure on the NHS (Pacey, 2010; Gudipati *et al*., 2013), in addition to causing an increase in the number of couples seeking donors from abroad and from the internet (Hudson *et al*., 2011), which can be time consuming and may increase unnecessary risks for these couples.

1.2.3.6 Solutions to the problem

Ultimately, the availability of sperm donors for women waiting for fertility treatment needs to be increased. The current guidelines recommend that IgG seropositive samples can be used on IgG seropositive recipients (Association of Biomedical Andrologists *et al*., 2008). This provides a small solution but there is no evidence that this practice is actively being carried out, despite this being in line with current practices during renal and liver transplantation procedures (Andrews *et al*., 2011). The use of serum antibody testing is heavily criticised, as it is a retrospective diagnostic tool: a positive IgG, or IgM, test does not necessarily indicate the donor is currently infectious and therefore, many donors that do not pose any risk are potentially being excluded from use. A more appropriate screening technique, which is able to definitively determine the presence of CMV in an individual donor would be required to overcome this problem. qPCR is used in the monitoring of the viral load in patients post organ-transplant (Emery *et al*., 2000), but this is not currently a recommended test for the screening of sperm donors. In addition, if there was better scientific evidence surrounding the relationship between CMV and sperm, it maybe possible to employ sperm washing techniques, as has been possible for some time in cases of HIV serodiscordant couples (Semprini *et al*., 1992), in order to make samples safer for use. Whilst there are many avenues to explore for improvement of

the current screening practices, allowing for more availability in sperm donors, firstly there needs to be a better understanding of the interactions between CMV and sperm on which to make clinical judgments.

1.2.4 Impact of pathogens on male fertility and reproductive potential

The presence of pathogens in semen has been widely reported over the years (reviewed in Garolla *et al*., 2013a). For example, Bezold *et al*., (2007) reported the presence of CMV, Human Papillomavirus (HPV), Human Herpesvirus-6 (HHV-6), Herpes Simplex Virus (HSV), Epstein-Barr Virus (EBV), and *C. trachomatis* in 18.7% of samples from 241 infertile patients with or without leukocytospermia. Furthermore, infections with multiple pathogens have been reported in the semen of 40.5% of men with an existing infection (Gimenes *et al*., 2014a). The presence of pathogens in semen raises many questions about transmission risks and effects on sperm production or function, which might affect reproductive potential (reviewed in Dejucq and Jegou, 2001; Gimenes *et al*., 2014b).

Whilst there is currently little evidence for the role of CMV in male infertility, there is a host of evidence surrounding other pathogens. Three different pathogens will be discussed in turn, focusing on the unique lesson that can be learnt from understanding their interactions with human sperm. After this, the current evidence for the interactions between the *Herpesviridae* family and sperm will be presented, before discussing what is already known about CMV.

1.2.4.1 Human Immunodeficiency Virus (HIV) - how can treatment be made safer?

It is known that semen is a vector for HIV transmission (Mermin *et al*., 1991) and it was originally thought that the main source of HIV in semen was due to the presence of T cells and macrophages, as opposed to direct infection of the spermatozoon (Mermin *et al.,* 1991; Quayle *et al.,* 1997; Pudney *et al*., 1999). However, it is now known that HIV can bind to (Figure 1.10a) (Dussaix putative sperm receptor is thought to be the mannose receptor (Liu *et al*.,

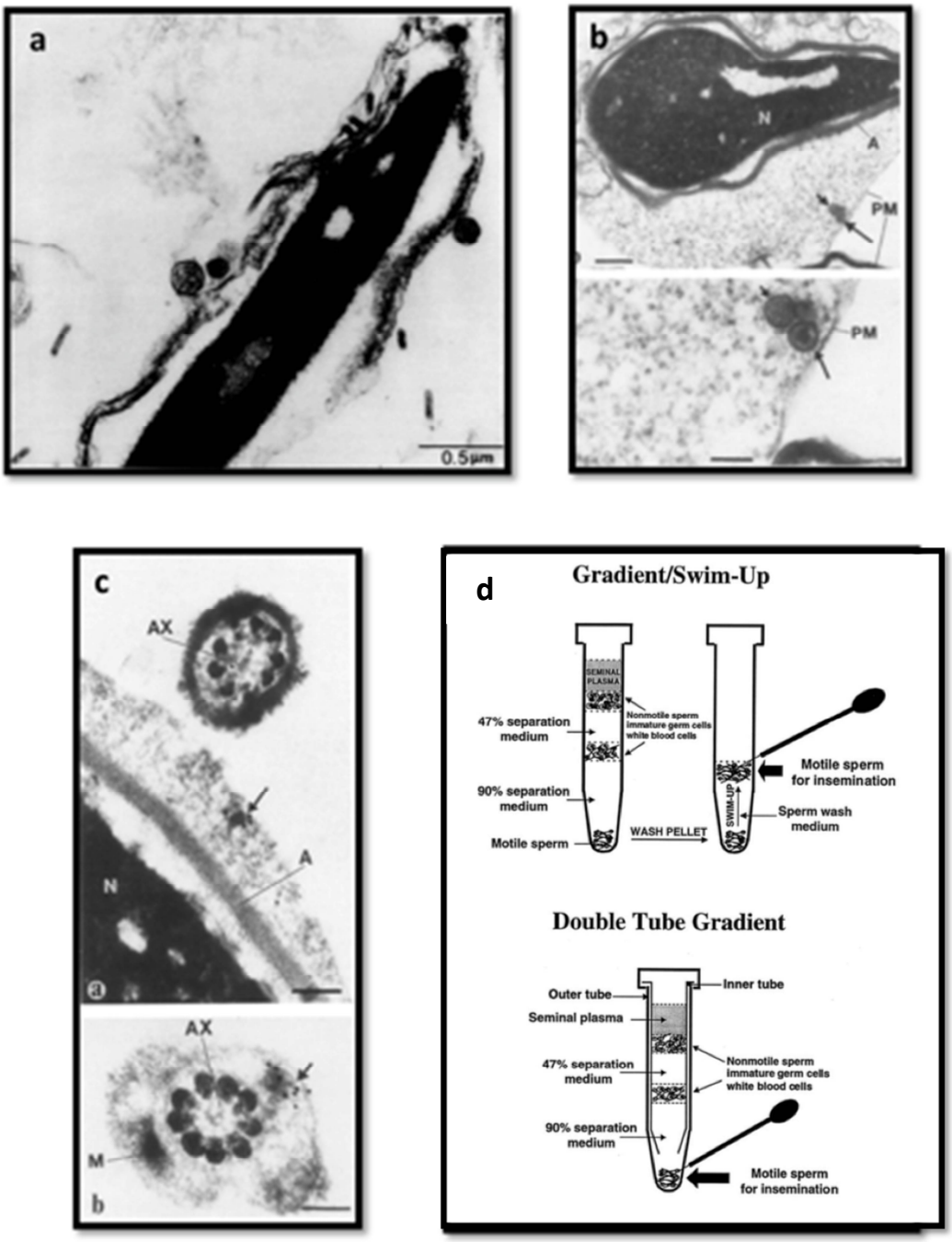

Figure 1.10: The relationship between sperm and HIV is shown with Panel (a) depicting an electron microscopy image of HIV interacting with the sperm head. Panel (b) shows HIV inside the sperm head by transmission electron microscopy (TEM) and Panel (c) shows HIV in the sperm tail via immunogold detection. Despite reported interaction between HIV and sperm, Panel (d) shows a modified version of density gradient centrifugation, which prevents re-contamination of the sperm pellet upon removal, and is efficient at removing >99.99% of HIV from infected semen samples. Figure Key: A = Acrosome, AX = Axoneme, M = Mitochondria, N = Nucleus, PM = Plasma Membrane. *Figure a was reprinted from (Dussaix et al., 1993) with permission from Elsevier. Figures b & c were reprinted from (Baccetti et al., 1994) with permission from The Rockefeller University Press, under the Creative Commons license NC-SA-2.0. Figure d was reprinted from (Politch et al., 2004) with permission from Elsevier.*

et al., 1993) and penetrate (Figure 1.10b,c) sperm (Baccetti *et al*., 1994). The 2004; Cardona-Maya *et al*., 2011), but this is still debated and reports of the involvement of other receptors, including Heparin Sulphate Proteoglycans (HSPGs) has been reported (Ceballos *et al*., 2011).

A direct interaction between HIV and sperm supports the findings that HIV infection effects sperm function and reproductive potential. Poor IVF outcomes have been correlated with high HIV RNA viral loads (Nicopoullos *et al*., 2004), which could be explained by the integration of viral DNA into sperm chromosomes (Muciaccia *et al*., 2007), leading to the possibility of vertical transmission (Wang *et al*., 2011b). In addition, HIV infection in men has been shown to be associated with decreased sperm concentration, progressive motility, and an increase in morphological abnormalities (Dondero *et al*., 1996), as well as increased DNA damage (Muciaccia *et al*., 2007). A correlation between a low number of CD4+ cells (>350/µl) and increased morphological abnormalities, and a decrease in progressive motility and concentration was also found (Dondero *et al*., 1996; Nicopoullos *et al*., 2004; Wang *et al*., 2014). Whilst the exact mechanism by which sperm is affected by HIV infection is still under investigation, evidence suggesting HIV binds directly to sperm provides strong evidence to suggest that the negative effects are due to a direct action of HIV on sperm.

The evidence supporting the role of sperm as a vector for transmission of HIV is clear, demonstrating the potential of both horizontal (Fanibunda *et al*., 2011) and vertical transmission (Baccetti *et al*., 1994). Due to the significant health risks associated with HIV infection, transmission of the virus has to be prevented between serodiscordant couples. Prior to any of the knowledge about how HIV and sperm interact, it was believed that HIV resided only as non-sperm cell associated virions. Based on this evidence, research focused on eliminating transmission by removing sperm from all other seminal components, in an attempt to eliminate HIV. Semprini *et al*., (1992) was the first to report the safe insemination of HIV negative women with sperm from a HIV seropositive man without seroconversion. Sperm were separated from infected seminal components by density gradient centrifugation, followed by

multiple washing steps and a final swim-up, resulting in an absence of HIV-infected cells in the motile sperm fraction (Semprini *et al*., 1992). Insemination of 29 women resulted in 17 pregnancies and no seroconversion occurred in any inseminated women, and no HIV infection was detected in any of the offspring.

However, some studies reported this technique as inefficient, with Marina *et al*., (1998) reporting the presence of HIV in 5.6% of samples after washing. Importantly, Politch *et al.,* (2004) observed that during the density gradient step of the sperm washing procedure, contamination occurred when harvesting the pellet after centrifugation. Using a "double tube gradient" method (Figure 1.10d), which reduces the risk of re-contamination, the authors demonstrated that HIV-1 RNA levels were significantly reduced (Politch *et al.*, 2004). Further studies using commercially available 'ProInsert tubes' reported the removal of HIV from 98.1% of samples from HIV-1 infected men (Fourie *et al*., 2015). The presence of HIV-1 proviral DNA after washing in 2/103 samples in this study highlights that whilst this method is clearly superior at removing the majority of HIV *in vitro* and *in vivo*, it is not foolproof.

The use of sperm washing to eliminate HIV from semen was used for a number of years and was shown to be safe and effective. A number of studies provided evidence to support this (Marina *et al*., 1998; Semprini *et al*., 2013), including a meta-analysis and systematic review which determined no horizontal or vertical transmission in >11,000 cycles of IUI/IVF/ICSI performed with washed semen (Zafer *et al*., 2015). However, this practice is no longer carried out in fertility clinics. It is now standard practice to monitor HIV RNA blood levels until undetectable, or <1500 copies/ml, at which point the risk of transmission is thought to be negligible (Castilla *et al*., 2005; Quinn *et al*., 2000). Current guidelines recommend that after 6 months of an undetectable viral load, it is safe for patients to have unprotected intercourse during ovulation in order to achieve pregnancy (NICE, 2013). Whilst this is now the currently accepted guideline, some studies have highlighted differences in the levels of viral load in semen compared to blood (Rinaldo *et*

al., 1992; Coombs *et al*., 1998; Gupta *et al*., 2000; Halfon *et al*., 2010; Lisco *et al*., 2012; Ferraretto *et al*., 2015). This evidence highlights differences between laboratory measures for detecting infections and epidemiology. Whilst this may not be of clinical concern currently, this is an interesting biological phenomenon that should be considered when investigating the shedding of other viruses in the semen of infected individuals.

Whilst the exact relationship between HIV and sperm is still unclear, what can be learned from this research is that steps can be taken to reduce the risks of transmission of the virus through an understanding of the relationship between the virus and sperm. In this case, whilst an understanding of the interaction between HIV and sperm was not needed to implement sperm washing for a number of years, an understanding of the shedding of HIV in the semen of infected individuals has ultimately led to better care for serodiscordant couples.

1.2.4.2 Human Papillomavirus (HPV) - can we understand the mechanism of interaction?

HPV is a highly prevalent DNA virus, which can lead to a range of different cancers, including anal and cervical. Due to this, the role sperm plays in the transmission of HPV has been intensely studied. Multiple types of HPV, including the 'high-risk' types responsible for causing cancer, have been found in semen samples from men attending fertility clinics (Kaspersen *et al*., 2011) and semen samples cryopreserved for donor insemination (Foresta *et al*., 2010a). The prevalence of HPV in semen is estimated to be ~2.3% in fertile men. However, in men experiencing fertility problems, the prevalence is higher, ~10 to 35.7% (Foresta *et al*., 2015) and in those with symptoms of HPV infection, the reported prevalence is as high as 53.8% (Foresta *et al*., 2010b).

The relationship between HPV infection and male infertility has been intensely studied with some studies suggesting there is no effect on sperm function (Schillaci *et al*., 2013; Golob *et al*., 2014; Luttmer *et al*., 2016), whereas others have reported an association with male accessory gland

infection (La Vignera *et al*., 2015), in addition to effects on sperm motility (Foresta *et al*., 2010c; Nasseri *et al*., 2015), sperm concentration (Gimenes *et al*., 2014a; Nasseri *et al*., 2015), morphology (Gimenes *et al*., 2014a; Cai *et al*., 2014) and increased levels of DNA damage (Kaspersen *et al*., 2011). An association with HPV infection and the presence of anti-sperm antibodies has also been reported (Garolla *et al*., 2013b).

Evidence also supports the transmission of HPV via sperm (Foresta *et al*., 2011a) and correlations with negative reproductive outcomes have been reported for both natural (Garolla *et al*., 2016) and assisted conception (Perino *et al*., 2011; Garolla *et al.*, 2016). Given this, it is clear that during assisted conception, every effort should be taken to remove HPV from semen samples. However, unlike HIV, it is apparent that conventional sperm washing is not able to eliminate HPV (Foresta *et al*., 2011b).

Through investigating the interaction between HPV and sperm, methods to improve the efficiency of sperm washing have been found. This research highlighted that HPV is capable of binding to sperm, with immunofluorescence studies supporting a binding at the equatorial segment of the sperm head (Figure 1.11a) (Perez-Andino *et al*., 2009; Kaspersen *et al*., 2011; Schillaci *et al*., 2013). Further investigation showed that HPV is able to bind to sperm through interaction with the HSPG, syndecan-1 (Figure 1.11b) (Foresta *et al*., 2011b). In sperm bound with HPV, there was a clear correlation with decreased DAPI staining, suggesting the DNA integrity had been compromised (Figure 1.11c). Initiation of the acrosome reaction does not abolish this interaction (Figure 1.11d), unlike the addition of Heparinase-III (Figure 1.11b). When Heparinase-III is added to the sperm washing preparation, it is able to completely eliminate HPV from semen samples (Garolla *et al*., 2012). Through an understanding of the direct interaction between HPV and sperm, it has been possible to design a way of eliminating this virus from semen samples, making assisted conception safer for those affected.

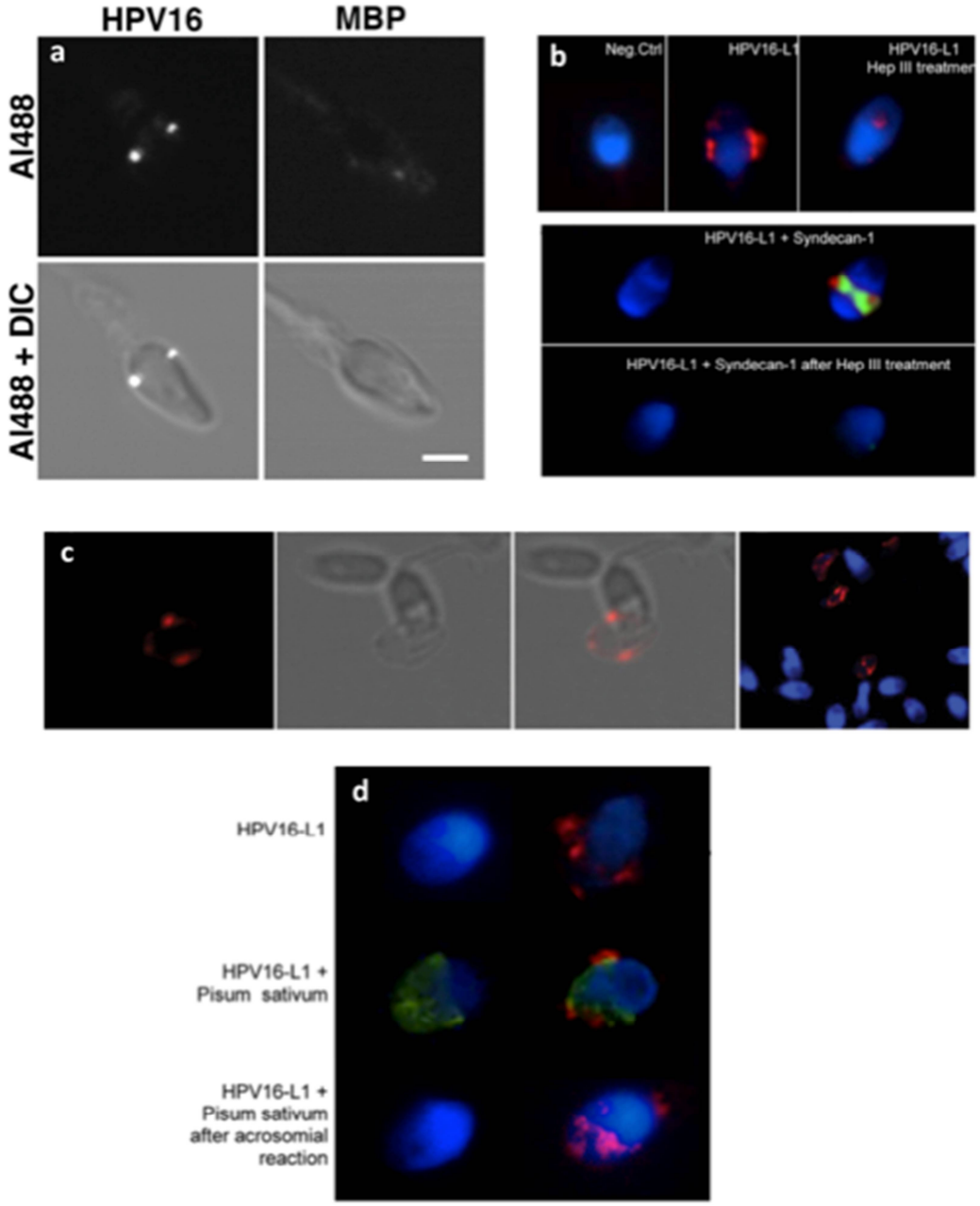

Figure 1.11: The relationship between HPV and sperm is depicted with Panel (a) showing HPV binding to the equatorial segment of the sperm head, which was not an artefact of the fluorescent label used (A1488), as specific fluorescence is absent when MBP (maltose binding protein) was used alone. Panel (b) demonstrates this interaction was due to the presence of a heparin sulphate proteoglycan on the sperm head surface, but this interaction was abolished in the presence of Heparinase-III (red=HPV-16 and green=syndecan-1). Panel (c) shows that when HPV was seen to be binding to sperm, the DAPI staining was reduced. Panel (d) shows that the interaction between HPV and sperm was not abolished when the acrosome reaction was induced (red=HPV-16, green=acrosome). *Figure a was reprinted from (Perez-Andino et al., 2009), Figures b & d from (Foresta et al., 2011) and Figure c from (Kaspersen et al., 2012). All were reprinted with permission from PLOS one under the Creative Commons license CC-BY-4.0.*

1.2.4.3 Chlamydia trachomatis - can we understand how sperm are affected?

For other pathogens, the focus has been to understand the mechanism of interaction with sperm, in order to eliminate it from semen samples. However investigations into the relationship between *C. trachomatis* and sperm, have led to a detailed understanding of how sperm can be affected by exposure to this bacteria.

It is well known that prolonged infection with *C. trachomatis* can lead to problems with fertility in both males and females due to inflammation of the reproductive tract (Malik *et al*., 2009; Mazzoli *et al*., 2010). The prevalence of the bacterium in semen varies significantly and the incidence seems to be correlated with a history of infertility, or symptoms of infection in the male. A range of ~2.5 to 38.6% prevalence in semen has been reported (Vigil *et al*., 2002; Bezold *et al*., 2007; Gimenes *et al*., 2014a).

Like other pathogens that are present in semen, investigations into whether there is a direct interaction with sperm have been performed. Electron microscopy studies initially suggested that *C. trachomatis* attached to the spermatozoon head (Erbengi, 1993) (Figure 1.12a) and evidence to support the penetration into the sperm tail was found (Figure 1.12b). Attempts to remove *C. trachomatis* by density gradient centrifugation supported the idea that the bacterium was bound to the sperm (Al-Mously *et al*., 2009), as in both naturally and artificially infected semen samples, infectious *C. trachomatis* was recovered after washing, possibly due to it being directly attached to the spermatozoon.

Infection with *C. trachomatis* is also known to affect sperm function. A correlation with reduced sperm count (Veznik *et al*., 2004; Gallegos *et al*., 2008; Mazzoli *et al*., 2010; Sellami *et al*., 2014), reduced motility (Hosseinzadeh *et al*., 2001; Veznik *et al*., 2004; Kokab *et al*., 2010; Mazzolli *et al*., 2010; Sellami *et al*., 2014), increased DNA damage (Gallegos *et al*., 2008; Sellami *et al*., 2014), and an increase in seminal leukocytes (Hosseinzadeh *et al*., 2004; Kokab *et al*., 2010) have been reported in men with an active infection.

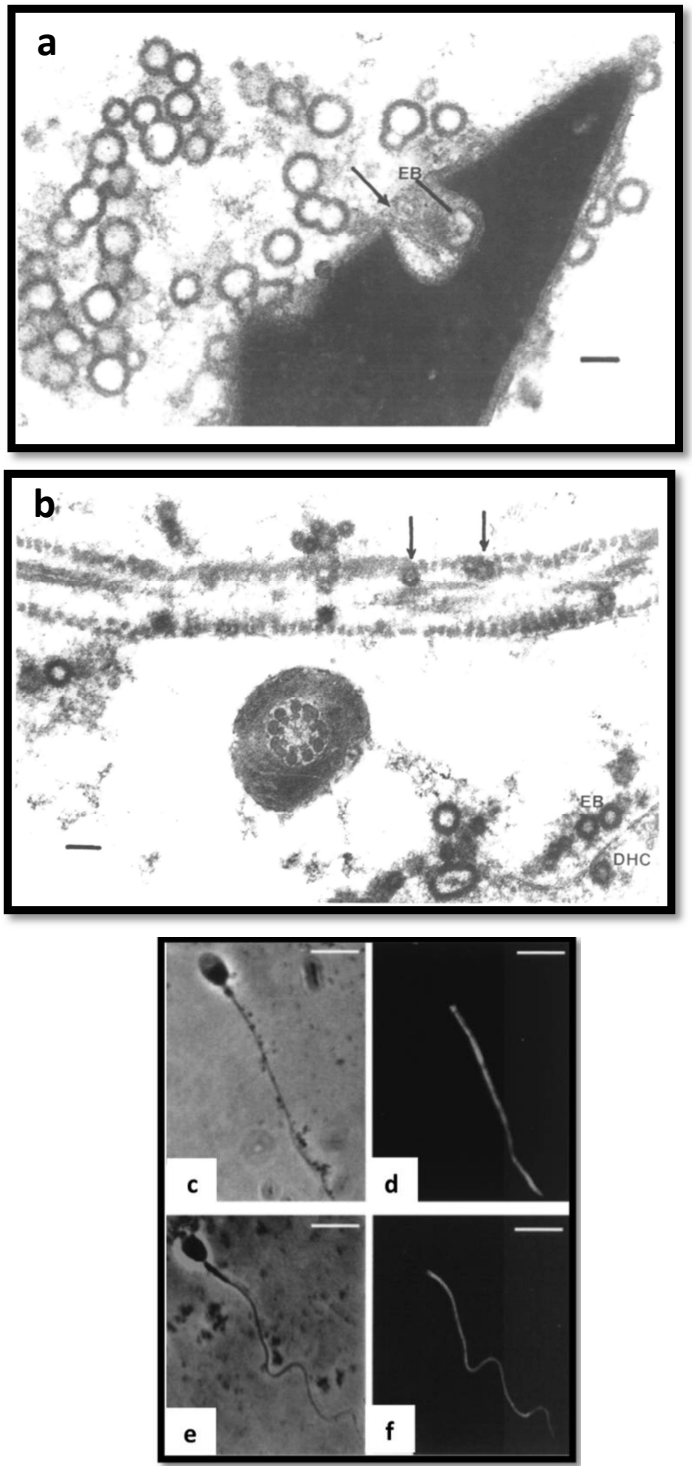

Figure 1.12: The relationship between *C. trachomatis* and sperm is shown with Panel (a) depicting the penetration of Chlamydial elementary bodies (EB) into the sperm head and Panel (b) into the sperm tail, as observed by electron microscopy. Panels (e&f) show that co-incubation of sperm with *C. trachomatis* elementary bodies results in phosphorylation of tyrosine residues along the sperm tail, in a manner analogous to the tyrosine phosphorylation patterns observed when sperm are capacitated, Panels (c & d). *Figure a & b were reprinted from (Erbengi, 1993), with permission from Oxford University Press. Figures c-f were reprinted from (Hosseinzadah et al., 2000) with permission from the American Society for Microbiology.*

An increase in apoptotic markers and loss of mitochondrial membrane potential has also been observed (Satta *et al*., 2006; Sellami *et al*., 2014). Unlike other pathogens the direct mechanisms by which *C. trachomatis* is able to affect sperm function have been uncovered. Biochemical evidence showed that co-incubation with *C. trachomatis* elementary bodies (EB) increased the levels of tyrosine phosphorylation along the sperm tail (Figure 1.12c) (Hosseinzadeh *et al*., 2000). Similarly, a correlation with infection and the percentage of acrosome reacted sperm was observed (Jungwirth *et al*., 2003). These biochemical alterations, in addition to an increase in sperm death (Hosseinzadeh *et al*., 2001) provide indirect evidence to support a receptor-mediated interaction between *C. trachomatis* and sperm, which is able to trigger intracellular signalling pathways, such as those that regulate apoptosis. The increase in sperm death when exposed to *C. trachomatis* has been shown to be due to a component of the bacterial cell membrane; lipopolysaccharide (LPS) (Hosseinzadeh *et al*., 2003), triggering apoptotic pathways and inducing cell death (Gorga *et al*., 2001; Eley *et al*., 2005a).

The exact method of interaction between *C. trachomatis* and sperm is still to be determined. However, the story of *C. trachomatis* highlights how biochemical evidence of how a pathogen can affect sperm function can lead to a deeper understanding of the mechanism of interaction between a pathogen and sperm.

1.2.4.4 The Herpesviridae family - what can we learn from similar viruses?

This section of the introduction has so far covered how an understanding of the relationship between pathogens and sperm can lead to changes in clinical practice, new methods for elimination and a deeper understanding of the molecular mechanisms of impairing sperm function. Before considering the current evidence for any interaction between CMV and sperm, it is important to consider the wider *Herpesviridae* family. The interaction between sperm and other members of this family, such as HSV, have been more intensely studied due to the immediate clinical complications associated with infection. With a high degree of homology between the

viruses (Mar Alba *et al*., 2001), it is likely that information about how other members of this family interact with sperm can be used to inform the discussion about CMV and sperm.

Interestingly, all eight human herpesviruses (HHV), except Varicella Zoster Virus (VZV) have been reported in semen at high frequencies ranging from 16.6 to 83.1% (Bezold *et al*., 2001; Kapranos *et al*., 2003; Bezold *et al*., 2007; Neofytou *et al*., 2009; Kaspersen *et al*., 2012; Michou *et al*., 2012; Chen *et al*., 2013), reviewed in (Kaspersen and Hollsberg *et al*., 2013).

A number of studies have investigated the presence of various HHV's in semen and a comparison of the most prevalent types can be seen in Table 1.1. It is clear that the prevalence of HHV in semen varies significantly and of the studies presented in Table 1.1, it is clear that the most prevalent HHV's are CMV, EBV and HSV, with one study finding a high prevalence of 66.8% for HHV-6 (Neofytou *et al*., 2009). However, very few studies have investigated the presence of VZV, HHV-7 and HHV-8. More studies might highlight that these HHV strains are just as prevalent as the others, however the current evidence suggests this is not that case. Table 1.1 also highlights that the presence of CMV and EBV is much higher in those infected with HIV (Howard *et al*., 1997; Rinaldo *et al*., 1992; Lisco *et al*., 2012). This is not surprising as it is widely reported that men infected with HIV often have co-infections with opportunistic pathogens, such as EBV and CMV (Lupton *et al*., 2013). Whilst the prevalence varies significantly for men seeking help with fertility, the presence is generally quite low in healthy sperm donors. With only HHV-6 being detected at 13.5% (Kaspersen *et al*., 2012), the remaining strains are detected at levels less than 6%.

Given the high prevalence of HHV's in the semen of men, the effect of these viruses on male fertility and sperm function have naturally been investigated. The majority of studies investigated this *in vivo* and in most cases concluded that the presence of HHV's did not have any significant effect on

Table 1.1: Details of HHV prevalence in semen from studies where the presence of multiple strains were found. (Blue=men attending fertility clinics, red=HIV positive men, green=health sperm donors).

Study	HSV1/2	VZV	EBV	CMV	HHV-6	HHV-7	HHV-8
Aynaud *et al.*, 2002 (n=111)	9%			6.3%			
Bezold *et al.*, 2001 (n=252)	3.2%	0%	7.1%	3.6%	4.0%	0.4%	0%
Bezold *et al.*, 2007 (n=241)	3.7%		0.4%	8.7%	3.7%		
Chen *et al.*, 2013 (n=153)	25.5%		3.9%	21.6%	2.0%		
Kapranos *et al.*, 2003 (n=113)	49.5%		16.8%	7.1%			
McGowan *et al.*, 1983	0% (n=210)			2.4% (n=170)			
Michou *et al.*, 2012 (n=109)	29%	0%	45%	43%	8.2%	3.6%	
Neofytou *et al.*, 2008 (n=172)	2.3%	2.3%	40.6%	56.9%	66.8%	0%	
Naumenko *et al.*, 2014 (n=232)			3.4%	5.2%	6.5%		
Howard *et al.*, 1997 (n=24)				83.3%			25%
Rinaldo *et al.*, 1992	0% (n=116)			33% (n=58)			
Lisco *et al.*, 2012 (n=50)	8%		56%	70%	2%	12%	6%
Lisco *et al.*, 2012 (n=28)	0%		3.5%	3.5%	6%	6%	
Kaspersen *et al.*, 2012 (n=198)	0.4%/0.1%	0%	6.3%	2.7%	13.5%	4.2%	0%
Howard *et al.*, 1997 (n=115)				3.5%			0%
McGowan *et al.*, 1983 (n=40)				2.5%			

volume, sperm count, motility, morphology, or levels of leukocytes and antisperm antibodies (Bezold *et* al., 2001; Bocharova *et al*., 2008; Neofytou *et al*., 2009; Eggert-Kruse *et al*., 2009; Naumenko *et al*., 2011; Michou *et al*., 2012; Pallier *et al*., 2002; Chen *et al*., 2013). Interestingly, no correlation was observed with even multiple infections of up to four different strains of HHV (Michou *et al*., 2012). However, infection with a HHV and another type of pathogen, such as *C. trachomatis* or HPV, might result in the production of abnormal sperm (Gimenes *et al*., 2014a), but evidence suggests infection with multiple types of HHV does not have such a synergistic effect on sperm abnormalities. A few studies have observed an effect on sperm concentration and motility, mostly in response to HSV infection (Kotrionas and Kapranos, 1998; Kapranos *et al*., 2003; Pallier *et al*., 2002). One study reported a correlation between the presence of CMV and HHV-6 with inflammatory urogenital tract diseases (Naumenko *et al*., 2014) and another study reported an effect on sperm concentration and motility but did not distinguish between HHV strains (Bezold *et al*., 2007). Pallier *et al*., (2002) investigated the effect on sperm motility *in vitro* and whilst they found no effect when co-incubating CMV with sperm, they did observe alterations in sperm kinematic movement in response to HSV co-incubation. When considering the role HHV infection plays in infertility, it is important to consider the evidence suggesting that men intermittently shed HHV's in their semen (Kaspersen *et al*., 2012). This evidence shows that results from studies correlating the presence of HHV and fertility issues might be underestimating the role HHV's play in infertility. Whilst at the time of the study the individual might not have been shedding virus, it does not mean they do not have an infection that could be affecting their fertility; it just might not have been detected at the particular time point of the study.

Whilst for most HHV's there appears to be no correlation with impaired sperm, there does appear to be a correlation with HSV. However, unlike HIV and *C. trachomatis*, little investigation into the mechanism of interaction and effect on sperm function has been conducted. Some studies have investigated the role of HSV thymidine kinase (HSV-*tk*) on spermatogenesis

in mice. They have observed that transgenic mice carrying the HSV-*tk* gene produced sperm with gross morphological defects, acrosomal abnormalities and a loss of germ cells due to apoptosis (Huttner *et al*., 1993; Cai *et al*., 2009). Further investigation showed gross abnormalities in the testes structure and a failure of the crucial Sertoli-germ cell interaction, which undoubtedly contributed to the failure of spermatogenesis to produce functional sperm (Cai *et al*., 2012). Whilst this evidence is interesting and shows how a viral gene can affect fertility, it is unlikely to be occurring in men. The mice used in these studies have been transgenically altered to express the HSV-*tk* gene and therefore the findings from these studies are difficult to translate to a human infection. Some studies using human sperm have suggested that HSV can interact with the sperm head (Figure 1.13a) and tail (Figure 1.13b) (Pallier *et al*., 2002), potentially penetrating the sperm head. The number of instances in this study is few but other studies showing the detection of HSV DNA inside the sperm head support this finding (Figure 1.13c) (Kotrionas and Kapranos, 1998; Bochorova *et al*., 2008). Interestingly, it has been reported that HHV-6 can also associate with sperm, but only when the acrosome is intact (Figure 1.13d) (Kaspersen *et al*., 2012).

Evidence to support interactions between HHV and sperm are further supported by evidence showing that sperm washing fails to remove most of these viruses from infected semen samples (Witz *et al*., 1999; Michou *et al*., 2012). Interestingly, Michou *et al*., (2012) did observe that HHV-6 and 7 could be removed from semen samples, contradicting the evidence from Kaspersen *et al*., (2012), which suggests HHV-6 interacts with the sperm head.

Whilst there is a wealth of evidence supporting the presence of HHV's in semen, few studies have attempted to investigate the mechanisms of interaction, which facilitate sexual transmission. The few studies that have been carried out provide contradictory evidence and the picture is far from clear.

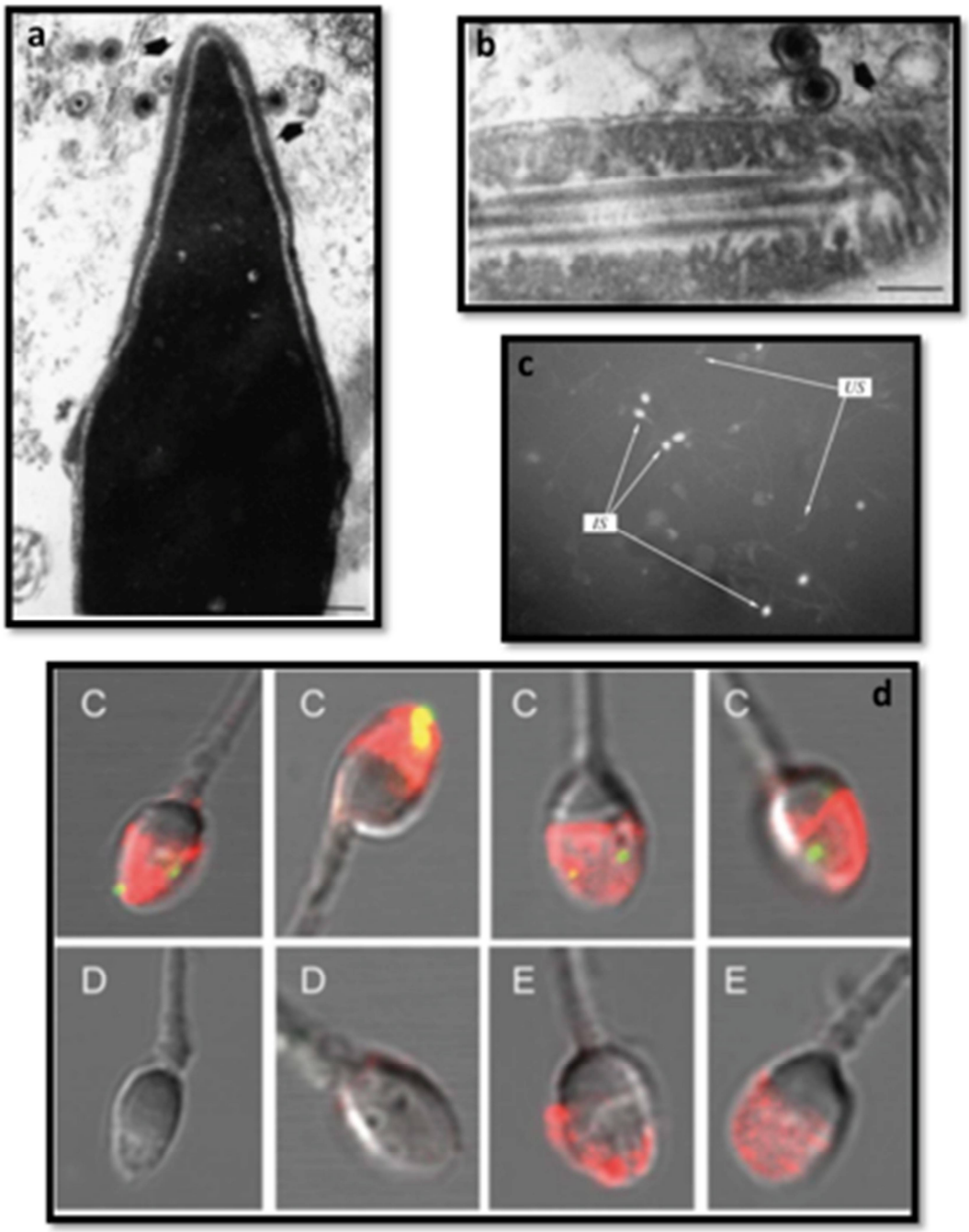

Figure 1.13: The relationship between sperm and *Herpesviruses* is shown with Panel (a) depicting an electron microscopy image with HSV-2 particles close to the sperm head and tail (Panel b), with the author reporting a direct interaction. Panel (c) shows *in situ* hybridisation demonstrating the presence of HSV DNA in the sperm heads of infected men. In Panel (d-c), co-incubation of HHV-6 with sperm showed that the virus (green) was able to bind to the sperm head in the presence of the acrosome (red). Panel (d-d) shows that when the acrosome reaction was induced the interaction was lost. (Panel d-e shows the acrosomal staining in the absence of HHV-6). Figure Key: IS = Infected sperm, US = Uninfected sperm. *Figures a & b were reprinted from (Pallier et al., 2002) with permission from Oxford University Press. Figure c was reprinted from (Bocharova et al., 2008) with permission from the Russion Journal of Developmental Biology. Figure d was reprinted from (Kaspersen et al., 2012) with permission from PLOS one under the Creative Commons license CC-BY-4.0.*

Despite being one of the most studied of the HHV's, as can be seen in Kaspersen and Hollsberg, (2013), investigations into the interactions between CMV and sperm are also limited.

1.2.4.5 Human Cytomegalovirus - what do we already know?

The presence of CMV in the semen of both symptomatic and asymptomatic men was observed as early as 1975 (Lang and Krummer, 1975). Furthermore, CMV has also been detected in semen samples cryopreserved for fertility treatment (Bresson *et al*., 2003; Mansat *et al*., 1997). The reported prevalence ranges from 0% to 56.9% (reviewed in Kaspersen and Hollsberg, 2013), and CMV infection can often be found in association with other HHV's (Table 1.2).

Few studies have directly investigated the interaction between CMV and sperm, and those that have do not provide convincing data. Pallier *et al*., (2002) reported the presence of a viral particle near the surface of the sperm head using electron microscopy (Figure 1.14a), but this was only observed in <5% of sperm sections analysed. Also, the authors report that the type of viral particle depicted 'interacting' with the sperm head is actually a non-infectious form (dense body), rather than a fully formed infectious virion. Another study reported a direct interaction with sperm, located at the equatorial segment (Figure 1.14c), in both samples infected *in vivo* and *in vitro*. However, despite using samples with high viral concentrations for immunoflourescent analysis, an association was only found with 2-6% of sperm observed (Naumenko *et al*., 2014). However, one interesting study documented the ability for CMV to infect immature germ cells *in vitro* (Figure 1.14b), which could lead to the production of mature sperm carrying CMV internally (Naumenko *et al*., 2011). This study found a reduction in germ cells in the presence of CMV infection, which could contribute to male infertility. Evidence suggesting sperm washing is inefficient at removing CMV from naturally infected semen samples (Witz *et al*., 1999; Michou *et al*., 2012; Naumenko *et al*., 2014) does support the possibility of a direct interaction

Table 1.2: Details of the prevalence of multiple HHV infections in men attending fertility clinics. Numbers shown as percentage prevalence and total number in brackets.

Study	E+C	E+H6	C+ H6	C+H	E+H	H+H6	E+H+C	C+H7	H+E+H7	E+C+H6	H+E+C+H6	E+C+H6+H7
Naumenko *et al*., 2014 (n=232)	0.43% (1)	0.86% (2)	1.3% (3)									
Aynaud *et al*., 2002 (n=111)				1.8% (2)								
Bezold *et al*., 2001 (n=252)	0.8% (2)				0.4% (1)							
Chen *et al*., 2013 (n=153)	1.6% (4)		5.9% (15)		0.4% (1)	0.8% (2)						
Kapranos *et al*., 2003 (n=113)				3.6% (4)	12.4% (14)		0.9% (1)					
Michou *et al*., 2012 (n=109)	4.8% (10)	1.8% (2)	1.8% (2)	3.7% (4)	5.5% (6)		16.5% (18)	0.9% (1)	1.8% (2)	1.8% (2)	1.8% (2)	0.9% (1)
Neofytou *et al*., 2009 (n=172)	27.9% (48)	31.9% (55)	44.1% (76)		2.3% (4)	2.3% (4)				22.6% (39)		

E=EBV
C=CMV
H=HSV
H6= HHV-6
H7= HHV-7
V=VZV

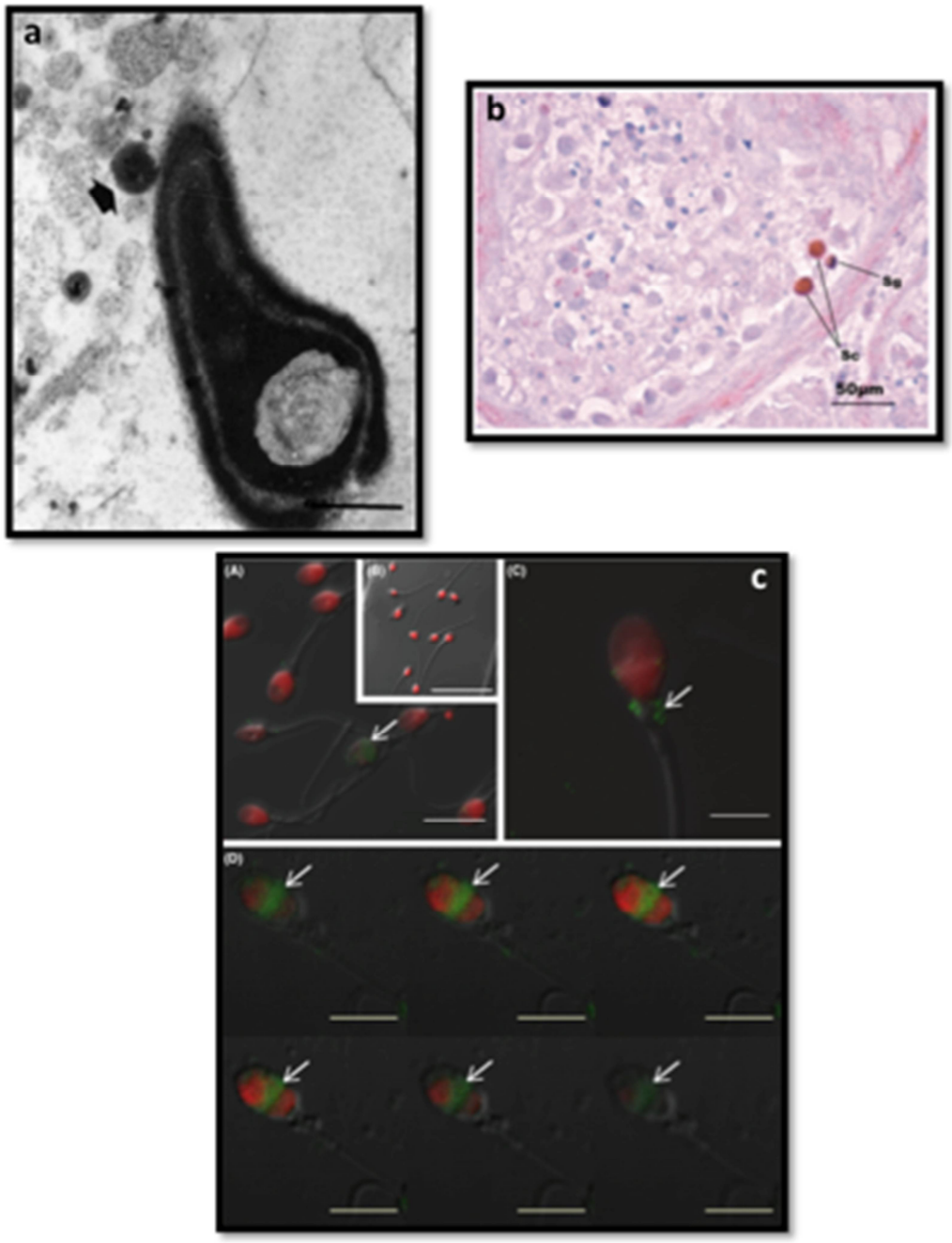

Figure 1.14: The relationship between CMV and sperm is shown, with Panel (a) displaying electron microscopic images showing a non-infectious CMV particle (dense body) close to the sperm head in <5% of sections analysed. Panel (b) depicts an organotypic culture using human testicular tissue samples infected with CMV. Using this method, it was observed that CMV was able to infect both spermatogonia and spermatocytes after 14 days of infection. In Panel (c), immunofluorescence of CMV pp65 protein shows CMV binding to the sperm head and mid-piece (c-a&c). The authors also noted a pattern of binding along the equatorial segment (c-d), which is similar to the pattern observed when using immunofluorescence to detect acrosome reacted sperm. Figure Key: Sc = Spermatocyte, Sg = Spermatogonia. *Figure a was reprinted from (Pallier et al., 2002) with permission from Oxford University Press. Figure b was reprinted from (Naumenko et al., 2011) with permission from Biomed Central under the Creative Commons license CC-BY-2.0. Figure c was reprinted from (Naumenko et al., 2014) with permission from Andrology.*

between CMV and sperm. However, the current evidence base is poor and does not provide a clear picture of how this interaction might be occurring.

1.3 Summary

Evidence from a variety of pathogens in addition to the wider *Herpesviridae* family clearly shows that sperm can act as a vector for viral transmission. The HSPG receptor appears to play an important role in this process, but as our understanding of the spermatozoon develops, the involvement of other receptors might be discovered. Methods of understanding interactions between pathogens and sperm have been developed through observing effects on sperm function, the efficiency of sperm washing, and by direct investigations of the molecular interactions. It is clear that the role sperm plays in the transmission of pathogens is something to be concerned about, given the severe health complications associated with some of the ones discussed in this chapter. A deeper understanding of the interactions that are occurring is essential in order to change clinical practice in the assisted conception field and make practices as safe as possible. This is no different for CMV. Whilst the risks of transmission of CMV are not as immediately apparent as for some of the other pathogens, such as HIV, the health consequences associated with infection can be just as severe. Given this, an understanding of how sperm can act as a vector for transmission of CMV should be given just as much concentration as other infectious diseases.

1.4 Aim(s) and objectives

It is clear that there is a gap in the knowledge regarding the relationship between CMV and sperm. The knowledge that CMV can have severe consequences if contracted *in utero* has had a negative impact on the assisted fertility sector, as it has affected who can donate sperm and possibly contributed to the falling numbers of sperm donors. Despite this, there is still no active investigation into solving the problem and the actual risks of contracting CMV through assisted conception are still not known. The first objective of this study is to understand how CMV is screened for in the

fertility clinic and whether a more in depth understanding of the relationship between CMV and sperm can better inform clinical practice with regards to the use of CMV positive sperm donors for assisted conception. This will serve as the context for investigating the main objective, which is to gain an insight into the relationship between CMV and sperm, firstly by investigating the effectiveness of sperm washing procedures at removing different quantities of virus from both naturally and artificially infected sperm and secondly, by assessing the effect of CMV infection on sperm parameters through co-incubating laboratory grown CMV with sperm.

1.4.1 Specific aims

1. Develop a survey to distribute to fertility clinics aimed at investigating the screening procedures for CMV in sperm donors and provision of fertility treatment.
2. To establish a system for culturing CMV *in vitro* and quantifying the viral load and infectious load by qPCR and the plaque assay, respectively.
3. To assess the efficiency of current sperm washing protocols at removing CMV from artificially and naturally infected semen samples.
4. To perform co-incubation experiments with spermatozoa to assess if CMV affects sperm function parameters.

Chapter 2

Materials & Methods

2.1 Biological safety, donor recruitment and ethics

Permission to grow and use CMV (AD169) was sought by the University of Sheffield biosafety committee and the project was approved on 9th May 2013 (Appendix I). Ethical approval to recruit voluntary sperm donors was granted on 28th February 2014 (Study No: SMBRER293) by the University of Sheffield ethics committee (Appendix II). Healthy men from the Yorkshire and Humber region aged between 18-65 were recruited through posters (Appendix III) and email advertisement and compensated £15 per sample in lieu of expenses. The recruitment and consent of donors was carried out by a laboratory research technician (Dr Sarah Waite), in order to exclude the possibility of coercion by researchers. Informed consent was obtained upon delivery of each sample to the laboratory and samples were anonymised with a unique donor number, before being handed over to be used in the experiments described in this thesis. In Chapter 3, a survey evaluating services was distributed to fertility clinics throughout the UK and abroad (Appendix IV). As this survey was designed to evaluate current practice in clinics, according to the National Health Service (NHS) Health Research Authority 'Defining Research' leaflet, this is defined as service evaluation and did not require ethical approval. This was confirmed by the Sheffield Teaching Hospitals research coordinator (Angela Driscoll), by telephone.

2.2 Strains, plasmids and growth conditions

Escerichia coli (DH5α), a kind gift from Professor Dave Hornby (University of Sheffield) was used for all molecular cloning techniques in Chapter 4, and was grown at 37°C in Luria-Bertani growth medium (LB). LB + agar (12g/L) was used to culture *E. coli* on agar plates, with or without 200µg/ml Ampicillin (Sigma-Aldrich, Dorset, UK). A laboratory strain of human Cytomegalovirus (CMV) AD169 (Health Protection Agency, Porton Down, Salisbury, UK) (NCBI: BK000394) was grown on Human Lung Fibroblast cells (MRC-5) (American Type Culture Collection, Middlesex, UK), as described in Chapter 4, and subsequently used in Chapters 5 and 6. CMV strains Towne (NCBI: AY446869), Merlin (NCBI: AY446894) and TB40/E (NCBI: AY446866), used

in Chapter 6, were provided by Dr Matt Reeves (University College London). Plasmid pRcRSV was used to clone the glycoprotein B gene, in Chapter 4, and was provided by Dr Neil Chapman (University of Sheffield).

2.3 Buffers and reagents

Details of the composition of all buffers, media and solutions used throughout this thesis are detailed in Table 2.1.

2.4 Tissue culture

MRC-5 cells were cultured in Eagle's Minimum Essential Medium (EMEM) (American Type Culture Collection, Middlesex, UK) with 10% (v/v) Fetal Calf Serum (FCS) (Lonza, Basel, Switzerland), 100U penicillin and 0.1mg/ml streptomycin (Sigma-Aldrich, Dorset, UK). Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Dorset, UK) was removed by centrifuging cells at 125*g* for 10 minutes in 2ml EMEM. Supernatant was removed and replaced with 2ml fresh EMEM in which the pellet was re-suspended. Cells were transferred to a T75 culture flask (Greiner Bio-one, Frickenhausen, Germany) with 13ml EMEM giving a 1:15 dilution.

