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Investigation into the vector competence of *Ixodes ricinus*
ticks to Hazara virus and Crimean-Congo Haemorrhagic
Fever virus

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Declaration

I, Stacey Louise Leech, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

A handwritten signature in black ink, appearing to read 'SL Leech', with a stylized flourish at the end.

Signed Miss Stacey Louise Leech

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Dedication

This PhD thesis is dedicated to my dad, Eric Leech. Thank you for believing in me, giving me your undivided encouragement, support and love. Most importantly, thank you for the numerous late night, three hour career advice lectures, (sorry chats), and always encouraging me to follow my dreams.

Abstract

Tick-borne pathogens represent a large threat to the UK and International Public Health authorities. Due to recent changes in legislation, an increase in animal & human movements and changing climate, the UK may now be at an increased risk of importing exotic tick species and their associated pathogens. It is vital to assess the susceptibility of UK tick species to these highly fatal tick-borne viral zoonoses. To date, studies investigating the interaction of many pathogens with their vectors have been hindered due to the lack of a suitable tick transmission model at high containment.

This thesis investigates the intrinsic ability of *Ixodes ricinus*, the most widely distributed tick in Europe and the UK, to acquire, replicate and transmit both Hazara virus (HAZV, a hazard group 2 surrogate for CCHFV) and Crimean–Congo haemorrhagic fever virus (a hazard group 4 pathogen) addressing their potential to act as competent vectors. During the last decade CCHFV has emerged in new areas within Europe and the principle tick vector of CCHFV has been detected within the UK.

The development of methods for use with ticks and highly pathogenic viruses within ticks was an essential part of this work. Firstly techniques for the handling, extraction and storage of RNA obtained from *I. ricinus* ticks were optimised and different endogenous controls were assessed for their ability to amplify mRNA transcripts for use as endogenous controls. The use of the immersion technique for use with *I. ricinus* nymphs was optimised and working procedures and protocols for handling ticks at containment level 2 and 4 were established.

Ixodes ricinus nymphs are susceptible to infection with HAZV with 100% becoming infected 13 days post-immersion. HAZV was able to establish itself within the key target organs of the tick midgut and salivary glands, produce infectious virus particles and transmit virus to 38% of mice. This artificial method of inoculation was optimised for use within the CL4 environment and was used to show that *I. ricinus* nymphs were not susceptible to CCHFV via immersion. In addition to horizontal transmission, *I. ricinus* ticks also demonstrated vertical transmission of HAZV through to the adult stage.

This is the first time *I. ricinus* ticks have been assessed for their susceptibility to HAZV and CCHFV and their use in establishing the first high containment *In vivo* tick feeding model in Europe.

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Abbreviations

Abbreviation	Meaning
ACDP	Advisory Committee of Dangerous Pathogens
BIG	Biological Investigations Group
β -ME	B-mercaptoethanol
CCHFV	Crimean Congo Haemorrhagic Fever Virus
CDC	Centre for Disease Control
CL2/3/4	Containment Level 2/3/4
DNA	Deoxyribonuclei acid
FCS	Fetal Calf Serum
FFU/ml	Focus Forming Units /ml
HAZV	Hazara Virus
HEPA	High-Efficiency Particulate Arrestance
HPRT	Hypoxanthine guanine phosphoribosyl transferase
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
MEM	Minimum Essential Medium
MG	Miligrams
MSC	Microbiological Safety Cabinet
MOI	Multiplicity of Infection
NP	Nucleoprotein
NTC	No Template Control
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU/ml	Plaque Forming Units /ml
PM	Peritrophic Membrane
qRT-PCR	Quantitative real-time reverse transcription PCR
R_0	Reproductive number
RNA	Ribonucleic Acid
RPM	Repetitions per Minute
SAT	Saliva Assisted Transmission
SD	Standard Deviation
SEM	Standard Error of the Mean
SGE	Salivary Gland Extract
SW13	Human Caucasian adrenal cortex adenocarcinoma cells
TBEV	Tick-borne Encephalitis Virus
UK	United Kingdom

Chapter 1. Introduction

1.1 Ticks

Ticks are obligate haematophagous ectoparasites of vertebrates relying solely on blood meals taken from vertebrate hosts. They are closely related to mites and both belong to the phylum Arthropoda, subclass Acari in the class Arachnida (Wall and Shearer, 2001). Ticks are thought to be one of the earliest terrestrial arachnids originating 144-165 million years ago during the Cretaceous period (Mans and Neitz, 2004) with 900 tick species currently described (Barker and Murrell, 2004). Adult ticks and nymphs, like all arachnids, are characterised as having four pairs of legs with larvae having 3 pairs, all ticks have mouthparts comprising the palps and chelicerae, an absence of wings and an antennae and the characteristic segmentation of the body; the anterior gnathosoma and the posterior idiosoma (Figure 1.1). Ticks differ from mites with the presence of a sensory Hallers organ located on the front tarsi containing chemoreceptors used during questing (host seeking), and an anchoring hypostome with backward facing barbs used during feeding (Anderson and Magnarelli, 2008).

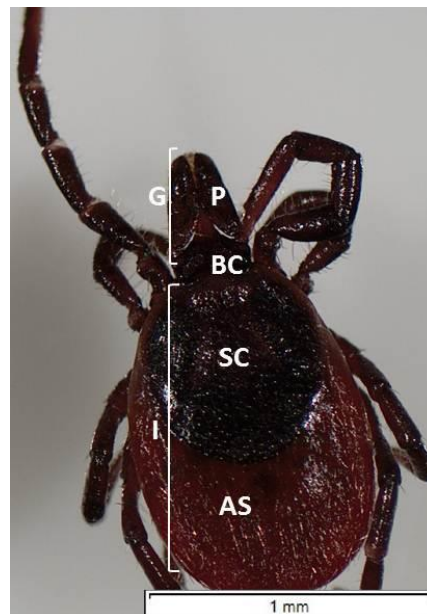


Figure 1.1: Representative picture of an unfed *Ixodes ricinus* adult female. The gnathosoma (G) comprises; the basis capituli (BC) which encloses the pharynx and anchors the mouthparts and the palps (P) which cover the paired chelicerae and the central hypostosome. The idiosoma (I) is consisted of an anterior podosoma comprising the walking legs and genital pore and the distinguishing feature of Ixodid ticks the hardened scutum (SC). The posterior opithosoma consists of the flexible alloscutum (AS), the spiracular plates and the anal aperture (not shown).

Ticks are represented by three families; the Ixodidae known as hard ticks; the Argasidae the soft ticks and; the Nutellidae which exhibit characteristics from both hard and soft tick species. The two main families, the hard and soft ticks, are distinguishable by their biological characteristics (Table 1.1). The hard seek hosts by a behaviour called questing whereby ticks crawl up the stems of grass or perch on the edges of leaves on the ground extending their front legs using their sensory hallers organ to detect biochemicals such as carbon dioxide and ammonia as well as heat and movement (Sonenshine and Roe, 2014). Ixodid tick species can be classified as one, two or three host species with the latter feeding once per life stage on a different host for several days ingesting large volumes of blood, several times their body weight.

Table 1.1: Biological characteristics of the Ixodid and Argasid tick families.

Feature	Ixodidae	Argasidae
Scutum	Present	Not present
Capitulum (mouthparts)	Anterior – visible from above	Ventral
Nymphal stages	One	Several
Adult engorgement time	Several days	30-60 minutes
Blood meals	One	Several
Egg laying events	One	Several
Total eggs laid	3,000-8,000	400-500

Finding a host is very important for the completion of the tick life cycle but also the transmission of pathogens. *Ixodes ricinus* is known to parasitise more than 300 vertebrate host species (Anderson, 1991) and are shown to follow a pattern of aggregation known as the Pareto principle whereby 20% of the host population harbour 80% of the tick population (Perkins et al., 2003). This localised on-host aggregation has been shown to be very important for pathogen transmission particularly with Tick-borne encephalitis virus (TBEV) where infection in the vertebrate hosts only lasts for 2-3 days (Randolph et al., 1999) requiring multiple tick hosts to be feeding at a pre-defined time in order to become infected and maintain the virus in nature. The influence of host choice also influences the moulting success of ticks as seen with *Ixodes scapularis* when parasitising white footed mice resulting in higher moulting rates of ticks compared to feeding on short tailed birds (Brunner et al., 2011). The way in which ticks identify suitable hosts is thought to be mediated by semio-chemical communication between the host and tick and between attached and non-attached ticks (Sonenshine, 2004). These are chemical mediators such as pheromones

which are secreted outside of the body and direct a specific behavioural response. These are secreted by attached and feeding ticks and have been shown to influence the attachment of unfed ticks to hosts (Alla and Sonenshine, 2002; Norval et al., 1989). Ticks appear to be able to select the most optimal questing sites within vegetation by occupying grass stems previously occupied by former questing ticks (Healy and Bourke, 2008). *Ixodes ricinus* ticks were shown to aggregate in female and male groups on plant stems in Northern Ireland (Healy, A, 2004) suggesting that aggregation is also directed by sex pheromones.

Kairomones are secreted by hosts and include chemical cues such as ammonia and CO₂ (Sonenshine, 2004). Secretion of other volatile substances such as indole, have been linked to the health status of hedgehogs with an increase in indole secretion with sick animals resulting in a high tick infestation density (Bunnell et al., 2011). Host selection is also determined by gender and size with larger male white footed mice preferred by *Dermacentor variabilis* nymphs compared to smaller females (Dallas and Foré, 2013; Dallas et al., 2012) both in the presence and absence of CO₂ again indicating sex based chemical aggregation. The site of tick attachment can vary between different life stages. Red deer in Europe harbour all life stages of *I. ricinus* ticks but each stage localises in different locations on host; nymphs attach on the ears, adults aggregate in the groin and on the neck and larvae are found on the legs (Myserud et al., 2014) and this is also seen on sheep (Ogden et al., 1998). This shows that host selection, attachment and feeding is an intricate and specific orientated behaviour.

1.1.1 ***Global importance of ticks***

Ticks have long been identified as parasites of vertebrates and for their related medical importance, with tick fever being described as early as 1550BC in an Egyptian papyrus scroll (Varma, 1993). Ticks parasitise a variety of vertebrate hosts including mammal, birds, reptiles (Sumrandee et al., 2014) and amphibians (Smith et al., 2008). Ticks transmit the greatest variety of pathogens compared to any other arthropod (Jongejan and Uilenberg, 2004) and can cause tick paralysis and toxicosis themselves (Hall-Mendelin et al., 2011). Ticks transmit debilitating and fatal pathogens within livestock such as Theileria a parasite which infects white and blood cells of cattle causing death costing the livestock industry hundreds of millions of dollars (Bishop et al., 2004),

Babesia canis, a protozoan haemoparasite affecting domestic dogs (Irwin, 2009), the Lyme disease spirochete causing a debilitating human disease and zoonotic haemorrhagic fever viruses are important human tick-borne pathogens (Whitehouse, 2004).

1.1.2 *Ticks as vectors of disease*

Ticks have a variety of characteristics that make them good at maintaining and transmitting diseases, one of which is their longevity and life cycle. The tick life cycle comprises four developmental stages; eggs, larvae, nymphs and adults with the entire life cycle measured in years (Labuda and Nuttall, 2004). Pathogens such as tick-borne viruses are shown to be maintained throughout the tick life cycle (Davies et al., 1986) making them significant reservoirs of disease.

The number of generations produced in one year varies between tick species, primarily influenced by climate (Gray et al., 2009). The warm climates of Europe harbour *Rhipicephalus sanguineus* ticks which may produce up to two generations per year remaining active throughout winter (Dantas-Torres, 2010). The cooler northern climates result in *I. ricinus* having two peaks of activity one in spring and autumn. This species is known for feeding only once per year, completing one life cycle in three years (HILLYARD, 1996). *Ixodes ricinus* can also over-winter if a suitable host is not found, prolonging the life cycle further (Kahl et al., 2002). *Dermacentor reticulatus* adopts an intermediate strategy with the adults overwintering but still resulting in one generation per year. These life history strategies make ticks good reservoirs of disease and efficient vectors, carrying viruses for long periods of time.

Each life history stage must acquire a blood meal from a suitable host in order to complete their life cycle. European tick species (*Ixodes*, *Rhipicephalus* and *Dermacentor*) are predominantly three host species with *Hyalomma* ticks being two host species whereby the larvae and nymphs feed on the same host (Sonenshine and Roe, 2014). The feeding behaviour of ticks therefore influences the vertebrate hosts that the pathogens come into contact with.

The feeding behaviour of ticks is also key in the transmission of pathogens with the route of pathogen transmission through saliva secreted during feeding (Kaufman and

Nuttall, 2003; Nuttall and Labuda, 2004). Ticks are "pool feeders", they hollow out an area of the host's skin which then becomes filled with blood from ruptured blood vessels. To accomplish blood feeding the ticks secrete a variety of bioactive compounds that compromise the host's ability to maintain haemostasis and counteract tissue damage (Kazimírová and Štibrániová, 2013). These include potent anticoagulants and anti-inflammatory proteins that inhibit platelet aggregation and facilitate blood flow, inhibit the coagulation pathway and suppress wound healing and inflammation. Immunoglobulin binding proteins suppress the ability of the host's antibody action and anaphylatoxins minimise inflammation (Reuben Kaufman, 2010).

1.1.3 *Ticks and Arboviruses*

Arthropod-borne viruses constitute the largest biological group of vertebrate viruses with more than 530 identified (Labuda and Nuttall, 2004). Arboviruses are transmitted by mosquitoes, sand flies, biting midges and ticks which transmit 25% of viral species comprising of at least 38 viruses from 6 different families (*Asafviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, *Bunyaviridae* and *Flaviviridae*) (Labuda and Nuttall, 2004). Tick-borne viruses are unique in that they are able to replicate in both vertebrate and non-vertebrate cells with little effect to the vector. Arboviruses are RNA viruses with the exception of African swine fever virus transmitted by *Ornithodoros* species which is shown to cause mortality in its tick vector (Endris et al., 1992). Only 10% of the 900 of tick species identified are implicated as virus vectors either indicating a highly specialised and specific relationship or alternatively may be due to the lack of vector competence studies using tick-borne viruses. Tick-borne viruses that are of significant importance to human health, such as tick-borne encephalitis virus and Crimean-Congo haemorrhagic fever virus, require handling within a high containment environment. To date, there are no established *In vivo* tick feeding models for use at high containment hindering the investigation of these pathogens with their vector and hosts.

1.1.4 *Vector competence*

Vector competence, the ability of biting arthropods to acquire, maintain, replicate and transmit infectious agents to their hosts is a multifaceted relationship between pathogen, vector and host influenced by both Intrinsic and extrinsic factors. Extrinsic

factors include the availability of hosts (Dobson, 2014), differences in types of habitat (Hoch et al., 2010) and the influence of climate (Gilbert et al., 2014). Intrinsic factors, most fundamentally, the physiological ability of vector tissue to become infected and maintain a particular infectious agent (Hardy et al., 1983) is extremely important when assessing the viability of a species in the propagation of a pathogen. This is the physiological ability of a tick to acquire, replicate and transmit infectious agents, most importantly the physiological capability of the tick gut cells to replicate virus (Ueti et al., 2007). In order for a tick to be deemed a competent vector a tick species must fulfil three criteria (Kahl et al., 2002); (i) a tick must acquire a pathogen via a natural blood meal when feeding on an infected host; (ii) the pathogen must replicate within the tick and be transmitted horizontally to a naive host and; (iii) vertically to one or more life stages. Investigation of vector competence therefore requires a natural feeding system.

Vector competence studies so far have focused on *Rickettsia* (Piranda et al., 2011), *Bartonella* (Reis et al., 2011), *Anaplasma* (Futse et al., 2003) and *Borellia* pathogens (Lane et al., 1994) which require handling at low containment. In order to assess vector competence of tick-borne viruses an *In vivo* feeding model for use at high containment needs to be established.

1.2 Tick fauna of the UK

In the UK there are 21 native tick species including 18 Ixodid species across four genera; *Ixodes*, *Dermacentor*, *Haemaphysalis* and *Rhipicephalus* (Jameson and Medlock, 2011). *Ixodes ricinus* is the natural vector for TBEV in Western Europe and CCHFV has been isolated from all other genus. Several British tick species are known to parasitise humans (Table 1.2).

Table 1.2: British tick species known to parasitise humans and their associated pathogens

Tick Species	Common British hosts	Records of human biting in Britain	Associated pathogens and diseases	References
<i>Ixodes hexagonus</i>	Hedgehog and foxes	Yes	TBEV (isolated)	(Jameson and Medlock, 2011; Krivanec et al., 1988)
<i>Ixodes ricinus</i>	Sheep, cattle, deer, dogs, small mammals and birds	Yes	<i>Borrelia burgdorferi</i> (Lyme disease), <i>Anaplasma phagocytophilum</i> , <i>Babesia divergens</i> , Louping ill virus and tick borne encephalitis	(Hudson et al., 1995; Jameson and Medlock, 2011; Rieille et al., 2014)
<i>Dermacentor reticulatus</i>	Dogs, cattle, sheep, horses	Yes	Omsk haemorrhagic Fever CCHFV (Isolated) TBEV	(Hoogstraal, 1979; Růžek et al., 2010; Wójcik-Fatla et al., 2011)
<i>Rhipicephalus sanguineus</i>	Dogs	Yes	CCHFV isolated <i>Babesia canis canis</i> <i>Rickettsia canis</i> (Rickettsiosis) <i>Coxiella burnetii</i> (Q fever)	(Bursali et al., 2011)
<i>Haemaphysalis punctata</i>	Sheep, goat, small mammals and birds	Yes	Q fever TBEV Lyme disease Tick paralysis	(HILLYARD, 1996)

In the UK there is a general consensus that tick abundance and distribution is changing (Scharlemann et al., 2008). Veterinary practices across Great Britain between March 2008 and October 2009 reported that more than 50% of dogs examined carried ticks (Smith et al., 2011). Environmental health officers have reported that cases of fleas and ticks have trebled over the last five years (Smith, 2011) and populations of *I. ricinus* ticks have increased in distribution by 17% and have increased at 77% of locations surveyed (Scharlemann et al., 2008). Tick surveillance conducted by Public Health England (PHE) has shown that increasing number of *Ixodes hexagonus* and *I. ricinus* ticks have been reported parasitising humans (Jameson and Medlock, 2011). An

established population of *Dermacentor reticulatus* ticks have been documented (Medlock et al., 2011) with previous records being associated with domestic travel and attachment to humans has also been shown (Jameson and Medlock, 2011). Importation of a number of tick species by returning travellers have also occurred including *Dermacentor marginatus* which is known to be associated with CCHFV (Pietzsch et al., Article in press) and the paralysis tick *Ixodes holocyclus* from Australia (Pietzsch et al., 2014).

Over the last 6 years the importation of exotic species to the UK has increased; *Hyalomma marginatum* (*H. marginatum*), the natural vector of CCHFV, have been collected from a horse imported from Portugal (Jameson and Medlock, 2011). This was the first report of an adult *H. marginatum* imported into the UK; all previous records have been immature stages collected from migratory birds and *H. marginatum* ticks have been shown to migrate from Africa to the UK during the spring months of March – May (Jameson et al., 2012). Due to harmonisation across the European Union regulations on the travel of companion animals has recently changed. Since the 1st January 2012 compulsory treatment for ticks from pets entering the UK from listed and non-listed EU and non EU countries (Roberts, H, 2011) was discarded. This is extremely important for the importation of tick species and their associated diseases into the UK. The brown dog tick; *Rhipicephalus sanguineus*, confined to the Mediterranean basin and associated with CCHFV (Bursali et al., 2011) has been imported on ten separate cases into the UK (Hansford et al., 2014) with high infestation levels found in domestic dwellings since the legislation was lifted (Hansford et al., 2015). Prior to the lifting of tick controls there was evidence of exotic ticks in quarantine kennels between 1976 and 2007 and on animals travelling to the UK under PETS between 2002 and 2009 (Jameson et al., 2010). Of particular note is the arrival of 53 *R. sanguineus* nymphs one of which was found parasitising a kennel worker.

1.2.1 *Ixodes ricinus*

Ixodes ricinus is the most geographically spread tick species in Europe present in Scandinavia, the UK and central and southern Europe (Figure 1.2 ECDC, 2015). The distribution of *I. ricinus* has expanded over the last few decades increasing in altitude (Daniel et al., 2003; Danielová et al., 2008; Materna et al., 2005) and latitude (Jaenson

et al., 2012) with changes attributable to an increase in temperature (Medlock et al., 2013) and roe deer populations (Scharlemann et al., 2008). *I. ricinus* ticks are shown to be increasing in urban environments in parts of Europe (Rizzoli et al., 2014) and the UK (Ogden et al., 2000; Rees and Axford, 1994; Smith et al., 2011) and are the most common tick species parasitising humans (Wilhelmsson et al., 2013). The distribution of *I. ricinus* is of interest as it encroaches on that of CCHFV with populations of ticks in a number of the Balkan countries.

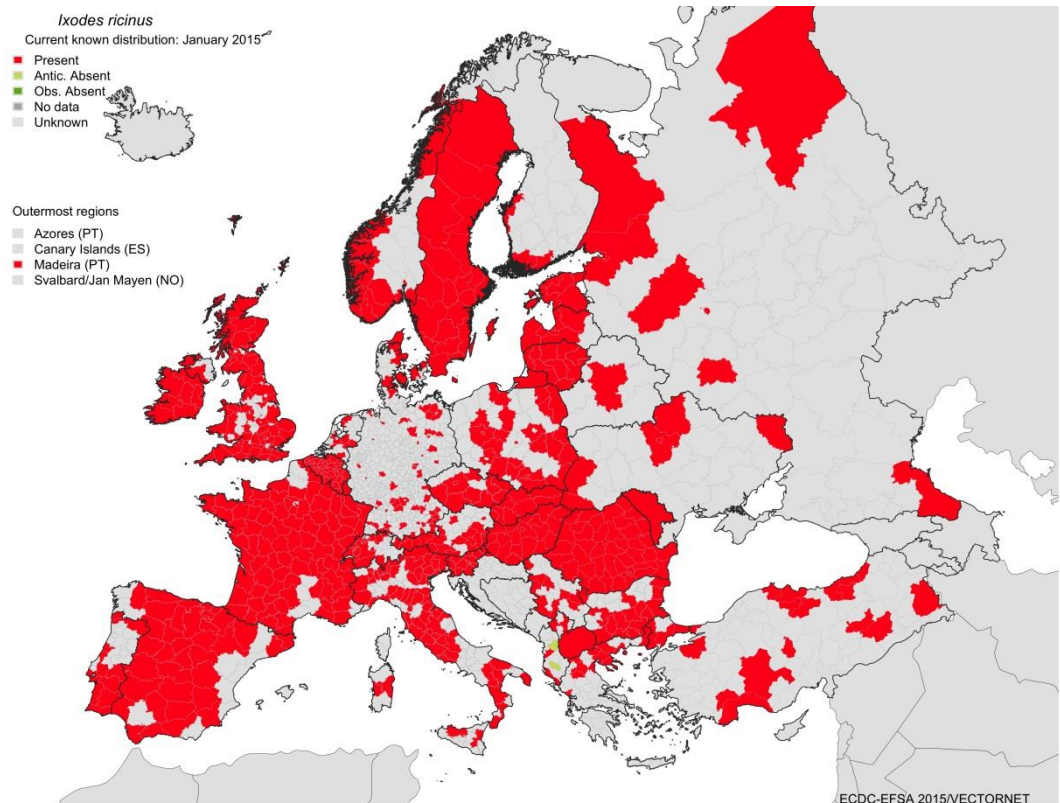


Figure 1.2: Distribution map of *I. ricinus* ticks within Europe (ECDC, 2015)

1.3 Crimean-Congo haemorrhagic fever

1.3.1 Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a highly pathogenic virus causing a fatal viral zoonosis carried and transmitted by ticks which causes the disease Crimean-Congo haemorrhagic fever (CCHF). CCHFV causes a severe disease in humans and has a fatality rate of between 10 – 80% (Ergönül, 2006; Hoogstraal, 1979; Yen et al., 1985). There is no commercial vaccine available, the disease is difficult to treat and control

and it is on the NIAID bioterrorism agent list making this an important human pathogen.

1.3.2 *Etiological agent*

CCHFV is a member of the *Bunyaviridae* which is comprised of 350 viruses within five genera; *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Hantavirus* and *Tospovirus*. The nairoviruses are predominantly transmitted by ticks and are separated into seven sero-groups with CCHFV and Hazara virus (HAZV) making up the CCHFV sero-group (Plyusnin and Elliott, 2011). Hazara virus (HAZV) was isolated from *Ixodes redikorzevi* ticks removed from a wild vole in the Hazara district of Pakistan (Begum et al., 1970). HAZV is considered to be non-pathogenic to humans and is handled at containment level 2 (CL2) and is used as a surrogate for CCHFV (Dowall et al., 2012).

The CCHFV virion is approximately 80-100nm in diameter, spherical in shape containing a single stranded negative sense RNA virus comprising a tripartite genome; with small, medium and large segments denoted by their size (Walter and Barr, 2011). The virion is constructed of a lipid layer containing structural membrane glycoproteins (Figure 1.3); Gn and Gc, encoded by the M segment, which are responsible for viral binding to cellular receptors. Each segment is enclosed within a nucleoprotein, encoded by the S segment, and the RNA-dependent RNA-polymerase (RdRp) encoded by the L segment (Walter and Barr, 2011).

