Honey as an antiviral agent against respiratory syncytial virus

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Abstract

Respiratory syncytial virus is the most frequent cause of hospitalization for viral respiratory infections in infants and young children worldwide. It also severely affects immunocompromised adults and the elderly, however, despite decades of efforts, there is no proven effective treatment for RSV infection and attempts at vaccine development have been hampered by several major obstacles.

A large amount of research has established the potent antibacterial activity of honey, but its activity against viral species has been the subject of only a small number of studies. These were with viruses which cause localised infections in which honey could be used topically. Recent studies demonstrating the safety of intrapulmonary administration of honey in sheep and humans raised the possibility of using honey to treat respiratory infections. The aim of this study, therefore, was to extend the knowledge obtained from previous studies of honey's antiviral activity to its action against RSV.

A variety of tests using cell culture were developed to evaluate the susceptibility of RSV to honey. Each test monitored and scored the development of morphological changes to the cells caused by RSV infection to determine whether the honey had any inhibitory effect on these changes. These included tests for: inhibition, where honey was used to treat infected cells; protection, in which the cells were treated with honey prior to infection; neutralisation, in which the virus was directly exposed to the honey for a defined period before being used to inoculate the cells.

Pre-treatment of the cells had no effect on the consequent development of cytopathic effect, while the inhibition and neutralisation experiments showed a significant inhibitory effect on the progression of infection, suggesting a direct effect on the virus rather than on the cells, however, further studies are required to confirm this.

A wide range of honey types were tested for their inhibitory and neutralising capabilities against RSV and the results suggested that the antiviral activity may be characteristic of more than one type of honey. The activity observed did vary, however, with some types of honey causing greater inhibition of RSV than others.

Enzyme-linked immunosorbent assays were also used to quantitatively measure the number of viral antigens in honey-treated and untreated cells. The results confirmed that treatment with honey had caused inhibition of viral replication, there being very little virus detected in honey-treated cells compared with untreated cells infected with RSV. Experiments using quantitative PCR also demonstrated the inhibitory effect of honey on RSV at the transcription level, with significant differences in the mRNA copy numbers of two out of the three viral genes examined.

Attempts at isolating the antiviral component in honey demonstrated that the sugar was not responsible for the inhibition of RSV, but that methylglyoxal may play a part in the greater potency of Manuka honeys against RSV.

It is concluded from the findings in this study that honey may possibly be an effective antiviral treatment for the therapy of respiratory viral infections, and provides justification for future *in vitro* studies and clinical trials.

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List of Abbreviations

°C	degree celsius
ABTS	(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid))
AM	adrenomedullin
bp	base pairs
BSA	bovine serum albumin
CPE	cytopathic effect
CD	cluster of differentiation
DEPC	diethylpyrocarbonate
DFA	direct fluorescent antibody
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EICAR	(5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide)
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	foetal calf serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GITC	guanidium thiocyanate
HEPES	N-2-(4-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid
HIV	Human immunodeficiency virus
HRP	horse radish peroxidase
Ig	immunoglobulin

IL	interleukin
IMPDH	inosine monophosphate dehydrogenase
kDa	kilo Dalton
MGO	methylglyoxal
MHC	Major histocompatibility complex
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NF-κB	nuclear factor kappa B
NK	natural killer cells
NPA	non-peroxide activity
ODN	phosphorothioate oligodeoxyribonucleotides
OPD	o-phenylenediamine dihydrochloride
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEFR	peaked expiratory flow rate
PIV	parainfluenza virus
RSV	respiratory syncytial virus
SB	sodium boric acid-based conductive medium
SDS	sodium dodecyl sulfate
siRNA	short interfering double-stranded RNA molecules
$TCID_{50}$	50% Tissue Culture Infective Dose
TE	Tris/EDTA
TEAC	Trolox equivalent antioxidant capacity
TLR	Toll-like receptor
$TNF\alpha$	Tumor Necrosis Factor α
Tris	(hydroxymethyl)aminomethane
Tween 20	polyoxyethylene 20 sorbitan monolaurate
UMF	Unique Manuka Factor

Chapter One

Introduction

Over the last few decades, there have been many investigations looking at the antibacterial properties of honey, but only a small number have investigated its antiviral properties. These few have demonstrated the antiviral effects of honey against a number of viruses for which current treatment may not be very effective or may be known to cause adverse side effects. It was thus desirable that the activity of honey be tested against other viral pathogens, to determine its potential as a treatment and to expand the field of knowledge in this area of study. One such pathogen is respiratory syncytial virus, a respiratory virus causing millions of hospitalisations and hundreds of thousands of deaths worldwide annually, and for which there is currently no vaccine or effective treatment. Ribavirin is sometimes used in hospitals, but only in the most severe cases, as studies have failed to establish its efficacy in infected individuals. A study demonstrating the safety of intrapulmonary administration of honey into sheep and humans indicated that using it as a therapy for RSV infection was a feasible prospect.

The second chapter of this thesis provides background information on respiratory syncytial virus and the history and uses of honey. Other early chapters describe experiments performed which aimed to observe the different aspects of the activity of honey against RSV including its inhibitory, protective and neutralising properties. These experiments involved utilising cell culture to perform a number of investigations which examined the effects of honey on the virus, and a number of molecular techniques were used to confirm the effects observed as changes in cell morphology.

Differences in the antiviral activity of different honey types were also investigated.

The later chapters of this thesis describe work in which it sought to determine the mechanism of action of honey, and experiments were performed to investigate the effects of honey on the replication kinetics of RSV. This was done by comparing the amount of transcripts for viral genes detected in RSV-infected samples which had been incubated with or without honey. Several experiments were also conducted with the aim of isolating the component in honey responsible for the observed activity. These included tests with the sugar, hydrogen peroxide and methylglyoxal components of honey.

The final chapter of this thesis discusses the relevance and importance of the findings from the previous chapters, and provides suggestions for future investigations in this area of study.

Chapter Two

Literature Review

2.1 Respiratory Syncytial Virus

2.1.1 Introduction and history

Respiratory syncytial virus (RSV) was first isolated in 1956 from a laboratory chimpanzee with an illness resembling the common cold and later identified to be a human pathogen. RSV is a member of the subfamily *Pneumovirinae* in the family *Paramyxoviridae*, order *Mononegavirales* (Cowton *et al.*, 2006). Worldwide, it is implicated in the majority of lower respiratory tract infections for which newborns require treatment and often hospitalization (Hall *et al.*, 1976). RSV disease manifestations include rhinitis, bronchiolitis and pneumonia as well as being a likely contributor to otitis media in older children (Ruuskanen *et al.*, 1989). RSV can also cause significant disease in the elderly and is associated with higher mortality rates than influenza in non-pandemic years (Falsey *et al.*, 1995). Even though maternal antibodies are usually present, disease caused by RSV is specifically severe in children between 1 and 3 months old (Kim *et al.*, 1973). Reinfection is also common in all age groups (Murphy *et al.*, 1994). Despite the importance of RSV being recognised for over 45 years, a licensed vaccine is still currently unavailable (Maggon & Barik, 2004). Several issues have been faced during its development, such as adverse reactions in the form of "vaccine-enhanced disease", of which will be discussed in a later section in this thesis.

2.1.2 Virion structure and viral proteins

RSV is an enveloped virus with a single-stranded, negative-sense, non-segmented RNA genome composed of approximately 15 222 nucleotides and 10 genes which encode 11 proteins (Falsey & Walsh, 2000) as exemplified in Figure 2.1. It ranges in size range from 120 to 300 nm and isolates can be segregated into two subgroups, A and B. In the RSV particle, the genome is contained within the helical viral protein N to form the nucleocapsid, packaged in a lipoprotein envelope acquired from the host cell plasma membrane during budding. The outer surface of the envelope contains a fringe of surface projections of around 12 nm (Collins, 2008). Human RSV lacks a hemagglutinin or a neuraminidase (Collins, 2008). The genome encodes three transmembrane surface proteins (F, G, SH), a matrix protein (M), the nucleocapsid protein (N), nucleocapsid-associated proteins (M2-1, P, L), a M2-2 protein, and two non-structural proteins (NS1 and NS2) (Maggon & Barik, 2004).

Figure 2.1 The human RSV RNA genome illustrated 3' to 5' as published by Leaman (2005)
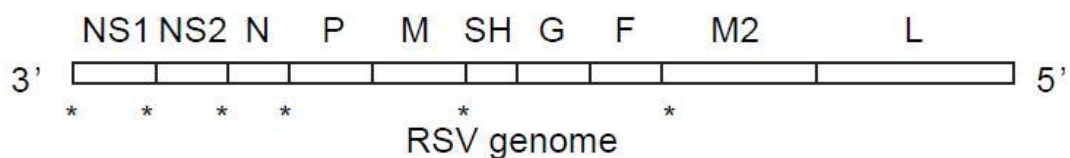
The G protein is a type II transmembrane protein with an N-terminal transmembrane domain. It is also found in a smaller secreted form that lacks the transmembrane region and is produced by a translation initiation site at a second AUG in the mRNA, followed by proteolytic trimming (Roberts *et al.*, 1994). The G protein is responsible for attachment of the virus particles to the host cell, although this function can also be provided by the F protein (Levine *et al.*, 1987; Karron *et al.*, 1997a). The F protein promotes entry of the

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virus particles into the host cell by fusion with the target cell plasma membrane, as well as the spreading of virus between cells by cell-to-cell fusion (Walsh & Hruska, 1983). The antigenic diversity in human RSV is associated with a high degree of amino acid heterogeneity in the G protein sequence with only 53% identity in sequences between the two subgroups (Johnson *et al.*, 1987). Within subgroups, the G protein is also highly variable, with 20% amino acid variability between subgroup A strains (Cane & Pringle, 1995) and 9 % variability between subgroup B strains (Sullender *et al.*, 1990).

The SH protein of RSV is a type II transmembrane protein containing 64 (subgroup A) or 65 (subgroup B) amino acid residues (Collins & Wertz, 1985; Olmsted & Collins, 1989; Collins *et al.*, 1990; Collins & Mottet, 1993). Some studies have suggested that the RSV SH protein may have a role in viral fusion (Heminway *et al.*, 1994; Techaarpornkul *et al.*, 2001) or in changing membrane permeability. However, it has been shown that RSV lacking the SH gene is viable, causes syncytium formation and grows just as well as the wild-type virus (Bukreyev *et al.*, 1997; Karron *et al.*, 1997a; Webster *et al.*, 2002), indicating that the SH protein is not necessary for virus entry into host cells or syncytium formation. Parainfluenza virus 5, a prototypical paramyxovirus, also encodes an SH protein which inhibits tumor necrosis factor alpha (TNF-α) signalling. In a study by Fuentes *et al.* (2007), recombinant PIV5 viruses without their own SH but containing RSV SH in its place (from A2 or B1 strains), and RSV lacking its own SH were generated and analyzed. The results indicate that the SH protein of RSV has a function similar to that of PIV5 SH and that it can inhibit TNF-α signalling.

The matrix protein M is an envelope-associated protein and is required for viral assembly. The nucleoprotein N, phosphoprotein P and large

polymerase fragment L are found in the nucleocapsid, and together with the co-factors M2-1 and M2-2 form the RNA dependent RNA polymerase complex (Yu *et al.*, 1995). M2-1 and M2-2 are both involved in viral RNA synthesis, but only M2-1 is essential (Fearns & Collins, 1999). NS1 and NS2 are non-structural viral products that accumulate in infected cells but are present only in trace amounts in the mature virion (Olmsted & Collins, 1989). They also antagonise the α/β IFN response in a species-specific manner (Schlender *et al.*, 2000; Bossert & Conzelmann, 2002; Bossert *et al.*, 2003).

2.1.3 Antigens and antigenic subgroups

Human RSV has a single serotype. However, isolates can be segregated into two serological subgroups, A and B (Kesson, 2007). Although the sequences of all 11 viral proteins differ to one extent or another between these two groups, the G protein shows the greatest divergence between prototype A and B viruses (Sugawara et al., 2002; Teng & Collins, 2002). Epitopes in F tend to be conserved while those for G are not, such that antigenic relatedness between the two subgroups is greater than 50% for the F protein compared to only 5% or less for the G protein. At the amino acid level, F is 89% identical between subgroups while G is the most divergent of the proteins and is only 53% identical. Other proteins range from 76% (SH) to 96% (N) identical. Thus, the two antigenic subgroups have substantial differences throughout the genome and represent two divergent lines of evolution. In a two-year window following infection, there was a 64% reduction in the incidence of infection by the same subgroup versus a 16% reduction against the heterologous subgroup. In epidemics, there is typically an alternating pattern with a 1-2 year interval with regard to the predominant subgroup (Collins & Graham, 2008). Several investigations have reviewed the regional and global epidemiology of these strains (Cane *et al.*, 1994; Sullender, 2000; Crotty & Andino, 2002; Hacking & Hull, 2002). The

impact of antigenic diversity on RSV epidemiology is not yet completely understood, but may partly explain the susceptibility to reinfection throughout life and also the yearly variation observed in the severity of epidemics within communities (Cane *et al.*, 1994; Sullender, 2000; Crotty & Andino, 2002).

2.1.4 Epidemiology and clinical presentation

Human RSV is recognised as an important pathogen in young children, immunocompromised adults and in the elderly. It is estimated by the World Health Organization (2010) to cause around 64 million infections and 160 000 deaths annually in the pediatric population. It infects up to 65% of babies in their first year of life and essentially all babies within their first two years of life (Maggon & Barik, 2004). Hospitalization rates in developed countries are approximately one in every 100 to 200 infections. RSV is responsible for the admission of 0.1% to 2% of babies under the age of 1 to hospital each year for bronchiolitis and among these, mechanical ventilation is needed in 2% to 5% (Leader & Kohhase, 2002). RSV infection also increases the risk for severe illness in children with underlying problems such as bronchopulmonary dysplasia, chronic lung disease, congenital heart disease and babies born prematurely (Cane, 2001; Maggon & Barik, 2004).

Primary infantile RSV infection typically presents as a winter upper respiratory tract infection followed by mild lower respiratory tract symptoms in about 40% of cases (Openshaw & Tregoning, 2005), however, there have been a number of studies on RSV in developing countries such as South Africa (Madhi *et al.*, 2003), Indonesia (Djelantik *et al.*, 2003), and the Gambia (Weber *et al.*, 2002), where RSV infections are usually seasonal and not necessarily most frequent at the coldest time of year (Stensballe *et al.*, 2003). High sibling number remains an important risk factor in all settings,

however, some risk factors play a more important role in developing countries and include, for example, the presence or absence of a flushing toilet and exposure to cooking fires (Weber *et al.*, 1999). Young children aged 8 to 30 weeks tend to experience more severe illness, however, RSV infections usually pass in less than a week (Openshaw & Tregoning, 2005). Very few previously healthy children suffer life-threatening infections, and deaths are mostly confined to immunocompomised individuals and those who have pre-existing cardiorespiratory disease (Ruuskanen & Ogra, 1993; Henrickson *et al.*, 2004).

Although more well-recognized as a pediatric pathogen, RSV is also known to cause life-threatening pulmonary disease in immunocompromised adults such as bone marrow transplant recipients and sufferers of cystic fibrosis. The elderly are also at high risk of pulmonary disease: RSV attack rates in nursing homes in the USA are approximately 5% - 10% per year with a 2% - 8% case fatality rate, amounting to approximately 10 000 deaths per year among persons over 64 years of age (Falsey & Walsh, 2005). RSV has a major impact on humans, claiming around 3 to 5 million live annually (Cane, 2001).

In experimental infections, RSV has an incubation period of 4 to 5 days. Virus is typically shed for 7 to 12 days, although sometimes shedding is longer and may continue after recovery. Most primary infections are symptomatic, with upper respiratory tract disease and sometimes, a fever. Twenty-five to forty percent of infections progress to the lower respiratory tract with the primary manifestations of serious disease being pneumonia or bronchiolitis (Collins, 2008). Symptoms include profuse rhinorrhea, coughing, fever, wheezing and middle-ear disease and seriously ill infants may experience severe coughing, wheezing, rapid respiration and hypoxemia, requiring the administration of humidified oxygen. RSV causes

yearly epidemics centered in the winter months in temperate climates, or in the rainy season in the tropics, though there may be some local variations to this pattern (Collins, 2008).

2.1.5 Pathogenesis

Inoculation occurs at the nasal or ocular mucosa via direct contact with secretions or infected fomites (Hall & Douglas, 1981; Hall *et al.*, 1981). Both droplet and contact transmissions are the main methods of spread, and thus hand washing, droplet isolation, and the use of personal protective equipment are all important in reducing the spread of RSV.

The virus first infects the epithelial cells of the upper respiratory tract and primarily replicates in the epithelial cells that line the lumen of the respiratory tract, as well as macrophages and dendritic cells. Infection then spreads to the epithelium of the bronchioles and from the bronchioles, the virus can then extend to the type 1 and 2 alveolar pneumocytes (Aherne *et al.*, 1970; Johnson *et al.*, 2007). RSV has a direct cytopathic effect on cells in the lung epithelium, leading to loss of specialized functions such as cilial motility and sometimes to epithelial destruction (Aherne *et al.*, 1970). A peribronchiolar mononuclear cell infiltrate also forms, accompanied by submucosal edema and mucus secretion. The inflammation leads to bronchiolar obstruction with patchy atelectasis and compensatory emphysema (Gardner *et al.*, 1970). Mucus, inflammatory cells and debris from dead infected cells often accumulate in the airways and cause obstruction, which is of special concern in infants due to the small diameter of their airways (Collins, 2008). RSV pathogenesis *in vitro* is normally characterised by syncytium formation which is seldom seen *in vivo*. However, this varies considerably from one strain to another (Shigeta *et al.*, 1968).

Human RSV has been shown to have an incubation period of 4 to 5 days in experimental infections (Collins, 2008). Virus is typically shed for 7 to 12 days, although sometimes shedding is longer and may continue after recovery. Most primary infections are symptomatic, with upper respiratory tract disease and sometimes, a fever. Symptoms may include rhinorrhea, coughing, fever, wheezing and middle-ear disease and seriously ill infants may experience severe coughing, wheezing, rapid respiration and hypoxemia, requiring the administration of humidified oxygen (Collins, 2008). Twenty-five to forty percent of infections progress to the lower respiratory tract with the primary manifestations of serious disease being pneumonia or bronchiolitis (Collins, 2008). A study has shown that children who experienced severe RSV bronchiolitis during infancy had a significantly higher rate of asthma than age and sex-matched controls (Sigurs, 2001). In this same cohort studied at 13 years of age, asthma had been diagnosed in 37% of the RSV bronchiolitics and only 5.4% of the control group (Sigurs *et al.*, 2005). The RSV group had a significantly higher occurrence of symptoms over the previous 12 months than among the control subjects, 43% versus 8% for asthma/recurrent wheezing and 39% versus 15% for allergic rhinoconjunctivitis. Sensitization to common inhaled allergens was also more frequent in the RSV group, judged by skin prick tests (50% versus 28%; p = 0.022), or by serum IgE antibodies (45% versus 26%; p = 0.038). The RSV group also showed mild airway obstruction both at rest and after bronchodilation, and had slightly more reactive airways compared with their control counterparts.

2.1.6 Immune response

RSV is an acute infection that is typically cleared completely by host immunity, although shedding may sometimes persist for weeks. Studies in experimental animals and in the clinic have indicated that immunity to the

virus is mediated via humoral and cellular effectors, including serum antibody, secretory antibody and major histocompatibility complex class I and class II-restricted cytotoxic T lymphocytes (Maggon & Barik, 2004). These same effectors are also thought to confer protection against reinfection. Protection conferred by cytotoxic T lymphocytes appears to diminish within several weeks or months and is more important in the short term. Virus-neutralizing secretory IgA antibodies are effective in restricting infection, however this response is also short-lived. Virus-neutralizing serum antibodies are thought to provide longer lasting protection, however, due to the poor method by which they gain access to the respiratory lumen, this protection is not thought to be very efficient (Maggon & Barik, 2004).

The innate immune response is the body's first line of defense against infectious diseases. A specific group of proteins that comprise the Toll or Toll-like family of receptors perform this role in vertebrate and invertebrate organisms. One study demonstrated that the F glycoprotein of RSV activated TLR4, most likely through interaction with the surface molecule CD14+, which is expressed primarily on monocytes (Kurt-Jones et al., 2000). In human monocytes stimulated *in vitro* with F protein purified from the RSV A2 strain, TLR4 was involved in the release of interleukin (IL)-6, IL-8 and IL-1b (Kurt-Jones et al., 2000). In addition, Haynes *et al.* (2001) report that RSV-challenged mice deficient in TLR4 experienced a decrease in CD14+ and NK cell trafficking to the lungs, impaired NK cell activity, reduced IL-12 expression, and decreased ability to clear virus compared to mice expressing TLR4. However, RSV-specific cytotoxic T lymphocyte responses were similar in both groups *in vitro* (Haynes et al., 2001), suggesting that mechanisms of viral clearance other than MHC class I perforin-mediated lysis of infected cells may be important in RSV infection control.

RSV infection exerts its effects on the TLR4/innate immunity pathway by triggering NF-κB nuclear translocation followed by the binding and activation of target genes (Haeberle et al., 2002). Nuclear extracts from the lungs of C3H/HeJ mice, lacking a functional TLR4 signaling pathway, and control mice were examined for NF-κB binding activity and it was found that early after inoculation, neither live nor UV-inactivated RSV induced significant nuclear translocation of NF-κB in TLR4-deficient mice. Conversely, nuclear translocation of NF-κB subunits RelA, p50 and c-Rel was detected in control mice 30 minutes after RSV infection and was reduced by the depletion of alveolar macrophages. Subsequently, late NF-κB binding was detected in TLR4-sufficient and -deficient mice at 24 hours only after live RSV inoculation, and was not affected by depletion of alveolar macrophages (Haeberle et al., 2002). These findings suggest that there may be at least two distinct RSV-inducible NF-κB responses in the lungs of RSV-infected mice: an early response that is TLR4- and AM-dependent and does not require virus replication and a later TLR4- and AM-independent response, probably affecting respiratory epithelial cells and requiring RSV replication (Haeberle et al., 2002). Considerable evidence suggests that innate immune inflammation plays a role in RSV pathogenesis, however, the role of the innate immune response during RSV infection requires further study.

The immune response during the first months of life has been shown to be reduced due to immunologic immaturity as well as the immunosuppressive effects of the maternal antibodies on humoral responses (Collins, 2008). This may facilitate reinfection in early life. The Th2-biased nature of immune responses in early infancy has also been speculated to contribute to human RSV disease and reduced immune responses. The tropism of RSV to the superficial epithelium may also be reducing its exposure to immune effectors and may also be providing reduced immune stimulation (Collins, 2008). The

lack of protection may also be explained by group specificity, since children with primary RSV infection develop a neutralising antibody of greater frequency and magnitude to the infecting strain than to a heterologous RSV strain (Maggon & Barik, 2004).