Cells were left to grow for seven days with a media change every 48 hours until confluent. Cells were passaged 1:3 by removing spent media and washing with 6ml Dulbecco's phosphate buffered solution (PBS) (Sigma-Aldrich, Dorset, UK). 3ml of trypsin (Sigma-Aldrich, Dorset, UK) was added to each T75 flask and incubated for 2-3 minutes to allow cells to detach from the flask surface. A 1ml aliquot of trypsinised cells were transferred to each T75 flask containing 13ml EMEM and incubated at 37°C.

Samples of cells were cryopreserved at each passage by trypsinising with 3ml of trypsin and centrifuging at 1,000*g* for 10 minutes. Supernatant was removed and the pellet re-suspended in 750µl EMEM + 10% (v/v) FCS. The cell suspension was divided into three cryovials and a 10% (v/v) DMSO solution (diluted in antibiotic free EMEM) was slowly added to the 250µl of

Table 2.1: Buffers, media and solutions.

	Composition	Amount (g)	Volume (ml)	Percentage composition (%)	Molarity (M)
LB	Tryptone	5			
	NaCl	5			
	Yeast Extract	2.5			
	Distilled Water		500		
LB Agar	Tryptone	5			
	NaCl	5			
	Yeast Extract	2.5			
	Agar	6			
	Distilled water		500		
SDS lysis buffer pH 7.4	Tris	0.61			0.02
	EDTA		2.5		0.5
	NaCl		7.5		5
	SDS		25	10	
	Distilled water		215		
Semi-solid overlay	Methyl Cellulose		25	1	
	2xEMEM		25		
	5% FCS		2.5	5	
	L-Glutamine		0.5		0.2
	Penicillin/Streptomycin Mix		0.5		
1% methyl cellulose	Methyl Cellulose	5			
	Distilled water	500			
5% formaldehyde	Formaldehyde	67.6		37	
	PBS	432			
Formalin	$NaHCO_3$	50			
	Formaldehyde		10	35	
	Distilled water		1000		
MOWIOL/ DABCO	Mowiol	2.4			
	Glycerol	6	4.76		
	H_2O		6		
	Tris		12		0.2
	DABCO	0.569		2.5 (w/v)	
TBS	TRIS				0.02
	NaCl				0.14
	H_2O		476		

MRC-5 cells giving a final 5% (v/v) DMSO concentration. The samples were cooled slowly using a Mr Frosty cooling box, stored at -80°C before being stored in liquid nitrogen at -196°C.

For visualisation of MRC-5 cells, cells were grown in 6 wells plates until various stages of growth. 500µl of 5% (v/v) formaldehyde (Table 2.1) was used to fix the cells for 30 minutes before being washed twice with 1x PBS. 500µl of 0.5% (w/v) crystal violet solution (Sigma-Aldrich, Dorset, UK) was added and left to stain the cells for 10 minutes before being washed off with 1x PBS twice. Cells were imaged using a 10X objective on an inverted Olympus CKX41 microscope (Figure 2.1).

2.4.1 Plaque assays

MRC-5 cells were grown until just confluent in 24-well Cell+ plates (Sarstedt, Nümbrecht, Germany). Once confluent, EMEM media was removed and cells were infected with either 100µl or 200µl of sample (amount and type of sample vary between experiments-precise details can be found in the relevant chapters). Samples were diluted in serum-free EMEM over a 2-fold or 10-fold dilution series and a different dilution was added to each well. Two negative control wells, containing serum-free EMEM only were included on each plate. The plates were incubated at 37°C for 1 hour with gentle manual rocking every 20 minutes (Plummer & Benyesh-Melnick, 1964). After 1 hour, 1ml of semi-solid overlay, composed of a 1:1 mix of 2xEMEM and 1% (w/v) methyl cellulose (Table 2.1), was added to each well in order to prevent virus from spreading and enable the identification of discernable plaques.

The plates were incubated at 37°C for 1-2 weeks, until plaques were discernable. Once plaques were visible, the cells were fixed by removing the overlay and adding 1ml 5% (v/v) formaldehyde (Table 2.1) to each well and incubating at room temperature for 30 minutes. The fixative was removed and plaques stained with 0.5% (w/v) crystal violet solution. The cells were left to stain for 10 minutes at room temperature. The stain was decanted, washed with 1xPBS and left to dry.

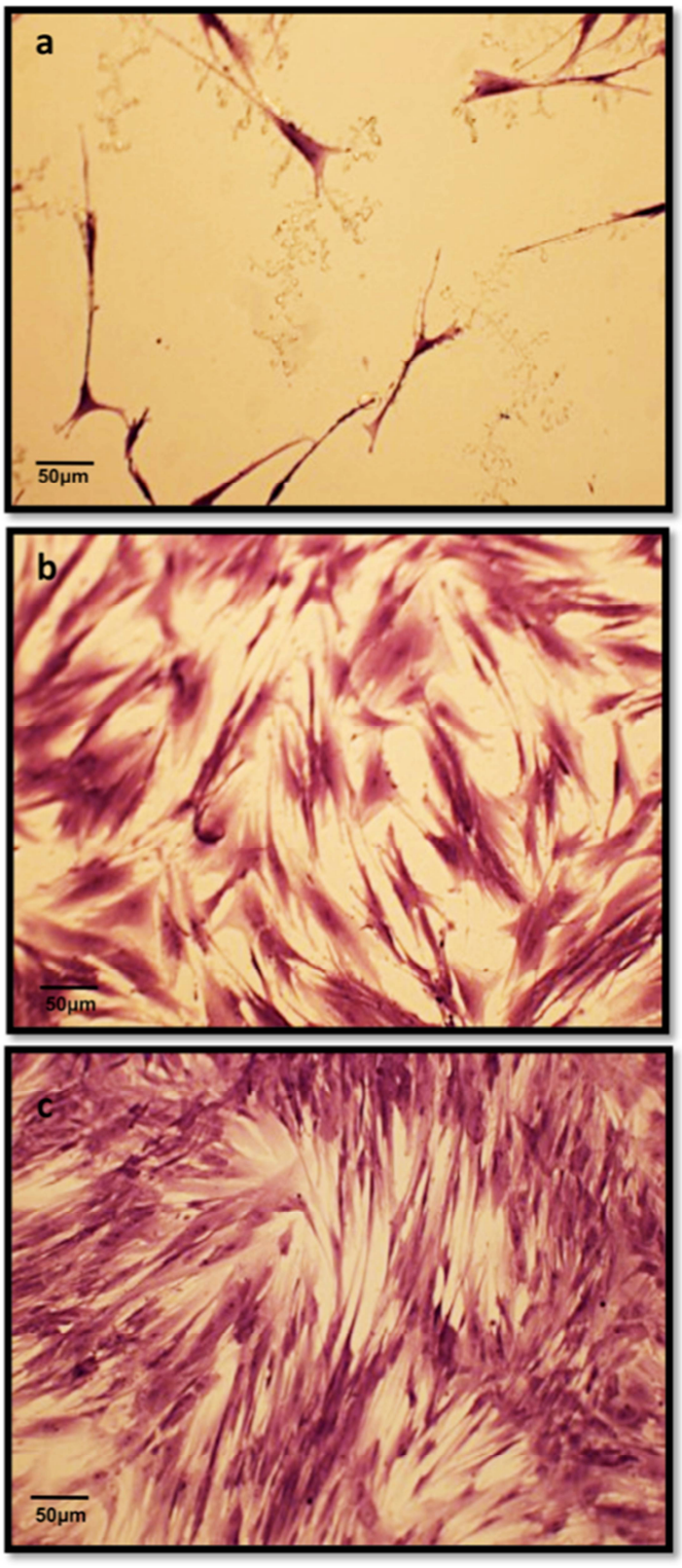

Figure 2.1: MRC-5 human lung fibroblast cells were grown in EMEM, fixed with 5% (v/v) formaldehyde and stained with 0.5% (w/v) crystal violet. Cells were imaged using an inverted Olympus CKX41 microscope on a 10X objective. Cell were fixed and stained at (a) 48 hours after passage, (b) when cells were at 80% coverage, or sub-confluency and (c) at an early stage of confluency. Scale bar shown=50 µm.

The cells were visualised on a microscope and the plaques were counted. The viral titre in PFU/ml was calculated by scaling up to determine the number of plaques present in 1ml and then multiplying by the dilution factor (Appendix V).

2.5 Semen analysis and sperm function tests

Semen samples from healthy donors aged between 18-65 were used for all experiments outlined in this thesis. Donors were asked to abstain for 2-3 days prior to producing the sample. Samples were produced at home in a collection pot (Sigma-Aldrich, Dorset, UK) and delivered to the Academic Unit of Reproductive and Developmental Medicine, Jessop Wing, Sheffield within 40 minutes of production. Samples were analysed, as outlined below, and used immediately.

2.5.1 Basic semen analysis

Sperm concentration and motility measurements were made according to methods described in WHO (2010), with modifications in sperm motility assessment as outlined in Bjorndahl *et al*., (2016). Briefly, a wet prep was prepared by adding 10µl of the ejaculate to a microscope slide and analysing under a 20x objective to determine the appropriate dilution of the sample to carry out. Sperm concentration was assessed by diluting the ejaculate with formalin (Table 2.1), and adding 10µl of the diluted sample to each side of an improved Neubauer haemocytometer. This was left to settle in a humidified chamber for 5-10 minutes. Upon analysis of the concentration, 200 sperm were counted on each side of the chamber before taking an average and determining the concentration of sperm in 10^6 per ml. The wet prep was used to analyse sperm motility by counting 200 sperm and categorising them into progressively motile (grades a and b), non-progressively motile (grade c) and non-motile (grade d). Duplicate measurements were not performed.

2.5.2 CASA motility analysis

Assessment of sperm motility and kinematics was carried out using the Sperm Class Analyzer software, version 5.4.0.0 (MicroOptic, Barcelona, Spain), in accordance with standards outlined in WHO (2010). Samples were observed on a Microtec LM-2 trinocular microscope (Mazurek, Warwickshire, UK) with a 20x objective and visualised using a Basler A312FC digital camera (Basler, Ahrensburg, Germany) at 25 frames per second. Briefly, 10µl of sample was added to a 20µm 2-chamber Microcell slide (Conception Technologies, San Diego, USA). The tracks of at least 200 motile sperm were obtained per sample, where possible, with a maximum of 400 sperm detected. Data collected included the percentage of progressive, non-progressive and immotile sperm and the percentage sperm velocities, categorised as rapid, medium and slow. In addition, sperm kinematic data focusing on sperm movement and velocity was also collected. The curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) were measured in µm/s. Ratios of sperm progression were measured in the form of linearity (LIN), straightness (STR) and wobble (WOB). The amplitude of lateral head displacement (ALH) was also measured in µM and the beat cross frequency (BCF) in Hz. (See Figure 2.2 for details on individual kinematic measurements). Finally, a measure of percent hyperactivation was collected. Hyperactivation is defined as sperm with VCL >150µm s^{-1}, LIN <50% and ALH >7.0 µm (Mortimer *et al*., 2015). Sperm kinematic data for each individual sperm analysed for each sample was collected via a Microsoft Excel (Microsoft, Washington, US) spreadsheet.

2.5.3 Density gradient centrifugation (sperm washing)

Motile sperm was recovered from seminal plasma and immotile sperm by density gradient centrifugation (Pertoft *et al*., 1978; Gorus and Pipeleers, 1981) using PureSperm (Nidacon, Sweden). Sperm were separated on an 80:40% gradient, created by gently layering 1ml 40% PureSperm onto a 1ml layer of 80% Puresperm, creating a clean and sharp meniscus (Figure 2.3a). 80% and 40% PureSperm was created by diluting 8ml PureSperm *100* (Nidacon, Sweden) with 2ml PureSperm Buffer (Nidacon, Sweden) and 4ml and 6ml, respectively. Between 0.5-1ml of semen (dependent upon

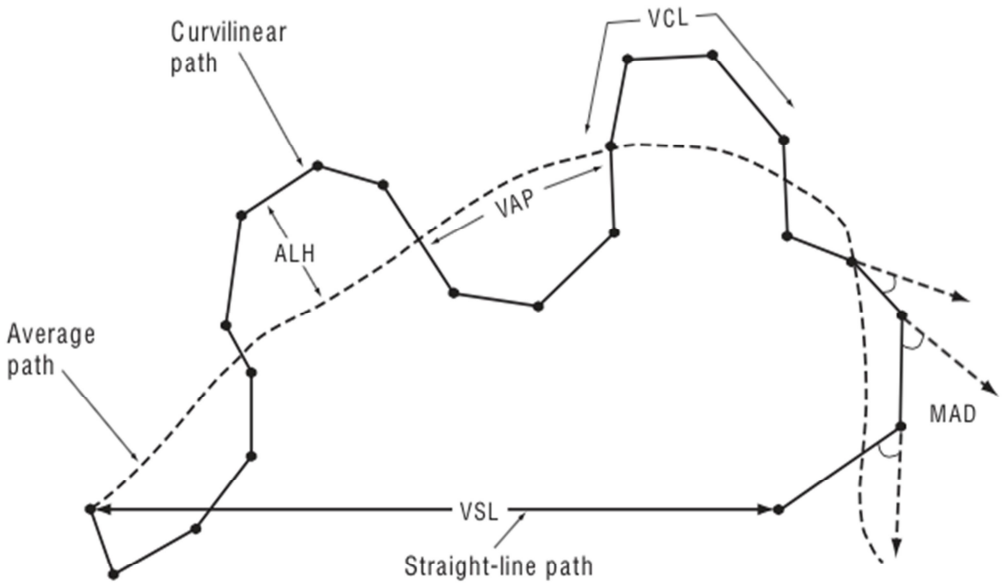

Figure 2.2: Diagram depicting some of the sperm kinematic parameters collected by CASA analysis in Chapter 6. The curvilinear velocity (VCL) calculates the distance travelled by the sperm along its curvilinear path corrected for time. The straight line velocity (VSL) calculates the distance travelled between the first and last points of the curvilinear trajectory, giving a measure of the net space gain with the period of time measured. The average path velocity (VAP) is the average trajectory of the sperm. The amplitude of lateral head displacement (ALH) is a measure of how far the position of the sperm head deviates from the average path (VAP) (Mortimer, 1994; Mortimer, 1997). The MAD stands for the mean angular displacement of the sperm head along its curvilinear trajectory. This parameter was not measured in this thesis. Figure has been adapted from (WHO, 2010), with permission to reprint from WHO Press.

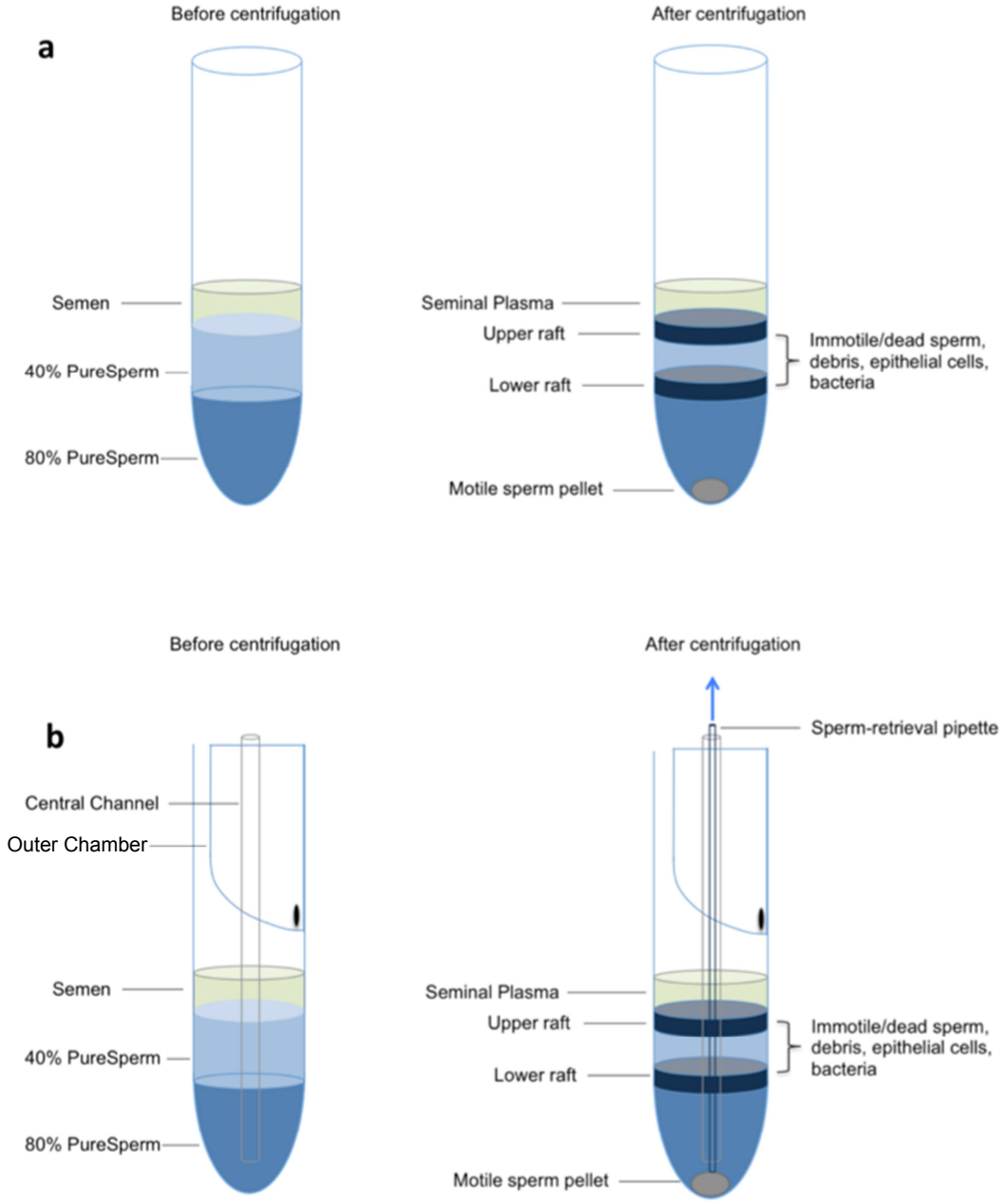

Figure 2.3: Diagrams highlighting the differences between density gradient centrifugation and the ProInsert tube™. Figure a depicts the conventional density gradient centrifugation method of separating seminal components from motile sperm. 40% PureSpem is layered onto 80% PureSperm and after centrifugation, immotile and dead sperm should remain in between the layers, leaving a pellet of motile sperm at the bottom of the 80% layer. The pellet is removed by aspirating off the upper layers and retrieving the pellet. In comparison, Figure b presents an adapted method aimed at preventing recontamination of the pellet upon retrieval. In this method, all components of the gradient are added via the other chamber and the pellet is retrieved via the central channel. This avoids recontamination of the pellet with debris from the upper layers as the central channel allows direct retrieval of the pellet.

experiment being performed) was then gently layered on the top of this gradient and centrifuged at 300*g* for 20 minutes. The remainder of the gradient and seminal plasma was gently removed in one swift motion and the pellet re-suspended by gentle agitation before being transferred via a glass Pastuer pipette to 2ml PureSperm Wash Buffer (Nidacon, Sweden). The sample was then centrifuged at 500*g* for 10 minutes before removal of most of the remaining wash buffer, leaving 300µl to re-suspend the pellet in.

An adapted version of the density gradient centrifugation technique was also used, using ProInsert tubes (Nidacon, Sweden). These tubes are designed to minimise recontamination of the pellet during retrieval by using a double tube system, similar to that of Politch *et al*., (2004). The density gradient, consisting of 1ml 40% PureSperm layered onto 1ml 80% PureSperm was prepared in the outer chamber (Figure 2.3b). Between 0.5-1ml of semen (dependent upon experiment being performed) was gently layered on top of the gradient and centrifuged at 300*g* for 20 minutes. The pellet was then retrieved using a sperm-retrieval pipette (provided with the tube), attached to a syringe. The pipette was passed through the central channel (Figure 2.3b) and the pellet aspirated. The pellet was then transferred to 2ml PureSperm Wash Buffer and centrifuged at 500*g* for 10 minutes before removal of most of the remaining wash buffer, leaving 300µl to re-suspend the pellet in.

2.5.4 Hypo-osmotic swelling test (HOST) and acrosome immunoflourescence

After exposure to CMV in Chapter 6, sperm were assessed for viability and acrosome status using the Hypo-osmotic Swelling Test (HOST) (Jeyendran *et al*., 1984), in combination with immunofluorescence detecting an acrosomal protein (Figure 2.4a) (Ellis *et al*., 1985; Moore *et al*., 1987). Briefly, 10µl of each incubate, containing ~$1.0x10^5$ sperm was added to 100µl HOST media (1:10 dilution) (FertiPro, Belgium) and incubated at 37°C for 30 minutes. Samples were smeared on to a poly-lysine microscope slide and left to air-dry overnight at room temperature. The slides were fixed by incubating for 1 minute in cold methanol before being allowed to air-dry and

stored at -20°C for analysis. Slides were warmed to room temperature before re-hydrating in 1ml TBS (Table 2.1) for 15 minutes. TBS was drained from the slides and 100µl of primary antibody targeted toward a protein present in the acrosome of the sperm was added (Ellis *et al*., 1985). The 18.6 mouse monoclonal antibody, a kind gift from Professor Harry Moore (University of Sheffield), was diluted 1:10 with PBS before being added to the slide and incubated at 37°C for 1 hour in a humidified chamber. The slides were washed twice with TBS and 100µl of secondary antibody [rabbit α-mouse IgG-fluorescin isothiocynate conjugated] diluted 1:100 with PBS was added to each slide and incubated at 37°C for 1 hour. Each slide was washed twice with TBS and the back of the slide dried. Slides were mounted using 1-2 drops of MOWIOL®4-88/1,4-diazobiycyclo-(2,2,2,)-octane (Sigma-Aldrich, Dorset, UK) (Table 2.1) and left overnight at 4°C in the dark. Slides were analysed on an Olympus BX41 with both x100 magnification phase contrast and epifluorescence objectives (UV filter 492 nm).

Acrosome fluorescence was determined by sperm displaying fluorescence over the entire acrosome classed as acrosome intact, and those with patchy fluorescence, a band of fluorescence around the equatorial segment or no fluorescence being classed as acrosome reacted sperm (Figure 2.4a). Initially, for each incubate, 200 spermatozoa were counted to measure viability using the criteria of sperm with curly/bent tails as viable and those with straight tails as non-viable, as outlined in Jeyendran *et al*., (1984) and as shown in Figure 2.4b. A further 200 viable (curly/bent tails) sperm were counted to determine the status of the acrosome, using definitions described above and the criteria outlined in (Zhu *et al*., 1994).

2.5.5 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

DNA damage was assessed via the TUNEL assay, using the Terminal deoxynucelotidyl transferase (TdT) enzyme (Gavireli *et al*., 1992; Sun *et al*., 1997). Briefly, ~5x10^6/ml sperm in a volume of 100µl, was added to a poly-

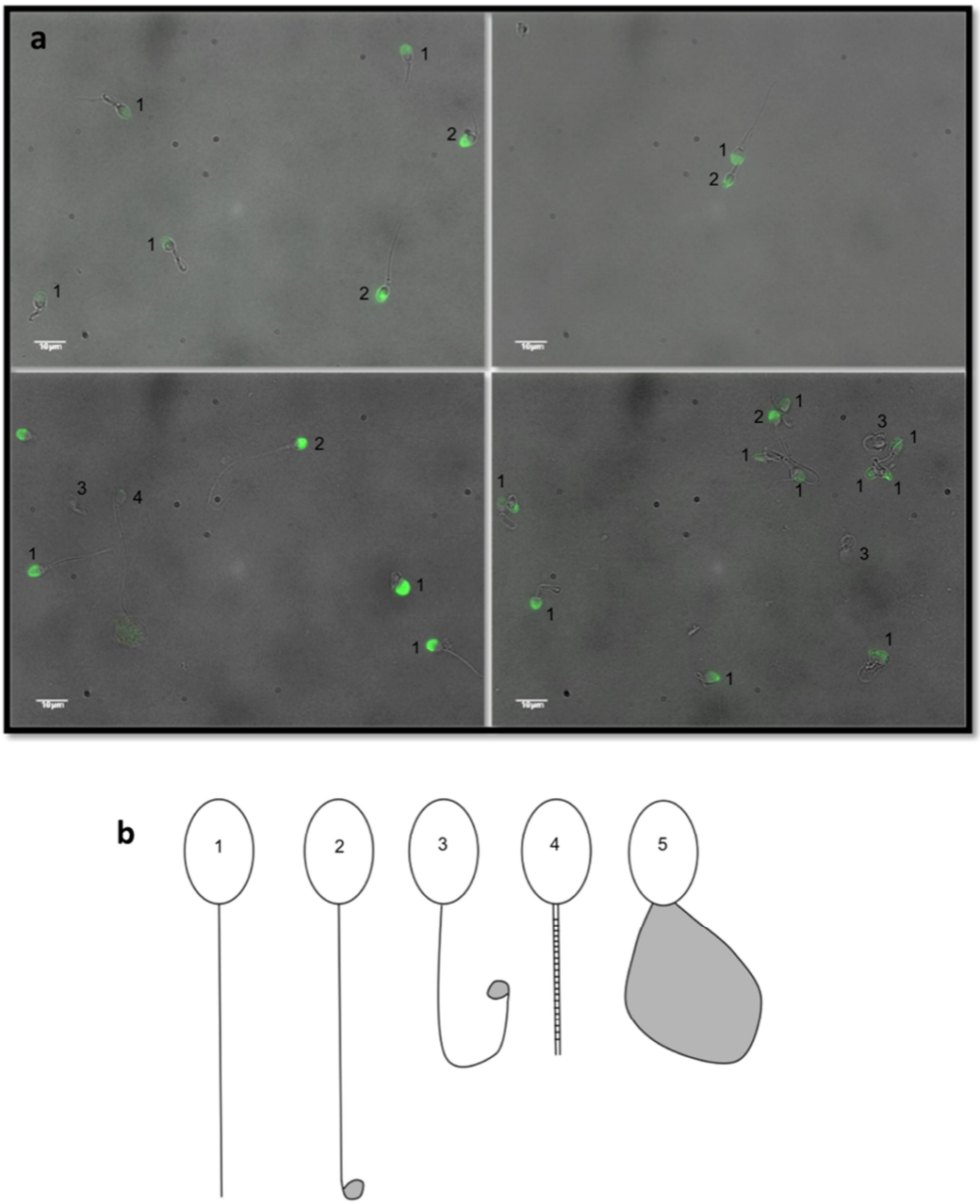

Figure 2.4: Assessment of acrosome status by immunofluorescence using an antibody targeted toward an acrosomal protein (a). Acrosome status was assessed by categorising those with fluorescence over the entire acrosomal region as being acrosome intact and those with patchy fluorescence, a band of fluorescence around the equatorial segment or no fluorescence determined as acrosome reacted. Acrosomal immunofluorescence was used in conjunction with the HOST test (b). Sperm with straight tails are considered to be dead (b.1). Sperm with varying degrees of bent tails (b.2-5) were considered to be viable (Jeyendran *et al.*, 1984). Using the two techniques together allows for identification of sperm that are acrosome intact and alive (a.1), acrosome intact and dead (a.2), no acrosome and alive (a.3) and no acrosome and dead (a.4). Examples of these categories can be seen in (a) (Zhu *et al.*, 1994). Sperm were visualised on an Olympus IX73 LED fluorescent microscope on a 60x oil immersion objective. Scale bar shown = 10µm.

lysine slide and left to air dry overnight at room temperature. The slides were fixed in methanol for 1 minute before storing at -20°C until analysis.

Slides were warmed to room temperature and re-hydrated using 1ml TBS for 15 minutes. Sperm were permeabilised by adding 200µl of 20µg/ml Proteinase K (Merck Millipore, California, USA) (2mg/ml Proteinase K was diluted 1:100 with 10mM Tris pH8) and incubating at room temperature for 5 minutes. The slides were washed 3 times with TBS and excess liquid tapped off. A positive control slide was included to control for specificity of the TdT enzyme. Briefly, 100µl of DNase I (Merck Millipore, California, USA) was added to the positive control slide and incubated at room temperature for 20 minutes. The slide was washed with 1ml TBS before removing excess liquid.

50µl of TUNEL reaction mix was prepared by adding 5µl TUNEL enzyme solution, containing TdT from calf thymus recombinant in *E. coli* (Roche, UK), to 45µl TUNEL label mix, constituting a 10% (v/v) mixture of enzyme (Sgonc *et al*., 1994). This reaction mix was added to the slides, covered with a coverslip and incubated at 37°C for 60 minutes in a humidified chamber. Coverslips were removed and slides washed in TBS twice for 1 minute at room temperature. All excess liquid was removed from the slide and the back of the slide dried.

Slides were mounted with one drop of Propidium Iodide (Vector Laboratories, Peterborough, UK) and the addition of a coverslip, sealed using nail varnish. Slides were kept in the dark at 4°C overnight and analysed on an Olympus BX41 with both x100 magnification phase contrast and epifluorescence objectives (UV filter 492 nm). For each incubate, the number of sperm with red fluorescence (no DNA damage), green fluorescence (DNA damage) or mixed fluorescence (partial DNA damage) were counted (Figure 2.5). 200 sperm per incubate were counted and the number of sperm in each category expressed as a percentage.

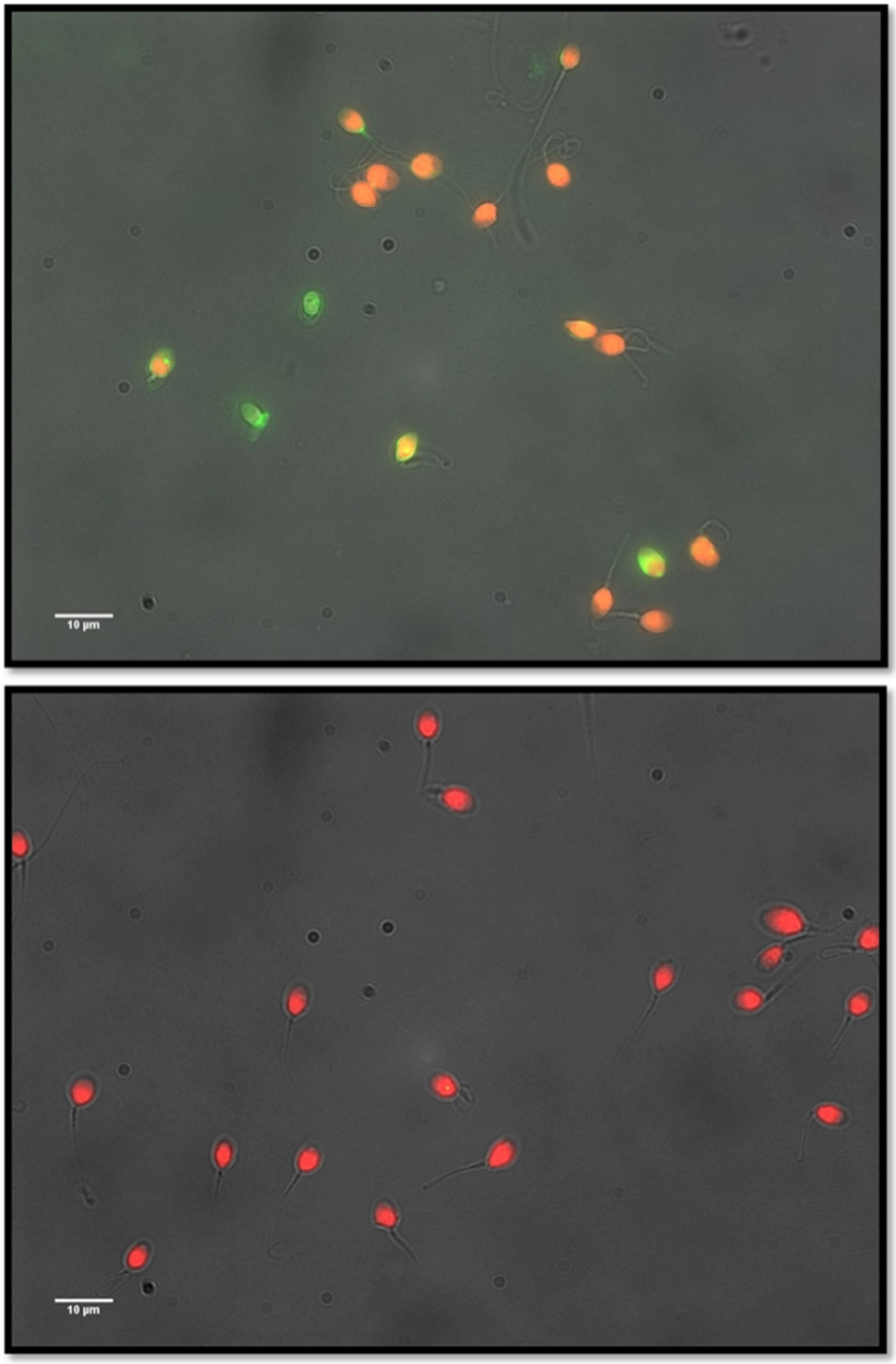

Figure 2.5: Terminal deoxynucelotidyl transferase dUTP nick end labelling (TUNEL) was carried out to assess levels of DNA damage in sperm. Staining and imaging was carried out as outlined in section 2.5.5. The top panel shows sperm incubated with the TUNEL enzyme and label mix. In comparison, the bottom panel shows a negative control, containing only the label mix. When the TdT enzyme is present, sperm with DNA damage are observed to fluoresce green, or yellow depending on the amount of DNA damage. This is in comparison to the negative control (lower panel), which shows in the absence of the TdT enzyme, all sperm fluoresce red only. Scale bar shown = 10µm.

2.5.6 Tyrosine phosphorylation immunofluorescence

Levels of tyrosine phosphorylation were measured by immunofluorescence using an α-phosphotyrosine mouse monoclonal antibody (clone 4G10) (Merck Millipore, California, USA), as outlined in Hosseinzadeh *et al*., (2000). A 100µl aliquot containing ~1.0x10^5 sperm was added to a poly-lysine microscope slide and left to air-dry overnight at room temperature. The slides were fixed in methanol for 45 minutes before storing at -20°C until analysis.

Slides were warmed to room temperature and re-hydrated using 1ml TBS for 15 minutes. The primary α-phosphotyrosine mouse monoclonal antibody (clone 4G10) was diluted 1:500 in antibody diluent (final concentration 0.5µg/ml) and 100µl was added to each slide. The slides were covered with a coverslip and incubated at 37°C for 1 hour in a humidified chamber. The slides were washed twice with TBS and 100µl of secondary antibody [rabbit α-mouse IgG-fluorescin isothiocynate conjugated] diluted 1:100 with PBS was added to each slide and incubated at 37°C for 1 hour. Each slide was washed twice with TBS and the back of the slide dried. Slides were mounted using 1-2 drops of MOWIOL®4-88/1,4-diazobiycyclo-(2,2,2,)-octane and left overnight at 4°C in the dark.

Slides were analysed on an Olympus BX41 with both x100 magnification phase contrast and epifluorescence objectives (UV filter 492 nm). For each incubate, 200 sperm were counted and the level of tyrosine phosphorylation fluorescence assessed. Sperm displaying tyrosine phosphorylation exhibit bright fluorescence along the entire length of the tail (Figure 2.6a). In comparison to a negative control (Figure 2.6c), which exhibits weak or a complete absence of fluorescence. Sperm exhibiting the fluorescence pattern seen in Figure 2.6a were scored as tyrosine phosphorylated and those exhibiting the pattern seen in Figure 2.6c scored as non-tyrosine phosphorylated. To demonstrate the specificity of the primary antibody, Figure 2.6b & d show no fluorescence when the secondary antibody is added alone.

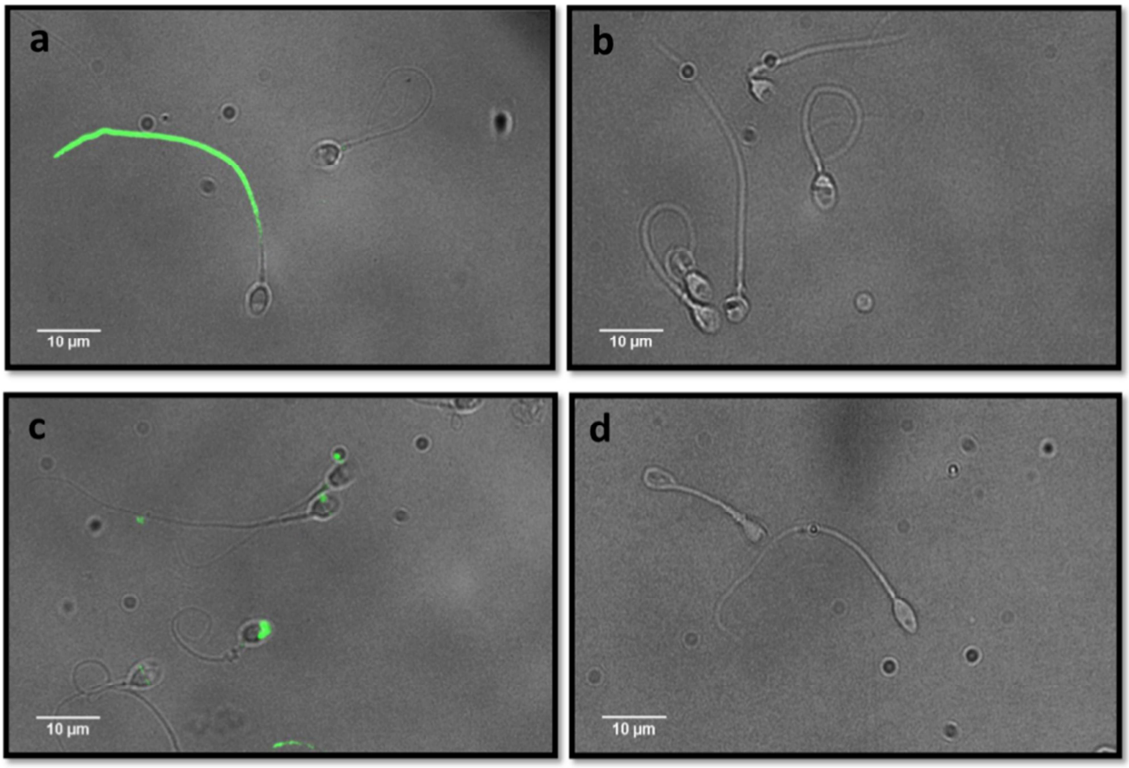

Figure 2.6: Measurement of tyrosine phosphorylation by immunofluorescence using an α-phosphotyrosine mouse monoclonal antibody (clone 4G10). When incubated with both primary and secondary antibody, as outlined in Section 2.5.6, capacitated sperm exhibit an intense fluorescence over the entirety of the sperm tail (a), in comparison to non-capacitated sperm which do not exhibit the same pattern of fluorescence (c). No fluorescence is observed when only the secondary antibody was used in either capacitated (b) or non-capacitated (d) sperm. Sperm were visualised on an Olympus IX73 LED fluorescent microscope on a 60x oil immersion objective. Scale bar shown = 10µm.

2.6 Molecular Biology

2.6.1 RNA extraction of MRC-5 cells

RNA was extracted from the cells using an E Z RNA isolation kit (Geneflow, Staffordshire, UK). Briefly, 0.5ml of denaturing solution was added to the culture flask and the cells were scraped from the flask surface using a cell scraper. The homogenate was stored at room temperature for 5 minutes before adding 0.5ml of extraction solution and vigorously shaking for 15 seconds. The sample was stored at room temperature for 10 minutes before centrifuging at 12,000*g* for 15 minutes at 4°C. The aqueous upper phase was transferred to a fresh Eppendorf tube (StarLab, Milton Keynes, UK) and 0.5ml of isopropanol added, mixed and stored overnight at -20°C. The following day the sample was centrifuged at 12,000*g* for 8 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 75% (v/v) ethanol by vortexing. The sample was centrifuged at 7,500*g* for 5 minutes at 4°C. The ethanol wash was removed and the pellet was left to air dry for 5 minutes before dissolving the RNA in 100µl of DEPC-treated water (Life Technologies, Paisley, UK). The purified RNA was quantified and the parameters of purity examined by adding 2µl to a nanophotometer (Geneflow, Staffordshire, UK).

2.6.2 cDNA synthesis

RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (BioRad, Hertfordshire, UK). Briefly, 1µg of RNA was added to a 20µl reaction volume composed of 4µl 5X iScript reaction mix, 1µl iScript reverse transcriptase and 12µl nuclease free water. A no reverse transcriptase control was also included, substituting 1µl of enzyme for 1µl of nuclease free water. The reaction was incubated for: 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. A 2µl aliquot of cDNA was used in subsequent PCR experiments.

2.6.3 DNA extraction of MRC-5 cells

MRC-5 cells were grown until confluent in a T75 culture flask. Cells were detached from the flask surface by adding 2ml trypsin and incubating at 37°C. Cells were washed from the cell surface and transferred to two 1ml Eppendorf tubes (StarLab, Milton Keynes, UK) before centrifuging at 1,000*g* for 10 minutes. The supernatant was discarded and 200µl of lysis buffer was added to the pellet on ice and incubated for 10 minutes at 37°C. One lysis reaction was centrifuged at 12,000*g* for 25 minutes and the cell lysate stored at -20°C for CMV analysis. DNA was extracted from the second lysis reaction by adding 200µl phenol chloroform and mixing well. The sample was centrifuged at 12,000*g* for 5 minutes at 4°C. The upper aqueous phase was transferred to a new Eppendorf tube and an equal volume of chloroform was added and mixed well. The sample was centrifuged at 12,000*g* for 5 minutes at 4°C, before transferring the aqueous upper phase to a new Eppendorf tube. The salt concentration was adjusted by adding 1/25 volume of NaCl and 1µl of glycogen was added. The solution was mixed well and exactly 2 volumes of ice cold 100% (v/v) ethanol added. The reaction was incubated on ice for 30 minutes before centrifuging at 12,000*g* for 10 minutes at 0°C. The supernatant was removed and 1ml of 70% (v/v) ethanol added and centrifuged at 12,000*g* for a further 2 minutes at 4°C. The supernatant was removed and the pellet was left to air dry before re-suspending in 50µl of DEPC-treated water.

2.6.4 DNA extraction of sperm

DNA was extracted from sperm using a QIAamp DNA Mini Kit (Qiagen, Manchester, UK). A 100µl aliquot of semen or sperm was extracted following the manufacturer's bodily fluids spin protocol. Briefly, 100µl of sample was added to 20µl QIAGEN proteinase K, to which 200µl of Buffer AL was added and mixed by pulse vortexing for 15 seconds. The sample was then incubated at 56°C for 10 minutes. Samples were briefly centrifuged (to remove drops from inside of the lid) and 230µl of 100% ethanol was added. This mixture was then applied to the QIAamp Mini spin column and

centrifuged at 6000*g* for 1 minute. The QIAamp spin column was removed, placed in a fresh collection tube and 500µl of Buffer AW1 was added. The columns were centrifuged at 6000*g* for 1 minute and the QIAamp spin column removed and placed in a fresh collection tube. 500µl of Buffer AW2 was added and samples centrifuged at 20 000*g* for 3 minutes. Following this final centrifugation step, the QIAamp column was removed and placed in a fresh Eppendorf tube. 100µl of Buffer AE was added directly to the column and incubated at room temperature for 1 minute. Samples were centrifuged for 1 minute at 6000*g* and the eluate retained for analysis. All DNA samples were analysed for quality and quantity by adding 3µl to a nanophotometer (Geneflow, Staffordshire, UK). The presence of genomic DNA was assessed by amplifying GAPDH by PCR (Section 2.6.5)

2.6.5 Polymerase Chain Reaction (PCR) conditions

All PCR reactions were carried out in a Sensoquest Labcycler (Geneflow, Staffordshire, UK). Two PCR master mixes were used throughout this thesis: (1) Promega PCR master mix (Promega, Southampton, UK) and (2) MyTaq™ HotStart Mix (Bioline, London, UK). The polymerase used was changed to MyTaq™ HotStart Mix as it was more efficient, needing fewer PCR cycles and less amplification time. Subsequently, this master mix was found to be more specific at amplifying the correct products and was therefore used in all later PCR reactions. The cycling conditions and reaction components for each amplification product and each PCR master mix are detailed below.

Thy1

Amplification to detect the presence of Thy1, a fibroblast specific gene, in MRC-5 cells. In a total reaction volume of 25µl, 2µl of cDNA, 25µM of both upstream and downstream primers (provided by Dr Sarah Waite, University of Sheffield) (Table 2.2) and 12.5µl of 2x PCR master mix (composed of 50 units/ml *Taq* polymerase, 400µM of each dNTP and 3mM $MgCl_2$), were added. The reaction was cycled 25 times for 2 minutes at 94°C, 25 seconds at 94°C, 30 seconds at 65°C, 45 seconds at 72°C and 5 minutes at 72°C.

Table 2.2: Primer sequences used throughout Chapter 4 in the construction of PcRsV-gB plasmid, detection of CMV and characterisation of MRC-5 cells.

Primer	Sequence 5'-3'
Thy1-forward	CTGGGTGCAGCAACCGGAGG
Thy1-reverse	TGCTCAGGCACCCCCACAGT
GAPDH-forward	GACATCAAGAAGGTGGTGAAG
GAPDH-reverse	GTCCACACCCTGTTGCTGTAG
gB1	GAGGACAACGAAATCCTGTTGGGCA
gB2	TCGACGGTGGAGATACTGCTGAGG
gBA	GCGAAGCTTCGACGCGCCTCATCGCTGCT
gBB	GTCTAGACCTCCTGGTTCAGACGTTCT
gBF	CTGAAGTCGGTATTTTCCAGC
gBR	GGGCGAGGACAACGAATC
QgBF	TGAAGTCGGTATTTTCCAGC
QgBR	GGGCGAGGACAACGAATC
gB3	CAATCATGCGTTTGAAGAGGTAGTCCACG

GAPDH

In a total reaction volume of 25µl, 2µl of MRC-5 DNA, 25µM of each upstream and downstream primer specific to a 200bp region of the GAPDH housekeeping gene (provided by Dr Neil Chapman, University of Sheffield) (Table 2.2) and 12.5µl of 2x PCR master mix (composed of 50 units/ml *Taq* polymerase, 400µM of each dNTP and 3mM $MgCl_2$) were added. The reaction was cycled 25 times for 2 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 53°C, 45 seconds at 72°C and 5 minutes at 72°C. For sperm DNA, in a total reaction volume of 25µl, 5µl of sperm DNA, 25µM of each upstream and downstream primer and 12.5µl of 2x MyTaq™ HS Mix were added. The reaction was cycled 25 times for 1 minute at 95°C, 15 seconds at 95°C, 15 seconds at 60°C and 10 seconds at 72°C.

Glycoprotein B

In a total reaction volume of 25µl, 2µl of MRC-5 DNA, 25µM of each upstream (gB1) and downstream (gB2) primer specific to a 149bp region of the CMV glycoprotein B gene (Mattes *et al*., 2004) (Table 2.2) and 12.5µl of 2x PCR master mix (composed of 50 units/ml *Taq* polymerase, 400µM of each dNTP and 3mM $MgCl_2$), were added. The reaction was cycled 30 times for 2 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 61°C, 45 seconds at 72°C and 5 minutes at 72°C.

An alternate set of primers was used in later experiments, gBF and gBR (designed using Primer3) (Table 2.2), specific to a 200bp region of the CMV glycoprotein B gene. These primers were used in conjunction with the MyTaq™ HS Mix, the reaction volume and components were the same as listed above but the reaction was cycled 25 times for 1 minute at 95°C, 15 seconds at 95°C, 15 seconds at 59.5°C and 10 seconds at 72°C.

Glycoprotein B-whole gene

The whole glycoprotein B gene was amplified for the purpose of cloning into a vector as a standard for quantitative PCR (Section 2.6.6). In a total reaction volume of 25µl, 0.6µl of CMV DNA (Health Protection Agency, Porton Down,

Salisbury, UK), 25µM of each upstream (gBA) and downstream (gBB) primers specific to the 2.8kB glycoprotein B gene (Temperton *et al*., 2003) (Table 2.2) and 12.5µl of 2x PCR master mix (composed of 50 units/ml *Taq* polymerase, 400µM of each dNTP and 3mM $MgCl_2$), were added. The reaction was cycled 30 times for 2 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 65°C, 3 minutes at 72°C and 5 minutes at 72°C. Primer design and PCR conditions were based on those published by Temperton *et al*., (2003).

2.6.6 Quantitative PCR (qPCR) conditions

In-house qPCR, reported in Chapter 4, was carried out on an Applied Biosystems 7900 Real-Time PCR machine, using a clear 384 well plate (StarLab, Milton Keynes, UK). Plates were sealed with an optically clear heat-sealing film (StarLab, Milton Keynes, UK). PCR set-up was performed in a PCR hood (Geneflow, Staffordshire, UK) and all plastic ware sterilised with UV light prior to use.

Absolute quantification using a TaqMan hydrolysis probe was carried out using the SensiFAST™ Probe Hi-ROX kit (Bioline, London, UK). In a total reaction volume of 20µl, 2.5µl of each flanking primers, QgBF and QgBR (Mattes *et al*., 2004) and hydrolysis probe (gB3) (labelled at the 5' end with 6-FAM and at the 3' end with TAMRA) (Mattes *et al*., 2004) (Table 2.2) were added at a final concentration of 125nM, 250nM and 300nM respectively. A no template control, constituting H_2O alone was included on each plate. 10µl of master mix was added in addition to 2.5µl of sample or standard. The reaction was cycled 40 times for 5 minutes at 95°C, 10 seconds at 95°C and 20 seconds at 60°C.

The Virus Detection Group at University College London performed a clinically validated PCR assay on all DNA samples in Chapter 6. This PCR assay was carried out in a clinical virology laboratory, which takes part in the EQA programme. The qPCR assay used is outlined in Mattes *et al*., 2004.

Chapter 3

How do fertility clinics diagnose and manage CMV infection in sperm donors?

3.1 Introduction

Since the introduction of recommended screening for CMV in sperm donors, by the British Andrology Society (Barratt *et al*., 1993), it has been a controversial issue. Most of the controversy surrounded the recommendation to exclude all CMV positive donors (British Andrology Society, 1999). This sparked much debate, with Curson & Karakosta (2000) pointing out the inevitable reduction in the number of available donors if all positive donors were excluded from donating sperm.

Further questions were raised regarding the practicality of this approach, with some asking “where do we draw the line” with sperm donor screening (Matson, 2001). The multiple possible sources of CMV infection, such as; sexual partners, infected children and reactivation of latent virus, led some to question the relevance of CMV screening, in particular relation to seropositive recipients (Liesnard *et al*., 2001). Furthermore, the evidence that infectious CMV is present in less than 5% of seropositive donors (Mansat *et al*., 1997) supports the argument that these donors might present a minimal risk level. Similar questions surrounding the need to screen for rare genetic disorders, such as hypertrophic cardiomyopathy (Maron *et al*., 2009), have also been debated and is an example of where the risk is considered too small to justify screening.

Despite these points, in a letter to the editor of *Human Reproduction* in 1999, then British Andrology chairman, Eileen McLaughlin, argued “it is not the size of the risk that is important but the fact that simple steps can be taken to reduce the risk” (McLaughlin, 2000). She also reiterated that the main aim of the 1999 guidelines was to “reduce as far as possible the risk of a child suffering from a serious disability, which could have been avoided” (McLaughlin, 2000). Whilst an ideal stand point, the 2008 guidelines took a more pragmatic view and recommend that whilst “it is always preferable to recruit CMV-negative donors”, this was not always feasible and “in situations where insufficient CMV-negative donors are available, CMV IgG positive (IgM negative) donors may be recruited but their use should be limited to

CMV IgG positive recipients". The guidelines also state "the decision to treat a patient with a seropositive donor should be a matter of clinical judgement" (Association of Biomedical Andrologists *et al*., 2008). This is an approach that is considered to be a practical and viable option (Liesnard *et al*., 1998).

The relevant UK regulatory bodies have made their view on CMV screening clear but there is a degree of variation across the world. For example, current UK guidelines fall short of the recommendations of clinics in the USA to carry out urine and throat cultures, to diagnose an active infection (American Society for Reproductive Medicine, 2013), and to assess IgG titre levels to diagnose a reactivation event (Dolan *et al*., 1989). Along with the UK guidelines, whilst the ASRM recommends seromatching, they acknowledge this is not without risk due to the risk of re-infection with an exogenous strain of CMV. In comparison, the EU Tissue Directive (Directive 2006/17/EC) does not require clinics in Europe to carry out CMV testing at all. This might add further complications to the issues surrounding CMV screening in the UK, as clinics rely on overseas donors from European sperm banks to meet demand for donors in the UK (HFEA, 2014). Despite routine CMV screening not being conducted in these clinics, the HFEA does stipulate that imported sperm should be screened as if it were sourced in the UK, from a UK donor.

It is clear therefore that there is wide variation in the recommended practice and opinions surrounding CMV screening within clinics in the UK and around the world. The lack of data regarding the risk CMV poses in donor insemination further confuses the issue and as pointed out in Liesnard *et al*., (2001), "it is urgent to investigate this risk and its consequences more deeply". Throughout this controversy and confusion, the actual approach clinics are taking to implement professional body guidelines has failed to be explored in a robust way. Given the apparent differing opinions, many questions need to be asked about what 'clinical judgements' are being made, for example, to what extent does seromatching donors and recipients actually occur? Through obtaining answers to these questions, it might be possible to identify any problems being caused by CMV screening and steps

to solve any issues could be taken to improve these procedures for clinics and patients.