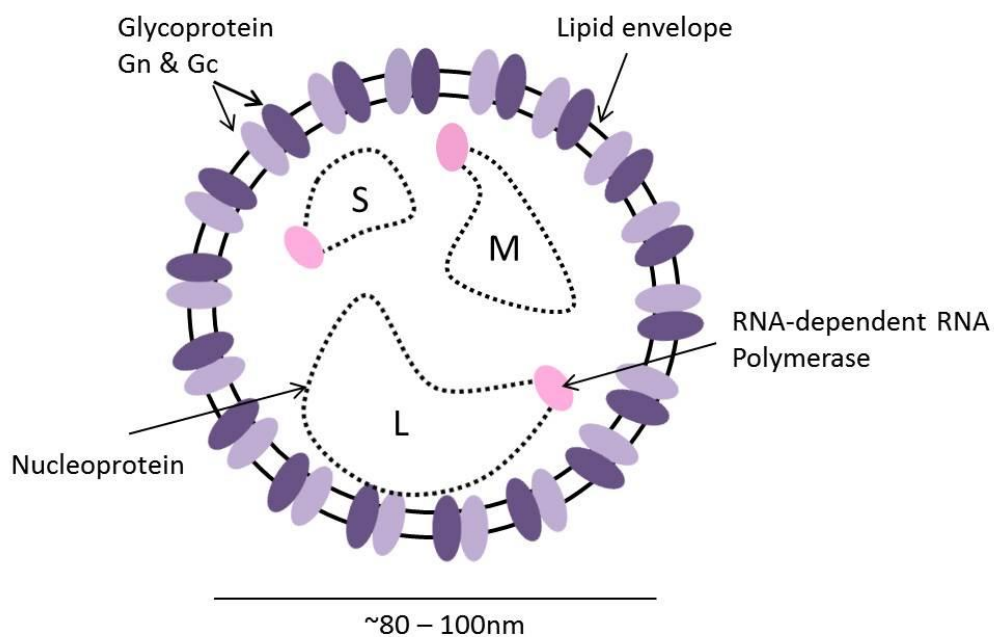


Figure 1.3: Schematic diagram of the CCHFV virion

Virus particles bind to cell surface receptors, currently unknown for CCHFV, and are internalised via clathrin-dependent, receptor mediated endocytosis (Simon et al., 2009). The viral envelope fuses with the endosome releasing the viral nucleocapsids into the cytoplasm where messenger RNA (mRNA) and complementary RNA (cRNA) are synthesised by the RdRp and mRNA translated into viral proteins. Viral RNA is generated from the cRNA and fuses with the produced viral proteins and RdRp to form new nucleocapsids. The endoplasmic reticulum is the site of glycoprotein translation where the precursors Gn and Gc are cleaved. These are then transported to the golgi apparatus for maturation where the newly formed nucleocapsids fuse with the glycoprotein and new virions bud from the plasma membrane (Bente et al., 2013).

1.3.3 *Clinical and pathological features*

Transmission of CCHFV is primarily associated with tick bite, but can also occur after contact with infected bodily fluids of infected patients and animals (Ergönül, 2006; Whitehouse, 2004). In human cases there are four distinct phases of CCHF infection; the incubation, pre-haemorrhagic, haemorrhagic and convalescence phase (Figure 1.4) (Hoogstraal, 1979). The incubation phase can vary in duration from 1 – 7 days with a maximum of 13 days and may be related to the route of inoculation (Ergönül, 2006; Swanepoel et al., 1989). Disease then presents itself in the form of a pre-haemorrhagic period by increasing viremia and a sudden onset of fever (39 – 41°C) which typically lasts 4-5 days accompanied by headaches, chills, photophobia, dizziness, abdominal and back pains as well as vomiting, diarrhoea and loss of appetite. Hyperemia (congestion of blood) can be seen in the face, neck and chest, the eyes can become bloodshot and conjunctivitis can be seen. This period lasts on average 3 days. The haemorrhagic phase is rapid and short and begins on the 3rd- 5th day of disease with haemorrhagic manifestations seen as small red or purple blotches on the skin (petechiae) or large haematomas on mucous membranes on the skin (Figure 1.5). Internal haemorrhagic can result in tar like stools and in the most severe cases cerebral haemorrhage is seen (Swanepoel et al., 1987). The most common sites for bleeding are the nose, gastrointestinal system, urinary tract and the respiratory tract. The convalescence period among survivors is between 10-20 days after the onset of disease.

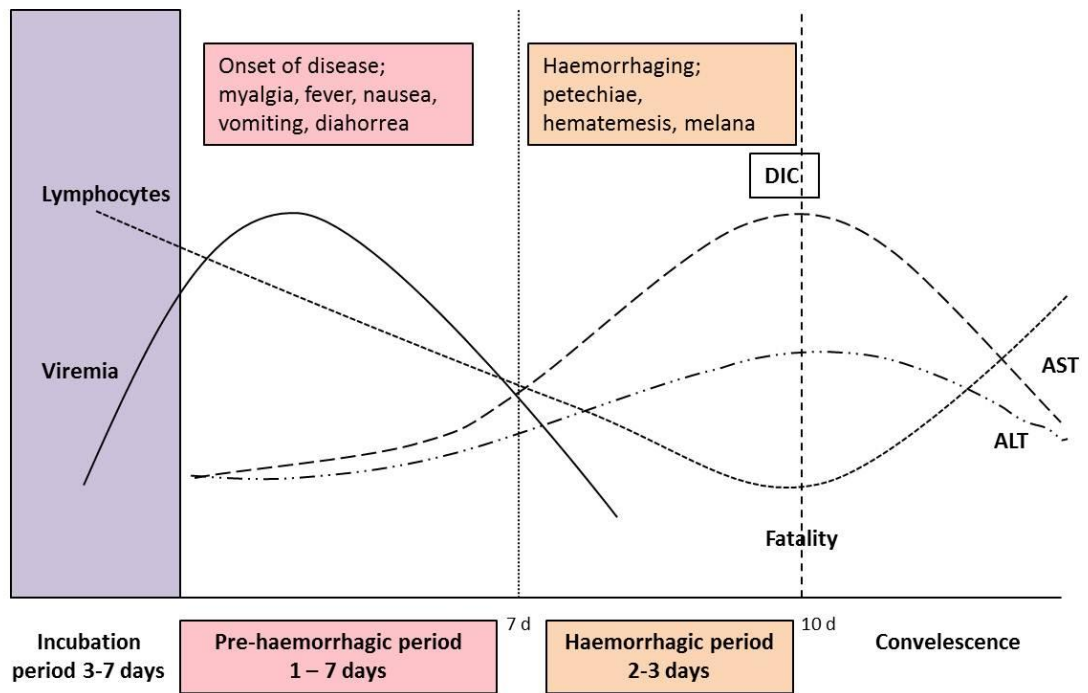


Figure 1.4: Clinical course of CCHFV adapted from Ergonul (2004). The levels of aspartate aminotransferase (AST) and alanine transaminase (ALT) are shown as is the time at which disseminated intravascular coagulation occurs (DIC).



Figure 1.5: Haemorrhagic manifestations in a patient with CCHFV. Bleeding from the gums and typical petechiae of the skin can be seen.

1.3.4 *Epidemiology*

Humans are dead end hosts for CCHFV and are the only vertebrate hosts that exhibit severe disease (Nalca and Whitehouse, 2007). CCHFV is maintained in nature in an enzootic cycle between the tick vector and vertebrate hosts and has been isolated in more than 30 tick species including *D. marginatus*, *I. ricinus* and *R. sanguineus* (Nalca and Whitehouse, 2007) all of which are present in the UK (1.2). Ixodid ticks, predominantly those of the *Hyalomma* species are the only ticks shown to be competent vectors of CCHFV with argasid ticks refractory to both artificial and natural infection via a blood meal from an infected host (Durden et al., 1993; Shepherd et al., 1989).

Once a tick has become infected with CCHFV via a natural blood meal, the virus replicates within the midgut and disseminates to various tissues, notably the salivary glands and reproductive organs where the highest viral titres are reached (Dickson and Turell, 1992). CCHFV is transmitted trans-stadially between different life stages; from larvae to nymph (Dohm et al., 1996; Logan et al., 1989), to co-feeding immature forms when fed alongside infected *Hyalomma impeltatum* in the absence of host viremia (Gordon et al., 1993), during copulation from males to females (Gonzalez et al., 1992) and to a number of domestic and wild animal species in nature. Serological analysis has been used to identify vertebrate hosts of CCHFV as animals do not exhibit clinical signs. These include cattle, goats, sheep camels and horses (Tantawi et al., 1981) which are parasitised by the adult forms and in hares from Bulgaria and South Africa and in rats, squirrels and gerbils (Nalca and Whitehouse, 2007).

Vector competence studies of ticks for CCHFV were conducted in the 1970s, 80s and 90s and little research has been done since. CCHFV is classified as an ACDP 4 pathogen within the UK and Europe and a BSL4 pathogen in the USA, requiring the necessary strict handling procedures and specialised laboratories. Currently within Europe there are 8 CL4 facilities in 6 different countries (Carla Nisii, 2013) however, there are no small animal tick transmission models for use at CL4 hindering the investigation of this pathogen and the tick vector.

1.3.5 *Discovery*

It is thought that the first documented description of CCHF was by a physician in the 12th century describing symptoms of blood in the urine, rectum, gums, vomitus, sputum and abdominal cavity from an area now known as Tajikistan (Hoogstraal, 1979). The associated arthropod was said to resemble a tick or louse and was seen parasitising a blackbird characteristic of the *Hyalomma* tick species (Hoogstraal, 1979). The first clinical cases of CCHF were during the Second World War in the former Soviet Union with 200 troops becoming ill with fever and haemorrhaging (Hoogstraal, 1979). Troops entered abandoned land where hares and other wild vertebrates, the hosts for *H. marginatum* ticks, had propagated and those involved in restoring the land suffered large numbers of tick bites. Association of the virus with ticks was confirmed by inoculation of human volunteers with filtered suspensions obtained from *H. marginatum* nymphs (Whitehouse, 2004). The resulting isolate was named the Drosdov strain and was used as the experimental prototype by Soviet researchers (Whitehouse, 2004). It was later discovered that the Drosdov strain was identical to a viral strain isolated from the Belgian Congo in 1956, it was proposed that these two virus strains should be classified as singular and was named CHF-Congo virus. The isolate known as IbAr 10200 isolated from a tick obtained from a camel in Nigeria was and is currently used by Western scientists (Causey et al., 1970).

1.3.6 *Distribution within Europe*

CCHFV is the most widely distributed of the medically important arboviruses and since the first clinical case over 70 years ago it is now detected in more than 30 countries throughout Africa; in Congo, Tanzania (Swanepoel et al., 1987) and Kenya (Dunster et al., 2002), Asia; China (Papa et al., 2002a; Yen et al., 1985), the Middle East; from Pakistan (Chinikar et al., 2010), Iraq (Majeed et al., 2012), and Afghanistan (Mustafa et al., 2011) and eastern Europe as discussed below (Figure 1.6).

In Europe CCHFV is currently only endemic in the Balkans. CCHFV was first detected in Bulgaria in the 1950's and became a notifiable disease in 1953 (Avšič-Županc, 2007). Between 1953 and 1974 over 1000 cases were reported, reducing to 279 cases after the introduction of a vaccination programme in 1974 (Papa et al., 2004) and a total of 1,500 cases have been detected between 1953 and 2008 predominantly from farmers

in the Eastern regions of the country with a case fatality rate of 17% (Papa et al., 2004). The seasonal peak of CCHFV from April through to May correlates to the feeding times of adult *H. marginatum* ticks and between 1950 and 1960 CCHFV was also isolated from *Rhipicephalus sanguineus* ticks. To date CCHFV has been detected in 2% of sampled ticks from Bulgaria (Gergova et al., 2012) and CCHFV antibodies are detected in 2.8% of the sampled population (Christova et al., 2013).

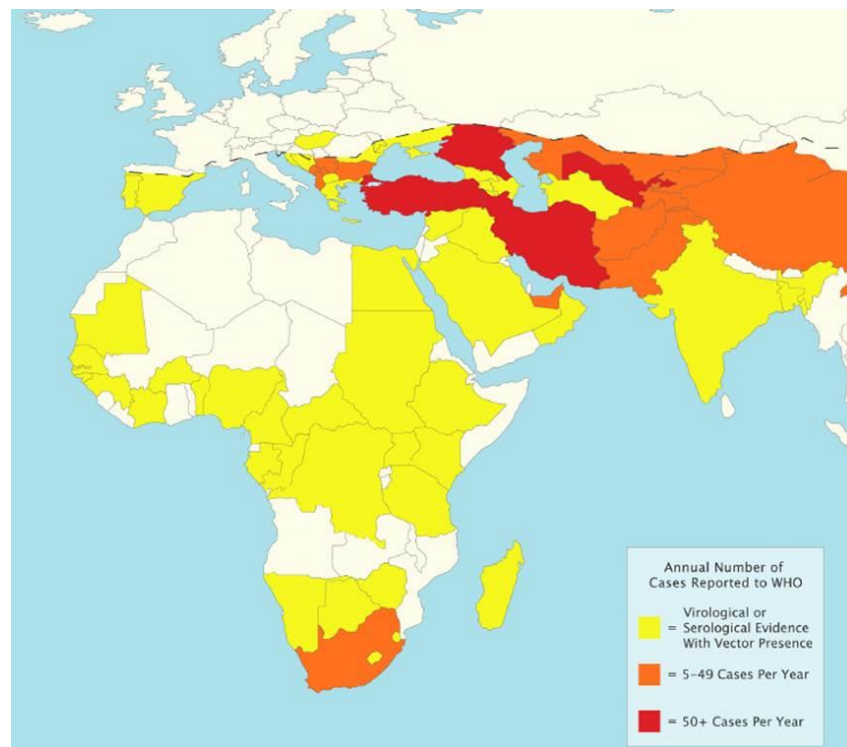


Figure 1.6: Distribution map of CCHFV (WHO, 2015)

During the last 10 years increasing outbreaks have occurred in areas of Europe; Albania (Papa et al., 2002b), Kosovo (Papa et al., 2002c) and for the first time in Turkey (Yilmaz et al., 2009) and Greece (Papa et al., 2010). In many cases serological evidence of CCHFV in humans has previously been detected but clinical manifestations have not been reported.

South of Bulgaria in Kosovo, CCHFV has been detected since the 1950's (Avšič-Županc, 2007) with 46 sporadic cases detected up to 1995 with continuing annual cases (Fajs et al., 2014). Antibodies to CCHFV have been detected in 4% of tested human sera and detected from cows, sheep and goats. CCHFV has been isolated from three tick species; *H. marginatum*, *I. ricinus* and *Rhipicephalus bursa* with CCHFV in the two former species closely related to the human isolates in Kosovo and the latter related to

the non-pathogenic AP92 strain (Sherifi et al., 2014). In 2001 the largest epidemic so far to occur in Kosovo resulted in a 23.3% case fatality rate with 58% of the cases related to a tick bite (Avšič-Županc, 2007). In the south western part of the Balkans in Albania, CCHFV was first recognised in 1986 with continuing annual cases (Papa et al., 2002b). In 2001 an outbreak associated with farmers and tick bite occurred in 8 human cases (Papa et al., 2002b) and in 2008 human sera from retrospective cases occurring between 2003 and 2006 were positive for CCHFV (Papa et al., 2009). Little is known about the tick fauna of Albania or the reservoirs or vectors of CCHFV, in 2009 *H. marginatum* ticks were tested for CCHFV with only 1 out of 32 pools testing positive with no antibody detection in cattle, hares or birds (Papa et al., 2009).

In south east Europe in Turkey, antibodies to CCHFV have been detected since the 1970's with increasing numbers of cases and fatalities from 133/6 in 2003 to 1300/62 in 2009 (Maltezou and Papa, 2010) with over 5000 diagnosed infections showing a mean fatality rate of 5% (Ozkaya et al., 2010). This may be severely underestimated as two thirds of patients are initially misdiagnosed (Tasdelen Fisgin et al., 2010). The majority of cases are associated with tick bites and with farmers, crop pastures and fields correlating with a high infestation rate of cattle (74%) and sheep (46%) with *H. marginatum* ticks compared to the usual 5% infestation rate (Tonbak et al., 2006). The development of detectable clinical disease within this area has been linked to the change in agricultural use whereby prior to 2002 highland areas had been abandoned and a hunting ban put in place which is still in force to day; leading to an increase in wildlife and hosts for ticks (Ozkaya et al., 2010). It has also been suggested that the increase in the number of days above 5°C in the years prior to the outbreak may also have played a part in the increase in tick density (Vatansever et al., 2007). CCHFV was first detected in the north of the country and has since spread to the east; it isn't known whether this is due to the spread of the tick vector or simply an increase in awareness. However, infected ticks have been detected in areas with no reported clinical cases. Another interesting finding is that a 10-19.6% sero-conversion rate has been observed in people showing no clinical signs of disease from areas where CCHFV cases have been reported (Ergönül et al., 2004; Koksal et al., 2014), which may be due to the viral dose or pathogenicity of the circulating strain. Turkey has approximately 25 ixodid and 7 argasid tick species (Vatansever et al., 2007) and CCHFV has been

detected in a diverse range of species. A sero-epidemiological survey of hard ticks for CCHFV antibodies resulted in a number of positive tick species including *I. ricinus* ticks with a detection rate of 12.06% (7/58 pools), higher than that of the natural tick vector *H. marginatum* with 8.49% detection (9/106 pools) with *Rhipicephalus* species predominating with 15.11% antibody detection (13/89) (Albayrak et al., 2010).

In 1975 CCHFV was isolated from infected *R. bursa* ticks removed from goats in Greece (Papa et al., 2010). Virus-specific antibodies were detected in the population however for the proceeding 33 years no clinical cases of CCHFV were reported and it was concluded that the AP92 strain was non-pathogenic to humans and is classified as the European 2 clade (Bente et al., 2013). The first human case was detected in 2008 in an area a few kilometres from the Bulgarian border and the virus was shown to cluster with other Bulgarian strains within the European 1 clade (Papa, A, 2008).

CCHFV has been detected in other areas of Europe; in Spain *Hyalomma lucitanicus* ticks have tested positive for CCHFV (Estrada-Peña et al., 2012) the first evidence of CCHFV in Spain as have migratory birds with the virus clustering with the African strains (Foley-Fisher et al., 2012). Seriological evidence of CCHFV has also been detected in hares from Hungary (Németh et al., 2013) and in sheep from Romania (Ceianu et al., 2012).

1.3.7 *CCHFV and the UK*

Currently CCHFV has not been detected within the UK but two imported cases of the virus have occurred within the last two years. In 2012 a man returning from a religious event in Kabul, Afghanistan reported to a local hospital in Glasgow with fever, diarrhoea, coughing and vomiting of blood (Atkinson et al., 2012b). The patient was placed within a negative pressurised room and serum samples were submitted for processing at Public Health England (UK). Three days later the man was flown to the high security infectious disease unit at the Royal Free hospital in London where he was given supportive therapy but unfortunately died on October 6th 2012, four days after hospital admission. The second case occurred in 2014 when an elderly man returning from a trip to the rural area of Burgas Province in Bulgaria, was admitted to a UK hospital with a fever, a petechial rash accompanied by low levels of; platelets, lymphocytes and neutrophils, elevated levels of alanine aminotransferase (ALT),

indicative of disease to the liver and exhibited a crackly chest (Lumley et al., 2014). The patient was isolated and treated with antibiotics as a precaution for infection with rickettsia whilst serum, urine and blood samples were submitted for processing at Public Health England (UK). The patient made a full recovery and was released from hospital 13 days after admission. The man initially became ill in Bulgaria on 22nd June and was admitted to hospital with muscle pain, headache, sore throat, coughing, sweating and a fever prior to travelling back to the UK. As expected the viral isolate obtained from the first patient clustered with the Asia 1 clade and the second case clustered with the Europe 1 clade. This illustrates the differences in pathogenicity of viral strains however currently no investigation into the pathogenicity of different CCHFV viral strains has been undertaken.

The immature stages of *H. marginatum* are known to be found on migrant birds flying to temperate Europe (Hoogstraal et al., 1961) and in late April and May of 2010 to 2011 the CCHFV tick vector *H. marginatum* was detected on Migratory birds entering Britain (Jameson et al., 2012). Ticks were removed from incoming birds that were routinely caught for ringing at one of the UKs first landfall sites for migrating birds in Dorset with four species infested with ticks, 21% of which were *H. marginatum*. Multiple nymphs were attached and it is thought that the numbers caught are only a small proportion of the migrating population and due to the high breeding rate of these bird species (Jameson et al., 2012) numbers could reach the tens of thousands entering the UK annually. However, this does not account for the number of birds passing through Britain before onward travel, of particular note was the species *Oenanthe oenanthe* harbouring the most number of ticks. A report produced by the European Food Safety Authority in 2010 disregarded this species as an important vector for tick importation as it was documented that these birds leave Africa in February before the active season of ticks however, later migrants of this species passing over in March could arrive infested (Jameson et al., 2012).

In 1979 Harry Hoogstraal highlighted *I. ricinus* as a 'prime candidate for investigation as a CCHFV virus reservoir and vector' (Hoogstraal, 1979). Despite this there have been no investigations into the vector competency of the most widely distributed tick in Europe to CCHFV.

1.3.8 *Animal models of disease*

After the initial discovery of the viral disease, research was hindered due to the lack of a laboratory animal model and inability to cultivate the virus. In 1967 however, it was discovered that newborn mice were susceptible to infection when inoculated via the intraperitoneal or intracerebral route with material from infected humans and ticks (Bente et al., 2013). CCHFV was shown to produce high titres of virus within the blood and liver and infect macrophages (Tignor and Hanham, 1993) however new born mice are unsuitable for studying the pathogenesis of CCHFV and *in vivo* research was severely hindered for the next 17 years. It was only after *in vitro* experiments showing that the interferon (IFN) innate immune responses were critical for the control of CCHFV (Andersson et al., 2006) that two mouse models were developed. Genetically modified mice lacking the IFN- α/β cell surface receptor (Bereczky et al., 2010) and mice lacking the signal transducer and activator of transcription (STAT)-1 protein (Bente et al., 2010) are both shown to rapidly develop a fatal disease with high levels of virus in the tissues and blood and show clinical manifestations as seen in human cases of CCHFV.

HAZV was first isolated in mice by inoculation via the intracranial and intraperitoneal route resulting in paralysis and death of mice (Begum et al., 1970). Subsequently a mouse model has been developed using mice lacking the IFN- α/β cell surface receptor which are shown to rapidly develop HAZV and succumb to disease 7 days post-inoculation (Dowall et al., 2012).

1.4 **PhD rationale**

This thesis focuses on the intrinsic susceptibility of *I. ricinus* ticks to HAZV and CCHFV with the rationale that if the pathogens and / or vectors were to enter the UK, would our native *I. ricinus* tick species be susceptible to infection.

1.5 **Hypothesis**

Ixodes ricinus ticks are susceptible to infection with HAZV and CCHFV

1.6 **Aims**

The aim of this thesis is to determine whether *I. ricinus* ticks are competent vectors for HAZV and CCHFV.

1.7 Objectives

The above aim will be addressed by the following objectives;

- Determine whether *I. ricinus* can acquire and replicate HAZV and CCHFV infection via artificial inoculation
- Determine whether *I. ricinus* nymphs can transmit virus horizontally to a susceptible vertebrate host and
- Determine whether *I. ricinus* can acquire the virus via a natural blood meal

Chapter 2. Materials and Methods

2.1 Introduction

This chapter details materials and methods used throughout this thesis. Where optimisation of methods occurred, this section details the final method used.

2.2 Cell lines and Virus strains

2.2.1 Cell culture

The Human epithelial cell line; Human Caucasian adrenal cortex adenocarcinoma (SW13) obtained from the European Collection of Cell Cultures (ECACC) at Public Health England, UK were used throughout this study. Cells were cultured in Minimum Essential Medium (MEM) with Earle's salts and L-glutamine (Sigma-Aldrich, UK) supplemented with 50:500ml heat inactivated fetal bovine serum (FBS; Sigma-Aldrich, UK) and 1:500ml penicillin and streptomycin (Life Technologies, UK).

2.2.2 Hazara virus

Stocks of Hazara virus (HAZV) (isolate JC280 obtained from J. Cassals in 1980, passaged once in suckling mice at Porton Down and sequenced M86624.1) were made for the purpose of this PhD. HAZV virus previously titred at 1×10^6 plaque forming units/ml (pfu/ml) was inoculated into SW13 cells at a multiplicity of infection (MOI) of 0.01. Virus was harvested after 2 days and quantified via plaque assay producing a viral stock at a titre of 1.7×10^6 pfu/ml. This was used in all subsequent experiments.

2.2.3 Crimean-Congo haemorrhagic fever virus

Crimean-Congo Haemorrhagic fever virus (CCHFV) (isolate IbAr10200, isolated from *Hyalomma anatolicum excavatum* ticks in Nigeria, (Causey et al., 1970), was amplified in suckling mouse brain followed by cultivation on SW13 cells at Porton Down. Virus was quantified via antibody staining and a viral stock of 1.12×10^6 focus forming units/ml (ffu/ml) was used in subsequent studies.

2.3 Ticks

Ixodes ricinus nymphs were used for all studies and were obtained unfed (Charles River Laboratories Ireland, UK). Ticks were reared for at least 2 generations on pathogen-free laboratory animals and were tested for *Borrellia*, *Anaplasma*, *Rickettsia* and *Babesia* pathogens by Charles River Laboratories.

2.3.1 ***Handling of ticks at CL2***

All tick manipulations were conducted within a class II microbiological safety cabinet (MSCII). Tubes containing ticks were placed onto ice prior to opening to reduce their motility and metabolism. Tubes were opened over a high rimmed plastic box placed onto white tissue covering the surface of the MSC II and ticks handled using 150mm long, straight fine-tipped forceps (Fisher Scientific, UK).

2.3.2 ***Maintenance of unfed non-infected ticks at CL2***

Ixodes ricinus nymphs were received in a 5ml plastic bijou with modified lids; the centre of the lid was removed and covered with 500micron mesh. These were housed in a tube rack within a Nalgene plastic desiccator (Scientific Laboratory Supplies Limited, UK) at a temperature of 17-20°C. The bottom of the desiccator was filled with a saturated solution of magnesium sulphate ($MgSO_4$; Scientific Laboratory Supplies Limited, UK), produced by diluting 400g of $MgSO_4$ with 500ml of de-ionised water, generating a relative humidity of 85-95%.

2.3.3 ***Immersion of *I. ricinus* nymphs with HAZV***

To infect ticks via immersion, nymphs were divided into 2ml O-ringed screw top tubes (Sarstedt, Germany) at a density of 25 ticks per tube and immersed in 0.5ml of Minimum Essential Medium (MEM) with Earle's salts and L-glutamine (Sigma-Aldrich, UK) containing 1.7×10^6 pfu/ml of HAZV. Tubes were placed into a tube rack and incubated at 37°C for 45 minutes. Ticks were re-suspended within the media every 10 minutes by pulse vortexing. After incubation, ticks were chilled on ice for 2 minutes and then centrifuged at 2000xg for 20 seconds, repeating as necessary, until all ticks were removed from the lid. The virus suspension was then removed by pipetting and the ticks washed once with 250µl chilled phosphate buffered saline (PBS; Life Technologies, UK), once with 250µl 70% ethanol (Sigma-Aldrich, UK) to surface sterilise the ticks and once with 250µl distilled water (Life Technologies, UK). One strip of 1.5 cm Whatman filter paper (GE Healthcare, UK) was added to the 2ml tube to absorb any excess moisture. After the immersion procedure was complete, modified lids comprising of multiple air holes, produced using a 3 gauge needle were placed onto the tubes (Figure 2.1).