2.1.7 Vaccines

Numerous obstacles have prevented the development of an effective RSV vaccine. In the 1960s, a formalin inactivated RSV vaccine was studied in human infants for the first time. Not only did it fail to protect against subsequent wild-type RSV disease, it also induced an exaggerated clinical response, causing as many as 80% of vaccinated children to be hospitalized and two infant deaths (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kapikian *et al.*, 1969; Kim *et al.*, 1969). Infiltration of excess eosinophils into the peribronchial spaces and deposits of non-protective antibodies complexed with virus in affected tissue were blamed for the vaccine-enhanced disease (Chin *et al.*, 1969; Fulginiti *et al.*, 1969; Kapikian *et al.*, 1969; Kim *et al.*, 1969; Murphy *et al.*, 1986; Polack *et al.*, 2002).

 As evidenced by natural reinfection, it is thought to be unlikely that a single vaccination will impart complete protection against RSV disease. The goal for a successful vaccine should therefore be to prevent serious RSV associated lower respiratory tract infections in those individuals at risk (Collins & Murphy, 2005). RSV vaccine development is currently focused on live, attenuated strains for intranasal administration. This strategy accomplishes several goals: it induces local mucosal and systemic immunity, the intranasal route partially escapes the suppressive effects of maternal serum antibodies (Crowe *et al.*, 1995), and, compared with inactivated vaccines, live intranasal vaccines are more immunogenic and provide broader protection (Johnson *et al.*, 1986).

The balance between attenuation and immunogenicity is critical in vaccine development. Live vaccine candidates have been developed using serial passages at decreasing temperatures (cold passage) and chemical mutagenesis to produce temperature sensitive mutants. These viruses will replicate at the low temperatures of the upper respiratory tract but not at the high temperatures of the lower respiratory tract (Collins & Murphy, 2005). Initial vaccine candidates developed using these attenuation methods were found to be either too reactive or overattenuated, and mutations were often unstable (Karron *et al.*, 1997b; Wright *et al.*, 2000). The latest strategy to effectively attenuate RSV is through reverse genetics (Collins & Murphy, 2005), which involves producing infectious virus from cloned complementary DNAs in cell culture and introducing targeted mutations to achieve more precise levels of attenuation while maintaining sufficient immunogenicity (Collins *et al.*, 1995; Firestone *et al.*, 1996).

Recombinant RSV A2 cp248/404/1030/ΔSH is a recombinant temperature-sensitive RSV with a deletion of the SH gene (Karron *et al.*, 2005). The SH protein has been shown to decrease T_H1 responses, inhibiting the host antiviral response as a result. A virus lacking the SH protein would therefore impart greater immunogenicity (Tripp *et al.*, 1999). It is the first vaccine candidate to be sufficiently attenuated for young infants. A phase 1/2a study is currently recruiting healthy children between the ages of 1 and 24 months to evaluate immunogenicity, viral shedding, safety, and tolerability (Clinicaltrials.gov, 2010). Other vaccine candidates under development using these attenuation strategies include recombinant RSV A2 cpts248/404/ΔNS2 and recombinant RSV A2 cpts530/1009ΔNS2, which include a deletion in the NS genes. The NS protein decreases type I interferon signalling, thus inhibiting host response (Ramaswamy *et al.*, 2006). Similar to SH deletions, virus lacking the NS proteins will be more immunogenic. There is still

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concern regarding the genetic stability of these vaccine candidates, however, highly attenuating gene deletion vaccines have been developed to address this. These include ΔNS1, ΔM2–2, and ΔM2– 2NS2 which all maintained a high level of immunogenicity when evaluated in chimpanzees (Ramaswamy *et al.*, 2006). They were also shown to induce protection after wild-type RSV challenge. Further evaluation in humans is needed (Teng *et al.*, 2000; Cheng *et al.*, 2001; Jin *et al.*, 2003).

An alternative method for overcoming genetic instability while maintaining immunogenicity is through the delivery of RSV proteins using viruses with greater growth and stability (Collins & Murphy, 2005). The vector vaccine candidate recombinant bovine/human parainfluenza virus type 3 (PIV3)/ RSV F2 delivers RSV F using a bovine/human chimeric parainfluenza type 3 genome. This candidate was shown to protect monkeys against challenge with wild-type RSV and generated high titers of RSV and human PIV3-neutralizing antibodies (Tang *et al.*, 2004). Safety was demonstrated in a phase 1 study of RSV-seropositive adults, however, further studies are needed to determine its safety in children (Tang *et al.*, 2008). Other viruses engineered to express RSV F and/or G glycoproteins include the Newcastle disease (Martinez-Sobrido *et al.*, 2006) and Sendai viruses (Takimoto *et al.*, 2004), both of which have demonstrated protection in rodent models.

Purified RSV F, G, and M proteins have been evaluated for their potential to induce neutralizing and protective antibodies. The following subunit vaccines have advanced to clinical trials: (1) Three RSV F subunit vaccines (purified F protein 1–3) (Wathen *et al.*, 1991; Simoesa *et al.*, 2001); (2) a combined subunit vaccine containing F, G, and M proteins (Sanofi Pasteur) (Falsey *et al.*, 2008); and (3) BBG2Na, a G peptide conjugated to streptococcal protein G (Power *et al.*, 2001). Only modest rises in antibody titers have been

observed in seropositive populations and their safety and efficacy in RSV-naive infants and young children is yet to be determined. Drawbacks to this vaccine approach include poor immunogenicity, immunosuppressive effects of maternally acquired antibodies, and potential for vaccine-enhanced disease (Empey *et al.*, 2010).

2.1.8 Potential therapeutics

Inhibitors of RSV that have recently been evaluated for efficacy fall into six groups classified according to their modes of action: those that inhibit attachment/fusion, oligonucleotides that target viral RNA, those that target the N-protein, those that inhibit some function of the virus RNA-dependant RNA polymerase, those that inhibit inosine monophosphate dehydrogenase (IMPDH), and also antibody preparations which interfere with pathogen binding, and facilitate opsonization, neutralization, and clearance of viruses. Currently, only ribavirin – an inosine monophosphate dehydrogenase inhibitor – is being used in hospitals. It is only used in the most severe cases as studies have been unable to prove its efficacy.

2.1.8.1 Attachment/fusion inhibitors

Fusion is a critical step in the life-cycle of RSV and the inhibition of this step has been shown to lead to a reduction in viral load and syncytia formation (Colman & Lawrence, 2003; Bonfanti & Roymans, 2009). The F and G proteins are involved in virus attachment and fusion, although it has been shown that the F protein alone is sufficient to promote attachment to cells, subsequently leading to a productive viral infection (Karron *et al.*, 1997a). The G protein may simply enhance attachment to a target cell, but the F protein is thought to bind to a specific receptor (Techaarpornkul *et al.*, 2001). On viral coalescence with target cell membranes, the F glycoprotein

undergoes a conformational change exposing hydrophobic pockets or epitopes (Lawless-Delmedico *et al.*, 2000). As a result, binding of these exposed targets by RSV fusion inhibitors prevents viral entry in the host cell (Zhao *et al.*, 2000). Several small-molecule fusion inhibitors have already been screened, each targeting a slightly different epitope within the F glycoprotein, however, attempts at development of these compounds have met an extremely high rate of failure, and only a few are still viable candidates for licensing. None are currently in advanced stages of clinical trials.

a) BTA9881

BTA9881 is a prototype compound from a new class of RSV fusion inhibitors (imidazoisoindole derivatives) belonging to Biota (AstraZeneca). The compound had very high oral bioavailability and favorable pharmacokinetics in phase I clinical trials, but failed to develop an acceptable safety profile (Luttick *et al.*, 2007). Rights to the compound have since been returned by AstraZeneca to Biota, who will attempt to develop more attractive derivatives (Welliver, 2010).

b) TMC-353121

TMC-353121, a compound under development by Tibotec (Johnson & Johnson), has previously been shown to have picomolar activity against various RSV strains when given to cotton rats by intravenous, oral and aerosol routes. This compound is a morpholinopropylaminobenzimidazole derivative of JNJ-2408068 (a benzimidazole with potent anti-RSV activity), with the revision of the compound prolonging its halflife (Bonfanti *et al.*, 2008). The derivative inhibits both virus–cell fusion and cell–cell fusion specifically for RSV, with no activity against other Paramyxoviridae (Andries

et al., 2003). The drug enters the lung and is active at 10 nmol/l concentrations, with very low levels outside the lung (Wyde *et al.*, 2003). The drug also inhibits the release of IL-6, IL-8 and RANTES from RSV-infected A549 cells (Andries *et al.*, 2003).

c) VP-14637

VP-14637 was one of the first fusion inhibitors to progress to Phase I clinical trials. It is a triphenolic compound which is thought to bind into the small hydrophobic cavity in the inner core of the F-protein, either preventing early transient Z conformation changes in the fusion process or by preventing formation of six-helix fusion core as the heptad repeats interact (Douglas *et al.*, 2005). VP-14637 is a broad-spectrum inhibitor of RSV strains, inhibiting the virus *in vitro* at concentrations of 2 nmol/l or less (Douglas *et al.*, 2005; Wyde *et al.*, 2005). In cotton rats, treatment by small droplet aerosol for 60 minutes significantly reduced mean lung virus titers (Wyde *et al.*, 2005). VP-14637 was in phase I trials prior to a decision by ViroPharma to discontinue its development, mainly due to strategic reasons and developmental costs (Welliver, 2010).

d) BMS-433771

Benzotriazole benzimidazoles represent another class of inhibitors that prevent fusion of RSV to host cell membranes. In particular, BMS-433771 (1-cyclopropyl-1,3-dihydro-3-[[1-(3-hydroxypropyl)-1H-benzimidazol-2-yl]methyl]-2H-imidazo[4,5-c]pyridin-2-one), an azabenzimidazolone derivative, targets the hydrophobic pocket within the trimer of hairpins of the F-1 protein, a class I fusion protein (Cianci *et al.*, 2004b). It is thought to interfere with the normal association of the N and C-terminal heptad repeats found within the binding pocket that occur as part of the fusion process

(Cianci *et al.*, 2005). BMS-433771 is active against multiple RSV strains, with a 50% inhibitory concentration of 20nM (Cianci *et al.*, 2004b). In a T cell-deficient BALB/c mouse model, the orally active compound also significantly reduced virus titers in the lungs and the compound was well tolerated (Cianci *et al.*, 2004c), however, it was found to be somewhat less inhibitory to lung virus replication in cotton rats (Cianci *et al.*, 2004a). It had progressed to Phase I/II clinical trials before being discontinued (Empey *et al.*, 2010).

e) RFI-641

RFI-641 is a biphenyl triazine anionic compound that is an analog of CL-309623, a previously identified dendrimer-like stilbene inhibitor with anti-RSV activity (Gazumyan *et al.*, 2000). Like its parent compound, RFI-641 inhibits RSV fusion mediated by the F protein by directly interacting with that protein (Razinkov *et al.*, 2002). The compound inhibited both A and B strains of RSV with EC_{50} values in the 20 nmol/l range. It was also found to be relatively non-toxic (Douglas, 2004). The compound has been evaluated extensively in small animal models (Huntley *et al.*, 2002), and in African green monkeys (Weiss *et al.*, 2003). In the mouse, RFI-641 at 1.3 mg/kg delivered intranasally 2 hours prior to virus exposure resulted in the reduction of lung virus titers by 1.5 $\log_{10}$ plaque-forming units. Using a similar prophylactic dosing regimen in cotton rats, doses up to 10 mg/kg appeared to reduce lung virus titers by >3 $\log_{10}$. RFI-641 prophylactically dosed at 6 mg/kg also appeared to reduce nasal virus titers by >3.4 $\log_{10}$ over a period of ten days in the primate. Using intranasal administration, lung virus titers were only substantially reduced after a two hour exposure to the compound (Weiss *et al.*, 2003). Nasal virus titers were also significantly reduced when the compound was given 24 hours after virus exposure with daily doses thereafter (Huntley *et al.*, 2002). RFI-641 reached Phase II clinical trials in 2000 to 2001, but has since been discontinued (Empey *et al.*, 2010).

f) MBX 300 (NMSO-3)

MBX 300 is a compound which targets the G protein and seemingly targets the attachment phase. It is RSV-specific (Kimura *et al.*, 2000) with EC_{50} values of 0.2 to 0.3 µmol/l (Douglas, 2004). Lung virus titers were significantly reduced in cotton rats when the compound was administered intraperitoneally at 100 mg/kg/day, 3 days post-virus exposure (Douglas, 2004). According to Microbiotix, MBX 300 has undergone preliminary toxicology studies, including testing in Cynomolgus monkeys and has specific and potent oral anti-RSV activity as well as an excellent safety profile (Douglas, 2004; Sidwell & Barnard, 2006; Empey *et al.*, 2010). It is still currently undergoing preclinical tests.

2.1.8.2 Oligonucleotides that target viral RNA (antisense/siRNA)

Much has been reviewed regarding the theory (Maggon & Barik, 2004), approaches used (Cramer, 2005), and attempts to develop antisense oligonucleotides (Leaman, 2005) as therapies for RSV disease. These attempts have included antisense phosphorothioate oligodeoxyribonucleotides (ODN) (Jairath *et al.*, 1997), short interfering double-stranded RNA molecules (siRNA) (Bitko *et al.*, 2005), and 2–5 °A antisense chimeras (Barnard *et al.*, 1999; Torrence, 1999). One phosphorothioate ODN targeted to repetitive intergenic sites with the RSVgenome appeared significantly more effective against the virus (Jairath *et al.*, 1997), however, development did not proceed into the clinic, due, in part, to side effects observed (Siddiqui-Jain *et al.*, 2002). Much study is underway with the siRNAs; a recent report has showed that intranasal instillation of an *in vitro*-active siRNA into RSV-infected mice was significantly inhibitory to the infection (Bitko *et al.*, 2005). Treatment begun four hours before the virus infection reduced the lung virus titers by 3 $\log_{10}$ and prevented pulmonary pathology from developing. When therapy began

after virus exposure, the antiviral effect was progressively less, but continued to lower the virus titers.

ALN-RSV01 is an siRNA directed against the mRNA encoding the N-protein of respiratory syncytial virus (RSV) that exhibits specific *in vitro* and *in vivo* anti-RSV activity. The results of two safety and tolerability studies with ALN-RSV01 involving 101 healthy adults (65 active, 36 placebo, single- and multiple dose, observer-blind, randomized dose-escalation) have been described. Intranasal administration of ALN-RSV01 was well tolerated over a dose range up through 150 mg as a single dose and for five daily doses. Adverse events were similar in frequency and severity to placebo (normal saline) and were transient, mild to moderate, with no dose-dependent trend. The frequency or severity of adverse events did not increase with increasing ALN-RSV01 exposure. All subjects completed all treatments and assessments with no early withdrawals or serious adverse events. Systemic bioavailability of ALN-RSV01 was minimal. ALN-RSV01 appeared safe and well tolerated when delivered intranasally (DeVincenzo et al., 2008). It has completed Phase I of clinical trials and Phase II trials are currently ongoing (Empey, 2010).

2.1.8.3 N-protein inhibitors

RSV-604 is a 1,4-benzodiazepine derivative (Kelsey *et al*., 2004), found to be unique in that it is thought to target the N-protein of RSV (Wilson *et al*., 2004). The *in vitro* inhibitory activity is in the submicromolar range for both A and B strains of RSV (Wilson *et al*., 2004). The compound was found to be safe and well tolerated without any serious adverse effects in Phase I clinical trials in the United Kingdom (Empey *et al*., 2010). Pharmacokinetic studies from this trial suggested that once daily dosing was feasible. The compound

has now entered Phase II clinical trials to evaluate the antiviral effect of nasal and oral administration versus placebo in post-stem cell transplant patients and to assess the safety of the product (Empey *et al.*, 2010).

2.1.8.4 RNA-dependent RNA polymerase inhibitors

A number of inhibitors were synthesized which targeted the guanylylation of viral transcripts (5′ cap) of the RSV ribonucleoprotein complex (Liuzzi *et al.*, 2005). These imidazo[4,5-h]isoquinoline-7,9-dione inhibitors may bind to a region in the L protein with similarities to nucleoside-diphosphate kinases (NDK) motifs, proteins which play a role in maintaining the balance of intracellular nucleotide pools. These compounds demonstrated inhibition of RSV replication in an ELISA-based assay, with EC_{50} values ranging from 0.021 to 2.1 µmol/l. Selectivity indices ranged from 30 to 400. In a mouse model, lung virus titers were reduced when the compounds were administered intranasally 3 and 6 hours after virus exposure, then three times daily for 3 days at 0.4 - 4.1 mg/kg/day. A novel benzazepine inhibitor of the L-protein was recently discovered from a large chemical library and designated as YM-53403 (Sudo *et al.*, 2005). This compound inhibited RSV replication at 0.2 µmol/l in a plaque-reduction assay, however, mutant viruses with single point mutations in the L protein were resistant to the antiviral effects of the compound (Sudo *et al.*, 2005). Timing studies suggested that inhibition was maximal at around 8 hours after virus exposure. YM-53403 is currently under ongoing preclinical trials (Empey *et al.*, 2010).

2.1.8.5 Inosine monophosphate dehydrogenase inhibitors

Ribavirin is the only antiviral drug currently approved for treatment of RSV infections. It has inhibitory effects on a very broad spectrum of viruses,

including RSV (Sidwell *et al.*, 1972). The mechanism of viral inhibition by the drug includes inhibition of Inosine-5′-monophosphate dehydrogenase (IMPDH), an enzyme which catalyzes the conversion of inosine monophosphate to xanthosine monophosphate. This reaction is an essential step in the *de novo* biosynthesis of guanine nucleotides leading to DNA and RNA synthesis. Inhibition of IMPDH thus reduces the amount of intracellular guanine nucleotides needed for the synthesis of RNA and DNA. This may consequently result in significant antiviral effects, although such effects may also be associated with inhibition of cell replication. It may also be involved in inhibition of the 5′ cap formation of mRNA, and inhibition of viral polymerase by the phosphorylated forms of the compound, although the specific mechanism by is not well documented (Sidwell, 1996). In early clinical studies, significant positive effects in RSV-infected infants were reported using ribavirin administered in a small-particle aerosol (Taber *et al.*, 1983; Hall *et al.*, 1983a; Hall *et al.*, 1983b; McIntosh *et al.*, 1984; Barry *et al.*, 1986). It is thought, however, that the outcome of the studies may have been affected by the use of water as the placebo, as this has a bronchoconstricting effect by itself. Subsequent trials using saline as the placebo did not demonstrate the positive effects initially observed (Broughton & Greenough, 2004).

Later studies have suggested that the aerosolized ribavirin may lessen post-bronchiolitic wheezing, reactive airway disease and reduce the viral load in the patient (Edell *et al.*, 1999; Guerguerian *et al.*, 1999; Rodriguez *et al.*, 1999; Edell *et al.*, 2002). However, it has not been shown to reduce duration of hospitalization. There is still limited evidence of its benefits, and along with potential side effects and the high cost of treatment (Glanville *et al.*, 2005), it is no surprise that its use has been limited to only severe cases.

A number of compounds in addition to ribavirin have exhibited significant anti-RSV activity, but these also carried their own range of harmful side effects. The compounds include VX-497, mycophenolic acid, mycophenolate mofetil, EICAR, pyrazomycin, viramidine, and LY253963 (Sidwell & Barnard, 2006).

2.1.8.6 Polyclonal and monoclonal antibodies.

RSV-IGIV, which consists of a high concentration of polyclonal, anti-RSV IgG antibodies purified from the plasma of healthy individuals (Siber *et al.*, 1994), became the first FDA approved agent for RSV disease prevention in 1996. The subsequent approval of palivizumab, a monoclonal antibody directed at the F glycoprotein, led many to question the superiority of one product over the other (Morris *et al.*, 2009). Despite similar reductions in hospitalizations (Morris *et al.*, 2009), RSV-IGIV had major disadvantages including significant adverse events in children with congenital heart disease, the need for intravenous access, and the risk of infectious disease transmission associated with human plasma–derived products. In 1998, the availability of palivizumab, a safe and effective alternative, led to the removal of RSV-IGIV from the US market that same year (Morris *et al.*, 2009). A new candidate IVIG product, RI-001 is also now being evaluated in phase 2 clinical trials in immunosuppressed, RSV-infected patients at risk for lower respiratory tract illness (Clinicaltrials.gov, 2010). Although human plasma products harbor certain risks, stringent purification requirements have been implemented for all human plasma–derived products to minimize the risk of infection transmission (Buchacher & Iberer, 2006). Polyclonal antibodies also contain a mixed population of antibodies targeting multiple viral epitopes, thus overcoming the mutagenic potential intrinsic among viruses. These

studies are ongoing, and the results have not yet been published (Empey *et al.*, 2010).

Monoclonal antibodies, on the other hand, target a single viral epitope. Palivizumab, the only FDA-approved monoclonal antibody for RSV, targets the highly conserved F glycoprotein, inhibiting viral entry into host cells (Weisman, 2009). It has demonstrated no clinical benefit for the treatment of RSV disease and is thus indicated only for RSV prevention. Motavizumab, a second-generation humanized IgG1 monoclonal antibody, was developed from palivizumab (Abarca *et al.*, 2009), with ~70-fold higher affinity for the RSV F glycoprotein and 20-fold greater neutralizing capacity (Wu *et al.*, 2007). In a rat model, motavizumab had 50–100 times greater anti-RSV activity tract compared with palivizumab in the lower respiratory tract (Wu *et al.*, 2005) and reduced RSV viral load in the upper airways, where palivizumab has minimal effect (Wu *et al.*, 2007). In a large phase 3 noninferiority study comparing motavizumab to palivizumab for RSV prevention in high-risk children, motavizumab demonstrated 26% fewer RSV hospitalizations and a 50% reduction in the incidence of RSV-specific outpatient lower respiratory tract infections (Carbonell-Estrany *et al.*, 2007). Motavizumab also significantly reduced viral load by day 1 after treatment in children hospitalized with RSV, suggesting it may be beneficial for RSV treatment and prevention *(Lagos et al., 2009)*. Motavizumab is currently pending FDA approval (Empey *et al.*, 2010).

While most RSV monoclonal antibody candidates target the more conserved F glycoprotein, recent evidence suggests that those targeting the G glycoprotein may impart dual anti-RSV activity (Empey *et al.*, 2010). The RSV G glycoprotein has been shown to induce lung inflammation by binding to

the chemokine receptor CXC3R1 and initiating a cascade of inflammatory mediators (Tripp *et al.*, 2001; Haynes *et al.*, 2003). Although still in early preclinical studies, a monoclonal antibody targeting the CXC3 motif on the RSV G glycoprotein (mAb 131–2G) was shown to reduce both lung inflammation and RSV titers in BALB/c mice (Haynes *et al.*, 2009).