3.2 Rationale

In light of the obvious pressure of reduction in availability of donors, possibly as a result of the requirement to screen for CMV, it was hypothesised that UK clinics may increasingly choose to ignore current screening guidelines, in order to increase the supply of donors. In turn, it was proposed that this would lead to variation and inconsistency in screening practices across the UK. Therefore, a simple service evaluation tool in the form of a survey was designed to understand the approaches currently used by UK clinics for the diagnosis of CMV and management of CMV positive donors. Clinics outside the UK were also approached to see how their procedures compared and whether they differed in any way.

The aim of the survey was to answer the following questions;

1. How is screening for CMV managed in fertility clinics, including; what type of laboratory test is performed, when this test is performed and how the results are interpreted.
2. How a clinic takes CMV screening into consideration when buying donor sperm from other centres.
3. How CMV infection in sperm donors is managed with regards to seromatching donors and recipients, and whether this causes issues with donor supply.
4. Identify if practices between UK and non-UK clinics differ and if this identifies any particular problems.
5. What are the views in clinics regarding CMV screening and the effects on their clinics.

3.3 Methods

A survey tool was developed to capture the screening practices in each clinic, in addition to capturing information about sperm donor recruitment and donor/recipient matching in the context of CMV infection. The questionnaire (Appendix IV) was divided into eight parts: (i) details of the clinic size, clinical activities and scale of the donor recruitment programme (if applicable); (ii) the clinic approach to CMV screening and how they managed the process including any use of (iii) serum antibody testing; (iv) PCR; or (v) viral culture. Part (vi) asked about the clinic policy of buying donor sperm from other centres and how the clinic assessed the CMV screening performed by the distributing centre (if applicable); and part (vii) asked about how the clinic matched CMV status of donors and recipients, with a view to understanding if they carry out seromatching and if CMV infection in donors was perceived as a problem. Each of the sections (i) to (vii) was comprised of a series of questions with a number of tick boxes to capture the responses, with a box at the end of each section where free-text responses were encouraged. The final part (viii) was a free text box where the respondent was asked to provide any further information about their views on CMV screening if the previous sections did not cover them.

In December 2013, the questionnaire was sent to 103 clinics listed on the HFEA website as being licenced to provide fertility treatment within the UK. In addition, in April 2014 the questionnaire was also circulated via Androlog (an internet user's group moderated by Craig Niederberger from the University of Illinois at Chicago and Andy Meacham at the University of Colorado at Denver) with over 1547 subscribers [Neiderberger, C., Personal Communication]. Recipients of the survey were encouraged to pass it on to colleagues in other clinics and we also circulated the questionnaire more opportunistically through a ‘Snowballing’ method often used to contact hard to reach groups (Faugier and Sargeant, 1997). Data collection ended at the end of November 2014 and any responses received after this date were not included.

Upon receipt, all responses were double entered into an Excel Spreadsheet (Microsoft, Washington, US) and checked for accuracy, before being summarised and examined using SPSS (Version 21, IBM corporation). Some data points were missing but were not excluded from analysis. Free text responses were examined using a thematic analysis approach outlined in Braun and Clarke (2006).

3.4 Results

A total of 52 responses were received from the 103 UK clinics approached by email (50.5% response rate) and a further 31 from non-UK clinics (response rate estimated at <10%). The majority of UK (84.6%) and non-UK clinics (77.4%) provided treatment with donor sperm, performing a median number of 64 (range 6-300) and 190 (range 5-2257) treatment cycles respectively in 2012. The majority of UK clinics (67.3%) recruited a median of 5 donors (range 0-79), compared to a median of 23 (range 0-16453) in 74.2% of non-UK clinics. The majority of UK (75.0%) clinics bought donor sperm from other centres, in comparison to only 64.5% of non-UK clinics. A summary of all the demographics of responding clinics can be found in Table 3.1.

3.4.1 How is CMV screening being performed and managed in clinics?

The majority of UK clinics and sperm banks that recruited donors in 2012 reported screening donors for CMV (97.1%), in comparison to only 65.2% of non-UK clinics and sperm banks. When screening donors, current guidelines recommend that semen samples should be quarantined for 180 days and 70.5% of UK clinics reported to follow these guidelines, in comparison to 80.0% of non-UK clinics (Table 3.2a). Clinics that did not report following the guidelines for quarantine stated that the use of NAT testing or instances of ‘known donation’ were situations where the quarantine period was shortened, or not carried out at all. When the quarantine period was performed, the majority of clinics screened donors both before and after quarantine (Table 3.2b). However, a minority of clinics in both UK and non-UK clinics stated they only screened before quarantine, 12.5% and 13.3%, respectively.

Table 3.1: Demographics of responding clinics.

Item	UK (n=52)	Non-UK (n=31)
Provide treatment with donor sperm	84.6% (n=44)	77.4% (n=24)
Recruitment of sperm donors	67.3% (n=35)	74.2% (n=23)
Buy sperm from other centres	75.0% (n=39)	64.5% (n=20)
Supply sperm to other centres	17.3% (n=9)	32.3% (n=10)
Number of cycles [1]	64.0 (6-300)	190.0 (2-2257)
Number of donors screened[1]	5.0 (0-79)	23.0 (0-16453)

[1]Median (Range)

A smaller percentage reported only screening donors after the quarantine period (3.1% and 6.7% respectively). The majority of UK clinics (94.1%) reported to screen donors by the recommended serum antibody test, in comparison to 100% of non-UK clinics (Table 3.2c). When performing this test, the majority of UK (78.1%) and non-UK (86.7%) clinics screened for both CMV IgG and IgM antibodies (Table 3.2d). However, a minority of clinics only screened for CMV IgG antibodies, with some clinics stating this was because IgM antibody testing was found to be unreliable.

Table 3.2 shows that the interpretation of serology test results varies between UK and non-UK clinics. An overview of how test results should be interpreted, in line with the current guidelines for the management of CMV positive donors can be seen in Figure 3.1. This flow chart also highlights how clinics are deviating from these guidelines based on findings from this survey. When interpreting CMV IgG antibody test results, 26.9% of UK clinics and 41.7% of non-UK clinics stated they would exclude an IgG positive donor after quarantine, if previously negative (Table 3.2g). Similarly, if the IgG antibody titre had increased after quarantine, only 3.8% of UK and 16.7% of non-UK clinics reported they would exclude a donor on this basis (Table 3.2h). The presence of IgM antibodies is indicative of an active infection and the guidelines are clear about how donors found to be CMV IgM positive should be managed. Despite this, only 76.0% of UK clinics stated they would exclude a donor if found to be IgM positive before quarantine (Table 3.2i) and 71.4% if positive after quarantine (Table 3.2j). In comparison 90.9% and 91.7% of non-UK clinics reported they would exclude in these scenarios, respectively. Despite not being recommended by the current guidelines, 26.5% of UK and 13.3% of non-UK clinics stated they used PCR to detect CMV (Table 3.2k). The majority of clinics used blood as the source for the test, however one clinic from each category reported to use semen (Table 3.2j). Approximately half of both UK and non-UK clinics stated they would exclude a donor if CMV DNA were present (Table 3.2n). Clinics were also asked if they performed viral culture as a test for CMV infection, in line with the current ASRM guidelines (American Society for Reproductive Medicine,

Table 3.2: How do clinics perform CMV screening in sperm donors?

Item		UK (n=34)	Non-UK (n=15)
Quarantine			
a. Quarantine samples for recommended period?		70.5% (n=24/34)	80.0% (n=12/15)
b. Time of Screening	Both:	81.3% (n=26/32)	73.3% (n=11/15)
	Before only:	12.5% (n=4/32)	13.3% (n=1/15)
	After only:	3.1% (n=1/32)	6.7% (n=2/15)
c. Serum Antibody Screening:		94.1% (n=32)	100.0% (n=15/15)
d. Type of antibody?	Both:	78.1% (n=25/32)	86.7% (n=13/15)
	IgG only:	15.6% (n=5/32)	13.3% (n=2/15)
	IgM only:	0.0%	0.0%
e. Exclude all positive donors (IgG or IgM)?		3.1% (n=1/32)	0.0%
f. Exclude IgG at start of quarantine?		3.3% (n=1/30)	0.0%
g. Exclude IgG at end of quarantine if originally negative?		26.9% (n=7/26)	41.7% (n=5/12)
h. Exclude IgG at end of quarantine if the antibody titre has increased?		3.8% (n=1/26)	16.7% (n=2/12)
i. Exclude IgM at start of quarantine period?		76.0% (n=19/25)	90.9% (n=10/11)
j. Exclude IgM at end of quarantine, if previously negative?		71.4% (n=15/21)	91.7% (n=11/12)
k. PCR:		26.5% (n=9)	13.3% (n=2/15)
l. Specimen used	Blood:	88.9% (n=8/9)	50.0% (n=1/2)
	Semen:	11.1% (n=1/9)	50.0% (n=1/2)
m. Type of PCR	Quantitative:	33.3% (n=3/9)	0.0% (n=1/2)
	Qualitative:	11.1% (n=1/9)	50.0% (n=1/2)
n. Exclude if CMV DNA is present?		55.6% (n=5/9)	50.0% (n=1/2)
o. Viral Culture:		0.0%	6.7% (n=1/15)

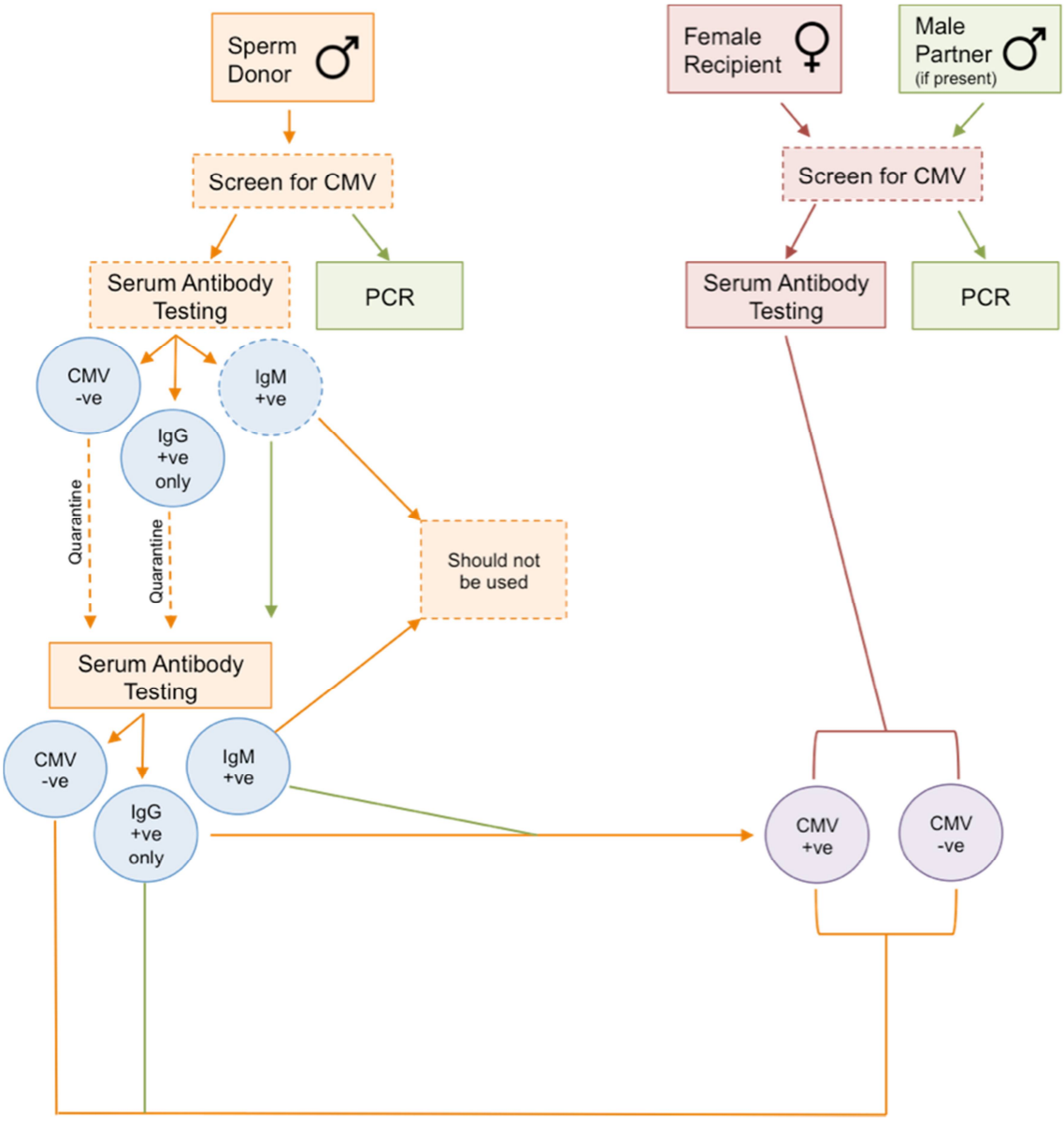

Figure 3.1: A flowchart summarising the screening process for CMV in sperm donors and those undergoing donor insemination. Briefly, current guidelines state all sperm donors should be screened for CMV by serum antibody testing. IgM positive donors should be deferred from donating but seronegative and IgG positive donors are allowed to be used, dependent upon continued IgM negative results after a quarantine period. Donors and recipients should be matched based on their CMV serostatus and CMV IgG positive donors should only be given to seropositive recipients. Shapes with dashed lines indicate procedures that are recommended but not always carried out. Green boxes and lines indicate procedures being carried out in clinics that are not current recommended. NB. Whilst no clinics actively reported offering IgM positive donors to recipients, this can be inferred by the lack of IgM testing in some clinics, the lack of exclusion of IgM positive donors after quarantine and the lack of sero-matching.

2013). No UK clinics reported to use this method, in comparison to one non-UK clinic (6.7%) (Table 3.2o). A graph summarising all of the types of exclusion criteria and the percentage of clinics following these criteria is shown in Figure 3.2.

3.4.2 How is CMV screening managed when sperm is imported from other centres?

Due to most clinics not being able to recruit enough donors to meet the demand in the UK (Hamilton *et al*., 2008), the majority of clinics that answered the survey also bought sperm from other centres. Fortunately, this survey has found that most UK clinics operate the same policy regarding CMV screening when buying sperm from other centres, as when recruiting and screening within their own clinics (Table 3.3). 92.3% of UK clinics reported to check that the donor had been screened for CMV in comparison to only 60.0% of non-UK clinics. However, only 23.1% of UK clinics stated that they checked how the screening had been performed, with a similar percentage of non-UK clinics stating the same. The majority (>80%) of both UK and non-UK clinics checked that the donors had been quarantined for the recommended time and >70% requested an official certificate. Analysis of the free-text responses in this section revealed that some clinics experienced problems when buying sperm from other centres, as not all non-UK based clinics have to screen for CMV. This means they have to request the additional information, which is sometimes difficult to obtain.

3.4.3 How is CMV infection in sperm donors managed in the fertility clinic?

In order to seromatch donors and recipients, as recommended in the current guidelines, the female recipient also has to be screened for CMV. According to the survey, the majority of UK clinics (88.6%) did screen the female recipient, in comparison to only 41.6% of non-UK clinics (Table 3.4a). Interestingly, 20.5% of UK and 29.1% of non-UK clinics also screened the male partner, if there was one (Table 3.4b).

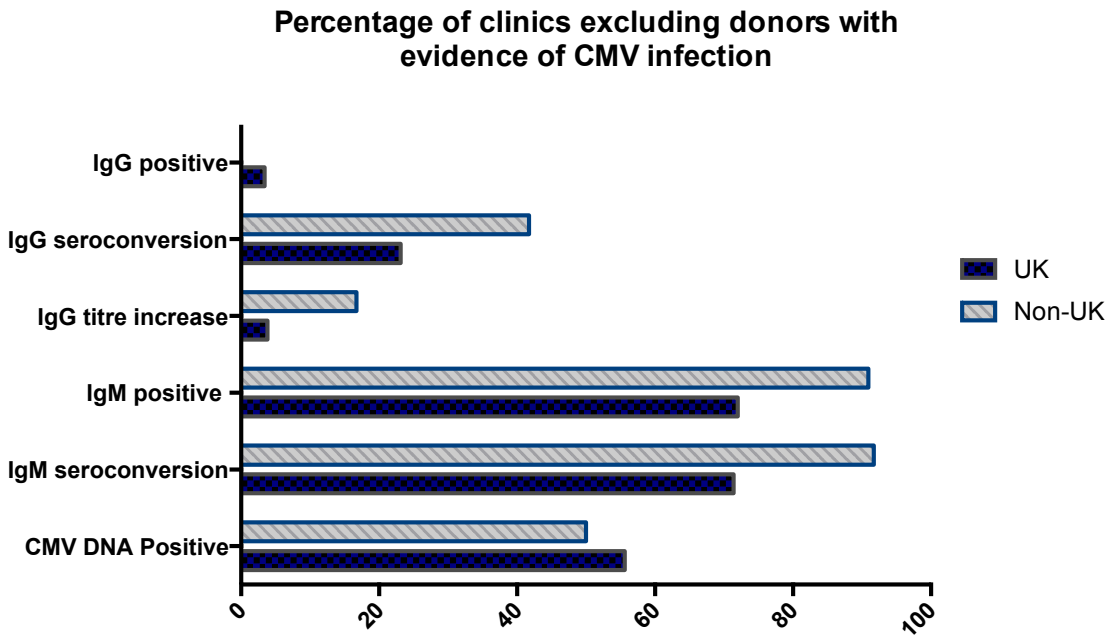

Figure 3.2: The percentage of clinics in the UK and non-UK stating they would exclude a sperm donor with test results indicating presence of CMV infection. Not all of the parameters included in this graph are criteria outlined in the current guidelines, but are all potential results from the current tests being performed in clinics that could indicate a current infection of CMV in a sperm donor. All of these test results could be associated with the presence of CMV in semen.

Table 3.3: How do clinics manage the documentation of CMV screening when buying from other centres?

Item	UK (n=39)	Non-UK (n=20)
Check they have been screened?	92.3% (n=36)	60.0% (n=12)
Check how they have been screened?	23.1% (n=9)	30.0% (n=6)
Check they have been quarantined for recommended time?	84.6% (n=33)	80.0% (n=16)
Request official copy of certificate?	76.9% (n=30)	70.0% (n=14)

Table 3.4: Management of CMV screening in clinics that provide treatment with donor sperm (including clinics that recruit their own donors and those that buy from other centres). *Please note, total numbers are based on the number of clinics screening either the donor or patient for clinics that recruit (UK n=33, non-UK n=9) and the number of clinics screening the patient only for clinics that do not recruit (UK n=7, non-UK n =4).

Item	UK (n=44)			Non-UK (n=24)		
	Recruit (n=34)	Buy sperm (n=10)	Average	Recruit (n=17)	Buy sperm (n=7)	Average
a. Clinics screening female patient for CMV?	94.1% (n=32)	70.0% (n=7)	88.6%	35.3% (n=6)	57.1% (n=4)	41.6%
b. Clinics screening male partner (if there is one)?	20.6% (n=7)	20.0% (n=2)	20.5%	29.4% (n=5)	28.6% (n=2)	29.1%
c. Clinics that seromatch CMV status of donor to recipient?*	93.9% (n=31)	85.7% (n=6)	92.5%	44.4% (n=4)	75.0% (n=3)	53.8%
d. Clinics that claim CMV screening causes donor supply problem?*	72.7% (n=24)	71.4% (n=5)	72.5%	44.4% (n=4)	25.0% (n=1)	38.5%
e. Clinics that inform recipient of theoretical risk of CMV transmission?*	75.8% (n=25)	85.7% (n=6)	77.5%	44.4% (n=4)	100.0% (n=4)	61.5%

UK clinics reported to seromatch donors and recipients based on CMV status more often than non-UK clinics, 92.5% and 53.8%, respectively (Table 3.4c). When asked to provide details on how seromatching was conducted, most provided a response that falls in line with current screening guidelines. However, clinics did report deviating from these guidelines by offering CMV positive donors to CMV negative recipients in instances such as a shortage of CMV negative donors or 'difficult to obtain phenotype or ethnic origin'.

When asked if they thought screening for CMV was contributing to a donor supply problem, only 38.5% of non-UK clinics reported that this was the case, in comparison to 72.5% of UK clinics (Table 3.4d). When asked to provide reasons for this, the majority of clinics reported that having to seromatch causes limited availability and choice for CMV negative patients. Some clinics felt that they no longer had a problem with sperm donor supply as they either had a sufficient pool of CMV negative donors, or the patients had sufficient choice from overseas donors. In some instances, relaxing of the guidelines with regards to CMV seromatching had relieved the problem.

When a seropositive recipient was offered sperm from a seropositive donor, the majority of clinics in the UK (77.5%) and 61.5% of non-UK clinics reported informing patients of the theoretical risk of infection (Table 3.4e). When clinics were asked to explain what the response was from recipients, some clinics stated that patients were generally not concerned, whilst others stated that CMV status was a big concern for patients as they were worried about the risk of transmission. However, the majority of clinics stated that in most cases, once the risks had been explained as 'small and theoretical', most patients would decide to then use a positive donor, if it provided the best match. Some clinics failed to see the relevance of the question as they reported to only use CMV positive sperm on CMV positive recipients, failing to acknowledge the risk of re-infection.

3.4.4 What are the views of clinics regarding CMV screening?

Analysis of all the free-text responses provided throughout the survey identified three key themes surrounding the process of screening and

Table 3.5: Details of individual themes identified in the thematic analysis and examples of representative comments made by clinics.

Themes (sub-themes)	Comments
(a) Relevance of CMV seromatching	
(a1) Risk of transmission	We screen all donors for CMV IgG and IgM but we do not screen recipients or their partners as we do not match based on CMV status. This was a fairly recent policy change (within the last year) based on the very small risk of transmission from a CMV IgG positive donor to a CMV IgG negative recipient. This has also had the significant benefit of increasing patients' choice of donors, which was previously very limited for CMV IgG negative recipients. [UK clinic]
(a2) External risk factors	As CMV is only tested for donor eggs and sperm, I feel it is a pointless test as couples for standard IVF neither partner is screened. This causes the patients extra anxiety as to why we are testing. CMV is around in the environment and can therefore be contracted any time via an outside source and therefore it can be proven whether it is from the gametes or not. [UK clinic]
(a3) Partner screening	I feel CMV screening is necessary but I do think that we should also test patients partners at the treatment stage especially if they are using sperm which the donor has had a past infection. [UK clinic]
(a4) Approach to seromatching	We continue to do so because of the guidelines. We are uncertain of the clinical significance particularly when the couples may be discordant themselves. We generally try to match a CMV negative donor with CMV negative recipient but will still offer treatment with CMV positive donor if this is all that is suitable/available otherwise following discussion with the recipient about the concerns. [UK clinic]

Themes (sub-themes)	Comments
(b) Consequences of CMV seromatching	
(b1) Availability of donors	Sometimes the number of CMV negative donors is reduced so this limits the choice of donors. [UK clinic]
(b2) Patient care	I think that without sufficient evidence to demonstrate any risk, this should not be a requirement. It only serves to reduce availability of donors, cause more anxiety and put more financial burden on patients. Individuals can be exposed to all sorts of other potential infections and toxins, and unless there is evidence for causing harm, then we need to draw the line. [UK clinic]
(b3) Patient concern	Of the CMV negative recipients we have discussed using CMV positive donors with, most are concerned about risks of transmission, but usually make the choice to continue when they understand that those risks are small. [UK clinic]

Themes (sub-themes)	Comments
(c) CMV screening methods	
(c1) Difference between UK/non-UK	The patients obtain sperm from CRYOS/European Sperm Bank and it appears that the UK is the only country who worries about it. [UK clinic]
(c2) Value of testing methods	IgM screening is not always particularly accurate. We have donors with low level IgM who over the space of 6 months are still showing levels of IgM in their blood work. In these cases, we have been informed that DNA PCR for CMV is a much more accurate test to determine whether or not an infection is current. [UK clinic]
(c3) Inconsistencies	We screen for total antibodies, as well as for CMV IgG and IgM. Screening is not the problem, interpretation is often an issue when we see a negative IgG and a positive IgM; or a positive IgG one time and then negative next screen. [Non-UK clinic]
(c4) Importance of testing	I think it is a valuable tool to try to minimise possibility of CMV infection during conception and early pregnancy. I do not believe that we should restrict CMV + sperm to treating only CMV+ patients, particularly if there is a problem with matching ethnicity. If the patient is counselled to the risks and are happy to proceed then I think we should. If a patient has a CMV+ partner and is CMV- herself then the risk of infection is the same. [UK clinic]
(c5) Screening standards	I believe we screen to the required standard, screening both patients and donors for CMV IgG and IgM antibodies. Any donor samples procured during a period of seroconversion would be discarded. This has happened once to date. [UK clinic]

managing CMV infection in sperm donors. The breakdown of the themes and representative quotes can be seen in Table 3.5. Briefly, the three major themes are (a) the relevance of CMV seromatching, (b) the consequences of CMV seromatching and (c) CMV screening methods. The relevance of seromatching is questioned on the basis of the theoretical risk of transmission, the risk of contracting CMV through external factors and the lack of screening of the male partner in non-donor assisted conception. The approach an individual clinic takes to CMV seromatching appears to be based upon these questions in addition to the consequences of seromatching. These include the impact on the availability and choice of donors, the effect it has on patient care, such as increasing waiting times, and the consequence for patient concern. Finally, when discussing the screening methods used, clinics were concerned about the discrepancies between UK and non-UK clinics and the problems this causes, the value of the current testing methods, and inconsistencies with testing results. However, clinics recognised the importance of testing for CMV infection in sperm donors and as such, the majority of clinics reported keeping high standards for screening that follow the current guidelines.

3.5 Discussion

This survey has shown that UK clinics are generally following UK screening guidelines. However, there are clear discrepancies with the management of CMV positive donors, with clinics openly reporting deviating from seromatching guidelines, by offering sperm from seropositive donors to seronegative recipients, in an attempt to relieve problems of supply. The outcomes of this survey provide evidence to show that CMV screening is causing problems in fertility clinics and has identified a number of issues, which need to be resolved.

Before discussing the results, it is important to evaluate the survey itself and the approach taken. To conduct this study, a pilot version of the survey was initially sent to 3 centres and feedback was given on how to improve the questions and format, after which amendments were made. A successful

response rate of 50.5% was obtained for UK clinics, however more responses from the non-UK clinics would have been preferable, to balance out numbers. Overseas clinics were approached opportunistically; therefore it was unlikely that any more replies would have been received. However, of the respondents from non-UK clinics, there is a wide spread across Europe, USA and Australia. Results from these clinics are therefore unlikely to be biased by any one set of alternative screening guidelines, such as the USA, where guidelines for CMV screening are stricter.

The survey itself was well designed with 'N/A' and 'Do not Know" options available for most questions. However, there were some issues with the data collected. In some instances, rather than choosing the N/A option, some clinics would answer 'No', when the question was actually not relevant to them, potentially affecting the results. This was a particular problem with regards to the seromatching question where some clinics chose the 'No' option, when they should have selected 'N/A', as they also stated they did not screen for CMV in female recipients. This is one area of the survey design that could have been clearer. Similarly, when answering the questions regarding how they would interpret screening test results (serology or PCR), the answers given were inconsistent. Some data points were missing throughout this section as clinics chose not to answer the question, or that they did not know. This was not thought to be due to the design of the survey. Rather it could have been because the wrong person at the clinic was filling out the survey, or they simply did not know as the test was performed elsewhere, or that they had never encountered that particular situation before. Despite this, the percentage of clinics reporting yes or no to a question is still important, regardless of what the other available options were. The findings highlight that there are inconsistencies with how tests are being performed, or at the very least, the lack of understanding clinics have regarding the tests being carried out.

This survey was successful in showing that the majority of UK clinics follow the current guidelines and screen for CMV. Whilst clinics reported to recognise the importance of screening, some clinics did question the need to

seromatch, due to other environmental exposure risks (Table 3.5a) (Liesnard *et al*., 2001). Whilst there are other potential sources of transmission to a pregnant woman, it is known that due to the short time frame of pregnancy, the 'natural' risk of acquisition of CMV in seronegative women in the USA is estimated to be as small as 1% (Colugnati *et al.,* 2007). Therefore, if the risk of contracting the virus is small elsewhere, every effort should be taken to avoid exposure during donor insemination. A second argument is the perceived small risk of vertical transmission, particularly for seropositive recipients, where the risk of transmission of the virus is lower than for seronegative mothers (Fowler *et al.,* 1992). Currently there is little evidence to refute this argument for CMV; however, a case has been reported of horizontal transmission of HSV-2 from a sperm donor to a seronegative recipient (Moore *et al*., 1989). No vertical transmission to the fetus was reported but infection with HSV-2 still has health complications for the female recipient (Moore *et al*., 1989). Unlike HSV-2, CMV is not generally associated with health issues in healthy individuals, however recent evidence suggests a link between increased levels of CMV IgG antibodies and increased mortality rates in seropositive individuals (Gkrania-Klotsas *et al.,* 2013; Simanek *et al*., 2011). Furthermore, infection with CMV is thought to deplete the number of naïve CD8+ T lymphocytes, due to repeated reactivation events, resulting in an inability for cellular immunity to respond to other infections acquired in old age (Almanzar *et al*., 2005). Given these health consequences associated with being CMV seropositive, every step should be taken to prevent horizontal transmission of CMV from a seropositive donor to recipient, regardless of the risks of vertical transmission. The only way to ensure this when following the current guidelines is to screen and seromatch based on CMV status.

Fortunately most clinics are conducting CMV screening in sperm donors and are doing so using the recommended method of serum antibody testing. However, there are limitations with this technique, as it is a retrospective test. It is able to detect if a person has previously been infected, but is not able to reliably detect current and active infections, due to the immune system taking

time to produce antibodies to an infection. Based on the evidence available at the time of writing the current guidelines, a combination of both CMV IgG and IgM antibodies was felt to be satisfactory in deciding whether to include or exclude a donor, with IgM testing highlighting those donors with a recent infection, a basis on which they should be excluded. However, this survey has shown that 15.6% of UK clinics are only screening donors for IgG antibodies (Table 3.2d). Therefore, these clinics are failing to rule out the possibility of a current infection, or a seroconversion event, in sperm donors prior to their use. This is concerning given the evidence that IgM positivity is strongly linked with an active infection, where high titres of infectious virus can be found in semen (Bresson *et al*., 2003). Analysis of the free text responses from this section highlighted that some clinics believed IgM testing to be unreliable. However, a study investigating the presence of CMV in HIV positive men found that the presence of IgM antibodies always correlated with the detection of CMV in semen. Conversely, in two instances, where CMV was found in semen, IgG tests were found to be negative (Lupton *et al*., 2013). This suggests that the presence of IgM antibodies is a better predictor for the presence of CMV in semen than IgG alone.

Whilst IgM positive donors should be excluded based on the potential risk they pose, it is thought that IgG positive (IgM negative) donors pose a minimal risk, as this combination of antibodies is thought to show a past infection only. However, IgG positivity in the absence of a positive IgM result does not always rule out an active infection. Multiple studies have found detectable CMV by PCR in the semen of IgG positive, IgM negative men (Bresson *et al*., 2003; Mansat *et al*., 1997; Witz *et al*., 1999). Also, Lupton *et al*., (2013), found that some IgG tests are unreliable. Men with CMV in their semen were initially found to be IgG negative, but upon re-testing with a different assay, they were IgG positive. Due to this, it cannot be said that the use of a CMV IgG positive, IgM negative donor is completely free of risk, as the current testing method is not reliable enough to predict the presence of CMV in the semen of a sperm donor.

Despite these limitations, serology is currently the most appropriate technique to use, based on the evidence available. However, clinics are further complicating the interpretation of the risks these results imply, by deviating from the current guidelines, as shown in the flowchart in Figure 3.1. Only a small percentage of clinics reported they would exclude an IgG positive donor after quarantine if they were previously negative, or if the antibody titre had increased (Table 3.2g,h). This highlights that the importance of IgG antibodies in detecting re-infection or reactivation events is clearly being underestimated. In post-transplant patients undergoing a secondary infection (re-infection or reactivation), IgG antibody titres increased in the absence of IgM antibodies (Dolan *et al*., 1989). Furthermore, infectious CMV was isolated from their urine samples, indicating these patients were infectious and therefore able to transmit CMV.

It is likely that clinics are not measuring antibody titre levels, as this is not currently recommended, but it is important for clinics to acknowledge differences in IgG antibody levels, as a failure to interpret these test results appropriately might result in a failure to detect an active infection. A more concerning finding is that not all clinics reported that they would exclude an IgM positive donor (Table 3.2i,j). As discussed above, IgM positivity is strongly linked with shedding of infectious virus (Bresson *et al*., 2003), and therefore poses a risk of transmission. The lack of clarity from clinics on this matter is alarming. Free text responses highlighted that clinics felt they were performing screening to the highest standards and that if unsure they would seek advice from consultant virologists. In addition, some clinics stated they could not provide an answer, as they had not encountered that particular situation. Regardless of this, the guidelines and the evidence are clear on how these tests should be interpreted.

It is interesting that some clinics report to use PCR to detect CMV in sperm donors, either in conjunction with serum antibody testing, or alone. When conducting PCR, most clinics reported to use blood as the source for the test (Table 3.2l). However, the choice of specimen to use for a test such as this is critical due to the phenomenon of compartmentalisation. It has been well

documented that some viruses can be present in higher loads in semen than blood, a finding which has been widely reported for HIV (Coombs *et al*., 1998; Gupta *et al*., 2000). Currently, there is no evidence to support that this is occurring with CMV, however, there is evidence to show men intermittently shed *Herpesviruses* in their semen (Kaspersen *et al*., 2012). This suggests that PCR on individual semen samples would be a better method for detecting an active infection than tests that use specimens of blood.

The importance of accurate screening for CMV lies in preventing horizontal, or vertical transmission of the virus. The current approach to ensuring no transmission occurs is to seromatch donors and recipients, if seropositive donors need to be used at all (Association of Biomedical Andrologists *et al*., 2008). Most clinics do report to follow these guidelines, however clinics also report deviating from these guidelines to offer sperm from seropositive donors to seronegative recipients. As justification for their decisions, clinics cited concerns over availability of donors, patient care and patient concern (Table 3.5b). The biggest contributing factor appeared to be the lack of availability of donors, with 72.5% of UK clinics reporting this as a problem (Table 3.4d). Conversely, only 38.5% of non-UK clinics reported the same, which is probably due to the lower percentage of clinics actually conducting CMV seromatching.

The discrepancy in sperm donor supply problem between UK and non-UK clinics is only one complication that arises from the different screening recommendations. The fact that non-UK clinics do not have to screen for CMV is an issue for UK clinics, as it presents difficulties when importing sperm from non-UK centres. Most clinics reported that they attempt to uphold the UK guidelines when importing donors, but often have difficulties obtaining the correct documentation from certain clinics due to differences in screening procedures. The Cryos International Sperm Bank list on their website that CMV screening is only carried out when the country the sperm is being shipped too requires it, such as the UK (Cryos International Sperm Bank, 2016). This presents a further problem clinics are experiencing in relation to CMV screening.

The choice of available donors, in relation to ethnicity, was also raised as a concern. Between 2009-2013, there were only 90 registered donors with an Asian ethnicity (HFEA, 2013b). As the prevalence of CMV is so high, this would clearly limit the number of available donors for seronegative recipients in this ethnic group. Whilst clinics recognise the potential risk of offering sperm from CMV positive sperm donors to CMV negative women and inform patients of the theoretical risk, this practice is not recommended in the current guidelines, as it is not known if it is safe. In fact, the guidelines clearly state that the use of "CMV IgG positive (IgM negative) donors may be recruited but their use should be limited to CMV IgG positive recipients" (Association of Biomedical Andrologists *et al*., 2008).

Even though seromatching donors and recipients based on CMV positivity is suggested as an option, it is not without risk, as an IgG positive (IgM negative) result does not always rule out the presence of CMV in semen. However, there appeared to be a lack of awareness regarding this. When clinics were asked if they informed patients of the risk when using seropositive sperm, some replied that "seropositive sperm was used for seropositive recipients only". This is of concern as prior immunity to CMV only infers partial protection. Re-infection with a different strain (Boppana *et al.,* 2001), or multiple strains (Yamamoto *et al*., 2010) can still lead to intrauterine transmission and symptomatic congenital infection. Moreover, in the USA, it is estimated that three quarters of congenital CMV infections are attributable to re-infection or reactivation events, as opposed to only one quarter of primary infections (Wang *et al*., 2011c). Therefore, the risk of re-infections and reactivation events in seropositive recipients of donor sperm should be of equal concern to clinicians as is the risk of primary infection in seronegative women.

It is clear to understand the complex decision a clinic faces in choosing to deviate from the guidelines. Clinics do recognise the importance of screening and stated on many occasions that they felt they were screening to the highest standards and consulting professional virologists if required. However, the need for screening is being questioned and although testing is

being carried out, the interpretation of test results varies significantly. An overall lack of understanding regarding the relationship between CMV infection and the risk it poses when using donor sperm is contributing to these problems. Better guidelines need to be written that are clearer and are able to give clinics concise, well-informed direction on the use of CMV positive donors in donor insemination programmes. To do this, better testing methods need to be used, to rule out the presence of CMV in an individual semen sample, improving safety and increasing the availability of sperm donors, which is the reason individual practice is becoming inconsistent. Before any improvements can be made to the current guidelines in order to alleviate these problems, more evidence needs to be gathered on which to base new decisions. The remaining chapters of this thesis will focus on increasing the understanding of the relationship between CMV and sperm, so as to provide a better evidence base on which to make informed decisions regarding the use of CMV positive sperm donors in fertility clinics.

Chapter 4

Development of a system to culture and quantify CMV *in vitro*

4.1 Introduction

It is clear from Chapter 3 that the management of sperm donors with CMV infection in fertility clinics around the world, but particularly the UK, is causing problems. Most of these issues arise from the blanket recommendation that CMV seropositive donors should not be used for seronegative recipients, given that they may pose a risk of infection (Association of Biomedical Andrologists *et al*., 2008). The lack of evidence to support or refute this statement is causing issues in clinics with regards to making clinical judgements on the safety of practice using seropositive donors. Better evidence surrounding the relationship between CMV and sperm is needed before changes to the guidelines can be made, or before the current guidelines can be fully accepted as the most appropriate method of screening for CMV.

There is a need to directly investigate how CMV and sperm interact, rather than just investigate its presence in semen (Bresson *et al*., 2003; Kapranos *et al*., 2003; Mansat *et al*., 1997) and how that correlates with fertility disorders (Naumenko *et al*., 2014). Throughout this thesis, laboratory grown CMV has been used to directly examine sperm-CMV interactions, in order to provide better evidence, which clinics can use in making judgements regarding CMV screening and the management of CMV positive sperm donors in the UK. To do this, techniques to grow and quantify CMV in the laboratory had to be established and this chapter will detail how the methods employed to do this were developed.

4.2 Experimental rationale

For the majority of viruses, permissive cells lines are required in order to grow them in the laboratory. Unlike bacteria, which are able to grow on nutrient-supplemented media, viruses are obligate intracellular organisms and can only be cultured in permissive vectors, or cell lines. These cell lines often differ from the cell type a virus would usually infect *in vivo* and for CMV, fibroblast cell lines are routinely used (Dolan *et al*., 2004). The cell line

chosen to use in this thesis are MRC-5, human lung fibroblast cells (Jacobs *et al*., 1970). This cell line has been used in clinics for diagnosis of CMV using patient samples and has been shown to be more sensitive to infection than other cell lines, such as WI-38 (Gregory & Menegus, 1983).

CMV was first isolated *in vitro* in 1956 from the liver biopsy of a child, now known as the Davis strain (Weller *et al*., 1957). Subsequently, multiple strains of CMV, from a variety of sources have been cultured *in vitro*, including CMV AD169, which was cultured from the adenoids of a 7-year old female (Rowe *et al*., 1956) and the Towne strain, isolated as a potential vaccine target (Plotkin *et al*., 1975). When comparing these high passage laboratory strains with low passage wild-type strains, such as Merlin, (Dolan *et al*., 2004), it is apparent that these older strains of CMV have acquired multiple mutations and alterations to the genome that are a direct result of multiple passages in culture. It is therefore considered that that no laboratory strain of CMV can be considered genetically intact, although low passage strains have a higher genetic similarity to wild-type CMV than high passage strains. Despite this, CMV AD169 was the strain of virus used for the majority of experiments throughout this thesis, given that this strain is more adapted to growth in fibroblast cells. A single round of replication takes less than 96 hours and distinctive plaques are formed within 7 days, a much shorter time frame than low passage strains of CMV (Prichard *et al*., 2001). However, in Chapter 6, a strain of Merlin, provided by Dr Matthew Reeves at UCL, was also used to compare effects on sperm function between a laboratory strain and a wild-type strain of CMV.

Once CMV was grown, it was essential to quantify the amount of virus, for the design of experiments, and to assess the success of some experiments. An effective quantitative PCR (qPCR) assay for CMV has been developed and is routinely used in clinical virology laboratories to diagnose infection in post-transplant patients (Mattes *et al*., 2004). Collaboration with the research group, at University College London (UCL), involved with pioneering this technique for CMV detection provided access to this as a method for quantification of CMV.

A common virology technique, known as a plaque assay, has been utilised throughout this thesis to determine the titre of infectious virus present in laboratory grown stocks of CMV. This technique was first described in 1969 for herpes simplex virus (Wentworth & French, 1969) and utilises the infection of a monolayer of cells with the addition of an overlay medium. The addition of an overlay medium is the unique feature of this assay as it prevents viral dissemination across the surface of the monolayer, creating localised areas of infection, which can be quantified. Both agarose and methyl cellulose have been reported to be used in a plaque assay overlay medium for CMV (Plummer & Benyesh-Melnick, 1964; Wentworth & French, 1970). This technique relies on the formation of discrete and distinguishable foci of viral replication and/or cell loss, which can be counted, and therefore the initial viral concentration can be calculated (Appendix V).

4.2.1 Specific aim(s):

1. Grow CMV (AD169) *in vitro* and establish a bank of viral stock to use in experiments throughout this thesis.
2. Develop a system for quantifying the amount of infectious virus (plaque forming units) in virus stocks and semen samples.
3. Develop a qPCR assay for determining total viral load in both virus stocks and semen samples.

4.3 Materials and Methods

This section will detail the experimental conditions for the characterisation of MRC-5 cells, prior to the growth of CMV (AD169) and methods for quantification of the virus, via two different methods, qPCR and the plaque assay.

4.3.1 Growth of CMV (AD169) on MRC-5 cells

4.3.1.1 Characterisation of MRC-5 cells

Before beginning to grow CMV, MRC-5 cells were grown as outlined in Section 2.4 and characterised as fibroblast cells. These cells were also

tested to ensure they were both CMV and *Mycoplasma* negative, as described below.

MRC-5 cells were grown until confluent in a T25 tissue culture flask and RNA was extracted as outlined in Section 2.6.1. The concentration of RNA was determined and reverse transcribed into DNA as outlined in Section 2.6.2. A 2µl aliquot of cDNA was amplified with primers specific to a 307bp region of the Thy1 gene, a gene present in all fibroblast cells (Moore-Morris *et al*., 2014), with a 2x Promega PCR master mix. In addition, detection of GAPDH and a no reverse transcriptase control were carried out as positive and negative controls (using 2µl of cDNA). Specific components and cycling temperatures of each reaction are outlined in Section 2.6.5.

The CMV status of MRC-5 cells was determined by detecting a 149bp region of the glycoprotein B gene (gB), as outlined in Section 2.6.5. MRC-5 cells were grown until 100% confluent in a T75 culture flask. The media was removed and kept for CMV analysis and DNA from the MRC-5 cells extracted as outlined in Section 2.6.3.

A 0.3µl aliquot of MRC-5 DNA (200ng/µl), a 0.6µl aliquot of MRC-5 cell lysate (190ng/µl) and a 0.6µl aliquot of spent media (concentration not determined) were amplified with gB1 and gB2 primers and a 2x Promega PCR master mix. 0.6µl of CMV viral DNA (6.6ng/µl) was used a positive control, along with GAPDH amplification. PCR components and conditions are outlined in Section 2.6.5.

Mycoplasma testing was performed using the E-Z PCR Mycoplasma test kit (Geneflow, Staffordshire, UK). Briefly, 1ml spent cell culture media from confluent MRC-5 cells was centrifuged briefly at 250x*g*. The supernatant was transferred to a fresh sterile tube and centrifuged at 15,000x*g* for 10 minutes. The supernatant was decanted and the pellet re-suspended in 50µl of Buffer Solution. The sample was incubated at 95°C for 3 minutes and stored at -20°C before analysis. The PCR reaction was carried out in a total volume of 50µl, constituting 35µl H_2O, 10µl of the Reaction Mix and 5µl of the test sample. The samples were cycled in a Sensoquest Labcycler for 30 seconds

at 94°C, then cycled 35 times for 30 seconds at 94°C, 120 seconds at 60°C and 60 seconds at 72°C. Finally, the reaction was incubated for 30 seconds at 94°C, 120 seconds at 60°C and 5 minutes at 72°C.

All PCR reactions in this section were resolved on a 1.5% (w/v) agarose gel (GeneFlow, Staffordshire, UK) at 80V for 45 minutes, using a High-Current Power Supply PowerPac™ (BioRad, Hertfordshire, UK).

4.3.1.2 Propagation of CMV using MRC-5 cells

MRC-5 cells were grown in EMEM until sub-confluent, approximately 80% coverage. The media was removed from two flasks and replaced with 5 ml of serum-free EMEM. One flask was infected with 200µl of virus stock and 200µl more serum-free EMEM added to the mock-infected flask. Both flasks were incubated at 37°C for 1 hour with gentle manual rocking from side to side every 20 minutes. After 1 hour, the flasks were replenished with 10 ml of 'maintenance medium', containing normal growth supplements and 5% (v/v) FCS. Flasks were incubated at 37°C until the infection process was complete.

The course of the viral infection was followed and when 60% of cells were exhibiting the typical cytopathic effect (CPE) of CMV, virus was harvested. Briefly, supernatant from both infected and mock-infected flasks was removed and centrifuged at 200*g* for 10 minutes to remove cellular debris. The supernatants were decanted and spread into 1ml aliquots and stored at -80°C. After the first viral harvesting, virus was harvested every 48 hours in the same manner until all the cells were infected and dying. At this stage, the flasks were frozen at -80°C and thawed, twice, to allow the cells to lyse. After the second freeze-thaw cycle, the cells were scraped from the surface of the flask with a cell scraper. The supernatant was then collected and treated in the same manner as previous harvestings and divided into 1ml aliquots.

Growth of CMV was confirmed by PCR analysis to determine the presence of a 200bp region of the CMV gB gene. DNA was extracted from MRC-5 cell spent media from both cells infected with CMV and mock-infected cells via

the protocol outlined in Section 2.6.3. 2µl of DNA was amplified with gBF and gBR primers and MyTaq™ HS Mix, using the components and conditions outlined in Section 2.6.5. 2µl of CMV viral DNA was used as a positive control, and 2µl of H_2O used as a negative control. The PCR reaction was resolved on a 1.5% (w/v) agarose gel at 80V for 45 minutes.

4.3.2 Quantification of infectious CMV via plaque assays

General details of how the plaque assay is performed can be found in Section 2.4.1. In this chapter, the plaque assay was performed to determine the infectious titre of virus stocks (in Chapter 5, the plaque assay is used in a different manner). The specific details of how the plaque assays performed in this chapter were carried out are outlined below.

Viral stocks were diluted 10-fold until a dilution of 10^{-6} was reached by adding 100µl of virus stock to 900µl of serum-free EMEM. A 200µl aliquot of each dilution was added in triplicate to confluent MRC-5 cells in 24-well plates. The plates were incubated and stained as outlined in Section 2.4.1. A worked example of how the viral titre in PFU/ml was calculated is outlined in Appendix V.

4.3.3 Development of a qPCR assay to quantify viral load

4.3.3.1 Construction of glycoprotein B plasmid

CMV glycoprotein B, 2720bp, was amplified using primers containing *Hin*dIII (AAGCCT) and *Xba*I (TCTAGA) restriction sites (Table 2.2) (Figure 4.1a) (Temperton *et al*., 2003). The PCR reaction and cycling conditions were performed as outlined in Section 2.6.5.

After resolving the PCR reaction on a 1% (w/v) agarose gel at 80V for 60 minutes, the 2.7kB band was extracted using a QIEX II gel extraction kit (Qiagen, Manchester, UK). To create sticky ends for ligating into a vector with corresponding restriction sites to the ones engineered into the 2.7kB construct, 14µl of purified DNA was digested with 0.3µl of *Hind*III (20U/µl)

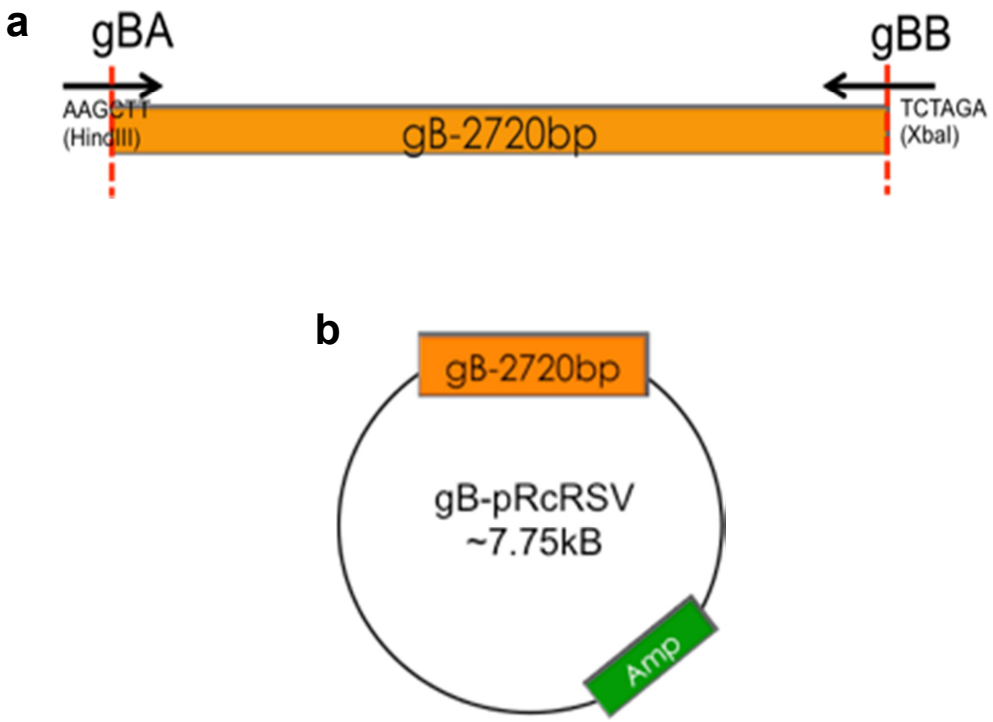

Figure 4.1: Construction of the gB-pRcRSV plasmid was carried out by amplifying the 2.7kB glycoprotein B gene (panel a) with flanking primers gBA and gBB and introducing corresponding *Hin*dIII and *Xb*aI sites. Panel (b) shows the final gB-pRcRSV product, constructed by ligation of the amplified gB gene into a linearised 5.3kB pRcRSV plasmid backbone.

and *Xba*I (20U/µl) (New England Biolabs, Ipswich, UK) in a 20µl total reaction volume containing 2µl NEBuffer 2 (10X) (New England Biolabs, Ipswich, UK) and 2µl BSA (1mg/ml) (New England Biolabs, Ipswich, UK). The reaction was incubated at 37°C for 60 minutes and resolved on a 1% (w/v) agarose gel at 80V for 60 minutes before extracting the band from the gel using a QIEX II gel extraction kit.

A 5.3kB plasmid, pRcRSV, containing a β-lactam ampicillin resistance gene, and a multiple cloning site was digested with *Hin*dIII and *Xba*I. Briefly, a 1µl aliquot of pRcRSV (2.5µg/µl) was digested with 0.3µl of each restriction enzyme (20U/µl) in a total reaction volume of 20µl, containing 2µl NEBuffer 2 (10x) and 2µl BSA. The reaction was incubated at 37°C for 60 minutes before adding 1µl of antartic phosphatase (5U/µl) (New England Biolabs, Ipswich, UK) and incubating for a further 15 minutes. The reaction was resolved on a 1% (w/v) gel at 80V for 60 minutes before the 5.3kB band corresponding to the linear plasmid backbone was extracted using a QIEX II gel extraction kit.

To perform the ligation step, 9µl of digested insert (24ng/µl) was added to 1µl of linear plasmid (25ng/µl) backbone in a total reaction volume of 20µl, containing 2µl T4 ligase buffer (New England Biolabs, Ipswich, UK), 1µl T4 ligase (400U/µl) (New England Biolabs, Ipswich, UK) and 7µl nuclease free water. The reaction was incubated overnight at 16°C. After ligation, 5µl of each ligation reaction was transformed into 45µl competent DH5α cells, along with 5µl digested vector, as a negative control. The transformation was placed on ice for 30 minutes then heat shocked at 42°C for 90 seconds and placed back on ice for 90 seconds. In order for the cells to recover, 100µl LB was added and incubated at 37°C for 60 minutes. Transformed cells were plated onto agar plates containing ampicillin (200µg/ml) and incubated overnight at 37°C.

Colonies were picked using a sterile tip and inoculated in 5ml LB and incubated overnight at 37°C. The following day, plasmid was extracted from the inoculated colonies, using a Qiagen QIAprep spin miniprep kit.