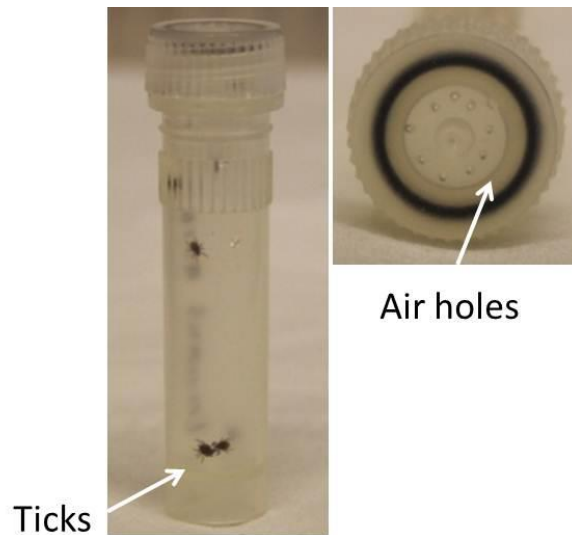


Figure 2.1: Immersion and post-immersion maintenance tubes with modified lids comprising of multiple air holes, produced by a 3 gauge needle.

2.3.4 *Post-immersion maintenance of ticks at CL2*

After immersion in HAZV, ticks were maintained at densities of 25 ticks per tube within the 2ml tubes used during the immersion procedure. Tubes were maintained within the desiccator as described in section 2.3.2. Modified lids on these tubes allowed gaseous exchange between the humidified air of the desiccator and the internal area of the tube.

2.3.5 *Handling of ticks at CL4*

All tick handling was conducted within a high containment cabinet line of interconnecting MSC III cabinets within a CL4 laboratory. A dedicated tick cabinet was prepared; cabinet surfaces were intact, impervious; light coloured and easy to clean. The working area of the cabinet was covered with highly visible yellow absorbent mat pads (New Pig, Glasgow), a clear plastic spill tray was placed on top and the edges surrounded by double sided sticky tape. HEPA filters were covered by 500micron nylon microfilament mesh, securely mounted at the filter face and fittings and fixtures covered with double sided sticky tape. A minimalistic approach was adopted with only necessary equipment present; a Minilys homogeniser (PeqLab, UK), a digital dry heat block (Flowgen Bioscience, UK), a micro centrifuge (SLS, UK) and two Sarstedt tube racks. Accountability and visualisation of ticks was essential; ticks were counted, verified and density recorded upon entry onto the cabinet line and before any manipulations throughout the immersion procedure.

2.3.6 Immersion of *I. ricinus* nymphs with CCHFV

One hundred and twenty *I. ricinus* nymphs were prepared at CL2; nymphs were divided into 24 2ml O ringed micro-centrifuge tubes (Sarstedt) at a density of 5 ticks per tube, triple contained and transferred to CL4. Ticks were immersed in two groups; group one: tubes 1-12 and group two: tubes 13-24 and immersed in 500µl of CCHFV diluted to 1.12×10^6 focus forming units/ml (ffu/ml) in L15 Media (Life technologies, UK). Tubes were removed from the tube rack, visually inspected and tick density recorded. If necessary, tubes were spun at 4000xg for 1 minute to remove ticks from the lid. Tubes were opened individually and 500µl of virus-media suspension added. Tubes were vortexed to ensure all ticks were covered in the virus-media suspension and placed into a digital dry heat block (Flowgen Bioscience, UK) at 37°C for 45 minutes. Temperature was monitored using a tube containing 500µl of media without ticks using a digital temperature probe (Digitron, Elektron Technology, UK). Tubes were removed from the heat block at ten minute intervals and vortexed to re-suspend ticks in the virus-media suspension and after 45 minutes tubes were removed and maintained on ice. Virus-media suspension was removed by pipetting, ticks were washed in 250µl of PBS, surface sterilised with 250µl of 70% ethanol and washed in 250µl of distilled water. Excess distilled water was removed using a 2cm piece of Whatman filter paper and the tubes sealed with a pre-prepared modified tube lid comprising of multiple air holes made with a 3 gauge needle as detailed in section 2.3.3.

2.3.7 Post-immersion maintenance of ticks at CL4

Immersed ticks were housed at a density of 5 ticks per tube within the 2ml tubes used for the immersion procedure with modified lids attached as described in section 2.3.3. Tick tubes were maintained within tube racks (Sarstedt, Germany) placed in a clip top O-ringed box with a saturated solution of MgSO₄ generating a relative humidity of 85-95%.

2.3.8 Histological processing of HAZV infected *I. ricinus* ticks

Histological processing of ticks was outsourced to Laudier Histology (USA). In brief; samples sent in 70% alcohol sanitiser gel were removed from the gel, rinsed with distilled water, placed into 4% Zinc-buffered formalin and fixed for 72 hours. Samples

were then rinsed with distilled water and transferred through various grades of low-water isopropyl alcohol (60%-100%) over a 24-hour period for free water dehydration of specimens. After dehydration, samples were placed into methyl benzoate for 24 hours to remove residual isopropyl alcohol. Tick specimens were removed from methyl benzoate and processed through three stages of a custom hydrophobic acrylic monomer. Samples were then embedded and polymerised to a hard resin block, sectioned at 2 microns using a Leica Microsystems rotary microtome (RM22665), mounted on electrostatic slides and dried over 48-hours at 35°C. Sections were stained for HAZV antibody using a polyclonal raised in sheep targeting the HAZV nucleoprotein (NP) (Rebecca Surtees, University of Leeds) and visualised using a horseradish peroxidase (HRP) enzyme method. Non-specific staining was blocked using a serum-free protein block. Non-immune sheep serum was used in lieu of the primary antibody to verify non-specific binding. IHC negative control tick sections were exposed to primary and non-immune serum for comparison. Anti-sheep secondary was used to detect primary antibody and HRP-diaminobenzidine (DAB) was used to visualize positive reaction in tissue sections. Section slides were counterstained for contrast using dilute toluidine blue. Section slides were imaged using a Zeiss AxioImager Z2 microscope and photographed using standard bright-field light and/or DIC optics with a Zeiss HR (high resolution) microscope camera.

2.4 Lysis, homogenisation and purification of RNA

Ticks processed at both CL2 and CL4 were flash frozen on dry ice for 5 minutes and stored at -80°C until further processing. Harvesting RNA from whole ticks and mouse tissues was performed as follows; samples for which RNA was simultaneously required for infectious virus quantification and genomic viral quantification, were homogenised in 500µl of PBS and RNA extracted using lysis buffer AVL via the QIAmp viral RNA Kit (Qiagen, UK). Individual and pooled ticks requiring only genomic RNA amplification and quantification and ticks used at CL4 were homogenised in 250µl of lysis buffer RLT supplemented with β-mercaptoethanol (β-ME). Mouse tissues stored in RNAlater were homogenised in 600µl of buffer RLT supplemented with β-ME via the RNeasy Mini Kit (Qiagen, UK) as per the manufacturer's instructions.

2.4.1 *Homogenisation of ticks and mouse tissue samples at CL2*

Ticks were homogenised individually or where specified in cohorts of 10. Ticks and mouse tissue samples were homogenised in the appropriate lysis buffer in 2ml reinforced Precellys tubes containing 2.8mm steel beads (PeqLab, UK). Samples were homogenised for 3 cycles at 4000xg for 5 seconds duration with a 30 second pause between each cycle within a precellys²⁴ (PeqLab, UK) beat beater. Tubes were allowed to stand for 2 minutes allowing any aerosols to settle. Homogenates were centrifuged at 13,000rpm for 2 minutes to remove any fluid from the lid and homogenates passed through a Qias shredder (Qiagen, UK) to shear any high molecular debris. Lysate was then purified using one of the techniques below.

2.4.2 *Homogenisation of I. ricinus ticks at CL4*

Ticks were homogenised as per section 2.4.1 with the following modifications: ticks were homogenised in 250µl of buffer RLT for 6 cycles at 5000rpm for 5 seconds duration with a 30 second pause between each cycle within a Minilys (PeqLab, UK) beat beater. Homogenates were allowed to stand in the RLT lysis buffer for 10 minutes before the addition of 250µl of 70% ethanol and samples were fumigated overnight, transferred to clean tubes at CL3 and RNA extracted at CL2.

2.4.3 *Purification of RNA using the QIAmp viral RNA kit*

Buffer AVL, containing chaotropic salts and detergent, was prepared by the addition of carrier RNA. Lyophilised carrier RNA was re-suspended in 310µl of buffer AVE and added to buffer AVL. The addition of carrier RNA improves the binding efficiency of RNA to the silica membrane and limits degradation by RNases. Homogenised lysate or cell free serum was added to buffer AVL; 140µl lysate into 560µl AVL or 70µl lysate into 280µl of AVL, samples were vortexed for 15 seconds and incubated at room temperature for 10minutes. RNA was then mixed with 70% ethanol and applied to a QIAmp spin column and centrifuged at 6000xg for 1 minute to allow RNA to bind to the membrane. The column was then washed twice to remove any contaminants; once with 500µl of buffer AW1 and centrifuged at 6000xg for one minute, and once with 500µl of buffer AW2 and centrifuged at 15,000xg for 3 minutes. To ensure all wash buffers were removed, the column was centrifuged at 15,000xg for a further one

minute. A double elution of RNA was performed using 2 x 40µl of RNase free water. RNA was stored at -80°C for downstream processing.

2.4.4 Purification of RNA using the RNeasy mini kit

RNA from homogenised samples was purified using RNeasy silica membrane spin columns (Qiagen) with on column DNase digestion using the RNase-free DNase kit (Qiagen). Samples were mixed with 70% ethanol, applied to an RNeasy spin column and centrifuged at 8000xg for 15 seconds. The column was washed with 350µl of RW1 buffer and centrifuged at 8000xg for 15 seconds. The DNase I incubation mix was prepared by adding 10µl of DNase stock solution to 70µl of buffer RDD, 80µl was added directly to the spin column membrane and incubated at room temperature for 15 minutes. The spin column was washed with 350µl of RW1 and centrifuged at 8000xg for 15 seconds. The spin column was washed a further two times in 500µl of buffer RPE, centrifuged at 8000xg for 15 seconds. The spin column was then centrifuged at 15,000xg for 2 minutes to remove any wash buffer remnants. RNA was eluted in 50µl of nuclease free water and RNA stored at -80°C for downstream processing.

2.4.5 Determination of RNA yield

RNA yield was determined using the Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, MA, USA). RNA has a known absorbance range of 260–280nm. The absorbance of each sample was determined by placing 1.5µl of sample onto a fibre optic receiver on the Nanodrop pedestal. The pedestal containing the fibre optic emitter is lowered and using a xenon flash lamp light is passed through the sample. The light that is received is measured and absorbance of the sample calculated and the yield of RNA present expressed as ng/µl.

2.4.6 Determination of RNA integrity

The Agilent 2100 bioanalyzer and the eukaryotic total RNA 6000 nano kit (Agilent) was used to assess the integrity of RNA obtained from whole ticks stored under different conditions. The integrity of the RNA was assessed by passing RNA through a polymer matrix gel and a voltage gradient causing molecules to separate according to size. Fluorescent dyes are then intercalated into the RNA strands, detected by lasers and translated data shown as an electropherogram. The 18s and 28s RNA fragments were

compared to a reference ladder and RNA assigned an RNA integrity number with 1 being the most degraded and 10 being the most intact. Upon arrival the RNA ladder was heat denatured at 70°C for 2 minutes, cooled on ice, divided into 2µl aliquots and stored at -80°C until use. The polymer matrix gel was prepared by placing 550µl of Agilent RNA 6000 Nano gel matrix into a spin filter (provided in the kit) and centrifuged at 1500xg for 10 minutes at room temperature. The resulting 65µl of filtered gel was placed into a 0.5ml RNase-free micro centrifuge tube (supplied with the kit) and 1µl of the fluorescence nano dye mix was added to the gel, thoroughly mixed and centrifuged at 13,000xg at room temperature for 10 minutes. A new 16 well RNA chip was placed onto the priming station (supplied with the kit), 9µl of the gel-dye mix was placed into the appropriate well, as detailed in the Agilent RNA 6000 nano kit guide, and air pressure via a plunger attached to the chip priming station was used to disseminate the gel within the RNA chip. The RNA nano marker was added to the appropriate well and all sample wells, followed by 1µl of sample RNA and 1µl of RNA ladder added into the appropriate well as detailed on the RNA Nano chip. The chip was then vortexed within an IKA vortex mixer (supplied with the kit) at 2400rpm for 60 seconds and then loaded onto the Agilent 2100 bioanalyzer.

2.5 Amplification and quantification of genomic RNA

RNA obtained from tick and mouse samples was amplified using a one-step Taqman quantitative real-time reverse transcription PCR (qRT-PCR) targeting the HAZV-NP, developed and optimised as per the CCHFV assay (Atkinson et al, 2012).

2.5.1 *Quantitative real-time RT-PCR (qRT-PCR)*

All qRT-PCR were performed using the Superscript III platinum quantitative real time RT-PCR kit (Invitrogen). Optimised forward and reverse primers were used at a concentration of 900nM and probes at 625nM. Components were mixed together in a final volume of 20µl and aliquoted into a FAST optical 96 well reaction plate (Applied Biosystems, Microamp). The PCR mixture contained 5µl of template, 10µl of 2xreaction mix, 1µl of each primer, 0.4µl of probe, 0.8µl of enzyme and 1.8µl of water. PCR amplification was performed over 45 cycles using the following cycling parameters; 2 minutes at 95°C activating the Hot start Taq DNA polymerase followed by three step

cycling for 45 cycles; denaturation for 10 seconds at 95°C; amplification and annealing of primers and probes for 40 seconds at 60°C and extension for 30 seconds at 40°C.

Fluorescence emitted by the reporter dye FAM, as a result of cleavage of the probe, was detected at a wavelength of 550 nm on the ABI 7500 FAST real-time machine. Normalisation of the fluorescent signal measured against background fluorescence was performed and this normalised reporter value (ΔR_n) was plotted against PCR cycle number.

2.5.2 *Design of Taqman primer and probe sets*

Primer and probe sets were generated for use with tick β -actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATA box binding protein (TBP) using Oligo Evaluator (Sigma-Aldrich, UK).

2.5.3 *Optimisation of Taqman primer and probe sets*

Primer and probe concentrations were optimised following manufacturer's guidelines with primer concentrations of 50nM, 300nM and 900nM tested (Table 2.1) and probe concentrations of 50nM, 100nM, 150nM, 200nM, 250nM and 625nM tested against optimised primer concentrations. A no-template control was used to ensure there was no contaminating cDNA present within the reaction. The negative control contained the 900/900nM forward and reverse primer concentrations and the ABI recommended optimal 250nM probe concentration, the RNA template was replaced with RNase free water.

Table 2.1: Optimisation of forward and reverse primers for use in Taqman real-time RT-PCR.

Reverse Primer (nM)	Forward Primer (nM)		
	50	300	900
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

2.6 Quantification of viral RNA

To enable accurate amplification and quantification of viral RNA an appropriate endogenous control was required for both tick and mouse samples.

2.6.1 Design and development of tick β -actin RNA transcript

Tick β -actin was used as both an endogenous control for amplification and quantification of RNA obtained from *I. ricinus* ticks. To enable quantification, standard curves were generated and run along-side samples on the qRT-PCR. The conserved target area of tick β -actin was synthesised (Geneart systems, Germany). This sequence was cloned into a PmK vector backbone plasmid with Kanamycin resistance using EcoRI and Ascl cloning sites downstream of a T7 promoter (Figure 2.2). The plasmid DNA was purified from transformed *E. coli* bacteria and concentration determined by UV spectroscopy. The plasmid was supplied as a glycerol stock and a 5 μ g lyophilised preparation for downstream processing.

The plasmid was linearised using EcoR1 and RNA transcription performed using the MEGashortscript high yield transcription kit (Ambion). Plasmid DNA was then digested with TURBO DNase and RNA purified using the RNeasy plus micro kit (Qiagen, UK). RNA was then diluted down to a stock of 2x10¹²copies/ μ l and ten-fold dilutions made. A total of 6 standard curve dilutions (10⁶ – 10¹) were run in triplicate alongside every sample tested via qRT-PCR.

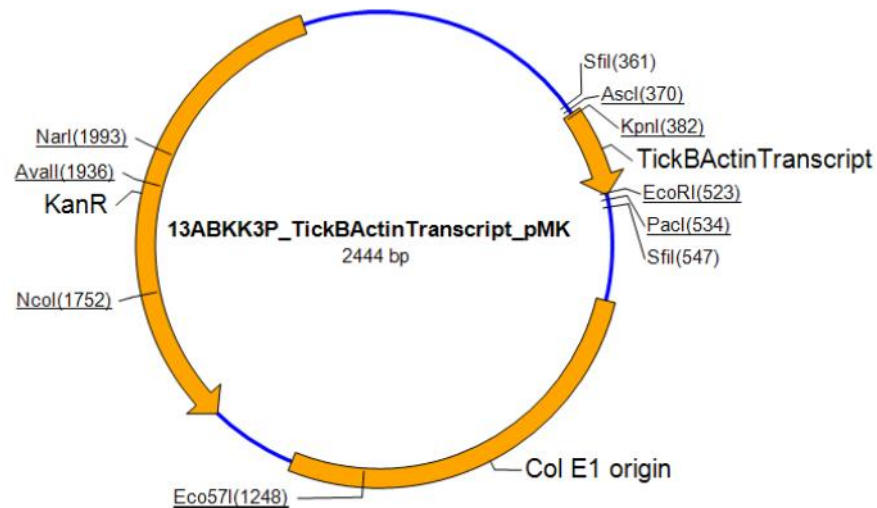


Figure 2.2: Diagrammatic representation of tick β -actin synthetic transcript cloned into a PmK vector backbone with associated restriction enzyme sites.

2.6.2 Generation of HAZV standard curve

A synthetic transcript previously made at PHE from the S segment of HAZV (KC344857) strain JC280 was used for real-time quantification. Ten-fold dilutions were made (10⁶ – 10¹) and were run in triplicate alongside every sample tested via qRT-PCR.

2.6.3 *Endogenous control for use with mouse samples*

The gene hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an endogenous control for RNA amplification and quantification from mouse samples. This one step singleplex qRT-PCR assay HPRT (NM_013556) was detected using a one-step qRT-PCR singleplex assay using the Quantifast probe assay from Qiagen using the cycling parameters detailed in section 2.5.1.

2.6.4 *Relative RNA quantification*

To quantify how much viral RNA is present in both tick and mouse samples, the level of mRNA detected relative to an endogenous control; tick β -actin or HPRT, was determined using the relative standard curve method. To normalise HAZV expression data, Ct values for HAZV and the endogenous control were inverted (45 minus the Ct value). The mean HAZV Ct per sample was divided by the mean Ct per sample of the endogenous control to give a ratio of target: endogenous control. This ratio was then divided by a calibrator sample, in the case of the immersion studies day 0 was used. The resulting data was expressed in a box and whiskers plot as fold change compared to the calibrator sample.

2.7 Quantification of infectious viral titres

2.7.1 *Titration of HAZV using plaque assay*

Viral titres of whole ticks, mouse; serum, spleen and liver samples were determined via titration on SW13 cell monolayers. Ticks and mouse tissue samples were homogenised in 500 μ l of serum-free MEM (Sigma, UK) supplemented with 1:500 penicillin and streptomycin (Sigma-Aldrich, UK) and homogenised as described in section 2.4.1. Mouse serum samples were diluted directly from frozen stocks. Serial ten-fold dilutions of each sample was performed and added to confluent 24 well cell culture plates of SW13 cells and incubated at 37°C for one hour. After absorption 0.5ml of overlay consisting of; carboxyl-methyl-cellulose (CMC) (Supplied by PHE media stores) supplemented with 2x MEM (Sigma-Aldrich, UK), 10:500 of FCS and 5:500 of penicillin and streptomycin, was added to each well. The cells were incubated at 37°C for 6 days, before fixing in 1ml per well of 20% formaldehyde, a 1:1 dilution in PBS, for at least 1 hour at room temperature. Formaldehyde was removed by washing with tap water and cells were stained with 1.15% v/w of crystal violet solution (VWR, International)

for 30 minutes at room temperature. Cells were washed in water and air dried. Plaques were quantified using a light box and data expressed as plaque forming units per ml (pfu/ml).

2.7.2 Titration of HAZV stocks using antibody staining

A vial of HAZV stock virus at a titre of 1.7×10^6 pfu/ml was thawed and ten-fold dilutions, (neat – 10^{-11}) were performed in 96 well dilution plates with serum-free MEM. Ten-fold dilutions of PBS were used as a negative control. 100 μ l of each viral dilution was added to a 96 well cell culture plate (Sigma-Aldrich, UK) of confluent monolayers of SW13 cells and incubated at 37°C for one hour. After absorption, the virus suspension was removed; cells rinsed with PBS and 200 μ l of MEM supplemented with 2% FBS added to each well. The cells were incubated at 37°C for 48 hours, fixed in 200 μ l of cold acetone (VWR, International) for 20 minutes and air dried for 30 minutes at room temperature. Cells were stained by indirect immunofluorescence with a polyclonal antibody raised in sheep targeting HAZV-NP (kindly gifted by Dr Rebecca Surtees, University of Leeds) at a 1:500 dilution with PBS. The cells were incubated for 60 minutes at 37°C, washed three times in PBS and stained with Alexa Fluor® 488 donkey anti-sheep FITC conjugated secondary antibody (Life Technologies, UK). Cells were incubated for 60 minutes at 37°C, washed three times in PBS and allowed to air dry. Florescence foci of cells were quantified using a fluorescence microscope at a wavelength of 425nm and expressed as ffu/ml.

2.7.3 Serological diagnosis of HAZV in mouse serum using indirect fluorescence-antibody test

A 175cm² vented cell culture flask (NUNC, UK) of sub-confluent SW13 cells was inoculated with HAZV at an MOI of 0.01. Cells were incubated for 1 hour at 37°C, virus culture media made of MEM supplemented with 2% FCS added and cells incubated at 37°C for 2 days. Supernatant was decanted and cells trypsinised with 0.25% trypsin-EDTA (Life Technologies, UK) and centrifuged at 3000xg for 10 minutes. The supernatant was discarded and the cell pellet re-suspended in 0.01% PBSA. Multispot microscope slides (Hendley Essex) were prepared by adding a 30 μ l droplet of cells onto each spot. After approximately 2 minutes the droplet of cells was removed by pipetting and reused for other slides. Slides were air dried within an MSCII for 10

minutes, fixed in 100% acetone (VWR, UK) on ice for 10 minutes and air dried. Slides were frozen at -80°C until used.

For staining, serum was diluted 1:10 and 1:100 with PBS. Approximately 10µl of serum was placed onto a multispot well on slides containing SW13 cells infected with Hazara virus. The slides were incubated for 60 minutes at 37°C, washed three times in PBS and stained with goat anti-mouse IgG FITC conjugated antibody (Sigma-Aldrich) diluted to 1:100 using PBS and supplemented with 3µl of Evans blue (Sigma-Aldrich). The slides were incubated for 60 minutes at 37°C, washed three times in PBS, air dried at room temperature and 2 drops of cover slip mountant (Citifluor, UK) added to each slide and covered with a cover slip. Serum obtained from mice intradermally inoculated with HAZV was used as a positive control at 1:10 and 1:100 dilutions.

2.8 *In vivo* techniques

2.8.1 *Mouse strains*

Three types of mice were used throughout this thesis; mouse strains A/J (Harlan, Laboratories, UK), 129 Sv/Ev mice (B&K Universals) and type-I interferon receptor deficient (A129 B&K Universals, UK) Interferon deficient mice were used for tick attachment and virus transmission studies.

Unless stated otherwise experiments were conducted within flexible film isolators under a climate controlled environment with food and water *ab libitum*. All animal procedures were conducted according to the UK animal Scientific Procedures Act 1986. All manipulations with animals were carried out by fully trained and competent Home Office licence holders.

2.8.2 *Attachment of on-host primary tick containment*

Mice were anaesthetised with 0.1ml of Alfaxan via the intraperitoneal route. The upper back of each mouse was shaved, a neoprene patch (RH Nuttall Ltd, UK) approximately 1cm² containing hole produced using a 6mm leather hole puncher (Amtech, UK) was applied to the skin using Copydex glue and anchored down by gluing the surrounding fur to the patch. Patches were allowed to dry before tick addition.

2.8.3 *Application of ticks onto mice*

Ticks were applied to the mice on the day of patch attachment once the patches had dried. Ticks were placed within the patch utilising the surface tension of a water droplet; 20-30µl of water was placed into the patch by pipetting and ticks were applied individually into the water droplet using tweezers. The patch was sealed with mesh and the water absorbed out using a piece of tissue.

2.8.4 *Removal of ticks from mice*

Where scheduled checks for fed and detached ticks were conducted, mice were anaesthetised with isoflurane, the mesh removed from the patch using tweezers and any ticks fed and detached were placed into labelled 2ml tubes. Alive fed and detached ticks were individually weighed to the nearest 0.1mg using an analytical balance (Sartorius, UK) before placing into individual 2ml modified tubes and maintaining as described in section 2.3.4 and allowing nymphs to moult to the adult stage. Dead ticks were discarded.

2.9 *Inoculation of mice with HAZV*

Mice were anaesthetised with 0.1ml Alfaxan via the intraperitoneal route and then inoculated with either 20pfu/ml or 2pfu/ml of HAZV diluted in PBS via intradermal injection.

2.10 *Mouse sample necropsy*

Upon specified sample dates or at the end of experimental studies mice were euthanised with 0.1 – 0.2 ml of Dolethal via the intraperitoneal route. For sample collection a separate set of instruments were used for each treatment group with negative controls processed first.