2.2 Honey

2.2.1 History and traditional uses of honey

Honey is a natural sweetener that has been used as a food and as a traditional medicine for at least six thousand years (Gunther, 1934; Ball, 2007). The earliest written records of honey used as medicine is in Egyptian papyri and Sumerian clay tablets dated from 1900 to 1250 BC (Stomfay-Stitz & Kominos, 1960). Aristotle (384-322 BC) referred to honey as being a good salve for sore eyes and wounds (Aristotle, 350 BC), while Dioscorides (50 AD) described honey as being good for all rotten and hollow ulcers, sunburn, "spots on the face", coughs and inflammation of the throat and tonsils (Gunther, 1934). Ancient Greeks are also reported to have used honey to treat fatigue, and athletes have been known to consume a mixture of honey and water before major athletic events (Wilson & Crane, 1975) The use of honey by the ancient Egyptians, Assyrians, Chinese, Greeks and Romans to treat wounds and infections of the gut has been described (Zumla & Lulat, 1989), emphasizing the role honey must have had in medicine before the advent of antibiotics.

Honey also has a role in present day folk medicine, where it is traditionally used to treat coughs and colds (Beck & Smedley, 1944) diseases of the eye (Fotidar & Fotidar, 1945), infected leg ulcers (Ankra-Badu, 1992), earache (Obi *et al.*, 1994) and in the eyes in measles to prevent corneal scarring

(Imperato & Traore, 1969). A study on the specific uses of honey in traditional medicine in the central part of Burkina Faso by Meda *et al.* (2004) also revealed that the most common applications that were known among individuals in different zones included the treatment of period pains, postnatal disorders, gastroenteric disorders and respiratory infections.

2.2.2 Antimicrobial activity of honey

Several species have been found to be sensitive to the antimicrobial activity of honey and include *Bacillus* sp., *Escherichia coli*, *Klebsiella* sp., *Micrococcus* sp., *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., *Sarcina* sp., *Seratia marcescens*, *Shigella* spp., *Staphylococcus* sp., *Streptococcus* sp. and *Vibria cholerae* (Molan, 1992). Several studies have also demonstrated the absence of a resistance mechanism to the antibacterial factors in honey. Farouk *et al.* (1988) showed that the antibacterial activities of 15 Sudanese honeys did not show selective inhibition when tested against *S. aureus*, *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Klebsiella aerogenes*. This was in contrast to four different antibiotics (ampicillin, cephradine, chloramphenicol and oxytetracycline) tested against the same organisms. Concentrations of 1 to 4% honey have also been shown to inhibit a collection of methicillin-resistant *S. aureus* (MRSA) strains (Molan & Brett, 1998), which also exhibit variations in sensitivity to antibiotics.

The reasons for the antibacterial activity of honey are diverse, and include factors such as an osmotic effect, acidity, the production of hydrogen peroxide, as well as the presence of additional non-peroxide factors.

2.2.2.1 Osmotic effect

According to folklore, honey is the only food that will not spoil (Ball, 2007). Its low moisture content and high osmotic pressure due to the high concentration of sugars are defining characteristics which prevent the growth and survival bacteria (Ball, 2007). Most of the carbohydrates in honey are monosaccharides, mainly fructose and glucose, usually in a ratio of 1.2:1.0, and together accounting for 85-95% of the total carbohydrate content (White, 1975a). Sucrose, higher sugars and disaccharides such as maltose, isomaltose, nigerose, turanose and maltulose are also often present (White, 1975b).

The antibacterial property of honey, first observed by van Ketel in 1892 (Dustmann, 1979), was assumed to be entirely due to the osmotic effect of honey. The low water activity of honey deprives bacteria of water needed for cell function, thus resulting in bacterial inhibition (Molan, 1992) Experiments using artificial honey, made using the same proportions of sugars found in natural honey, also demonstrated the same inhibiting properties, however, it appeared to be less potent in a couple of studies looking at its activity against *P. aeruginosa*. The minimum inhibitory concentration of artificial honey was found to be 20% (v/v) compared with 8% (v/v) for a lime honey (Postmes *et al.*, 1993) in one study and 22% (v/v) artificial honey compared with 5.6% (v/v) honey in another study (Cooper & Molan, 1999).

2.2.2.2 Hydrogen peroxide

Although honey's high sugar content was initially thought to be the reason for its antibacterial activity, honey was found to have increased antibacterial action upon dilution, even beyond the point where osmolarity would have been inhibitory (Cooper *et al.*, 1999). This increased activity was found to be

due to an enzyme within honey, glucose oxidase, which produces hydrogen peroxide when diluted, as shown in Figure 2.2. It is almost inactive in full strength honey (White *et al.*, 1963).

Figure 2.2 Reaction producing hydrogen peroxide.
This reaction occurs when bees secrete glucose oxidase from their hypopharyngeal glands into the nectar during honey formation (source: White, 1975a; White, 1975b)

$$\text{Glucose} + \text{Oxygen} \; \rightarrow \; \text{Hydrogen peroxide} + \text{Gluconolactone}$$
$$\textit{Glucose oxidase} \qquad\qquad\qquad \downarrow$$
$$\text{Gluconic acid}$$
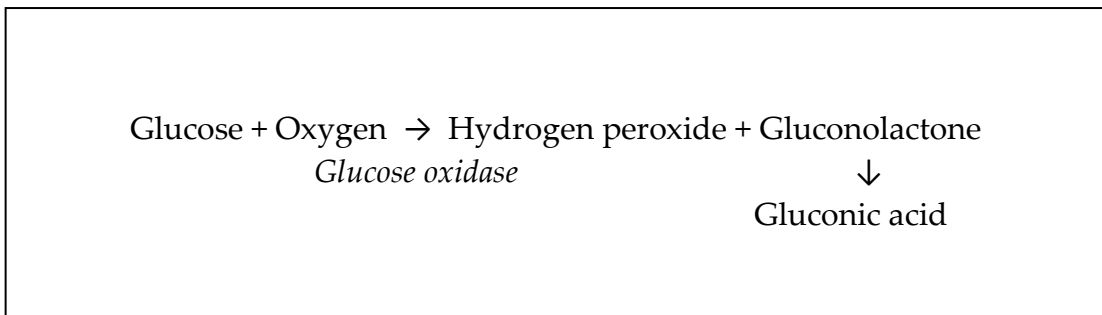
Hydrogen peroxide acts as a powerful antiseptic and has been used for its antibacterial properties in clinical practice, but is only produced when honey is diluted as glucose oxidase is almost inactive in full strength honey (Harcourt, 2005). It is also harmful to tissues and can cause inflammation and damage, however, these effects are reduced in honey due to the inactivation of free iron which catalyses formation of free radicals by hydrogen peroxide (Buntting, 2001). The evident antimicrobial activity in diluted honey has implications for its use in healthcare due to the dilution effects of bodily fluids such as pus, lymph, plasma or stomach acids (Harcourt, 2005).

2.2.2.3 Acidity and non-peroxide antibacterial components

In addition to high osmotic pressure, and hydrogen peroxide, antibacterial activity was also thought to be due to the acidic pH of honey, which, as well as providing inhospitable conditions for bacteria, may also be aiding the increased lymphocyte and phagocytic activity of honey (Ryan & Manjno,

1977). Honey has a characteristically low pH of around 3.2 to 4.5 due to the presence of gluconolactone/gluconic acid which are produced enzymatically during the ripening of the nectar into honey (White, 1975b). The pH of most honeys is lower than the maximum a number of key pathogenic species can tolerate, however, one study looking at the relationship between pH and the antibacterial activity of honey failed to find a correlation (Thimann, 1963; Molan, 1992).

Other components such as flavonoids, phenolic acids, caffeic acid, and ferulic acid have also been identified and isolated from honey. These substances have proven antibacterial activity, however, they are present in such low concentrations that they are not thought to have any significant effects (Weston, 2000).

2.2.3 Methylglyoxal

The removal of hydrogen peroxide by catalase reveals an additional antibacterial agent present in some honeys (Allen *et al.*, 1991) referred to as the unique manuka factor or UMF. This unique activity has been found to be due to the presence of methyglyoxal, which is derived by the non-enzymatic conversion of dihydroxyacetone (Adams *et al.*, 2008). The level of activity varies between different honeys, as shown in a study by Molan and Russell (1988) which found that a range of honey samples had varying levels of this activity from nil to almost whole of the activity in some manuka honeys. This variation was found to be due to the dilution of manuka honey with nectar derived from other floral sources, and also the different varieties of manuka (*Leptospermum scoparium*) harvested (Stephens, 2006).

2.2.4 More recent uses of honey

Honey is used clinically to treat a wide variety of ailments today. Its use in wound care, in particular, has recently been rediscovered after being displaced by antibiotics in the 1940s. Its effectiveness in comparison to conventional wound care practices has been studied extensively, and honey was shown to be more effective in several studies including 17 randomised controlled trials involving a total of 1965 participants, and five other clinical trials and 16 trials on a total of 533 wounds on experimental animals (Molan, 2006b). Honey has also shown to promote the healing of superficial to severe burns more effectively compared to the control treatment using silver sulfadiazine (Molan, 2006a).

An advantage to using honey in wound care is that allergic reactions to it are very rare (Kiistala *et al.*, 1995) and it has very few adverse effects. Numerous reports of honey being used clinically on open wounds exist where no adverse reactions have been reported other than a transient stinging sensation described by some patients which could be due to the acidity of honey as stinging has not been noted after the acidity has been neutralized (Betts & Molan 2001).

Honey has also been used therapeutically for a variety of ulcers including gastric ulcers, diabetic foot ulcers, leg ulcers and decubitus ulcers (Somal *et al.*, 1994; Oluwatosin *et al.*, 2000; Van der Weyden, 2003; Eddy & Gideonsen, 2005). In the cancer setting, honey has been found to be effective for radiation-induced oral mucositis, periodontal gum disease, stomatitis, radiotherapy-induced skin reactions, malignant ulcers and infected lesions in paediatric oncology patients (Chiba *et al.*, 1985; Smirnova *et al.*, 2000; Biswal *et al.*, 2003; English *et al.*, 2004; Moolenaar *et al.*, 2006; Simon *et al.*, 2006).

Honey has also been found to be effective in expediting healing and reducing pain in tonsillectomy patients (Ozlugedik *et al.*, 2006).

2.2.5 Previous studies on the antiviral activity of honey

The possibility of honey having antiviral as well as antibacterial activity has been looked at in a number of studies. One study which investigated the effect of honey on Rubella virus survival *in vitro* compared with thyme showed that honey had anti-rubella activity while thyme did not (Zeina *et al.*, 1996). A similar study investigated, *in vivo*, the effect of topically applied honey on recurrent attacks of labial and genital herpes lesions compared with acyclovir (Al-Waili, 2004). Sixteen adult patients with a history of recurrent attacks of herpetic lesions, 8 labial and 8 genital, were treated by topical application of honey for one attack and acyclovir cream for another attack. Results showed that for labial herpes, the mean duration of attacks and pain, occurrence of crusting, and mean healing time with honey treatment were 35%, 39%, 28% and 43% better, respectively, than with treatment with acyclovir. For genital herpes, the mean duration of attacks and pain, occurrence of crusting, and mean healing time with honey treatment were also 53%, 50%, 49% and 59% better, respectively, than with acyclovir. Two cases of labial herpes and one case of genital herpes remitted completely with the use of honey. The lesions crusted in 3 patients with labial herpes and in 4 patients with genital herpes. None of the attacks remitted with acyclovir cream, and all the lesions, labial and genital, developed crust. No side effects were observed with repeated applications of honey, whereas 3 patients developed local itching with acyclovir. It was therefore concluded that application of honey was safe and effective in the management of symptoms associated with recurrent herpes lesions, however, it was unclear whether the honey had a direct antiviral effect or merely reduced symptoms by means of reducing inflammation, or both.

To confirm the presence of antiviral activity, an *in vitro* study was conducted in order to observe the effects of honey on several strains of herpes simplex virus and adenovirus (Littlejohn, 2009). The study showed that the antiviral effect of honey could not be attributed alone to the methylglyoxal content. It was also shown that honey exerted its antiviral effects by more than one mode of action. Protection experiments showed less CPE after pre-treatment of the cells with honey before inoculation with virus while the neutralization experiments showed that treatment of the viral isolate with honey before using it to inoculate the cells resulted in a slower development of CPE compared to untreated virus. The experiments looking at prevention of spread of infection showed that honey had an effect against the spread of virus from infected cells to uninfected cells, as shown by the lower levels of viral CPE observed with the honey treatments compared with the untreated virus over the course of observation.

The direct effect of the honey on the virus may be being caused by the honey targeting internal or external proteins of the viral particle, which causes unsuccessful attachment to, penetration of, or replication within the cell. It was also suggested that perhaps honey affects the cells by causing an antiviral response in the cells which then works on the viruses after infection. It was also thought that honey could be coating the cellular receptors preventing the virus from attaching to the cell. The shedding of the cell membrane would therefore explain the eventual development of CPE observed even after treatment with honey. The toxic nature of honey, thought to slow cellular growth, was also considered to be a factor as viruses replicate most efficiently in actively dividing cells.

2.2.6 Administration of honey into the lungs

A study by Al-Waili (2003b) was conducted to investigate the effects of intrapulmonary administration of honey in sheep. Fasting blood sugar was estimated in six sheep after 16 hours of fasting and repeated after 30, 60, 90, 120, 150 and 180 min without any intervention. After 1 week, sheep were subjected to 15 min of inhalation of distilled water using a medical ultrasonic nebulizer (nebulizer rate: 0-3 ml/min; particle size: mass median aerodynamic diameter = 4.7 μm; air volume: maximum of 17 l/min). A mask was fixed around the upper and lower jaw including the external nasal opening. Sheep inhaled through the external nasal opening while the mouth was closed. Fasting blood sugar was estimated before inhalation, and blood sugar was estimated at 30 min intervals for 3 hours. After 1 week, the same procedure was repeated except that instead of distilled water, sheep inhaled honey in distilled water (1.2 ml of honey dissolved in 1 ml of distilled water) for 15 minutes. Another comparable two groups of sheep, six sheep each, inhaled a lower concentration of diluted honey in distilled water; 0.75 ml of honey dissolved in 1 ml of distilled water was delivered by nebulizer to one group and 0.5 ml of honey dissolved in 1 ml of distilled water delivered by nebulizer to sheep in the second group for 15 minutes. Blood sugar estimation was performed before inhalation and after 30, 60, 90, 120, 150 and 180 minutes. Close monitoring of respiratory rate, level of consciousness, development of dyspnea, coughing, tachypnea, rhonchi, crepitation, and bluish discolouration of lips and tongue was performed during and after inhalation. All the animals completed the experiment, and none of the sheep developed adverse effects during and after inhalation of water or various concentrations of honey. Blood sugar estimation was insignificantly changed in control sheep. Water inhalation did not cause any significant changes in blood sugars compared with fasting blood sugar and control values. Honey inhalation, on the other hand, caused significant lowering of blood sugar as

compared with control values, and values obtained after water inhalation. Greater effects were obtained when higher concentrations of honey were delivered by nebulizer.

The safety and effect of intrapulmonary administration of 60% honey solution, 10% dextrose or distilled water on blood sugar, plasma insulin and C-peptide, blood pressure, heart rate, and peaked expiratory flow rate (PEFR) in normal and diabetic human subjects were also studied by Al-Waili (2003a). Results showed that in normal subjects, distilled water caused mild elevation of blood glucose level, mild lowering of plasma insulin, and significant reduction of plasma C-peptide. Dextrose inhalation caused mild reduction of plasma insulin and C-peptide and unremarkable changes in blood glucose level. No significant changes were obtained in blood pressure, heart rate or PEFR after distilled water or 10% dextrose inhalation but inhalation of honey caused lowering of blood glucose level and elevation of plasma insulin and C-peptide, mild reduction of blood pressure and up to 11 and 16 percent increase in PEFR. Honey inhalation also significantly reduced random blood glucose level, and fasting blood glucose level three hours post-inhalation. Intensity of hyperglycemia was significantly lowered in glucose tolerance test when patients received honey inhalation. Systolic and diastolic blood pressure was reduced by honey inhalation in hypertensive patients; significant changes were obtained at 60 and 120 min after inhalation. No adverse effects were observed with inhalation of distilled water, 10% dextrose and 60% honey solution except for nasal watery discharge experienced by all subjects and mild cough that was experienced by seven subjects after honey inhalation. Overall, the results demonstrated that honey inhalation in humans was safe, and that it was effective in reducing blood glucose level in normal and diabetic subjects. It also

improved the glucose tolerance test, elevated plasma insulin, C-peptide and PEFR, and reduced elevated blood pressure in hypertensive patients.

The results of this study suggest that the intrapulmonary administration of honey in humans is a feasible concept. If indeed proven to have antiviral activity against RSV, this method would be a way through which honey may be applied into the lungs of a patient, however, extensive clinical trials would need to be performed to assess any adverse effects the long-term inhalation of honey could have on humans.

2.3 Outline of the study in this thesis

The aim of this study is to determine whether honey does in fact have antiviral activity against RSV. This will be performed using cell culture assays and qualitative scoring of cytopathic effect (CPE). It also aims to determine its mode of action, whether it acts directly on the virus, the cells or both, in experiments that will look at the protective and neutralising capabilities of honey. Any results showing an antiviral effect will then be confirmed by enzyme-linked immunosorbent assay (ELISA), a quantitative approach used to detect the amount of viral antigen present in treated and untreated samples.

Various honey types will then be tested for their ability to inhibit the development of CPE. This aims to determine whether their antiviral activity correlates with their antibacterial activity and phytochemical profiles. This study aims to then characterise the observed antiviral activity by looking at the effect of honey on the replication kinetics of RSV using real-time quantitative PCR. This would give a better indication of how exactly the

honey affects the virus as it will provide some information on changes in the transcription of viral genes and thus, protein synthesis.

Lastly, the study also aims to determine the specific component in honey responsible for the observed antiviral effect by testing the activity of several components found within honey. This will also lead to a better understanding of the mechanism behind the antiviral activity.

Chapter Three

Initial Experiments

3.1 Introduction

Initial experiments were performed in order to validate methods to be used in further experiments. They involved evaluating growth of the cell line and of the viral isolate, testing the presence of the appropriate viral isolate, as well as measuring the density, antibacterial activity and antioxidant capacity of the different types of honey used during this study.

To detect the presence of viral infection, the A549 cell line was used as it had previously exhibited the development of characteristic CPE caused by RSV (Kumar *et al.*, 1987; Spann *et al.*, 2004). By examining viral CPE, observations were inexpensive as they could be made visually using phase contrast microscopy. The CPE was qualitatively measured by visually comparing the level of infection against a specifically developed CPE scoring system. This scoring system was derived from visual observations of infected cell culture from the point of infection up to 5 days after infection as this was considered to be a feasible time period during which cultures could be observed daily. The observed CPE development was then confirmed and visualised prior to performing further experiments. This was accomplished by using a direct immunofluorescent antibody technique.

Once the presence of the virus was confirmed, the number of viral particles in a suspension was determined by performing a 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay, an endpoint dilution assay that is commonly

used in clinical research applications. This assay tests for the highest dilution of the suspension which produces a CPE in 50% of the cell cultures inoculated.

Determining the density of each honey used in this thesis was required so that concentrations could be standardised between honey types. The antibacterial activity of the different types of honey was also determined in order to make comparisons with any antiviral activity observed.

3.2 Materials and Methods

3.2.1 Honey

A number of New Zealand honeys with varying phytochemical profiles were included to test for any high correlation between phytochemical composition and antiviral effect: Manuka honey with high non-peroxide antibacterial activity (NPA), known to contain methylgyloxal (MGO) and phenolics was obtained from Summer Glow Apiaries, Te Kowhai, and Watson and Son, Ltd., Masterton; Rewarewa honey with a high level of antibacterial activity due to hydrogen peroxide, high iron-binding antioxidant activity and phenolics from Lorimers Apiaries; Honeydew honey which shows a high level of antibacterial activity due to hydrogen peroxide and antioxidants from Tom Penrose Rangiora; and clover honey which shows no antibacterial activity sourced from Waitemata Honey Redvale.

These honeys show a range of antibacterial levels (see Section 3.3.5) and were selected to allow comparisons to be made between phytochemical profiles and antiviral activity.

3.2.2 Viral isolate

The wild-type RSV isolate used in this study was acquired from a Waikato Hospital patient, these were samples that had been cultured in the Virology Laboratory certified for use in research. They were obtained as swab samples maintained in a cell culture medium.

3.2.3 Cell culture materials

The A549 cell line (human lung carcinoma epithelial cells) was kindly provided by the Dr. Ray Cursons (Waikato University). This cell line was cultured in RPMI 1640 containing HEPES (N-2-(4-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid), obtained from Invitrogen. This culture medium was supplemented with foetal calf serum (FCS) from ICP Biologicals at 10% or 1% to make growth or maintenance medium respectively, with the following antimicrobials added: Penicillin/Streptomycin from Gibco at a final concentration 50 µg/ml, and Fungizone (Gibco) at a final concentration of 2.5 µg/ml. Complete medium was filter sterilised using a 0.22 µm syringe filter into a sterile Schott bottle before being added to cells. All cell culture work was performed in a biosafety cabinet.

3.2.4 Growth, maintenance and freezing down of cell line

A549 cells were grown in a tissue culture flask in growth medium containing 10% FCS (37°C, 5% CO_2) for a minimum of 24 hours. Once a confluent layer of cells had been obtained, the culture was split by detaching the adherent cells from the flask by adding pre-warmed 1 mmol/l 0.05% trypsin-EDTA (Invitrogen) (2 ml for 25 ml flask) into the flask and incubating it at 37°C for 2-3 minutes, while gently rocking the flask at intervals. Once cells were detached from the bottom of the flask, half of the cell suspension was aspirated from the flask, and 4 ml of growth medium was added to the

remaining suspension. The flask was then put back in the incubator and the cells left to grow.

To prepare a plate for experiments, once the cells had detached from the bottom of the flask, the cell suspension containing detached cells was diluted with an appropriate amount of growth medium and distributed into the wells of the plate at an average density of 4.1×10^5 cells per ml.

To prepare a storage culture, after detaching the cells, 6 ml of growth medium was added to the cell suspension and the suspension was loaded into 1.5 ml Eppendorf centrifuge tubes and centrifuged for 2 minutes at 6 000 rpm. The cells were re-distributed using a Pasteur pipette in 1 ml freezing medium (10% dimethyl sulphoxide (DMSO) in FCS), and transferred to 1.5 ml freezing cryovials. The cells were brought down to a low temperature by first placing in a cell freezing chamber, and then stored indefinitely at -80°C.

To revive the stored cells, cryovials were thawed in a 37°C waterbath for 5 minutes before the contents were transferred into a 25 ml cell culture flask containing 4 ml pre-warmed growth medium.

3.2.5 Growth, maintenance and freezing down of viral isolates

To establish viral stocks, cells were detached from the tissue culture flask and supplemented with growth medium as described in Section 3.2.4. The cell suspension was distributed in appropriate volumes to a number of cell culture flasks and was incubated overnight at 37°C. Once the cells were confluent, the medium was replaced with maintenance medium and 200 μl of the appropriate viral isolate from the samples obtained from the Waikato Hospital Virology Laboratory was added. The cells were further incubated at 37°C and observed daily for characteristic morphological changes caused by

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the virus (CPE). Once a moderate level of CPE was reached (see Section 3.3.1), the culture medium was distributed into a number of Eppendorf centrifuge tubes and frozen at -80°C until required.