Clones were screened for the presence of the gB insert by digesting 2µl of extracted plasmid with 0.2µl of each *Hin*dIII and *Xba*I in a total reaction volume of 20µl for 60 minutes at 37°C. Restriction digests were resolved on a 1% (w/v) agarose gel at 80V for 60 minutes. Plasmids exhibiting two bands corresponding to a 2.7kB insert and the 5.3kB plasmid backbone (Figure 4.1b) were sent for sequencing. Those clones exhibiting the AD169 (BK000394) glycoprotein B sequence were considered successfully ligated plasmids and one was chosen for future qPCR work. The yield of plasmid recovered from the miniprep protocol was 295ng/µl, as determined by a nanophotometer. To increase the amount of plasmid available to work with, a Qiagen MaxiPrep kit was used to isolate plasmid from 100ml of competent DH5α cells, in comparison to 5ml used for the miniprep protocol. This increased the yield significantly, to 3935ng/µl.

The plasmid copy number was determined using the following equation from the Integrated DNA Technologies website (Integrated DNA Technologies, 2013).

Number of copies= amount of DNA (ng/µl)*$6.022x10^{23}$/bp*660*$1x10^{9}$

$6.022x10^{23}$=Avogadro's Constant-used to determine the number of molecules of the DNA template per gram.

660=average weight of a base pair in Daltons.

$1x10^{9}$=used to convert the number of copies of template into ng.

4.3.3.2 qPCR assay development

The components and cycling conditions of the qPCR assay are as outlined in Section 2.6.6. The primer concentrations were optimised to 125nM (qgBF) and 250nM (qgBR) by titrating across a 1000nM-125nM concentration range. The combination of primer concentrations with the earliest C_T, highest ΔRn and latest C_T in the corresponding no template control (NTC) were chosen as the most optimal.

To construct the standard curve, a 10-fold dilution series of the plasmid gB-pRcRSV was performed. Given that the plasmid stock had $4.6x10^{11}$ copies/µl (3935ng/µl) this was initially diluted to 900ng/µl in sterile H_2O, to obtain a copy number of $1x10^{11}$. A starting copy number of 10^9 for the 10-fold dilution was found to be most optimal as in higher concentrations non-specific bands were detected by conventional end-point PCR, due to the high abundance of DNA present in the reaction. For each standard curve, a plasmid copy number range from 10^1 to 10^9 copies was established by transferring 10µl of each concentration into 90µl sterile H_2O until the desired dilution was reached. In a 20µl total reaction volume, 2.5µl of each plasmid concentration was combined, in triplicate, with 2.5µl qgBF, 2.5µl qgBR, 2.5µl gB3 and 10µl master mix. For the NTC, 2.5µl of H_2O was added, instead of the plasmid. The reaction was then cycled as outlined in Section 2.6.6. Replicates that had an undetermined result were eliminated from analysis and not included in the construction of the standard curve.

Once the standard curve was constructed, the ability of the assay to accurately detect samples was measured. A standard curve was set up in the manner outlined above and 20 DNA samples were analysed for the presence of CMV. 2.5µl of each sample was added instead of 2.5µl of plasmid and the reaction cycled in the same manner as outlined above. Aliquots of the same 20 samples were analysed by the validated assay at UCL for direct comparison.

4.4 Results

4.4.1 Growth of CMV (AD169) on MRC-5 cells

4.4.1.1 Characterisation of MRC-5 cells

MRC-5 cells were successfully grown and characterised as fibroblast cells by the expression of Thy1, a fibroblast specific gene (Moore-Morris *et al*., 2014). RNA was extracted from MRC-5 cells and reverse transcribed into cDNA. Expression of Thy1 was analysed by PCR and a band of 307bp (Figure 4.2a) was amplified (Lane 2), showing that the MRC-5 grown cells express Thy1

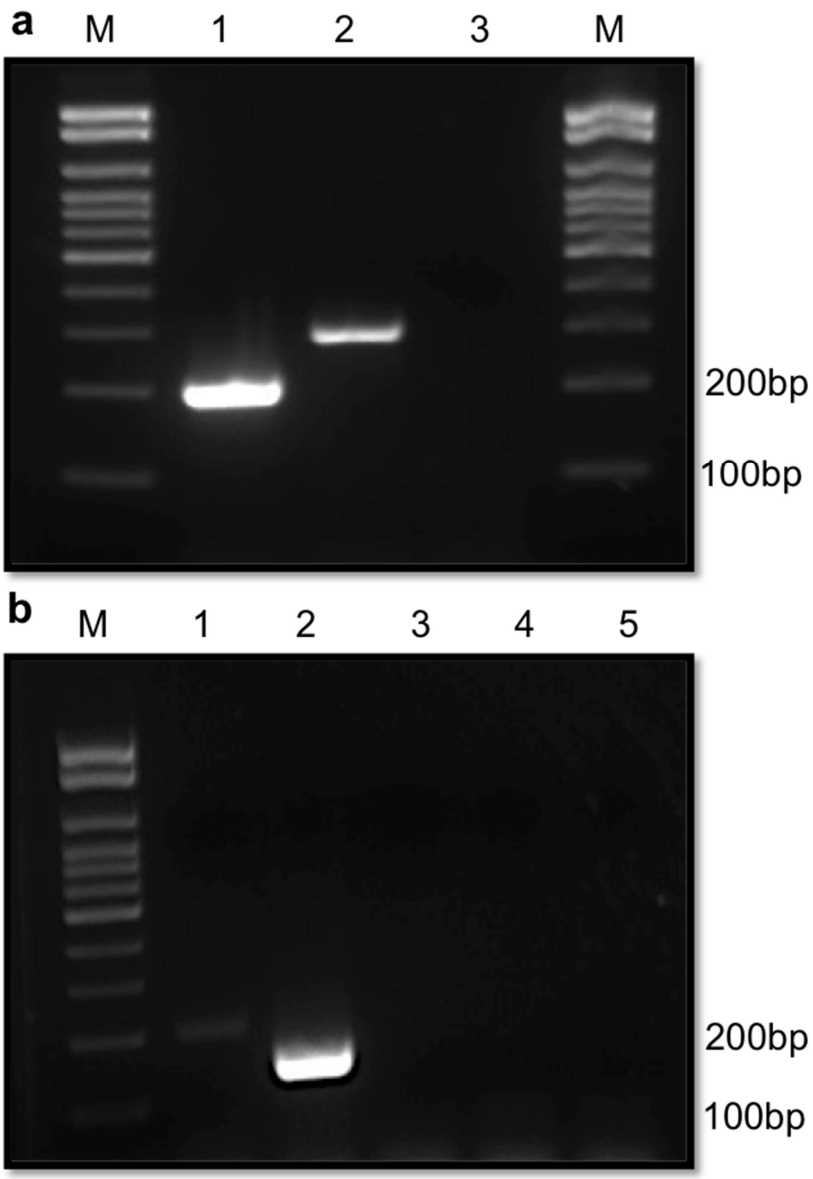

Figure 4.2: Characterisation of MRC-5 cells as a fibroblast cell was performed by detection of the fibroblast specific gene, Thy1 (Panel a). A 200bp band corresponding to the amplification of the housekeeping gene, GAPDH, was used as a positive control for RNA extraction and cDNA synthesis (Lane 1). Lane 2 displays a 300bp band specific to the Thy1 gene and Lane 3 is a no reverse transcriptase (RT) control. The absence of a band in the no RT control confirms the absence of genomic DNA contamination. Panel (b) shows the characterisation of MRC-5 cells as CMV negative by PCR amplification of a 149bp region of the glycoprotein B gene (gB). A 200bp band corresponding to the amplification of the housekeeping gene, GAPDH, was used as a positive control (Lane 1). CMV (AD169) DNA was used a positive control (Lane 2). MRC-5 DNA (Lane 3), cell lysate (Lane 4) and spent media (Lane 5) were all negative for the presence of CMV gB. Both reactions were resolved on a 1.5% (w/v) agarose gel and calibrated with 10µl of Norgen DNA LowRanger ladder.

and are therefore fibroblast cells and susceptible to infection by CMV AD169. The presence of a 200bp band in Lane 1, corresponding to GAPDH, a constitutively expressed housekeeping gene, confirms the successful extraction of RNA and cDNA synthesis. No band corresponding to GAPDH was observed in the no reverse transcriptase control (Lane 3), ruling out genomic DNA contamination, confirming the presence of Thy1 is due to expression, as opposed to the presence of the gene alone.

Prior to growing CMV, it was important to establish that MRC-5 cells were not previously infected by CMV and potentially producing a different strain. To be confident that any virus stock produced was homogenously AD169, cells were analysed for the presence of a 149bp region of the glycoprotein B gene. DNA extracted from MRC-5 cells, along with cell lysate and spent media were amplified by PCR (Figure 4.2b). CMV (AD169) DNA purchased from NCPV was used as a positive control, to ensure the primers gB1 and gB2 amplified the correct region (Lane 2). Amplification of GAPDH from the extracted DNA was used as a control for the successful extraction of DNA from the cells (Lane 1). No detection of CMV glycoprotein B was detected in any of the MRC-5 samples in comparison to the positive control (Lanes 3-5), confirming that MRC-5 cells grown in culture are CMV negative. The cells were also tested for the presence of *Mycoplasma* and were confirmed as negative (Appendix VI).

4.4.1.2 Propagation of CMV using MRC-5 cells

Sub-confluent MRC-5 cells were infected with an unknown titre of virus and the process of infection observed until the first signs of CMV CPE were apparent (Figure 4.3b), in comparison to a mock-infected flask (Figure 4.3a). Early signs of CMV infection present as dark spots in the cells, representing formation of inclusion bodies, which are areas of replicating virus in the nuclei of the cell (Andrade *et al*., 2004). As the infection progresses, the accumulation of virus inside the cells causes them to become swollen and eventually large areas of the monolayer become devoid of cells, indicating the beginning of cell death (Figure 4.3c).

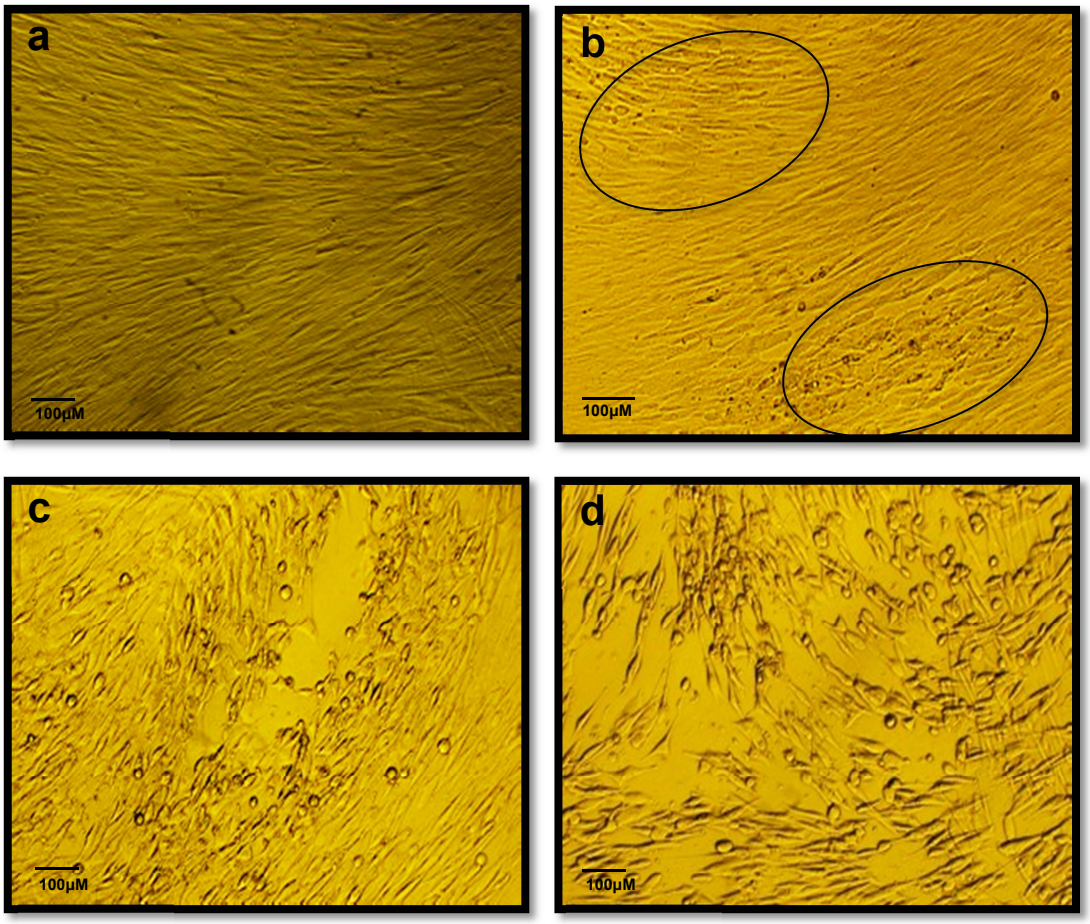

Figure 4.3: The effect of CMV on MRC-5 cells can be seen in panels b-d, in comparison to panel (a), which shows the normal appearance of uninfected MRC-5 cells. Panel (b) shows early signs of CMV infection, with the black circles identifying patches of MRC-5 cells showing typical signs of infection, with areas of swollen and darkened cells. Panel (c) shows that as the infection progresses, cells become more swollen due to intracellular viral replication and areas of cell loss start to appear. Finally, panel (d) shows that ultimately the majority of cells become infected and the cell monolayer becomes depleted. All images were taken on an inverted Olympus CKX41 microscope on a 10X objective. Scale bar = 100µm

Once 60% of the monolayer was exhibiting typical CMV CPE, the first harvest of virus was taken. Subsequently, virus was harvested every 48 hours until the majority of cells were showing signs of infection (Figure 4.3d). At this point, a final harvest of virus was taken and the cells lysed to release intracellular virus. Typically, 12ml of virus stock was stored per harvest. To confirm the presence and therefore successful growth of CMV, DNA was extracted from the spent media of MRC-5 cells infected with CMV and mock-infected cells and analysed by PCR analysis for the presence of CMV glycoprotein B gene (Figure 4.4). CMV (AD169) DNA purchased from NCPV was used as a positive control and H_2O used as a negative control. The presence of a 200bp band corresponding to the region of the glycoprotein B gene amplified by primers gBF and gBR was present in both the positive control (Lane 1) and CMV-infected spent media (Lane 2). No band was present in the mock-infected spent media (Lane 3) and the negative control (Lane 4). These results confirm the growth of CMV in MRC-5 human lung fibroblast cells.

4.4.2 Quantification of infectious CMV via plaque assays

For each stock of virus used in the experiments throughout this thesis, the number of infectious viral particles, the 'plaque forming units' (PFU) per ml, was determined using a plaque assay. This was used in conjunction with the total viral load, determined by qPCR, which is detailed in Section 4.4.3. After incubating MRC-5 cells with a serial dilution of virus stock for 1-2 weeks, various stages of plaque formation were observed. If the optimal dilution was reached, distinct areas of swollen and deeply stained cells, characteristic of CMV CPE were visualised (Figure 4.5a) . With the larger plaques, cell loss can be observed in the central area of the plaque, characteristic of what has previously been reported for CMV plaque assays. These changes can be seen clearly when compared to a mock-infected layer of cells (Figure 4.5b). If the dilution was too low, these plaques merge together (Figure 4.5c) making it hard to quantify the amount of virus present. Similarly, if the dilution was too great, no plaques are observed (Figure 4.5d).

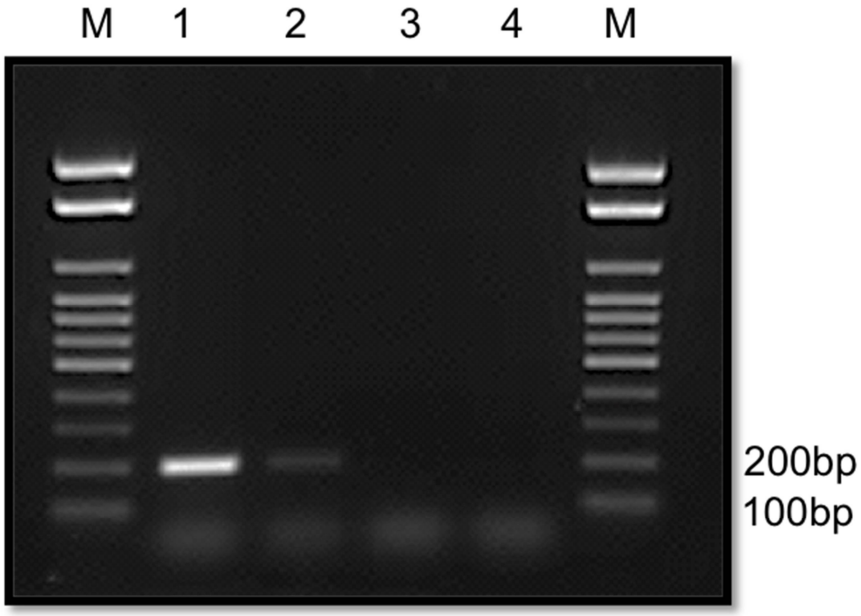

Figure 4.4: Confirmation of growth of CMV by extraction of DNA from the spent media of infected and non-infected MRC-5 cells. CMV (AD169) DNA was used a positive control (Lane 1) and H_2O as a negative control (Lane 4). The presence of a 200bp band in Lanes 1&2 confirms the presence of CMV glycoprotein B gene in the infected MRC-5 cells and the absence of the same band in Lanes 3&4 confirms the absence of CMV in non-infected cells and the absence of any contamination in the PCR reaction. Resolved on a 1.5% agarose gel and calibrated with 10µl Norgen DNA LowRanger ladder.

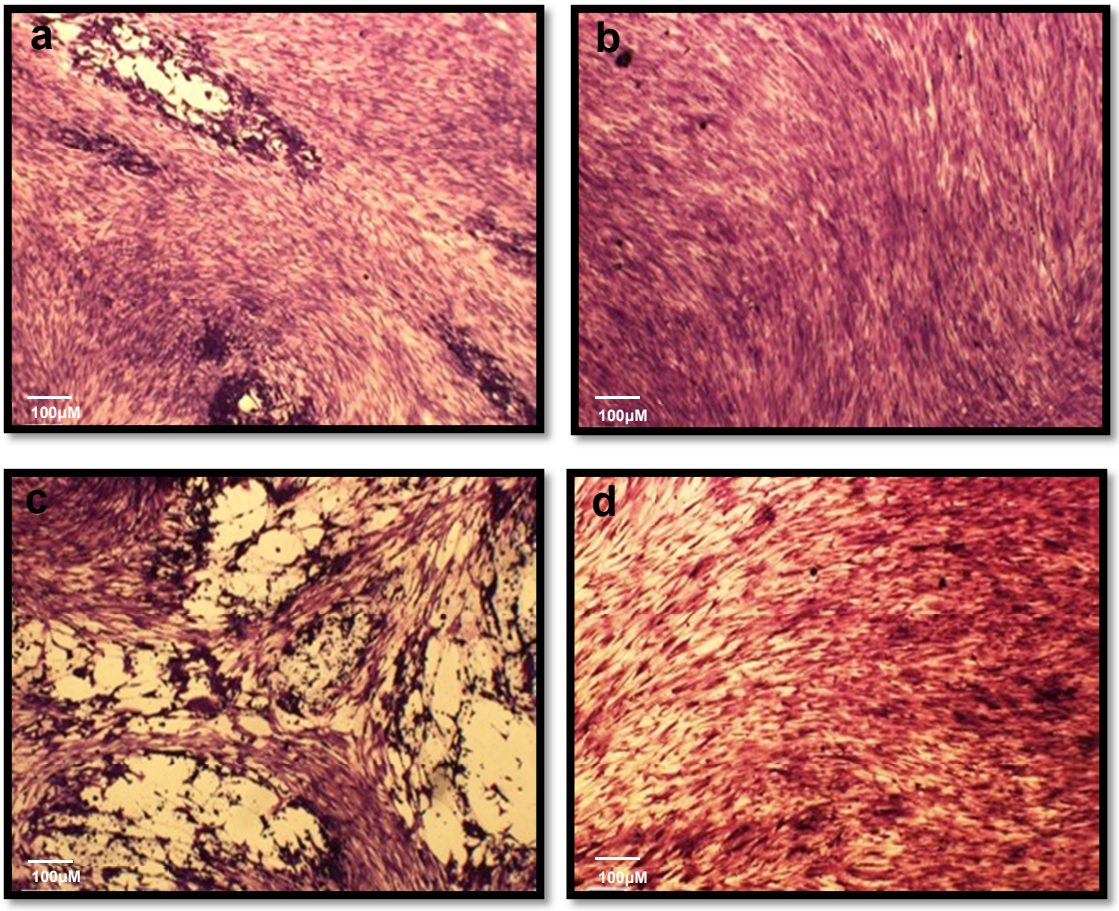

Figure 4.5: Images displaying typical 'plaques', or areas of cells loss (Panel a) are caused by the infection of a single virus particle and subsequent cell loss. These effects of viral replication are apparent in comparison to a mock-infected cell monolayer (panel b). Panel (c) shows that if too much virus is present the plaques are too concentrated and merge together and similarly, panel (d) shows that if there is a low concentration of virus, no plaques are observed. All images were taken on an inverted Olympus CKX41 microscope on a 10X objective. Scale bar = 100µm

The type of dilution series performed is paramount to the success of the plaque assay and for stocks of virus harvested from MRC-5 cells in the manner outlined in the previous section, a 10-fold dilution series was found to be most optimal. By performing the plaque assay under these conditions, the viral titre of the three different virus stocks used in this thesis could be determined. The individual viral titres were calculated as $2.2x10^4$, $4.3x10^4$ and $1.7x10^5$ PFU/ml, respectively.

4.4.3 Development of a qPCR assay to quantify viral load

4.4.3.1 Construction of glycoprotein B plasmid

The construction of a plasmid containing the 2.7kB gene encoding for one of the cell surface receptors, glycoprotein B (gB) was carried out in order to generate a qPCR assay for accurate quantification of viral load. The plasmid was used to generate a standard curve of known plasmid concentrations and copy numbers of gB gene.

The glycoprotein B gene was amplified using primers, gBA and gBB, incorporating restriction sites *Hin*dIII and *Xba*I (Temperton *et al*., 2003). A plasmid containing a multiple cloning site containing the same restriction sites was linearised. The cloned gB fragment was digested and ligated with the linearised plasmid backbone, in order to generate a 7.75kB vector containing the gB gene. Upon transformation into DH5α cells, Ampicillin resistance clones were recovered, indicating successful ligation. Plasmid DNA was then isolated and restricted with *Hin*dIII and *Xba*I to confirm the presence of the insert. All clones screened exhibited two bands, one corresponding to the 2.7kB insert and the 5.0kB plasmid backbone (Figure 4.6). Successfully cloned plasmids were analysed for the correct AD169 gB sequence via DNA sequencing and the concentration of the chosen plasmid determined to be 3935ng/µl. Subsequently, the plasmid copy number was determined to be $4.6x10^{11}$copies/µl.

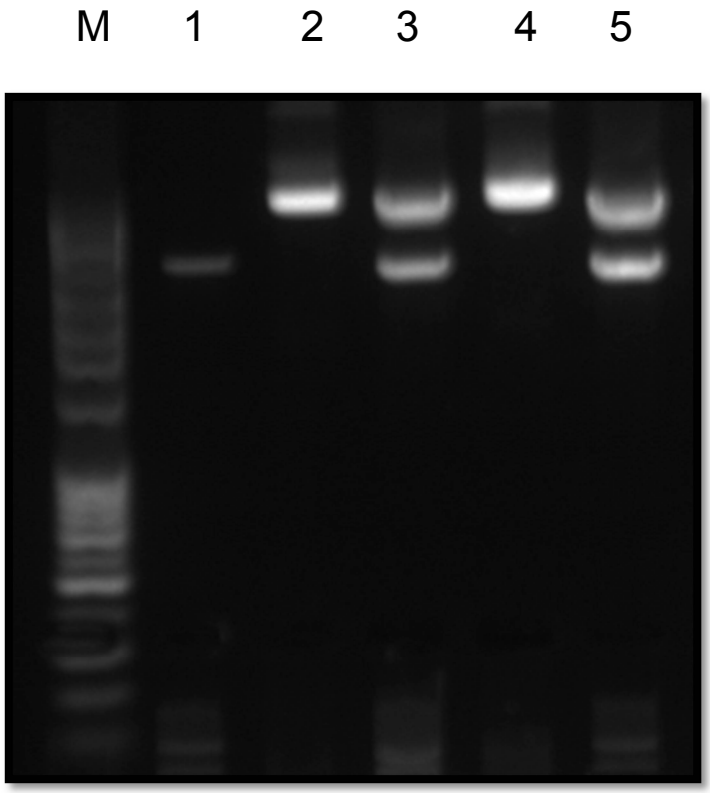

Figure 4.6: Confirmation of successful construction of gB-pRcRSV, with Lane 1 showing a band corresponding to the 2.7kB insert. Lanes 2 & 4 are unrestricted plasmids from two bacterial clones. Lanes 3 & 5 are double digested plasmids exhibiting two bands corresponding to the insert and the plasmid backbone. The gel was calibrated with Norgen HighRanger DNA ladder.

4.4.3.2 qPCR assay development

A qPCR assay using the absolute method of quantification was developed to determine CMV viral load. This method determines the number of copies of viral DNA in an unknown sample against pre-determined standards, The gB-pRcRSV plasmid constructed in Section 4.4.3.1 was used to generate a standard curve of known concentrations of the gB gene. The standard curve is designed to plot the gene copy number (as determined by the amount of plasmid added) against the C_T value, which is the number of cycles in the PCR reaction it takes for the amount of fluorescence detected to cross a pre-defined threshold level. The C_T value is therefore proportional to the amount of starting template in the sample. Using the standard curve, samples of unknown viral load can be quantified by determining the C_T value and plotting on the standard curve. From this, the number of copies of gB in the starting template/sample can be determined and the viral load/titre can be calculated.

Certain parameters relating to the standard curve determine the efficiency and reproducibility of the assay. The MIQE guidelines set out the minimum requirements when constructing an assay (Bustin *et al*., 2009) and details of specific values are reported in (Taylor *et al*., 2010). For this assay, a standard curve was constructed (Figure 4.7) with a slope of -3.14, which lies within the acceptable range of -3.58 to -3.10. This corresponds to a PCR efficiency of 90-110%, which is within the acceptable range. The R_2 value of 0.913 falls below the expected value of 0.999, highlighting a lack of reproducibility. The Y-intercept was calculated to be 39.7, which provides a theoretical limit suggesting this assay would not be able to reliably detect values above this C_T value, which corresponds to a DNA copy number of $>1x10^1$copies/ml.

When the accuracy of this assay was compared to a clinically validated assay, routinely performed at UCL, there were some discrepancies between viral loads for the same samples. A series of DNA samples determined to be both positive and negative for the presence of CMV as per the validated assay were analysed through the in-house assay (Table 4.1).

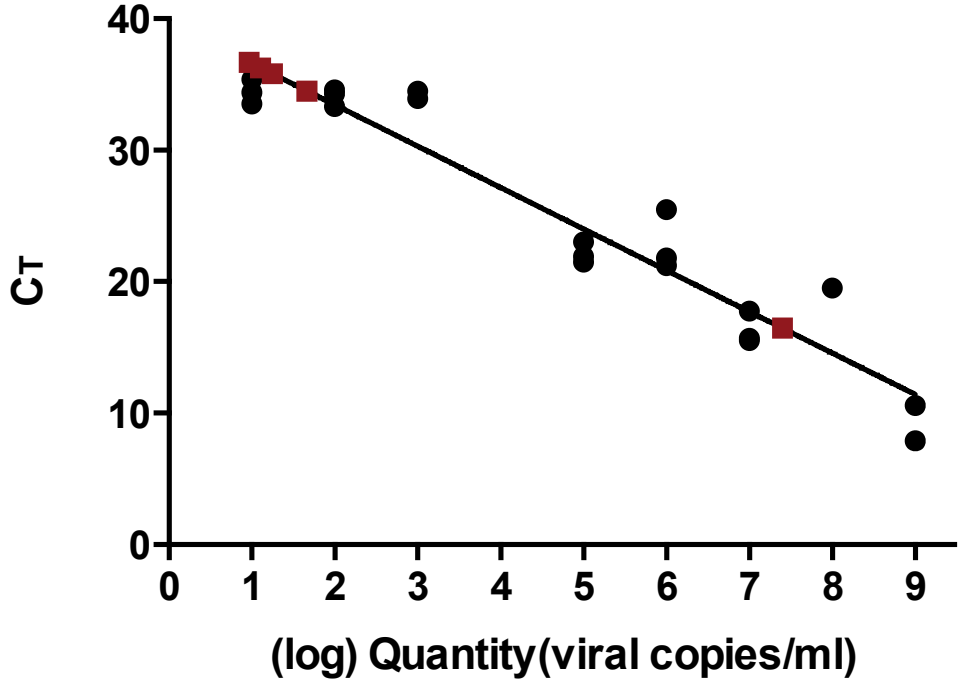

Figure 4.7: Construction of quantitative PCR standard curve using gB-pRcRSV plasmid. A plasmid copy number range of 10^1-10^9 was used (black circles) and plotted against the C_T (PCR cycle at which the signal crosses a threshold level) value. The C_T of unknown samples (red squares) can be plotted on the graph to determine the viral load (copies/ml). The slope of the standard curve was calculated as -3.15, the Y-intercept value is 39.73 and the R_2 value is 0.913. Repeats that were undetermined were removed from analysis.

Table 4.1: Table outlining the amount of CMV in viral copies/ml of 20 DNA samples detected in an in-house assay developed for this thesis in comparison to a clinically validated assay.

Sample	Validated Assay	In-House Assay
1	$7.8x10^9$	$3.13x10^8$
2	$3.0x10^8$	NEG
3	54584	NEG
4	24391	NEG
5	11472	37.6
6	8034	NEG
7	6640	11.9
8	2861	NEG
9	1750	NEG
10	754	NEG
11	490	NEG
12	185	NEG
13	NEG	NEG
14	NEG	5.4
15	NEG	NEG
16	NEG	NEG
17	NEG	NEG
18	NEG	8.0
19	NEG	NEG
20	NEG	NEG

Only 3/12 positive samples were also found to be positive by the in-house assay and the viral loads determined by the in-house assay were incorrect by 1-2 orders of magnitude. Also, this assay incorrectly determined 2/8 negative samples as positive.

Given the discrepancies between the two assays, it was decided to use the clinically validated assay for all qPCR analysis of CMV viral load throughout this thesis for more accurate and reliable results. Using this assay, the individual viral loads of the stocks of virus used throughout this thesis were calculated as $2x10^8$, $5.4x10^7$ and $4.7x10^9$ copies/ml, respectively.

4.5 Discussion

The aim of this chapter was to develop the fundamental virology techniques needed to grow and quantify CMV in order to carry out *in vitro* experiments to examine the relationship between the virus and sperm. These include examining the effects of co-incubation with CMV on sperm function (Chapter 6) and whether sperm washing techniques are able to remove CMV from semen samples (Chapter 5). To do this MRC-5 cells were infected with a laboratory strain of CMV, allowing for viral propagation and the creation of homogenous stocks of virus. Also, viral stocks were quantified via the use of a plaque assay, which determined the infectious titre of viral stocks and attempts were made to develop an in-house qPCR assay to detect viral load.

In this chapter, CMV AD169 was successfully grown and propagated in culture, producing multiple viral stocks for use in subsequent experiments. The AD169 strain of CMV was chosen due to the extensive genomic information available, as this was the first CMV strain to be fully sequenced (Chee *et al*., 1990). This strain is also easier to grow, with short cycles of replication (96 hours) (Prichard *et al*., 2001) and produces higher yields of virus after growth in fibroblast cells, in comparison to other strains (Dargan *et al*., 2010). These are all desirable features when wanting to establish large stocks of virus for subsequent use in other experiments.

When considering the choice of CMV strain chosen, it is important to note the genetic differences between AD169 and other strains such as Merlin (reviewed in Prichard *et al*., 2001) (Figure 4.8). Due to multiple passages *in vitro*, laboratory strains such as AD169 and Towne, have acquired multiple genetic alterations that are more favourable for growth in culture. The most notable for AD169 is the loss of 19 genes (UL133-150) in the unique long (U_L) region of the genome (Cha *et al*., 1996) (Figure 4.8a). In comparison, Merlin, a low passage strain does not have these substantial alterations, but has acquired a single point mutation through growth *in vitro* (Figure 4.8b), although it is still highly genetically similar to clinical isolates of CMV (Dolan *et al*., 2004) From this evidence, it is clear that no laboratory strain of CMV can be considered as genetically intact.

Another important point to consider when evaluating the choice of strain used is that the loss of the genes in the U_L region changes the cell tropism of the virus. Normally, CMV has a broad host cell range *in vivo* (Sinzger *et al*., 1995), however, laboratory strains are no longer able to infect endothelial or epithelial cells (Hahn *et al*., 2004; Sinzger *et al*., 2008; Wang & Shenk, 2005) and can only be grown in fibroblast cell lines. This is thought to be due to the role these genes play in mediating cell entry by endocytosis, which is required for entry to epitheliail/endothelial cells (Ryckman *et al*., 2006), as opposed to fusion-mediated entry, which is the mechanism of entry employed when infecting fibroblast cells (Compton *et al*., 1992). The deletion of these genes in AD169 results in the loss of receptors involved in the endocytosis-mediated entry mechanism, but retains the ability to bind and enter via fusion of membranes, allowing this strain to continue to infect fibroblast cells.

As it is only the mechanism of entry to cells that is affected by these alterations, this is not of concern for how this strain of CMV will be used throughout this thesis. The experiments conducted are concerned with binding events between CMV and sperm, rather than entry and subsequent replication, as it is unlikely that CMV would be able to replicate inside sperm,

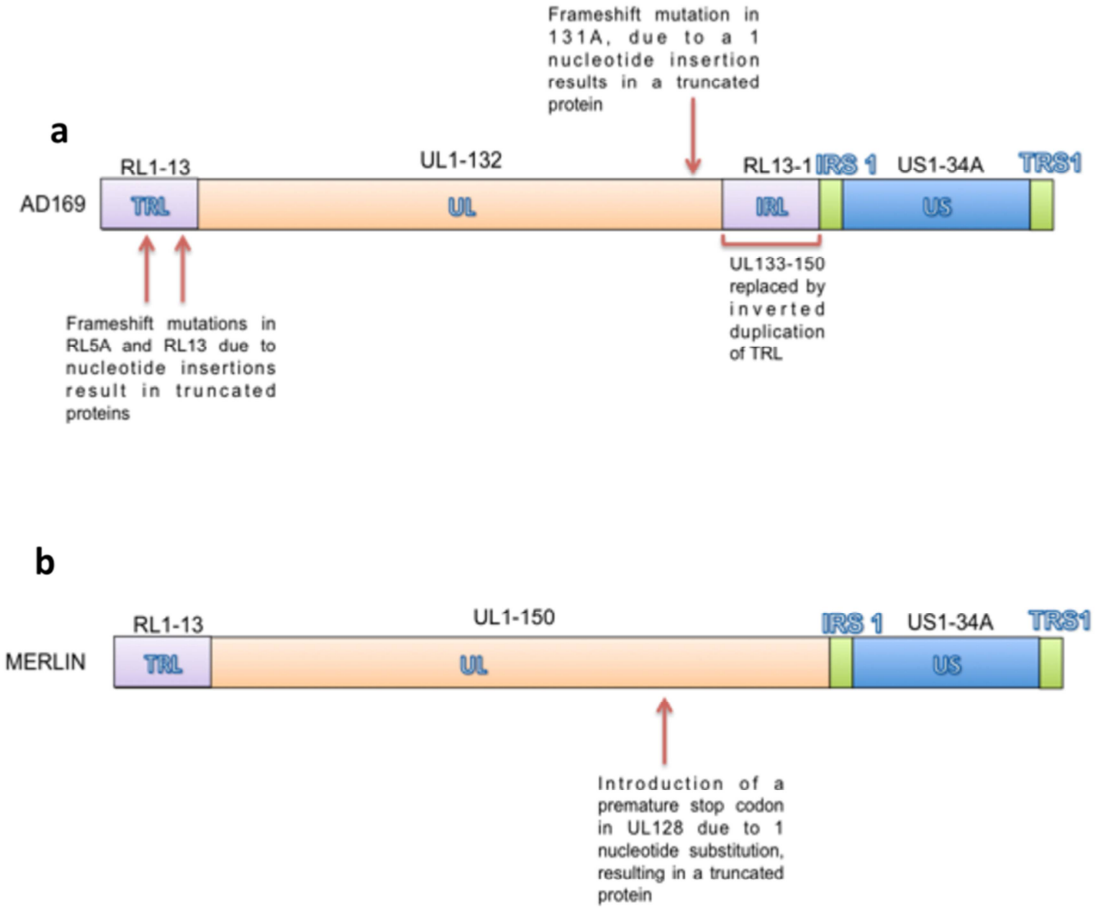

Figure 4.8: Schematic diagrams of the genomes of CMV AD169 and Merlin showing sites of known mutations (Dolan *et al*., 2004). Panel (a) shows the known AD169 mutations including the replacement of genes UL133-150 by an inverted duplication of the RL region of the genome. These genes are all involved in the penetration and entry of CMV into epithelial cells (Ryckman *et al*., 2006). There are mutations in the TRL region, including frameshift mutations in RL5A, RL13 and UL131a, resulting in truncated proteins (Akter *et al*., 2003; Davison *et al*., 2003; Yu *et al*., 2002). In comparison, Panel (b) shows that Merlin only possesses one known mutation in the UL128 gene due to a one-nucleotide insertion resulting in the incorporation of a stop codon. Evidence suggests UL128 has a chemokine like domain and therefore may play a role in the infectivity of CMV.

due to the condensed nature of the sperm genome. Furthermore, CMV is known to make initial contact with cells via HSPGs (Compton *et al*., 1993), which are also present on sperm, suggesting this might be a mechanism by which CMV might bind to sperm, as has been shown for HPV (Foresta *et al*., 2011a). Given that the receptors involved in this initial interaction via HSPGs remain intact in AD169 (Chee *et al*., 1990), the genetic alterations in this strain are not of concern to the primary objective of this thesis. However, these fundamental differences are important to remember and consider when analysing results.

Typically, when viral preparations are made in the manner outlined in this thesis, subsequent concentration and purification steps are normally performed. This can be carried out by ultracentrifugation and serves to separate infectious viral particles from aberrant products produced during the lytic cycle (Lou and Zhou, 2007), such as dense bodies and non-infectious enveloped particles (NIEP) (Talbot & Almeida, 1997; Irmiere & Gibson, 1983). These particles are defective and non-infectious as both lack any viral DNA (Craighead *et al*., 1972; Irmiere & Gibson, 1983; Stannard *et al*., 1989). They are produced in excess of the mature infectious virion and are thought to act as a decoy for the immune system, as they are still capable of binding to and entering cells (Topilko & Michelson, 1994), distracting the immune system and allowing for survival of the infectious particles. Given this, the presence of these impurities in viral preparations may induce adverse effects on experimental outcomes; therefore virus preparations are usually purified to eliminate the presence of these erroneous factors. Due to technical limitations, the preparations of CMV used throughout this thesis were not purified. Consequently, these viral stocks still contain impurities associated with propagation in MRC-5 cells, such as cellular debris, in addition to dense bodies and NIEP's. However, low speed centrifugation of the virus-infected media after harvesting from MRC-5 cells was performed and is thought to be sufficient at removing cellular debris (Talbot & Almeida, 1977). Confirmation of this could have been carried out by analysing viral preparations by western blotting for the presence of host cell markers, such as calnexin and actin, as

was performed in Zhou *et al*., (2015). However, no purification step to remove dense bodies and NIEP's was performed and due to the lack of viral DNA present in these particles, they are unquantifiable by qPCR or plaque assays, making them an unknown entity within un-purified viral preparations. As a result of this, it is probable that the concentration of virus used within the individual experiments throughout this thesis is a gross underestimation of the actual number of viral particles being added to each experiment. Despite this, given that dense bodies and NIEP's are by-products of the normal viral replication process, it is reasonable to assume that they would be present in an infection within a male *in vivo*, therefore their presence could be considered to reflect the *in vivo* situation. As outlined earlier in this section, this thesis is primarily concerned with binding events, rather than viral entry and replication, so the presence of these non-infectious particles appears appropriate in this context.

In order to quantify virus infectivity for viral stocks grown in this chapter and experiments conducted throughout this thesis, the viral plaque assay was used. This technique relies on the addition of viral serial dilutions to a monolayer of permissive cells and the formation of 'plaques'. These plaques arise from the presence of a single infectious particle and by counting the number of plaques present at a given dilution, the number of 'plaque forming units' (PFU) can be calculated, which is a measure of virus infectivity. Another tissue culture based method of quantification is the end point dilution ($TCID_{50}$) assay (Gray, 1999), which determines the highest dilution of virus to result in CPE in 50% of cells. It is thought that the plaque assay method is thought to be more precise in quantifying infectivity (Boeckh & Boivin, 1998).

These traditional tissue culture based methods are known to be time consuming and have low sensitivity but are well-established methods that are able to provide an estimate of CMV quantity (Boeckh & Boivin, 1998). Variations in technique are known to result in poor reproducibility, including the type of semi-solid overlay used (Plummer & Benyesh-Melnick, 1964; Wentworth & French, 1970), the method of viral adsorption employed (Chou & Scott, 1988), the dye used to visualise plaques, and the number of cells

used to seed the plates (Wentworth & French, 1970). In this case, the chosen combination of overlay medium and stain, methylcellulose and crystal violet, did not appear to affect the identification of discernable plaques. In addition, no plaques were ever observed in the absence of inoculum, suggesting the assay was specific and no cross-contamination was occurring. However, plates were not seeded with a particular number of cells and plates were not centrifuged to increase viral adsorption (Chou *et al*., 1988), which may have affected the accuracy of quantification, which cannot be ruled out as validation tests were not performed. Regardless of the limitations of this technique, the plaque assay is able to give an estimate of the quantity of infectious virus present, which can be considered useful when used in conjunction with qPCR data.

Two main experimental limitations encountered with the plaque assay were related to the large sets of dilutions required, in order to obtain the right dilution at which an accurate number of plaques can be counted, and the long incubation period, which often resulted in bacterial and/or fungal infection. These are two well-known disadvantages of tissue culture based methods of viral quantification (Boeckh & Boivin, 1998).

Replacement of these traditional techniques with more rapid and effective tests has been previously investigated and would bypass the main limitations of the plaque assay. An immunofluorescence test to detect the presence of CMV by targeting a monoclonal antibody to an immediate early antigen (Stinski *et al*., 1981) was determined to have a specificity and sensitivity of 100% and 80%, respectively, only 24 hours after infection (Griffiths *et al*., 1984). It was also shown to produce comparable titres of infectious virus to the plaque assay after only 20 hours of incubation, as opposed to 2 weeks (Chou *et al*., 1988). Incorporation of a similar method into the design of this study would have bypassed the two main limitations outlined above and might have provided more accurate quantitation of the virus. However, these assays have their own limitations with regards to antibody specificity and sensitivity.

Tissue culture based methods of CMV quantification are thought to be less accurate than methods that detect viral DNA (Boeckh & Boivin, 1998), such as qPCR. Detection of CMV by qPCR was proven to be sensitive enough to detect low titres of virus and allowed accurate measurement of the amount of virus in a sample (Lao *et al*., 1997), in addition to reducing the time to diagnosis when using tissue culture based methods. This method has been shown to be effective at monitoring CMV levels post organ transplant in order to provide anti-viral drugs prior to onset of clinical symptoms (Emery *et al*., 2000).

Since one of the objectives of this thesis was to identify men with CMV present in their semen, it was clear that a qPCR assay would be best method to do this due to its sensitivity and accuracy. A collaboration was established with a CMV research group at University College London (UCL) who perform this qPCR assay routinely. Advice was sought and attempts to replicate the Mattes *et al*., (2004) qPCR assay 'in-house' were made. Repeated problems were encountered during the optimisation of the assay with regards to DNA contamination. Despite using a PCR set up hood and decontaminating all plasticware prior to use with UV light, the no template control (NTC) samples consistently produced a peak at a late C_T. Adjustments to primer concentrations and annealing temperatures were made in order to optimise the assay and eliminate primer dimer formation but ultimately, contamination was always observed in the no template controls (NTC).

The parameters of the final standard curve constructed were close to the desired values, suggesting the assay was reliable. The efficiency of the 'in-house' assay lies within the acceptable range of 90-110%, suggesting it should produce accurate and reliable results. However, the viral load values calculated by the in-house qPCR assay were significantly lower than those predicted by the clinically validated assay at UCL (Table 4.1). One explanation of this could be the presence of primer dimers, which were difficult to rule out in this assay and are known to effect the efficiency of probe-based PCR assays (Bustin *et al*., 2009). The presence of primer dimers could also explain the apparent contamination in the NTC samples.

It is likely that the inconsistency between the 'in-house' and clinically validated assays is due to a poor R_2 value. This is a measure of how well the data fit the curve and is an indicator of an error with intra-assay variance. Ideally, the R_2 value should be around 0.999, but the value for this assay was 0.914. The variance could be due to pipetting error, inconsistencies with the qPCR machine or problems with the plasmid standard. Using electronic pipettes controlled for pipetting error, however inconsistencies with the qPCR machine could not be ruled out and it is possible that the preparation of plasmid was not homogenous, possibly contributing to the intra-assay variance. Inaccurate quantification of the initial plasmid concentration could explain why the values calculated by the clinically validated assay were all approximately one order of magnitude higher than the in-house calculated values. Also, it is unlikely that each plasmid within the plasmid population contained the viral gene, which might also contribute to inaccurate quantification.

The Y-intercept for the assay was 39.7, which corresponds to the theoretical limit of detection for the reaction. However, given the problems with contamination and that the NTC samples consistently amplified a signal around $C_T = 36$, the detection limit was actually much lower. The Minimum Information for Publication of Quantitative PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009) state that amplification in a NTC can be ignored if the $C_T \geq 40$ and if the highest unknown value is $C_T<35$. Therefore, the actual limit of detection for this assay should be around $C_T = 31$, corresponding to a viral load of approximately 10^3 copies, which does not give the assay a high level of analytical sensitivity. The continual presence of amplification in the NTC samples suggests the assay also does not have a high level of analytical specificity, conferring yet another area of criticism.

Given the inaccuracies outlined above with regards to specificity, sensitivity and repeatability, it was decided to have all of the samples collected during the work outlined in this thesis analysed by the validated UCL assay. Despite the work put into developing an in-house assay, it was clear that using the

validated assay would be the best method for obtaining accurate, clinically meaningful and reliable results.

It is clear that the infectious titre (PFU/ml), calculated by the plaque assay, and the total viral load (copies/ml), calculated by the qPCR assay, differ by at least 3-4 orders of magnitude (Sections 4.4.2 & 4.4.3). This is because CMV (AD169) has a high particle-to-PFU ratio, due to the large number of particles present in the viral preparation that are unable to establish an active infection, such as dense bodies and NIEPs (Zhou *et al*., 2015). For calculation of the amount of virus to include in each experiment throughout this thesis, the copies/ml value was used, rather than the number of PFU/ml, as the potential binding of any viral particle, regardless of whether it can establish an infection or not, is of interest in studying the interactions between CMV and sperm.

In summary, a laboratory strain of CMV (AD169) has been successfully grown and methods for quantification established. It is important to recognise that whilst there are criticisms with the chosen methods and strain of virus used, these do not affect the use of this strain in investigating the overall objective of this thesis, which is to examine the relationship between CMV and sperm. Many of the limitations outlined in this discussion pertain to studies investigating the replication and pathogenesis of CMV, which is not important at this stage of investigation in this study. The techniques for growing and quantifying CMV outlined in this chapter can now be used for incubating semen and sperm with CMV *in vitro* and evaluating the outcomes of these experiments. The next chapter will use this laboratory grown CMV to examine if sperm washing is effective at removing CMV from artificially infected semen samples. The methods for quantification developed in this chapter will allow the efficiency of these techniques to be evaluated for samples infected both artificially and naturally.

Chapter 5

The efficiency of density gradient centrifugation in removing CMV from donor semen samples

5.1 Introduction

Sperm washing is a technique used in fertility clinics to separate spermatozoa from seminal components (WHO, 2010). Prior to performing assisted conception techniques, sperm has to be separated from seminal plasma, as the presence of prostaglandins in semen can result in uterine cramping during IUI (Barwin, 1974; Tarlatzis *et al*., 1991). Also, prolonged exposure to seminal components can affect the fertilising capacity of sperm (Rogers *et al*., 1983). Simple dilution and subsequent centrifugation steps are two simple ways to wash sperm, however there is no selection for good quality sperm using this technique. Techniques involving a sperm selection step, such as sperm migration through a culture medium (Mortimer, 1994) were shown to be more successful with regards to IVF outcomes (reviewed in Mortimer, 1991). These techniques are known as the swim up or swim down methods.

Further techniques were developed utilising the method of density gradient centrifugation. With the aid of a well-known cell separation media, known as Percoll, which contains polyvinylpyrrolidone (PVP) colloidal silica particles (Pertoft *et al*., 1978), sperm were shown to separate from seminal components based on their own density, known as an isopycnic point (Gorus & Pipeleers, 1981). Sperm washing by density gradient centrifugation has been reported to increase clinical pregnancy rates (Guerin *et al*., 1989). However, it was found that whilst this technique improved the concentration of sperm recovered, the swim-up technique allowed for selection of better quality sperm (Ng *et al*., 1992). Percoll was withdrawn from use as a sperm separation medium in 1996, primarily due to endotoxin contamination (Mortimer, 1994), but it has also been shown to have detrimental effects on sperm (Claassens *et al*., 1998). This resulted in the development of numerous other media that could replace Percoll as a sperm separation agent. A replacement product, containing saline-coated silica particles, known as PureSperm, was found to be equally as effective and efficient at sperm separation in comparison to Percoll, as were other saline-coated silica particle based solutions (Claassens *et al*., 1998).

As well as being used in the preparation of sperm for IUI or IVF, sperm washing was also found to be useful in the elimination of HIV from semen samples of infected men (Semprini *et al*., 1992). No horizontal transmission of HIV was recorded in 367 couples inseminated with semen from HIV-positive men when density gradient centrifugation, in combination with the swim-up technique, was performed (Semprini *et al*., 2013). In addition, a systematic review found no vertical transmission and no effect on clinical pregnancy rates (Zafar *et al*., 2015), providing evidence that this technique is safe and effective in helping HIV serodiscordant couples conceive.

Similar studies have been carried out investigating other pathogens, including *C. trachomatis* (Al-Mously *et al*., 2009). This study concluded that, unlike HIV, sperm washing was not effective at removing bacteria from semen samples. Other research groups investigating the role sperm washing plays in removing different pathogens have modified the techniques further, including the addition of Heparinase-III for the removal of HPV (Garolla *et al*., 2012). In an attempt to rule out potential re-contamination of the sperm pellet upon retrieval from the bottom of the tube, the double tube gradient method was developed by Politch *et al*., (2004) and further developed into a commercially available product, known as the ProInsert™ tube (Figure 2.3b), which was found to be effective at removing HIV (Fourie *et al*., 2015).

Little investigation into the role sperm washing plays in the removal of CMV from semen samples has been performed. One study investigated the removal of CMV in men attending fertility clinics using density gradient centrifugation with PureSperm. The authors reported that CMV DNA was detected in the sperm pellet in 89% of CMV positive samples after washing (Michou *et al*., 2012). Similarly, Naumenko *et al*., (2014) reported detection of CMV in the sperm pellet of 92% of ejaculates washed by density gradient centrifugation. The presence of CMV after sperm washing at such high rates, suggests that CMV might be tightly associated with the spermatozoa. However, apart from these studies, no other investigations into the efficiency of sperm washing by density gradient centrifugation, or other methods, at removing CMV from semen samples has been carried out.

5.2 Experimental rationale

The aim of this chapter was to determine if sperm washing is able to remove CMV from semen samples. As sperm washing is already routinely carried out in fertility clinics, evidence supporting its role in CMV removal would be further beneficial. It would provide a simple and immediate solution to some of the problems associated with the management of CMV positive sperm donors outlined in Chapter 3.

In addition, the efficacy of sperm washing might provide insight in into the potential interactions between CMV and sperm, as has been shown for HPV (Foresta *et al*., 2011b; Garolla *et al*., 2012). The failure to remove a pathogen from a semen sample by density gradient centrifugation indicates a potential direct interaction. Due to the effects on sperm tyrosine phosphorylation levels when co-incubated with *C. trachomatis* (Hosseinzadeh *et al*., 2000), a direct receptor mediated interaction was proposed. A tight association between the bacterium and sperm would explain why density gradient centrifugation fails to remove *C. trachomatis* from semen samples (Al-Mously *et al*., 2009).

The efficiency of both conventional sperm washing techniques, currently used in UK fertility clinics, and modified experimental techniques will be explored. This chapter will primarily follow the *in vitro* approach used throughout this thesis, using laboratory grown CMV to answer this question. One advantage of this is the novel addition of the plaque assay to the experimental design, as outlined in Chapter 4. After washing, samples will be subject to detection of viral DNA by qPCR and detection of infectious virus via the plaque assay. This will provide evidence to show whether any virus remaining after washing is able to establish an infection or not. Given the argument that any virus remaining after washing might be due to an interaction with sperm, this might also provide evidence to show that sperm act as a vector for CMV transmission. The addition of an assay to detect live virus after sperm washing is a unique aspect of this study, which has not been investigated before.