2.10.1 *Blood and serum*

Whole blood was obtained via cardiac puncture and 100µl placed into an RNA Protect Animal Blood Tubes (Qiagen), gently inverted 8-10 times and frozen at -80°C. The remaining blood was placed into a SST™ serum separation tube (BD Vacutainer, Life Technologies), allowed to stand at room temperature for a minimum of 30 minutes and centrifuged at 1300xg for 15minutes in a fixed angle centrifuge. The supernatant was removed and divided into two 1.5ml RNase free tubes and frozen at -80°C for

further processing. RNA was purified using the QIamp Viral RNA Kit (Qiagen) as detailed in section 2.4.3.

2.10.2 *Tissue samples*

A skin biopsy at the site of tick attachment, directly underneath the patch location was taken and placed into 1.6 ml of RNAlater (Life Technologies, UK) and stored at -80°C until further molecular processing. It is recommended that tissue samples are stored in 5-10x volume of RNAlater which equates to 10µl per 1mg of tissue. Liver and spleen tissue samples were both divided into thirds; one third was placed into a 2ml tube containing serum-free MEM supplemented with 5:500ml penicillin / streptomycin; one third into a 2ml tube containing 350µl of RNAlater for spleen samples and 1.6ml of RNAlater for liver samples; and one third into 10% non-buffered formalin for histological processing and maintained at room temperature until processed.

2.10.3 *Histological processing of mouse tissues*

Histological processing was performed (Histology Department, PHE) on mouse samples fixed in 10% neutral-buffered formalin for two days and processed routinely to paraffin wax. Samples were sectioned between 3-5 µm, stained with haematoxylin and eosin (HE) and examined microscopically.

2.11 **Statistical analysis**

All statistical analysis was performed using GraphPad Prism (Version 5.01, GraphPad Software, USA). The significance threshold of 0.05 for P values was used for each experiment.

When analysing data from three or more small sample size groups or animal data (n=3) where data is not normally distributed, the non-parametric Kruskal Wallis test was used. The Dunns multiple comparison test was also performed to compare data between all groups. When comparing the means of two groups the Mann-Whitney U test was performed (Bente et al, 2010; Dowall et al, 2012).

Linear regression was used to assess the efficiencies of qRT-PCR assays. Linear regression reports the best fit value of the slope from raw data which is required for determining qRT-PCR assays.

Kaplain-meier survivorship curves were used to illustrate the survival ratios of one or more treatment groups. Survival proportions were determined using the log rank Mantel-Cox test and the log rank test for trend.

Chapter 3. Development and optimisation of methods for use with *Ixodes ricinus* RNA and whole ticks

3.1 Background

3.1.1 *Use of endogenous controls in tick research*

There are four common methods used for quantification of messenger RNA (mRNA) gene expression levels (Bustin, 2000): Northern blot; microarrays; *in situ* hybridisation; and quantitative real-time PCR (qRT-PCR). Northern blot analysis is the most common method used and can provide information on transcript size and integrity. This method requires large amounts of RNA and requires multiple steps involving electrophoresis which can cause degradation of RNA compromising quantification (Streit et al., 2009). Microarrays are a useful tool when analysing numerous genes but do require the generation of a gene library which is difficult in ticks as genome information is not readily available for all species. *In situ* hybridisation uses hybridisation probes to detect specific RNA in cells or tissues but can require supplementation with PCR for accurate quantification.

Quantitative real-time PCR is a highly specific, fast and reliable method capable of amplifying low quantities of mRNA for quantification (Bustin, 2002). This has proved beneficial for tick-borne diseases allowing the development of rapid diagnostic assays to new emerging pathogens such as Severe Fever with Thrombocytopenia Syndrome Virus (Sun et al., 2012) and highly pathogenic re-emerging zoonosis such as Crimean-Congo haemorrhagic fever virus (CCHFV) (Atkinson et al., 2012a). Real time PCR can be used for quantification of mRNA transcripts but can be difficult as many sources of variation exist. Principally, PCR requires DNA as a template; however, many viruses have RNA genomes and gene expression profiles use mRNA. An additional step is incorporated to reverse transcribe RNA into cDNA, which can provide a source for contamination. However, the use of one-step reagents such as SuperScript III (Invitrogen) can reduce the risk of pre PCR contamination by reducing the number of handling steps. RNA itself poses issues which must be acknowledged for the successful interpretation of results and the requirement for biological controls. RNA is inherently less stable than DNA especially once extracted. Care must be taken to ensure the integrity of the RNA, which can be done pre-PCR by ensuring the correct storage of

samples prior to processing, ensuring efficient lysis of samples and removal of contaminating nucleases and genomic DNA. The heterogeneity of samples such as tissues, blood and whole organisms can also produce variation in RNA quantity and quality. RNA extractions from tissues containing a variety of cell populations or whole organisms at different stages of differentiation may have different levels of RNA expression.

In order to control for inhibitors, sample loading, variation in reverse transcription levels, and even minor differences in the amount of starting material, a PCR assay can be normalised with reference data (Bustin, 2002). This data is most commonly obtained from using a housekeeping gene; those that have stable expression across all tissues to maintain cellular function (Butte et al., 2001). However, in order for a gene to be suitable as a reference gene it must also exhibit low variation within tissues under defined experimental conditions (Bustin, 2002). These reference genes undergo the same processing as the target and are subjected to the same errors in cDNA preparation and other steps as the target gene, it can therefore be used as a baseline for comparison with test samples to firstly ensure that test material has been added to the reaction and to correct sample-to-sample variation. A variety of genes expressed within Eukaryotic organisms have been proposed as reference or housekeeping genes, the most common including: the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase (GAPDH); the cytoskeletal protein β -actin; and ribosomal RNA (Arya et al., 2005). The assumption of these genes is that they have constant expression throughout all cells under all conditions. However, recent studies have casted doubt on the use of some genes; both β -actin and GAPDH have been shown to be degraded by microRNA (Sikand et al., 2012). No ideal housekeeping gene has yet been identified and it has been suggested to use multiple internal control genes where possible (Bustin, 2000) but qRT-PCR is an expensive technique and it is not always feasible to do this.

Within tick-borne disease research, there are currently three papers that have assessed the expression stability and variation of a cohort of genes within tick samples. Nijhof *et al* evaluated nine commonly used tick reference genes including; β -actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATA box binding

protein (TBP) throughout all developmental stages of *Rhipicephalus microplus* and *Rhipicephalus appendiculatus* ticks (Nijhof et al., 2009). Their stability and variation in expression across all developmental stages resulted in GAPDH and TBP showing the highest overall stability and elongation factor 1 α (EF1 α) and ribosomal protein L4 with the least variation. Individual developmental stages did show differing optimal genes with β -actin, TBP and GAPDH having high expression stability in the unfed nymph and fed nymph stages. Browning *et al* also identified β -actin and GAPDH as having the most stable expression and lowest degree of variation across all life stages of *Amblyomma maculatum* (Browning et al., 2012). EF1 α was ranked as the least stable in salivary gland tissues but as the most stably expressed in adult midguts. It has also been shown that in engorging *Ixodes scapularis* adult females the ribosomal proteins S4 and L13A were shown to be the most stable specifically within the salivary glands and synganglia (the tick brain) with GAPDH and TBP ranking second (Koci et al., 2013). These studies illustrate the species specific, developmental and physiological specific differences in gene expression within and between ticks. Therefore it must be demonstrated that the chosen reference gene is suitable for the species, sample and experimental conditions to be used.

The selection and testing of putative reference genes is easier where sequence data is available. In 2012, the first tick genome was sequenced for *Ixodes scapularis* (Megy et al., 2011) which has formed the basis of many sequence extrapolations for other tick species. Currently a validated reference gene for *Ixodes ricinus* (*I. ricinus*), the most widely distributed tick in Europe, has not been reported.

3.1.2 **Artificial inoculation techniques of ticks**

Tick-borne viruses are transmitted to vertebrate hosts during feeding via the salivary glands by exploiting the biochemical make up of tick saliva (Nuttall and Labuda, 2004). Investigations into the intricate relationship between pathogen, vector and host would ideally need the use of a mammalian host. However, the maintenance and rearing of ticks can be a time consuming process and where no *In vivo* feeding model exists, optimisation is required. In lieu of this a number of artificial routes of inoculation are currently used: injection, capillary feeding, membrane feeding and immersion.

Injecting pathogens via several routes including intra-anal (Gonzalez et al., 1991), intra-coleomic (Shepherd et al., 1989) and into the front tarsi of ticks allows the quantification of infective dose: however, high mortality rates are observed (Rechav et al., 1999). These methods also require the use of sharp fine-tipped needles and expert precision to ensure accurate delivery requiring close contact with the vector and with a high titre of pathogen. Capillary feeding was first described in the 1950s and used to infect ixodid ticks (Burgdorfer, 1957), and more recently used to infect a number of tick species with various pathogens including *Borrelia burgdorferi* (Broadwater et al., 2002), *Rickettsia* (Baldrige et al., 2007), Dugbe virus (Booth et al., 1991), Bluetongue virus (Bouwknegt et al., 2010) and most recently for use in screening potential anti-tick vaccine candidates (Lew-Tabor et al., 2014). Capillary feeding requires the tick hypostome to be inserted into glass capillaries filled with a pathogen with the tick secured into place until feeding is completed. This requires direct handling of infectious inoculum in glass tubes, the direct handling of tick mouthparts and also requires ticks to complete feeding upon an animal host. However, this is representative of the natural route of infection and allows quantification of ingested inoculum. The use of artificial membranes made from silicone or a range of animal skin samples provides a natural route of infection, the ability to quantify infective dose and has the potential to infect a large number of ticks simultaneously. This is an intricate system to set up and can require extra stimuli to be added to enhance attachment such as CO₂ (Voigt et al., 1993), tick faeces and synthetic pheromones (Kuhnert et al., 1995). The use of immersion for infecting ticks with pathogens was first described by Policastro and Schwan (Policastro and Schwan, 2003) and has since been used to immerse ticks in bacterial pathogens and viruses from the flaviviridae (Table 3.1).

Table 3.1: Summary of studies using the method of immersion in ticks. Details of species, developmental stage, the inoculated pathogen and associated titre and the success rate are detailed. Percentage infectivity was determined by amplification of viral RNA and immunofluorescence assay for detection of *Borrelia* (Policastro). LGTV Langat Virus, TBEV Tick-borne encephalitis Virus

Tick species	Developmental stage	Pathogen	Titre	Infected (%) (DPIM)	Survival (%)	Ref
<i>I. scapularis</i>	Larvae	<i>Borrelia burgdorferi</i>	10 ⁸ spirochetes/ml	65 (45 minutes)	ND	(Policastro and Schwan, 2003)
<i>I. scapularis</i>	Larvae	LGTV	10 ⁷ pfu/ml	96 (15)	ND	(Mitzel et al., 2007)
<i>I. ricinus</i>	Adults	TBEV	10 ⁷ pfu/ml	21 (20)	100	(Belova et al., 2012)
<i>I. ricinus</i>	Larvae	<i>Borrelia burgdorferi</i>	10 ⁷ pfu/ml	65 (nymphs)	ND	(Fiserová et al., 2008)

The immersion technique provides a method of infecting large numbers of ticks simultaneously with a defined stock of known concentration. The lack of specialised equipment and requirement for needles or animals to complete feeding provides minimal contact with the vector and direct handling of pathogenic material. Immersion provides a convenient and simple method for use in both a low and high containment setting.

3.1.3 Chapter aims

The aims of this chapter are to; (1) optimise techniques for the use of RNA obtained from *I. ricinus* ticks; (2) due to their high stability throughout all life stages of certain tick species and specifically within the nymph stage, GAPDH, TBP and β -actin will be assessed for their ability to amplify *I. ricinus* mRNA transcripts and therefore their suitability as endogenous controls and; (3) determine and optimise the quantification of viral levels within tick samples and optimise the use of artificial inoculation of *I. ricinus* nymphs via immersion.

3.1.4 *Chapter objectives*

The above aims will be addressed via the following objectives;

- Determine the optimal handling, extraction and storage parameters of *I. ricinus* RNA
- Identify the most suitable housekeeping gene for use with *I. ricinus* nymphs and optimise for use in a real-time RT-PCR assay.
- Determine the efficacy of the immersion technique to infect *I. ricinus* ticks
- Optimise *I. ricinus* survival during and post-immersion

3.2 **Methods**

3.2.1 *Maintenance of I. ricinus ticks*

In all experiments laboratory reared *I. ricinus* were maintained in a Nalgene plastic desiccator at 17-20°C. The bottom of the desiccator was filled with a saturated solution of magnesium sulphate, generating a relative humidity of 85-95%. Unless stated otherwise all ticks were used within 4 months of emergence and were in an unfed state.

3.2.2 *Optimisation of the handling, extraction and storage of tick RNA*

3.2.2.1 Measurement of RNA Integrity

The RNA quality and integrity of samples was assessed by inspection of the 18s and 28s fragments using an Agilent 2100 Bioanalyzer (Bustin and Nolan, 2004) as detailed in section 2.4.6. Fluorescent dyes are intercalated into the RNA strands, detected by lasers and translated data shown as an electropherogram (Figure 3.1) depicting the 18s and 28s ribosomal peaks. The base width of each peak is measured and this is used to assign each sample an RNA integrity number (RIN) with 1 being the most degraded sample and 10 being the most intact.

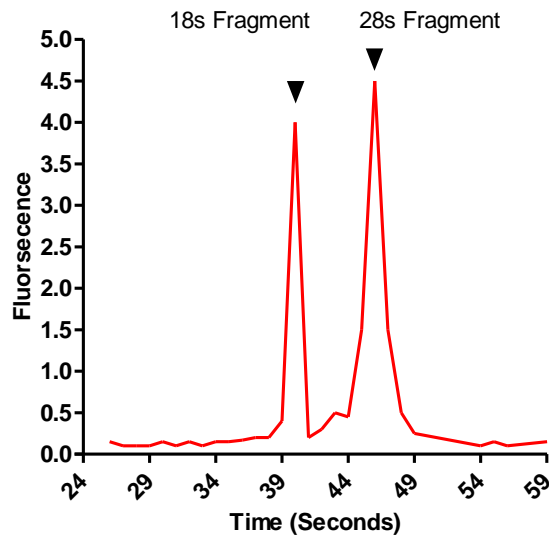


Figure 3.1: Electropherogram depicting the 18s and 28s regions that are indicative of RNA quality.

3.2.2.2 Individual and pooled tick RNA

An initial analysis of RNA obtained from ticks stored at -80°C and homogenised individually or in a cohort of 10 was performed. Samples were prepared for analysis using the Agilent 6000 RNA Nano kit following the method detailed in section 2.4.6.

3.2.2.3 Heat denaturation of tick RNA

The standard heat denaturation of RNA samples prior to analysis allows the hydrogen bonds holding the RNA together to dissociate. In some cases secondary structures can form and appear as one peak on the electropherogram. To ensure that this was not occurring with the tick RNA, RNA was both non-denatured and denatured at 70°C for 2 minutes and compared.

3.2.2.4 DNase digestion of tick RNA

Contaminating genomic DNA can cause spikes on the electropherogram and can appear as a single wide peak. Tick RNA samples ($n=20$) were individually homogenised (2.4.1) and RNA extracted using the RNeasy mini kit (2.4.4), ten samples underwent on column DNase digestion during RNA extraction and ten samples remained undigested. RNA was then pooled for each condition and analysed using the Agilent 2100 Bioanalyser.

3.2.2.5 Storage of tick RNA

Four storage conditions were compared to determine if the storage conditions of whole ticks prior to homogenisation and RNA extraction are affecting RNA quality.

Forty unfed, non-infected *I. ricinus* nymphs were divided into 4 storage groups (n=10) as per Table 3.2.

Table 3.2: Storage conditions of homogenised ticks. Group 1, homogenisation of live ticks with immediate RNA extraction; Group 2, ticks were flash frozen on dry ice for 5 minutes and stored at -80°C overnight; Group 3, ticks flash frozen on dry ice and placed into RNA Later and stored at -80°C overnight; and Group 4, ticks stored at -80°C overnight.

Group	N	Storage condition			
		Alive / Dead	Flash Frozen	-80°C O/N	RNAlater
1	10	A			
2	10	D	X	X	
3	10	D	X	X	X
4	10	D		X	

Ticks were homogenised in 250µl of buffer RLT and individually extracted using the RNeasy Mini kit (Qiagen) with on column DNase digestion. RNA yield (ng/ul) and integrity (RIN) were assessed.

3.2.3 Viability testing of tick homogenisation on RNA yield and quality

Homogenisation, by its nature, is a highly disruptive process. The precellys²⁴ bead beater uses reinforced tubes containing 2.8mm metal beads to homogenise samples between 5000 – 6200rpm. It is important to ensure that the homogenisation process itself does not cause loss of RNA yield or quality affecting downstream processes such as quantitative real time PCR.

3.2.3.1 Homogenisation speed and tick RNA yield

I. ricinus nymphs that were flash frozen on dry ice and stored at -80°C overnight were divided into three groups (n=3 per group) and homogenised individually as detailed in section 2.4.1, with three cycles at their designated speed; 6200, 5500 or 5000 rpm. RNA was purified using the RNeasy mini kit (Qiagen) with on column DNase digestion

as detailed in section 2.4.4. RNA yield of all samples was assessed by quantification using a UV spectrometer (2.4.5) and expressed as ng/ μ l.

3.2.3.2 Homogenisation duration and tick RNA yield

I. ricinus ticks that were previously flash frozen on dry ice and stored at -80°C were divided into four groups (n=3 per group). Ticks were homogenised individually as previously described (2.4.1) with three cycles at 6200rpm for; 5, 10, 15 or 20 seconds with 30s between each cycle. RNA was then extracted using the RNeasy mini kit (Qiagen) with on column DNase digestion. RNA yield of all samples was assessed by quantification using a UV spectrometer and expressed as ng/ μ l.

3.2.3.3 Homogenisation speed and viral titre

Twelve ticks were divided into three groups according to homogenisation speed; 5000rpm, 5500 rpm and 6200rpm for groups 1, 2 and 3 respectively. Ticks were homogenised individually in 500 μ l of Hazara virus (HAZV) at a titre of 1.7×10^6 plaque forming units/ml. Ten-fold dilutions were performed in Minimum Essential Media (MEM) immediately after homogenisation and viral titre was estimated by plaque assay on SW13 cell monolayers as detailed in section 2.6.2. Infectivity was expressed as plaque forming units (pfu) per ml.

3.2.4 ***Development of a Taqman Real-Time RT-PCR for use with tick endogenous controls***

Previously published sequences for β -actin, GAPDH and TBP reference genes were downloaded from Genbank (Table 3.3) and aligned using the Megalign and Clustal Wallis programme (DNASStar). A conserved area of sequence was identified, as seen in Figure 3.2, and Taqman primer and probe sets were designed using the web based software programme; Oligo Evaluator provided by Sigma-Aldrich. Multiple primer and probe sets were generated for each gene (Table 3.4) and the chosen combination determined following the guidelines generated for designing Taqman hydrolysis real-time RT-PCR assays as detailed in section 2.5.2.

Table 3.3: Available sequences downloaded from Genbank and used for the design of Taqman primer and probes.