3.2.6 Direct fluorescent antibody (DFA) test

Cells were grown on round 13 mm coverslips in 24-well cell culture plates as in Section 3.2.4. Once confluent, the cells were infected with 200 µl of viral stock suspension for 1 hour at 37°C. The cells were washed once with sterile PBS (8 g/l sodium chloride, 1.21 g/l dipotassium phosphate, 0.34 g/l potassium dihydrogen phosphate made up to 1 litre with water and filter-sterilised, pH 7.3) and the supernatant was then replaced with maintenance medium. The plates were then incubated at 37°C in a 5% CO_2 incubator and observed daily for CPE development. Cells that were not infected with RSV served as controls. For this study, a Millipore Light Diagnostics RSV DFA Kit (Catalog no. 3125, Lot no. JH1643661) was used.

When the CPE in the infected cells had reached a score of 3, the medium was aspirated off and the cells washed once with wash buffer prepared using materials provided in the kit (100 X Tween 20/Sodium Azide Solution (Catalog No. 5037) diluted 1:100 in PBS buffer (Catalog No. 5087)). After aspirating the wash buffer, the cells were then fixed by adding 80% acetone to each well. The acetone was left on for 10 minutes before it was aspirated off and the plate thoroughly air-dried. Once dry, the cover slips were removed from the wells and put on a petri dish. A drop of antibody solution was applied on top of each cover slip. The petri dish was then put in a 37°C incubator for 20 minutes. After 20 minutes, the cover slips were rinsed with wash buffer three times and rinsed gently with water and dried before being mounted onto a glass slide with mounting fluid (Catalog No. 5013), also included in kit. The slides were then viewed under a fluorescent microscope.

3.2.7 Determining viral titre

A tube of viral stock suspension prepared as described in Section 3.2.5 was thawed at room temperature. Once thawed, 100 µl of the stock viral solution was serially diluted from 10^{-1} to 10^{-6} in maintenance medium. Confluent A549 cells prepared in 24-well plates were inoculated with 1 ml of 10^{-1} to 10^{-6} dilutions in quadruple. The plates of inoculated A549 cells were then incubated at 37°C (5% CO_2) and observed daily for development of characteristic CPE.

The lowest dilution of viral stock suspension that could still induce a CPE was taken as the maximum dilution that would lead to infection. This was termed the $TCID_{50}$ as it was the minimum concentration of virus required to detect viral CPE, and gave a rough indication of the overall concentration of virus present within the stock suspension.

3.2.8 Measuring the density of honey

To determine the density of each type of honey, 5 ml of distilled water was measured into a 25 ml measuring cylinder and 5 g of honey was added at to the cylinder, all at room temperature. The new volume within the measuring cylinder was recorded.

3.2.9 Measuring the antibacterial activity of honey

The antibacterial activity, with and without catalase added to remove activity due to hydrogen peroxide, was measured in an agar well diffusion assay as described by Allen *et al*. (1991). The activity measured by this method is expressed as the concentration of phenol with equivalent antibacterial activity.

3.2.10 Measuring the antioxidant activity of honey

The antioxidant activity of the different honey types was measure by a modification of the method of Baltrusaityte (2007) as described in Brangoulo & Molan (2011), in which the removal of the coloured ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) radical was measured spectrophotometrically. The trolox equivalent antioxidant capacity (TEAC) values were expressed as mmol trolox per kg of honey.

3.2.11 Determining the A549 cell line tolerance to honey

Adding high concentrations of honey to the media can cause changes to the cells such as shrinking as a result of osmotic action. Thus, the maximum concentration of honey at which the cells were displaying no morphological changes was determined. This was to ensure that all morphological changes recorded were due to the activity of the virus and not the honey.

A549 cells were seeded in 24-well plates as described in Section 3.2.4 and left in a 37°C incubator overnight. Once confluent, 0% to 4% honey solutions in maintenance medium were prepared using various honeys using the density outlined in Section 3.2.4. These were mixed and filter-sterilized before being used to replace the growth medium in the cell culture plates. The plates were then incubated at 37°C (5% CO_2) for a few days and observed daily for changes in cellular morphology. Evidence of morphological change was recorded using the CPE scoring system outline in Figure 3.1. This experiment was performed in triplicate and repeated on a different day.

3.3 Results

3.3.1 Cytopathic effect (CPE) scoring

This investigation aimed to monitor the progression of infection in cells caused by RSV and classify the observed morphological changes into a scoring system that can be used in subsequent experiments. As outlined in Section 3.2.4, A549 cells were cultured in 24-well plates. Once confluent, the growth medium was removed and replaced with 1 ml viral suspension diluted to 10^{-1} in maintenance medium. Cells serving as controls were not inoculated. The plate was then further incubated at 37°C (5% CO_2) and observed daily for CPE development. This experiment was performed in triplicate and repeated in triplicate twice on different occasions.

The CPE scoring system is illustrated and presented in Figure 3.1. The results of the experiment following the progression of RSV infection are shown in Figure 3.2. Controls did not show any visible changes in morphology, and no differences were found between replicates. The bars in the graph represent the mean of triplicates from three different days of experimentation. All replicates demonstrated a lack of CPE after 24 hours, however low levels of CPE became evident two days after infection. Within 3 days, cell cultures were showing moderate CPE and by day 4, high levels of CPE were observed with most of the cells being detached from the bottom of the wells at this point.

Figure 3.1 CPE development in A549 cells after RSV infection.
a) CPE 0, b) CPE 1, c) CPE 2, d) CPE 3, e) CPE 4, f) CPE 5
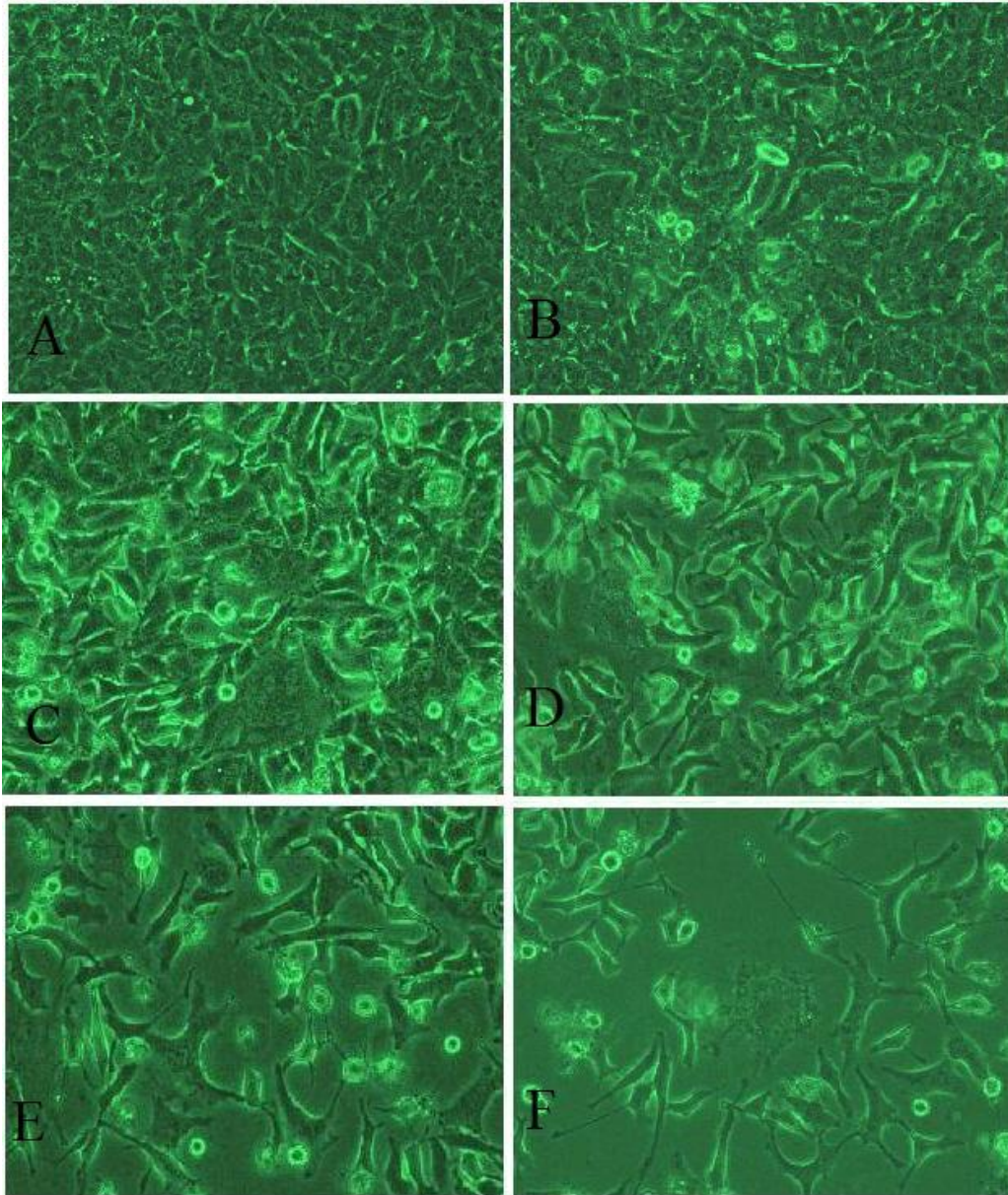
Morphology	Score
100% of monolayer intact	0
90% of monolayer intact, 10% dead	1
80% of monolayer intact, 20% dead	2
75% of monolayer intact, 25% dead (cells also start to appear elongated)	3
50% of monolayer intact, 50% dead	4
25% of monolayer intact, 75% dead	5

Figure 3.2 CPE resulting from infection of A549 cells with RSV.
Each bar represents the mean ± SEM of triplicates on each of the three days of
experimentation.

Based on the results from this experiment, it was concluded that tests looking at treatment effects on CPE development would have to be run for at least 4 days as this was how long it took the isolate to cause severe CPE on A549 cells.

3.3.2 Confirmation of presence of virus using direct fluorescent antibodies

As outlined in Section 3.2.6, DFA tests were performed on normal A549 cells and cells infected with RSV to confirm that the CPE observed is caused by the appropriate virus. The antibody solution included in the Millipore Light Diagnostics RSV DFA kit used for this study comprises four different antibodies to ensure that it detects all strains of RSV including all A and B types. The various antibodies together recognize the fusion protein, the glycoprotein and the nuclear protein of the RSV. The antibodies which bind the viral antigen are labelled with fluorescein isothiocyanate (FITC) which fluoresces apple-green when illuminated with ultraviolet light, while uninfected cells stain dull red due to the presence of Evans blue. This experiment was performed in triplicate.

Figure 3.3 Cells with no virus (left) and cells infected with RSV (right) stained using direct fluorescent antibodies
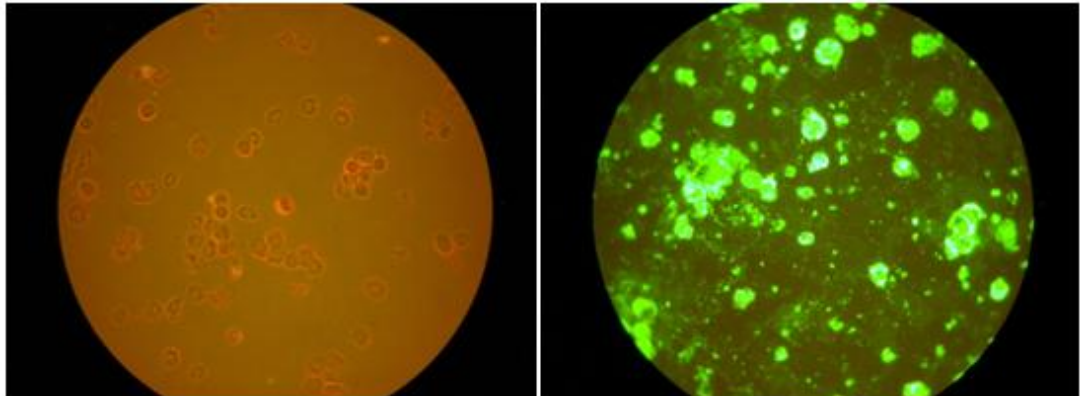
The DFA tests confirmed the presence of RSV virus in cells exhibiting CPE. The control cells which were uninfected and exhibited no CPE looked dull red, showing the absence of virus in the sample. This technique was a very easy and fast way to confirm the presence of virus.

3.3.3 Viral Titre

As described in Section 3.2.5, viral stock suspension was thawed at room temperature and used to serially inoculate confluent A549 cells on a 24-well plate. The cells were then incubated at 37°C and observed daily for the development of characteristic CPE development. This experiment was performed in triplicate. The results of the CPE scores observed with serial dilutions of each virus are shown in Figure 3.4.

Figure 3.4 CPE resulting from serially diluted RSV at 24, 48, 72, 96 and 120 hours after infection
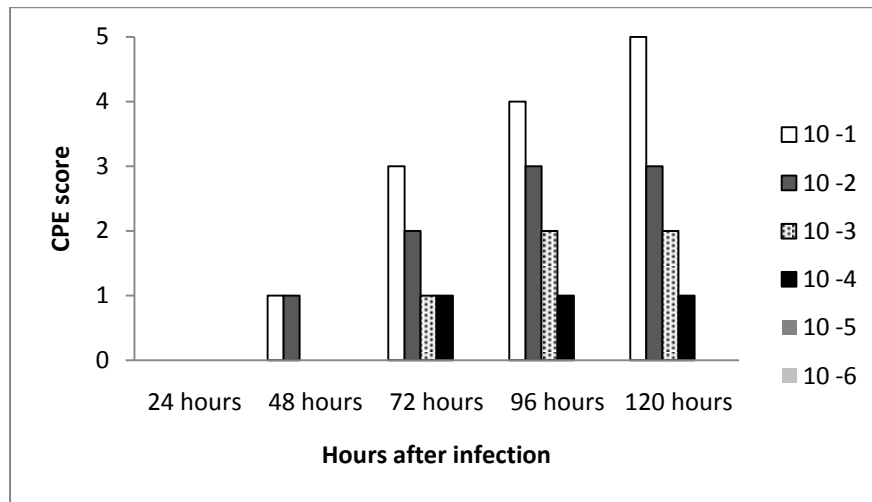
The results from the plates were then analysed using the Reed-Muench method for calculating TCID$_{50}$. The data shown in Table 3.1 was used to demonstrate the calculation by this method.

Table 3.1 Infectivity of RSV at various dilutions

Log of virus dilution	Infected test units	Cumulative infected (A)	Cumulative non-infected (B)	Ratio of A/(A+B)	Percent infected
-1	5/5	20	0	20/20	100%
-2	5/5	15	0	15/15	100%
-3	5/5	10	0	10/10	100%
-4	5/5	5	0	5/5	100%
-5	0/5	0	5	0/5	0%
-6	0/5	0	10	0/10	0%

The dilution in the table that corresponds to the 50% end point lies somewhere between the 10^{-4} (100% infected) and 10^{-5} (0% infected) dilutions.

The proportionate distance between these two dilutions was calculated in the following manner (Mahy & Kangro, 1996):

$$\frac{(\% \text{ positive above } 50\%) - 50\%}{(\% \text{ positive above } 50\%) - (\% \text{ positive below})} = \text{Proportionate distance}$$

Therefore: (100% - 50%) / (100% - 0%) = 0.5

Given that the log of the dilution above 50% is -4, the proportionate distance is 0.5 and the log of the dilution factor is -1 (serial 10-fold dilutions were used), the 50% end point is calculated using the following equation:

(log dilution above 50%) + (proportionate distance x log dilution factor) = log ID_{50}

(-4) + (0.5 x -1.0) = -4.5

The 50% endpoint is calculated to be $10^{-4.5}$. The virus suspension (1 ml) will contain the reciprocal of the calculated dilution, which is $10^{4.5}$ $TCID_{50}$ units.

3.3.4 Honey density

As described in Section 3.2.8, the density of each honey type was established using a displacement method. The different types of honey used included Manuka honeys M117, M115 and M157, Rewarewa honey RE32, Honeydew honey HD19 and clover honey CL24. All the honeys measured gave a volume increase of 3.5 ml when added to water. The following formula was used to calculate their density:

Density = Old weight/ (New volume - Old volume)

= 5/(8.5 – 5)

= 1.43 g/ml

The density of all the honeys tested was therefore concluded to be 1.43 g/ml.

3.3.5 Antibacterial and antioxidant activities of the honeys used

The antibacterial and antioxidant activities found for the honeys used in this study are shown in Table 3.2.

Table 3.2 Antibacterial and antioxidant activity of the honeys used
TA= total antibacterial activity, NPA= non-peroxide antibacterial activity, TEAC= trolox equivalent antioxidant capacity.

Honey	TA (w/v) phenol %	NPA (w/v)	TEAC mmol/kg
M157	23.2	21.9	0.62
M115	15	15.8	1.2
M117	27	25.9	1.6
RE32	21.9	0	2.2
CL24	0	0	0.48
HD19	15.1	0	1.73

The Manuka honeys have generally high total (the majority of which is non-peroxide) and non-peroxide antibacterial activity. The other honeys generally have lower levels of total antibacterial activity and do not have non-peroxide antibacterial activity, however, they have been found to have characteristically high levels of antioxidant capacity.

3.3.6 Cell tolerance to honey

The results from the investigation determining the maximum concentrations of honey the A549 cells tolerated before showing changes in morphology are shown in Figure 3.5.

Figure 3.5 A549 cell line tolerance to a range of honey types
The results represent the mean ± SEM of triplicates from experiments performed on two different days.
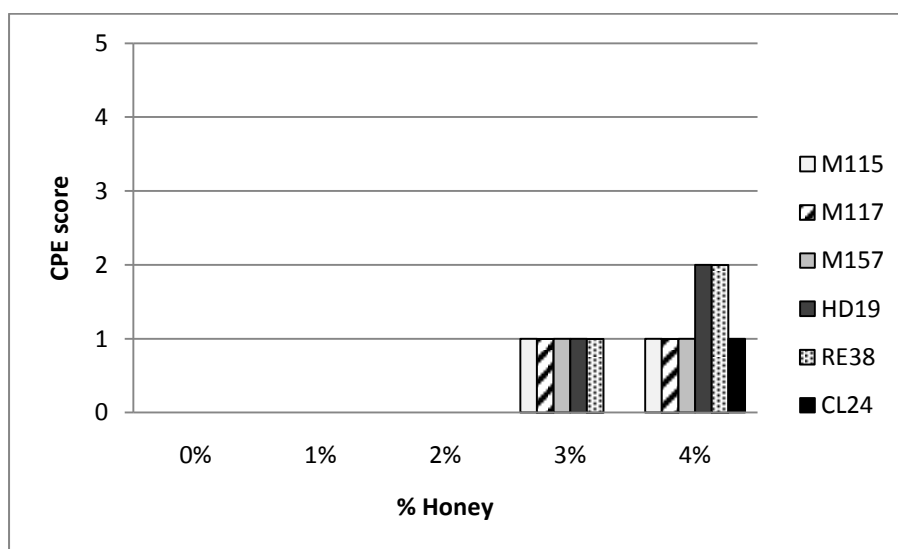
It was concluded that the maximum concentration the cells could tolerate from most honeys was 2%. Cells were able to tolerate clover honey (CL24) up to 3%.

Chapter Four

The antiviral activity of honey

In order to evaluate the antiviral activity of honey, a range of methods were used to examine different aspects of the hypothesised antiviral effect. The methods aim to investigate the inhibitory, protective, and neutralising capacities of honey, with the goal of gaining a better understanding of the underlying nature of its antiviral activity.

These primary experiments used cell culture and one type of honey to investigate the feasibility of methodology, and find methods that were most suitable to be used in subsequent work with a range of honey types.

4.2 Inhibition of development of viral infection

4.2.1 Introduction

This experiment aimed to test whether incubation of RSV- infected cells with honey could prevent or slow down the rate of CPE development compared with infected cells without honey. This type of investigation may possibly show whether honey can be used as a treatment for viral infections once infection has taken place. It was decided that, to mimic real-life situation, the cells would first be inoculated with virus for 1 hour before honey is applied. However, it was discovered that the rate of CPE development was much slower than previously observed (Section 3.3.1) due to the shorter inoculation time. A number of methods were then tested to determine the best way to ensure the fast development of CPE so that experiments could run for as little time as possible.

4.2.2 Materials and Methods

a) First experiment

A549 cells were seeded into a 24-well plate and incubated at 37°C overnight as described in Section 3.2.4. Once confluent, the growth medium was removed from the cells. Viral stock suspension was diluted ten-fold in maintenance medium and 200 µl was added to each well. The plate was then put in a 5% CO_2 incubator set at 37°C for one hour. After one hour, the inoculum was aspirated off, the cells were washed once with sterile PBS (see Section 3.2.6) which was then replaced with 1 ml of maintenance medium containing varying concentrations of M115 honey. The plate was then incubated and observed daily for CPE development. This experiment was performed in triplicate.

b) Second experiment

The second experiment was carried out in the same way as the first, but after the suspension was added to each well, the plate was then centrifuged at 20°C at 2 000 rpm for 15 minutes before continuing with the rest of the procedure.

c) Third experiment

The third experiment was carried out in the same way as the first, but undiluted viral stock suspension (200 µl) was added to each well instead of diluted viral stock suspension.

d) Further observations on the inhibitory activity of honey against RSV

Further observations on the inhibitory effect of honey against RSV were made using the method which was found to result in a more rapid development of CPE. This experiment was performed in triplicate and repeated on a different day.

4.2.3 Results

The results of the experiments are presented as plots of developing CPE in Figures 4.1 to 4.4. The controls of each honey concentration without virus did not cause any visible morphological changes of the cells over the observation period. The different inoculation methods all resulted in different rates of CPE development, with the fastest being shown in Figure 4.3, where 200 µl of undiluted viral supernatant was used to inoculate the cells for one hour. Results from further observations using this method are shown in Figure 4.4 and demonstrate an inhibition of RSV as a result of treatment with honey.