It is important to investigate the presence and shedding pattern of CMV in naturally infected men and the efficiency of sperm washing in removing CMV from these naturally infected semen samples. This will provide evidence to support the role sperm washing might play in removal of CMV from semen samples in fertility clinics.

A combination of *in vitro* data, incorporating the plaque assay, and *in vivo* data showing the presence of CMV in semen, will provide a strong basis to show if sperm washing is effective at removing CMV and how this might be incorporated into the fertility clinic. By answering this one question, this study will also provide insight into the interaction between CMV and sperm and the potential impact sperm washing might have on improving the process of screening and management of CMV positive sperm donors.

5.2.1 Specific aim(s)

1. Investigate the efficiency of conventional density gradient centrifugation methods at removing CMV from naturally and artificially infected samples.
2. Assess if modified versions of sperm washing, such as Proinsert™ tubes, are more effective.
3. Investigate the presence of CMV in semen of infected men over an extended donation period.

5.3 Materials and Methods

To achieve the objectives outlined above, the efficiency of sperm washing in removing CMV was investigated in a number of different ways. Initially, the efficiency of conventional density gradient centrifugation was assessed *in vitro*, using laboratory grown CMV. The dose of virus used and the length of co-incubation period with sperm were investigated. An alternative technique, thought to reduce viral contamination, known as the Proinsert™ tube (Figure 2.3b) was also utilised. In addition to these *in vitro* experiments, the efficiency of density gradient centrifugation to remove CMV from naturally infected

semen samples was also investigated. In these men, the presence and shedding pattern of CMV was also examined.

For all of the experiments outlined in this section, semen samples were assessed for sperm concentration and motility, as outlined in Section 2.5.1, prior to density gradient centrifugation, as outlined in Section 2.5.3. All samples were used regardless of the quality, provided there was a sufficient volume for the specific experiment being performed, which ranged from 1.5-2.5ml and is indicated in the relevant sections below. In order to prevent any inconsistent changes in the rheological properties of the semen, the same volume of virus was added to each sample. As the concentration of virus varied dependent upon the concentration of sperm in a sample, the concentration of virus was adjusted prior to addition to the semen, in order to keep the volume of virus added consistent across all experiments.

After the initial set up of each experiment, all of the samples were incubated in the same manner and the same methods were used to assess the efficiency of the different sperm washing techniques used. These conditions are outlined in Section 5.3.5, and the individual experimental conditions are outlined in each relevant section below.

5.3.1 Efficiency of conventional density gradient centrifugation techniques at removing CMV from artificially infected semen samples

Semen samples from 5 healthy donors, with an initial semen volume of >2.0ml were divided into 4 x 500µl aliquots in 4 x 5ml polystyrene round-bottom tubes (VWR, Pennsylvania, USA). Two 500µl aliquots were infected with 500µl CMV (AD169)-infected supernatant at a ratio of 1 virus particle to 1 sperm. The other two aliquots were mock-infected with 500µl serum-free EMEM. One incubate of each condition was incubated at 37°C 5% CO_2 for either one hour or six hours prior to sperm washing with standard density gradient centrifugation, as outlined in Section 2.5.3.

5.3.2 Efficiency of Proinsert™ tubes at removing CMV from artificially infected semen samples

Semen samples from 9 healthy donors, with an initial semen volume of >1.5ml were divided into 2 x 750µl aliquots in 4 x 5ml polystyrene round-bottom tubes. One 750µl aliquot of semen was infected with 750µl CMV (AD169)-infected supernatant at a ratio of 1 virus particle to 1 sperm. The second aliquot was mock-infected with 750µl serum-free EMEM. Samples were incubated for one hour at 37°C 5% CO_2 prior to sperm washing. In this experiment, 500µl of each condition was loaded onto either a conventional density gradient column or loaded onto a gradient created in a ProInsert™ tube and washed as outlined in Section 2.5.3.

5.3.3 Use of naturally infected semen samples to investigate the efficiency of sperm washing and the viral shedding of CMV in semen

A total of 102 normozoospermic semen samples from 41 men were washed by adding 500µl of semen to a conventional density gradient centrifugation column and washing as outlined in Section 2.5.3. Pre-wash samples were analysed for the presence of CMV by qPCR and if positive, the corresponding post-wash samples were also examined by PCR. Any positive pre-wash samples were used to investigate the viral shedding pattern of CMV in semen and the paired positive pre-wash/post-wash samples used to analyse the efficiency of sperm washing. All DNA extracted from samples were analysed for the presence of genomic DNA by GAPDH, as outlined in Section 2.6.5 to confirm successful extraction of DNA. Samples negative for GAPDH were excluded from analysis.

5.3.4 Effect of viral dose on the efficiency of conventional density gradient centrifugation techniques at removing CMV from artificially infected semen samples

Semen samples from 7 healthy donors, with an initial semen volume of >2.5ml were divided into 5 x 500µl aliquots in 4 x 5ml polystyrene round-bottom tubes. A 10-fold dilution series of CMV (AD169)-infected supernatant was performed with an initial ratio of 1 virus particle to 1 sperm. 10µl of this initial concentration was added to 90µl serum-free EMEM to obtain dilutions

of 10^{-1}, 10^{-2} and 10^{-3}. These four concentrations of virus were added to four of the 500µl aliquots and the final aliquot was mock-infected with 500µl serum-free EMEM. The samples were incubated for one hour at 37°C 5% CO_2 before washing by standard density gradient centrifugation, carried out as outlined in Section 2.5.3.

5.3.5 Incubation conditions and sample analysis

For the experiments outlined in Section 5.3.1, 5.3.2 and 5.3.4, after the initial incubation period, 200µl was removed for detection of CMV viral load by qPCR and 200µl was removed for detection of infectious virus, or plaque forming units (PFU), by performing a plaque assay, for each incubate. In addition, 10µl was removed to analyse sperm motility and 40µl used to measure sperm concentration. For Section 5.3.3, no plaque assay was performed and no incubation period was carried out. Semen analysis was performed on the raw semen sample, as outlined in Section 2.5.1, and 200µl removed for qPCR analysis.

For all experiments, after washing, the sperm pellet was resuspended in ~500µl PureSperm wash buffer and the same aliquots outlined above taken again for post-wash analysis.

DNA was extracted from all samples as outlined in Section 2.6.4. For each batch of samples extracted, a negative H_2O control was included. If the negative control tested positive for CMV DNA, the samples were extracted and tested again, or excluded from the final analysis. The concentration of DNA and optical density (OD) ratios of the DNA extracted in each experiment can be found in Appendix VII. The OD260/280 ratio should be in the range of ~1.8 for DNA and the majority of sets of data were calculated to be in that range. The OD260/230 ratio should be in the range of ~2.0-2.2. None of the sets of data collected were within this range, with all of them <1.5. This suggests that the samples contained a type of contaminant that absorbs at 230nm. The presence of some reagents in the final elution, such as EDTA might account for this lower absorption. However, no inhibitory effects on

qPCR were observed and all negative samples were confirmed as true values by GAPDH analysis.

5.3.6 Statistics

For each data set, a test of normality was performed using GraphPad Prism (Version 6.0c). For data sets with at least 8 repeats, the D'Agostino Pearson omnibus normality test was used, those with at least 7 repeats, the Shapiro Wilk test was used and for those with at least 5 repeats, the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefor P Value test was performed. For those data sets with less than 5 repeats, a non-Gaussian distribution was assumed and a non-parametric test performed.

When comparing pre-wash to post-wash samples for a significant difference, a paired test was used (details of specific tests used for each data set can be found in corresponding figure/table legends). For sperm concentration, viral load and infectious virus, a one-tailed test was performed as these parameters could only decrease after washing. However, for sperm motility, a two tailed test was performed as this could increase or decrease. For comparison of samples of the same type, such as post-wash density gradient centrifugation vs Proinsert™, a two-tailed independent test was used.

5.4 Results

5.4.1 Do conventional density gradient centrifugation techniques remove CMV from artificially infected semen samples and is this affected by the length of time sperm are exposed to CMV?

Semen samples from five healthy sperm donors with a mean volume of 2.76±0.24ml, a mean sperm concentration of 50.3±10.10x10^6/ml and a mean progressive motility of 56.3±6.11% were incubated with CMV (AD169). After either one or six hours incubation, samples were washed using an 80:40% Puresperm density gradient and the amount of virus remaining determined by qPCR (n=5) and a plaque assay (n=3).

There was a statistically significant difference (P<0.05) in the viral load present before and after washing when samples were incubated for both one and six hours (Figure 5.1a). The median percentage of CMV remaining after washing was 0.7 (range 0.1-19.6)% and 1.1 (range 0.7-17.6)% of the initial load, respectively. Although the same trend was also observed when analysing the number of plaque forming units (PFU), this difference was not statistically significant, perhaps due to the smaller number of repeats and the spread of data (Figure 5.1b). No differences were observed in the viral load or PFU between the two different incubation periods, either before or after washing. This shows that the efficiency of sperm washing is not affected by the amount of time semen samples are exposed to CMV. A negative control of serum-free EMEM was included in all experiments. All controls were found to be negative for CMV DNA, except for one repeat, which produced some positive results, thought to be due to a naturally occurring infection as this donor also was found to be positive in Section 5.4.3. No PFU were observed in any of the negative control samples.

Analysis of the quality of sperm after washing shows that motility was generally increased after a one-hour incubation period with CMV. However, the post-wash motility was decreased significantly (P<0.05) after a six-hour incubation period in both the infected samples and in a negative control (Table 5.1). The post-wash sperm concentration decreased significantly in all conditions, as would be expected. However, the post-wash concentration after a one-hour incubation with CMV appeared to be much lower (P<0.01).

5.4.2 Are Proinsert™ tubes more effective than density gradient centrifugation at removing CMV from artificially infected semen samples?

The conventional density gradient centrifugation method is criticised due to the risk of re-contamination of the pellet upon its retrieval from the bottom of

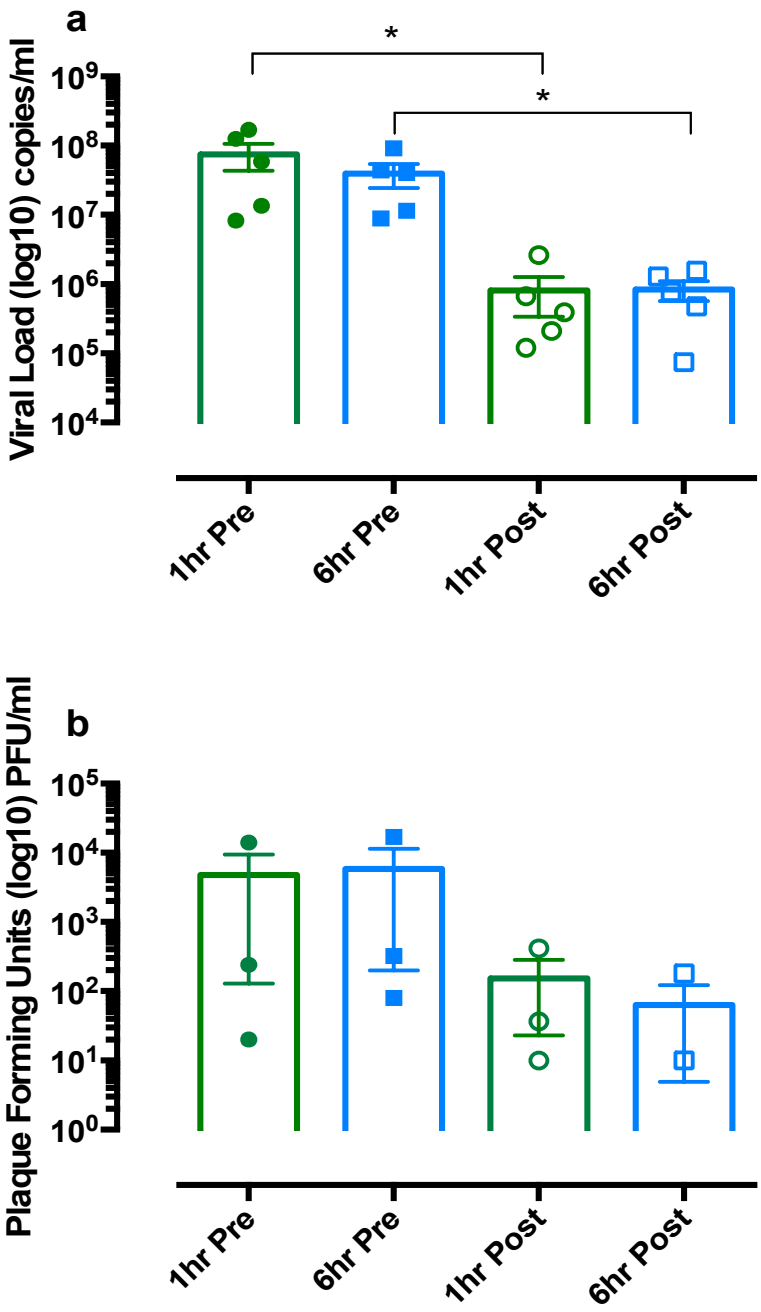

Figure 5.1: Details of CMV (AD169) viral load (a) and PFU (b), present before and after sperm washing by density gradient centrifugation. Semen samples from five (a) and three (b) healthy donors were incubated with CMV (AD169) at a ratio of 1 virus particle to 1 sperm. Samples were incubated with CMV or with EMEM for either one hour or six hours prior to washing. Data shown are mean ± SEM and circles and squares represent individual replicates for each experimental condition. Statistical significance was determined using a one-tailed paired t-test for comparing pre-wash samples to post-wash samples. A two-tailed independent t-test was used when comparing 1hr and 6hr pre-wash samples and 1hr and 6hr post-wash samples separately. The symbol (*) indicates a difference at the significance level of $P < 0.05$.

Table 5.1: Details of sperm motility and concentration before and after density gradient centrifugation incubated with and without CMV (AD169). Data shown are the mean±SEM of incubations with semen from five healthy sperm donors for either one hour or six hours.

Condition	Motility (%)*	Concentration ($x10^6$/ml)$^+$
1hr Pre Infected	28.5±7.6	19.5±0.6
1hr Pre Control	31.7±9.0	19.1±2.7
1hr Post Infected	40.7±12.5	0.9±0.3 ***
1hr Post Control	33.9± 8.4	2.0±0.6 **
6hr Pre Infected	9.6±3.5	19.6±4.2
6hr Pre Control	7.2±3.2	16.5±3.5
6hr Post Infected	2.7±2.2 *	1.3±0.3 **
6hr Post Control	4.5±3.0 *	1.5±0.4 **

* Statistical significance was analysed using two-tailed paired and independent t-tests on log transformed data. The symbol (*) indicates a difference between the pre-wash and post wash samples at a significance level of P <0.05.

$^+$ Statistical significance was analysed using a one-tailed paired t-test and a two-tailed independent t-test. The symbol (*) denotes a difference between the pre-wash and post wash samples with two symbols (**) indicating a significance level of P <0.001 and three symbols (***) indicating a significance level of P <0.001.

the gradient. To investigate this, a comparison between density gradient centrifugation and a modified method, the Proinsert™ tube, designed to reduce the risk of re-contamination, was performed.

Semen samples from nine healthy sperm donors with a mean volume of 3.1±0.31ml, a mean sperm concentration of 76.4±25.9x10^6/ml and a mean progressive motility of 54.0±4.6% were incubated with CMV (AD169) for one hour. Samples were then washed using either density gradient centrifugation or the Proinsert™ tube. The amount of virus remaining after washing was determined by qPCR (n=9) and a plaque assay (n=5).

In line with the experiment performed in section 5.4.1, there was a significant reduction (P<0.05) in the amount of virus present after washing when using the density gradient centrifugation method. In comparison, the Proinsert™ tube appears to reduce the amount of virus further (P<0.01). There was also a significant difference in the amount of virus present after washing between the two different methods (P<0.05) (Figure 5.2a), with the median percentage of CMV remaining after washing being only 1.6 (range 0-101)% when using the Proinsert tube, in comparison to density gradient centrifugation, where the median percentage of CMV remaining after washing was 15.2 (range 0.6-61)%. Interestingly, when examining the amount of infectious virus present, the Proinsert™ tube was better at removing infectious CMV than density gradient centrifugation (P<0.01), as no PFU's were observed in the post-wash samples using the Proinsert™ tubes (Figure 5.2b). All negative controls were found to be negative for CMV DNA, except for three positive results, with low values (322-2434 copies/ml), thought to be due to low-level contamination, or due to a naturally occurring infection. No PFU were observed in any of the negative control samples.

After washing with the Proinsert™ tube, sperm motility was generally higher than when density gradient centrifugation was performed (Table 5.2). However, the sperm concentration was generally lower when using the modified sperm washing procedure, although the concentration of sperm present after washing was significantly different in all conditions (Table 5.2).

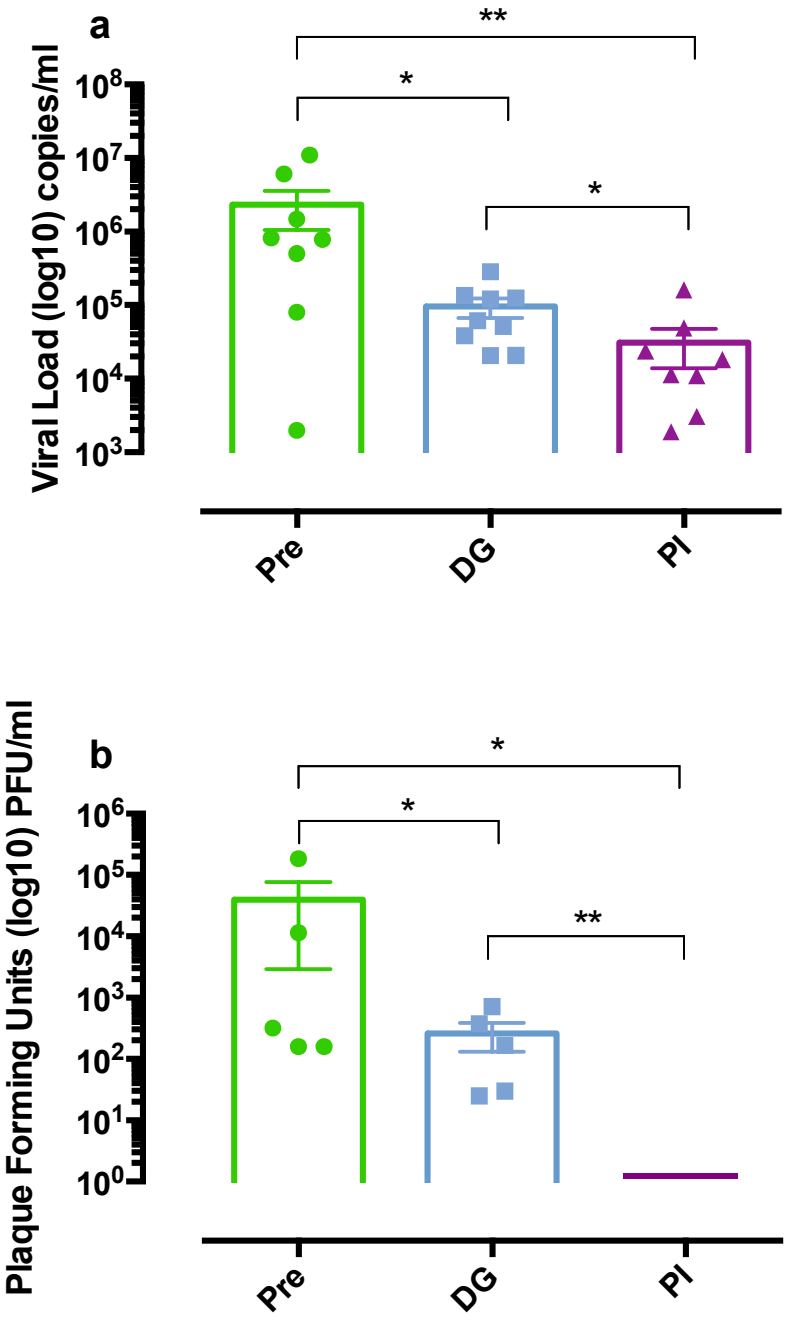

Figure 5.2: Details of CMV (AD169) viral load (a) and PFU (b), present before (pre) and after sperm washing by either density gradient centrifugation (DG), or the ProInsert™ method (PI). Semen samples from nine (a) and five (b) healthy donors were incubated with CMV (AD169) at a ratio of 1 virus particle to 1 sperm. Samples were incubated with CMV or with EMEM for one hour. Data shown are mean ± SEM and circles, squares and triangles represent individual replicates for each condition. Statistical significance was analysed using a one-tailed Wilcoxon matched-pairs signed rank test for comparing pre-wash samples to post-wash samples. A two-tailed Mann Whitney test was used when comparing DG with PI. The symbol (*) indicates a difference at the significance level of $P<0.05$ and the symbol (**) indicates a difference at the significance level of $P<0.01$.

Table 5.2: Details of sperm motility and concentration before (pre) and after washing with either density gradient centrifugation (DG) or ProInsert™ (PI) tubes. Semen samples were incubated with or without CMV (AD169) for one hour. Data shown are the mean±SEM of incubations with semen from nine healthy sperm donors.

Condition	Motility (%)*	Concentration ($x10^6$/ml)+
Pre Infected	42.4±5.0	21.16±5.6
Pre Control	42.6+5.3	17.92±4.7
DG Post Infected	51.0±7.8	5.4±1.7 **
DG Post Control	51.4±8.1	5.3±1.4 **
PI Post Infected	68.0±10.4 *	4.9±1.4 *
PI Post Control	63.3±10.8	3.4±1.0 **

* Statistical significance was analysed using two-tailed paired and independent t-tests on log-transformed data. The symbol (*) indicates a difference between the pre-wash and post wash sample at a significance level of P <0.05.

+ Statistical significance was analysed using a one-tailed paired t-test and a two-tailed independent t-test. The symbol (*) denotes a difference between the pre-wash and post wash samples at a significance level of P <0.05 and two symbols (**), a significance level of P <0.01.

5.4.3 Is sperm washing effective at removing CMV from naturally infected samples?

To investigate if the same findings for semen infected *in vitro* were found in naturally infected men, 102 ejaculates from 41 normozoospermic donors with a mean volume of 3.1±0.14ml, a mean sperm concentration of $59.0\pm4.9x10^6$ /ml and a mean progressive motility of 48±2.1% were washed by density gradient centrifugation. After washing, the mean sperm count was $10\pm1.3x10^6$ /ml and a mean progressive motility of 50.3±2.8% was observed.

DNA extracted from samples before washing were analysed for the presence of CMV DNA and 21/102 (20.6%) were positive. For all positive samples, the corresponding post-wash samples were analysed to check for the presence of CMV after washing and 5/21 (23.8%) were positive.

A H_2O control was included for each extraction to determine if any contamination occurred during the extraction process. On one occasion, this negative control tested positive, therefore the 15 samples included in this batch of extraction were excluded from the analysis. Similarly, all samples were analysed for the presence of the housekeeping gene, GAPDH, by PCR. This analysis was carried out to ensure that any negative results were not due to an absence of genomic DNA because of an extraction error. Eight samples were negative for the presence of GAPDH and were excluded.

After excluding these 23 samples, 79 ejaculates from 35 donors were included in the final analysis. The number of pre-wash CMV positive samples in the final analysis was 13/79 (16.5%), with only 2/13 (15.4%) samples positive after washing. The mean viral load pre-wash was 19,416±11,568 copies/ml and post-wash 146.9±109.7 copies/ml, giving a statistically significant difference of $P<0.001$ (Figure 5.3). In comparison to the *in vitro* experiments outlined previously, the median percentage of CMV remaining after washing was slightly lower, at 6.2 (range 2.2-10.2)% of the initial amount.

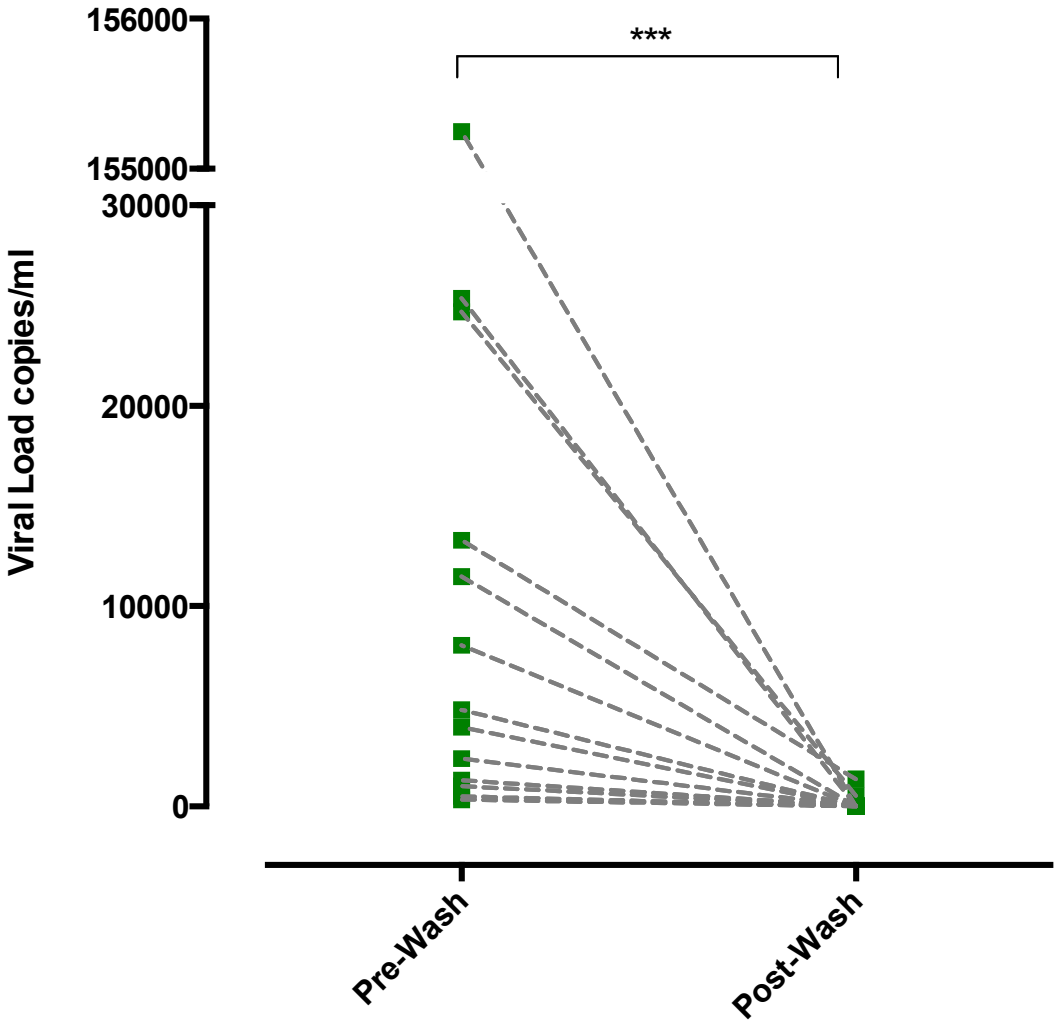

Figure 5.3: Details of the CMV viral load of 13 ejaculates from naturally infected men before and after washing. Semen samples were washed on an 80:40% Puresperm gradient and the presence of CMV before and after washing was analysed by qPCR. Data shown are mean ± SEM. Statistical differences were examined using a one-tailed Wilcoxon matched-pairs signed rank test. The symbol (***) indicates a difference at the significance level of P <0.001.

Furthermore, there were no significant differences in semen volume, sperm concentration or progressive motility between CMV positive and CMV negative ejaculates (Table 5.3).

5.4.4 Is the dose of CMV important in the efficiency of removal of CMV from artificially infected semen samples?

From observing the viral load in naturally infected men, it was clear that the amount of virus used in the experiments outlined in Sections 5.4.1 and 5.4.2 were not representative of what was occurring *in vivo*. The mean viral load included in the first two experiments was $7.5x10^7 \pm 3.148x10^7$ and $2.3x10^6 \pm 1.261x10^6$ copies/ml, respectively, in comparison to the 19,416±11,568 copies/ml found *in vivo*. Therefore, to investigate if the dose of virus sperm is exposed to effects the efficiency of sperm washing, a dilution series of different amounts of virus was carried out.

Semen samples from seven healthy sperm donors with a mean volume of 3.6±0.26ml, a mean sperm concentration of $64.5 \pm 18.2x10^6$/ml and a mean progressive motility of 57.3±8.0% were incubated with CMV (AD169) for one hour. Samples were then washed by density gradient centrifugation and the amount of virus remaining determined by qPCR (n=7) and a plaque assay (n=4).

In line with the experiments performed in Sections 5.4.1 and 5.4.2, the amount of virus present after washing was significantly reduced when a ratio of 1 virus particle was incubated with 1 sperm ($P<0.05$) (Figure 5.4a). The same was observed when the amount of virus was diluted 10-fold (0.1). However, when dilutions of 100 (0.01) and 1000-fold (0.001) were used in co-incubations, there was no statistically significant reduction in viral load after washing. The median percentage of CMV remaining after washing, at the two highest concentrations, was 5.1 (range 0.76-18.3)% and 17.4 (range 5.4-34.5)%, respectively. However, with a 100-fold dilution, 17.8 (range 0-241)% of CMV remained after washing. When a 1000-fold dilution was used, the amount of virus was slightly higher after washing, with a mean of 217.6±167 copies/ml detected post-wash in comparison to a mean of

Table 5.3: Comparison of sperm concentration, progressive motility and semen volume between CMV positive and negative ejaculates.

	CMV Negative (66)	CMV Positive (13)
Sperm Concentration ($x10^6$/ml)*	58.2±5.3	72.6±18.7
Progressive motility (A+B) %+	48.9±2.4	52.8±5.1
Semen Volume (ml)+	3.3±0.2	2.6±0.2

Data are shown as mean±SEM

* Statistical significance was analysed using the Mann-Whitney test on log-transformed data. No statistically significant difference was found.

+ Statistical significance was analysed using a two-tailed independent t-test. No statistically significant difference was found.

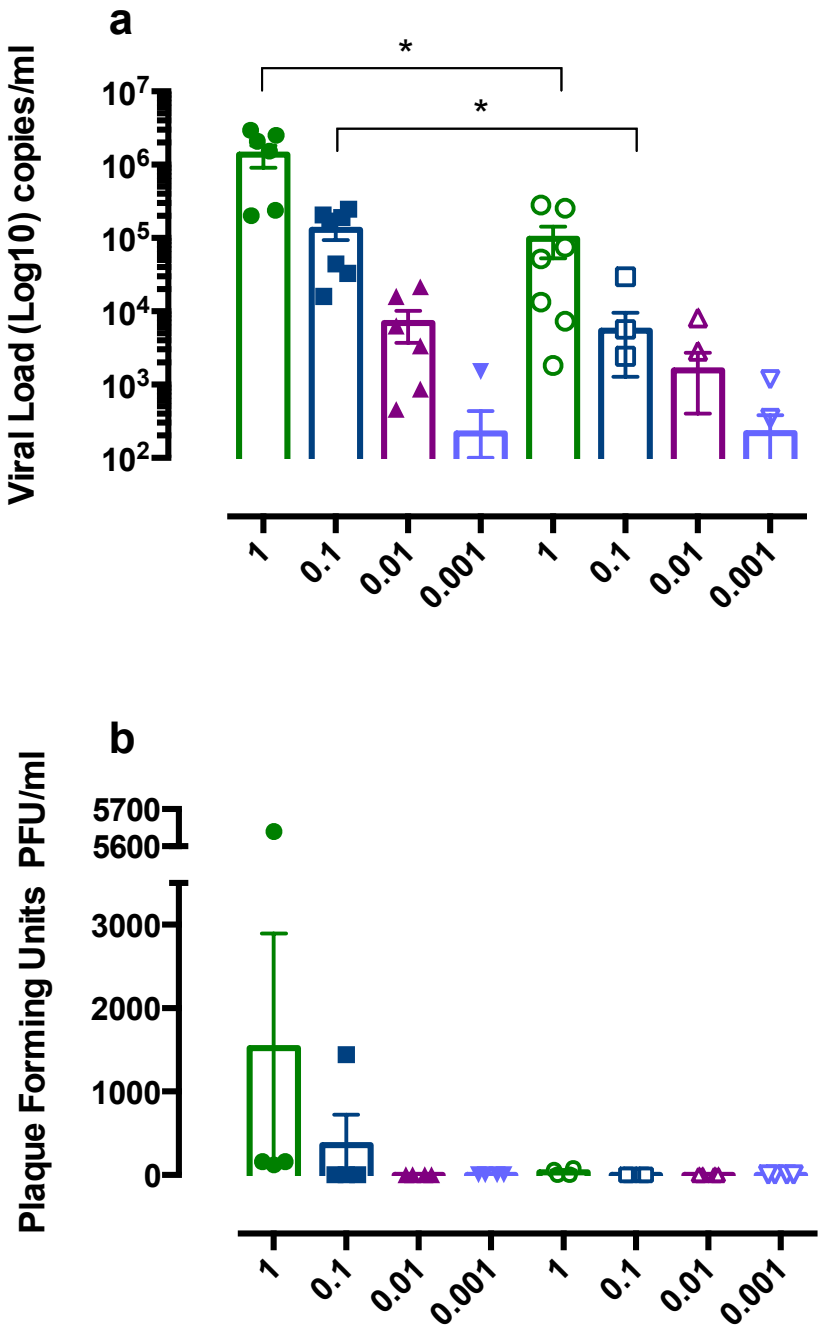

Figure 5.4: Details of CMV (AD169) viral load (a) and PFU (b), present before and after sperm washing by density gradient centrifugation. Semen samples from seven (a) and four (b) healthy donors were incubated with CMV (AD169) at various doses. A ratio of 1 virus particles to 1 sperm was the starting dose, after which a 10-fold dilution was carried out until a dilution of 0.001 (1000-fold). Samples were incubated with CMV or with EMEM for one hour prior to washing. Data shown are mean ± SEM with symbols indicating individual replicates for each dilution. Statistical significance was analysed using a one-tailed Wilcoxon matched-pairs signed rank test. The symbol (*) indicates a difference at the significance level of P <0.05.

215.5±215.5 copies/ml detected before washing. This discrepancy could be due to contamination, as some of the negative control samples tested positive after washing (with values in the range of 1027-2729 copies/ml). Alternatively, the presence of CMV due to a natural infection was not ruled out and could have adversely affected the results at these low concentrations.

No statistical differences were observed upon analysis of the amount of PFU (Figure 5.4b), although the amount of virus was reduced for the starting concentration, but this was not statistically significant. Unfortunately, for the higher dilutions, the small amount of virus present pre-wash was not detected by this assay and therefore no comparison can be made.

Progressive sperm motility was significantly increased ($P<0.05$) for each condition, except for the negative control (Table 5.4). Conversely, sperm concentration was significantly decreased ($P<0.05$) for each condition, except for the negative control, which had a significant reduction of $P<0.01$ (Table 5.4).

5.4.5 Does removal of CMV by sperm washing have a place in the fertility clinic?

The evidence outlined so far in this chapter suggests that sperm washing by density gradient centrifugation is effective at significantly reducing the viral load in naturally infected men. However, it was important to establish the significance of these findings for a fertility clinic. Currently, upon screening for CMV in sperm donors, no analysis of the individual semen sample is performed; therefore the impact of sperm washing on the removal of CMV is unknown. This section will investigate if there is a requirement for testing individual ejaculates, as has been suggested previously (Kaspersen *et al*., 2012), and whether this would influence the impact of removal of CMV by sperm washing in the fertility clinic.

PCR data from the 79 semen samples analysed for the presence of CMV in Section 5.4.4 was used to investigate the shedding of CMV in semen.

Table 5.4: Details of sperm motility and concentration before (pre) and after (post) density gradient centrifugation incubated with a 10-fold dilution series of CMV (AD169) and a negative control. Data shown are the mean±SEM of incubations with semen from seven healthy sperm donors.

Condition	Motility (%)*	Concentration ($x10^6$/ml)+
1:1 Pre	30.7±6.7	15.4±4.7
1:1 Post	46.0±.0 *	2.7±0.9 *
10^1 Pre	30.9±6.6	13.4±4.0
10^1 Post	54.7±10.5 *	4.4±1.4 *
10^2 Pre	33.9±7.5	14.8±4.6
10^2 Post	58.1±9.3 *	3.3±1.1 *
10^3 Pre	32.6±7.1	17.2±5.0
10^3 Post	54.4±10.0 *	2.8±0.9 *
Pre Control	33.1±8.0	18.1±5.4
Post Control	48.8±10.8	3.4±1.2 **

* Statistical significance was analysed using two-tailed paired and independent t-tests. The symbol (*) indicates a difference between the pre-wash and post wash sample at a significance level of P <0.05.

+ Statistical significance was analysed using a one-tailed paired t-test. The symbol (*) denotes a difference between the pre-wash and post wash samples at a significance level of P <0.05 and two symbols (**), a significance level of P <0.01.

In Section 5.4.4, each ejaculate was tested by PCR for the presence of CMV and Figure 5.5 shows the spread of positive and negative samples for each donor, over their entire donation period. Out of 79 ejaculates, 66 were negative (83.5%) and 13 were positive (16.5%). Whilst the majority of donors (23/35) only produced samples negative for CMV, 11 donors produced one positive sample, with one donor producing two positive samples. Of the 12 donors that produced at least one positive sample, 9 intermittently shed CMV in their semen (Donors 7, 18, 20, 23, 25, 26, 27, 30, 31). Interestingly, the time period between producing a positive sample and a negative sample was quite short in some instances. For example, Donor 25 produced a positive sample on day 52, but then provided a negative sample three days later, on day 55. Likewise, Donor 23 produced a positive sample on day 70 and a negative sample one week later, on day 77. Donor 26 provided 5 samples over a period of 71 days (~2.5 months) and was the only donor to produce more than one positive sample. The first sample produced was negative, followed by a positive sample on day 9, a further two negative samples on days 20 and 48, and finally a positive sample on day 71 (Figure 5.5).

Analysis of the individual viral loads of the 12 men who produced at least one positive sample shows the range of concentrations of CMV detected in semen (Figure 5.6). The viral load ranged from 337 to 155,247.5 copies/ml with an average viral load of 19,416±11,568 copies/ml. Interestingly, Donor H had the highest viral load in any sample (Figure 5.6), but after producing this sample, only 3 days later a negative sample was produced (Donor 25 in Figure 5.5).

Figure 5.7 shows that there was no correlation between the amount of virus present in a semen sample and the sperm concentration (Figure 5.7a) or motility (Figure 5.7b). This suggests that there is no relationship between semen quality and viral load.

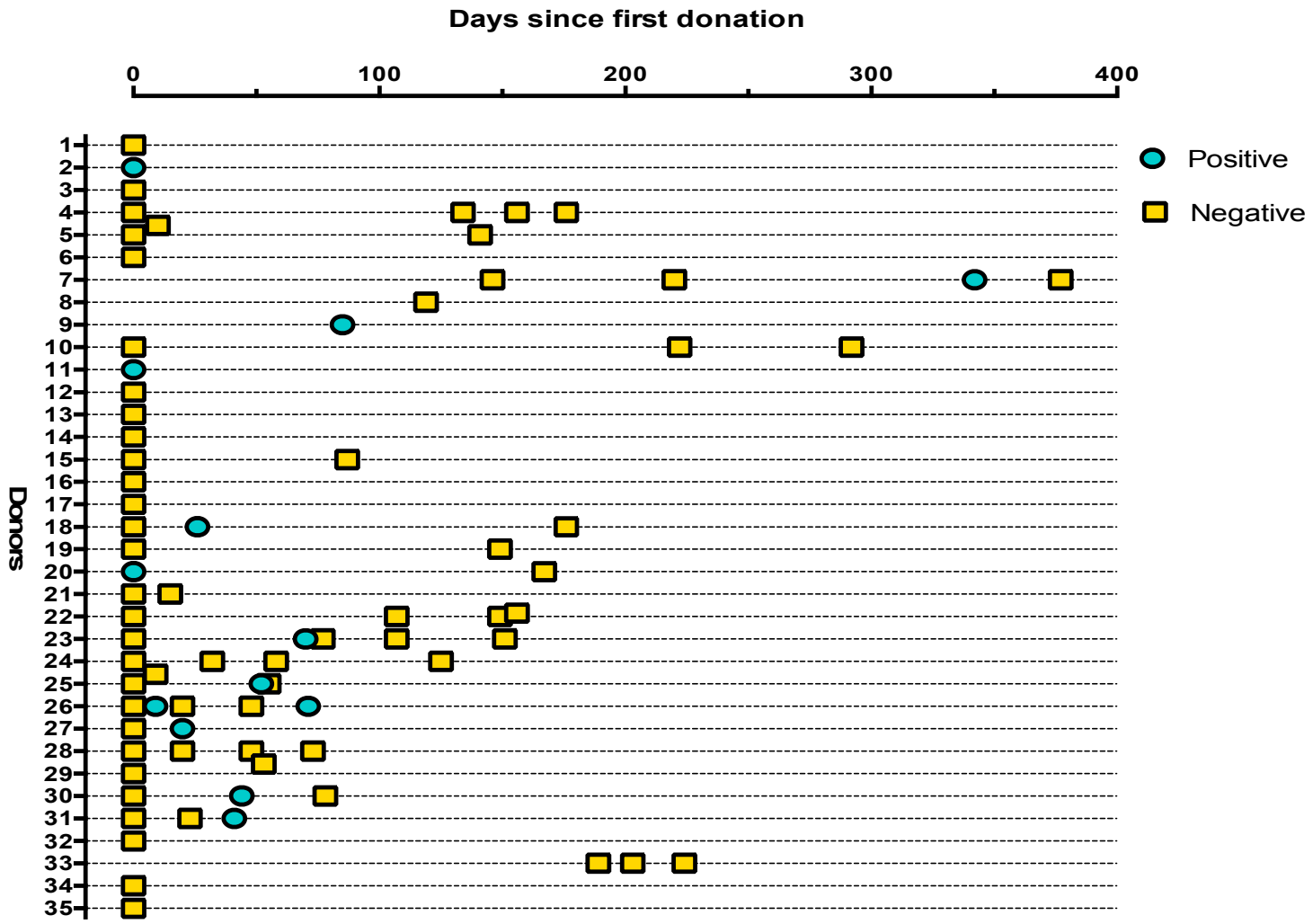

Figure 5.5: Details of the viral shedding of 35 different donors. The viral shedding in each ejaculate is shown against the days since an individual donor made their first donation. The CMV status of each ejaculate was confirmed by qPCR. Donors 7, 8, 9 and 33 do not have data points at time point 0 due to the exclusion criteria outlined in Sections 5.3.3 & 5.3.5.

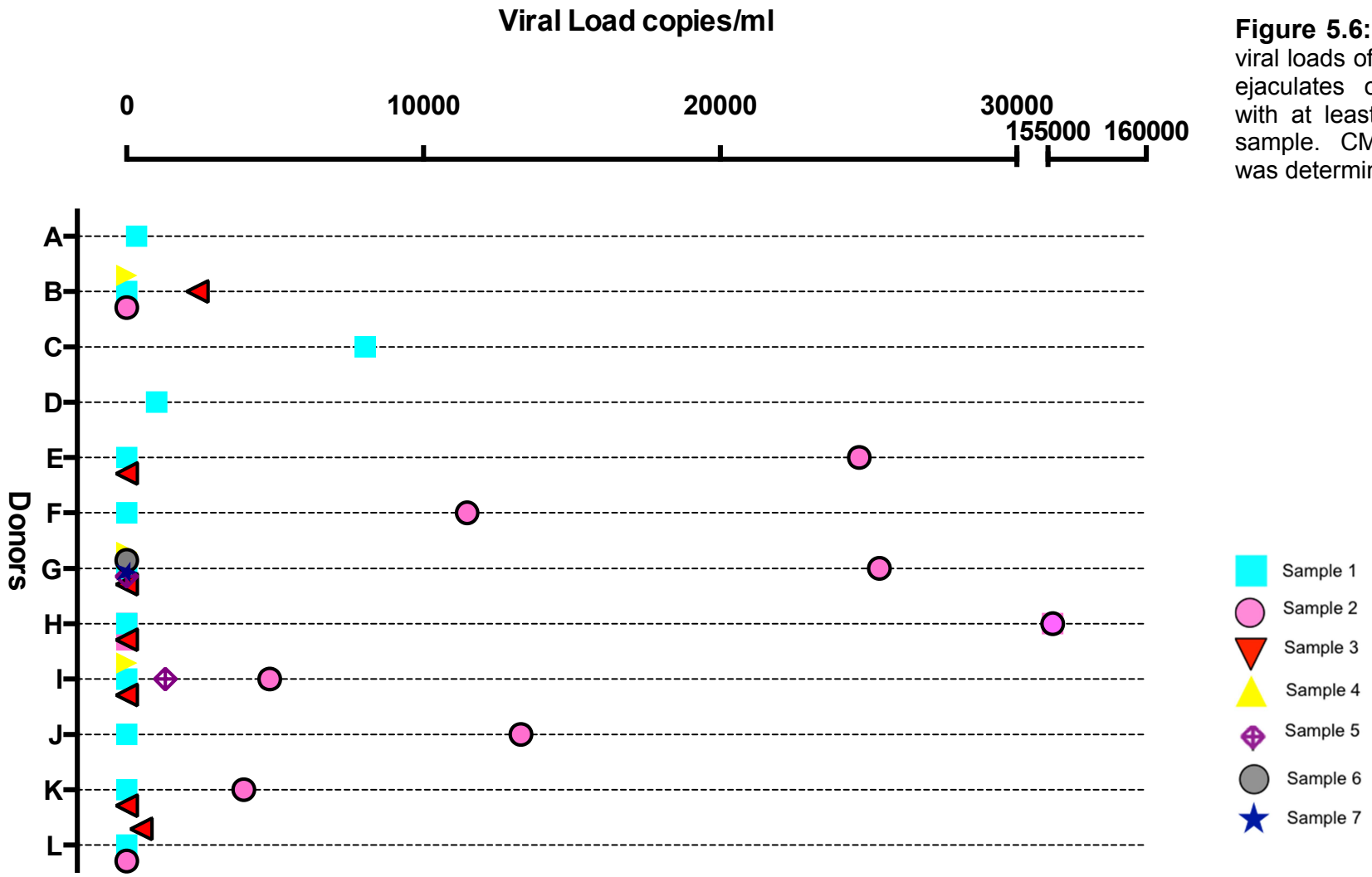

Figure 5.6: Details of the viral loads of the individual ejaculates of 12 donors with at least one positive sample. CMV viral load was determined by qPCR.

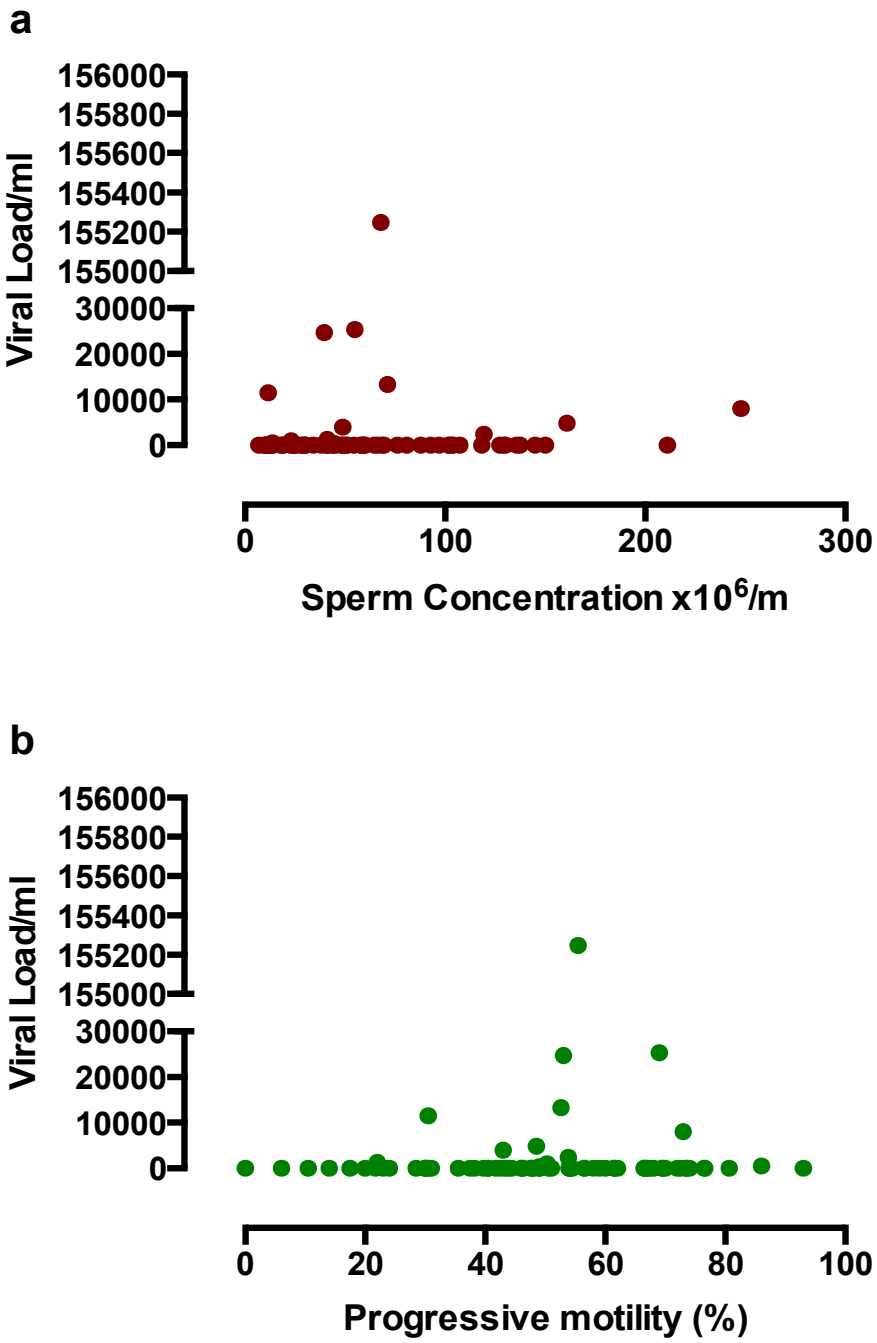

Figure 5.7: Correlations between the pre-wash CMV viral load and pre-wash sperm concentration (a) and progressive motility (b), of 79 ejaculates from 33 healthy sperm donors.

5.5 Discussion

The overall aim of this chapter was to investigate the efficiency of sperm washing to remove CMV from both *in vitro* and *in vivo* infected semen samples. This has provided evidence to support the role of sperm washing in the management of sperm donors with a CMV infection, and has provided insight into the interactions between CMV and sperm. This study was novel in its design due to the incorporation of the plaque assay in addition to qPCR, which acts as a model for transmission *in vivo*. Incorporating this technique showed that virus remaining after washing is able to establish an infection. However, overall sperm washing appeared to be mostly effective at removing CMV from semen samples, and was more efficient with naturally infected samples than those artificially infected.

Despite sperm washing by density gradient centrifugation being broadly effective at removing CMV from semen samples infected *in vitro*, CMV does persist after washing at low levels (<17.8%). Furthermore, the plaque assay shows that the remaining virus is able to establish an active infection (Figure 5.1). These findings are similar to those reported for *C. trachomatis*, where sperm washing was found to be ineffective at removing the bacteria from both artificially and naturally infected semen samples (Al-Mously *et al*., 2009). The average Chlamydial load remaining after washing was 4.02%, a slightly lower value than that observed for CMV in this study. The failure of sperm washing to completely remove CMV also corresponds with previous reports found *in vivo*. In these studies, CMV DNA is detected in the sperm pellet in 89.36% and 92% of semen samples purified by density gradient centrifugation (Michou *et al*., 2012; Naumenko *et al*., 2014). Unfortunately, these papers do not report the viral load remaining after washing, so the efficiency of removal of CMV cannot be directly compared to this study.

The efficiency of sperm washing was not affected by the amount of time the sperm were exposed to CMV (Figure 5.1). As little is known about the interaction between CMV and sperm, the amount of time needed to establish a direct interaction, if one was occurring, was not known. It is known that

during a normal replication cycle of CMV in human foreskin fibroblast cells, immediate early proteins are detected with 3 hours (Stinski *et al*., 1981). This suggests that binding, entry and initial gene expression would have to occur within those first few hours. Most studies allow for an initial attachment period of 60 minutes (Griffiths *et al*., 1984), including studies involving sperm (Pallier *et al*., 2002). However, some studies investigating mechanisms of binding use a period of 90 minutes (Compton *et al*., 1993). Given that any potential mechanism of binding to sperm is not known, two incubation periods were chosen: one and six hours. If CMV is binding to sperm via a mechanism taking longer than one hour to establish, it would be expected that the amount of virus remaining after washing would be increased after a six hour incubation. This was not observed, showing that the amount of time sperm are exposed to CMV does not affect the efficiency of sperm washing. This is an important consideration in the fertility clinic with regards to sample delivery and preparation. For example, if preparation of the semen sample prior to washing took longer than one hour, this would not affect the outcome of the procedure. However, after six hours, sperm motility would be considerably decreased (Table 5.1), which would affect the quality of sperm recovered after washing, and be more problematic for assisted conception techniques.