Gene	Gene name	Function	Tick Species	Genbank Accession no.
β-actin	Beta actin	Cytoskeletal structural protein	<i>Ixodes ricinus</i>	HQ682101 AJ889837.1
			<i>Dermacentor variabilis</i>	EF488512.2
			<i>Rhipicephalus microplus</i>	AY255624.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxireductase in glycolysis and gluconeogenesis	<i>Ixodes scapularis</i>	XM_002434302
			<i>Dermacentor variabilis</i>	EU999993
			<i>Rhipicephalus microplus</i>	CK180824
			<i>Rhipicephalus appendiculatus</i>	CD791831
TBP	TATA box binding protein	Transcription factor	<i>Ixodes scapularis</i>	XM_002402081

```

D.variabilis      AAGTACCCCATGAGCACGGTATCGTCACCAACTGGGACGACATGGAGAA
I. ricinus        AAGTACCCCATCGAGCACGGCATCGTGACCAACTGGGACGACATGGAGAA
B.microplus       AAGTACCCCATGAGCACGGTATCGTCACCAACTGGGACGACATGGAGAA
I. ricinus        AAGTACCCCATCGAGCACGGCATCGTCACTAACTGGGACGACATGGAGAA
*****
D.variabilis      GATCTGGCACCACACTTTCTACAACGAGCTGCGTGTGGCCCCCGAGGAGC
I. ricinus        GATCTGGCACCACA CTTCTACAACGAGCTCCGCGTGGCCCCCGAGGAGC
B.microplus       GATCTGGCACCACACCTTCTACAACGAGCTGCGTGTGGCTCCCGAGGAGC
I. ricinus        GATCTGGCACCACACCTTCTACAACGAGCTCCGCGTGGCCCCCGAGGAGC
*****
D.variabilis      ACCCCGTTCTGCTGACCGAGGCCCCCTGAACCCCAAGGCCAACCGTGAG
I. ricinus        ACCCCGTTCTGCTGACTGAAGCCCCCTGAACCCCAAGGCCAACCGGGAG
B.microplus       ACCCCGTCCTGCTCACCGAGGCCCCCTGAACCCCAAGGCCAACCGCGAG
I. ricinus        ACCCCGTCCTGTTGACCGAGGCCCCCTGAACCCCAAGGCTAACCGCGAG
*****
D.variabilis      AAGATGACCCAGATCATGTTTGAGACCTTCAACACGCCCGCCATGTACGT
I. ricinus        AAGATGACTCAGATCATGTTTCGAGACCTTCAACACCCCGCCATGTACGT
B.microplus       AAGATGACGCAGATCATGTTTCGAGACCTTCAACACGCCCGCCATGTACGT
I. ricinus        AAGATGACCCAGATCATGTTTCGAGACCTTCAACACCCCGCCATGTACGT
*****

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Figure 3.2: Conserved sequence of tick β-actin identified across all available sequences. Forward and reverse primer sequences are highlighted in yellow and probe binding site shown in purple.

Table 3.4: Characteristics of Taqman primer and probe sets selected for real-time RT-PCR. The position of the forward (F) and reverse (R) primers, and probes, along the RNA sequence are described by the first base at the 3'. The oligonucleotide (oligo) size, GC %, melting temperature (Tm), GC %, GC clamp, Cross Dimer, Self dimer, hairpin and PCR product size are also indicated.

	Position	Oligo Size (bp)	GC %	TM	PCR product size (bp)	GC clamp	Cross dimer	Self dimer ΔG	Hairpin ΔG
β-Actin									
F Primer	239	18	50	60.7	139	1	-3.4	-2.4	0.0
R Primer	377	18	50	61.0		1		-3.2	0.0
Probe	260	22	54.5	68.4				0.0	0.0
GAPDH									
F Primer	1056	18	50	61.6	149	1	-2.4	-5.1	-0.9
R Primer	1205	18	50	60.5		1		-0.8	-0.8
Probe	1082	21	57.1	70.1				0.0	0.0
TBP									
F Primer	377	18	44.4	60.0	156	1		0.0	0.0
R Primer	533	18	50	61.1		1		0.0	0.0
Probe	405	20	60	69.0				-2.1	0.0

3.3.4.1 Specificity and optimisation of designed primers and probes

Specificity of the designed primer and probe sets were tested against their target genes; tick β -actin, GAPDH and TBP using tick RNA isolated from a pool of ten unfed whole *I. ricinus* nymphs. RNA was extracted using the RNeasy mini kit (Qiagen) and amplified using the one-step qRT-PCR Real-time assay described in section 2.5.1. Concentrations of both forward and reverse primers and probe were optimised for the chosen target gene as per the ABI guidelines (2.5.3) with final concentrations of 50, 300 and 900 nM of both forward and reverse primers assessed for the most sensitive real-time RT-PCR assay. Probe concentrations of 50, 100, 150, 200, 250 and 625 nM were added to the real-time RT-PCR reactions using optimal primer concentrations of 900 nM.

3.3.5 Amplification efficiency of the designed Real-Time RT-PCR

The efficiency of PCR amplification of each transcript was determined by generating ten-fold serial dilutions of HAZV and tick β -actin RNA. Dilutions of 1×10^6 to 10^{-1} were run in triplicate to account for sample variability generating a standard curve for both

targets. The use of 5 sets of log dilutions is thought to generate 100% efficiency with a slope of 3.3, as each log is separated by 3.3 cycle threshold (Ct). The R^2 value is also observed, the closer R^2 is to 1 the more accurately the Ct value can be used to predict the number of target copies in a sample.

The Ct values obtained from the amplification curves were plotted against RNA concentration. To determine if the efficiency of the PCR amplification of the target transcript was equal to the reference transcript the difference of the Ct values was calculated and plotted.

3.3.6 *Immersion of I. ricinus nymphs in HAZV*

I. ricinus nymphs were used in a preliminary experiment assessing the efficacy of a published immersion method (Mitzel et al., 2007; Policastro and Schwan, 2003). Fifty nymphs were divided into two 2ml screw top tubes (n=25) and immersed in 0.5ml of MEM containing 1.7×10^6 pfu/ml of HAZV and incubated at 37°C for 45 minutes. Ticks were re-suspended within the media every 10 minutes by pulse vortexing. After incubation ticks were chilled on ice for 2 minutes and then centrifuged at 2000g for 20 seconds, repeating as necessary, until all ticks were removed from the lid. The virus suspension was then removed by pipetting and the ticks washed twice with chilled Phosphate buffered saline (PBS) by centrifugation at 2000g for 30seconds, twice in 70% ethanol to surface sterilise the ticks and twice in distilled water. Several strips of 1.5 cm Whatman filter paper (GE Healthcare) were added to the 2ml tubes to absorb any excess moisture. Ticks were then transferred to a modified 5ml universal tube and maintained at 95% relative humidity (RH) until sampled. Ten *I. ricinus* nymphs were immersed in PBS as per above and used as a negative control.

After 2 and 6 days post-immersion ten ticks were sampled and on day 13 thirty ticks were sampled. *I. ricinus* ticks were placed into a 2ml cryovial tube and snap frozen on dry ice and stored at -80°C for molecular processing.

Upon processing, ticks were removed from the freezer and placed into individual reinforced 2ml Precellys tubes (PeqLab, UK) containing 2.8mm metal beads and homogenised as detailed in section 2.4.1. Briefly; samples were homogenised in 250µl

of RLT lysis buffer, homogenate passed through a Qias shredder (Qiagen) to shear any high molecular weight debris and RNA extracted using the RNeasy mini kit (Qiagen).

3.3.7 Effect of HAZV on *I. ricinus* survival

To determine if the presence of virus during immersion affected tick mortality, immersion of ticks in both HAZV and PBS was conducted and survival compared. Twenty *I. ricinus* ticks were divided into two groups (n=10); group A ticks were immersed in 0.5 ml of HAZV and group B ticks were immersed in 0.5 ml of PBS. Ticks were immersed in the groups of 10 and remained within the same tube after immersion. A modified lid, consisting of multiple air holes made with a 3 gauge needle was placed onto the tubes which were then placed back into the desiccator and maintained as described in section 2.3.4. Survival was monitored over ten days.

3.3.8 Effect of the immersion protocol on tick survival

The immersion protocol was broken down into 4 sections and each parameter investigated for its effect on tick survival. The parameters investigated were; (i) the age of ticks used; (ii) the effect of mechanical centrifugation and vortexing compared to manual inversion; (iii) the amount of wash and disinfectant added and; (v) the incubation temperature used.

Ticks were immersed in 0.5ml of PBS with the following modifications; nymphs were divided into five treatment groups (n=10) and placed into 2ml screw top tubes. The conditions used for each group are detailed in Table 3.5. Conditions in bold are those altered from the original method. All other methods remained the same as in section 3.2.6.

Table 3.5: Ticks were divided into 5 different treatment groups (n=10) to address the following factors; Age of ticks, the effect of centrifugation and vortexing, the amount of wash and disinfectant added and the incubation temperature. Group 1; Immersed as described above using newly emerged nymphs (5-6 weeks). Group 2; Newly emerged ticks immersed in 250ul of PBS and subsequent wash and disinfection steps. Vortexing was replaced with manually inversion of tubes, ticks incubated at 28°C for 45 minutes. Group 3; As group 2 but incubated performed at 37°C for 45 minutes. Group 4; As group 2 but incubated at 37°C for 30 minutes. Group 5; As group 1 but using nymphs emerged > 4 months. B Survival proportions days post-immersion.

Treatment Group	Age	Incubation Temperature	Method of suspension	Volume of wash and disinfection solutions		
				PBS	70% ETOH	Distilled water
1	5-6 weeks	37°C 45mins	Mechanical	500µl	500µl	500µl
2	5-6 weeks	28°C 45mins	Manual	250µl	250µl	250µl
3	5-6 weeks	37°C 45mins	Manual	250µl	250µl	250µl
4	5-6 weeks	37°C 30mins	Manual	250µl	250µl	250µl
5	>4 months	37°C 45mins	Mechanical	500µl	500µl	500µl

3.3.9 *Surface sterilisation of immersed ticks*

To ensure that the surface sterilisation step of the immersion protocol is effective and any detected signal of HAZV is due to internalised virus and not that remaining on the surface of the tick, 20 ticks were divided into four groups (n=5), as described in Table 3.6, and the volume and number of ethanol washes assessed. Ticks previously flash frozen and stored at -80°C were placed into 2 ml screw top tubes and immersed as previously described but with the following modification; ticks were immersed in the virus for 1 minute at room temperature compared to 37°C for 45 minutes. This eliminated the effect of the heat on the tick cuticle minimising the chance of the virus becoming internalised. Ticks were washed in PBS as described with modification of the ethanol step only. Ticks were then individually homogenised.

Table 3.6: Ethanol (Etoh) disinfection of HAZV immersed ticks.

Group	Sample Size (n)	Etoh volume (μ l)	Number of washes
1	5	500	1
2	5	500	2
3	5	250	1
4	5	250	2

3.3.10 *Optimisation of post-immersion tick density on tick survival*

To determine if tick density affects tick survival post-immersion 140 *I. ricinus* nymphs > 4 months emerged were immersed in PBS as per Group 5 in Table 3.5 and randomly allocated into four post-immersion aggregation groups: group 1, n=10; group 2, n=20; group 3, n=25; and group 4, n=35. After the immersion procedure was complete, modified lids comprising of multiple air holes, produced using a 3 gauge needle were placed onto the tubes. Ticks were maintained in a desiccator at a temperature of 17 – 20°C at 85-95% RH and survival observed daily.

3.4 Results

3.4.1 *Optimisation of the handling, extraction and storage of tick RNA*

3.4.1.1 Individual and pooled tick RNA

Initial testing of tick RNA extracted from individual ticks (A) and pooled ticks (B) produced a RIN of 1.70 and 2.20, respectively. Only one peak is detected at the location of the 28s rRNA (Figure 3.3).

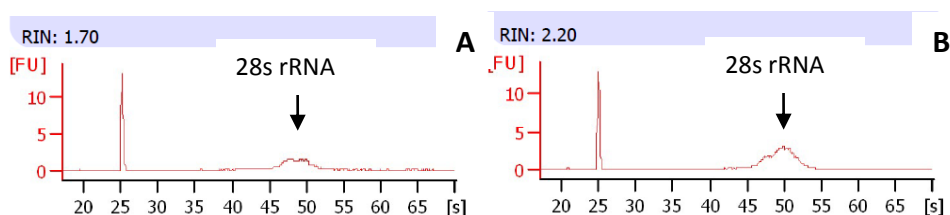


Figure 3.3: Bioanalyser electrophoresis plot of individually extracted RNA (A) and pooled RNA from ten ticks (B). RNA appears of poor quality due to the lack of the 18s rRNA peak.

3.4.1.2 Heat denaturation of tick RNA

Heat denaturation of RNA samples prior to analysis on the bioanalyser generates the same peak profile as those not heat denatured. A similar RIN is calculated as of that generated for the pooled ticks (Figure 3.4).

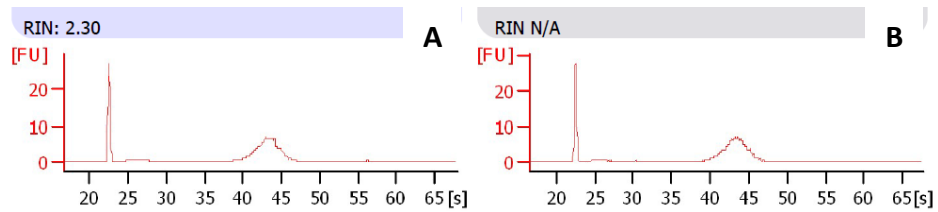


Figure 3.4: Bioanalyser electrophoresis plot of non-heat denatured RNA (A) and denatured RNA (B) at 70°C for 2 minutes.

3.4.1.3 DNase digestion of tick RNA

The effect of on column DNase digestion of tick RNA was compared to non-DNase digested tick RNA. Similar RIN values were obtained for both samples, indicating that the downstream processing of extracted RNA is not the source of these low readings or abnormal plots (Figure 3.5).

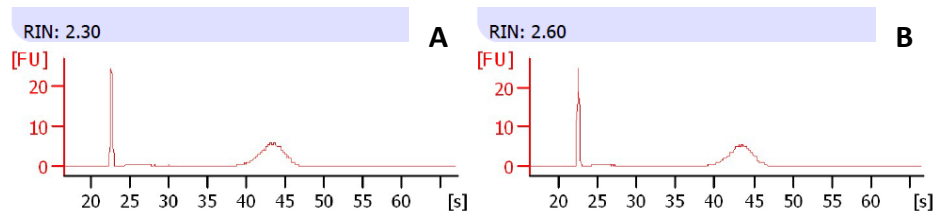


Figure 3.5: Bioanalyser electrophoresis plot of genomic DNA depleted RNA (A) and non-genomic DNA depleted RNA (B).

3.4.1.4 Storage of tick RNA

Tick RNA was stored at four different conditions before homogenisation. It can be seen from Figure 3.6 that a significant difference in RNA yield between all storage conditions was observed ($P=0.0001$ Kruskal Wallis). Samples that were stored at -80°C overnight produced the lowest RNA yield (mean 10.60 ± 1.485 SD) closely followed by those snap frozen and stored in RNA later at -80°C (group 3) with a mean of 10.58 ± 1.073 SD, and those snap frozen and stored at -80°C overnight (group 2) with a mean of 11.38 ± 1.733 SD. Ticks which were homogenised immediately with no storage (group 1) produced the highest yield of RNA (mean 14.88 ± 1.543 SD).

A statistically significant difference ($P=0.05$, Kruskal Wallis) was observed between ticks homogenised immediately and those flash frozen and stored at -80°C overnight ($P=0.05$, Kruskal Wallis) and a highly significant difference was seen between group 1 and those flash frozen, stored in RNA later at -80°C overnight ($P=0.01$, Kruskal Wallis) and fresh tick homogenates and those stored at -80°C overnight ($P=0.01$, Kruskal Wallis). No significant difference was seen between groups 2, 3 and 4.

No significant difference in RIN was seen across the different storage groups (P=0.1300, Kruskal Wallis). When looking at the raw data there is a storage dependent decrease in RIN and increase in standard deviation; group 1 mean 5.025 ± 0.7312 SD, group 2 mean 4.895 ± 1.337 SD, group 3 mean 4.120 ± 1.489 and group 4 mean 3.631 ± 1.668 (Figure 3.7).

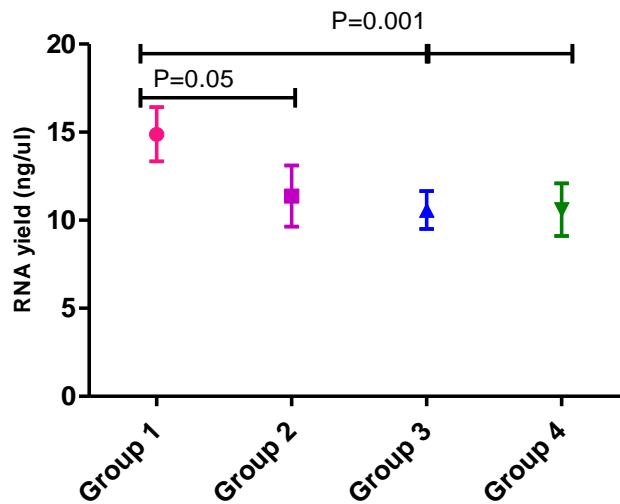


Figure 3.6: RNA yield (ng/ μ l) of tick RNA stored under four different conditions (n=10 per group). Group 1, homogenisation of live ticks and immediate RNA extraction; group 2; ticks were flash frozen on dry ice for 5 minutes and stored at -80°C overnight; group 3 ticks were flash frozen on dry ice and placed into RNA later and stored at -80°C overnight; and group 4, ticks were stored at -80°C overnight. Error bars denote standard deviation.

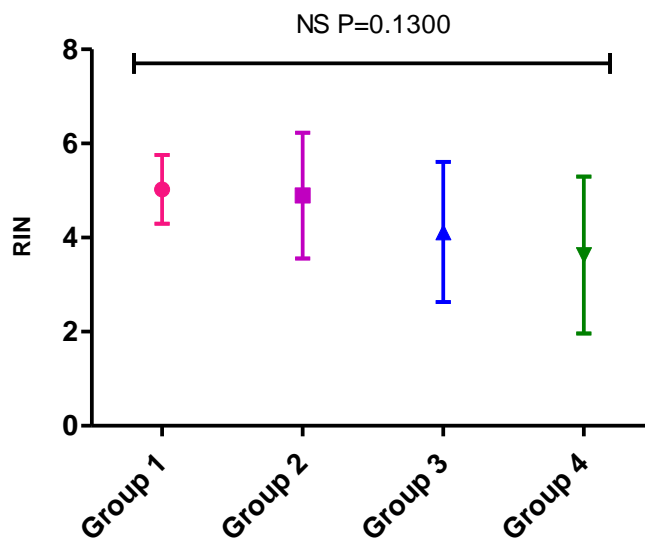


Figure 3.7: RNA integrity number (RIN) of tick RNA stored under four different conditions (n=10 per group). Group 1, homogenisation of live ticks and immediate RNA extraction; group 2; ticks were flash frozen on dry ice for 5 minutes and stored at -80°C overnight; group 3 ticks were flash frozen on dry ice and placed into RNA later and stored at -80°C overnight; and group 4, ticks were stored at -80°C overnight.

3.4.2 Viability testing of tick homogenisation on RNA yield and quality

3.4.2.1 Homogenisation speed and tick RNA yield

There was no statistically significant difference in RNA yield obtained between the three groups ($P=0.4268$, Kruskal Wallis). However, when looking at the raw data it can be seen that decreasing homogenisation speed is accompanied by a decrease in RNA yield (Figure 3.8). Ticks homogenised at a speed of 6200 rpm generated a mean yield of $16.03\text{ng}/\mu\text{l} \pm 2.71$ SD, samples homogenised at a speed of 5500 rpm produced a mean yield of $12.93\text{ng}/\mu\text{l} \pm 2.66$ and those homogenised at 5000rpm produced a mean yield of $12.93\text{ ng}/\mu\text{l} \pm 0.96$ SD.

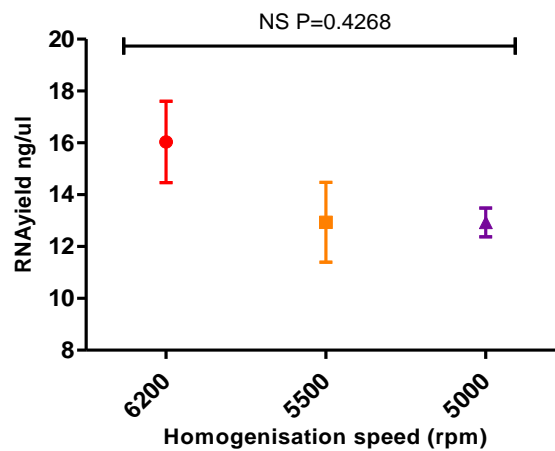


Figure 3.8: RNA yield ($\text{ng}/\mu\text{l}$) obtained from ticks ($n=3$ per group) homogenised at speeds of 6200, 5500 and 5000 rpm using a precellys²⁴ bead beater. Error bars denote standard deviation.

3.4.2.2 Homogenisation duration and tick RNA yield

There was no statistically significant difference in RNA yield obtained between the varying homogenisation time points ($P=0.2361$, Kruskal Wallis (Figure 3.9)). However a trend showed mean RNA yield decreasing as homogenisation time increased with a duration of 5 second producing a mean of $16.03\text{ng} \pm 2.71$ SD and with 15 seconds producing a mean of $11.36\text{ng} \pm 1.35$ SD and 20 seconds per cycle producing a mean yield of $12.60\text{ng} \pm 3.43$.

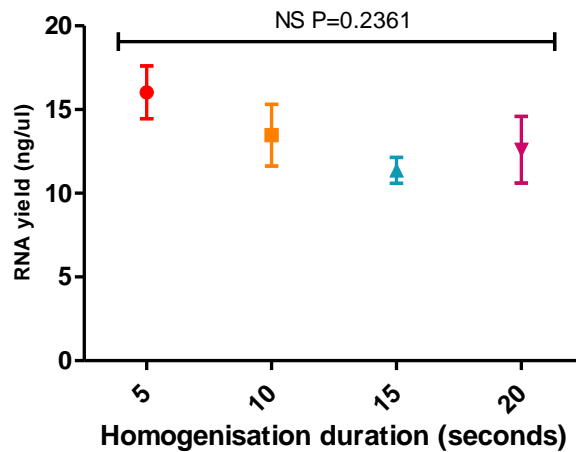


Figure 3.9: RNA yield (ng/μl) obtained from ticks (n=3 per group) homogenised at a speed of 6200 rpm for a duration of 5, 10, 15 and 20 seconds using a precellys²⁴ bead beater. Error bars denote standard deviation.

3.4.2.3 Homogenisation speed and viral titre

To ascertain whether homogenisation speed affected viral titre, a standard stock of HAZV was tested at different speeds. A significant difference in the mean titres across all homogenisation speeds was observed (P=0.0249, Kruskal Wallis) (Figure 3.10). It is seen that as homogenisation speed increased the viral titre of HAZV increased from a mean of 2.33×10^6 pfu/ml at 5000 rpm to 3.4×10^6 pfu/ml at 5500 rpm and 4×10^6 pfu/ml when homogenised at 6200 rpm. This is a fold increase of 1.3, 2 and 2.3 of Pfu/ ml of the original stock at 1.7×10^6 pfu/ml.

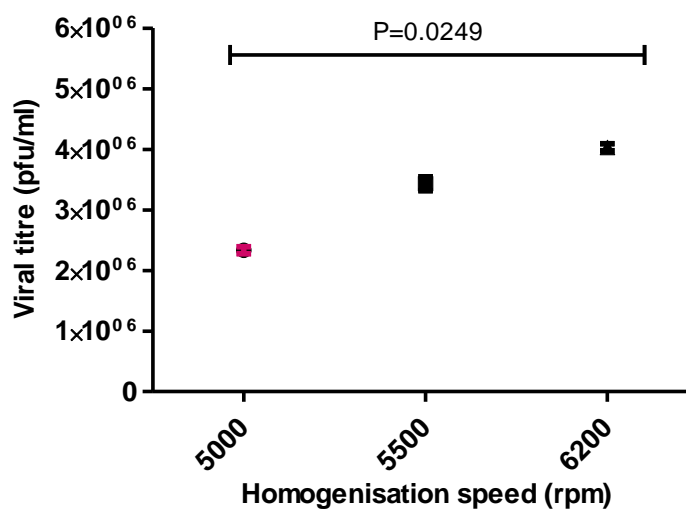


Figure 3.10: Viral titre of 500μl of Hazara virus homogenised in the presence of a tick (n=4) at varying speeds using the Precellys²⁴ bead beater.

3.4.3 Development of a Taqman real-time RT-PCR

The specificity of each reference gene to *I. ricinus* RNA was determined. Amplification of tick β -actin was detected after 24 cycles, GAPDH was detected at cycle 39 and TBP produced no detectable amplification (Figure 3.11). No amplification of no template controls (NTC) occurred confirming that the reagents were not contaminated with tick RNA, which would have resulted in false positive amplification.

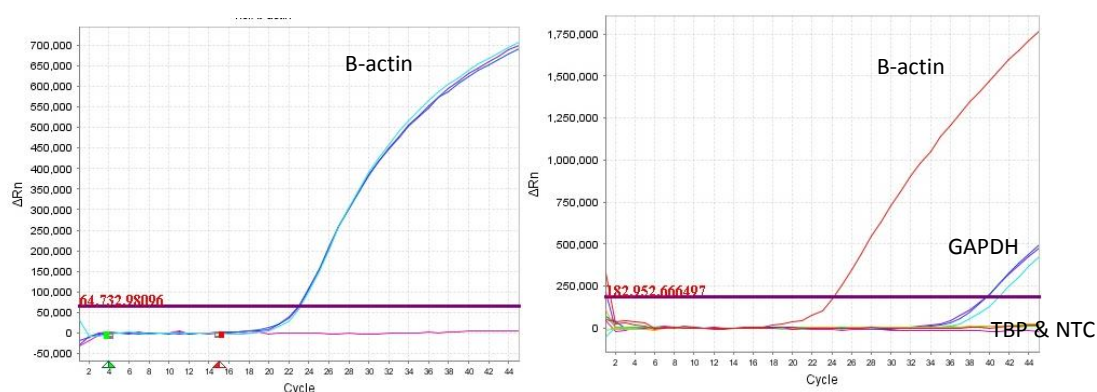


Figure 3.11: Taqman real-time RT-PCR amplification plots. Tick RNA was amplified by Taqman real-time RT-PCR targeting; β -actin, GAPDH and TBP transcripts. The fluorescent intensity of JOE at each cycle was measured and normalised to background fluorescent (ΔR_n). The horizontal line indicates the threshold at which the normalised fluorescent intensity rises exponentially. NTC = no template control of all reactions.