Figure 4.1 CPE development in A549 after exposure to viral supernatant diluted to 10^{-1} for 1 hour prior to addition of honey solution
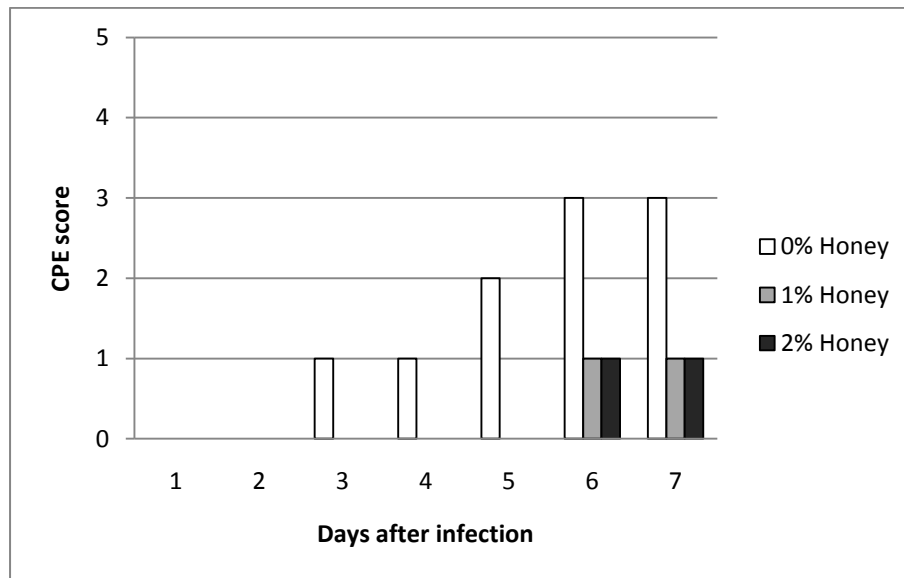
Figure 4.2 CPE development in A549 cells after inoculation with viral supernatant diluted to 10^{-1} and centrifuged for 15 minutes prior to addition of honey solution
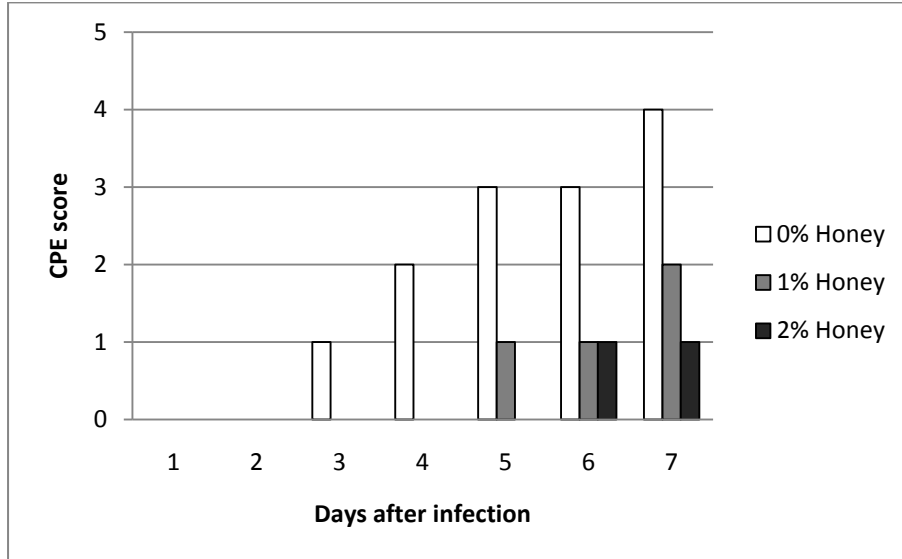
Figure 4.3 CPE development in A549 cells after exposure for 1 hour to undiluted viral supernatant prior to addition of honey solution
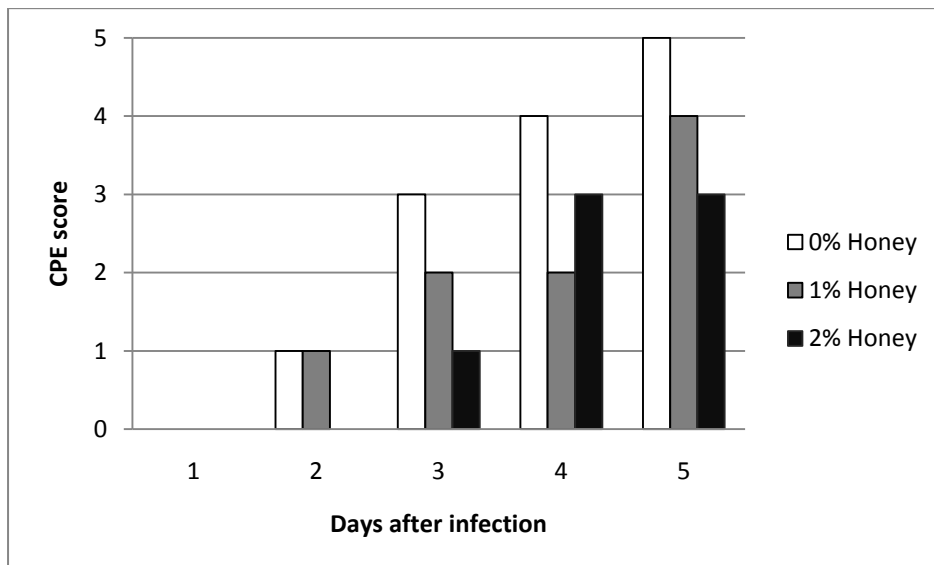
Figure 4.4 Further observations on the inhibitory effect of honey on CPE development in RSV-infected A549 cells
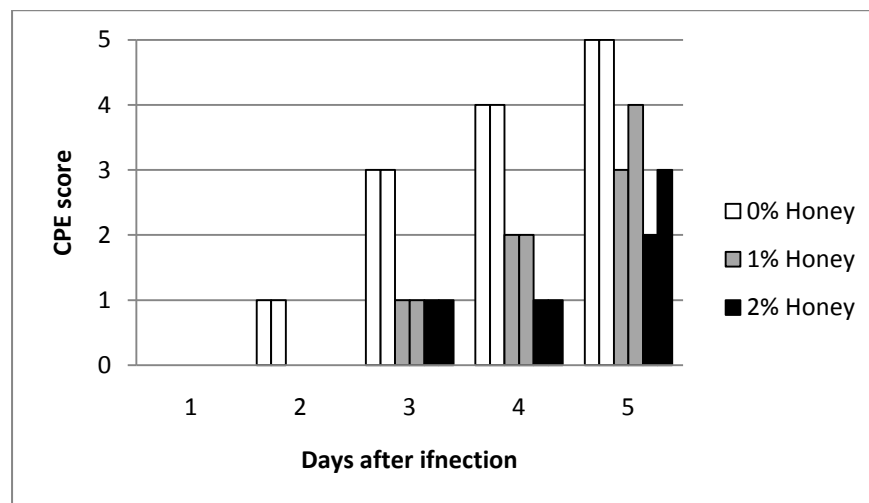
4.2.4 Discussion

The first three experiments all demonstrated the inhibitory effect of honey on RSV, however, differences in results were observed depending on the method of inoculation with virus. Diluting the viral supernatant ten-fold and having shorter inoculation times resulted in slow CPE development, with cells showing only moderate infection at 7 days post-infection. This suggests that this strain of RSV requires a long inoculation time. Using undiluted virus increased the rate of CPE development, resulting in high levels of CPE by 5 days post-infection and was thus employed in further experiments.

The honey was shown to have an effect against the progression of viral infection, as shown by the lower levels of viral CPE observed with the honey treatments compared with the untreated virus over the course of observation. Each honey treatment suppressed the severity of viral CPE, with the higher concentration having the greatest effect.

The delayed CPE development in honey-treated cells suggests that the honey could have more than one mode of action. It could be acting directly on the virus to neutralise it, or it could be acting on the cells, possibly causing changes in the expression of genes involved in the antiviral response, such as NF-κB – or both. To determine the mode of action of honey, further experiments looking at the protective and neutralising abilities of honey were performed.

4.3 Protection of the cells from viral infection

4.3.1 Introduction

The ability of honey to protect the cells from infection by viruses was evaluated through the culturing of confluent A549 cells within honey-containing growth medium, followed by removal of this medium before inoculation of the cells with the viral isolate. This approach aimed to test whether pre-treatment of the cells with honey can prevent the development of viral CPE following inoculation of the cells with virus. A positive result obtained in such an investigation can provide support towards the use of honey as a preventative or prophylactic treatment for individuals at risk of such viral infections. This experiment also sought to determine whether honey acts on the cells directly to cause an antiviral response, resulting in the slower development of CPE previously observed in Section 4.2.

4.3.2 Materials and Methods

Growth medium was prepared containing 0%, 1%, and 2% concentrations of Manuka honey M115 and was filter-sterilised. Stocks of A549 cells were prepared for seeding into 24-well plates as described in Section 3.2.4. Appropriate volumes of the cell suspension were mixed with the honey solutions based on the size of the flask, and were then distributed in volumes

of 1 ml into a 24-well plate. The plate was incubated overnight at 37°C (5% CO_2). Once the cells were confluent, the honey-containing growth medium was removed and replaced with 200 µl of viral suspension and incubated at 37°C (5% CO_2) for 1 hour. After 1 hour, the inoculum was removed and the cells were washed once with PBS. Maintenance medium (1 ml) was added to each well. The plate was returned to the incubator and observed daily for CPE development. As controls, cells treated with each concentration of honey were also tested without subsequent addition of virus. The experiment was performed in triplicate within each plate and repeated on a different day.

4.3.3 Results

The results of the experiments are presented as plots of developing CPE in Figure 4.5.

Figure 4.5 CPE development from exposure to RSV after pre-treatment of the cells with various concentrations of honey
Each bar represents the mean ± SEM of triplicates from each of the two days of experiments.
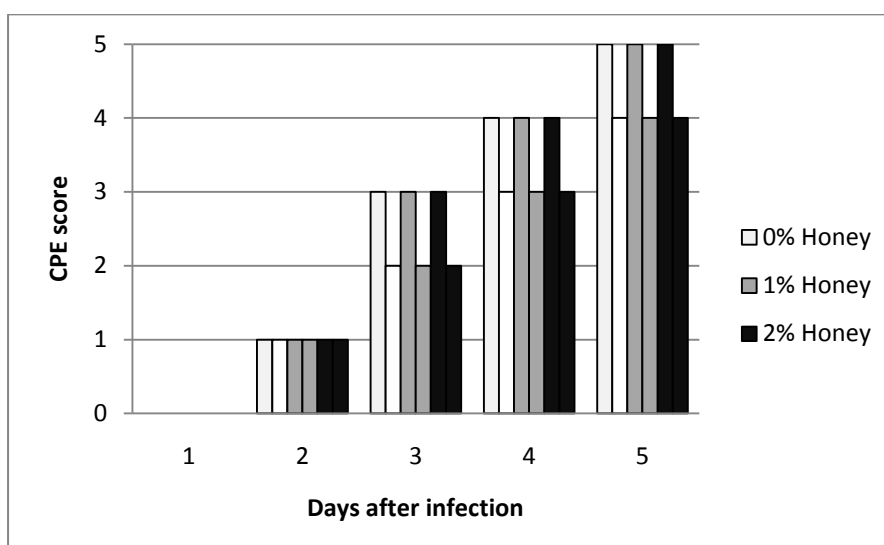
The results from the replicates were identical for each day, but differed between days. No treatment effect was observed. The controls of each honey concentration without virus did not show any visible morphological changes of the cells over the observation period.

4.3.4 Discussion

This experiment has demonstrated that the honey was unable to protect the cells from RSV infection. These results are contrary to previous findings by Littlejohn (2009), whose similar study on herpes simplex virus and adenovirus showed that incubation of A549 cells with honey prior to infection resulted in a slower development of CPE. This indicates that different viruses may be affected by honey differently. It also suggests that perhaps honey acts on the virus particle directly, rather than causing changes to the cell which indirectly inhibit its replication. This includes changes such as upregulation or downregulation of specific genes involved in the antiviral response, however, it is also possible that an infection needs to be present in the first place for honey to be able to illicit an antiviral response in the cells. Future studies would benefit from looking at changes in the expression of genes involved in the antiviral response to determine if honey has any effect on them.

Based on the results obtained from this experiment, it is unlikely that honey would be an effective prophylactic agent for individuals at high risk of RSV infection, however, experiments with primary cell cultures and animal models would give a better indication of this.

4.4 Neutralisation

4.4.1 Introduction

This experiment aimed to test whether honey would has any neutralising activity against RSV. It is hypothesised, based on the results from Section 4.2 and 4.3, that honey affects the virus directly to inactivate it, thus causing inhibition of CPE development. In this experiment, the viral isolate was exposed to various concentrations of honey for a one hour before being used to inoculate the cells. CPE development was then monitored on the days following inoculation of the cells to determine whether neutralisation, observed as the absence or delay of viral CPE development, has occurred. If neutralisation is found to occur with a short period of exposure of virus to honey, this experiment will show that it may be possible for the virus to be inactivated between being shed and infecting other cells.

4.4.2 Materials and Methods

A549 cells were prepared and grown overnight in 24-well plates at 37°C until confluent as described in Section 3.2.4. Sterile bijoux bottles were used to prepare filter-sterilised honey solutions containing 0%, 1% and 2% honey in maintenance medium. In an Eppendorf microcentrifuge tube, viral supernatant (200 μl) was added to each honey solution (800 μl). These solutions, as well as honey solutions without virus serving as controls, were incubated with shaking for 1 hour using a shaking incubator at level 1 set at 37°C. At the conclusion of the neutralisation period, the growth medium was removed from the cells which were then incubated with 1 ml of each of viral-honey solution for 1 hour at 37°C. After one hour, the inoculum was removed, the cells washed once with PBS and 1 ml maintenance medium was added. The plates were left in a 37°C, 5% CO_2 incubator and observations were made daily to record the development of CPE until a high

level of CPE had developed with the untreated virus (0% honey). Each experiment was completed in triplicate and repeated in time.

4.4.3 Results

The results from the neutralisation experiment are shown in Figure 4.6. It shows a treatment effect caused by honey, where CPE development in cells infected with honey-treated virus was much slower compared to that treated with maintenance medium only.

Figure 4.6 CPE development in A549 cells infected with RSV pre-treated with 0%, 1% and 2% M115 hone
Each bar represents mean ± SEM of triplicates from one day of experimentation
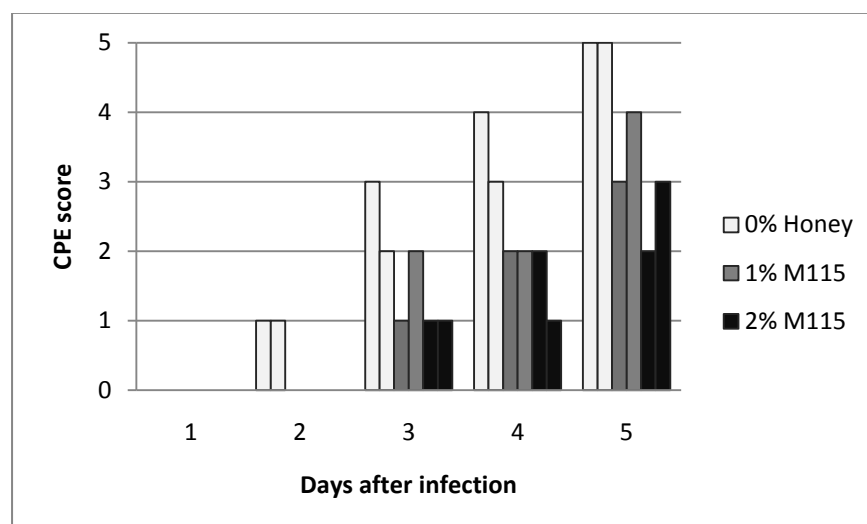
Treatment with honey at 2% resulted in the greatest inhibition, while 1% honey caused moderate inhibition.

4.4.4 Discussion

These experiments indicate that honey had a significant inhibitory effect on RSV as demonstrated by the slower progression of CPE development in treated virus compared with untreated virus. In each experiment,

suppression of viral CPE was observed with the honey treatments, and further evidence of neutralisation was seen with increasing concentrations of honey. The resulting CPE levels reached during the duration of the experiments are similar to the levels observed in the first tests looking at the prevention of spread of virus. The findings from this experiment therefore support the hypothesis that the honey could be acting on the virus directly, however, the effects observed may have also been due to the presence of honey, which would have been in contact with the cells for one hour.

The results from this experiment suggest that honey can potentially be used as an antiviral agent to aid the immune system in response to viral infection by RSV. It may slow down the progression of infection by neutralising viral particles that have been shed, allowing the body's own defences to help eliminate the virus.

4.5 Addition of honey to moderately infected cells

4.5.1 Introduction

This experiment aimed to determine whether would be an effective treatment for patients already suffering from the symptoms of an RSV infection. This was evaluated by observing the effects of adding honey to cells exhibiting a moderate level of CPE. If found to be effective, this experiment would highlight the potential of using honey to treat otherwise healthy individuals who are likely to seek medical help only once the symptoms of infection had become apparent.

4.5.2 Materials and Methods

A549 cells were prepared and grown overnight in 24-well plates at 37°C until confluent as described in Section 3.2.4. Once confluent, growth medium was

aspirated off and the cells, apart from the controls, were inoculated with 200 μl of viral supernatant for 1 hour in a 37°C, 5% CO_2 incubator. After one hour, the inoculum was removed and replaced with 1 ml of maintenance medium. The plate was then placed back in the incubator and the cells were observed daily for CPE development. Once the infected cells had reached a CPE score of 3, the supernatant was removed and replaced with 0%, 1% and 2% M115 honey solutions. The plate was then placed back in the incubator and observed daily for CPE development. The experiment was performed in triplicate and repeated on a different day.

4.5.3 Results

The results showing the effects of adding honey to moderately infected cells is shown in Figure 4.7. The asterisk indicates the day maintenance medium was replaced with honey solutions containing various concentrations of honey, which was when most of the cells were showing signs of moderate RSV infection (CPE 3).

Figure 4.7 Effects of adding honey to moderately infected cells
Asterisks indicate when maintenance medium was replaced with honey solutions.
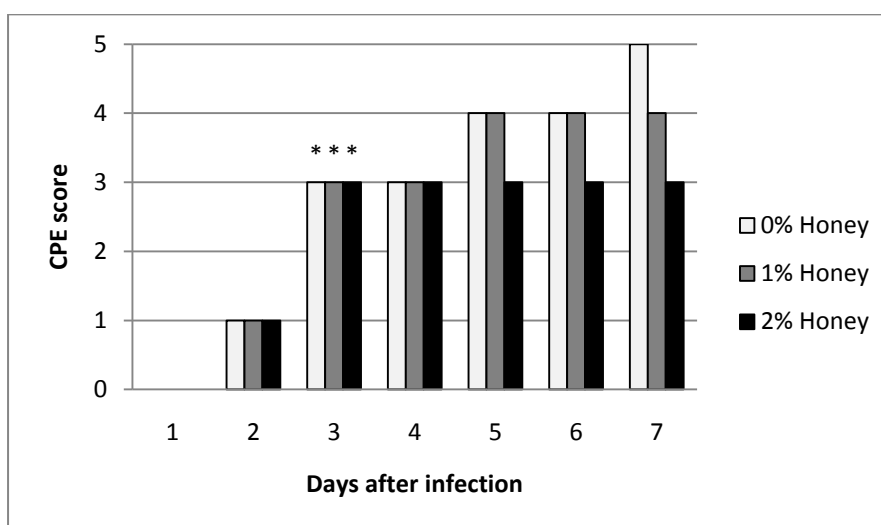
The normal development of CPE was found to be slower in this experiment, with the untreated cells reaching CPE 5 only after 7 days. The results from the experiment show that the addition of honey slows down any further development of CPE, with the addition of 2% honey halting its development for the duration of the experiment. No differences were found between replicates and repeats, and no morphological changes were observed in control cells not infected with virus.

4.5.4 Discussion

The slower development of CPE observed even in the control cells is likely to be due to the replacement of maintenance medium, thereby removing a large proportion of viral particles present in the culture. The results obtained from this experiment have demonstrated the ability of honey to inhibit further progression of infection in cells already exhibiting moderate CPE levels. Considering that most healthy individuals will only seek professional medical help for RSV infection once symptoms are already present, the results from this experiment demonstrate its potential benefits for use as a therapeutic. The use of honey to suppress the progression of RSV infection in humans may have implications on the morbidity and mortality rate of patients, allowing the immune system some time to eliminate the virus, possibly resulting in a quicker recovery. Further *in vivo* investigations would need to be performed to see whether the same effect will be observed in animal models.

Chapter Five

The antiviral activity of different honey types

5.1 Inhibition of development of viral infection by different types of honey

5.1.1 Introduction

It has been established by the current study that honey can temporarily suppress the development of CPE. It was desirable to test a range of honeys with varying phytochemical profiles to determine whether some honeys can cause greater suppression or neutralisation of the virus than others. It was hypothesised that the observed antiviral activity can be linked to a characteristic property within the honey, such as high levels of hydrogen peroxide, phenolics, antioxidants or non-peroxide activity due to the presence of methylglyoxal.

5.1.2 Materials and Methods

A549 cells were seeded into 24-well plates and incubated at 37°C overnight as described in Section 3.2.4. Once confluent, the growth medium was removed from the cells. Viral supernatant (200 μl) was added to each well. The plate was then put in a 5% CO_2 incubator set at 37°C for one hour. After one hour, the inoculum was aspirated off using a plastic Pasteur pipette, washed with sterile PBS (see Section 3.2.6) and replaced with 1 ml of maintenance medium containing 2% of different types of honey. The honeys used were Manuka honeys M117, M157 and M115, Rewarewa honey RE32, Honeydew honey HD19 and clover honey CL24. These honeys were chosen based on their distinct phytochemical profiles as outlined in Section 3.2.1.

The plate was then returned to the incubator and observed daily for CPE development. This experiment was performed in triplicate on a 24-well plate and repeated in triplicate on a different day. Controls without honey and without virus were included.

5.1.3 Results

The results are presented in Figures 5.1 as plots of CPE development. Manuka honey M117 was observed to have the greatest inhibitory effect on RSV, with treated cells exhibiting only a CPE score of 1 at Day 5 post-infection. Treatment with Manuka honeys M115, M157, Rewarewa RE32 and Honeydew HD19 caused similar levels of inhibition, with treated cells showing moderate levels of infection by Day 5 (CPE 2 to 3). Clover honey CL24 was observed to have the least effect on RSV, only resulting in mild inhibition, and treated cells displaying moderate to severe levels of CPE at the conclusion of the experiment (CPE 3 to 4).