The efficiency of sperm washing was also not affected by the dose of virus the sperm were exposed to (Figure 5.4). As the dose of virus in men with natural infection was not known prior to performing the initial experiments, a dose of 1 virus particle to 1 sperm was chosen. This ratio follows a pattern similar to a multiplicity of infection (MOI) dose used in other studies such as Al-Mously *et al*., (2009). However, in this instance, as the viral load (copies/ml) was used to calculate the amount of virus added, rather than the PFU, the term MOI cannot be used. When the amount of virus in naturally infected men was subsequently found to be much lower (Figure 5.6) than the dose of virus used in the initial experiments in this chapter, a dose response experiment was carried out (Figure 5.4). At a dose of 1 and 0.1, there was a significant reduction in the amount of virus present after washing (Figure

5.4), in line with other experiments carried out. However, at the lower doses, of 0.01 and 0.001, there is no significant reduction, and importantly, some virus still remained. Unfortunately, the ability of virus in these lower doses to establish an active infection could not be determined due to detection limits of the plaque assay. Whilst the higher doses of virus did not affect the efficiency of sperm washing, this experiment does shows that the more virus present prior to washing, the more virus will remain after washing (Figure 5.4). This suggests, that semen samples with a higher dose of virus *in vivo* are likely to retain a larger amount of virus after washing.

From these initial experiments, it was clear that density gradient centrifugation was able to remove the majority of CMV in a semen sample, regardless of incubation time or dose of virus. However, the retention of some virus after washing suggests that CMV could be binding to sperm. Conversely, the presence of CMV in the sperm pellet could be due to recontamination of the pellet with virus that gets trapped at the interface between the two PureSperm layers. Modified methods of sperm washing have been adopted to bypass this problem, including the double tube gradient method (Politch *et al*., 2004). The procedure works by having an inner tube, through which the pellet is retrieved, without having to pass through the interface layer, located in the outer tube (Figure 2.3b). This thesis used a commercially available version of this, the ProInsert™ tube, which was found to be more effective at removing CMV from semen samples *in vitro* (Figure 5.2). A median percentage of 1.6 (range 0-101)% of CMV remained after washing when using the ProInsert™ tube, in comparison to 15.2 (range 0.6-61)% when density gradient centrifugation was used. Interestingly, no infectious virus was detected after washing with the ProInsert™ method.

The average amount of CMV remaining after washing with the ProInsert™ method was only ~60,000 copies/ml lower than when density gradient centrifugation was used, but infectious virus was still detected with the latter method, but not the modified method. It could be argued that the lack of plaques observed could be due to detection limits of the plaque assay.

However, some of the values of CMV remaining after washing with the ProInsert™ method were higher than those found after conventional washing, within the same repeat, but plaques were not observed, suggesting this finding is not due to the detection limits of the plaque assay.

The presence of DNA without corresponding plaques could be due to DNA contamination of the ProInsert™ samples, or ineffective DNA extraction, some limitations of the techniques used. Alternatively, it could be due to the presence of exogenous viral DNA in the preparation of virus used, a limitation discussed in Chapter 4. Exogenous DNA is known to bind directly to sperm (Zani *et al*., 1995), and could therefore be present in the sperm pellet, contributing to the presence of viral DNA after washing, as detected by qPCR. If only viral DNA were present in the pellet, this would explain the lack of plaques observed. It is possible that the ProInsert™ tubes are better at separating full virions from the sperm pellet than density gradient centrifugation but is not able to filter out exogenous DNA.

Despite these limitations, it is clear that the ProInsert™ method is more effective at removing CMV from semen samples. Importantly, viable sperm were present in the sperm pellet, which is important if this technique is to be used in fertility clinics. Sperm motility was higher after washing compared to the density gradient method, although less sperm are recovered (Table 5.2). It is important to note that some virus is still detected after washing, though this was not determined as infectious. Despite this, the presence of CMV after washing with this modified method provides evidence to suggest a small proportion of CMV is binding directly to sperm. As re-contamination of the pellet is ruled out with this method, the presence of CMV in the sperm pellet could be explained through a direct interaction.

The *in vitro* experiments outlined so far have shown that sperm washing is able to remove over 80% of CMV from semen samples. This is a promising finding when considering the role sperm washing might play in the removal of CMV *in vivo*, in the context of donor assisted conception.

To investigate this, semen samples were analysed for the presence of a natural CMV infection. Out of 79 ejaculates, 13 were found to be positive, resulting in a 16.4% prevalence of CMV. This rate of CMV presence in semen is slightly higher than what has previously been reported (reviewed in Kaspersen & Hollsberg, 2013), which could be due to the use of qPCR to detect viral load, rather than other techniques, which are known to be less sensitive than qPCR.

CMV DNA was found in the sperm pellet of only 2/13 (15.4%) positive samples after washing, with only 6.2% of the virus present before washing remaining. This was statistically significant, but directly conflicts with some values reported in the literature, which have found up to 92% of naturally infected samples remaining positive after washing (Michou *et al*., 2012; Naumenko *et al*., 2014). However, other studies have reported values similar to those found in this study, with only 15% (Witz *et al*., 1999) and 12.8% (Naumenko *et al*., 2011) of samples remaining positive after washing.

Evidence presented in this chapter suggests sperm washing by density gradient centrifugation is more efficient on *in vivo* infected samples compared to experimentally inoculated ones. These findings are encouraging for the potential use of sperm washing in fertility clinics as a way of better managing sperm donors with a CMV infection. As discussed in Chapter 3, sperm donors are currently screened by serum antibody testing, which gives no indication of the presence of CMV in their semen. Under these current testing conditions, the removal of CMV by sperm washing could not be utilised as a tool to improve practice, as the presence of CMV in the individual ejaculate is not tested for. The evidence that sperm donors intermittently shed CMV in their semen (Figure 5.5), suggests that it may be prudent to screen every ejaculate as such rapid changes in the shedding of virus, sometimes as short as 3 days, will not be detected by antibody testing.

The evidence that men intermittently shed CMV in their semen is an interesting observation that has been reported before (Kaspersen *et al*., 2012). This thesis has built upon these previous reports by showing short

bursts of reactivation and clearance of CMV from semen that was not reported in the Kaspersen *et al*., (2012) study. This has previously been reported for HSV in oral and anogenital swabs (Mark *et al*., 2008). Samples taken four times a day over a six day period observed approximately 44% of HSV reactivation events lasted ≤12 hours. Moreover, in 23% of cases, the reactivation event lasted ≤6 hours (Mark *et al*., 2008). This rapid clearance is thought to be due to the persistence of HSV specific $CD8^+$ T lymphocytes in genital skin after initial infection (Johnston *et al*., 2012; Zhu *et al*., 2007). One limitation of this finding is the lack of serological data to accompany the PCR data. It would have been ideal to know if the shedding pattern of CMV correlated with a donor's CMV serostatus, as this would have shown if serum antibody testing is a valuable tool in determining if a sperm donor is safe to use or not. However, this was not done, as when the study was designed, the concept of intermittent shedding was not known. The finding that men intermittently shed CMV in their semen brings into question how screening for CMV in sperm donors is currently being performed and will be discussed in more detail in Chapter 7.

The findings outlined in this chapter provide promising prospects for alleviating some of the problems with the screening and management of CMV positive sperm donors outlined in Chapter 3. However, the limitations of the techniques used in this chapter, such as inefficient DNA extraction and DNA contamination of samples, need to be acknowledged. The method employed for DNA extraction was not optimised for use in sperm. This could be problematic, as in comparison to somatic cells sperm have a more condensed nucleus. Due to this, it is possible not all of the sperm DNA present in a given sample was extracted. This is an inevitable problem when working with sperm (Silva *et al*., 2014; Yuan *et al*., 2015) but reasonable steps were taken to confirm that DNA was extracted by testing for the presence of GAPDH, and any negative samples were excluded from analysis to eliminate any false negative results. Also, no correlation was observed between the sperm concentration and viral load, showing that the amount of

sperm DNA present did not influence the amount of viral DNA recovered, eliminating the concern of inefficient DNA extraction.

DNA contamination was a continual problem throughout this study even though all obvious potential sources of contamination were eliminated. To control for this a negative H_2O control was included for each batch of samples extracted and if found to be positive, samples were repeated or discarded from analysis, ruling out the presence of false positives, where possible. Whilst the qPCR assay has limitations, it has been reported that the validated assay used is accurate to within one C_t (Atkinson, C., Personal Communication). Therefore, with the inclusion of the controls to rule out false positives and negatives, it is believed the values reported in this chapter are accurate and correct.

Similarly, the plaque assay is not without limitations, as has been discussed previously in Chapter 4. A smaller number of repeats for the plaque assay were reported in this chapter, in comparison to the qPCR assay, due to these limitations. Briefly, these include difficulties in determining the right dilution to use, problems with infection and inaccuracies in detecting the true amount of infectious virus present in a sample. Despite these problems, the inclusion of the plaque assay has presented an interesting and novel finding in that CMV remaining after washing is able to establish an infection. Also, it has shown that sperm are able to transmit CMV to permissive cells and therefore could potentially act as a vector for transmission of CMV *in vivo*. Given the findings that sperm washing is effective at removing CMV *in vivo*, the findings pertaining to the presence of infectious virus *in vitro* seem unimportant. However, this evidence provides support for the theory that CMV is able to bind to sperm, resulting in its continued presence in the sperm pellet.

In summary, the findings outlined in this chapter provide evidence to support the fact that sperm washing is generally effective at removing CMV from semen samples. It appears to be more effective when used on men experiencing a natural infection, as opposed to the *in vitro* conditions used in these experiments. Sperm washing may therefore provide a solution to the

problems experienced in relation to the management of CMV positive sperm donors in UK clinics. Interestingly, the evidence showing that CMV persists after sperm washing *in vitro*, even with a modified tube aimed to rule out contamination, and that virus is able to establish an active infection provides evidence to support an interaction between CMV and sperm. The next chapter will focus on investigating this in more detail.

Chapter 6

Effects of *in vitro* co-incubation of CMV on human spermatozoon functional parameters

6.1 Introduction

The previous chapter in this thesis provided evidence to show that CMV might be interacting directly with sperm as some virus remained after washing by density gradient centrifugation. This suggests a direct interaction between CMV and sperm might be occuring, as has been shown previously for *C. trachomatis* (Al-Mously *et al*., 2009) and HIV (Politch *et al*., 2004). To investigate this potential interaction further, this chapter will examine if exposure to CMV *in vitro* has any effect on sperm function.

Many studies have examined if CMV has an effect on male fertility by investigating sperm parameters in men with or without a CMV infection (Bezold *et al*., 2001; Bezold *et al*., 2007; Chen *et al*., 2013; Eggert-Kruse *et al*., 2009; Kapranos *et al*., 2003; Michou *et al*., 2012; Naumenko *et al*., 2011; Neofytou *et al*., 2009), which provide an insight into how an infection with CMV could affect male fertility. However, *in vitro* studies are able to provide a better understanding of the direct mechanism for how CMV might affect sperm directly by eliminating other factors that could damage sperm, such as the immune system. This has been studied for other pathogens, such as *C. trachomatis*, HIV and HPV and has shown many potential mechanisms by which sperm function can be affected by exposure to infectious agents.

C. trachomatis is known to induce apoptosis in sperm (Eley *et al*., 2005a) and increases tyrosine phosphorylation (Hosseinzadeh *et al*., 2000). This provides indirect evidence for a receptor-mediated interaction (Eley *et al*., 2005b), which could cause alterations in cell signalling pathways. As of yet, a receptor responsible for the interaction between *C. trachomatis* and sperm has not been found. However receptors have been identified for HIV (Cardona-Maya *et al*., 2011) and HPV (Foresta *et al*., 2010), which explains how these pathogens are able to affect sperm motility (Dondero *et al*., 1996; Foresta *et al*., 2011) through binding directly to spermatozoa.

Some *in vitro* studies have been conducted for examining the relationship between CMV and sperm. A recent study observed a pattern of staining for a CMV protein along the equatorial segment of the sperm (Naumenko *et al*.,

2014), possibly suggesting a direct interaction, however, this was only observed in ~2% of sperm. The authors of this paper claim to have shown "successful infection of spermatozoa *in vitro*" and demonstration of intracellular localisation of CMV. However, they provide no explanation of why they believe the localisation of the CMV proteins to be intracellular, rather than only being present on the outer surface of the sperm head. Intracellular localisation of CMV in spermatogonia has been shown previously in an *in vitro* model (Naumenko *et al*., 2011), resulting in a reduction in the number of immature germ cells. If this was occurring *in vivo*, this alteration in immature germ cells might lead to a reduction in sperm concentration, as has been documented in CMV positive men (Naumenko *et al.*, 2014).

These recent studies provide evidence to suggest that CMV could be interacting directly with sperm, possibly through receptor-mediated binding. However, what these studies lack is a direct investigation of if an infection with CMV affects sperm function parameters, such as motility or viability. They also lack any deeper understanding of the molecular basis for any effects the virus might be having on the function of the sperm. By gaining an understanding of the exact mechanism by which CMV and other pathogens are able to affect sperm function, as has been demonstrated with *C. trachomatis*, it will allow for a greater understanding of how CMV infection can contribute to male infertility.

6.2 Experimental rationale

To begin investigating if infection with CMV has an effect on sperm function, an *in vitro* approach was used to examine the effects on certain sperm function parameters, such as motility, viability and the acrosome reaction. Other parameters that might affect sperm function, such as levels of DNA damage and tyrosine phosphorylation were also assessed. By co-incubating laboratory grown CMV with sperm *in vitro*, this controls for the presence of other organisms present in an ejaculate, which might also have an effect on sperm. Similarly, the use of ejaculated sperm, purified from all seminal

components will control for any *in vivo* biological processes/factors, which might have an adverse effect on the spermatozoon. These erroneous factors might contribute to inconsistencies observed in *in vivo* correlation studies. Elimination of these factors allows for direct investigation of whether CMV is capable of affecting the function of the spermatozoon.

In order for sperm to successfully deliver the paternal genome to the oocyte, they need to be motile in order to reach the egg (Suarez and Pacey, 2006), be able to penetrate the zona pellucida, which requires an intact acrosome (Yoshinaga & Toshimori, 2003) and be able to hyperactivate (Stauss *et al*., 1995). Investigating whether exposure to CMV has an effect on these functional parameters will directly assess the fertilisation potential of the sperm. Other molecular aspects of sperm function have also been linked to a reduction in fertilisation potential, such as the levels of tyrosine phosphorylation and DNA damage. Phosphorylation of tyrosine residues on the spermatozoon is an essential part of sperm capacitation (Viscont *et al*., 1995), hyperactivation (Nassar *et al*., 1999) and the acrosome reaction (Sebkova *et al*., 2012). The precise activation of these mechanisms at the most optimal point in the female reproductive tract is necessary for successful fertilisation. Therefore, premature activation of these events might lead to a reduction in fertilisation potential. To investigate this, the levels of tyrosine phosphorylation and hyperactivation will be assessed. Similarly, sperm DNA damage has been linked to an increase in pregnancy loss due to the role the sperm genome plays in early embryogenesis (Robinson *et al*., 2012). DNA damage that occurs in regions of the sperm genome accessible to enzyme modification (i.e regions bound by histones, not protamines) (Figure 6.1) is thought to have an impact on the success of fertilisation and embryonic development as these areas are thought to be most active during the first hours of fertilisation (Hammand *et al*., 2009; Ward *et al*., 1999). The TUNEL assay is able to measure DNA damage in these areas of the sperm genome and will be used in this chapter to assess if exposure to CMV compromises the integrity of sperm DNA.

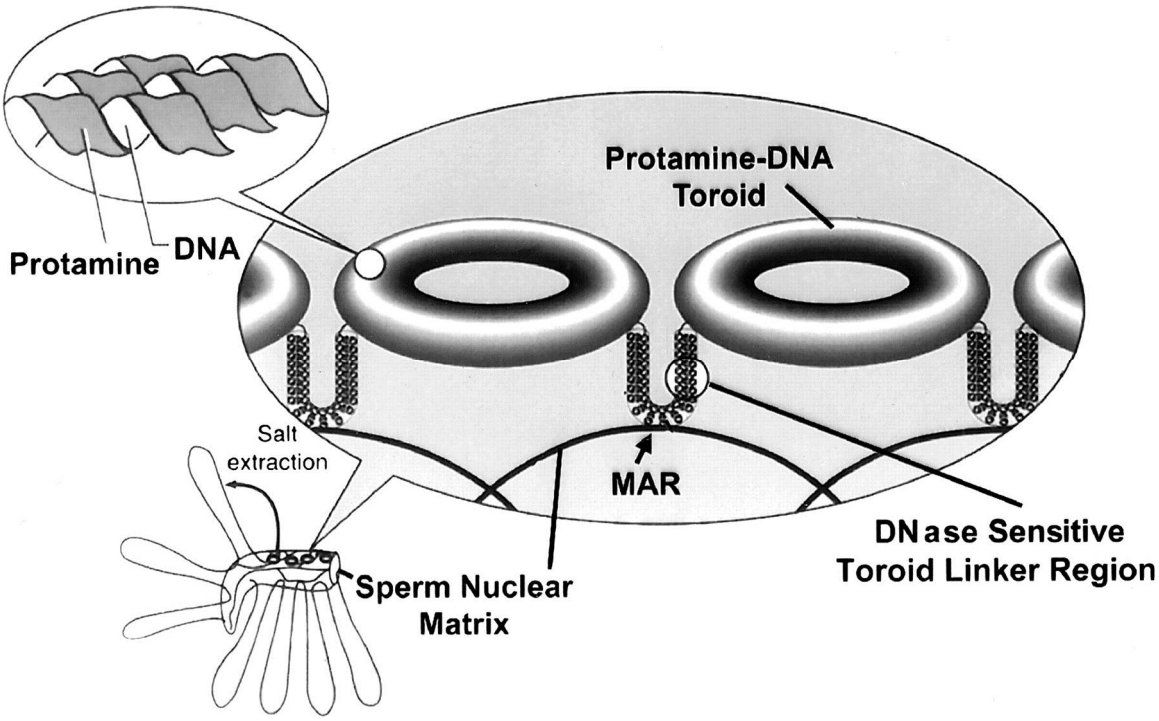

Figure 6.1: Donut-loop model for sperm chromatin structure. During spermiogenesis, histones bound to DNA are replaced by protamines, which fold the DNA into tightly packaged toroids. The donut model proposes that a toroid linker region, which is composed of chromatin that is more sensitive to DNA damage, connects each protamine toroid (Sotolongo *et al*., 2003). These regions are thought to house the remaining histones left over after modification, located at the MAR (matrix attachment regions). It is in these regions that is thought most of the sperm DNA damage occurs (Shaman and Ward, 2006). The TUNEL assay works by detecting DNA damage present in the toroid linker regions. Unlike other DNA damage assays, such as the Sperm Chromatin Structure Assay (SCSA), or the COMET assay, the TUNEL assay has no salt extraction step, which would remove the protamines. *Reprinted from Sotolongo et al., (2003) with permission from the Society for the Study of Reproduction.*

The aim of this chapter is to determine the fertilisation potential of sperm after exposure to CMV by assessing sperm parameters associated with reproductive success. By measuring the viability, motility, acrosome status, levels of DNA damage and tyrosine phosphorylation, this will be indirectly measuring if sperm exposed to CMV are capable of reaching, binding and fertilising the oocyte, as well as possessing the genomic integrity to produce a viable zygote. Any alteration in these parameters would provide evidence for the role *in vivo* CMV infection might play in male infertility and would provide evidence to support a direct interaction between CMV and sperm.

6.2.1 Specific aim(s):

1. Determine if co-incubation of sperm with CMV affects sperm motility, viability and acrosome status over a 6-hour time period.
2. Determine if the dose of CMV has an incremental effect on basic sperm function parameters, measured *in vitro*.
3. Investigate if there are different effects on sperm function between laboratory and wild-type strains of CMV.
4. Investigate the effects of CMV exposure on molecular sperm responses, such as DNA damage and tyrosine phosphorylation over a 6-hour time period.

6.3 Materials and Methods

To achieve the objectives outlined above, washed sperm were co-incubated with different strains of CMV at different doses, over a six-hour time period. Different parameters of sperm function were assessed in order to determine if exposure to CMV was having an effect on sperm function. Sperm motility, viability and acrosome status were assessed in the first part of this chapter. Following this, molecular markers of sperm function were assessed, in the form of DNA damage and tyrosine phosphorylation. Finally, sperm kinematics were investigated to analyse if exposure to CMV had any subtle effects on sperm movement.

For all of the experiments outlined in this section, semen samples were assessed prior to use for motility and the sperm concentration of each sample was measured, as outlined in Section 2.5.1. Samples were washed by density gradient centrifugation, as outlined in Section 2.5.3. All samples were coded to prevent observer-introduced bias of the results obtained.

After the initial set up of each experiment all samples were incubated at 37°C at 5% CO_2 for 6 hours. For time course experiments (Sections 6.3.1.1, 6.3.1.3, 6.3.1.5, 6.3.2 and 6.3.3), aliquots were removed for analysis at 0, 1, 3 and 6 hours. However in the dose response experiment (6.3.1.4), aliquots were only removed at 6 hours. The aliquots removed for analysis were: 10µl to assess sperm motility (see Section 2.5.1); 10µl added to 100µl of HOST media for assessment of sperm viability and acrosome status (to reach a final sperm concentration of ~$1x10^6$/ml) (Section 2.5.4); 35µl diluted in 65µl H_2O (to reach a final sperm concentration of ~$5x10^6$/ml) for the TUNEL assay (Section 2.5.5); and 10µl diluted in 90µl H_2O (to reach a final sperm concentration of $1.18x10^6$/ml) for tyrosine phosphorylation assessment (Section 2.5.6). Statistical differences were examined using the one-way ANOVA test on log-transformed data using the Tukey's test for multiple corrections.

6.3.1 Effects of co-incubation of different strains of CMV on sperm motility, viability and acrosome reaction

6.3.1.1 CMV (AD169) time course co-incubation

Washed sperm from six healthy donors with an initial sperm concentration of >$60x10^6$/ml were used to prepare 3 x 300µl aliquots of highly motile sperm with a concentration of ~$18x10^6$/ml and a percent motility >60%. To each aliquot, either 100µl of virus-infected supernatant, mock-infected supernatant or *E. coli* lipopolysaccharide (LPS) (026:B6) were added, giving a final sperm concentration of ~$13.5x10^6$/ml and a total sperm count per incubation of ~$5.4x10^6$. Virus-infected supernatant was added at a viral load of $5.4x10^7$copies/ml, giving a final number of viral particles of $5.4x10^6$, and a 1:1 ratio of viral particles to sperm. Mock-infected supernatant was added as a

negative control to control for erroneous factors in the growth conditions of the virus, such as cell debris and exogenous DNA. This was added at the same concentration of virus-infected supernatant (100µl, undiluted). 100µl of *E.coli* LPS was used as a positive control, as this has been shown to effect sperm function parameters at a final concentration of 50µg/ml (Hosseinzadeh *et al*., 2003).

6.3.1.2 LPS control test

Washed sperm from six healthy donors with an initial sperm concentration of >60x10^6/ml were used to prepare 4 x 100µl aliquots of highly motile sperm with a concentration of ~10x10^6/ml and a percent motility of >60%. To each aliquot, 20µl of *E.coli* LPS (055:B5) (Sigma-Aldrich, Dorset, UK), at a final concentration of 150µg/ml, 100µg/ml or 50µg/ml was added, along with 20µl of H_2O as a negative control. The samples were incubated at 37°C in 5% CO_2 for 6 hours and sperm motility was assessed at the end of the incubation period.

6.3.1.3 CMV Towne VS CMV AD169 time course co-Incubation

Sperm from six healthy donors with an initial sperm concentration of >60x10^6/ml were used to prepare 3 x 300µl aliquots of highly motile sperm with a concentration of ~18x10^6/ml and a percent motility of >60%. To each aliquot, either 100µl of CMV (AD169)-infected supernatant (AD169), purified CMV (Towne), or serum-free EMEM (negative control) were added, giving a final sperm concentration of ~13.5x10^6/ml and a total sperm count per incubation of ~5.4x10^6. CMV (AD169)-infected supernatant was added at a viral load of 5.4x10^7copies/ml, giving a final number of viral particles of 5.4x10^6, and a 1:1 ratio of viral particles to sperm. CMV (Towne) was supplied at a titre of 3x10^6 PFU/ml. As the total viral load was not supplied, the viral load in copies/ml was matched with CMV (AD169)-infected supernatant by matching the PFU/ml. Therefore, since 100µl of CMV (AD169) contains 4300 PFU/ml, the same amount of CMV (Towne) was added by diluting 14.5µl of CMV Towne stock in 985.5µl serum-free EMEM, to give a final concentration of 4.3x10^4PFU/ml. By matching the virus

preparations based on the PFU titre, a measure of infectious virus, the total viral load should be approximately the same. Whilst not exact, due to the probability of circulating exogenous DNA in an unpurified preparation of virus, the number of live virus particles should be very similar.

6.3.1.4 Dose-response co-incubation

Washed sperm from six healthy donors with an initial sperm concentration of >60x10^6/ml were used to prepare 13 x 100µl aliquots of sperm with a concentration of ~20x10^6/ml and a percent motility >60%. To each aliquot different concentrations of either 100µl of CMV (AD169)-infected supernatant, mock-infected supernatant, purified CMV (Towne) or serum-free EMEM (negative control), were added. A two-fold dilution series of both CMV (AD169)-infected supernatant and purified (Towne) was performed to achieve final viral loads of 2.0x10^7, 1.0x10^7, 0.5x10^7 and 0.25x10^7copies/ml, giving final virus to sperm ratios of 2:1, 1:1, 0.5:1 and 0.25:1. A two-fold dilution series of mock-infected supernatant was also performed to control for any erroneous factors in the growth conditions of the virus, such as cellular debris and exogenous DNA. The final sperm concentration in each experimental incubation was ~10x10^6/ml with a total sperm count per incubation of ~2.0x10^6.

6.3.1.5 Wild-type time course co-incubation

In this experiment, different strains of purified CMV were used, all supplied by Dr Matthew Reeves (UCL, London). CMV (Towne) and TB40E were used as laboratory strains and Merlin as a wild-type strain of CMV. Sperm from six healthy donors with an initial sperm concentration of >60x10^6/ml were used to prepare 4 x 300µl aliquots of highly motile sperm with a concentration of ~18x10^6/ml and a percent motility >60%. To each aliquot, either 100µl of CMV (various strains) or serum-free EMEM (negative control) were added, giving a final sperm concentration of ~13.5x10^6/ml and a total sperm count per incubation of ~5.4x10^6. Once again, the virus preparations were not supplied with a total viral load value (in copies/ml). Therefore, the concentration was matched using the value of 4300 PFU/ml to achieve a final

total virus to sperm ratio of 1:1, as outlined in Section 6.3.1.3. To each aliquot of sperm, 100µl of each virus preparation, containing 4300 PFU/ml was added (which is estimated to be approximately $5.4x10^6$ total virus in copies/ml).

6.3.2 Effects of co-incubation of different strains of CMV on molecular markers of sperm function

6.3.2.1 Analysis of DNA damage levels

In the wild-type time course co-incubation experiment detailed in Section 6.3.1.5, at each time point, 35µl was taken to perform the TUNEL assay (as outlined in Section 2.5.5).

6.3.2.2 Tyrosine phosphorylation time course

Sperm from six healthy donors with an initial sperm concentration of $>66x10^6$/ml were used to prepare 5 x 200µl aliquots of highly motile sperm with a concentration of $\sim16x10^6$/ml and a percent motility >60%. To each aliquot, either 100µl of CMV (AD169)-infected supernatant, purified CMV (Towne), purified wild-type CMV (Merlin), serum-free EMEM (negative control), or *E. coli* LPS (R515) (Enzo, Exeter, UK) were added, giving a final sperm concentration of $\sim11.6x10^6$/ml and a total sperm count per incubation of $\sim3.6x10^6$. All strains of CMV were diluted prior to addition to the reaction to reach a concentration of $3.6x10^7$copies/ml, giving a final number of viral particles of $3.6x10^6$ and a 1:1 ratio of viral particles to sperm. As in Section 6.3.1.3, the viral load of CMV strains Towne and Merlin were estimated based on the PFU/ml value provided with the viral preparations. LPS (R515) was added at a final concentration of 50mg/ml+2%FCS.

6.3.3 Effects of co-incubation of different strains of CMV on sperm kinematics

In the tyrosine phosphorylation time course experiment outlined in Section 6.3.2.2, at each time point 10µl of each incubation was taken to assess

sperm motility and kinematics on a CASA machine, as outlined in Section 2.5.2.

6.4 Results

6.4.1 Co-incubation with CMV has no significant effect on sperm parameters *in vitro*

6.4.1.1 CMV (AD169) time course co-incubation

The results from this initial co-incubation indicated that at a 1:1 ratio of virus particles to sperm, there was a significant effect on sperm motility at 3 hours (Figure 6.2a), in comparison to the mock-infected supernatant control ($P<0.05$), but this statistical difference was not seen at 6 hours post-incubation. However, there was no significant effect on sperm viability (Figure 6.2b) or the percentage of acrosome reacted sperm (Figure 6.2c) with the preparation of virus-infected supernatant, even after 6 hours.

Further analysis of the individual motility categories (Figure 6.3) shows that over the 6-hour co-incubation the percentage of category a spermatozoa (i.e those swimming at >25μm/second) decreases. There is a significant difference between virus-exposed sperm and both the mock infected sperm and LPS control at 1 ($P<0.001$ and $P<0.01$, respectively) and 3 hours ($P<0.01$ and $P<0.001$, respectively). However, this significance was not observed after 6-hours co-incubation with the only significant difference remaining between the mock-infected control and LPS control ($P<0.05$) (Figure 6.3a). Similarly for category b sperm (those swimming between 25-5μm/second), there is a slight increase over the 6-hour co-incubation period. There are significant differences between sperm co-incubated with virus and both mock-infected samples and sperm co-incubated with 50μg/ml LPS at 1-hour post incubation ($P<0.001$ and $P<0.01$, respectively), and a small significance of $P<0.05$ between mock-infected control and LPS control at 3 hours post-incubation. Once again, this significance was no longer seen at 6 hours post-incubation (Figure 6.3b). For category c sperm (those swimming at <5μm/second) there is a general increase over the 6-hour incubation

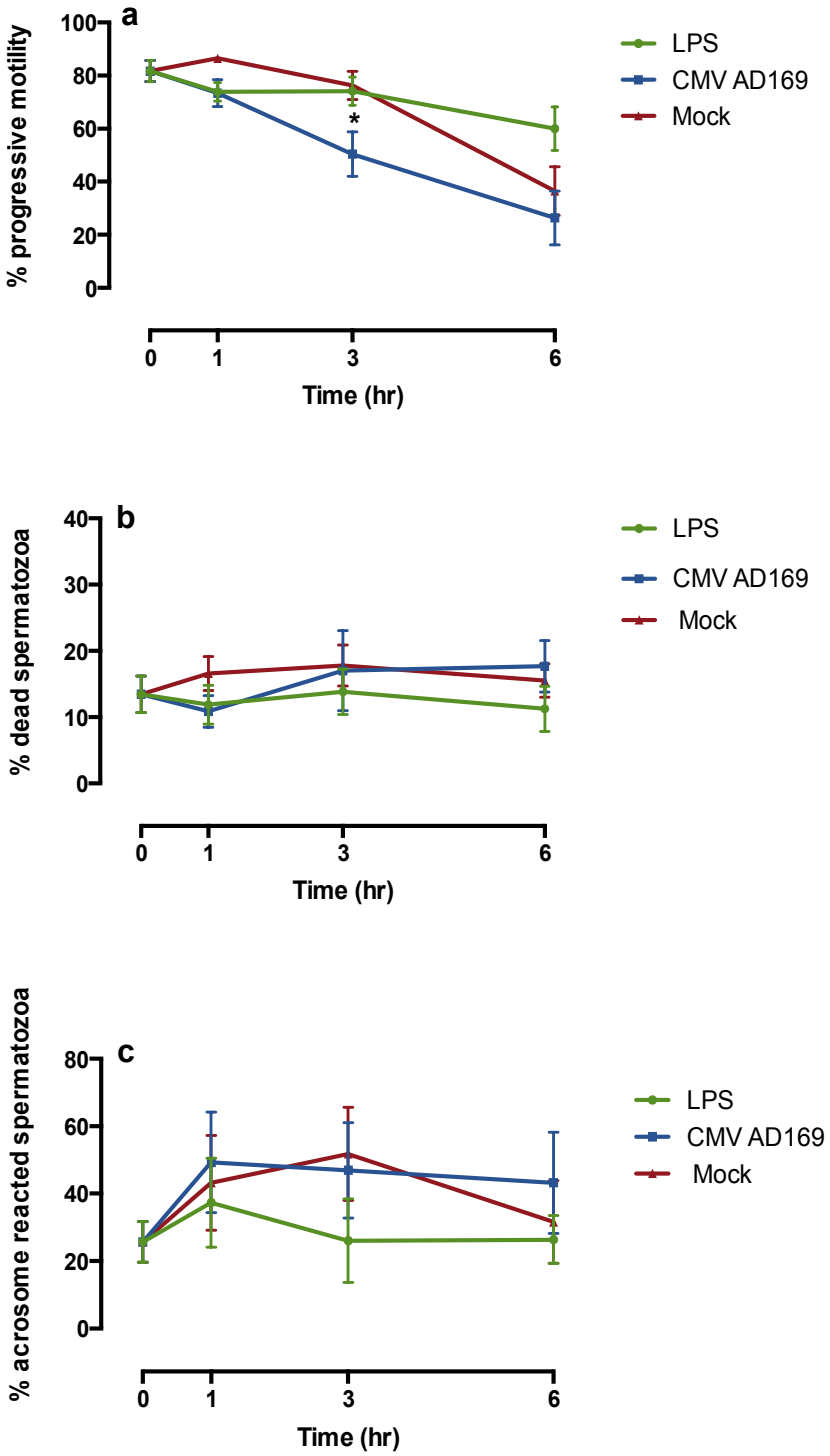

Figure 6.2: CMV (AD169) time course. Details of percentage: (a) sperm motility; (b) viability; and (c) acrosome-reacted sperm over a six-hour co-incubation period with $5.4x10^6$ CMV particles, mock-infected supernatant or 50µg/ml LPS positive control. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. The symbol (*) indicates a difference between the CMV experimental group and the mock-infected control at a significance level of $P<0.05$.

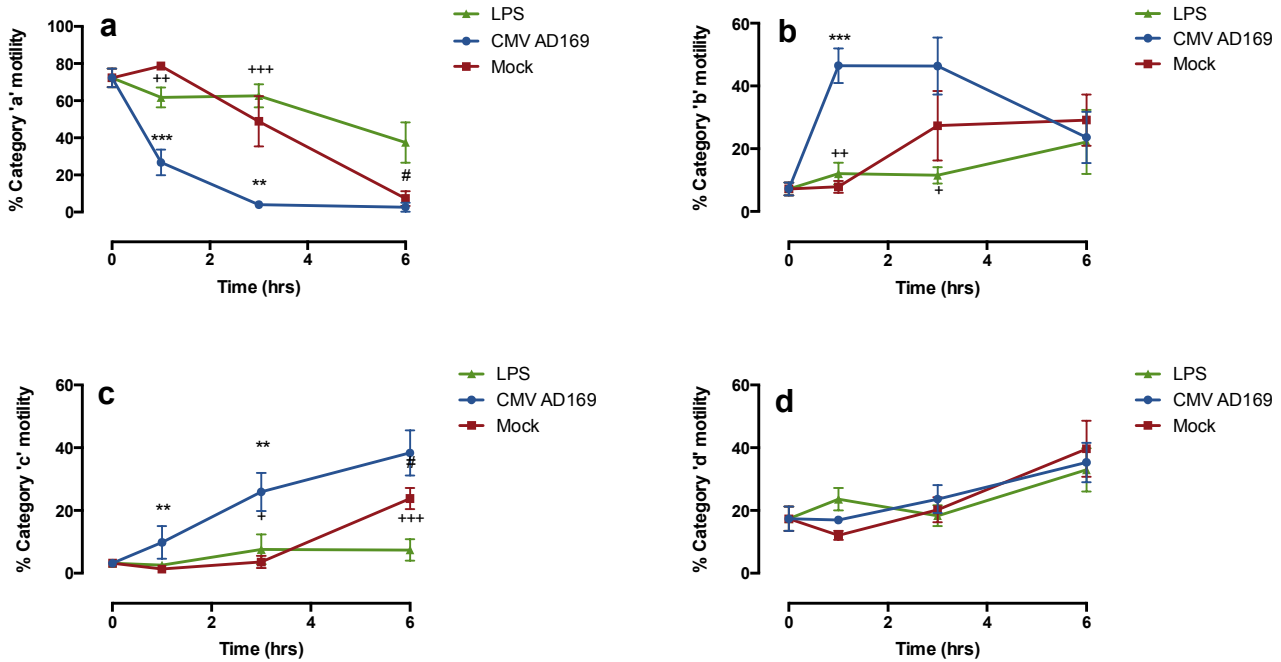

Figure 6.3: Details of individual motility categories over a six-hour co-incubation period with $5.4x10^6$ CMV particles, mock-infected supernatant or 50µg/ml LPS positive control. Figures a, b ,c , and d correspond to the four different motility categories, respectively. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. The symbol (*) indicates a difference between the CMV experimental group and the mock-infected control. The symbol (+) indicates a difference between the CMV experimental group and the LPS control. The symbol (#) indicates a difference between the mock-infected control and the LPS control. One symbol indicates a significance level of P = <0.05, two symbols a significance level of P<0.01, and three symbols a significance level of P<0.001.

period. There are significant differences between sperm co-incubated with virus and the mock-infected control at both 1 hour (P<0.01) and 3 hour (P<0.01) post-incubation, but this significance was not observed at 6 hours post-incubation. At 6 hours post incubation, there are significant differences between the virus-exposed sperm and LPS control (P<0.001) and LPS control and mock-infected control (P =0.05) (Figure 6.3c). Over the 6-hour incubation period, there was an increase of category d sperm (immotile), but there were no significant differences between any of the conditions (Figure 6.3d).

It is clear that the positive control of LPS was not effective in this set of experiments as there was only a moderate decline in motility over the 6-hour period (Figure 6.2a), despite much higher effects being observed at the same concentration by Hosseinzadeh *et al*., (2003). Similarly, there were no obvious effects of LPS on viability and the percentage of acrosome-intact sperm (Figure 6.2b,c). Clearly, in order to use LPS as a positive control, the LPS should be having a more noticeable effect on these sperm function parameters. After altering the dose of LPS used (Figure 6.4) from 50µg/ml to 150µg/ml, there was no significant effect on sperm motility, in comparison to a negative control. However the dose that seemed to have the most effect on decreasing the percentage of progressively motile sperm, and increasing the percentage of immotile sperm was 50µg/ml, the same dose as reported to be effective in the literature.

This set of experiments does not clearly identify if exposure to CMV has an effect on sperm function, as there is no consistent statistically significant difference between sperm co-incubated with virus and those incubated under control conditions. This could be due to the dose of the virus, the strain of the virus, or factors present in the viral supernatant, which are also present in the mock-infected supernatant, such as exogenous DNA. The subsequent experiments were designed to address these issues.

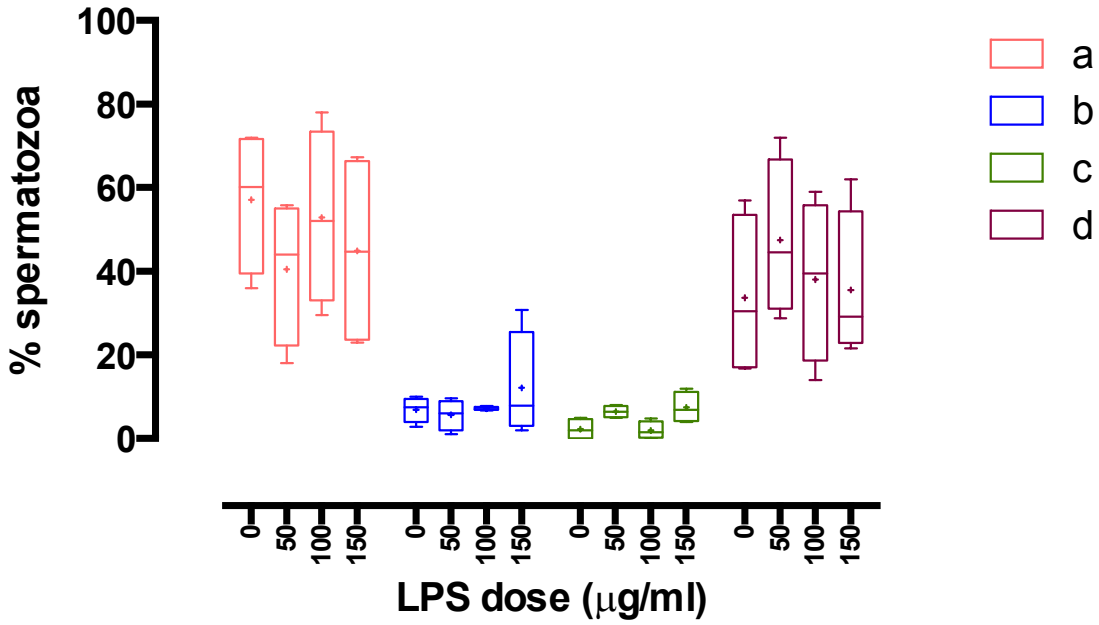

Figure 6.4: Details of individual motility categories of four healthy donors over a six -hour incubation period with an increasing dose of *E. coli* LPS (055:B5). A H_2O negative control was used and is shown at a value of zero. Box and whisper plots depict the interquartile range, 5th percentile and 95th percentile. The mean is shown as a '+'. Statistical significance was analysed using a one-way ANOVA test on log-transformed data with differences between each group examined using Tukey's multiple comparison test. No statistical differences were found.

6.4.1.2 CMV (AD169) VS TOWNE time course co-incubation

To determine if the lack of effect on sperm function parameters using CMV (AD169) is due to the lack of purification of the AD169 virus preparation, a time course was performed using a purified laboratory strain of CMV (Towne), supplied from Dr Matthew Reeves (UCL, London). Also, rather than a mock-infected supernatant control, a media-only (EMEM) control was used as this is a more suitable control for the purified virus.

The results are shown in Figure 6.5 and in summary there were no significant difference between sperm co-incubated with CMV Towne and a media-only control on sperm motility (Figure 6.5a), sperm viability (Figure 6.5b) or the percentage of acrosome reacted sperm (Figure 6.5c). Similarly, there were also no significant differences between CMV Towne and CMV AD169 in the percent of viable sperm or the proportion which had undergone the acrosome reaction. However there was a significant ($P<0.05$) reduction in sperm motility when exposed to AD169 in comparison to Towne and a media control after 6 hours incubation (Figure 6.5a).

Detailed analysis of the individual motility categories (Figure 6.6) shows a significant difference in the proportion of grade a sperm co-incubated with CMV AD169 and both CMV Towne and a media control after 6 hours ($P<0.05$) (Figure 6.6a). No significant differences were observed between the three conditions for either category b or d sperm (Figures 6.6b & 6.6d). However there was a significant difference ($P<0.01$) after 6 hours co-incubation between sperm incubated with CMV AD169 and CMV Towne (Figure 6.6c), for category c sperm.

This significant decrease in category a sperm and increase in category c sperm after co-incubation with AD169 is similar to what is observed in Figure 6.2c and suggests that whilst CMV AD169 is not causing an increase in the percentage of immotile sperm, it is reducing the motility of the sperm. As this is not observed with the purified strain of CMV (Towne), this difference could be explained by the presence of external factors, such as exogenous DNA,

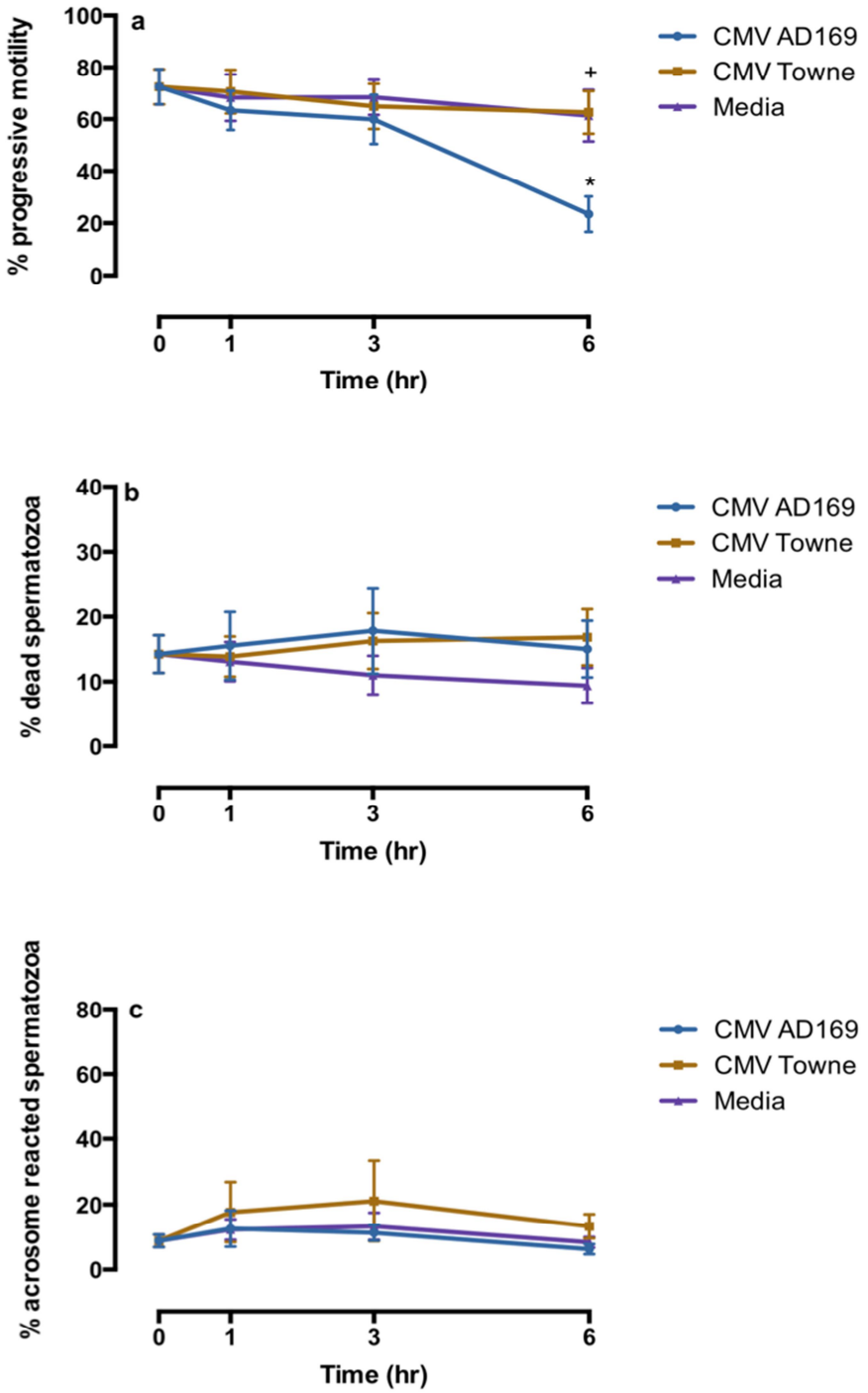

Figure 6.5: CMV (AD169) vs CMV (Towne) co-incubation. Details of percentage: (a) sperm motility; (b) viability; and (c) acrosome-reacted sperm over a six-hour co-incubation period with 5.4×10^6 CMV AD169 and Towne particles or a serum-free EMEM negative control. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. The symbol (*) indicates a difference between the CMV AD169 group and CMV Towne group. The symbol (+) indicates a difference between the CMV AD169 group and the media control, at a significance level of $P<0.05$.

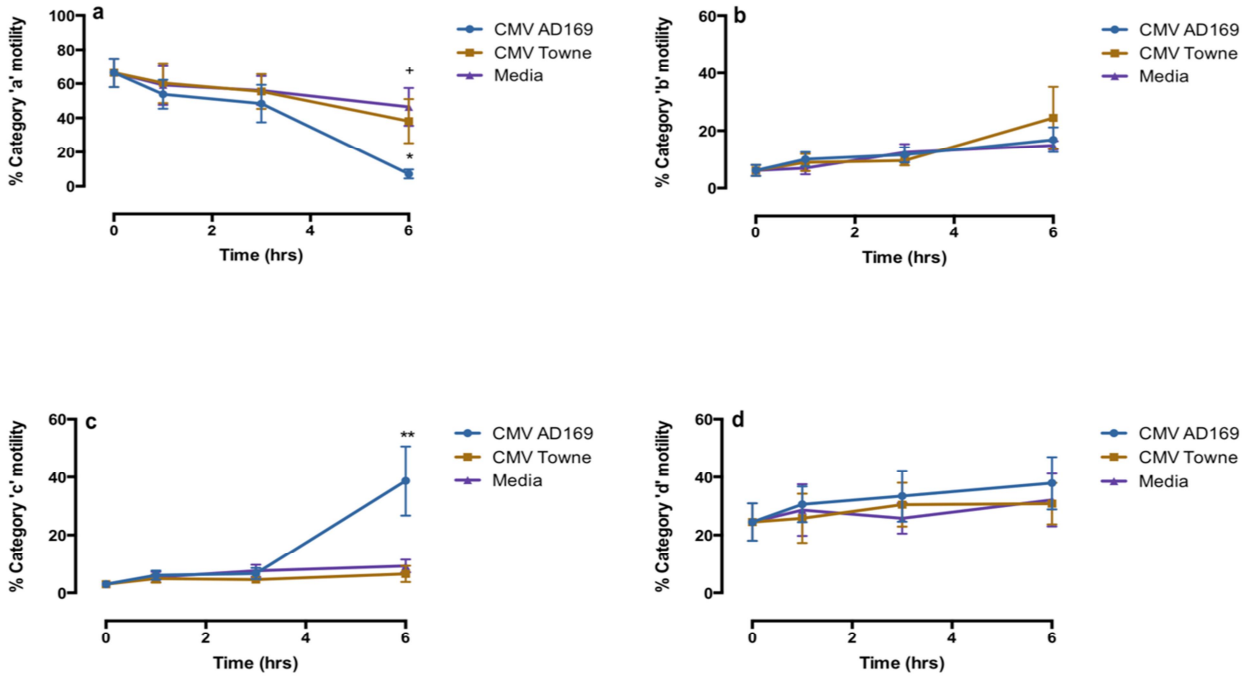

Figure 6.6: Details of individual motility categories over a six-hour co-incubation period with $5.4x10^6$ CMV AD169 or Towne particles, or a serum-free EMEM negative control. Figures a, b ,c , and d correspond to the four different motility categories, respectively. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. The symbol (*) indicates a difference between the CMV AD169 group and CMV Towne group. The symbol (+) indicates a difference between the CMV AD169 group and the media control. One symbol indicates a significance level of $P<0.05$ and two symbols a significance level of $P<0.01$

present in the un-purified AD169 preparation, which are absent in the purified Towne preparation.

6.4.1.3 CMV (AD169) & CMV Towne dose response co-incubation

To investigate if the lack of effects observed in the first two sets of experiments is due to the dose of virus used (a 1:1 ratio), a dose response was carried out. A range of viral concentrations from 2:1 virus particles to sperm down to 0.25:1 were carried out, with a media-only (EMEM) control.

In summary, there was no significant effect of co-incubation of CMV AD169 or Towne at any dose on sperm motility (Figure 6.7a), sperm viability (Figure 6.7b) or the percentage of acrosome reacted sperm (Figure 6.7c), in comparison to a mock-infected and negative control.

This set of experiments shows that the lack of effects observed with CMV AD169 and Towne at a ratio of 1 virus: 1 spermatozoon is not due to the dose of virus used. When the dose was increased 2-fold, no significant effects were observed.

6.4.1.4 Wild-Type CMV time course co-incubation

The strains of CMV used in the previous experiments (Section 6.4.1.1-6.4.1.3) were laboratory strains, characterised by the inability to infect the type of cells CMV would infect *in vivo*. This is caused by mutations in the genes responsible for epithelial/endothelial cell tropism. Given this, a further set of experiments were performed with a wild-type strain of CMV (Merlin). In addition, another high passage strain of CMV (TB40E) was incorporated to analyse inter-strain differences.