3.4.3.1 Primer optimisation

Out of the three Taqman primer and probes sets designed, only tick β -actin transcripts were detected using real-time RT-PCR assay. Therefore only optimisation of the primer and probes used to detect β -actin were carried out to further enhance the sensitivity.

Real-time RT-PCR sensitivity altered according to primer concentration used (Table 3.7), with increasing primer concentrations the Ct value decreased, indicative of increased sensitivity. Concentration of 900nM showed the highest Ct values with the smallest standard deviation between replicates and was therefore considered the most suitable for use in subsequent real-time RT-PCR assay.

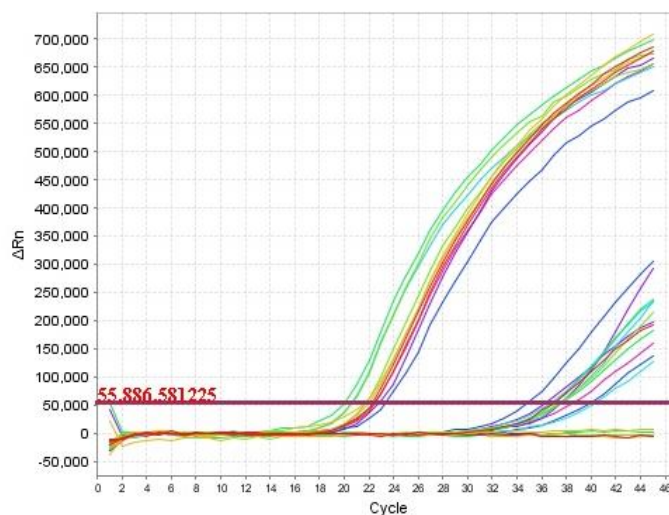


Figure 3.12: Optimisation of tick β -actin primer concentration. Amplification curves were plotted from the normalised fluorescent data which were collected after each real-time PCR cycle. Reactions were performed in triplicate and the mean Ct value and standard deviation determined for each concentration.

Table 3.7: Optimisation of tick β -actin primer concentration. The average Ct value + standard deviation for each combination of primer concentration was determined at the point where real-time RT-PCR amplification became exponential and crossed the set threshold ($n=3$). F&R= forward and reverse primers; NTC= no template control; UD= undefined Ct values.

	Primer conc (nM) (F&R)	Average Ct ($n=3$)
1	50/50	UD
2	50/300	39.40 ± 1.44
3	50/900	37.41 ± 1.20
4	300/50	37.19 ± 0.39
5	300/300	23.15 ± 0.33
6	300/900	22.03 ± 0.23
7	900/50	36.80 ± 1.59
8	900/300	22.22 ± 0.48
9	900/900	20.44 ± 0.35
NTC	250	UD

3.4.3.2 Probe optimisation

The real-time RT-PCR sensitivity altered according to probe concentration used (Figure 3.13; Table 3.8). With increasing probe concentrations the Ct value decreased, indicative of increased sensitivity. A probe concentration of 625 nM showed the highest Ct value and is the most suitable for an efficient real-time RT-PCR assay.

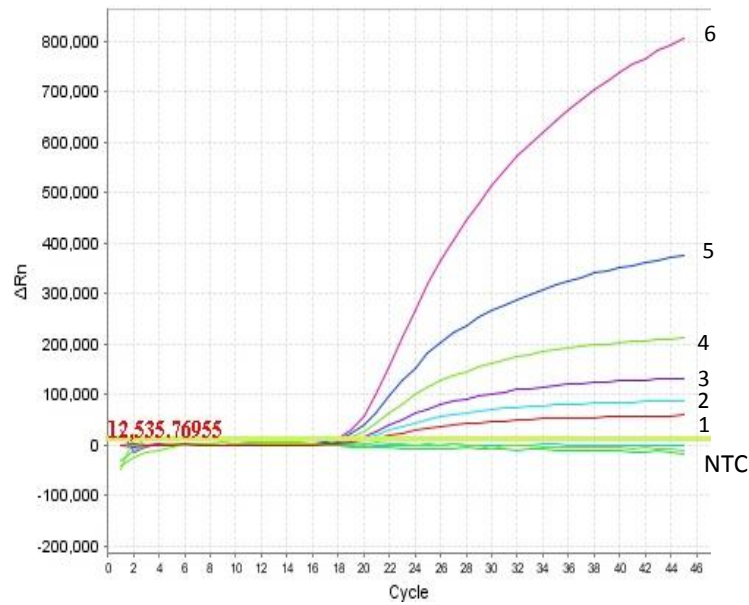


Figure 3.13: Optimisation of tick β -actin probe concentration. Amplification curves were plotted from the normalised fluorescent data which were collected after each real-time PCR cycle. Reactions were run in triplicate and a representative curve is illustrated. NTC = no template control.

Table 3.8: Optimisation of tick β -actin probe concentration. The average Ct value \pm standard deviation, for each combination of probe concentration, was determined at the point where real-time RT-PCR amplification became exponential and crossed the set threshold ($n=3$). F/R= forward and reverse primers; NTC= no template control; UD= undefined.

	Probe conc (nM)	Average Ct (n=3)
1	50	21.13 \pm 0.26
2	100	19.89 \pm 0.30
3	150	19.38 \pm 0.39
4	200	18.85 \pm 0.16
5	250	18.37 \pm 0.27
6	625	17.67 \pm 0.24
NTC	250	UD

3.4.4 Amplification efficiency of the designed real-time RT-PCR

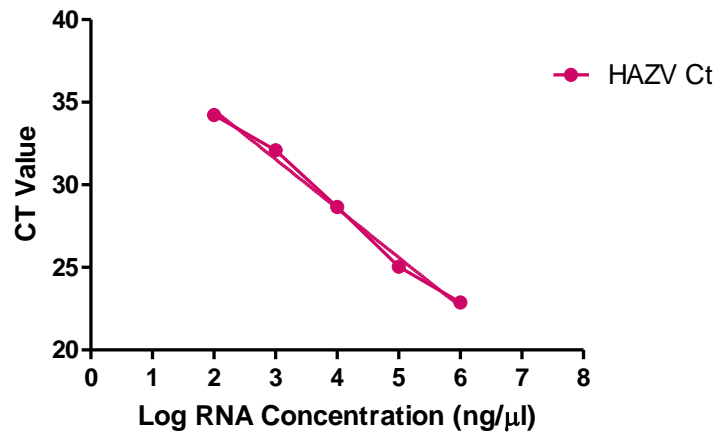
3.4.4.1 Real-Time RT-PCR amplification efficiencies of synthetic transcripts

The Ct values obtained from amplification curves using synthetic transcripts were plotted against RNA concentration (Figure 3.14). The standard curve for HAZV had

0.9914 % efficiency with a slope of 2.973. This means that the assay is over efficient separating each log by 2.9 Ct instead of 3.32.

Standard curves of tick β -actin produce 0.9999% efficiency with a slope of 3.4 indicating a slight under efficiency.

A.



B.

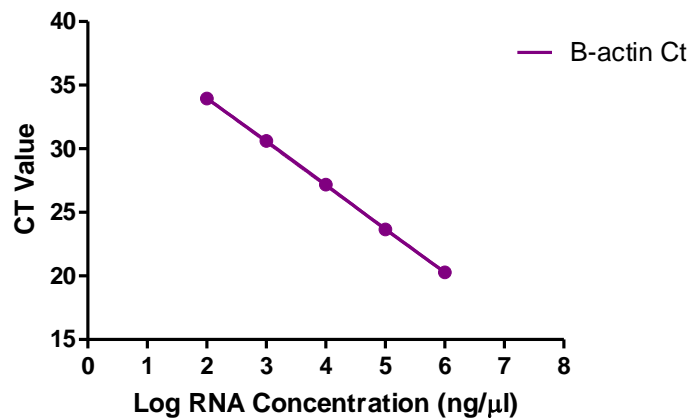


Figure 3.14: Amplification efficiency of target and reference gene. Synthetic RNA transcripts were serially diluted and optimal primer and probe concentrations used to amplify targets. Mean Ct values (n=3) were plotted against log RNA concentration for both Hazara (A) and β -actin (B)

Comparisons between these two assays show they do not have the same individual efficiency (Figure 3.15). Variation in efficiencies determined by the differences in the slope of the curves should be between +0.1 and -0.1 for the PCR amplification

efficiencies to be considered similar. In this case the slopes of HAZV and tick β -actin were 0.29 and 0.33, outside of the defined range to be considered similar.

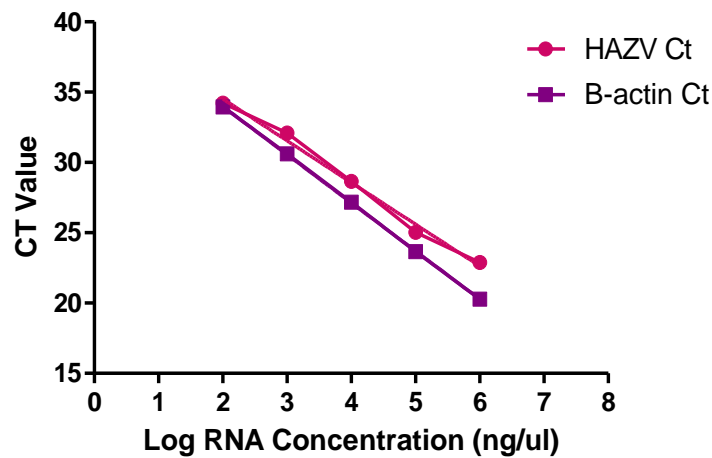


Figure 3.15: PCR amplification efficiency of target and reference transcripts. HAZV and Tick β -actin synthetic transcripts were serially diluted and optimal primer (900 nM) and probe (625 nM) concentrations were added to the real-time RT-PCR. Standard curves were generated on the ABI 7500 FAST RT-PCR real-time machine and average Ct values ($n = 3$) for each RNA concentration were plotted for all target and reference transcripts.

3.4.5 Immersion of *I. ricinus* nymphs in HAZV

To determine the efficacy of the immersion technique to infect *I. ricinus* nymphs, individual ticks were analysed for the presence of HAZV RNA using real-time RT-PCR on days 2, 6 and 13 post-immersion.

HAZV RNA was detected in homogenised whole ticks as early as 2 days post-immersion with fifty percent (3/6) of ticks sampled producing a mean Ct of 15.36 ± 0.73 SD.

Immersion success varied according to day PIM with a decreasing detection rate of 30% (3/10) on day 6 PIM with a mean Ct of 9.22 ± 5.16 SD but increasing to 100% detection (30/30) on day 13PIM with a mean Ct of 15.77 ± 1.12 SD. There was no significant difference in Ct value between D2, D6 and DAY 13 PIM ($P=0.0688$, Kruskal Wallis). All ticks immersed in PBS tested negative via real time PCR as did the negative PCR controls. The positive control of HAZV stock tested positive with a Ct value of 24.

3.4.6 Effect of HAZV on tick survival

I. ricinus nymphs that were either immersed in HAZV or PBS both exhibited a 90% mortality rate on day 1 post-immersion (Figure 3.16) P=1, Mantel Cox Survival Analysis).

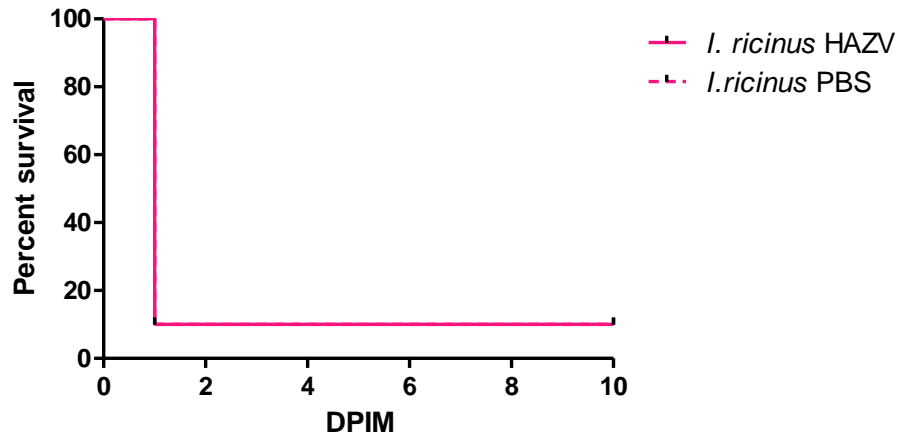


Figure 3.16: The effect of immersion in Hazara virus on tick survival. *I. ricinus* nymphs (n=10) immersed in 500 μ l of 1.7×10^6 pfu/ml of HAZV and 500 μ l of PBS and survival monitored over 10 days post immersion (DPIM). A mortality rate of 90% was observed in both treatment groups with mortality occurring on day 1 post-immersion.

3.4.7 Optimisation of the immersion protocol on tick survival

The immersion protocol was investigated to determine its effect on tick survival. Four parameters were investigated; (i) the age of ticks used; (ii) the effect of mechanical centrifugation and vortexing compared to manual inversion; (iii) the amount of wash and disinfectant added and; (v) the incubation temperature used. Mortality was seen in all treatment groups albeit to differing degrees. Age appeared to have the biggest effect on tick survival (P=0.0046, Mantel cox test) with ticks emerged more than 5 months having higher survival rates than those of 5-6 weeks emergence. Replacement of the vortex step with manual inversion and reduction of wash and disinfection volumes did not appear to affect survival (P=0.6171, Mantel cox test) and variation in temperature also did not have an effect on the survival of ticks (28°C for 45 minutes vs. 37°C for 45 minutes, P=0.8464; 28°C for 45 minutes vs. 37°C for 30 minutes, P=0.3243; 37°C for 45 minutes vs. 37°C for 30 minutes, P=0.2679).

In all groups mortality occurs within the first five days post-immersion. Figure 3.17 illustrates the Kaplan Meier survival curves of each group and survival proportions of each group.

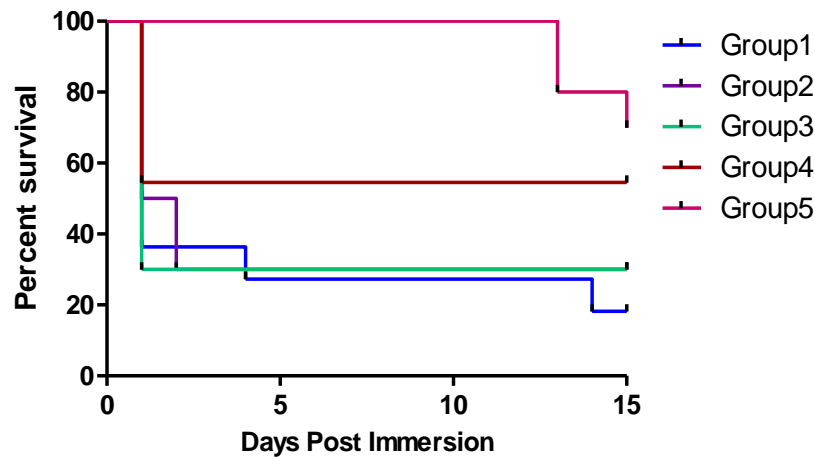


Figure 3.17: Ticks were divided into 5 different treatment groups (n=10) to address the following factors; age of ticks, the effect of centrifugation and vortexing, the amount of wash and disinfectant added and the incubation temperature. **Group 1;** Immersed as described above using newly emerged nymphs (5-6 weeks). **Group 2;** newly emerged ticks immersed in 250µl of PBS and subsequent wash and disinfection steps. Vortexing was replaced with manually inversion of tubes, ticks incubated at 28°C for 45 minutes. **Group 3;** as group 2 but incubated performed at 37°C for 45 minutes. **Group 4;** as group 2 but incubated at 37°C for 30 minutes. **Group 5;** as group 1 but using nymphs emerged > 4 months. **B** Survival proportions days post-immersion.

3.4.8 *Surface sterilisation of immersed ticks*

To ensure residual virus was removed from the surface of the tick, ticks were either washed once or twice in 500µl or 250µl of 70% ethanol. No amplification of HAZV was observed in surface sterilised ticks. All samples produced detectable amplification of the endogenous control tick β -actin and the positive control of Hazara viral stock produced a detectable Ct of 24.

3.4.9 *Optimisation of post-immersion tick density on nymph survival*

Ticks were housed at 4 different densities post-immersion; group 1, 10 ticks per tube; group 2, 20 ticks per tube; group 3, 25 ticks per tube and; group 4, 35 ticks per tube. Survival was monitored over a 30 day period. Analysis of survival curves across the four groups shows that post immersed nymph survival is affected by tick density (P=0.0001, Log rank test) and there is a distinct trend across all groups (P=0.0001, Log rank test for trend).

There was a significant difference in survival between ticks kept at densities of 10 per tube compared to 20 per tube (P=0.0001, Log rank test). There was no significant

difference between ticks kept in groups of 20 and 25 ($P=0.7892$, Log rank test) or groups of 25 and 35 ($P=0.1497$, Log rank test).

All groups exhibited mortality within the first 5 days post-immersion. When looking at Figure 3.18, density-dependent survival can be seen. Groups 2 & 3 resulted in the same survival rate on day 30 PIM but the onset of mortality appeared at a later date in group 3. Optimal survival was seen in ticks kept at densities of 35 (group 4).

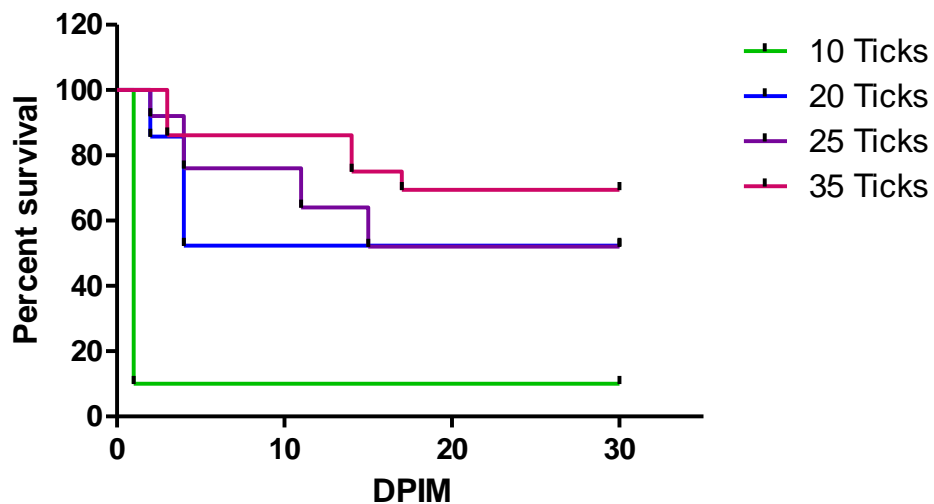


Figure 3.18: Ticks were divided into 4 post-immersion density groups to investigate the effect on post-immersion survival. Ticks were maintained in 2ml Eppendorf tubes used during immersion and modified lids, comprising of multiple air holes, were placed onto the tubes to allow air exchange. Tubes were maintained at room temperature at 85-95%RH. **Group 1**, 10 ticks per tube; **Group 2**, 20 ticks per tube; **Group 3**, 25 ticks per tube; and **Group 4**, 35 ticks per tube.

3.5 Discussion

3.5.1 *Optimisation of the handling, extraction and storage of tick RNA*

To preserve the integrity of RNA for successful downstream applications endogenous RNases must be immediately inactivated. Ribonucleases (RNase) are highly robust enzymes found ubiquitously in prokaryotes and eukaryotes which fragment RNA. This has a necessary function in cells removing unwanted RNA molecules and is also involved in the maturation of all RNA molecules for the production of proteins. RNase activity has been shown to inhibit protein and gene expression and has been utilised to

examine the loss of function in certain genes in eukaryotic cells. This is known as RNAi silencing and also proved useful in elucidating the function of genes at the tick–pathogen interface (de la Fuente et al., 2007; Zhang et al., 2011). RNase molecules act as an antiviral defence system against viral RNA, causing inactivation and fragmentation of RNA and can lead to inaccurate quantification of viral mRNA transcripts (Goncharova et al., 2011). There are three effective methods of RNA stabilisation; (i), samples can be homogenised immediately after harvesting using a lysis agent containing chaotropic agents; (ii), Samples can be flash frozen in liquid nitrogen or on dry ice and must be stored at -80°C and not allowed to thaw during homogenisation; and (iii), RNAlater stabilisation solution can be added to the samples which must be thin enough for the solution to permeate the sample fully. During RNA recovery samples can also be DNase treated to remove contaminating genomic DNA.

The storage method that produced the highest yield of RNA was that of freshly homogenised samples immediately after harvest, closely followed by those flash frozen on dry ice and stored at -80°C. Storage in RNAlater did not appear to protect RNA from degradation possibly due to incomplete permeation through the whole tick. Direct storage at -80°C produced the lowest yield of RNA and this may be due to cold stress via slow freezing. To ensure consistent processing of all samples, ticks will be flash frozen on dry ice followed by storage at -80°C for further downstream processing.

When performing gene expression analysis it is important to have high quality starting material which will reduce inaccurate quantification and time and cost in repeating results. RNA integrity of tick samples was assessed using Agilent's bioanalyser which determines a RIN number based on measurements of the 18srRNA and 28srRNA peaks and is considered the gold standard. Initial testing of tick samples resulted in low RNA integrity numbers and inclusion of the recommended heat denaturation and DNase digestion did not cause any significant increase. This, according to Agilent, is indicative of degraded RNA. There was a storage dependent decrease in RIN number with those freshly homogenised immediately after harvesting producing the highest closely followed by the flash frozen samples stored at -80°C.

The development of the bioanalyser is based on a library of ribosomal length variations detailed for human, mice, zebrafish, *Drosophila melanogaster*, yeast, bacteria and plant

chloroplasts (2005). Despite the full tick genome of *I. scapularis* being made available, values for arachnids and specifically ticks are not yet documented in the literature or on Agilent's RIN database (http://www.chem.agilent.com/rin/_rinsearch.aspx). Therefore, the Agilent Bioanalyser has not been optimised for use with these samples. If a sample produces a low RIN number it is considered to be too degraded for use especially when looking at gene expression on a microarray. However, it may still provide good RNA template for amplification using real-time RT-PCR which only requires amplification of ~150bp. It is therefore recommended that the scope of the intended study should be taken into account when interpreting such values. As this study requires that amplification of only 135bp of HAZV and 185bp of tick β -actin tick RNA will be stored and treated with DNase digestion.

Sufficient homogenisation is required to ensure optimal recovery of RNA from samples. Optimisation of homogenisation speed and duration will also aid in the recovery of high quantity and quality RNA. Methods routinely used to obtain nucleic acid from whole ticks involve crushing the tick in liquid nitrogen, homogenisation using ceramic or steel beads in a homogeniser or cutting the tick. Homogenisation using 2.8mm ceramic beads within reinforced tubes using the Precellys bead beater was chosen as it causes sufficient homogenisation of the tick cuticle in the presence of a chaotropic agent. This method has minimal contact time between the user and the tick and pathogen before it is lysed. The use of liquid nitrogen poses a risk to the user and use within a microbiological safety cabinet would not be feasible at high biological containment. The cutting of ticks involves the use of sharps, again posing a risk to the user and requires close contact with the tick and the virus. The cutting action may also induce RNase activity via cell stress causing RNA degradation with loss of yield and quality.

A comparison of homogenisation speeds using whole ticks showed that with increased speeds the yield of RNA also increased. The optimal speed of 6200 rpm was then shown to produce the highest yield of RNA at 5 second intervals compared to 10, 15 or 20. High speed homogenisation for a short duration was deemed the most optimal method of extracting tick RNA.

A tick was homogenised with a 500µl aliquot of HAZV at a titre 1.7×10^6 PFU.ml at varying homogenisation speeds. The viral titre was shown to increase as speed increased with a 2.3 fold increase in titre, compared to the stock virus, when homogenised at the optimal speed of 6200rpm for 5 seconds. A potential explanation could be that the virus particles form aggregations and the increased homogenisation is allowing them to separate (Galasso and Sharp, 1965). As there was no detrimental effect to the virus this method of homogenisation was used for all tick homogenisations.

3.5.2 *Development of a Taqman real-time RT-PCR*

Quantitative real-time polymerase chain reaction (qRT-PCR) has become an important research tool for vector-host-pathogen interactions. Despite its common use only three papers have evaluated the use of suitable reference genes for quantification (Nijhof et al, 2010; Browning et al, 2012; Koci et al, 2013). Such data still does not exist for many tick species, including *I. ricinus*.

Three commonly used housekeeping genes were assessed for their specificity to *I. ricinus*. Tick β -actin produced exponential amplification on cycle 24 however, GAPDH produced amplification on cycle 39 and TBP produced no detectable amplification by real-time RT-PCR. GAPDH sequences were downloaded from Genbank for three tick species; *Ixodes scapularis*, *Dermacentor variabilis* and *Rhipicephalus appendiculatus* and appear to be highly species specific in their sequence with 72.2 – 82.02 % identity of the *I.scapularis* sequence to all other species and a conserved area could not be identified.