Figure 5.1 CPE development in A549 cells in the presence of various types of honey after infection with RSV.
Experiment was performed twice on different days (A and B). Each bar represents mean ± SEM of triplicates from one day of the experiment.
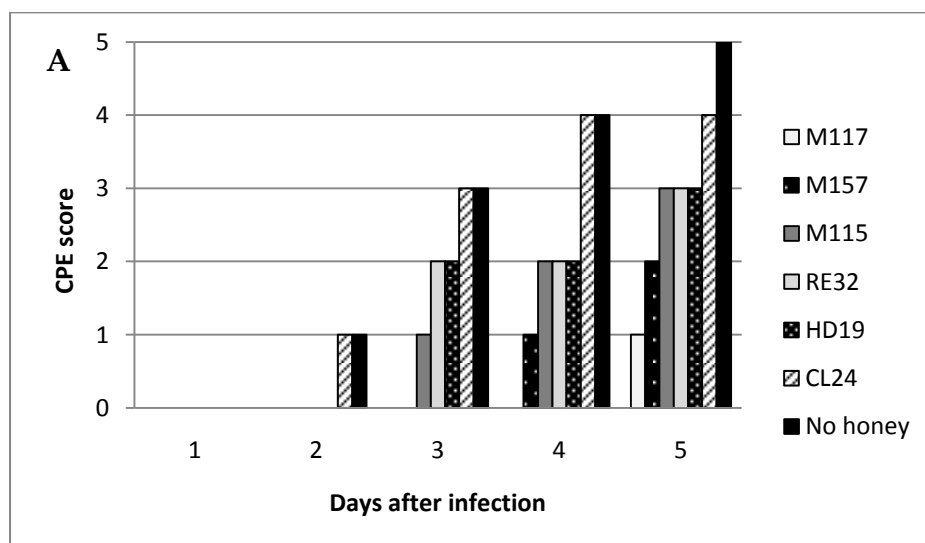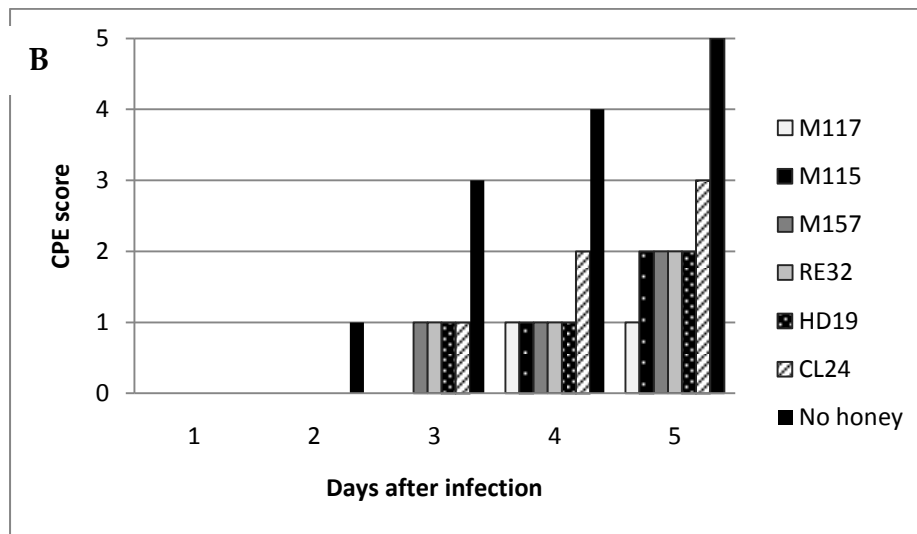
No morphological changes of the cells were observed in the controls without virus.

5.1.4 Discussion

In this experiment, all honeys were shown to have some inhibitory effect against RSV. The Manuka honey with high-NPA activity, M117, had the greatest effect on the resulting severity of viral infection. Manuka honeys M157 and M115, which had mid-range NPA, also slowed down CPE development, however, their effect was similar to two of the other honeys tested, Rewarewa RE32 and Honeydew HD19, which do not have NPA. Clover honey CL24 had the least effect, with the cells exhibiting moderate to severe CPE by Day 5.

The results from this experiment suggest that there is some correlation between a honey's antibacterial activity and its antiviral activity. The honeys shown to have the greatest effect in this experiment were honeys which had the highest total antibacterial activity ratings as outlined in Section 3.3.5. Similarly, CL24, which was shown to have the least effect on development of

CPE, had no observable antibacterial activity. The other honeys which were shown to have similar effects on reducing viral CPE have different antibacterial activity ratings, however, due to the limitations of the CPE scoring system, the differences in their activity against RSV may be more difficult to determine by eye. A quantitative test such as an enzyme-linked immunosorbent assay (ELISA) would allow for a more thorough analysis to determine the potency of each honey type.

Looking at the different types of Manuka honey used in this experiment, it is possible that antiviral activity may be directly correlated with the amount of methylglyoxal (MGO) present in that type of honey. Investigating the effect of adding MGO to low activity Manuka honeys will provide a better look at its role in inhibiting viruses. High levels of phenolic compounds are also found in Manuka honey, and may also be a contributing factor in the observed antiviral effect (Littlejohn, 2009). It would therefore be of value to look at the inhibitory effects of adding phenolics, as well as other components – such as hydrogen peroxide, antioxidants and proteins – abundant in the non-Manuka honeys which also exhibited an antiviral effect.

5.2 Neutralisation of RSV using different types of honey

5.2.1 Introduction

This experiment aimed to investigate the neutralisation capabilities of various honey types. It is hypothesised that the ability of each honey to neutralise RSV will reflect its ability to suppress the development of CPE as observed in Section 5.1.3.

5.2.2 Materials and Methods

A549 cells were grown overnight as described in Section 3.2.4. Bijoux bottles were used to prepare honey solutions containing 2% honey in maintenance medium. The honeys used were as described in Section 5.1.2. In an Eppendorf microcentrifuge tube, viral supernatant (200 µl) was added to each honey solution (800 µl). These solutions, as well as honey solutions without virus serving as controls, were incubated with shaking for 1 hour using a shaking incubator at level 1 set at 37°C. At the conclusion of the neutralisation period, the growth medium was removed from the cells which were then incubated with 1 ml of each of viral-honey solution for 1 hour at 37°C. After one hour, the inoculum was removed and replaced with 1 ml maintenance medium. The plates were left in a 37°C, 5% CO_2 incubator and observations were made daily to record the development of CPE until a high level of CPE had developed with the untreated virus (0% honey). Controls with each honey solution without virus were also included. Each experiment was completed in duplicate and repeated in duplicate on a different day.

5.2.3 Results

The results from the experiment looking at the neutralisation capabilities of different types of honey are shown in Figure 5.2.

Figure 5.2 CPE development in A549 cells infected with RSV which had been pre-treated with different types of honey at 2%
Experiment was performed twice on different days (A and B). Each bar represents mean ± SEM for triplicates from one day of the experiment.
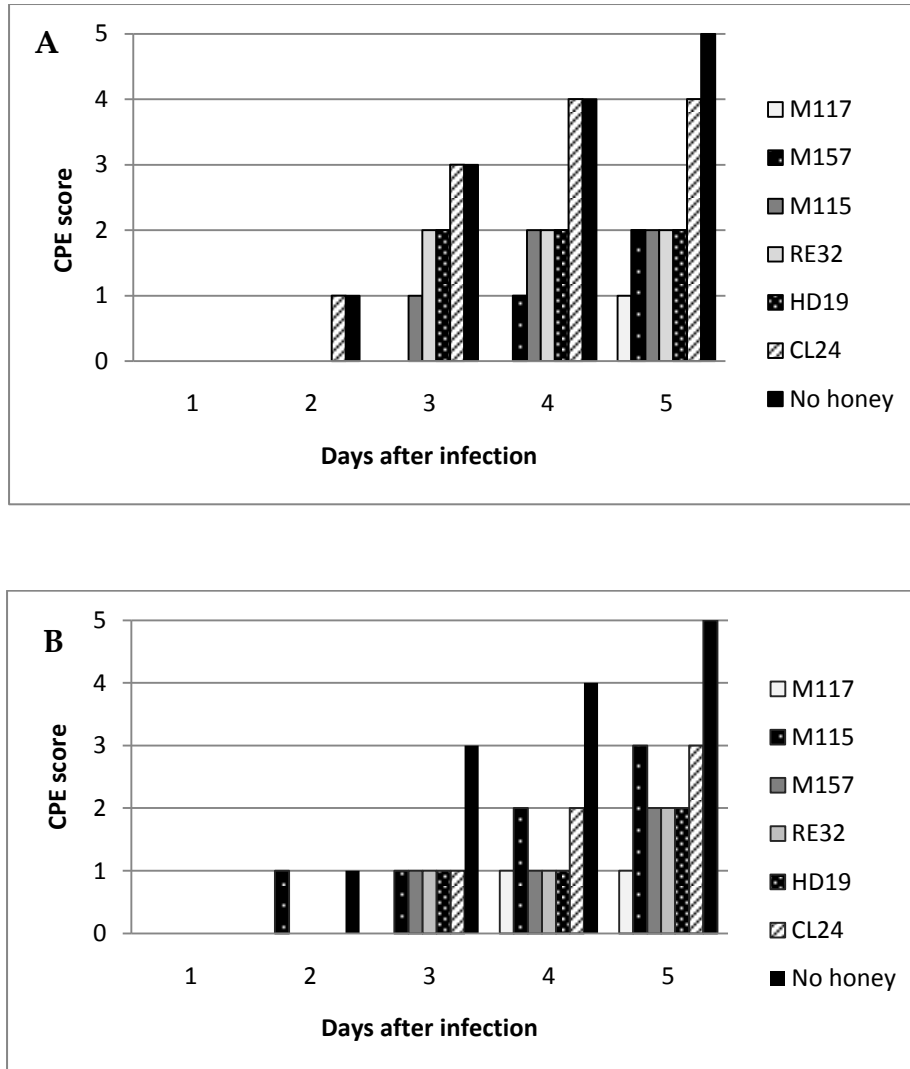
The high-NPA Manuka honey M117 had the greatest effect on the virus, while clover honey CL24 had the least effect. The mid-range Manuka honeys caused similar levels of inhibition to the Rewarewa honey and Honeydew honey examined.

5.2.4 Discussion

This experiment looked at the neutralisation capabilities of different types of honey. The results observed were similar to those observed in Section 5.1.3 where the honey treatment was added after inoculation with the virus. The high-NPA Manuka honeys generally had a greater inhibiting effect, while the other honeys exhibited similar levels of antiviral activity. The clover honey used in this experiment had little inhibitory effect on RSV, with the cells showing moderate CPE by Day 5. As previously discussed in Section 5.1.4, the methylglyoxal in Manuka honeys may be the component responsible for the greater inhibition observed in these honeys compared with non-Manuka honeys. Future experiments will need to confirm this. The antiviral activity observed in the other honeys tested may be due to a number of factors, since each one has a phytochemical profile distinct from the others. Other components found in these honeys, such as hydrogen peroxide, antioxidants and phenolics should also be examined in future investigations.

The experiments in this chapter have demonstrated that high-NPA Manuka honeys would be the best candidates for use as treatment in RSV infection, clinical studies are required to verify this.

Chapter Six

A quantitative study on the antiviral activity of honey

6.1 Introduction

Enzyme-linked immunosorbent assays (ELISA) can be used as a sensitive measure to detect the amount of a specific antigen within a sample. The whole ELISA experimental process includes several steps, including the blocking of binding sites, the immobilization of an antibody on the plate, the binding between the immobilized antibody and its antigen and the detection of binding signals.

Ideally, the enzymes used to label the antigen or antibody should be stable, safe and inexpensive. These enzymes, when reacted with the substrate molecules, act as the catalysts and can convert a colourless substrate to a coloured product which can be easily detected using a plate reader at a specific wavelength for this substrate. Many enzymes are used for ELISA labelling and Table 6.1 lists some examples of the most commonly used enzymes and their corresponding substrates.

Table 6.1 The most commonly used enzymes in ELISA and their substrates

Enzymes	Substrates
Horse Radish Peroxidase	3,3′,5,5′-Tetramethylbenzidine (TMB) ortho-phenylenediamine dihydrochloride (OPD)
Alkaline Phosphatase	p-N itrophenyl Phosphate (pNPP)
Beta-Galactosidase	o-nitrophenyl-β-D-Galactoside (ONPG)

Several different types of ELISA have been developed and studies that mostly differ in the sequence of addition of antigens or antibodies to the solid plate:

• Direct ELISA

• Indirect ELISA

• Competitive ELISA

• Sandwich ELISA

The indirect ELISA technique used during this study requires the addition of a primary antibody that will bind to antigen molecules adsorbed on to the plate. The binding of these two molecules can then be recognized by an enzyme-labelled secondary antibody, which will then convert a specific substrate to a coloured product to be measured (Figure 6.1).

Figure 6.1 Indirect ELISA technique (source: Gong, 2009)
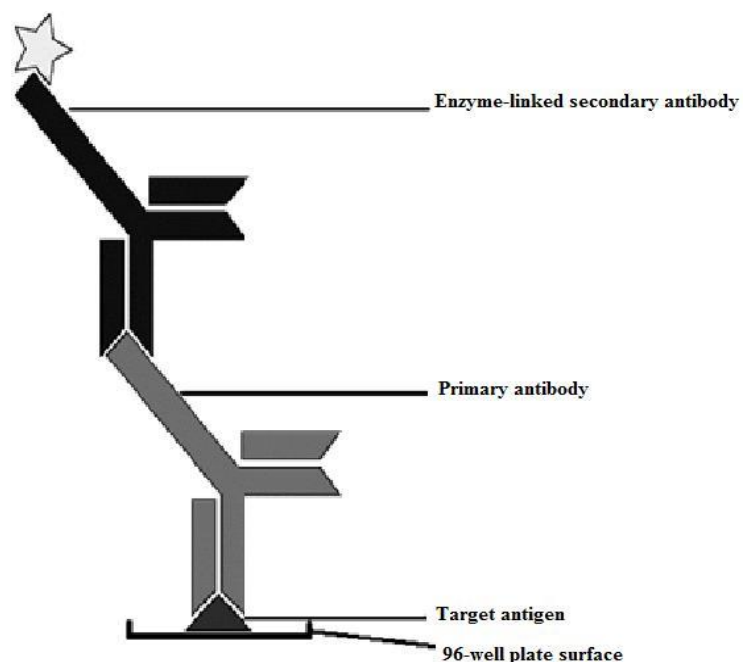
The aim of using the ELISA technique is to enable quantitative measurement of the effects of honey treatment on the number of viral antigen present in a sample. It is hypothesised that honey treated samples will contain less of the antigen compared to untreated samples.

6.2 Experiments

6.2.1 Determining optimal dilutions of primary antibody

6.2.1.1 Materials and Methods

A549 cells were grown in ten of the wells on a 24-well cell culture plate overnight as described in Section 3.2.4. Once confluent, growth medium was removed via aspiration. Viral stock suspension (200 µl) was added to five of the wells, while the other five remained uninfected to serve as controls. The plate was then put in a 5% CO_2 incubator set at 37°C for one hour. After one hour, the supernatant was aspirated off and all wells supplemented with 1 ml maintenance medium. The plate was then incubated and observed daily for CPE development. Once the infected cells had reached a CPE score of 3, the medium was removed and each well was washed once with wash buffer (0.05% Tween 20 in PBS pH 7.3 (see Section 3.2.6)). The cells were then fixed with 80% acetone for 10 minutes, and air dried. Once dry, the cells were treated to destroy any endogenous peroxidase activity present. This was done by adding 1 ml of solution containing 3% hydrogen peroxide in methanol and incubating the plate at 37°C for one hour. After one hour, the solution was aspirated off and the cells were washed once with wash buffer. Blocking buffer (1% bovine serum albumin (BSA) from Sigma prepared in PBS pH 7.3) was then added to each well in 200 µl aliquots and the plate was incubated at 37°C for 30 minutes. After incubation with the blocking buffer, the wells were washed 3 times with wash buffer and air dried. The primary antibody used in this experiment, Mouse Anti-Respiratory Syncytial Virus

monoclonal antibody (1 mg/ml, Millipore, Catalog no. MAB8262, Lot no. LV1700732) was prepared in PBS (pH 7.3) and 0.05% Tween 20 and diluted to various concentrations. They were then added in 200 µl aliquots to each well. The concentrations used were 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml. The plate was then incubated at 37°C for 1 hour. After incubation with the primary antibody, the wells were washed 3 times with wash buffer for 5 minutes each time, then 200 µl of the stock secondary antibody solution (Goat anti-mouse IgG, HRP conjugate polyclonal antibody from Millipore, Catalog no. 12-349, Lot no. DAM 1698735) (1 mg/ml) rehydrated in PBS (pH 7.3) was added to each well (using a dilution of 1:10,000 as suggested by manufacturer) and incubated at 37°C for 1 hour. After incubation with the secondary antibody, the wells were washed 3 times with wash buffer for 5 minutes each time. During the last wash the substrate was prepared by mixing 30 ml of substrate buffer (3.73 g citric acid and 5.04 g di-sodium hydrogen phosphate made up to 400 ml water, pH 5) with a 30 mg Ortho-phenylenediamine dihydrochloride (OPD) tablet (Sigma). Once the tablet had dissolved, 200 µl of 6% H_2O_2 was added. Substrate was added to each well in 200 µl aliquots and incubated at 37° for 30 minutes in the dark. After incubation, 50 µl 2 mol/l H_2SO_4 was added to each well to stop the enzyme action. The absorbance was then read at 450 nm using a BMG FLUOstar Optima plate reader.

6.2.1.2 Results

The results of the assay determining the optimal dilutions of the primary antibody are shown in Fig 6.2. There was no decrease in absorbance readings observed with decreasing concentration of primary antibody for both the cells with no virus and cells infected with RSV.

Figure 6.2 Absorbance values recorded from an ELISA using various dilutions of the primary antibody with a 1:10 000 dilution of the secondary antibody
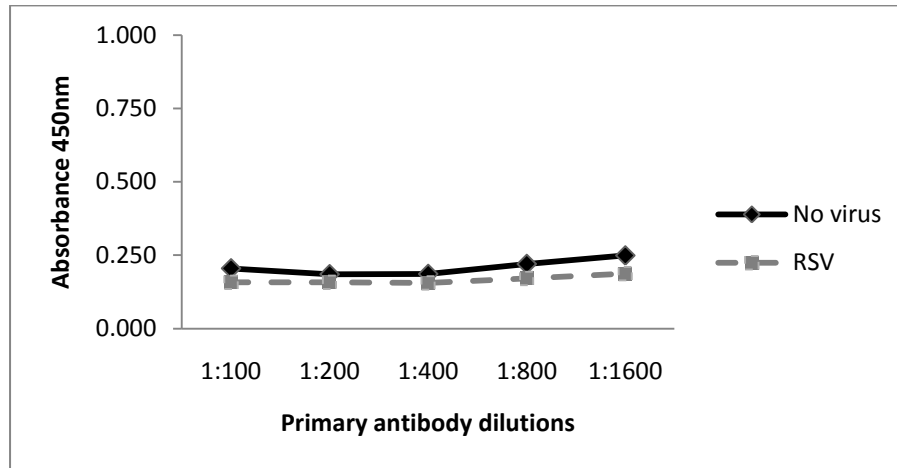
6.2.1.3 Discussion

Although the manufacturer's instructions were followed, the absorbance readings were too low to provide any useful quantification of the antigen present (0–0.2). From the readings it was concluded that there was either a very small amount of viral antigen present on the plate, or that the secondary antibody was too dilute. A separate experiment was performed in which higher concentrations of secondary antibody were used with a primary antibody dilution of 1/200 (Section 6.2.2).

6.2.2 Determining optimal dilution of secondary antibody

6.2.2.1 Materials and Methods

A549 cells were grown overnight in a 24-well plate as outlined in Section 3.2.4. The method as described in Section 6.2.1.1 was used with the following changes: 200 µl of the primary antibody (prepared in PBS pH 7.3 and 0.05% Tween 20) was added to each well at a dilution of 1/200 (5 µg/ml) and incubated at 37°C for 1 hour. After incubation with primary antibody, the

wells were washed 3 times with wash buffer for 5 minutes, and 200 μl of the secondary antibody prepared in PBS (1 mg/ml) was added to each well (using dilutions of 1/200, 1/400 and 1/800 and 1/1600) and incubated at 37°C for 1 hour. The washing, enzyme reaction and reading of absorbance were carried out as in the preceding experiment (Section 6.2.1.1).

6.2.2.2 Results

The results from the assay determining optimal dilutions of the secondary antibody are shown in Figure 6.3. The absorbance readings were positively correlated to the secondary antibody dilution, and the RSV-infected cells gave much higher absorbance readings than the controls.

Figure 6.3 Absorbance values from an ELISA using a 1:200 dilution of the primary antibody and various dilutions of the secondary antibody
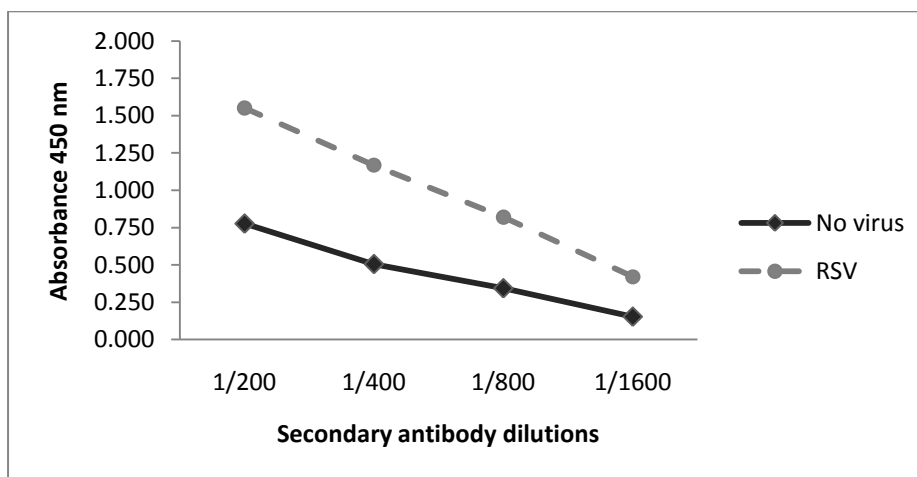
6.2.2.3 Discussion

This experiment demonstrated that the previous problems encountered with the lack of signal were in fact due to the low concentration of secondary antibody used. Much higher concentrations were used in the experiment compared with the manufacturer's recommendation for ELISA of a dilution

of 1:10,000. Here a positive correlation between concentration of secondary antibody and absorbance reading can be seen, and the RSV-infected wells clearly exhibited higher absorbance readings. However, it is evident that there was some non-specific binding occurring between the secondary antibody and the plate as the negative controls also exhibited some colour change, with the absorbance readings also decreasing with lower concentrations of secondary antibody. For further experiments, the successful dilution of 1:200 of the primary antibody and 1:400 of the secondary antibody was used, with a longer incubation time with blocking buffer to minimise the non-specific binding.

6.2.3 Further observations on the effect of honey

6.2.3.1 Materials and methods

A549 cells were grown overnight in a 24-well plate as outlined in Section 3.2.4. Once confluent, cells were inoculated with RSV for 1 hour before the medium was replaced with 0% and 2% M117 honey and left in a 37°C, 5% CO_2 incubator. Once the untreated cells (0% honey) had reached a CPE score of 3, the medium was removed and each well was washed once with wash buffer (0.05% Tween 20 in phosphate buffered saline (PBS) pH 7.3). The method as described in Section 6.2.2.1 was used with some changes. Blocking buffer was left on for 1 hour instead of 30 minutes. Primary antibody (prepared in PBS pH 7.3 and 0.05% Tween 20) was added to each well in 200 μl aliquots at a dilution of 1/200 (5 μg/ml) and incubated at 37°C for 1 hour. After incubation with primary antibody, the wells were washed 3 times with wash buffer and 200 μl of the secondary antibody prepared in PBS was added to each well (at a dilution of 1/400) and incubated at 37°C for 1 hour. The washing, enzyme reaction and reading of absorbance were carried out as described in Section 6.2.1.1.

6.2.3.2 Results

The results from the assay measuring the effects of honey on the amount of detected viral antigens are presented in Figure 6.4.