Briefly, no significant differences were observed in sperm motility (Figure 6.8a), sperm viability (Figure 6.8b) or the percentage of acrosome reacted sperm (Figure 6.8c), between any of the strains of CMV and a negative control over a 6-hour co-incubation period. A general trend of decline in motility was observed over the 6-hour period for all of the conditions, except

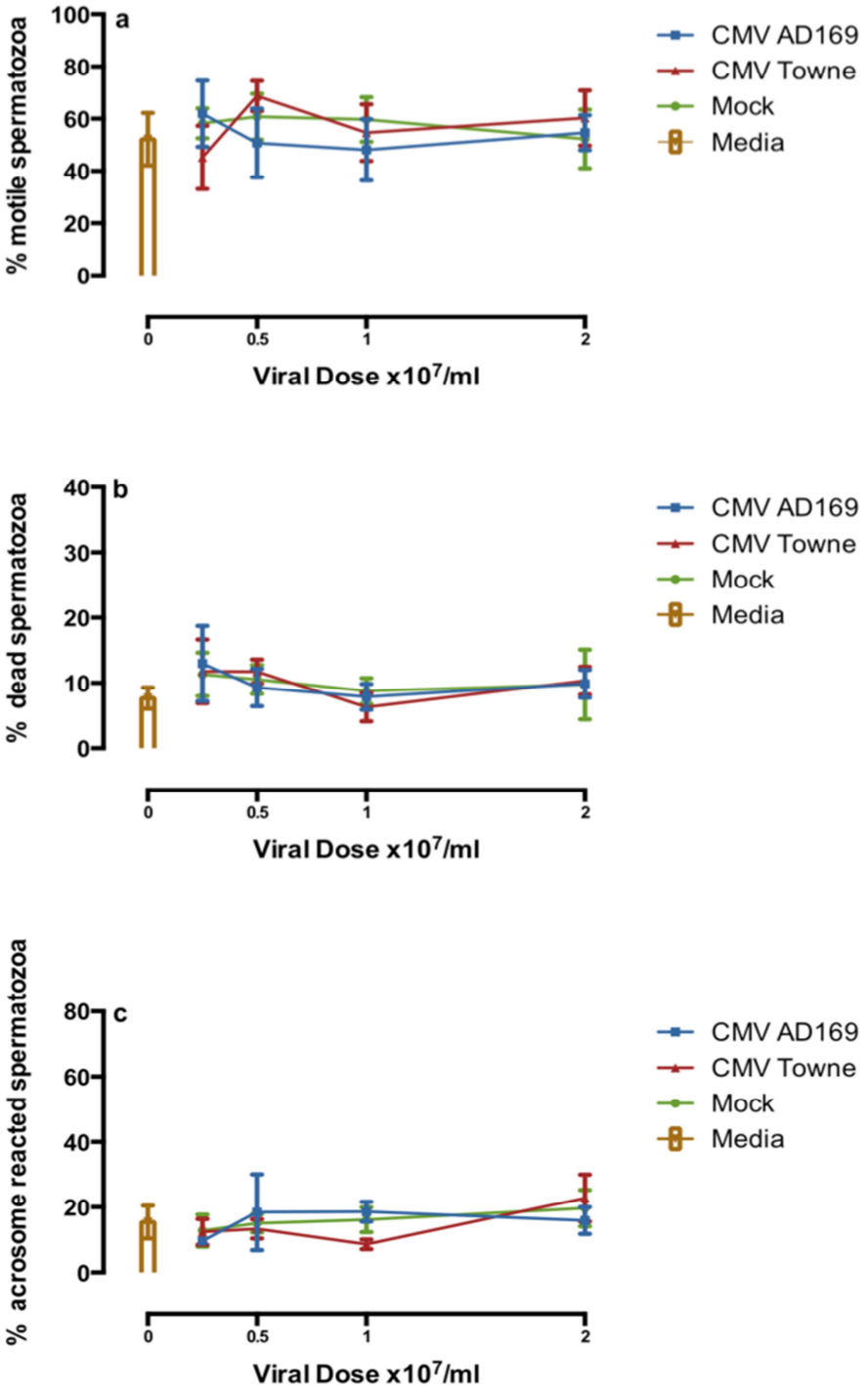

Figure 6.7: CMV dose response co-incubation. Details of percentage: (a) sperm motility; (b) viability; and (c) acrosome-reacted sperm after a six-hour co-incubation with $2.0x10^7$/ml, $1.0x10^7$/ml, $0.5x10^7$/ml, $0.25x10^7$/ml CMV AD169/Towne, or a serum-free EMEM negative control. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. No statistically significant differences were observed.

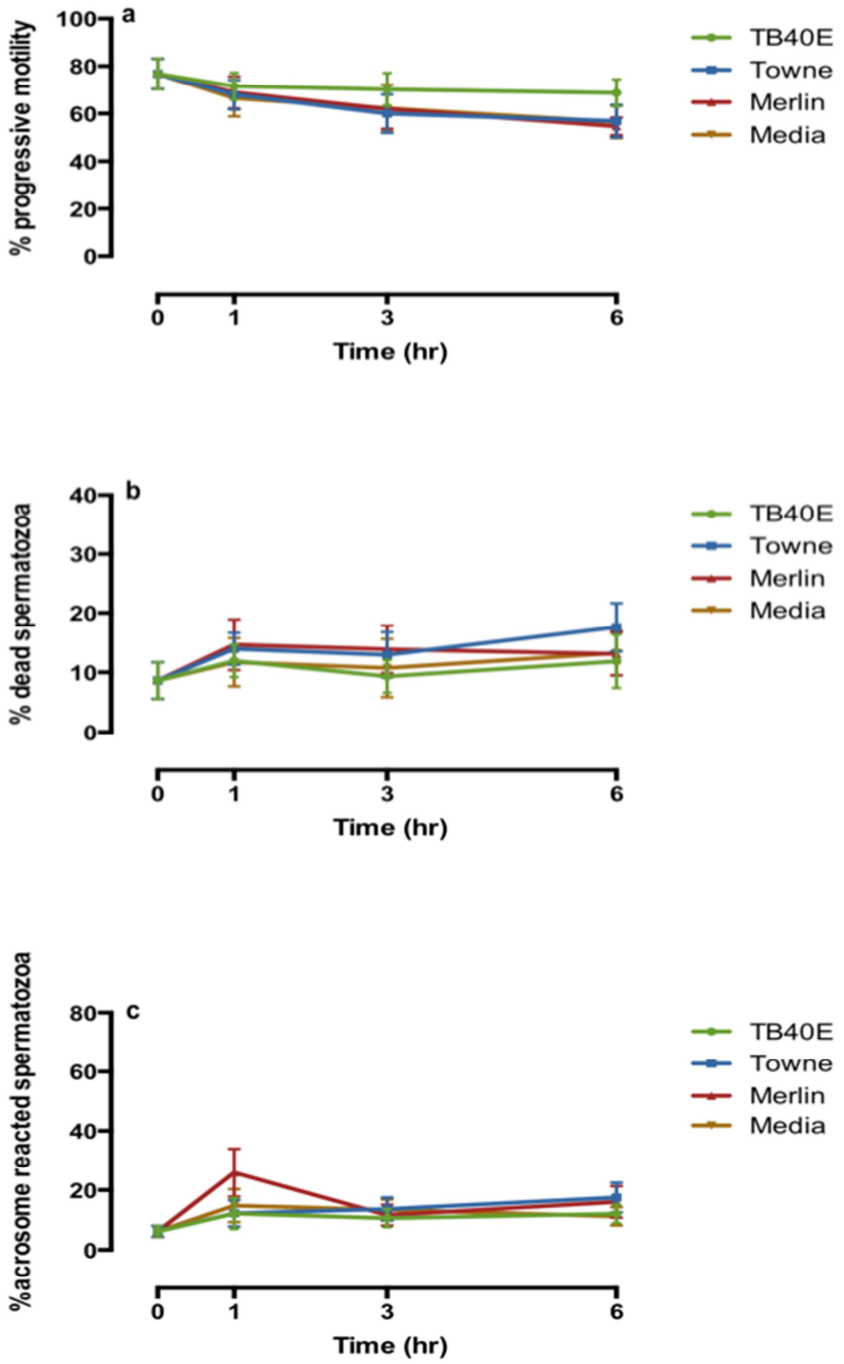

Figure 6.8: Wild-type CMV vs laboratory strain CMV co-incubation. Details of percentage: (a) sperm motility; (b) viability; and (c) acrosome-reacted sperm over a six-hour co-incubation period with $5.4x10^6$ Towne, Merlin, TB40/E particles or a serum-free EMEM negative control. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. No statistically significant differences were observed.

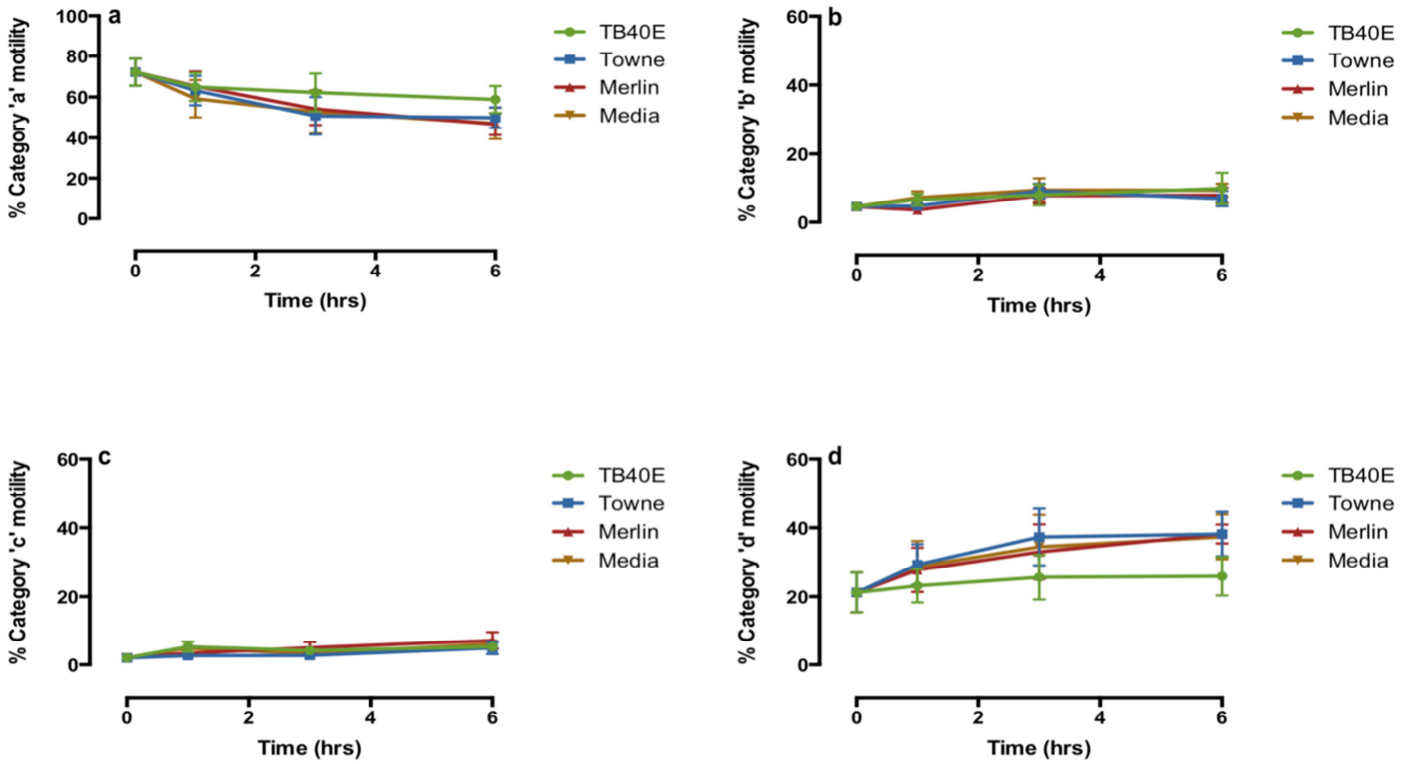

Figure 6.9: Details of individual motility categories over a six-hour co-incubation period with $5.4x10^6$ Towne, Merlin, TB40/E particles, or a serum-free EMEM negative control. Figures a, b ,c , and d correspond to the four different motility categories, respectively. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. No statistically significant differences were observed.

TB40E, but this was not statistically significant between the different strains of CMV used.

Similarly, analysis of the individual motility categories (Figure 6.9) revealed no significant differences between the virus strains and a media-only control for any of the sperm motility categories. There are slightly less category d (Figure 6.9d) spermatozoa in the TB40E infected samples, but this was not statistically significant.

From this set of experiments, it can be concluded that different strains of CMV, including a wild-type strain, have no effect on functional parameters of washed sperm at this concentration.

6.4.2 Co-incubation with CMV has no effect on molecular markers *in vitro*

As no obvious effects on sperm function, were observed in the experiments outlined in Section 6.4.1, further molecular aspects of sperm function were considered to investigate if these parameters were affected by exposure to CMV. In a similar experimental design, the levels of DNA damage and tyrosine phosphorylation were examined after exposure to various strains of CMV over a 6-hour co-incubation.

Briefly, over 6-hours, no changes were observed in the levels of sperm DNA damage (Figure 6.10a). There were no significant differences between any of the strains of virus used, or the negative control. Similarly, no significant differences were observed between the strains of CMV used and either control when analysing the levels of tyrosine phosphorylation (Figure 6.10b). The levels of tyrosine phosphorylation generally increased over the 6-hour incubation, as would be expected, but there was no difference between the different incubations. Whilst there was no significant difference between the positive control (LPS) and the experimental conditions, incubation with LPS did lead to the highest percentage of tyrosine-phosphorylated sperm after 6 hours incubation.

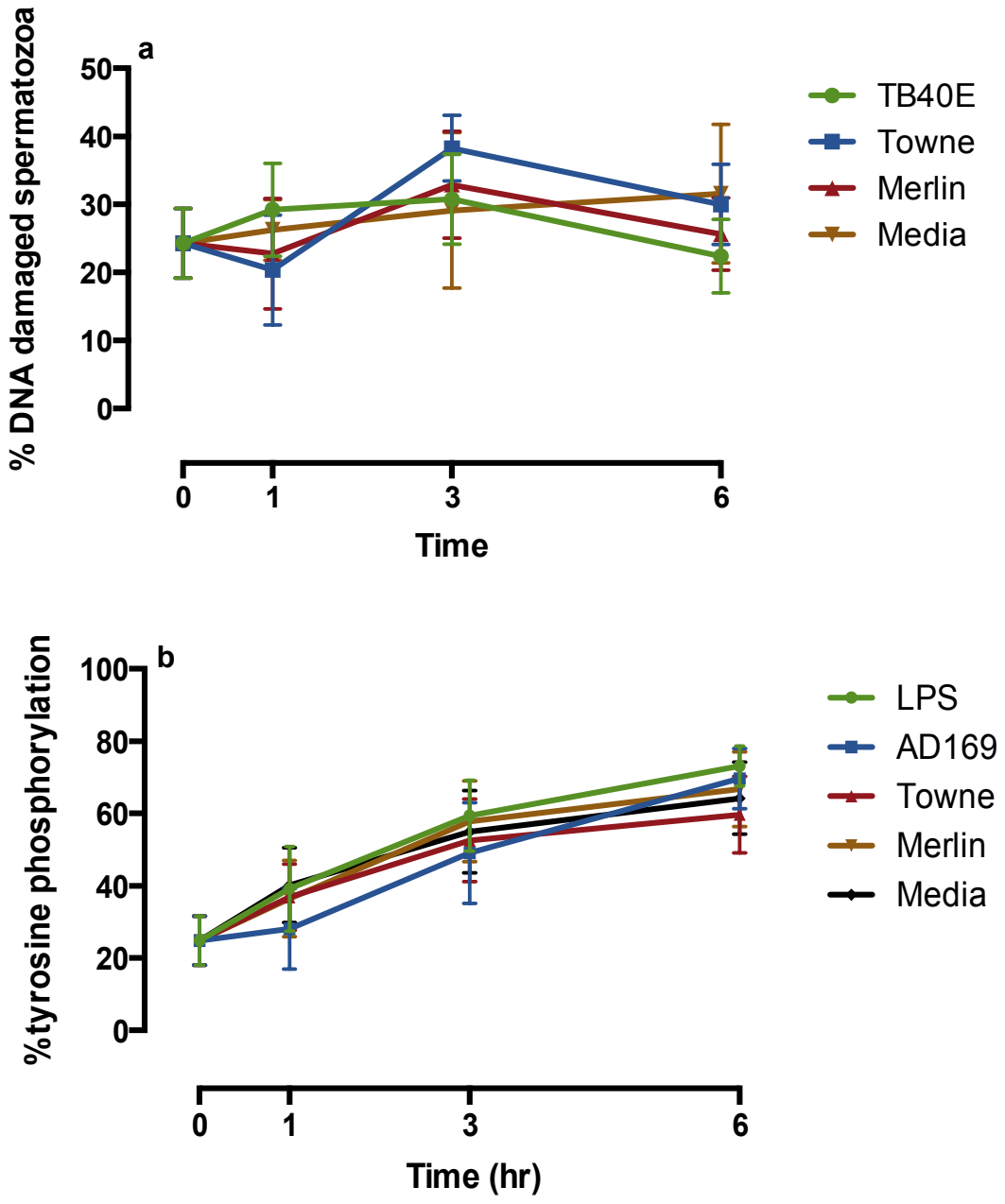

Figure 6.10: Details of percentage: (a) DNA damaged sperm; and (b) tyrosine phosphorylated sperm over a six-hour co-incubation period. Samples in (a) were incubated with $5.4x10^6$ Towne, Merlin, TB40/E particles or a serum-free EMEM negative control. Samples in (b) were incubated with $3.6x10^6$ AD169, Towne or Merlin particles or a serum-free EMEM negative control. *E. coli* LPS (R515) was added as a positive control at a final concentration of 50mg/ml + 2%FCS. Data shown are the mean ± SEM of incubations with sperm preparations from six healthy donors. Statistical significance was assessed using a one-way ANOVA test on log-transformed data with differences between groups examined using Tukey's multiple comparison test. No statistically significant differences were observed

From these two experiments, it can be concluded that exposure to CMV also has no effect on molecular aspects of sperm function at a dose of 1 virus particle to 1 sperm.

6.4.3 Co-incubation with CMV has no effect on sperm kinematics *in vitro*

Throughout the experiments outlined in Section 6.4.1, some changes in the proportion of sperm swimming in individual motility categories were observed, particularly with regard to the percentage of category a and category c sperm (Figures 6.3 & 6.6). To investigate these differences further, the motility of sperm exposed to CMV were analysed by a CASA machine. Briefly, no statistically significant differences were observed between any of the strains of CMV used and the two controls for the individual sperm motility categories (progressive, non-progressive or immotile). Similarly, when analysing the speed of sperm (rapid, medium or slow), no differences were observed (Table 6.1).

Upon analysis of the parameters of velocity, including the curvilinear velocity, average path velocity and straight-line velocity, no differences were observed (Table 6.2). Similarly, the progression ratios of sperm movement, the amplitude of lateral head movement (ALH) and beat cross frequency (BCF) showed no differences between the groups. Finally, the percentage of hyperactive sperm was not affected by co-incubation with CMV, or the positive control (Table 6.2).

In conclusion, co-incubation with various strains of CMV over 6 hours does not affect any aspects of sperm motility, progression or movement.

Table 6.1: Details of sperm motility parameters, measured by a CASA machine, over a 6-hour incubation with $3.6x10^6$ AD169, Towne, or Merlin particles or a serum-free EMEM negative control. *E. coli* LPS (R515) was included as a positive control at a final concentration of 50mg/ml+2% FCS. Data are shown as mean±SEM. Statistical significance was analysed using a one-way ANOVA test on log-transformed data.

Condition	Motility			Speed		
0hr	Progressive (P)	Non-Progressive (NP)	Immotile (IM)	Rapid	Medium	Slow
All	69.3±8.2	10.7±1.4	19.9±6.8	66.6±8.0	9.6±1.3	3.9±0.9
1hr	Progressive (P)	Non-Progressive (NP)	Immotile (IM)	Rapid	Medium	Slow
LPS	72.4±3.4	11.6±1.9	16.1±1.9	70.9±3.7	9.5±2.6	3.5±0.4
CMV AD169	71.5±1.4	10.0±0.7	18.5±1.6	57.6±12.6	7.7±0.5	3.4±0.4
CMV Towne	63.7±8.4	11.1±1.1	25.2±7.6	62.9±8.4	7.7±0.9	4.2±0.8
CMV Merlin	68.4±7.4	9.0±1.4	22.6±6.7	67.3±7.9	6.8±1.7	4.4±0.8
EMEM	75.0±1.8	9.6±1.6	15.4±1.6	73.7±1.8	7.3±1.8	3.6±0.6
3hr	Progressive (P)	Non-Progressive (NP)	Immotile (IM)	Rapid	Medium	Slow
LPS	76.1±3.7	9.3±1.4	14.6±3.0	75.0±4.2	7.0±1.1	3.3±0.7
CMV AD169	70.2±6.6	10.1±1.6	19.7±5.1	69.6±6.6	6.8±0.6	3.9±1.4
CMV Towne	75.1±2.2	8.9±1.0	16.0±1.9	74.4±2.2	6.3±0.7	3.2±0.5
CMV Merlin	66.0±7.4	11.4±1.5	22.7±6.1	64.4±7.4	9.6±1.6	4.9±1.3
EMEM	68.3±5.2	9.7±1.1	22.0±4.8	67.2±5.3	7.4±0.9	3.6±0.6
6hr	Progressive (P)	Non-Progressive (NP)	Immotile (IM)	Rapid	Medium	Slow
LPS	46.7±9.5	25.4±6.9	27.9±5.2	45.5±9.9	17.4±5.1	9.2±2.2
CMV AD169	57.1±6.8	12.3±1.4	31.8±6.8	56.6±6.8	8.0±1.3	4.8±0.4
CMV Towne	60.1±8.9	14.9±3.4	25.0±8.3	59.1±8.9	10.0±3.0	5.9±1.1
CMV Merlin	56.1±9.9	16.6±3.6	7.3±7.0	55.5±10.0	12.6±3.6	6.8±2.0
EMEM	61.1±10.2	13.6±2.0	23.1±9.4	60.1±10.3	10.6±1.3	4.1±1.3

Table 6.2: Details of sperm motility parameters, measured by a CASA machine, over a 6-hour incubation with $3.6x10^6$ AD169, Towne, or Merlin particles or a serum-free EMEM negative control. *E. coli* LPS (R515) was included as a positive control at a final concentration of 50mg/ml+2% FCS. Data are shown as mean±SEM. Statistical significance was analysed using a one-way ANOVA test on log-transformed data.

Condition	Velocity Parameters			Progression Ratios			Motion	Vigor	Hyperactivity
0hr	VCL µm/s	VSL µm/s	VAP µm/s	LIN %	STR %	WOB%	ALH µm	BCF Hz	%Hyperactive
All	61.2±5.2	23.4±4.5	34.7±4.1	36.6±3.6	65.2±4.3	55.6±1.9	2.3±0.1	9.9±1.0	7.7±3.7
1hr	VCL µm/s	VSL µm/s	VAP µm/s	LIN %	STR %	WOB%	ALH µm	BCF Hz	Hyperactive
LPS	67.6±7.9	23.3±5.7	37.5±5.3	33.4±4.4	55.0±9.0	55.2±2.2	1.9±0.3	10.0±2.1	5.1±4.1
CMV AD169	70.0±5.3	22.3±3.6	38.3±4.1	31.8±3.9	57.5±5.9	54.8±1.5	2.1±0.2	9.8±1.2	4.9±3.9
CMV Towne	65.5±4.7	25.2±6.4	38.3±4.8	36.9±6.6	62.2±7.5	57.7±3.2	2.2±0.2	10.9±1.5	6.1±4.7
CMV Merlin	66.6±3.4	23.5±2.7	37.1±1.8	35.7±3.8	57.4±5.0	56.1±1.8	2.3±0.2	10.7±0.9	7.0±2.9
EMEM	65.9±3.1	24.0±4.5	37.7±2.9	36.1±5.6	62.0±6.8	57.1±2.6	2.1±0.1	10.7±1.4	5.7±4.8
3hr	VCL µm/s	VSL µm/s	VAP µm/s	LIN %	STR %	WOB%	ALH µm	BCF Hz	Hyperactive
LPS	74.4±7.7	25.8±4.3	41.6±4.6	34.2±3.5	56.3±8.3	55.9±1.5	2.3±0.2	11.4±1.2	6.1±3.7
CMV AD169	76.6±6.8	26.3±3.3	42.7±3.5	34.4±3.3	61.0±4.5	56.0±1.2	2.4±0.2	11.1±0.8	5.8±3.3
CMV Towne	79.8±7.0	23.5±2.2	42.3±3.1	29.5±1.4	55.2±2.1	53.2±0.9	2.4±0.1	10.6±0.5	3.7±1.4
CMV Merlin	65.5±2.2	26.1±4.2	38.8±2.5	39.7±5.6	60.8±5.9	59.1±2.7	2.3±0.2	10.5±0.6	9.1±4.5
EMEM	71.4±5.4	26.3±4.4	40.7±3.4	36.0±4.1	57.5±6.4	56.8±1.4	2.3±0.2	9.5±1.7	6.6±3.8
6hr	VCL µm/s	VSL µm/s	VAP µm/s	LIN %	STR %	WOB%	ALH µm	BCF Hz	Hyperactive
LPS	54.1±10.3	18.7±5.4	30.2±6.4	31.2±4,2	52.7±6.7	54.2±2.4	1.9±0.4	7.2±1.8	3.4±2.8
CMV AD169	66.7±7.1	23.9±4.3	33.5±2.2	35.0±2.8	62.7±4.7	55.8±1.0	2.5±0.4	9.9±1.1	5.9±4.2
CMV Towne	65.6±7.4	20.8±2.8	35.9±3.8	31.6±1.7	57.3±2.4	55.0±1.0	2.2±0.1	10.1±0.7	2.4±1.0
CMV Merlin	57.8±5.5	17.8±2.7	30.8±3.5	30.0±2.6	52.2±6.4	51.0±2.3	2.1±0.3	7.8±1.2	3.6±1.2
EMEM	59.3±5.1	19.4±2.0	33.6±2.4	33.2±3.6	53.1±7.2	57.2±2.3	2.1±0.2	8.3±1.8	2.6±1.5

6.5 Discussion

The aim of this chapter was to investigate if exposure to CMV had any effect on sperm function parameters, such as motility and kinematics, viability, acrosome status and the levels of DNA damage and tyrosine phosphorylation. The results show that CMV does not have any effect on these parameters when co-incubated with ejaculated, washed sperm *in vitro*. Different doses of virus, lengths of incubation, and strains of CMV have been investigated, none of which exhibit any effect on sperm. Based on findings discussed in Chapter 5, which suggested that CMV might be interacting directly with sperm, due to it not being washed off by density gradient centrifugation, the primary objective of this chapter was to investigate if any interaction between CMV and sperm was potentially receptor mediated and could therefore affect sperm function. This was not observed but does not rule out a potential interaction between CMV and sperm, which does not trigger biological effects.

Sperm might encounter CMV in several places throughout the male reproductive tract, either pre-ejaculation due to exposure in the testis (Naumenko *et al*., 2011), during ejaculation through contaminated seminal components from the prostate gland (Rapp *et al*., 1975) or the seminal vesicle and vas deferens (Decucq and Jegou, 2001), and finally post-ejaculation exposure can occur in the female reproductive tract. The *in vitro* approach taken in this chapter to investigate the effects of exposure to CMV on sperm function emulate a post-ejaculation exposure scenario. Other factors that could affect sperm, such as the production of ROS due to an inflammatory response (Aitken *et al*., 1992) or the presence of other infections, would be occurring in the male reproductive tract in a pre/peri-ejaculation exposure scenario and therefore a direct investigation of how CMV itself is able to affect sperm function can not be performed. In order to determine if a direct interaction between CMV and sperm is occurring that might affect sperm function, these confounding factors had to be eliminated, so an *in vitro* approach was taken. Despite this, it was not possible to determine that donors used for these experiments were not infected with

other pathogens, including CMV. However, the use of multiple repeats in each experiment would help to mitigate this limitation.

Given that these experiments represent how sperm might be affected by exposure to CMV post-ejaculation, extrapolation of the findings of how CMV might affect sperm function pre-ejaculation and the consequences this has for male fertility is difficult to interpret. It is apparent that CMV does not have an effect on sperm that has been ejaculated and separated from seminal components. However, given the complex environment in which sperm are made and the natural immune response that occurs upon infection, the effect of an *in vivo* infection on sperm function cannot solely be determined from this set of experiments. However, the results from these experiments are able to provide an insight into the relationship between CMV and sperm and provide information for consideration when using CMV positive sperm donors.

An aspect of the experimental design throughout this chapter that needs to be considered is the amount of virus added to each co-incubation. Traditionally when studying viral infection of a cell, the number of plaque forming units' (PFU) would be used in order to maximise the amount of virus present that is able to establish an infection. However, this study is only concerned with binding events, and potential interactions, not an ability to establish an infection. Given this, the total viral load in copies/ml, was used as a measure of how much virus to add, rather than the PFU/ml. However, as discussed in Chapter 4, the preparation of virus used throughout this thesis still contains non-infectious particles, such as dense bodies and NIEPs, which are still able to bind to cells, but cannot be quantified by qPCR. Given this, for the experiments using the un-purified AD169 strain, the number of viral particles added to each experiment is likely to be a significant underestimation of the number of particles potentially able to bind to and affect the function of a spermatozoon.

Initial investigation with the AD169 strain of CMV showed there was no effect on any parameters analysed. Although there was an initial significant

difference in motility after 3 hours co-incubation between the virus infected sample and the mock-infected control, this was not apparent after 6-hours (Figure 6.2a), due to the mock-infected sample also exhibiting an effect on sperm motility. The effect on motility by the mock-infected control is likely because of MRC-5 cell debris or exogenous cellular DNA present in the inoculum. This is also likely to be present in the virus-infected sample but in larger quantities due to the cell death that occurs as a result of viral infection. This would explain why a difference is observed after 3 hours co-incubation, but not after 6 hours, as the larger quantities of impurities in the virus-infected samples could affect sperm motility faster than the smaller quantities in the mock-infected samples. When analysing sperm motility, the four categories recommended in WHO (1999) were used, as opposed to the updated recommendation of WHO (2010) to use the three categories of sperm motility; progressive, non-progressive and immotile. The use of four categories in this thesis is consistent with the current Association of Biomedical Andrologists (ABA) guidelines (Association of Biomedical Andrologists, 2012) and a recent paper outlining a checklist for acceptability of studies based on human semen analysis (Björndahl *et al*., 2016).

In this set of experiments, an *E. coli* LPS control was included as a positive control, however, it did not exhibit the same marked effect on sperm function as has been shown previously (Hosseinzadeh *et al*., 2003). Initially the source of LPS used (Section 6.4.1.1) was from a different strain of *E. coli* than that reported in Hosseinzadeh *et al*., (2003). Further investigation with the correct strain showed that the dose at which the maximum effect on sperm motility was observed was 50µg/ml (Figure 6.4), as has been previously reported, but this was not significant enough to be used as positive control. The incorporation of a positive control was considered important to show that sperm could be affected by a biological agent within the experimental conditions designed for this study. It is known that LPS requires FCS to access LPS-binding protein (Tobias *et al*., 1986) and trigger the immune responses that result in damage to the cell. Therefore, a final attempt to incorporate LPS as a positive control was conducted in Section

6.3.2.2 (Figure 6.10b) and even with the addition of FCS; there were still no significant differences on the parameters tested, in comparison to the negative control. However, the LPS control did have the most effect on increased tyrosine phosphorylation levels, showing sperm can be negatively affected within the experimental conditions of this study.

Despite observing some slight differences in sperm motility upon exposure to CMV (AD169) in the initial experiment outlined in Section 6.4.1.1, there were no sustained differences between samples infected with CMV and the negative control samples over the 6-hour co-incubation period. Due to the preparation of CMV AD169 not being purified, it cannot be ruled out that the slight effects observed are not due to impurities present in the virus preparation, such as viral and cellular exogenous DNA. This could contribute to the slight differences observed, as sperm are known to bind and uptake exogenous DNA (Zani *et al.,* 1995), which has been shown to decrease sperm motility and viability in bovine sperm (Canovas *et al*., 2010). To investigate this further, sperm were co-incubated with a purified strain of CMV (Towne) (Figure 6.5) and effects on motility, viability and acrosome reaction compared to the un-purified strain (AD169). No effect on any of these parameters was observed with Towne, although a similar decline in sperm motility, and an increase in category c sperm (Figure 6.6c), was observed with AD169. The absence of any effect with Towne suggests that these effects with AD169 could be due to factors present in the viral preparation, as opposed to direct action of the virus itself.

Further investigation by altering the dose of CMV used in the co-incubation experiments also showed no effect on any of the sperm parameters tested (Figure 6.7), ruling out the possibility that the lack of effects observed in the previous experiments were due to a low dose of virus used (Figure 6.8). Similarly, when a wild-type strain of CMV was used, as opposed to the three laboratory strains used throughout this chapter, there was also no effect on any parameters (Figure 6.9). The differences in cell tropism for laboratory and wild-type strains, outlined in Chapter 4, might have explained the lack of effect observed when sperm were incubated with laboratory strains of CMV.

However, no effect was observed when incubated with a wild-type strain, therefore ruling out this explanation.

From this set of experiments, it is clear that various strains of CMV at different doses have no effect on these three basic sperm function parameters over a 6-hour incubation period. Currently, there is little *in vitro* work to compare these findings to. One study that investigated the effect of both HSV-2 and CMV (Pallier *et al*., 2002) found no effect on motility upon co-incubation of each virus with sperm, despite adding a high concentration of virus (10^4 to 10^6 PFU for 10^5 sperm). All of this evidence together suggests CMV does not have any effect on sperm function when exposed after ejaculation. Interestingly, most *in vivo* studies do not report any correlation between CMV infection and reduced sperm function, including sperm motility, concentration and the presence of antisperm antibodies and white blood cells (Eggert-Kruse *et al*., 2009; Kapranos *et al*., 2003; Michou *et al*., 2012; Naumenko *et al*., 2011; Neofytou *et al*., 2009), suggesting pre-ejaculatory exposure does not have any effect either. These findings for CMV differ in comparison to other *Herpesviruses*, such as HSV and EBV, which does appear to affect sperm function and quality pre-ejaculation (Bezold *et al*., 2001; Bezold *et al*., 2007; Kapranos *et al*., 2003; Neoftytou *et al*., 2009). However, this *in vivo* work fails to answer if the effects observed are due to a direct interaction between the virus and sperm, which is what this study investigated.

Despite CMV not having an effect on basic sperm function parameters, if CMV was binding to sperm through receptor mediated events, it is possible that molecular signalling pathways would be affected, as has been shown for other pathogens, such as HPV and *C. trachomatis*. Decreased DAPI staining of the sperm nucleus after exposure to HPV suggests this exposure might have compromised the integrity of the DNA (Kaspersen *et al*., 2011). Increased levels of DNA fragmentation were also observed after *in vitro* co-incubation with *C. trachomatis* (Eley *et al*., 2005b; Satta *et al*., 2006), thought to be as a result of apoptosis mediated events. However in this chapter, the same findings were not found with CMV, as after six-hours co-incubation with

multiple strains of CMV, the levels of DNA damage were not significantly increased (Figure 6.10a). This suggests that CMV is not able to induce DNA damage in washed sperm post-ejaculation, but damage could occur during ejaculation or post-ejaculation in the female reproductive tract, due to the presence of ROS produced from leukocytes present in semen (Lee *et al*., 2014). Likewise, damage could occur pre-ejaculation in the testis through CMV infection of spermatogonial cells (Naumenko *et al*., 2011). Activation of CMV immediate early genes has been shown to have a mutagenic effect on cellular DNA (Shen *et al*., 1997), suggesting if damaged sperm survived the 'quality control' abortive apoptotic processes in the testis (Rodriguez *et al*., 1997; Aitken and Baker, 2013), mature sperm might be produced with DNA damage as a direct result of CMV infection.

The TUNEL assay was used to measure the levels of DNA damage, however the use of this assay for sperm has previously been criticised, due to problems with accessing DNA breaks because of the condensed nature of the sperm chromatin. Others have suggested the addition of a step to fully digest the proteins surrounding sperm DNA in order for the TUNEL assay to efficiently detect all DNA damage, such as the addition of DTT (Mitchell *et al*., 2011). The assay used in this thesis did not incorporate such a step but was still able to detect DNA damage in sperm treated with DNase I, after treatment with Proteinase K. It is probable that not all DNA damage was detected using this method, but this is a known limitation of this assay and it is thought that TUNEL is a better predictor of IVF outcomes (Sun *et al*., 1997; Robinson *et al*., 2012; Garolla *et al*., 2015) because it can only detect damage in these accessible regions of the genome.

Another molecular signalling pathway that could be affected by co-incubation with CMV is the level of tyrosine phosphorylation, which is affected by co-incubation with *C.trachomatis* (Hosseinzadeh *et al*., 2000), thought to be due to a receptor mediated induction of this intracellular signalling pathway. However, after a 6-hour co-incubation with multiple different strains of CMV, no changes were observed in sperm tyrosine phosphorylation levels (Figure 6.10). CMV is known to inhibit tyrosine phosphorylation of a major

component of the JAK/STAT pathway responsible for the activation of Interferon (IFN)-ϒ in somatic cells (Baron & Davignon, 2008). This shows CMV is able to alter tyrosine phosphorylation in some cell types, however, this chapter did not show any such effect on sperm.

Despite co-incubation with CMV not affecting any of the parameters examined so far, there were some subtle differences in the individual motility categories (Figures 6.3 & 6.6) that were not apparent when just reporting the percentage of progressive motility. To investigate this further, a CASA machine was used to measure subtle differences in sperm movement. It has been reported that decreased average path velocity (VAP) and straight-line velocity (VSL) correlate with poor reproductive outcomes (Irvine *et al*., 1994; Macleod & Irvine, 1995). In comparison, when samples were analysed with the standard WHO guidelines for motility assessment, this did not correlate with reproductive success (Macleod & Irvine, 1995). In addition, all parameters of sperm movement, except amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) correlated with the time taken to achieve pregnancy (Irvine *et al*., 1994). Given this evidence it is clear that subtle changes in sperm movement, that are not adequately described by the standard four category assessment, might have an impact on male fertility. It has previously been reported for HSV-2, that whilst no differences in the percentage of motile sperm were observed after co-incubation, alterations in all sperm kinematic parameters, except the VSL, were observed (Pallier *et al*., 2002). Despite this, analysis of sperm velocity and kinematic parameters in this chapter showed no differences in any of the kinematic parameters measured by CASA upon exposure to multiple strains of CMV (Table 6.1 & 6.2).

In summary, this chapter has shown that co-incubation with CMV does not have any obvious effects on the function of ejaculated, washed sperm, however, this does not rule out the possibility that CMV could affect sperm function when exposed pre/peri-ejaculation. Similarly, it does not rule out a direct interaction between CMV and sperm. Indirect evidence presented in Chapter 5 of this thesis provides evidence to suggest CMV could be

attaching to sperm. Furthermore, evidence from a study using immunofluorescence has shown CMV localises to specific areas of the spermatozoon, suggesting a direct interaction (Naumenko *et al*., 2014). It is plausible that CMV might still be binding to sperm without inducing any adverse effects. CMV is a complex virus that has co-evolved with its host for many years. As such, it has developed extensive mechanisms for evading detection by cellular surveillance responses, ensuring its propagation amongst the human population. This idea will be explored further in the next chapter.

Chapter 7

Discussion

7.1 Summary of study aim(s) and main findings

The main aim of this study was to better understand the mechanisms of interaction between human Cytomegalovirus and human sperm. Many fertility clinics have identified a problem with sperm donor supply and this thesis has highlighted that most staff attribute some of this problem to the strict CMV screening guidelines (Chapter 3). Whilst it is possible to change screening practices to alleviate some of these problems, it is first essential to increase the evidence base on which informed decisions can be made. Ultimately, a screening procedure that permits the use of seropositive donors for all recipients would be the ideal scenario. This would alleviate problems with availability and choice for seronegative recipients, in addition to reducing waiting times and stress for couples waiting for a sperm donor. However, this can only be conducted with a full understanding of the transmission risk a seropositive donors poses. To do this, it is essential to better understand the relationship between CMV and sperm.

Evidence presented throughout this thesis has shown there is an alternative way to screen sperm donors for CMV, by screening individual ejaculates, rather than each donor and by incorporating sperm washing to increase the pool of donors that are safe to use. This alternative approach would not only alleviate the problems outlined above, but would also be safer, based on what has been learnt about the relationship between CMV and sperm. However, there are still questions to be answered, particularly regarding the specific molecular interactions between CMV and sperm. Despite this, this thesis has provided some clarity on the situation that can prove useful, particularly given the apparent problems CMV screening is causing in the fertility clinic.

Throughout this thesis, an *in vitro* approach was used to investigate the aims. By establishing a system in which CMV could be cultured and quantified *in vitro*, it has been possible to examine the relationship between CMV and sperm directly, without many of the confounding factors found in *in vivo* studies, which can complicate the interpretation of the findings. This has

shown that CMV does not affect any of the sperm function parameters associated with reproductive success, including motility, viability, acrosome status, DNA damage and tyrosine phosphorylation. This is supported by the many studies that have been conducted *in vivo*, which document no damage to sperm function in the presence of a CMV infection (Bezold *et* al., 2001; Neofytou *et al*., 2009; Eggert-Kruse *et al*., 2009; Naumenko *et al*., 2011; Michou *et al*., 2012; Chen *et al*., 2013). This study also showed that neither, the concentration of CMV used, the exposure time, nor the strain of virus used had any effect on sperm function. However, this does not rule out an effect on the male reproductive tract in response to CMV infection. Inflammatory responses triggered in response to an infection could compromise reproductive potential, a point that will be discussed in more detail in Section 7.3.

Whilst this study has shown that CMV infection in men is not an immediate concern to the functional capacity of sperm, the shedding pattern of CMV in semen should be of concern to fertility clinics. This was not known prior to the design of this study but has been reported since, by Kaspersen *et al*., (2012). This thesis was able to build on this by showing the extent of the rapid turnaround in viral shedding that has not been previously documented for CMV. This finding raises concerns about the current screening practices conducted in fertility clinics, as it is unlikely that the recommended test of serum antibody testing is able to detect such rapid fluctuations in the shedding of CMV in semen. Further complications arise from the apparent deviations from the recommended guidelines, reported in Chapter 3. Fortunately, this study has shown that sperm washing is mostly effective at removing CMV from semen samples infected *in vivo* and *in vitro.* As sperm washing is routinely conducted in fertility clinics, this is potentially an easy solution to the problems many clinics are facing. These findings can be used to better inform current screening practices, a point which will be explored in detail in this chapter. An alternative approach to the current screening process, based on this new scientific evidence, will be proposed.

One thing this study has not been able to provide evidence to definitely answer, is whether CMV can bind directly to sperm through a receptor mediated event, as has been shown for HIV (Cardona-Maya *et al*., 2011), HPV (Foresta *et al*., 2011a), and *C. trachomatis* (Hosseinzadeh *et al*., 2000). If an effect on sperm function were observed, this would have opened channels for exploration into investigating the type of interaction occurring. This was not observed and therefore, this was not investigated directly, but some observations were made that could help to increase our understanding of this interaction.

7.2 What do we now know about the interactions between CMV and sperm?

Whilst this study has not been able to definitively answer if a direct interaction between CMV and sperm is occurring, it is still an important question to consider. An understanding of this interaction would help in determining the risks of transmission during donor-assisted conception, but the true risks can only be fully understood through epidemiological studies. However, evidence from this study allows a hypothetical model for how an interaction might be occurring.

Given that CMV does not affect sperm function parameters when co-incubated *in vitro*, it could be argued that CMV is not binding to sperm. It would be expected that certain intracellular pathways, such as those responsible for initiating cell death cascades would be triggered upon binding of CMV. As has been shown for *C. trachomatis*, activation of these apoptotic pathways in response to bacterial LPS results in alterations in sperm motility, viability and levels of DNA damage (Eley *et al*., 2005a; Gorga *et al*., 2001; Hosseinzadeh *et al*., 2000; Hosseinzadeh *et al*., 2001; Hosseinzadeh *et al*., 2003; Jungwirth *et al*., 2003). This is not observed in sperm exposed to CMV, and therefore it could be argued that this is because an interaction is not occurring.

Also, Chapter 5 of this study has shown that sperm washing is mostly effective at removing CMV from semen samples, both *in vivo* (>95%

removal) and *in vitro* (~>90%-98% removal). This evidence does not appear to support the theory of a direct interaction occurring between CMV and sperm. If this were occurring, it could be argued that a greater number of CMV particles would be recovered in the motile sperm pellet. However, other studies have not observed the same level of efficiency with sperm washing, with Naumenko *et al*., (2014) observing the majority of CMV remaining after washing, providing evidence to support a direct interaction between CMV and sperm. This is a plausible scenario given that sperm do possess receptors to which CMV is known to bind in somatic cells, such as Heparin Sulphate Proteoglycans (HSPGs) (Foresta *et al*., 2011a).

One criticism of this theory would be the lack of effect on sperm function upon exposure to CMV. This could be explained by the ability of CMV to modulate responses by the sperm that could result in cellular damage, such as cell death. This thesis has previously discussed in section 1.2.2.3, how adept CMV is at manipulating the host cell immune response by overriding mechanisms, such as the cell death cascade, in order to support viral growth and reproduction. Clearly CMV has the capability to do this in sperm, which could explain why no effect on sperm function was observed. However, this would rely on the assumption that CMV was able to penetrate the sperm cell and modulate genes involved in these inflammatory response-signalling pathways. Current evidence from this study does not support this theory. If CMV is able to penetrate and reside inside a sperm, this is most likely to occur at the spermatogonial cell stage, as shown *in vitro* in Naumenko *et al*., (2011). At this stage, the cell could be manipulated to reproduce daughter viral progeny, which is the main aim of any virus upon infecting a cell. A fully differentiated mature spermatozoon could not be manipulated in such a manner due to the condensed nature of the genome (Brewer *et al*., 2002; Dadoune, 2003). Also, the chance of exposure to CMV over a sustained period of time for spermatogonial cells is greater than for mature sperm, due to the nature of swift transit through the reproductive system, once fully formed. Finally, as discussed in section 1.2.2.1, multiple receptors are required for membrane fusion, in order to allow CMV to penetrate a cell (Kari

and Gehrz, 1992; Huber and Compton and Feire, 1997). It is not sufficient to just have one putative receptor, to which an initial interaction is made. Currently there is no evidence to support the existence of such a system that would allow CMV to penetrate a spermatozoon. This cannot be completely ruled out, as it has been shown to occur with other viruses, such as HIV (Dussaix *et al*., 1993; Baccetti *et al*., 1994). However, at this time there is no convincing evidence for CMV being capable of doing this in mature sperm. Given this, it is unlikely that the lack of effect on sperm function is due to down regulation of immune responses by CMV.

The simplest explanation for how an interaction might be occurring, without the expected measurable corresponding impact on sperm function, is that CMV only binds to a small proportion of sperm within a given ejaculate. Naumenko *et al*., (2014) reported that CMV only bound to ~2% of cells, despite a high viral load being determined in the individual semen samples. If CMV were only binding to a small proportion of sperm, any negative effects on sperm at the single cell level would not be observed in the experiments performed in this thesis. Given the number of sperm counted per repeat, no statistical significance would be observed due to the significant number of unaffected sperm present in each experiment. This theory is supported further by the small percentage of CMV remaining after washing, which was found to be between ~0.7-17.8% of the initial viral load. This is consistent with CMV only interacting with a small proportion of the sperm in the ejaculate.

The capability of CMV only binding to a small proportion of the sperm population can be explained by considering the role the acrosome might play in this interaction. The acrosome has previously been implicated in interactions between viruses and sperm, with the direct interaction between HHV-6a and sperm being abolished once the acrosome reaction is induced (Kaspersen *et al*., 2012). Similarly, the equatorial segment (ES) has also been implicated as a region of the sperm head important in interactions between viruses and sperm, particularly HPV (Foresta *et al*., 2010c; Foresta *et al*., 2011a; Kaspersen *et al*., 2012). This is interesting as it is known that

receptors revealed at the ES, after the acrosome reaction occurs, are involved in the binding of sperm to the oocyte membrane (Cho *et al*., 1998). Furthermore, syndecan-1, a HSPG receptor involved in HPV interactions with sperm is localised to the ES region (Foresta *et al*., 2011a). From this study, it is not clear if this receptor is only exposed once the acrosome reaction occurs, as the authors did not report the percentage of sperm exhibiting syndecan-1 expression. However, the authors did report that binding of HPV to sperm was only observed for ~25% of sperm cells. This suggests that in 75% of sperm, HPV was not able to bind, which could be explained by the putative receptor not being present because the acrosome was intact.

With this evidence in mind, there are two possible models for how CMV could bind to sperm. The first is that CMV binds to a receptor on the sperm plasma membrane situated around the acrosomal region, in a manner similar to HHV-6a (Figure 7.1, Model A). The second and more likely model, based on the evidence presented in this thesis, is that CMV binds to a receptor exposed at the ES once the acrosome reaction occurs (Figure 7.1, Model B). Model B is the more likely scenario given that throughout this study, <15% of sperm were observed to be acrosome reacted (Chapter 6), giving only a small percentage of cells to which CMV could bind. Not all sperm recovered after washing are acrosome intact (Brandeis and Manuel, 1993; Matas *et al*., 2011), suggesting acrosome reacted sperm are present in the sperm pellet. Therefore, this could explain the small amount of CMV remaining after washing and would also support the apparent lack of effect on sperm function, as a statistically significant difference would not be observed. In further support of this model, Naumenko *et al*., (2014) observed a binding pattern of CMV that is similar to the pattern of syndecan-1 binding at the sperm ES, observed in Foresta *et al*., (2011a). This pattern of fluorescence is a hallmark of acrosome reacted sperm and supports the theory that CMV is only able to bind to acrosome reacted sperm.

The consequence of this is that sperm could act as a vector for viral transmission. This could result in sperm carrying CMV into the cervix of a

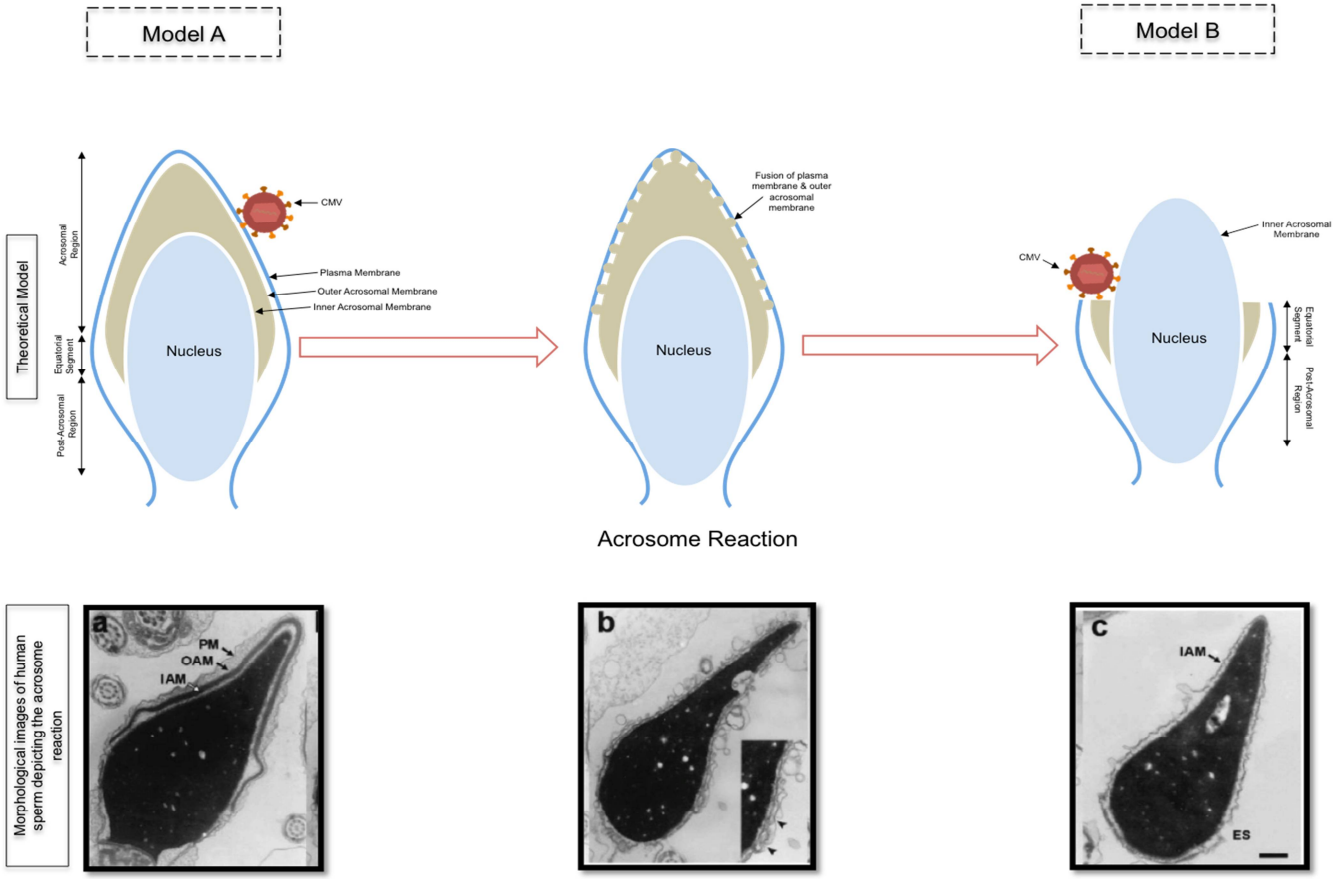

Figure 7.1: Two hypothetical models of how CMV may be interacting with sperm. Morphological images presenting the biological process of the acrosome reaction are included to demonstrate the actual process, depicted in the theoretical model. Model A depicts a scenario, in which CMV binds to a receptor on the surface of the plasma membrane (PM) that surrounds the acrosome. During the process of the acrosome reaction, this portion of the PM is lost, exposing receptors at the equatorial segment (ES) on the surface of the inner acrosomal membrane. Model B depicts a scenario where CMV is able to bind to receptors exposed at this surface. Morphological images reprinted with permission from Michaut *et al*., 2000, with permission from PNAS. Figure Key: PM = Plasma Membrane, OAM = Outer acrosomal membrane, IAM = Inner acrosomal membrane, ES = Equatorial Segment.

female, or at the very least the vagina, which could lead to the virus ascending to the uterus (Coonrod *et al*., 1998). This would result in a genital tract infection, potentially leading to health complications and transmission of CMV to the fetus. The likelihood of this and the potential outcomes will be discussed in more depth in the next section. Another concern would be the ability of sperm to deliver CMV to the egg, potentially creating a fetus with systemic CMV infection.