In these studies tick β -actin was used as an endogenous control and the optimal primer concentration of both forward and reverse primers of 900nM and probe concentration of 625nM gave the most efficient real-time PCR assay targeting tick β -actin. Actin is an important structural component of cells and has a variety of roles including cellular motility, intracellular transport and provides cells with their structure and shape (Kabsch and Vandekerckhove, 1992). β -actin is one of two isoforms found in the cytoplasm and is highly conserved among eukaryotes (Bunnell and Ervasti, 2011) and ticks (da Silva Vaz et al., 2005). Published tick β -actin sequences have been used as either quantification controls or loading controls in many tick studies. These include:

functional gene studies in *I. scapularis* cells and whole *D. variabilis* ticks infected with *Anaplasma marginale* (de la Fuente et al., 2007); as a loading control investigating the migration of *Borrelia burgdorferi* within *I. scapularis* nymphs (Zhang et al., 2011); and as a reference control gene for optimising the conditions of RNAi in tick cell lines (Barry et al., 2013).

To quantify how much viral RNA is present in tick samples, the level of mRNA compared to the levels of tick β -actin was determined using the relative standard curve method. There are two types of relative quantification; the $2^{-\Delta\Delta}$ method and the standard curve method (Arya et al., 2005). The $2^{-\Delta\Delta}$ method uses mathematical equations to calculate relative mRNA expression levels, it reduces the need to run standard curves on every plate therefore reducing cost and time. However, in order for accurate and reliable quantification the real-time amplification efficiencies, determined by the slope of the amplification curves, of both the target and the endogenous control must be within +0.1 and -0.1 of each other (Arya et al., 2005). Analysis of HAZV amplification efficiencies against tick β -actin efficiencies determined that these two amplicons did not have similar efficiencies and the standard curve method will be used for all future mRNA expression studies.

3.5.3 ***Optimisation of the immersion protocol on tick survival***

The current methods used to artificially inoculate ticks with pathogens all add a level of intricacy and technical difficulty especially if applied to hazard group 3 and 4 viruses. It was shown that immersion of *I. ricinus* nymphs is a very successful method for use with Hazara virus. Using real-time RT-PCR viral RNA was detected in 100% of immersed ticks at 13 days post-immersion compared to other studies (Policastro and Schwan, 2003)(Belova et al., 2012; Mitzel et al., 2007; Policastro and Schwan, 2003). Early life stages such as nymphs have an under developed cuticle compared to adults. Nymphs only possess a layer called the epicuticle which is 1 μ m in thickness in contrast to adults which possess an endocuticle and the epicuticle (Hackman, 1982). This may explain why nymphs appear to be more susceptible to infection via the immersion method compared to adults (Belova et al., 2012).

After immersion a mortality rate of 20% was observed in HAZV-immersed ticks. An additional study showed no differences in mortality between HAZV -infected and

mock-infected groups 10 days post-immersion (DPIM) indicating that immersion in virus does not lead to reduced survival. To determine the source of the observed mortality the individual stages of the immersion procedure were investigated. The age of the ticks appeared to significantly affect post-immersion survival ($P=0.0046$, Mantel cox test) with nymphs more than 4 months emerged showing higher survival (70%) than those 5-6 weeks emerged (18.1%). It has been suggested that newly emerged nymphs are unable to regulate their water content as efficiently as older nymphs due to the gut residue not being discharged (Lees, 1946). Mortality is also seen in ticks which have remained in a fasting state for a prolonged period of time and may be due to loss of nutritional stores (Lees, 1946). It has also been demonstrated that damage or even removal of this lipid layer by abrasion or solvents accelerates the rate at which water is lost (Lees, 1946).

Within this study mortality was consistently observed within the first 5 days after immersion, notably on the first and second days during the density dependent studies. This mortality may be representative of those individuals unable to reach their original weight once placed back into the 85 – 95%RH environment. Ticks, notably *I. ricinus* are very susceptible to desiccation with development ceasing at humidities lower than 80% (Lees, 1946). A tick can spend up to 90% of its life cycle off host and in order to survive must be able to regulate its water content. This is achieved via a number of methods; uptake from humid air, absorption of water in contact with the cuticle, active drinking and absorption via a blood meal (Macleod, 1935). The loss of water also occurs via the cuticle and via excretion. In addition to the age and nutritional status of a tick prior to immersion, exposure to high temperatures may compromise the ticks ability to regulate its water content once returned to a controlled environment. Lees (1946)(Lees, 1946) showed that *I. ricinus* ticks maintained at a desiccated temperature of 50°C for two days and then returned to a 95%RH environment were shown to increase their water content by 5-10% per day, reaching their original weight by 2-4 days. When ticks were placed back into 100% saturated air, water uptake was very rapid up to 10-20% water content obtained per day with ticks reaching their original weight in 2-3 days.

A second predisposing factor may be body size, more specifically the surface area to volume ratio. It is known that smaller organisms such as ticks have a high surface to volume ratio meaning the area through which water can be lost is increased relative to the volume of water that can be stored inside the body. Therefore, it is hypothesised that ticks with a smaller mass, therefore body size, would be more susceptible to desiccation and would struggle to regulate their water content. It was observed that not all of the ticks obtained from Charles River Laboratories were the same size and this is due to the heterologous nature of ticks.

The effect of differing post-immersion densities on tick survival was investigated showing ticks in aggregates of 10 having the lowest survival rate (10%) compared to aggregates of 20, 25 and 35. Aggregation is another strategy employed by arthropods to mitigate water loss (Benoit and Denlinger, 2010). Formation of a group has been shown to increase local relative humidity, suppressing water loss for members of the group (Yoder et al., 1992). Bed bugs have been shown to form aggregates in protective harborages near their hosts; as group size increases, metabolic rate drops and water conservation is enhanced. In the initial immersion study, it was noted that ticks were observed aggregating near to the top of the maintenance tubes.

Considering all of the above factors, if a tick is newly emerged or has been in a fasting state for a prolonged period of time and has utilised most of its nutrient supplies and it is then subjected to high temperatures causing changes in cuticle structure and therefore water balance, it may be unable to regulate its water content once returned to a humidity controlled environment. It is inevitable that some mortality will occur but to mitigate this, future ticks will not be used less than 4 months post emergence. Where this is not possible an increase in sample size will be required to account for mortality rates.

3.5.4 **Summary**

Here I have identified the optimal handling, extraction and storage parameters of RNA obtained from *I. ricinus* ticks. To ensure rapid inactivation of endogenous ribonucleases within whole tick samples and to ensure consistent processing of all samples, ticks will be flash frozen on dry ice and stored at -80°C. Samples will be homogenised at high speeds of 6200rpm for short durations of 5 second to obtain

optimal RNA yield and not to negatively impact viral titre. Optimal primer concentrations of 900nM and a probe concentration of 625nM will be used for tick β -actin amplification.

To ensure maximal survival of immersed ticks, ticks will be maintained at densities of 35 where possible, within the same tube after immersion and continued use of a modified lid perforated with multiple air holes. This will reduce the handling and therefore the damage that maybe imposed on the cuticle from use of tweezers and will allow the exchange of humidified air between the tube and the desiccator.

Chapter 4. Immersion of *Ixodes ricinus* nymphs with Hazara and Crimean-Congo haemorrhagic fever virus

4.1 Introduction

The immersion technique has previously been used to infect adult *Ixodes ricinus* ticks with tick-borne encephalitis virus (Belova *et al*, 2012) and *Ixodes scapularis* larvae with Langat virus (Mitzel *et al*, 2007) with variable success. In the previous chapter I have shown the immersion procedure to be very efficient in infecting nymphs with Hazara virus (HAZV) with 100% viral detection on day 13 post-immersion. To determine the susceptibility of *I. ricinus* nymphs to HAZV and therefore the ability to act as a suitable model for future tick – virus studies, the kinetics and dissemination of HAZV within *I. ricinus* will be investigated. The use of immersion at containment level 4 (CL4) will also be investigated immersing *I. ricinus* nymphs in Crimean-Congo haemorrhagic fever virus. Particular attention will be placed on the ability of HAZV to establish an infection within the midgut cells and salivary glands as these are the gateway keepers of viral establishment, replication and dissemination within the tick vector. Artificial inoculation allows us to determine whether important organs are susceptible to infection without the constraints imposed by blood meal digestion. Using artificial inoculation combined with natural feeding, areas of virus localisation and interaction within the tick vector have been identified for a number of tick-borne viruses (Table 4.1).

Table 4.1: Localisation of virus in the known tick vector. SG: Salivary glands, MG: mid-gut, GO: Genes organ, He: Hemolymph. Table adapted from Nuttall *et al*, 1994. * indicates data obtained from artificial inoculation, ** indicates detection after the start of feeding.

Virus	Tick	Localisation	Reference
Crimean-congo haemorrhagic fever*	<i>Hyalomma truncatum</i>	SG	(Dickson and Turell, 1992)
Powassan	<i>Dermacentor andersoni</i>	MG, SG, GO**	(Chernesky and McLean, 1969)
African Swine Fever	<i>Ornithodoros moubata</i>	MG, He	(Greig, 1972; Kleiboeker et al., 1999)
Thogoto	<i>Rhipicephalus appendiculatus</i>	SYG, SG**	(Booth et al., 1989) (Kaufman and Nuttall, 2003)
Dugbe	<i>Amblyomma variegatum</i>	He SG**	(Booth et al., 1991)
Langat*	<i>Ixodes scapularis</i>	MG	(Mitzel et al., 2007)
Tick-borne encephalitis	<i>Ixodes ricinus</i>	SG	(Slovák et al., 2014)

Infections of the midgut and salivary glands are considered key factors in determining vector competence. Pathogens are naturally acquired via a bloodmeal and as a result have to overcome a number of barriers in order to infect the tick vector (Figure 4.1). These are described as; (i) the mid-gut infection barrier, the ability of a virus to enter and replicate within mid-gut epithelial cells; (ii) the mid-gut escape barrier, the ability of progeny virus to exit epithelial cells and disseminate via the haemolymph either to secondary organs for amplification or directly to the salivary glands; (iii) the salivary gland infection barrier and (iv), the salivary gland escape barrier (Hardy et al., 1983).

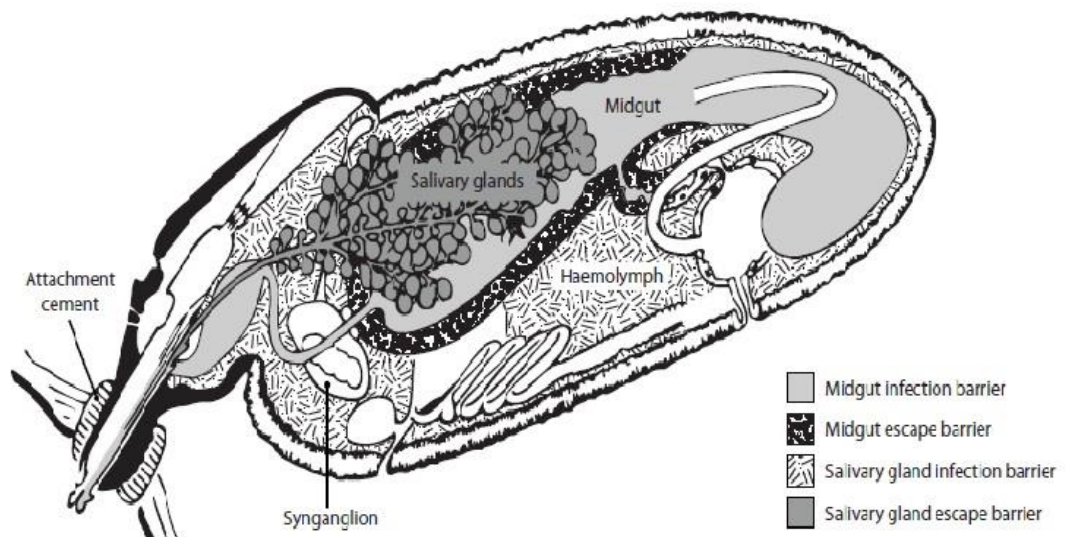


Figure 4.1: Diagrammatic representation of the internal barriers to arbovirus infection and transmission in the tick vector. Image courtesy of Dr Patricia Nuttall.

Pathogens obtained via a blood meal undergo a different route of infection compared to those artificially inoculated. Blood digestion in ticks is a slow intracellular process and once a tick had fed on an infected vertebrate host the blood meal, containing virions and infected cells, can be stored within the gut lumen for long periods of time (Nuttall and Labuda, 2003). This can be advantageous for pathogens as the lumen lacks digestive enzymes allowing intracellular virus particles to replicate, possibly increasing the infective dose. Red blood cells are broken down within the lumen via enzymes, releasing heme and haemoglobin, a process known as hemolysis (Lara *et al*, 2005) with digestive cells taking up the haemoglobin into vesicles via clathrin coated pits via enzyme-mediated endocytosis. These fuse with lysosomes containing proteases released by the endoplasmic reticulum digesting the haemoglobin (Lara *et al*, 2005). Pathogens must be able to withstand this process and the generation of oxidative reactive substances due to the release of heme (Graca-Souza *et a*, 2006) and withstand host derived antimicrobials contained within the ingested host blood (Kopáček *et al*, 2010).

Pathogens must also face the peritrophic membrane; an uneven layer of chitin and glycoprotein lattice structures (Hegedus *et al*, 2009) separating the gut lumen and the epithelial cells (Zhu *et al*, 1991). In *I. ricinus* nymphs the PM is 0.03-0.79µm thick (Zhu

et al., 1991). The presence and development of the PM during the feeding of ticks and the timing of pathogen acquisition from a blood meal may play a part in the ability of pathogens to infect the epithelial cells. During feeding of *I. ricinus* ticks the membrane increases in thickness and can reach 13-16µm in parts (Zhu et al., 1991), decreasing in size during the rapid stages of engorgement but increases again during the first 12 hours after detachment. Interestingly the PM of *I. ricinus* remains intact for at least 11, 30 and 10 days after engorgement in larvae, nymphs and adults (Zhu et al., 1991) and may explain the initial decrease in viral replication immediately after a blood meal (Chernesky et al, 1969). There is no PM in *I. ricinus* unfed larvae, nymphs, adults or fed males and may explain the success of artificial inoculation techniques. The peritrophic membrane acts as a mechanical barrier to infection as seen in the infection of *Ixodes dammini* with *Babesia microti* that develop a specialised organelle, the arrowhead, during metamorphosis within the mid-gut lumen, allowing only those Babesia containing this organelle to pass through the PM (Rudzinska et al., 1983). It has also been shown that some vectors susceptible to infection with viruses have a reduced level of chitin within the PM reducing in the structural integrity of this layer allowing penetration of viruses into the epithelial cells of the gut (Levy et al., 2011). These factors are considered the first barrier of pathogen infection in the tick vector and comprise the 'mid-gut infection barrier', overcoming these is deemed a determinant of vector competence. Some viruses that are able to overcome this barrier such as; Qalyub virus (Miller et al., 1985), Powassan virus (Chernesky and McLean, 1969) and African swine fever virus (Kleiboeker et al., 1999) replicate within the mid-gut cells.

In order for viruses to disseminate within the tick they must be released from the tick mid-gut, overcoming the 'mid-gut release barrier'. This is demonstrated by the susceptibility of *R. appediculatus*, the non-vector tick species, to Dugbe virus when fed on infected blood compared to inoculation of virus into the haemocoel, bypassing the gut. Blood fed ticks were susceptible to infection via a natural blood meal with 71% of ticks testing positive for infectious virus 21 days post engorgement illustrating the establishment and replication of the virus within the tick. The artificial inoculation of Dugbe virus into the haemocoel also resulted in viral replication. After an eclipse phase, the period in which neither intracellular nor extracellular virus particles are detected, lasting for 2-3 days rapid replication occurred with titres reaching $4.4 \log_{10}$

PFU/tick 12 days after engorgement. Upon moulting ticks artificially inoculated carried over viral infection into adults but virus was not detected in adults from blood fed ticks and inoculation of tick homogenate into suckling mice confirmed the absence of virus (Jones *et al*, 1989b), demonstrating the ability of Dugbe virus to establish an infection in the mid-gut via the natural route of infection but the inability to release this from the mid-gut and carry this over to the next developmental stage. Viruses that do overcome this barrier such as Dugbe virus within its natural tick vector; *Amblyomma variegatum* exit the gut without replication and migrate via the hemolymph, and via the hemocytes, to the salivary glands where they localise prior to feeding also seen with Crimean-Congo Haemorrhagic Fever virus and Tick-Borne Encephalitis virus (Table 4.1). Other viruses such as Thogoto virus are only detected in the salivary glands after feeding has commenced (Booth *et al*, 1989).

The salivary glands are an important organ as they are the interface between the tick and the vertebrate host. Tick saliva contains many molecules that are used to modulate the host response to tick infestation such as; vasodilators, platelet aggregation inhibitors, anticoagulants and immunomodulators (Kazimirova *et al*, 2010; Kazimirova *et al*, 2013). Pathogens are able to exploit this biochemical make up to enhance their transmission to a host, known as saliva activated transmission (SAT; Nuttall and Labuda, 2004). This has been shown experimentally by enhanced transmission of virus to uninfected ticks feeding on hosts inoculated with the pathogen plus salivary gland extract (SGE) and was first described for Thogoto virus (Jones *et al*, 1989). Here, ten-fold more *R.appendiculatus* ticks were infected when feeding on guinea pigs inoculated with Thogoto virus and SGE compared to hosts inoculated with virus alone as also seen with TBEV (Labuda *et al*, 1993), *Borellia* (Pechova *et al*, 2002; Horka *et al*, 2009), CCHFV with indirect transmission from infected ticks to co-feeding ticks on the same host (Gordon *et al*, 1993) as well as Bhanja virus (Labuda *et al*, 1997) and Louping ill virus (Jones *et al*, 1997). Tick saliva and their associated compounds play an important part in pathogen transmission and are a key site for virus – vector – host interaction.