Figure 6.4 Means ± SEM of absorbance values obtained from ELISA detecting the amount of viral antigens present in controls and RSV-infected cells incubated with or without 2% M117 honey
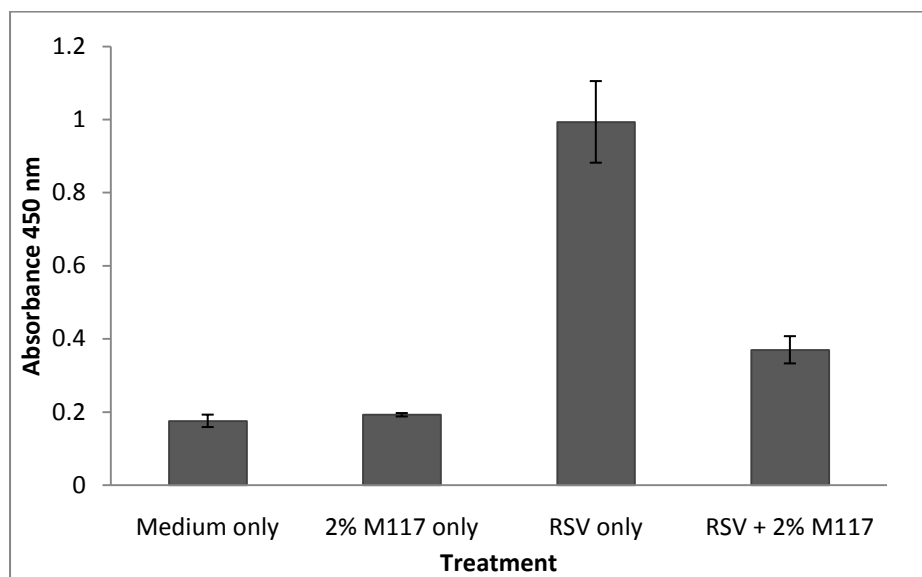
The absorbance readings obtained from RSV-infected cells were significantly higher than those recorded for RSV-infected cells treated with 2% M117 honey. No differences were observed between the control cells.

6.2.3.3 Discussion

This experiment confirmed the results from the previous experiments observing CPE development that honey has an inhibitory effect on the development of RSV infection in A549 cells. It was shown in this study that RSV-infected cells which were treated with 2% M117 honey had a lower number of viral antigens detected compared with untreated cells. This suggests that honey somehow prevents viral replication, resulting in lower

numbers of viral particles and slower development of CPE which was observed in the previous experiments.

How honey affects RSV replication remains unclear. It is possible that honey deactivates, or inhibits the viral particles from entering the cells, resulting in suppression of infection as observed in the earlier neutralisation experiments. Although less likely, another possibility is that the viral particles are entering the cells, but a cellular response elicited by honey may be inhibiting the transcription or translation of viral protein. An experiment looking at viral gene transcription in RSV-infected cells incubated with or without honey would be desirable as it would narrow down the stages of infection at which honey may be acting on the viral particles. Further studies on the effects of honey on the upregulation of genes involved in the antiviral response in A549 cells would also be of value.

Chapter Seven

The effect of honey on RSV replication

7.1 Initial experiments

7.1.1 Introduction

The aim of this chapter was to investigate the effect of honey on viral replication kinetics in RSV-infected cells. Findings from preceding chapters have confirmed a reduction in virus titres using ELISA, a method which detects the amount of viral antigen present in a sample, however, it was desirable to determine whether the effects of honey could also be observed at the transcription level, and to rule out the possibility of the occurrence of "abortive" replication, where there is an increase in the expression of viral transcripts and genome replication that does not lead to the production of detectable progeny virus (Boukhvalova *et al.*, 2007).

Initial experiments look at cells that have been incubated with or without virus. They were performed as a way to validate the methods to be used, and to determine the specificity of the designed primers before performing further experiments looking at the treatment effect of honey. Conventional RT-PCR analysis was used to detect whether mRNA for specific genes of interest were present. This enabled a number of genes to be screened for further quantification with real-time PCR.

7.1.2 Materials and Methods

7.1.2.1 Preparation of cells

A549 cells were seeded into cell culture flasks and incubated at 37°C overnight as described in Section 3.2.4. Once confluent, the growth medium was removed from the cells. Viral stock suspension (1 ml) was added to one flask and the other served as a control. The flask was then put in a 5% CO_2 incubator set at 37°C for one hour. After one hour, the inoculum was aspirated off and replaced with 4 ml of maintenance medium. The flasks were then left to incubate and were observed daily for CPE development.

7.1.2.2 Lysis of cells and RNA extraction

Once a CPE score of 3 had been reached with the RSV-infected cells, the growth medium was poured off the flasks and 700 µl of 5 M guanidium thiocyanate (Roche) was added to each. The flasks were then rocked gently and left at room temperature for 5 minutes for further lysis. The lysed cells from each flask were then transferred into a 1.75 ml Eppendorf microcentrifuge tube and 75 µl of 2 mol/l sodium acetate buffer (pH 4.0) was added. The tube was inverted to mix the contents before adding 750 µl of water-saturated phenol. The contents were mixed and the tube left for 5 minutes at room temperature. After 5 minutes, 200 µl of chloroform was added and the tube was vortexed for 20 seconds and then left on ice for 10 minutes.

After 10 minutes on ice, the tube was then centrifuged for 15 minutes at 16 000 rpm at 4°C using an Eppendorf bench microcentrifuge. The clear supernatant containing the RNA was then transferred to a new tube and the process repeated from the addition of 75 µl of 2M Sodium Acetate. After the tube had been centrifuged for 15 minutes for the second time, the clear

supernatant was again transferred to a new tube and an equal volume of isopropanol was added. The tube was inverted to mix the contents and then was stored at room temperature for 20 minutes to precipitate the RNA.

After 20 minutes, the tube was centrifuged for 10 minutes at 16 000 rpm at 4°C in a bench centrifuge. The supernatant was then removed carefully, avoiding touching or moving the pellet. Ethanol (1 ml of 70% ethanol) was added to the tube which was then mixed briefly and centrifuged for 4 minutes at 7 500 rpm at 4°C. The tube was then taken out of the centrifuge and the ethanol removed carefully, avoiding the RNA pellet. The tube was briefly re-spun for 5 seconds to bring down any remaining ethanol which was then also aspirated off. The tube was then left open on the bench to air-dry the RNA pellet for about 5 minutes before adding 20 µl of 1 X Tris $MnCl_2$ buffer (10 mmol/l Tris, 0.5 mmol/l $MnCl_2$, pH 7.8). The tube was vortexed to re-dissolve the pellet prior to addition of 1 µl of DNAse (Promega) to remove any residual DNA. The tube was then incubated for 30 min in a thermomixer (37°C). The DNAse was then inactivated by adding 1 µl of DNAse Stop Solution (Promega) and the tube incubated for 10 min at 65°C in a thermomixer. RNA quality was analysed and the sample frozen at -80°C until required.

7.1.2.3 Checking RNA quality

To check the quality of the extracted RNA, 5 µl of RNA from each tube was run in a 1% agarose gel (see Section 7.1.2.4) in 1X SB buffer (5.6 g boric acid, 1 g NaOH made up to 2 litres with distilled water, pH 8.5). The samples electrophoretically separated at 70 volts for 60 minutes and the gel was viewed under UV light. RNA integrity was indicated by the presence of sharp, clear 28S and 18S rRNA bands. Partially degraded RNA and primer

dimers would appear as a low molecular weight smear. RNA purity was checked by measuring the absorbance ratio of samples at 260 nm/ 280 nm (A_{260}/A_{280}) using a NanoDrop ND-1000 Spectrophotometer. Values of 1.8– 2.0 were considered acceptable.

7.1.2.4 Preparation of a 1% agarose gel

Agarose (0.3 g) was added to 30 ml 1 X SB buffer and microwaved on high for 2 minutes. Once the agarose was dissolved, the flask was cooled by swirling the flask, and 2 µl of ethidium bromide (10 mg/ml stock solution) was added. Approximately 30 ml was poured into a gel caster (Horizon® 11.14, GibcoBRL), an eight lane comb was added, and the gel was left to set for around 30 minutes. For 2% agarose gels, 0.6 g of agarose was added to 30 ml 1 X SB buffer and prepared in the same manner.

7.1.2.5 First-strand cDNA synthesis

After extraction of purified RNA (as described in Section 7.1.2.2), cDNA was prepared from this for conventional RT-PCR according to a method adapted from Jobin *et al*. (1998) and Bierhaus *et al*. (1997). For each cell sample, 3 µl of RNA sample was placed in a 1.5 ml Eppendorf tube along with 1 µl random hexamers (Bio-Rad Laboratories) and 4 µl DEPC-treated water. Random hexamers are used to ensure the efficient amplification of all RNA in a sample. The contents were mixed by vortex. The tubes were then incubated for 5 min at 70°C in a PCR machine (MJ Research PTC-200 Peltier Thermal Cycler) to separate secondary structures from the RNA. The tubes were then put on ice for 10 min to enable the random hexamers to anneal to single-stranded RNA.

In a separate 1.5 ml Eppendorf tube, 4 µl of 5 X First Strand Reverse Transcriptase buffer (Invitrogen) was placed with 1 µl of 0.1 mol/l DTT (Invitrogen) along with 1 µl of dNTP mix (Invitrogen, 10 mmol/l each), 3 µl DEPC-treated water and 1 µl Superscript III Reverse Transcriptase™ (Invitrogen). The contents of this tube were added to the tube containing the RNA and random hexamer mix. The samples were then incubated in a PCR machine at 25°C for 10 minutes, 50°C for 60 minutes and the reaction was terminated at 85°C for 5 minutes. The cDNA samples were then stored at -80°C until required.

7.1.2.6 Primers

Primer pairs for the RSV genes investigated were designed using the Primer3 version 0.4.0 (Rozen & Skaletsky, 2000) software and are listed in Table 7.1.

Table 7.1 Primer sequences for RSV F, M, L and NS1 genes and housekeeping gene (GAPDH)

Gene	Primer sequence (forward and reverse) 5'-3'	Primer (bp)	Product (bp)	Tm (°C)
F	CAAGAACCGACAGAGGATGG	20	245	64.6
	GCATGACACAATGGCTCCTA	20		63.7
M	ATGGGGCAAATATGGAAACA	20	214	63.7
	AGGTCCATTGGGTGTGGATA	20		64.0
L	AAAGGTTACGGCAGAGCAAA	20	246	63.5
	TTAACAACCCAAGGGCAAAC	20		63.5
NS1	ATGGGGTGCAATTCACTGAG	20	202	64.9
	CAGGGCACACTTCACTGCT	19		64.2
GAPDH	GAGTCAACGGATTTGGTCGT	20	238	63.9
	TTGATTTTGGAGGGATCTCG	20		63.8

A primer pair for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used based on previous publications (Parekkadan *et al.*, 2007; Ylösmäki *et al.*, 2008). All primers were purchased from Sigma-Aldrich.

7.1.2.7 PCR with iStar® Taq

The PCR process involves multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences. iStar® *Taq* DNA polymerase (Intron Biotechnologies) was used for PCR amplifications. An antibody complexed to *Taq* polymerase acts to block polymerase activity until after the first 94°C denaturation step during PCR cycling. Each PCR reaction contained 20 µl of Master Mix, 0.5 µl of forward and 0.5 µl of reverse primers, 1 µl cDNA and 1 µl DNA polymerase.

After pre-incubation for 2 min at 94°C (MJ Research PTC-200 Peltier Thermal Cycler), the target DNA was amplified with 40 cycles, each cycle consisting of a denaturation step at 94°C (20 sec melting), annealing for 20 sec at 60°C (optimised for the specific genes, as presented in Table 7.1), and extension at 68°C for 35 sec.

7.1.2.8 Electrophoresis of DNA

To check whether the cDNA had been successfully synthesised, a 2% agarose gel was prepared (Section 7.1.2.4) and run containing 5 µl of amplified cDNA mixed with 2 µl of loading buffer (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) for each sample. A 100 bp DNA ladder (Invitrogen) was also loaded on each gel to enable the base pair size of the DNA products to be determined. Electrophoresis was carried out in Horizon tanks (Invitrogen) containing SB (1 X) buffer, at 110 volts for approximately 25

minutes until separation was achieved. DNA was illuminated using UV, photographed and analysed using the Scion Image software.

7.1.2.9 DNA sequencing

The PCR products from the initial experiments were sent to the Waikato DNA Sequencing Facility for validation. To prepare the samples for sequencing, the products had to be purified. This was performed by adding 40 µl of each PCR product into a 1.7 ml Eppendorf tube containing 40 µl of 20% polyethylene glycol (PEG) solution and vortexing the tube before leaving at room temperature for 15 minutes. The tube was then vortexed again and centrifuged at 16 000 rpm for 10 minutes. The supernatant was aspirated off and 1 ml 100% ethanol added to the remaining pellet. The tube was vortexed and centrifuged again for 5 minutes. The ethanol was aspirated off and replaced with 1 ml 70% ethanol, and the tube centrifuged for 5 minutes. All remaining liquid was aspirated off and the tubes left to air dry. Once dry, 15 µl of TE buffer (10 mmol/l Tris pH 8, 1 mmol/l EDTA, pH8) was added and the amount and quality of the products checked with the NanoDrop ND-1000 Spectrophotometer. Primers were diluted to 5 pmol/l and were also sent away with the templates for sequencing.

7.1.3 Results and Discussion

Figure 7.1 shows the gel electrophoresis run of PCR products acquired from cells that have been incubated with or without RSV. A 100 bp ladder was included as a guide to determine whether the products observed are of the correct size (based on Table 7.1). Clear bands were seen for the housekeeping gene in both infected and uninfected cells.

Figure 7.1 2% agarose gel of PCR products.
(From left: 100 bp ladder, F, M, L, N and GAPDH from RSV-negative cells and F, M, L, N and GAPDH from RSV-positive cells)
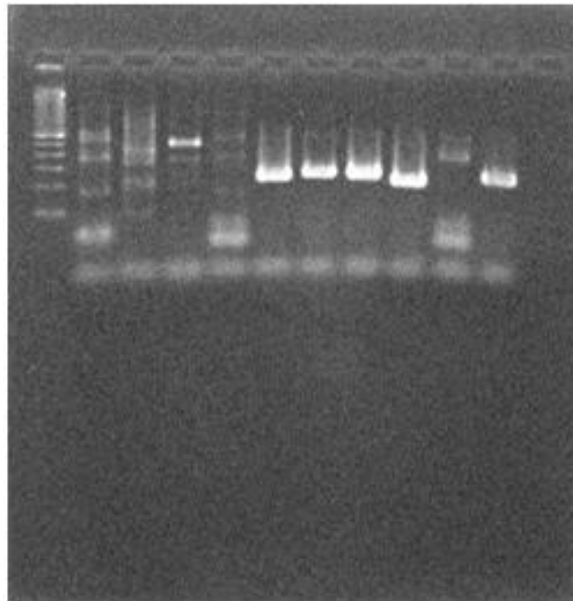
Clear bands representing the PCR products from RSV-infected cells for RSV genes F, M and L can also be seen. There was no clear band observed for the RSV N gene in the infected cells and this was therefore excluded from further experiments. There was also some non-specific binding of primers seen in the uninfected cells, as illustrated by the presence of faint bands and smearing, which was minimised in further experiments by raising the melting temperature from 55°C to 60°C.

7.2 Quantitative Real-Time RT-PCR

7.2.1 Introduction

Real-time PCR was used to provide a quantitative measure of the transcription of mRNA for specific proteins of interest. A fluorescent marker is hybridised to the cDNA between two PCR primers for a single gene of interest. As PCR proceeds, the 5′ nuclease activity of the *Taq* polymerase cleaves the probe, releasing the tag such that the fluorescence is proportional

to the amount of cDNA present. SYTO82® (Roche) was used as a fluorescent marker allowing any double-stranded DNA generated during PCR to be detected. Specificity of the PCR products was confirmed by melt curve analysis and electrophoresis on a 2% agarose gel.

7.2.2 Materials and Methods

After extraction of purified RNA (as described in Section 7.1.2.2), it was prepared for real-time PCR according to a method adapted by Dr Ray Cursons (University of Waikato) from that described by Konnai *et al.* (2003). All steps prior to the addition of cDNA were carried out in a PCR cabinet.

Real-time qPCR was performed using a Corbett Research qPCR machine, including fluorescent emission and detection of the signals. Reactions were carried out in a total volume of 20 µl. A stock master mix solution was made which contained (for 10 ml); 1 ml 10X buffer (500 mmol/l KCl, 100 mmol/l Tris HCl (pH 9.0 at 25°C), 15 mmol/l $MgCl_2$, and 1% Triton X-100), 2 ml $MgCl_2$ (25 mmol/l), 7 ml distilled water, 20 µl of each dNTP (10 mmol/l) and 4 µl SYTO82 nucleic acid stain (Invitrogen). This stock solution was distributed into 1.5 ml Eppendorf tubes and each tube was treated with 10 µl of DNAse for 1 hour. The DNAse was then inactivated by heating the tubes at 75°C for 10 minutes in a thermomixer. Each real-time PCR reaction mixture contained 18.6 µl master mix, 0.13 µl each of the forward and reverse primers, 0.15 µl of *Taq* DNA polymerase and 1 µl of cDNA. Each sample was tested in duplicate, and the experiment was repeated twice on different days.

The qPCR protocol using Corbett Rotor-Gene 6000 (Corbett Life Science, NZ Ltd) consisted of pre-incubation for 2 min at 95°C followed by amplification of target cDNA with 40 cycles, each cycle consisting of a denaturation step at

95°C for 20 sec, annealing for 20 sec at 60°C, extension at 68°C for 30 sec, and determination of fluorescence at 80°C for 10 sec. The annealing temperature was raised to 60°C from 55°C as previously used in order to minimise the presence of non-specific products. To check if there was any contamination with genomic DNA, a negative control was done for every reaction set by performing the RT-PCR reaction with sterile water instead of cDNA.

Melting curve analysis was performed which resulted in single product-specific melting temperatures above 80°C for each primer pair, confirming specificity of each primer pair. The samples were analysed for both the target genes and an endogenous control gene, GAPDH. The expression levels of the target genes were reported relative to the expression levels of GAPDH using the comparative quantitation method in the Rotor-gene 6000 software. It is recognised that housekeeping genes are differentially expressed under various experimental conditions (Rogler *et al.*, 2004).

After analysis of the real-time PCR results, an end-product sample of DNA from the reaction for each gene was analysed by 2% agarose gel electrophoresis (as described in Section 7.1.2.8). This enabled the presence of any DNA contamination to be seen and the size of the products to be determined by comparison with a 100 bp ladder. The agarose gel was prepared as described in Section 7.1.2.4.

Student's t-test was used for statistical analysis.

7.2.3 Results

The results from the qPCR experiments are shown in Figure 7.2.

Figure 7.2 Expression of viral genes in RSV-infected cells that have been treated with or without 2% M117 honey.
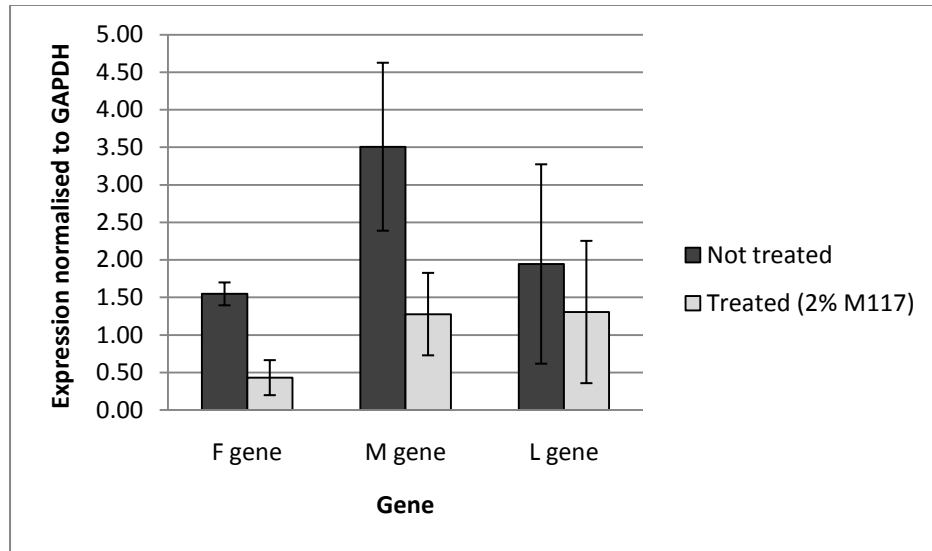
This graph shows the expression of viral genes in non-treated and treated samples normalised to GAPDH. Significant differences in detected levels of F gene and M gene transcripts were observed, however, there was no significant difference in the levels of L gene transcripts detected. Correct products and presence of contamination were checked by melt curve analysis and gel electrophoresis. A representative gel is shown on Figure 7.3.

Figure 7.3 A representative gel showing PCR products from controls and RSV-infected cells incubated with or without 2% M117 honey.
a) cells only, b) cells in 2 % honey, c) RSV-infected cells, d) RSV-infected cells treated with 2% honey
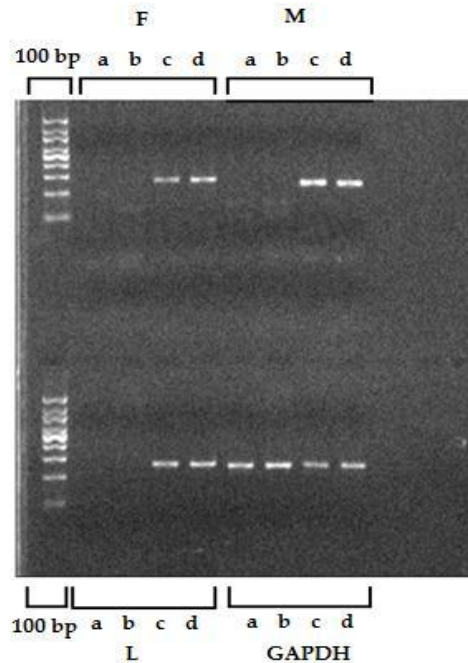
7.2.4 Discussion

A reduction in the number of viral antigens present in RSV-infected cells treated with honey has been previously shown in the ELISA experiments in Chapter 6. This chapter, in turn, has illustrated that not only has honey inhibited the replication of RSV at the translation level, as shown by the amount of protein detected in the previous ELISA experiments, but also at the transcription level, as demonstrated by the reduced number of viral mRNA detected in the treated samples in this experiment. Significant differences were observed in the levels of F and M gene transcripts detected. No significant differences were found between the amount of L gene transcripts acquired from treated and untreated samples, however this could be a reflection on the small sample number. A greater number of repeats of this experiment are needed to confirm the findings presented here.

The results from this study support the hypothesis that honey acts on the virus particle directly by either preventing its entry into the cell or inhibiting its replication within the cell. In future studies, it would be of value to monitor the transcription of all RSV genes from the start of infection and follow its progress in honey-treated and untreated cells. It would also be of importance to perform similar experiments on cellular genes involved in the antiviral response, to determine what role they play in this inhibition and how honey affects them.

Chapter Eight

Determining the antiviral component in honey

8.1 Testing the antiviral activity of the sugar content of honey

8.1.1 Introduction

This experiment aimed to test whether the sugar content of honey is responsible for the slowing of the rate of CPE development caused by RSV when cells are treated with honey, as shown in Chapter 4. The same method was used as described in Section 4.2.2c, with artificial honey used instead of natural honey. Manuka honey M117 was also used as a control.