However, both models for CMV-sperm interaction presented in this discussion rule out this possibility. During the acrosome reaction, the plasma membrane and the outer acrosomal membrane fuse together in an exocytotic process, which culminates in the release of the acrosomal contents (Michaut *et al*., 2000). As a result of this, the plasma membrane in this region of the sperm head is lost (Figure 7.1b). Therefore, if CMV were binding to a receptor in this region, as is proposed in Model A, this interaction would be lost prior to penetration of the ZP, ruling out its passage into the egg, but not ruling out infection of the mother and subsequent vertical transmission to the fetus. Similarly, with Model B, sperm carrying CMV would no longer be able to penetrate the ZP, due to having already undergone the acrosome reaction. Once again, this model of interaction would not allow sperm to deliver CMV directly to the oocyte. Finally, given the theory that CMV only binds to a small percentage of sperm cells, it is likely that a sperm carrying CMV would not reach the oocyte, as most sperm are lost during the transit through the female reproductive tract (Suarez and Pacey, 2006). Therefore, it is unlikely that sperm are able to deliver CMV directly to the oocyte. The only scenarios by which transmission straight to the oocyte would be of concern, is if CMV was internalised into the sperm head, for which there is no current evidence to support. Also, it is possible that sperm carrying CMV might be chosen for ICSI, which could lead to the transmission of CMV directly to the egg, as has been shown for HBV (Ali *et al*., 2005). Whilst the presence of CMV in semen and any potential interaction does not appear to pose a risk directly to the oocyte, this does not rule out effects on the male reproductive tract and the female reproductive tract, which may affect fertility. Also, the consequences

of transmission of CMV to a fetus via infection of the uterus and placenta need to be considered. The consequences of this for male reproductive potential and assisted conception will be discussed further in the next two sections.

7.3 Is CMV infection a concern for male reproductive potential?

Whether CMV interacts directly with sperm or not, it is clear that this does not effect sperm function when exposed post-ejaculation, an observation found in this study, and others (Pallier *et al*., 2002; Naumenko *et al*., 2011). However, this does not give an accurate picture of the effect on sperm through pre/peri-ejaculation exposure due to the secondary responses that occur in the male reproductive tract in response to infection, and the possibility that CMV could infect spermatogonial cells in the testis (Naumenko *et al*., 2011). Viruses are able to cause inflammatory disorders of the male reproductive tract, as they are able to infect the epithelial cells lining the reproductive system through the blood stream (Keck *et al*., 1998). This is important, as the role inflammation of the male genital tract plays in contributing to male infertility cannot be ruled out.

Inflammation of the reproductive tract often results in increased production of leukocytes (Comhaire *et al*., 1999), which correlates with a history of infertility (Wolff and Anderson, 1988). In turn, the levels of IL-8, a pro-inflammatory cytokine activated by NF-κB are increased, in line with a rise in the levels of leukocytes present in semen (Eggert-Kruse *et al*., 2001). IL-8 has been implicated as playing a key role in male genital tract inflammation (reviewed in Lotti and Maggi, 2013). CMV is known to significantly up-regulate the expression of IL-8 in fibroblasts (Craigen *et al*., 1997) and monocytes (Muruyama *et* al., 1997; Murayama *et al*., 1998), which serves to promote viral replication and modulate antiviral activity of host cells through inhibiting interferon (Muruyama *et al*., 1994; Murayama *et al*., 1998). The link between IL-8 playing a role in male genital tract infection and CMV being able to up regulate IL-8 expression supports a role of CMV in causing inflammation, a correlation that has been observed *in vitro* (Naumenko *et al*., 2014).

Inflammation in the male genital tract is detrimental to reproductive health due to the secondary effects on sperm and spermatogenesis. The presence of monocytes, in response to infection, activates cytokines such as IL-8, leading to the recruitment of B-lymphocytes (Seshadri *et al*., 2012). This results in the production of anti-sperm antibodies, which are detrimental to sperm function (Eggert-Kruse *et al*., 2002). Furthermore, the presence of granulocytes results in the production of ROS, which induces DNA damage (Kodama *et al*., 1997; Twigg *et al*., 1998) and lipid peroxidation on the spermatozoon (Aitken *et al*., 1993; Aitken *et al*., 1995).

CMV infection mostly presents as an asymptomatic infection, which is likely to mean that inflammation of the genital tract will remain sub-clinical. Consequently, CMV infection will remain undiagnosed and untreated. Intermittent shedding of CMV over a sustained period of time (Kaspersen *et al*., 2012), will likely lead to multiple instances of inflammation, resulting in repeated episodes of damage to sperm. Despite this, most *in vivo* studies did not observe any correlation between semen quality and the presence of CMV. Also, this study did not observe any significant differences in the semen volume, sperm concentration or sperm motility between the 13 CMV positive samples and the CMV negative samples (Table 5.4). Effects of inflammation on sperm function are only likely to occur during active shedding of CMV. As this study has shown, men intermittently shed CMV, with a rapid rate of clearance. This would have an effect on studies investigating the role CMV plays in infertility, and likely contributes to the negative findings. Similarly, these studies do not consider the length of time of CMV infection. This would be difficult to determine, but prolonged infection with multiple bouts of reactivation might have a more permanent effect on male infertility and could also be a compounding effect in *in vivo* studies.

When considering the role CMV infection of the male reproductive tract might play in male infertility, it is important to consider the effects of transmitting CMV to the female and any consequences this might have on reproduction. If CMV is binding directly to sperm, it will be transmitted to the female reproductive tract. Given that this does not appear to affect sperm function,

sperm would be able to travel through the cervix into the uterus. Even if CMV is not binding to sperm, it could still be transmitted to the female reproductive tract via carriage in the seminal fluid. This would allow the virus to establish an infection in the genital tract, which might affect the outcomes of pregnancy.

One argument against this is that the immune system present in the female reproductive tract would combat the virus and prevent an infection establishing. The mucosal immune system of the female reproductive tract mediates this immune response, acting through TLR's on the surface of cells, which form the innate immunity. This results in the production of cytokines/chemokines, necessary to mount a full immune response against an infectious agent (reviewed in Wira *et al*., 2005). Whilst these mechanisms exist, this thesis has already discussed how well CMV is adapted to evade the host immune response. Therefore it is possible that CMV could establish an infection throughout the female reproductive tract, despite the immune system present. This is supported by evidence that CMV can be detected throughout the entire female reproductive tract in women with pelvic inflammatory disease, including the cervix, endometrium, Fallopian tubes and ovaries (Clarke *et al*., 1997b).

Transmission of CMV via semen could be one cause of infection in the female reproductive tract, either through sperm directly carrying CMV into the upper genital tract, or through ascension of the virus from the cervix. It has been shown in mice that viral infection of the cervix during pregnancy can result in the ascension of bacteria into the uterus, compromising pregnancy (Racicot *et al*., 2013). The authors hypothesised that a down-regulation of TLR ligands in the murine cervix when infected with virus might be responsible. Interestingly, this study also suggested that the sex hormones that regulate changes to the cervix throughout the menstrual cycle might also make the cervix more susceptible to viral infection (Racicot *et al*., 2013). All of this evidence points to the fact that transmission of CMV through semen could establish an infection throughout the female reproductive tract, and affect fertilisation and pregnancy outcomes. This is supported by *in vitro*

evidence that shows shedding of CMV in the reproductive tract correlates with increased miscarriage rates (Tanaka *et al*., 2006).

Whilst the immune system in the female reproductive tract is there to protect from infection, it is a fine balance between doing this and sustaining the life of the allogenic fetus. Unfortunately, when challenged with an infectious agent, this can often result in 'rejection' of the developing embryo/fetus. TLR-2 is thought to play a key role in the immune response against pathogens in the cervix (Lashkari *et al.,* 2015). It has been shown *in vitro* that activation of the TLR-2 ligand during pregnancy can result in a failure for the developing embryo to implant (Sanchez-Lopez *et al*., 2014), therefore negatively effecting pregnancy outcome.

This evidence clearly shows CMV is able to establish an infection in the reproductive tract of both males and females, which can have negative outcomes on reproductive potential. When considering the role CMV plays in donor assisted conception, it is important to consider how this translates to the risk of transmission of CMV to a fetus. Evidence shows that the presence of CMV in the genital tract correlates with women giving birth to babies born with congenital CMV infection, in comparison to mothers positive for CMV in their saliva, blood or urine (Kaye *et al*., 2008). Infection of the endothelial cells that line the uterus is thought to be the cause of transmission to the fetus, through direct contact with the placental cytotrophoblasts (Maidji *et al*., 2002). Therefore, this evidence shows that infection in the female genital tract can lead to transmission of CMV and this could be mediated by transmission of the virus via semen.

It is clear that whilst CMV might not have any direct effects on the function of a spermatozoon, its presence in the male reproductive tract can have consequences for the reproductive potential of a man. This can be due to the effect on the male genital tract or through transmission to the female reproductive tract, which has a myriad of consequences on the success of fertilisation and pregnancy in itself. Clearly, these concerns do not only apply to natural conception, but also to assisted conception. Therefore the next

section will consider if these potential negative outcomes on fertility and pregnancy should be of concern to fertility clinics.

7.4 Should CMV infection in men be a concern for fertility clinics?

Whether a direct interaction between CMV and sperm is occurring, or not, it is clear from this study that sperm washing is mostly effective at removing CMV from semen samples. Of the initial viral load, the majority of CMV is removed (>93%) from samples infected *in vivo* (Section 5.4.3), with a similar rate of removal observed for samples infected *in vitro* (Sections 5.4.1, 5.4.2 and 5.4.4). Given this evidence, it appears that this technique is more effective than has been previously reported in the literature (Michou *et al*., 2012; Naumenko *et al*., 2014). Given that fertility clinics routinely carry out sperm washing during the process of preparing a sample for assisted conception, it is likely that CMV is removed from most semen samples, regardless of the inadequacies of current screening methods.

Despite the current guidelines stating that only seronegative donors should be recruited whenever possible, Chapter 3 of this thesis shows that the majority of clinics report to actively recruiting seropositive donors. This is concerning due to evidence suggesting that the current methods for screening sperm donors might be ineffective. This is based on the evidence that men intermittently shed CMV, which is likely to be left undetected using the current diagnostic test, serum antibody testing. Therefore, if clinics do deviate from guidelines and use seropositive donors, it is probable that semen samples from donors actively shedding CMV are being used for therapeutic use. Furthermore, evidence from the survey conducted shows that some clinics are using seropositive donors for seronegative women. In this scenario, there is the potential that a semen sample, which contains CMV, would be given to a woman with no pre-existing immunity, which could result in substantial health consequences to any resulting fetus. However, this study has shown that for 84.6% of ejaculates, sperm washing was able to remove CMV from these samples. Therefore, it is likely that if CMV were present in any semen samples from seropositive men used for donor

insemination, the virus would have been removed during the routine sperm-washing step.

However, it must be noted that sperm washing is not 100% efficient. In 15.4% of ejaculates, CMV remained after washing and there was an overall 6.4 (range 2.2-10.2)% of CMV viral particles remaining after washing. As has been discussed, this could be due to a direct interaction between CMV and sperm, or due to internalisation of the virus into the sperm head. Therefore, in the 15.4% of cases where CMV is not removed by sperm washing, there is the potential for horizontal transmission to the female recipient. Given the complications associated with CMV infection of the female reproductive tract outlined in the previous section, this is something fertility clinics should continue to be concerned about.

The aim of this thesis was to develop the understanding around CMV and sperm in order to alleviate some of the problems the requirement to screen for CMV causes. Whilst it has been documented in this thesis, in Section 3.4.3, that the use of seropositive donors for all recipients, regardless of serostatus, is being performed in some clinics, the risks of doing this are currently not known. Fortunately, evidence showing sperm washing is mostly effective at removing CMV supports a scenario in which this practice could be routinely used in the process of screening and managing CMV positive sperm donors. However, this technique is not 100% effective, therefore, before this can be considered as a routine alternative to only using seropositive donors for seropositive women, other factors need to be considered, in order to ensure the safety of this approach.

7.5 How can screening for CMV and the management of CMV positive donors be improved?

It is clear that the current screening process and management of CMV positive donors can be improved. Rather than seromatching donors and recipients based on their CMV serostatus, this thesis has suggested that the use of sperm washing might be an effective way of rendering samples from seropositive donors safe to use for seronegative recipients. However, to do

this, the method of screening donors needs to be changed. A test that is better suited to detecting fluctuations in the seminal viral load, such as qPCR could be incorporated. Also, in order to incorporate sperm washing into the CMV screening and management process, the testing of individual semen samples needs to be conducted, rather than testing the individual. Currently, testing donors for CMV infection via serology only gives an indication of a past infection. Likewise, using qPCR on any sample other than semen will not give an accurate indication of the viral load in semen due to the phenomenon of compartmentalisation.

Sperm washing is not 100% effective; therefore it is essential to ensure a sample is negative for CMV after washing, as this study has shown CMV remaining after washing is able to establish an infection *in vitro*. Therefore, if CMV is present in the motile sperm pellet, it could cause an infection in the recipient. Given this, it is essential that each sample be tested before and after washing for the presence of CMV to avoid horizontal transmission. This can only be carried out with a method of testing which would detect CMV in the individual semen sample, such as qPCR. However, it might not be practical to test the sperm pellet after washing due to the turn around time on obtaining the qPCR results. Given this, it would make more sense to screen all ejaculates at the point of freezing and to discard all the positive samples. However, as DNA technologies become more advanced, it might be possible to develop a rapid 'on the spot' test for CMV detection that would bypass this problem.

Currently, the incorporation of this process into sperm donor screening is hindered by the lack of a validated qPCR test for the detection of CMV in semen, but this could easily be resolved. There are a number of benefits for fertility clinics in altering the screening process to incorporate these suggested changes, which will be presented in a new model for screening in the next section.

7.6 How could changing screening methods impact sperm donor recruitment?

Evidence from this study clearly supports a change in the way current screening for CMV in sperm donors and management of those donors is conducted. This section will propose a new model for screening donors. The feasibility of this approach, in terms of cost will be discussed, alongside the benefits of this approach and how it might help alleviate some of the problems currently facing fertility clinics.

7.6.1 Model assumptions

In order to fully explain this model, it is necessary to explain a number of assumptions, rules and sources of data.

1. The numbers of donors used throughout is based upon the number of new donor registrants in 2013, as per the HFEA donation statistics 2012-2013 (HFEA, 2013a). Currently, there are no official statistics on the total number of sperm donors available in any given year, so the availability of donors has been calculated based on the number of new donor registrants in one year.
2. Based on statistics from the HFEA, (2013a), the average number of IVF/DI cycles each woman has is 1.6. This value is based on the number of heterosexual couples, same sex couples and single women undergoing IVF with donor sperm, or donor insemination. This comes to a total of 4,461 women having 7,138 cycles of IVF/DI (HFEA, 2013a), equating to 1.6 cycles per woman.
3. Using the figures outlined in assumption 2, it is estimated that each donor is able to donate to 16 cycles, based on the criteria that each donor can donate to a maximum of 10 families, and that each family undergoes, on average, 1.6 cycles. The actual number of donations per donor might be as high as 45, as reported in Paul *et al*., 2003, but only the samples to be used for therapeutic use will be considered in this model. This model will look at the number of

cycles and the number of available donors in one year to calculate the total cost of the different screening approaches in one year alone.

4. The number of available donors for the number of cycles conducted is based upon the number of new registrants. Clearly, there are other donors available from previous years, but this is off-set by the assumption that not every single donor will donate to ten families.
5. The number of available overseas donors is not taken into consideration, as these donors are not screened for CMV by UK clinics.
6. This model will only consider the use of CMV seronegative sperm donors for use in donor-assisted conception. Despite evidence in this thesis showing clinics are using seropositive donors, it is not something that can be measured for the purpose of this model. Therefore, this model will consider the recommended approach and assume that only seronegative donors are being used on a routine basis.
7. The only costing taken into consideration for this model is the costing for serum antibody testing and qPCR. This is because all factors that can be assigned a monetary value will stay the same (i.e costs for quarantine). It does not take into consideration the cost of administrative duties to the respective screening protocols, or any other similar costs. Costs for qPCR are estimated at £50 per sample via personal communication with Claire Atkinson at the Virology Unit of Royal Free Hospital, London [Atkinson, C, Personal Communication]. Serum antibody testing costs are estimated at £35 per sample (one at the beginning of quarantine and one at the end) from information available on the Bristol Centre for Reproductive Medicine (BCRM) website regarding charges for patients (BCRM, 2016).

7.6.2 The model

In the UK, there were 391 new UK sperm donor registrants in 2013. As per the findings of this study (Section 3.4.1), only 97.1% of clinics screened their donors for CMV. Assuming an equal distribution of donors across all clinics in the UK, this would amount to 380 donors being screened via serology, at a total cost of £13,300 (i.e £35x380). As can be seen in Figure 7.2, assuming clinics are following the current recommendation of excluding all seropositive donors (Assumption 6) this would immediately result in approximately 60% of donors being excluded on the basis of being seropositive for CMV as this is the prevalence of CMV in the general population. This would leave 163 seronegative donors available for use, in addition to the 11 donors that were never screened for CMV, as only 97.1% of clinics screen for CMV. In addition, 26.5% of UK clinics reported carrying out PCR in conjunction with serum antibody testing. Of the 380 donors that would actually be screened, this suggests 100.7 would be further screened using PCR. Assuming the same cost of £50, this equates to a total extra cost of £5,035.

The semen samples then go through a quarantine period, which is subject to various practical and administrative costs. After this, the donors are screened once again to ensure they have not seroconverted to being CMV seropositive during the quarantine period, equating to a total cost of £5,705. There is no current estimate as to how many donors seroconvert and how many samples are discarded due to this, so for the purpose of this model, it is assumed all donors are viable for use.

In addition to screening the sperm donor, the female recipient is also screened. In 2013, an estimated 4,461 women had treatment with donor sperm (HFEA, 2013a). Results from this study showed that 88.6% of clinics screened the female recipient. Once again, assuming an equal distribution of donor cycles across the clinics, this would equate to 3,952 women having CMV serology testing conducted. Also, 20.4% of clinics reported to screen the male partner of the female recipient, if there was one.

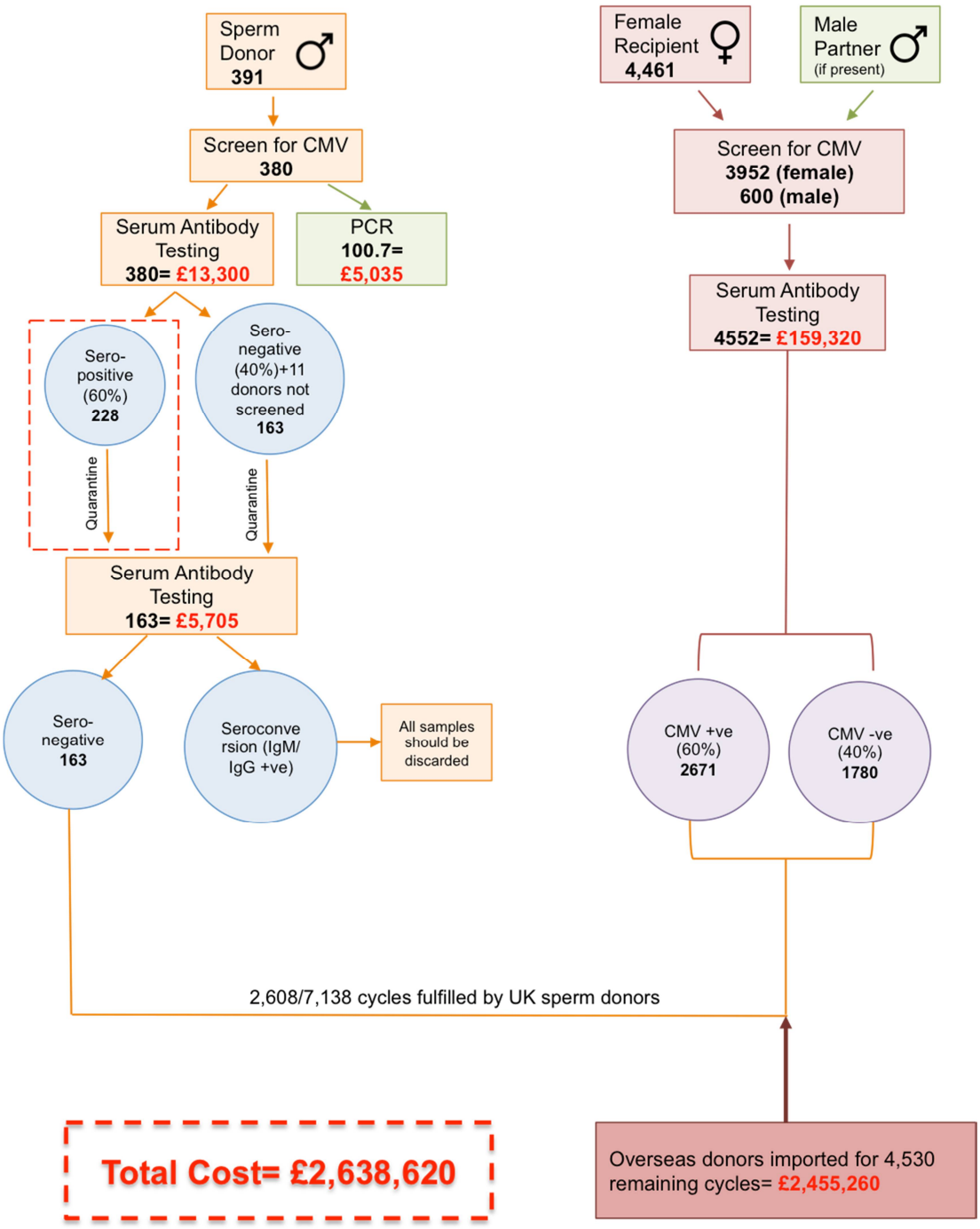

Figure 7.2: A flowchart depicting the current process (Model A) for CMV screening of sperm donors in UK fertility clinics**.** The number of donors available at each step in the process is included, along with the cost associated with any screening steps. In addition, the number of women receiving donor sperm and the number of cycles of IVF/DI are included. The numbers used throughout are from the HFEA Donation and Fertility Treatment Statistics from 2013.

In 2013, approximately two thirds of women accessing fertility treatment with donor gametes had a male partner (although no distinction was made between donor sperm, eggs or embryos, the majority of cycles using donor gametes used donor sperm) (HFEA, 2013b). This equates to 600 male partners also being screened for CMV antibody status. Conducting this screening activity is for the sole purpose of being able to seromatch donor and recipient and equates to a total cost of £159,328.

For a model that incorporates qPCR into the screening process, the key distinction is the requirement to screen the individual ejaculate. This would increase the practical and administrative workload, as rather than processing one sample from one donor, this model estimates that on average, a minimum of 16 samples would need to be processed (see assumption 4) (Figure 7.3). Any extra administrative costs associated with this are not quantified in this model. It is also important to consider that screening in this way might produce extra wastage costs, due to the turnaround time in qPCR results. This would result in a total screening cost of £312,800.

However, based on evidence from this study, it is estimated that 83.5% of semen samples will be negative for CMV, leaving 327 donors available for use for any recipient, regardless of their CMV serostatus. It is important to note here that this completely eliminates the need for: (a) screening for CMV in the female recipient: (b) quarantine for CMV seroconversion (although this would still be conducted for the detection of other infectious agents); and (c) no further screening after the quarantine period. For the remaining 64 donors that test positive for CMV, there is an option to wash these samples and re-test for the presence of CMV after washing. This would be at a total cost of £51,600, but would yield a further a further 54 donors available for use, based on a 15.4% rate of positivity after washing (as per the findings in Chapter 5 of this thesis). One advantage of screening samples in this manner would be that samples could be stored in separate locations based upon their CMV positivity. This would eliminate any risks or concerns regarding cross-contamination of samples during storage in liquid nitrogen

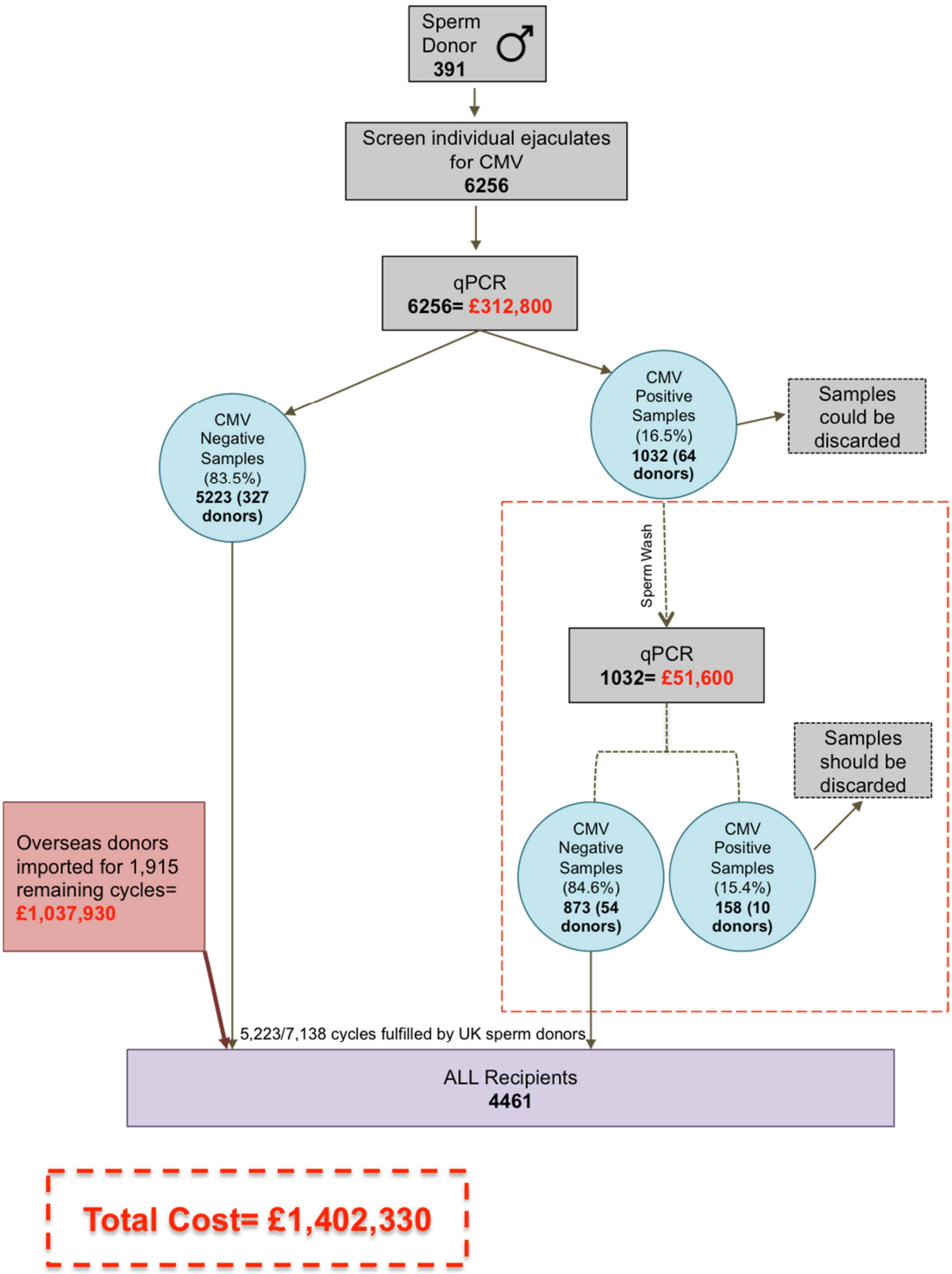

Figure 7.3: A flowchart depicting a proposed new model (Model B) for CMV screening of sperm donors. This includes an alternative method of testing, using qPCR, instead of serum antibody testing. The number of donors available at each step in the process is included, along with the cost associated with any screening steps. In addition, the number of women receiving donor sperm and the number of cycles of IVF/DI are included. The section outlined in a red box is an optional section of the flowchart. The numbers used throughout are from the HFEA Donation and Fertility Treatment Statistics from 2013.

Table 7.1: The total cost of screening sperm donors using the current screening process (Model A), in comparison to a new proposed model (Model B).

Step in Screening Process	Model A Cost (£)	Model B Cost (£)
Initial screening sperm of donor/ejaculate	18,335	312,800
Screening female recipient (and male partner)	159,320	Nil
Screening after quarantine/sperm washing	5,705	51,600
Cost of importing overseas donors to fulfil short fall in demand	2,455,260	1,037,930
Total Cost	2,638,620	1,402,330

(Foresta *et al*., 2010a). However, given the increase in number of CMV negative donors that could be used (327 vs 163), in comparison to the current model, this step could be considered as optional. However, this model will include the extra cost of this step in order to showcase the full extent to which findings from this study can be implemented in the CMV screening process.

For clinics following the guidelines and only recruiting seronegative donors, there would be twice the number of available donors to use, which would be a huge benefit. However, for clinics already recruiting and using seropositive donors, the major saving point is the lack of requirement to screen the female recipient, as seromatching is no longer required, at a total cost of £159,328 (Table 7.1). To compare the benefits of the increase in available donors to clinics recruiting only seronegative donors, in relation to the cost, it is necessary to consider the number of cycles of IVF/DI these donors would be able to donate too. A total of 7,138 cycles of IVF/DI were conducted with donor sperm in 2013 (HFEA, 2013b). Assuming each donor donates to 16 cycles, the current model would allow for 2,608 cycles of IVF/DI to be conducted. This leaves a short fall of 4,530 cycles, which would need to be fulfilled with sperm from overseas donors. The estimated cost of this based on the current European Sperm Bank charges would be £542 per semen sample (European Sperm Bank, 2016), equating to a total cost of £2,455,260. In comparison, with the new model, there would only be 1,915 cycles short of an available sperm donor, which would equate to a cost of £1,037,930. This leaves a total cost for the current model of £2,638,620, in comparison to £1,402,330 for the new model (Table 7.1). Potentially, if all ejaculates from each donor were screened, estimated to be 45 (Paul *et al*., 2003), there could be an extra cost of £728,808 (cost of screening an additional 12,512 samples, in addition to an additional 2,064 after washing) on top of the current estimate in for the new model. This would still be ~£500,000 less than the current model based on the assumptions made and the estimates calculated.

To determine the full benefits of each model, it would be beneficial to conduct a cost effective analysis, which includes all of the costs associated with each approach. Due to limitations of access to such information, it was not possible to conduct such an analysis in this thesis. However, it is clear to see from Figures 7.2 and 7.3 that the proposed model is simpler than the current approach, as there is no requirement to seromatch donor and recipient, and significantly increases the number of available donors. This would ultimately translate to needing to recruit fewer donors from overseas, saving a significant amount of money. What this model is not able to estimate is whether the administrative costs of recruiting, screening and storing donors in the UK outweighs the cost of buying semen samples from overseas sperm banks. This could be worked into a cost effective analysis to fully evaluate the feasibility of screening for donors via the proposed model. One clear advantage of the proposed model of screening is that it removes the added complication of seromatching donors and recipients. By determining that each individual sample is negative for CMV, and therefore safe to use, the serostatus of the recipient is irrelevant. This therefore removes the need for the costly screening process of all recipients undergoing assisted conception with donor sperm.

Another factor to consider is the number of babies born every year as a result of IVF with donor sperm, or donor insemination and whether this constitutes a big enough risk to warrant a change in screening. In 2012, a total of 1215 babies were born from 6846 cycles of IVF/DI using donor sperm (HFEA, 2013b) (654 births from 2372 cycles of IVF using donor sperm, and 561 births from 4474 cycles of DI). The overall incidence of CMV infection in neonates on a population level is 0.7% (Dollard *et al*., 2007), resulting in 8.5 babies being at risk of infection. Furthermore 25.5% of these babies will exhibit symptoms of CMV infection (Dollard *et al*., 2007), resulting in the possibility of ~2 babies every year being born with symptoms associated with CMV infection. Whilst any infection in this group of infants could not be directly attributed to a risk from using donor sperm, due to other

environmental factors, there are still enough babies being born using donor sperm every year to warrant the risk being taken seriously.

Whilst the model presented in this section has its limitations based on the assumptions made, it is clear that there may be a number of benefits to incorporating a screening process that screens each ejaculate, as opposed to the individual donor. These include:

1. A two-fold increase in the number of available donors.
2. The option to segregate positive samples from negative, ruling out cross-contamination risks.
3. Removal of the costly requirement to seromatch donors and recipients.
4. Reducing the need for overseas donors.
5. Increased safety, as samples can be definitively determined as safe (within the limits of the qPCR test performed).
6. Overall reduced cost (£1,402,330 vs £2,638,620).

7.7 The future & further research

Overall, the evidence presented in this thesis and the model discussed in this chapter, supports a change in the way sperm donors are screened for CMV in the UK. Screening each individual ejaculate for CMV via qPCR would significantly increase the number of available sperm donors, as has been shown in Section 7.6. This approach would also increase safety, as there would be a higher-level confidence that an individual sample from a CMV positive donor was negative for CMV and therefore safe to use with a CMV negative recipient. Ideally, the serostatus of each donor used throughout this study would have been determined. If this information had been gathered, it would have been possible to determine if a CMV positive sample came from an IgG or IgM seropositive donor. Therefore it would have been possible to investigate the effectiveness of the current method of screening over the proposed model, at accurately quantifying the risk a donor poses. Similarly, paired serology and semen results would have highlighted if serum antibody testing was able to detect the rapid fluctuations in seminal shedding of CMV. This was not conducted, as the phenomenon of intermittent shedding of CMV in semen was not known prior to the design of this study. A robust study investigating this in sperm donors would add significant weight to the argument that the model proposed in this thesis should be used for screening of CMV in sperm donors.

Whilst qPCR could be incorporated into screening practices, it is not without limitations. Due to its detection limits, it is often not possible to definitely determine if a sample is negative or not. Often virology clinics will report a negative value as less than a certain number of viral copies (for this thesis, the detection limit was 200 copies/ml). This is likely to change in the future due to more sensitive techniques being developed for the detection of DNA, such as digital droplet PCR (Sedlak *et al*., 2014). However, it would be ideal if no screening needed to be performed for CMV, as it is likely that the cost of screening for CMV will always outweigh the risks. Unfortunately, without epidemiological studies to determine the actual risk of transmission involved

with using CMV positive sperm donors, it is not likely the risks will ever be fully understood.

One way this could be avoided, is if it were possible to completely eliminate CMV from semen samples. This thesis has shown that sperm washing is mostly effective at removing CMV, but that it is not 100% efficient. It has also been argued that whilst CMV does not affect sperm function, this does not rule out a direct interaction between CMV and sperm. If the mechanism of interaction between CMV and sperm could be fully understood, it may be possible to develop a mechanism to completely remove CMV from semen samples, by interfering with the receptors involved with the interaction, as has been shown for HPV (Garolla *et al*., 2012). Further research into the site of binding, as discussed in Section 7.2, might shed some light on the situation. A similar experiment to that of Kaspersen *et al*., (2012) could be conducted to determine the role the acrosome plays in this interaction. By inducing the acrosome reaction and examining the effects on sperm function, or by using immunofluorescence to determine binding, it would be possible to determine on which membrane surface the putative CMV receptor was present. This would allow candidate receptors to be proposed and systematic investigation of their involvement in the interaction with CMV. Robust elimination of CMV from semen samples might remove the need to test for CMV at all, completely removing all concerns over donor supply problems, problems with seromatching, and the expense of conducting such tests.

It is clear that the current method for screening sperm donors is causing problems in fertility clinics and this study has provided evidence to explore alternative screening strategies, which could significantly decrease the current burden on them. Furthermore, it has provided additional avenues for exploration to completely eliminate these problems and change the future landscape of sperm donor screening in the UK. Without epidemiological studies that rule out transmission to a fetus via a sperm donor, CMV should always be a concern to fertility clinics. This does not mean it always needs to be a burden. This thesis has shown that with a better understanding of the relationship between CMV and sperm, methods can be proposed to alleviate

the problems clinics are currently facing. With further research, these problems could be eliminated all together, whilst still ensuring no donor conceived child is ever born with congenital CMV as a result of infection through a sperm donor.

Chapter 8

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Appendices

Appendix I: Biosafety approval letter

The University Of Sheffield.

Health

& Safety.

Dr Allan Pacey
Department of Human Metabolism
Academic Unit of Reproductive and
Developmental Medicine

Biological Safety Officer
Cliona Boyle

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WT2013_05
09 May 2013

Dear Dr Pacey

AUTHORISATION TO COMMENCE HAZARD GROUP 2 WORK

Your Hazard Group 2 Project entitled:

Investigating interactions between Cytomegalovirus and human spermatozoa

: - has been assessed project and approved by the Biosafety Committee.

The above project is now authorised to commence subject to the following conditions being met in advance of the project commencing:

a) ~~That all workers involved in the project have been assessed as "medically fit" by the Staff Occupational Health Service prior to commencing their involvement on the project.~~

b) ~~That the laboratory (or laboratories) to be used for the project **[JW4 64, JW4 74]** has been assessed as suitable for work with Genetically Modified Organisms.~~

c) ~~That the Principal Investigator updates relevant databases with changes to personnel or laboratories involved in the above project as soon as practicable following changes;~~

d) That the Principal Investigator inform Health & Safety in the event of a change, or planned change, to the Project Proposer;

e) That the Principal Investigator submit a new or revised project to Biosafety Committee in the event of any changes to the experimental details (e.g. changes to the organisms involved, etc), or to the Hazard Classification of the Project;

f) That the project is carried out in full compliance with the requirements of the *Control of Substances Hazardous to Health: Schedule 3* and *Hazardous Wastes Regulations 2005*;

g) ~~That the project has been notified to the HSE and a letter of approval received from HSE for the project to commence; (strike through if Class 2 project).~~

Yours sincerely

Cliona Boyle
Health & Safety Date:…09 May 2013

Appendix II: University of Sheffield ethical approval letter

The University Of Sheffield.

The Medical School.

28 February 2014

Dr Allan Pacey
Academic Unit of Reproductive &
Developmental Medicine
The Jessop Wing
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Research Ethics Administrator
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Email: j.lazenby@sheffield.ac.uk

Dear Dr Pacey

PROJECT TITLE: 'Studies on human sperm function and seminal contents' – SMBRER293

I am pleased to inform you that on 28 February 2014 the School's Ethics Reviewers **approved** the above-named project on ethics grounds, on the basis that you will adhere to and use the following documents that you submitted for ethics review:

i) Ethics application form [approved – 28 February 2014]
ii) Amended Information Sheet [approved – 28 February 2014]
iii) Amended Consent Form [approved – 28 February 2014]
iv) Blanket Email [approved – 28 February 2014]
v) Participant Enrolment Log [approved – 28 February 2014]
vi) Sample Production & Experimental Allocation Log [approved – 28 February 2014]
vii) Sample Tracking Log (nmr) [approved – 28 February 2014]
viii) Sample Tracking Log (DNA) [approved – 28 February 2014]
ix) Sample Tracking Log (Pathogens) [approved – 28 February 2014]
x) Sperm Poster [approved – 28 February 2014]

If during the course of the project you need to deviate from the above-approved documents please inform me. The written approval of the School's Ethics Review Panel will be required for significant deviations from or significant changes to the above-approved documents. If you decide to terminate the project prematurely please inform me.

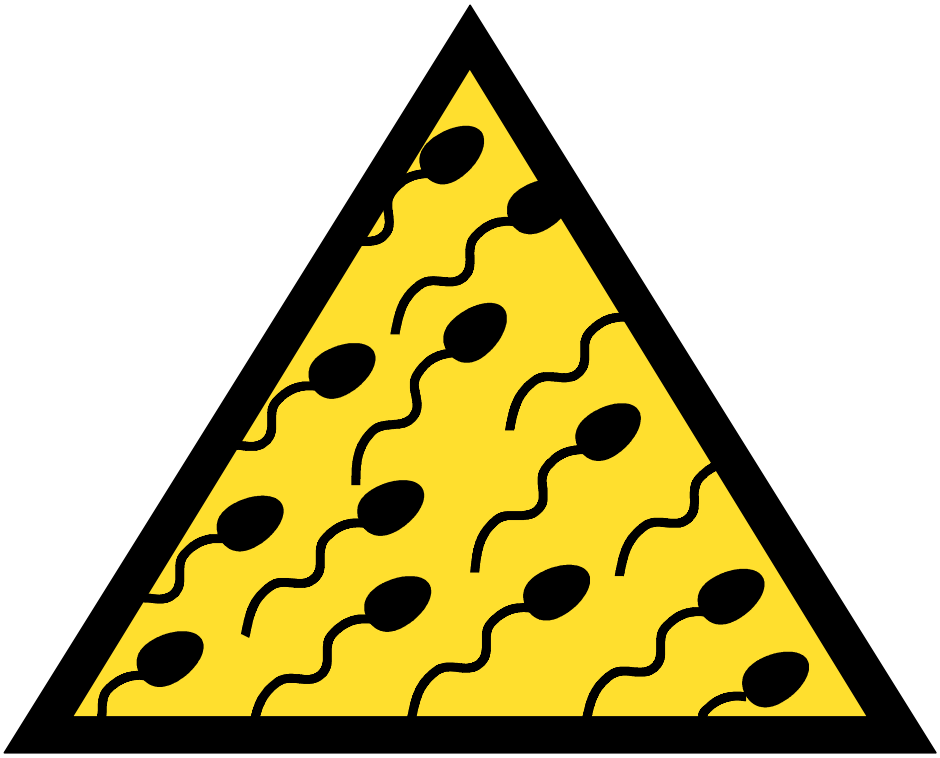
Yours sincerely

J. Lazenby

Jean Lazenby
School Research Ethics Administrator

Appendix III: Poster used for sperm donor recruitment

ATTENTION



DID YOU KNOW THAT YOU CAN DONATE YOUR SPERM FOR SCIENTIFIC RESEARCH?

If you are interested in participating please email

spermresearch@sheffield.ac.uk

for more information.

Appendix IV: Survey evaluating CMV screening in fertility clinics

The University Of Sheffield.

CYTOMEGALOVIRUS (CMV) SCREENING QUESTIONNAIRE

This questionnaire has been designed by researchers at the University of Sheffield to try and understand the approaches currently used in UK fertility clinics for diagnosing Cytomegalovirus (CMV) in sperm donors and the provision of fertility treatment using donor sperm. We would be grateful if you could take the time to fill out this questionnaire and return it to the address below. Thank you in advance for your help.

Kind Regards,

Allan Pacey and Katrina Williams

Please return completed questionnaire to: Allan Pacey, Academic Unit of Reproductive and Developmental Medicine, Level 4. The Jessop Wing, Tree Root Walk, Sheffield, S10 2SF, United Kingdom. (A.pacey@sheffield.ac.uk)

CYTOMEGALOVIRUS (CMV) SCREENING QUESTIONNAIRE

Contact details *Please give the name and contact details of the person filling out this form so that we can contact you to clarify any of your responses if required (please note any responses will be anonymous and without attribution to named individuals)*

Title:

Name:

Address:

Postcode:

Telephone:

E-mail:

Role: ☐ Doctor ☐ Nurse ☐ Embryologist ☐ Andrologist ☐ Other

Section 1: About your clinic.

1.1 Do you offer fertility treatment with donor sperm?

☐ YES ☐ NO

1.2 Do you recruit sperm donors?

☐ YES ☐ NO

1.3 Do you obtain donor sperm from other centres?

☐ YES ☐ NO

1.4 Do you supply donor sperm to treatment centres?

☐ YES ☐ NO

If you answered no to all of these questions then please go straight to section 8, but if you answered yes to any please tell us more in the relevant sections below.

1.5 How many cycles of treatment using donor sperm did your centre perform in 2012?

……. cycles ☐ N/A

1.6 If you recruited donors, how many donors did you screen in 2012 (regardless of whether they were finally accepted)?

……. donors ☐ N/A

Section 2: About your clinic's approach to CMV screening.

2.1 Do you screen for CMV in female patients receiving fertility procedures with donor sperm?

☐YES ☐NO ☐N/A *(Don't treat patients)*

2.2 Do you screen for CMV in the male partner of the woman receiving fertility treatment (if she has a male partner)?

☐YES ☐NO ☐N/A *(Don't treat patients)*

2.3 Do you screen sperm donors you recruit for CMV?

☐YES ☐NO ☐N/A *(Don't recruit donors)*

2.4 If you recruit your own donors, do you always quarantine sperm samples for >180 days as advised by the ABA, ACE, BAS, BFS, RCOG (2008) guidelines?

☐ YES ☐ NO ☐ Don't know ☐ N/A

If answered no, please explain why not:

2.5 If you screen your own donors for CMV, do you screen them:

☐ Before quarantine begins ☐ After the quarantine ☐Both ☐ Don't know

☐N/A

2.6 In the space below please tell us your views about CMV screening at your centre:

Section 3: CMV screening via serum antibody testing.

3.1 Do you screen donors/patients for CMV by serum antibody testing?

☐ YES ☐ NO *(Go to section 4)*

3.2 Do you carry out serum antibody testing for:

☐ IgG levels ☐ IgM levels ☐ Both ☐ Other ☐ Don't know

3.3 Do you exclude ALL seropositive donors (IgG or IgM)?

☐ YES ☐ NO ☐ Don't know

3.4 Do you exclude sperm donors with positive IgG at the start of the quarantine period?

☐ YES ☐ NO ☐ Don't know

3.5 Do you exclude sperm donors with positive IgG at the end of the quarantine period, if;

a) previously negative**?** ☐ YES ☐ NO ☐ Don't know

b) the IgG antibody titre has increased? ☐ YES ☐ NO ☐ Don't know

3.6 Do you exclude sperm donors with positive IgM at the start of the quarantine period?

☐ YES ☐ NO ☐ Don't know

3.7 Do you exclude sperm donors with positive IgM at the end of the quarantine period, if previously negative?

☐ YES ☐ NO ☐ Don't know

3.8 Please use the space below to tell us your views about CMV screening using serum antibody testing:

Section 4: CMV screening via PCR.

4.1 Do you screen donors/patients for CMV via PCR?

☐ YES ☐ NO *(Go to section 5)*

4.2 Which biological specimen do you carry out PCR analysis on?

☐ Blood ☐ Urine ☐ Semen ☐ Saliva

4.3 Do you carry out qualitative PCR?

☐ YES ☐ NO ☐ Don't know

4.4 Do you exclude sperm donors with the presence of CMV DNA?

☐ YES ☐ NO ☐ Don't know

4.5 Do you carry out quantitative PCR?

☐ YES ☐ NO ☐ Don't know

4.6 If yes, do you determine the viral load of CMV?

☐ YES ☐ NO ☐ Don't know

4.7 Do you have a threshold value of viral load for excluding CMV positive donors / samples?

☐ YES ☐ NO ☐ Don't know

If yes, what is the threshold value?.....

4.8 Please use the space below to tell us your views about the use of PCR to screen for CMV:

Section 5: CMV screening via viral culture.

5.1 Do you screen donors/patients for CMV via viral culture?

☐ YES ☐ NO *(Go to section 6)*

5.2 Which biological specimen do you carry out viral culture on?

☐ Blood ☐ Urine ☐ Semen ☐ Saliva

5.3 Do you exclude samples presenting CMV cytopathic effect after 2-3 days?

☐ YES ☐ NO ☐ Don't know

5.4 In the case of an initially negative result, do you continue the culture to confirm negativity?

☐ YES ☐ NO ☐ Don't know

5.5 Please use the space below to tell us your views about the use of viral culture to screen for CMV:

Section 6: About buying donor sperm from other centres.

6.1 When obtaining donor sperm from other centres, do you check that they have been screened for CMV?

☐YES ☐NO ☐N/A *(don't buy from other centres-Go to section 7)*

6.2 If you obtain donor sperm from other centres, do you check HOW the donors have been screened for CMV?

☐ YES ☐ NO

6.3 Do you know how the recruitment centres screens for CMV (i.e. Serology, PCR or culture)?

☐ YES ☐ NO

6.4 If you obtain donors from other centres, do you check that they have been quarantined for >180 days as advised by the ABA, ACE, BAS, BFS, RCOG (2008) guidelines?

☐ YES ☐ NO

6.5 Do you always request the official copy of the screening results for each donor?

☐ YES ☐ NO ☐ Don't know

6.6 Please use the space below to tell us your views about buying in sperm from other centres with regard to CMV testing:

Section 7: CMV Matching.

7.1 Where applicable, do you match donors with recipients with regards to their CMV status?

☐YES ☐NO ☐N/A *(Don't treat patients-Go to section 8)*

7.2 If yes can you please describe in the space below how you do this?

7.3 Does matching for CMV status cause supply problems with regard to the availability of donors?

☐YES ☐NO ☐Don't know

7.4 If yes, can you please describe this in more detail in the space below?

7.5 When providing treatment to women with a CMV positive donor, do you inform her of the theoretical risk of CMV transmission to the fetus?

☐YES ☐NO

7.6 If yes, what is your experience of women's response to this information? Is this a major concern to them?

Section 8: Your views about CMV and donor sperm treatments.

Please use the space below to tell us your views about the requirements for CMV testing and seromatching that are not covered elsewhere in this questionnaire:

Thank you for taking your time to complete this questionnaire. If you would like to see a copy of the summary results then please tick this box ☐

Appendix V: Example calculation of infectious load (PFU/ml)

1. Volume of virus-supernatant added to plate= 100µl
2. Number of plaques counted= 20
3. Dilution of virus in well= 10^{-3}
4. 20 x 10^{3}= 20,000 plaques in undiluted sample
5. 20,000 x 10= 200,000 plaques in 1ml undiluted sample
6. Infectious load = **$2x10^{5}$ PFU/ml**

Appendix VI: MRC-5 Mycoplasma test results

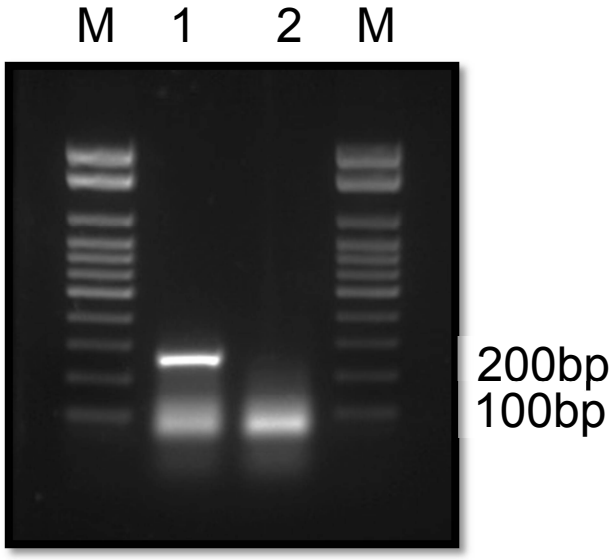


Figure: PCR results showing a negative result for Mycoplasma detection in MRC-5 cell supernatant. Lane 1 shows 1µl of Positive Template Control, used to test the efficiency of the PCR reaction and Lane 2 shows 5µl of MRC-5 cell culture supernatant amplified with 10µl of reaction mix. In comparison to the positive control, the cell culture supernatant reaction does not display a band at ~270bp, confirming that this cell line is not contaminated with Mycoplasma.

Appendix VII: DNA concentration and OD ratios of DNA samples extracted from semen samples in Chapter 5

	Pre-Wash			Post-Wash		
Experiment/Conditions	DNA (ng/µl)	OD 260/280	OD 260/230	DNA (ng/µl)	OD 260/280	OD 260/230
Time (Section 5.4.1)						
1-hour Infected	9.9±5.0	1.8±0.07	0.9±0.4	2.8±1.5	2.2±0.1	0.2±0.1
1-hour Control	8.0±2.5	1.6±0.11	0.7±0.3	2.0±0.7	1.7±0.1	0.5±0.3
6-hour Infected	11.4±6.5	1.9±0.2	0.8±0.3	1.4±0.2	1.8±0.3	0.2±0.1
6-hour Control	14.7±9.4	1.9±0.04	0.8±0.2	3.0±0.5	2.9±0.5	0.4±0.2
ProInsert™ (Section 5.4.2)						
ProInsert™ Infected	9.6±3.9	2.0±0.3	1.4±0.6	6.9±3.7	3.2±1.0	0.3±0.1
ProInsert™ Control	7.1±3.3	1.9±0.1	0.9±0.4	3.6±0.9	2.8±0.4	0.3±0.1
Density Gradient Infected	9.6±3.9	2.0±0.3	1.4±0.6	1.6±0.4	2.0±0.1	0.2±0.1
Density Gradient Control	7.1±3.3	1.9±0.1	0.9±0.4	1.6±0.2	2.3±0.3	0.1±0.0
Naturally Infected (Sections 5.4.3 & 5)						
102 semen samples*	9.3±1.1	1.8±0.05	1.0±0.2	3.0±1.4	1.9±0.2	0.3±0.04
Dose (Section 5.4.4)						
1:1	4.6±0.1	1.9±0.2	0.4±0.1	1.0±0.5	3.0±0.0	0.1±0.1
0.1	5.6±1.7	2.0±0.1	1.6±0.8	3.3±1.9	4.0±1.0	0.2±0.0
0.01	6.0±2.0	1.7±0.1	0.6±0.2	3.6±1.3	2.2±0.6	0.4±0.2
0.001	3.4±0.5	2.0±0.2	0.4±0.2	0.8±0.3	3.0±1.0	0.1±0.1
Control	5.4±1.6	1.6±0.1	0.7±0.2	1.8±0.3	1.8±0.3	0.4±0.3

Data are shown as mean±SEM

*For samples from naturally infected men, 102 samples were included pre-wash and 13 samples post-wash