8.1.2 Materials and Methods

Artificial honey was made according to a recipe described by White (1975a), which was formulated using the average composition of at least 400 samples of major commercial honey types in the USA. It contained 17.2% water, 38.4% fructose, 30.3% glucose, 1.3% sucrose, 8.6% maltose and 1.4% maltodextrin at neutral pH. The syrup was stored at 4°C and solutions were made up as described for natural honey in previous chapters.

A549 cells were seeded into 24-well plates and incubated at 37°C overnight as described in Section 3.2.4. Once the cells were confluent, growth medium was removed from the cells. Viral stock suspension (200 μl) was added to each well and left to incubate at 37°C (5% CO_2) for 1 hour. After one hour, the inoculum was aspirated off and replaced with 1 ml of maintenance medium containing varying concentrations of either M117 honey or artificial honey. The plate was then incubated at 37°C (5% CO_2) and observed daily

for CPE development. The experiment was performed in triplicate within one plate and was repeated, again in triplicate, on a different day.

8.1.3 Results

The results of the experiment investigating the antiviral effects of artificial honey are presented as plots of developing CPE in Figure 8.1.

Figure 8.1 CPE development in RSV-infected A549 cells incubated with varying concentrations of honey and artificial honey
The results are presented as mean ± SEM of triplicates for each of the two days of experiments.
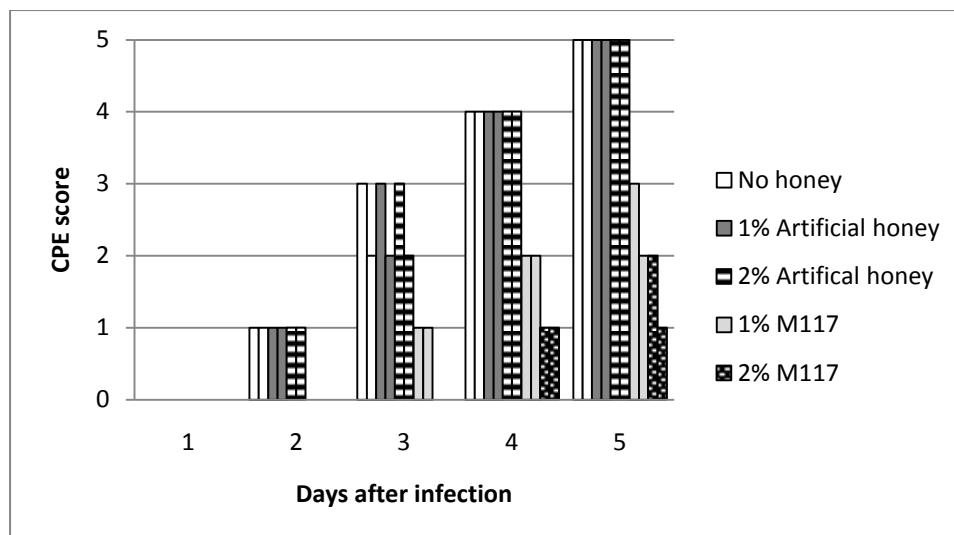
The controls of each honey and artificial honey concentration without virus were shown to not cause morphological changes of the cells over the observation period.

8.1.4 Discussion

This experiment aimed to determine if artificial sugar, at concentrations equivalent to that found in 2% natural honey, would have any antiviral activity against RSV. The results obtained demonstrate that artificial sugar

by itself has no detectable effect on the development of CPE caused by RSV in A549 cells. The development of CPE in cells treated with 1% and 2% artificial honey was similar to that observed in non-treated cells, and much faster than in cells treated with 1% and 2% Manuka honey. Although it can be concluded from this experiment that the sugars in honey alone do not have any antiviral effects, it is possible that they have a role to play in the overall observed antiviral activity when combined with other components found in whole honey.   Future experiments would need to confirm this.

8.2 Testing the antiviral activity of hydrogen peroxide in honey

8.2.1 Introduction

An enzyme within honey, glucose oxidase, produces hydrogen peroxide when honey is diluted. Hydrogen peroxide is a well known and widely used antiseptic, and although present in small quantities in diluted honey, it is possible that it plays a role in the antiviral activity exhibited in previous experiments.

Tests were therefore performed to test the antiviral activity of diluted honey and diluted honey with catalase, an enzyme which functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. This experiment aimed to determine whether the antiviral effect of honey was solely or partly due to the presence of hydrogen peroxide upon dilution. A honey was used which had a high level of production of hydrogen peroxide.

8.2.2 Materials and Methods

Confluent A549 cells were prepared as described in Section 3.2.4.   Bovine liver catalase (Sigma) was made to 2 mg/ml in maintenance media and was filter sterilised. A solution of 4% Rewarewa (RE32) honey was prepared in

maintenance medium. The 4% honey solution was diluted down to 2% honey by mixing with the catalase solution, then filter-sterilised. Once confluent, the growth medium was aspirated off the cells which were inoculated with 200 μl viral stock for 1 hour at 37°C (5% CO_2). The viral solution was then removed and replaced with 1 ml of maintenance medium containing either honey or honey mixed with catalase. Medium without honey or catalase was included as a control.

8.2.3 Results and Discussion

Despite filter-sterilisation of all materials used on the cells for this experiment, what seemed to be a bacterial contaminant remained persistent in all catalase-containing media. No results were therefore acquired from this experiment due to time constraints. Future studies should include this experiment to determine the role hydrogen peroxide plays in the observed antiviral activity of whole honey.

8.3 Testing the antiviral activity of methylglyoxal

8.3.1 Introduction

It was found in earlier experiments that the Manuka honeys tested generally had higher antiviral activity compared with the other honeys tested. Manuka honey is known to contain high levels of methylglyoxal (Adams *et al.*, 2008). Although a previous study by Littlejohn (2009) found that methylglyoxal at concentrations found in 5% and 10% honey had no effect on CPE development caused by adenovirus and herpes simplex virus, it was desirable to examine its effects on RSV. The aim of this experiment was to use methylglyoxal, in similar levels found in 2% honey, in a neutralisation assay, in order to determine whether methylglyoxal is wholly or partly responsible for the observed antiviral effects of whole honey on RSV.

The effect of methylglyoxal alone on the cell line was first tested to determine if there was any cytotoxic effect. This was performed both visually and by performing an MTT assay, a technique used to measure the activity of enzymes, found in living cells, that reduce MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). These reactions only take place when reductase enzymes from the cells are active, therefore, the conversion is used as to measure the number of viable cells present in a treated sample.

8.3.2 Materials and Methods

a) MTT assay

A549 cells were grown overnight in 24-well plates as described in Section 3.2.4. A stock solution of methylglyoxal was made by diluting commercially available 40% methylglyoxal (5 500 mmol/l) solution (Sigma Aldrich) by 1:10,000 in maintenance medium. This was to create a stock solution that, when diluted, will reach methylglyoxal concentrations around and including that found in 2% whole high-activity manuka honey (~0.2 mmol/l) (Littlejohn, 2009). From the stock solution (0.55 mmol/l), solutions of methylglyoxal at concentrations of 0.4, 0.3, 0.2 and 0.1 mmol/l were made. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was made by adding 125 mg of MTT salt (Sigma) to 25 ml sterile PBS (see Section 3.2.6). The solution was then sterilised by filtration and stored at 4°C. Lysing buffer was prepared by dissolving 10 g sodium dodecyl sulfate (SDS) in 20 ml distilled water, 20 ml dimethylformamide (DMF) and 1 ml acetic acid to reach a pH of 4.7.

Once the cells were confluent, growth medium was replaced with various concentrations of methylglyoxal and incubated at 37°C for 1 hour. After 1 hour, the methylglyoxal was removed and the cells were washed once with

PBS (pH 7.3). MTT solution (125 μl) was added to each well including control wells and the plate was incubated for 2 hours at 37°C, 5% CO_2. After 2 hours, the supernatant was removed and 1 ml of lysing solution was added. The plate was left at 37°C in a 5% CO_2 incubator overnight. The following day, the absorbance (at 600 nm) of the wells was measured in a BMG Fluostar Optima plate reader using the lysing solution as the blank. The experiment was repeated twice on different days. Student's t-test was used for statistical analysis.

b) Methylglyoxal neutralisation assay

A549 cells were grown overnight in 24-well plates as described in Section 3.2.4. A stock solution of methylglyoxal was made by diluting 40% methylglyoxal solution by 1:10 000 in maintenance medium. From the stock solution, solutions of methylglyoxal at concentrations of 0.4, 0.3, 0.2 and 0.1 mmol/l were made. Viral supernatant (200 μl) was added to 800 μl of methylglyoxal solution of various concentrations in Eppendorf microcentrifuge tubes which were then placed in a shaking incubator (Level 1, 37°C) for one hour. After one hour, growth medium was aspirated off the cells and replaced with 1 ml of each of the solutions of methylglyoxal with virus and left for 1 hour in the 37°C, 5% CO2 incubator to inoculate. After inoculation, the supernatant was removed and replaced with maintenance medium, and the plate returned in the incubator. Daily observations were made and the development of CPE was recorded until a high level of CPE had developed with the untreated virus. The experiment was conducted in triplicate and repeated in triplicate on a different day.

8.3.3 Results

The results from the MTT assay looking at the effects of methylglyoxal on cell viability are shown on Figure 8.2.

Figure 8.2 Effect of methylglyoxal on the viability of A549 cells.
The absorbance recorded from an MTT assay performed on cells incubated with or without methylglyoxal for one hour is shown as mean values ± SEM.
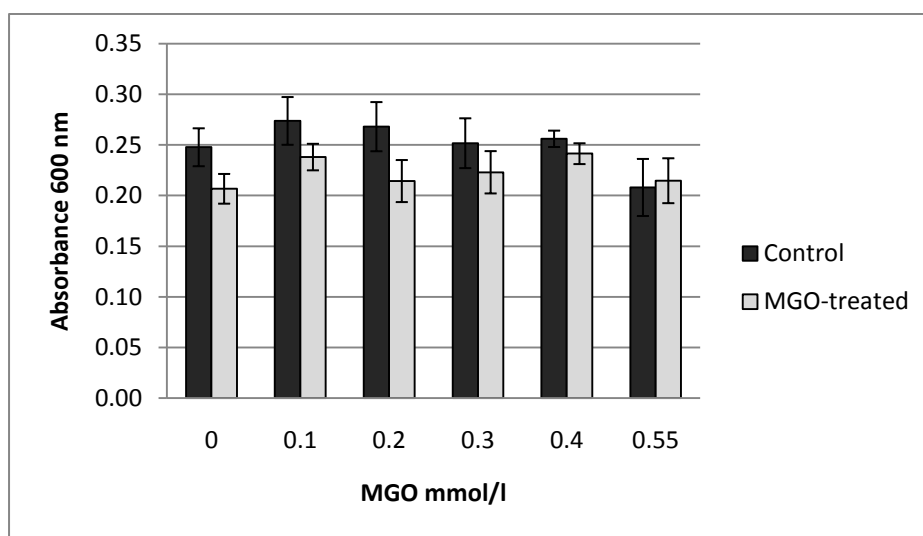
No significant differences in absorbance values were found in MGO-treated cells compared with the controls. The results from the methylglyoxal neutralisation assay are shown in Figure 8.3.

Figure 8.3 Effects of pre-treatment of RSV with methylglyoxal on the development of CPE in A549 cells
The CPE score is shown for successive days of incubation of cells infected with RSV that had been previously treated with varying concentrations of methylglyoxal for 1 hour. The graph represents the means ± SEM of all replicates and repeats.
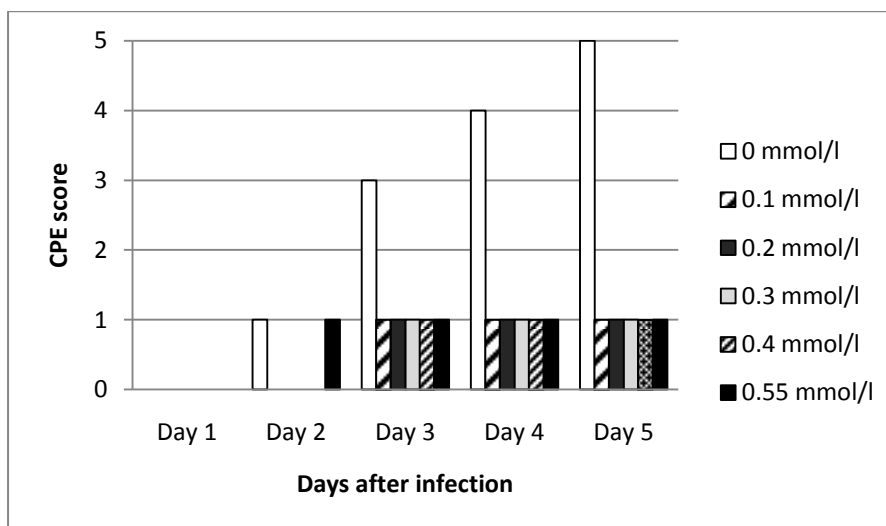
All triplicates and repeats for the neutralisation assay gave identical results and demonstrated the inhibitory activity of methylglyoxal against RSV. By Day 5, it was observed that RSV-infected cells without methylglyoxal had reached a CPE score of 5, while RSV-infected cells treated with methylglyoxal at concentrations as low as 0.1 mmol/l only had a CPE score of 1.

8.3.4 Discussion

This experiment has shown that methylglyoxal, even at low concentrations, had antiviral properties against RSV. By Day 5, cells inoculated with RSV that had been incubated with medium only had reached a CPE of 5 while the other cells inoculated with RSV that had been treated with various concentrations of methylglyoxal ranging from 0.1 mmol/l to 0.55 mmol/l had by this stage developed a CPE score of only 1. Methylglyoxal used at 2 mmol/l has been shown to reduce the TCID of influenza virus by 50%

(Tiffany *et al.*, 1957), however, findings from a study by Littlejohn (2009), where methylglyoxal at concentrations of 0.11 to 2.4 mmol/l was unable to suppress the development of CPE in adenovirus and had little effect on herpes simplex virus. So although methylglyoxal is an effective antiviral agent, it appears as though different strains and types of viruses vary significantly in their reaction to the substance.

The finding that methylglyoxal is an effective antiviral agent against RSV may explain the greater activity seen previously in manuka honeys compared with other honeys, as these contain varying levels of methylglyoxal. It must be noted, however, that it is not likely to be wholly responsible for this observed activity, as other honeys which do not contain methylglyoxal (such as Honeydew and Rewarewa) also have antiviral properties.

It would be of great importance in future studies to test the other components found in non-manuka honeys, such as antioxidants and phenolics, for their neutralising capabilities.

Chapter Nine

General discussion and recommendations for future research

9.1 Summary and General Discussion

The effects of honey on respiratory syncytial virus were investigated in the preceding chapters of this thesis. In Chapter 4, it was found that honey had an inhibitory effect on the development of CPE caused by RSV in A549 cells, except in the protection experiment (Section 4.3), where culturing the cells in honey failed to slow down the subsequent progression of viral infection. Previous studies have shown that honey (0.000025 – 0.1%) can cause changes in gene expression in cells, such as upregulation of a wide range of mediators including TNF-α, IL-1β, and TGF-β (Harcourt, 2005). TNF-α in particular is thought to be important in the host response to viral infection - a study looking at the effect of TNF-α treatment on HEp-2 cells before RSV infection showed that pre-treatment of the cells with TNF-α inhibited RSV replication as determined by CPE (Neuzil *et al.*, 1996). RSV-infected BALB/c mice treated with antibody to TNF-α also had greater maximal weight loss and slower recovery time than control mice, further suggesting a protective role for TNF-α in RSV infection (Neuzil *et al.*, 1996). Although no direct effect of honey on the cells were visually observed in this experiment, they cannot be ruled out until further investigations are conducted. It is possible that the washing away of the honey prior to adding the virus could have allowed diffusion of the protective component of honey, though this is unlikely in the period between aspiration of the honey solution and addition of virus (~ 2 minutes).

As there is strong evidence of honey being able to cause upregulation of genes in other types of cells (Harcourt, 2005), future studies should include determining the effects of honey on the expression of TNF-α and other genes involved in the host antiviral response such as type 1 interferons (Muller *et al.*, 1994) and Toll-like receptors (Bowie & Haga, 2005) in A549 cells.

The progression of infection in cells inoculated with RSV that had been pre-treated with honey (Section 4.4) showed similar rates of CPE development as in a previous experiment looking at the inhibitory properties of honey (Section 4.2), indicating that either the honey is acting on the virus particle directly or that the honey present in the inoculum may be having an effect on the cells, despite the cells only being in contact with the honey (present in the inoculum) for one hour. There was no feasible method available to enable the separation of the viral particles from the honey solution. To confirm the neutralising activity of honey, any effects on the cells as a result of being incubated with honey for one hour must be determined. This could be done by performing timed qPCR experiments on cells that have been treated with honey for one hour, again looking at genes involved in the host antiviral response. Based on the results acquired from these experiments, it is unlikely that honey would be an effective prophylactic agent for individuals at risk of RSV infection, however, as discussed, further investigations are required to confirm this. In contrast, addition of honey to moderately infected cells was observed to halt the progression of infection, thus showing the potential benefit of using honey as a treatment in individuals already experiencing symptoms of infection.

In Chapter 5, experiments testing the antiviral effect of a range of honey types demonstrated significant differences which somehow correlated to their phytochemical profiles and antibacterial activity. The Manuka honeys

were found to have the greatest antiviral effect, with the high-NPA honeys causing the greatest inhibition. The Rewarewa and Honeydew honeys showed inhibitory effects comparable to M115, a mid-range UMF honey. The Rewarewa honey and the Honeydew honey used both had a high level of antibacterial activity due to hydrogen peroxide, high iron-binding antioxidant activity and phenolics. In Chapter 8, it was shown that sugar, in similar levels found in 2% natural honey, is not an effective antiviral agent. In contrast, further investigations determined that methylglyoxal had potent antiviral activity even at very low concentrations, and may therefore be the component responsible for the greater inhibition seen in the high-NPA honeys. The component or components responsible for the observed effect in non-Manuka honeys are yet to be confirmed but is possible that a number of factors determine the level of antiviral activity a particular type of honey may have. Antioxidants, in particular, have been researched thoroughly over the years for their antiviral activity against a wide range of viruses and have been proven to be effective against them. Resveratrol (Faith *et al.*, 2006), glutathione (Tian *et al.*, 2010) and cinnamaldehyde (Ding *et al.*, 2010) in particular have been associated with the suppression of NF-κB, thereby inhibiting viral replication of herpes simplex virus, dengue virus and coxsackievirus B3, respectively. Phenolics have also been shown to have some antiviral activity. One study evaluated the ability of (-)Epigallocatechin gallate (EGCg) and theaflavin digallate (TF3) to inhibit the infectivity of both influenza A virus and influenza B virus in Madin-Darby canine kidney (MDCK) cells *in vitro* (Nakayama et al., 1993). Study by electron microscopy revealed that EGCg and TF3 agglutinated influenza viruses, and that they prevented the viruses from adsorbing to MDCK cells. EGCg and TF3 also more weakly inhibited adsorption of the viruses to MDCK cells and inhibited haemagglutination by the influenza viruses (Nakayama et al., 1993). Another study showed that semi-synthetic catechin derivatives

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exerted inhibitory effects for all six influenza subtypes tested, as well as H2N2 and H9N2 avian influenza virus. The compounds strongly inhibited adsorption of the viruses on red blood cells and also restricted the growth of avian influenza virus *in ovo* (Song *et al.*, 2007).

Chapter 6 of the current study confirmed using ELISA, a quantitative assay, the observed treatment effects of honey. This reinforced the qualitative observations made in the preceding chapters. This was a necessary measure to eliminate any bias that may have occurred during the subjective scoring of CPE in these experiments. This experiment enabled statistical analysis of the quantitative data acquired, showed significant differences between honeytreated and untreated samples, and also provided some insight into the mechanism of action of honey by showing that treatment with honey resulted in a decrease of viral proteins produced. The qPCR experiments in Chapter 7 confirmed this, and demonstrated that the decrease in viral proteins produced stemmed from a decrease or inhibition in the transcription of viral genes. Future experiments could benefit from using the ELISA to track and compare the increase in viral titre in honey-treated and untreated samples from the point of inoculation over the course of a few days. It must be noted, however, that a limitation to this suggestion is the fact that at severe CPE levels, cells begin to detach from the plate surface, making performing an ELISA on severely infected cells an unreliable tool for measuring the amount of viral antigen present. Experiments could be performed on the combined supernatant and cells instead. They could also be performed using qPCR, to gain more information on the transcription of viral genes over the course of the experiment.

Overall, this study has shown that honey has antiviral activity against RSV that is likely to be a result of a direct effect on the virus particle. Previous

studies suggest other roles that honey may play if used as a treatment for viral infections. As well as having antibacterial activity which would protect patients from secondary infections, honey has also been found to have some immunostimulatory effects (Tonks *et al.*, 2003; Tonks *et al.*, 2007), which would also augment the direct antiviral action and therefore contribute to the clearing of virus and healing of infection by the body's own defences.

The results obtained from this study present strong justification to test the effect of honey on the virus in more complex models.

9.2 Recommendations for future work

This study looked at several aspects of the antiviral activity of honey against RSV and has demonstrated its potential therapeutic benefits, however, further research is needed to increase knowledge in this area. Future investigations should include, firstly, determining the mechanism of action of honey. This could be achieved by chromatographic separation of the honey into fractions, to test the antiviral activity of specific components. It would also be ideal to perform this test on a range of honeys with varying phytochemical profiles, as activity may be due to the presence of a number of compounds, not just one. Studies could also focus on determining the effects that honey may have on the expression of genes in cells which are involved in the antiviral response, such as NF-κB. Its role in the clearing of RSV infection by stimulating the body's immune system should also be investigated as stimulation of B- and T-cell lymphocytes and activation of neutrophils by honey has been previously reported (Abuharfeil *et al.*, 1999), as has the stimulation of monocytes in cell culture to release TNF-α and IL-1 and IL-6 (Jones *et al.*, 2000). These cytokines activate many facets of the immune response to infection (Benjamini *et al.*, 1996).

Once the mechanism has been determined, studies may then move on to determining the efficacy of honey on more complex models including primary cell cultures. It will also be vital to investigate its effects *in vivo* using animal models as the internal lung environment is very complex and involves an array of biological processes, for example, mucus production, which may interfere with the intrapulmonary administration of honey.

It is likely that honey may also have antiviral activity against a range of other RNA and respiratory viruses, however, these will need to be tested individually as honey does not appear to affect all viruses similarly, as demonstrated by the differences in the results observed from this study compared with previous studies. A wider range of honey types should also be tested for their antiviral activity, to determine which honeys have the greatest effect as this knowledge would be of benefit therapeutically.

If clinical trials prove the effectiveness indicated by the results in the current study, it is possible that honey may be a better therapeutic option for the treatment of RSV infections compared with other treatments available today.

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