4.2 Chapter aims

The aims of this chapter are to determine the susceptibility of *I. ricinus* nymphs to both Hazara and Crimean-Congo haemorrhagic fever virus and to characterise viral infection after artificial inoculation.

4.3 Chapter objectives

The above aims will be addressed via the following objectives;

- Determine the viral kinetics of HAZV and CCHFV within whole infected nymphs
- Determine the dissemination and localisation of virus within *I. ricinus* nymphs

4.4 Methods

4.4.1 *Viral kinetics of HAZV within I. ricinus ticks*

To determine the viral kinetics within infected ticks and to determine when ticks produce infectious virus particles, *I. ricinus* nymphs were immersed in HAZV as described in section 2.3.3 and sampled over a period of 97 days.

One hundred and fifty ticks were divided into 6 tubes at a density of 25 ticks per tube and immersed in 500µl of 1.7×10^6 pfu/ml Hazara virus. One hundred ticks were sampled over the experimental period and the remaining fifty ticks acted as a contingency for the mortality rate observed when ticks are maintained in groups of 25 (3.3.9). Ten ticks were sampled at the following time points; day 0, 5, 10, 13, 20, 30, 40, 50, 63 and 97. Ticks were flash frozen on dry ice for 5 minutes and then stored at -80°C until processed. Whole ticks were homogenised as described in section 2.4.1 and viral RNA extracted using the Qiamp viral RNA min kit (Qiagen). RNA was detected via qRT-PCR, normalised to tick β -actin and calibrated to day 0 as described in section 2.6.4. Viral titre was determined via plaque assay 2.7.2.

4.4.2 *Establishment of in vitro tick work at CL4*

The use of *I. ricinus* nymphs at high containment and inoculation with CCHFV was sought from the Health and Safety Executive and all procedure and protocols were developed in consultation with those performing the *in vitro* CL4 work (the High Containment Microbiology Group).

4.4.3 *Optimisation of the immersion technique for use at CL4*

The immersion technique was first performed within an MSCIII cabinet in a containment level 3 laboratory (CL3) to mimic the working environment at CL4. As a result, modifications were made to the equipment used during the immersion procedure to accommodate the decrease in work space; a mini centrifuge, vortex and a Minilys homogeniser were sourced; the density of ticks was reduced to 5 ticks per tube for ease of visualisation and accountability and blunt nosed tweezers (Agar Scientific) replaced fine point tweezers for manipulation of dead ticks.

4.4.4 *Immersion of I. ricinus ticks with CCHFV*

One hundred and twenty *I. ricinus* nymphs were prepared at CL2; nymphs were divided into 24 2ml O ringed micro-centrifuge tubes (Sarstedt) at a density of 5 ticks per tube and immersed in two groups; group one: tubes 1-12 and group two: tubes 13-24. Tubes were transferred into CL4 and immediately placed on ice for 15 minutes, reducing tick metabolism and mobility. Any tubes with ticks located in the lid were centrifuged for 1 minute at 6000rpm using a micro centrifuge (SLS, UK). Ticks were immersed in 500µl of CCHFV diluted to 1.12×10^6 ffu/ml in L15 tissue culture medium as detailed in section 2.3.6. Acting as a positive control for the immersion procedure, ten ticks were immersed in 500µl of HAZV at a titre of 1.7×10^6 pfu/ml and were sampled on day 20 post-immersion. Immersed tubes were maintained within a clip top box.

Ten ticks, two tubes, were sampled on the following days post-immersion; 0, 10, 20, 30, 40, 50, 62, 70, 80, 90. Ticks were killed by placing tubes on dry ice for 5 minutes. Using blunt nosed forceps, ticks were individually placed into a 2ml Precellys bead tubes (PeqLab) and homogenised in 250µl of buffer RLT as described in section 2.4.2. After homogenisation samples were left to stand in the RLT lysis buffer for 10 minutes before addition of 250µl of 70% ethanol. Samples remained within the CL4 laboratory and were fumigated overnight. Inactivated samples were transferred into a clean 2ml tube at CL3 and RNA was extracted using the RNeasy mini kit at CL2 as described in section 2.4.4. A 500µl aliquot of both the CCHFV and HAZV virus media suspension was frozen at -80°C for back titration.

4.4.5 *Dissemination of HAZV within the whole tick*

Dissemination of HAZV within *I. ricinus* nymphs was determined using immunohistochemistry. Twenty three *I. ricinus* nymphs were immersed in HAZV and sampled on days 0, 13 and 63 post-immersion and prepared for histological processing. Time points were chosen to clarify the route of virus inoculation via the cuticle (day 0), the target organ (s) for HAZV replication (day 13) and the production of infectious virus particles (day 63) prior to infestation on a mouse. Live ticks were placed into individual 2ml starstedt tubes and suspended in a 70% alcohol based hand gel (Tesco, UK) and stored at 4°C until all time points had been sampled. Ticks were sent to Laudier Histology (New York, USA), an established arthropod histologist for histological processing and immunohistochemistry (IHC). Nymphs not exposed to HAZV were used as a negative control for IHC work up.

4.5 **Results**

4.5.1 *Viral kinetics of HAZV within I. ricinus ticks*

The efficacy of the immersion procedure to infect *I. ricinus* nymphs was determined by the detection of viral RNA up to 97 days post-immersion (Figure 4.2). Hazara nucleoprotein (NP) was detected from the day of immersion (day 0) through to 97 days post-immersion. Viral replication was first detected on day 13 with a mean 9.38 fold increase in HAZV RNA compared to day 0. Over the subsequent seven days virus detection decreased to a mean 6.97 fold difference compared to day 0, dropping to a 1.80 fold difference on day 30 compared to day 0. On day 40 a 6 fold increase in viral RNA detection was seen, indicating a secondary replication event. HAZV exhibits a cyclical pattern of viral increase and decrease approximately every 20 days, with the last observed peak on day 63.

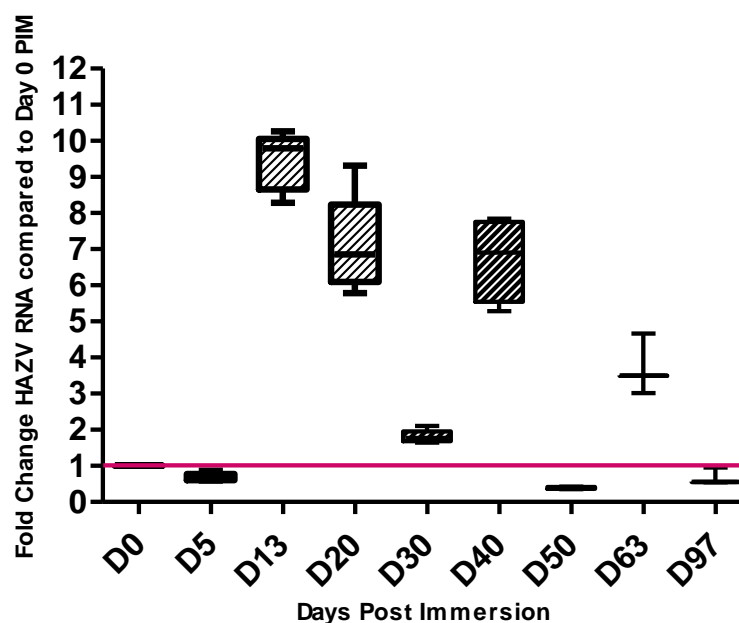


Figure 4.2: Relative fold change of HAZV normalised to tick- β actin endogenous control and calibrated to day 0. Minimum to maximum values are shown. Each time point represents a pool of ten nymphs and 5 replicates performed via real-time PCR. Viral RNA was detected at all time points. The solid pink line indicates a fold change of 1, values above this indicate a fold increase in viral RNA detection relative to day 0 and values below this indicate a fold decrease in viral RNA detection relative to day 0.

Table 4.2: Data represents the mean of 5 replicates at time points 0 – 63 and 3 replicates for day 97. All time points were plotted in the box and whiskers graph (**Figure 4.2**)

Days post-immersion	Mean HAZV NP quantity	Mean Tick β -actin quantity	Mean Normalised ratio of HAZV NP : Tick β -actin	Calibrated mean fold change of HAZV NP to Day 0
0	9.20×10^4	3.45×10^5	0.26622325	1
5	1.18×10^5	6.61×10^5	0.178906014	0.672014986
13	5.64×10^5	2.26×10^5	2.498230088	9.383966594
20	6.02×10^5	3.24×10^5	1.856966708	6.975223632
30	5.67×10^4	1.18×10^5	0.481294941	1.807862163
40	2.52×10^5	1.43×10^5	1.792537005	6.733209822
50	1.59×10^4	1.53×10^5	0.104552753	0.392725852
63	1.26×10^5	1.31×10^5	0.991196923	3.723179406
97	5.3×10^4	2.0×10^5	0.259464939	0.683399457

To determine if infectious virus was being produced, viral titre was estimated from homogenates of ten ticks from each time point. Infectious virus particles were detected on day 63 and persisted until day 97 post-immersion at a titre of 2pfu/tick (Figure 4.3).

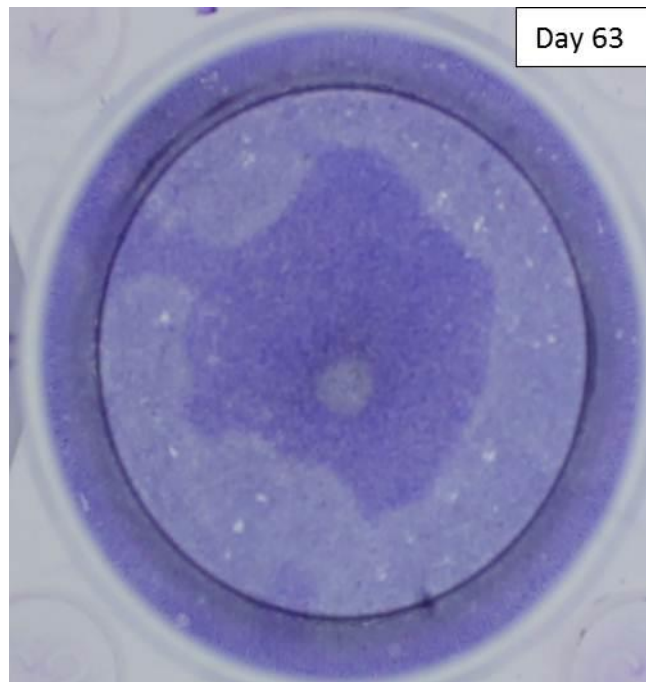


Figure 4.3: Viral titres of neat nymph homogenates 63 days post-immersion. Viral titres of 20pfu/time point equating to 2pfu/tick were obtained.

4.5.2 *Dissemination of HAZV in the whole ticks*

To ascertain where the virus was localised within *I. ricinus* nymphs, ticks were histological sectioned and stained with a polyclonal antibody targeting the Hazara NP on days 0, 13 and 63 post-immersion. Non infected nymphs were used for the work up of IHC to ensure both the primary antibody targeting HAZV NP and the secondary HRP conjugated anti-sheep detector did not produce non-specific binding ensuring no false positives were detected (Figure 4.4 and Figure 4.5). Sagittal sections were obtained from 4 ticks at each time point and a representative image is shown in Figure 4.6- Figure 4.8. All ticks sampled from all groups tested positive for HAZV as indicated by the brown colouration with slides counterstained for contrast using dilute toluidine blue. There was viral dissemination throughout the tick body from day 0 as seen in figure 4.6 with virus detected in key target organs; the midgut and salivary glands. An increase in HAZV intensity within the midgut cells on day 13 (Figure 4.7) correlates with an increase in viral RNA detection via qRT-PCR. The detection of infectious virus particles on day 63 correlates with an increase in viral detection within the midgut and salivary glands (Figure 4.8 and Figure 4.9). Another striking difference over time is the intensity of staining within the sub-cuticular layer as day post-immersion increases

(Figure 4.10 – 4.12). Virus is detected on the epicuticle of ticks on day 0 with virus accumulating at the subcuticular layer with virus infection of dermal cells within the epithelial cell layer already evident on day 0 (Figure 4.10). On day 13 an increase in staining intensity indicative of an increase in viral detection can be seen within both the outer endocuticle and subcuticular layers (Figure 4.11) and by day 63, viral detection has intensified within the tick cuticle and subcuticular layers with detection within the cellular duct of a dermal gland (Figure 4.12). Interestingly there was no viral detection within the pre-oral cavity or the pharynx of immersed ticks on day 0 post-immersion (Figure 4.6, 4.13 and 4.14).

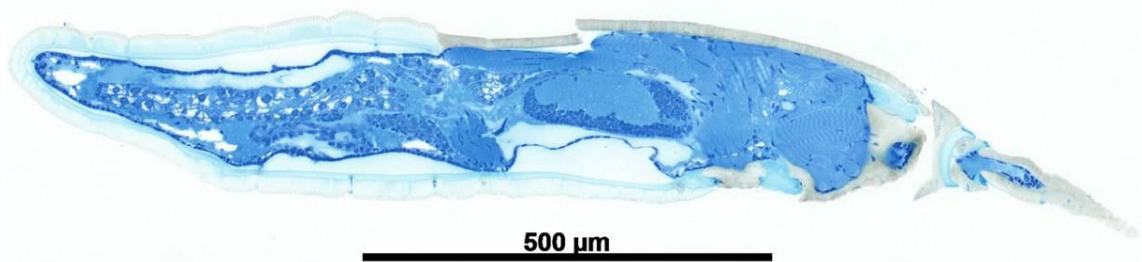


Figure 4.4: Testing of the primary antibody for non-specific binding to tick tissues. An *I. ricinus* nymph not exposed to HAZV was stained as per treatment samples with polyclonal antibody targeting the HAZV nucleoprotein and detector antibody with the HRP conjugated anti-sheep detector added. The primary antibody did not exhibit non-specific binding. Tick sections were visualised under standard bright-field light under 50x magnification.

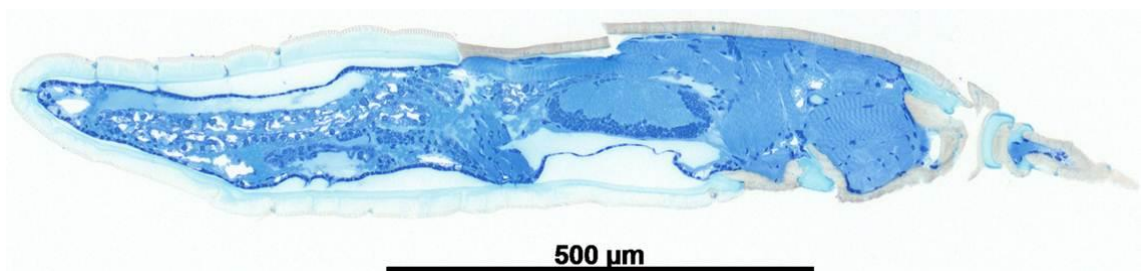


Figure 4.5: Testing of the secondary detector antibody for non-specific binding to tick tissues. An *I. ricinus* nymph not exposed to HAZV was stained with negative sheep serum and stained with the HRP conjugated anti-sheep detector antibody. Non-specific binding was not detected. Tick sections were visualised under standard bright-field light under 50x magnification.

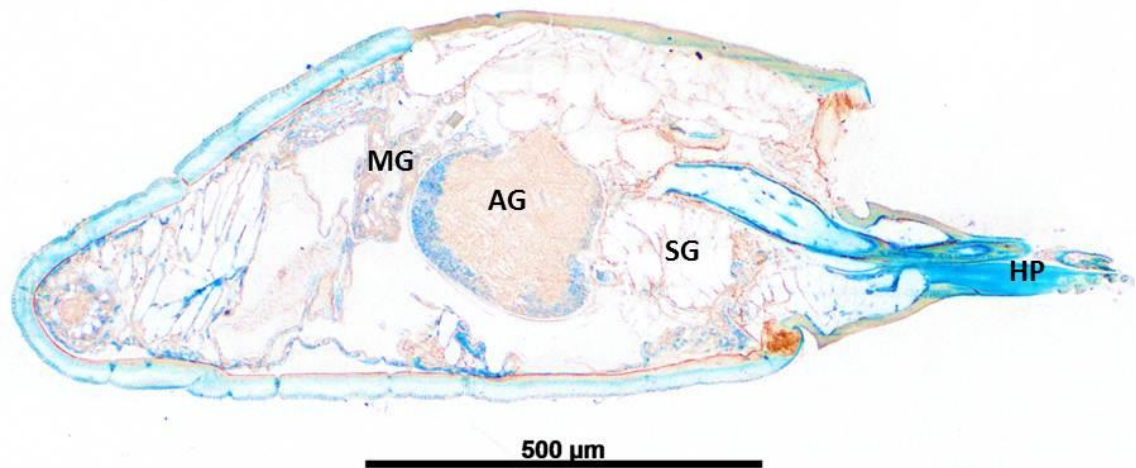


Figure 4.6: A sagittal section of a day 0 post-immersion *I. ricinus* nymph immersed in HAZV. Positive viral detection is shown by staining with a polyclonal antibody targeting the HAZV NP and detected using an anti-sheep horseradish peroxidase secondary antibody. Viral RNA was detected throughout the tick tissues; MG: midgut; SG: salivary glands; AG: accessory gland; HP: hypostome. Tick sections were visualised under standard bright-field light under 50x magnification.

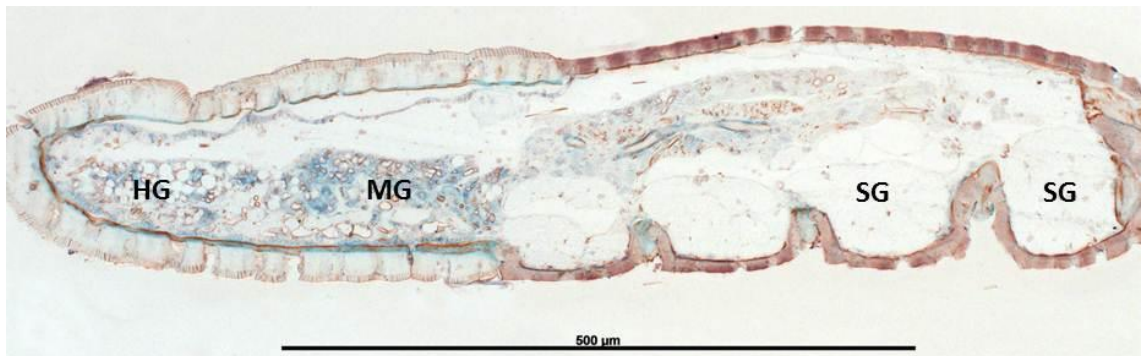


Figure 4.7: A sagittal section of a day 13 post-immersion *I. ricinus* nymph immersed in HAZV. Positive viral detection is shown by staining with a polyclonal antibody targeting the HAZV NP and detected using an anti-sheep horseradish peroxidase secondary antibody. Increased viral RNA intensity was observed within the midgut (MG) and hindgut (HG) tissues. Image was visualised under 50x magnification.

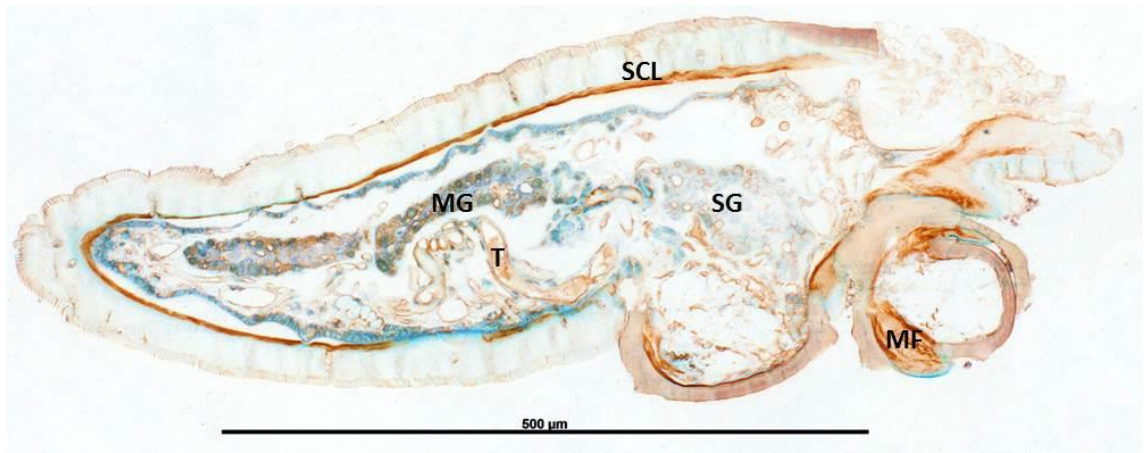
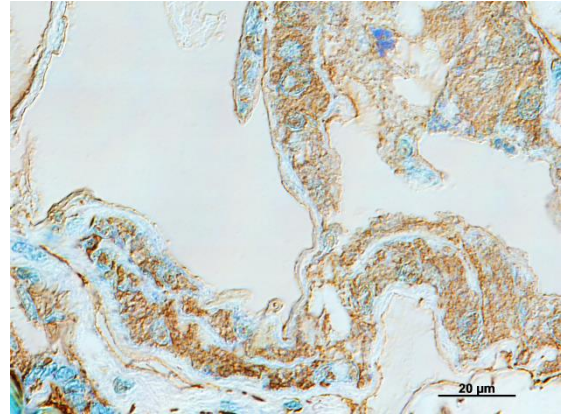
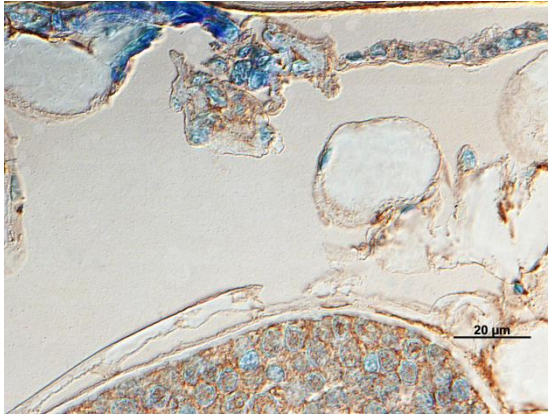


Figure 4.8: A sagittal section of a day 63 post-immersion *I. ricinus* nymph immersed in HAZV. Positive viral detection is shown by staining with a polyclonal antibody targeting the HAZV NP and detected using an anti-sheep horseradish peroxidase secondary antibody. The intensity of viral RNA replication within the subcuticular layer (SCL) between timepoints can clearly be seen. An increase in viral RNA detection within the midgut (MG), salivary glands (SG), trachea (T) and muscle fibres (MF) of the appendages can be seen. Image was visualised under 50x magnification.

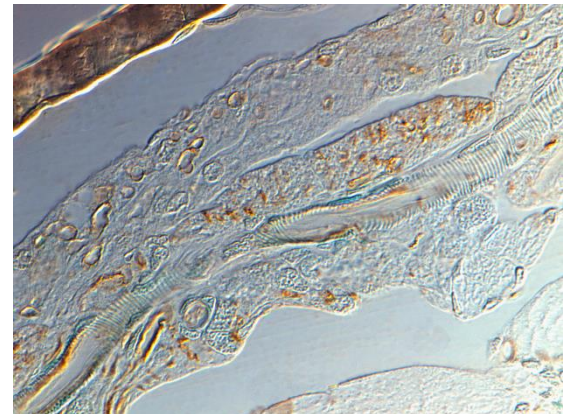
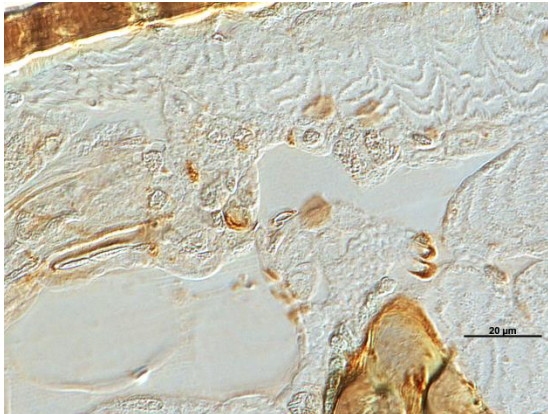
Salivary glands

Midgut

DAY 0



DAY 13



DAY 63

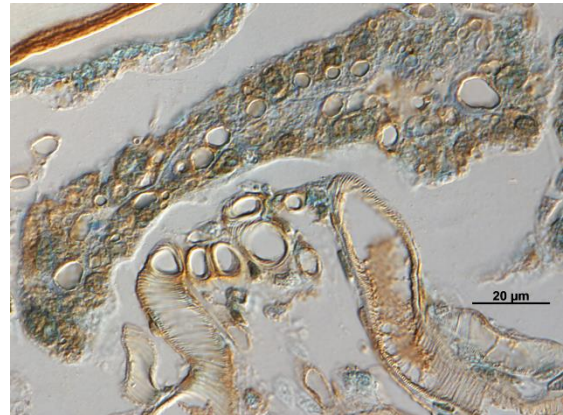
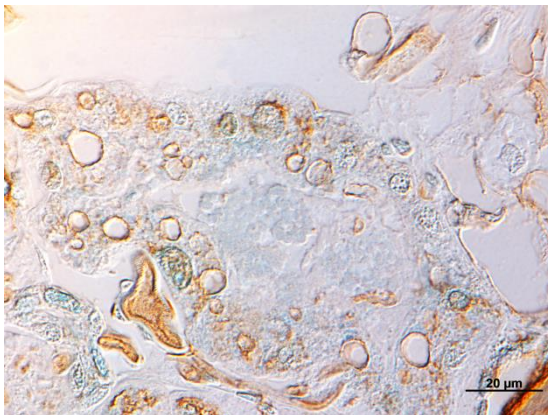


Figure 4.9: Hazara virus detection in the salivary glands (left column) and midgut (right column) of *I. ricinus* nymphs immersed in HAZV. Ticks were sectioned and visualised under polarised light at a magnification of 630x.

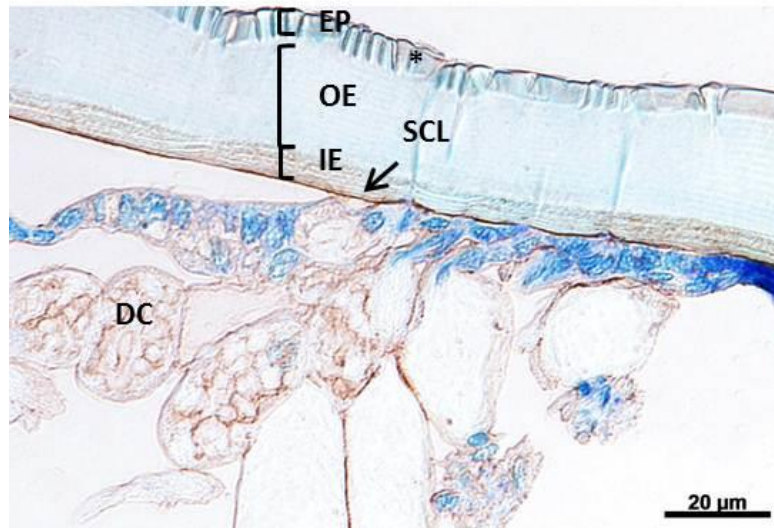


Figure 4.10: Day 0 post-immersion sagittal section of tick cuticle from an *I. ricinus* nymph immersed in HAZV. EP: epicuticle; OE: outer endocuticle; IE: inner endocuticle; SCL: subcuticular layer; DC: dermal cell. Small areas of viral staining (*) can be observed on the tick epicuticle (EP) and within the inner endocuticle with higher intensity staining observed within the subcuticular layer. Dermal cells within the epithelial layer are also seen to contain HAZV. Images were visualised at 400x magnification.

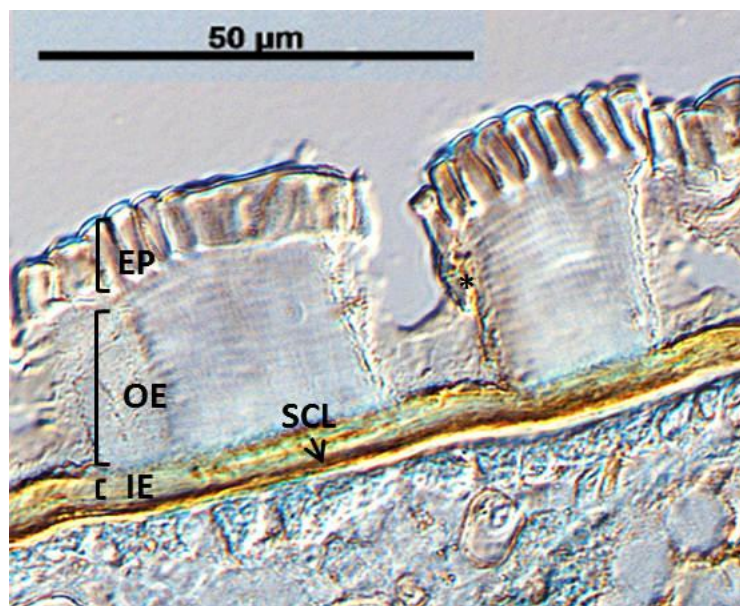


Figure 4.11: DAY 13 post-immersion sagittal section of tick cuticle from an *I. ricinus* nymph immersed in HAZV. EP: epicuticle; OE: outer endocuticle; IE: inner endocuticle; SCL: subcuticular layer. HAZV is detected within the outer endocuticle (*), inner endocuticle and subcuticular layer. An increase in staining intensity indicative of an increase in virus detection can be seen compared to day 0. Images were visualised at 6300x magnification.

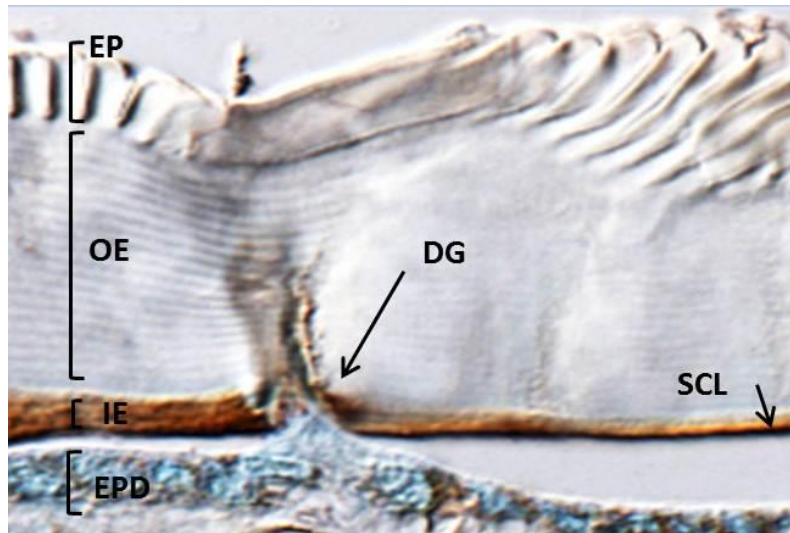


Figure 4.12: Day 63 post-immersion sagittal section of tick cuticle from an *I. ricinus* nymph immersed in HAZV. EP: epicuticle; OE: outer endocuticle; IE: inner endocuticle; SCL: subcuticular layer; EPD: epidermal layer; DG: dermal gland. The highest intensity of viral staining within the tick cuticle is observed with viral detection within the outer endocuticle layer and within the cellular duct of the dermal gland. Images were visualised at 6300x magnification.

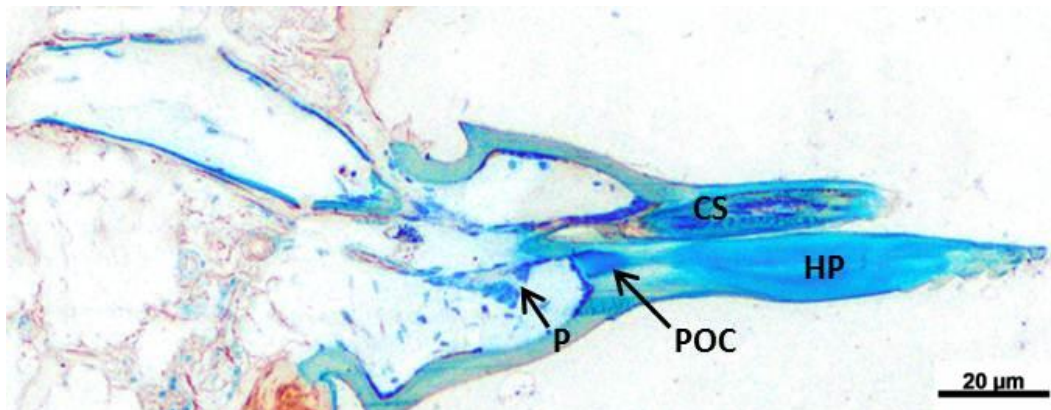


Figure 4.13: Day 0 mouthparts from *I. ricinus* nymph immersed in HAZV. No viral staining was observed within the pre-oral canal (POC), or on the surface of the hypostosome (HP) or within the pharynx (P). The celicerae sheath is also seen (CS). Image was observed at 50x magnification.

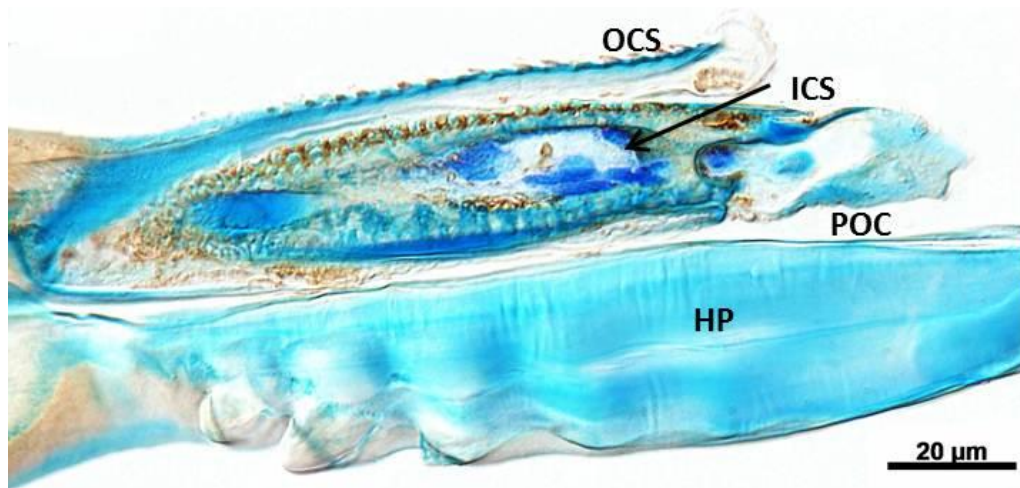


Figure 4.14: Day 0 mouthparts from *I. ricinus* nymph immersed in HAZV. Small amounts of viral staining were observed within the inner (ICS) and outer chelicerae sheath (OCS) but no viral staining was observed within the pre-oral cavity (POC) or on the surface of the tick hypostosome (HP). Image was observed at 400x magnification

4.5.3 *Efficacy of the immersion technique to infect I. ricinus with CCHFV*

Ixodes ricinus nymphs were not susceptible to infection with CCHFV via immersion. All time points were negative for CCHFV RNA and positive for the endogenous control tick β -actin tested via qRT-PCR (Figures 4.15 and 4.16). *I. ricinus* nymphs immersed in HAZV acting as a positive control for the immersion technique were susceptible to HAZV infection with all producing detectable amplification of HAZV nucleoprotein (Figure 4.17).

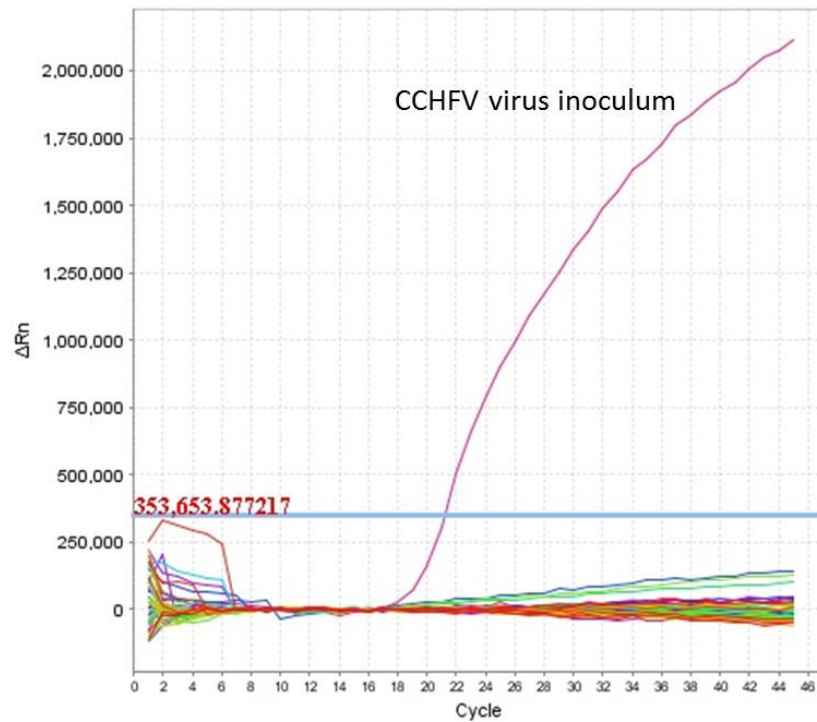


Figure 4.15: Taqman real-time RT-PCR amplification plot targeting CCHFV nucleoprotein (NP) mRNA transcripts within *I. ricinus* nymph homogenates DAY 13 and DAY 63 post-immersion. CCHFV RNA was amplified by Taqman real-time RT-PCR. The fluorescent intensity of FAM at each cycle was measured and normalised to background fluorescent (ΔR_n). The horizontal line indicates the threshold at which the normalised fluorescent intensity rises exponentially. CCHF viral inoculum used to immerse ticks produced detectable Ct value of 21.23. All samples from DAY 13 and DAY 63 did not produce detectable Ct values.

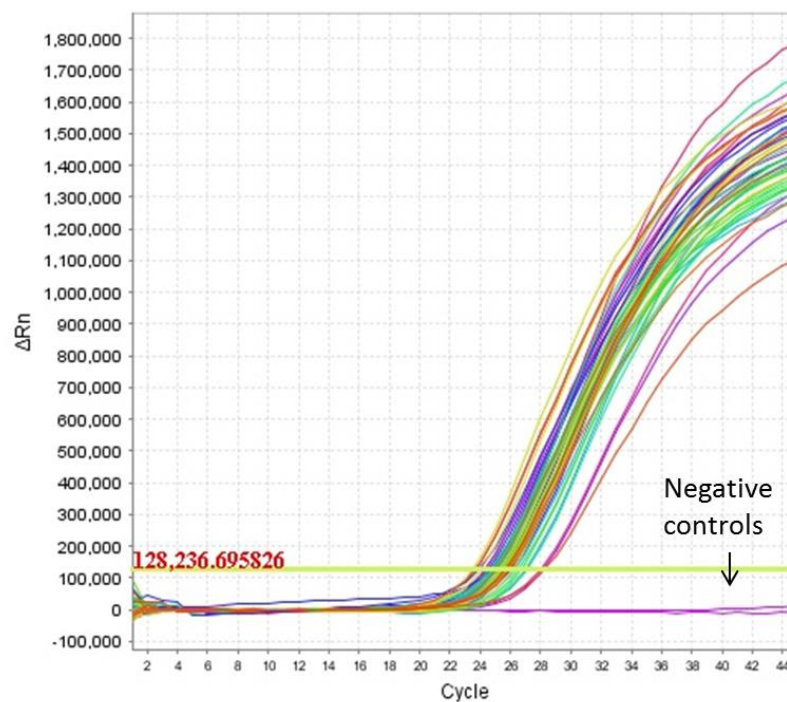


Figure 4.16: Taqman real-time RT-PCR amplification plot targeting tick β -actin mRNA transcripts within *I. ricinus* nymph homogenates DAY 13 and DAY 63 post-immersion. All samples from DAY 13 and DAY 63 produced detectable Ct values for the endogenous control tick β -actin. Negative controls did not produce detectable amplification.

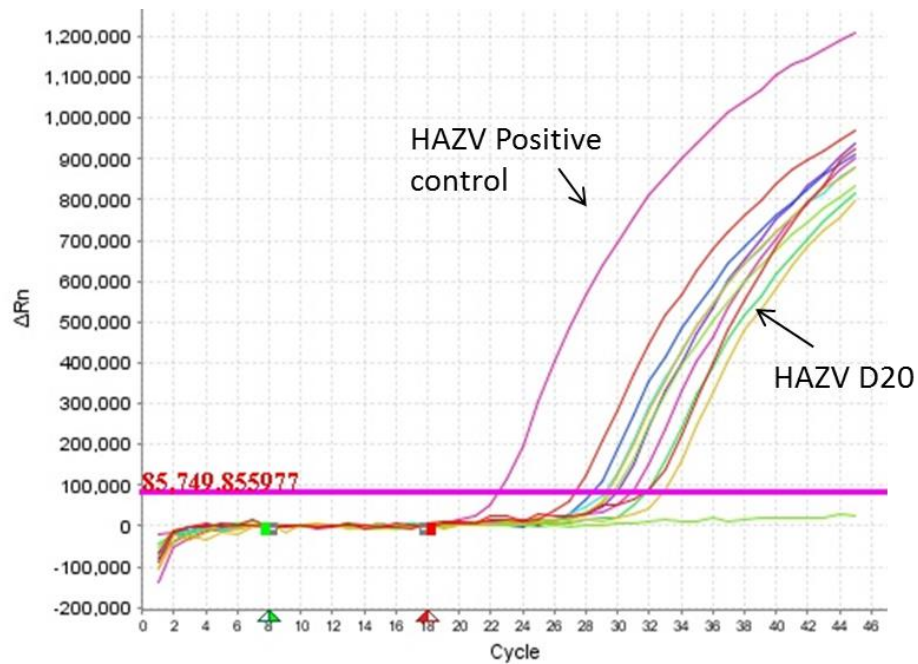


Figure 4.17: Taqman real-time RT-PCR amplification plot targeting HAZV mRNA transcripts within *I. ricinus* nymph homogenates D20 post-immersion (n=10). A representative sample from each tick on D20 is shown. All samples produced detectable amplification and negative controls did not produce detectable amplification.

To enable a direct comparison of HAZV and CCHFV titres used during the immersion procedure, the focus forming unit (FFU) titre of Hazara stock virus was determined. The FFU assay resulted in a two log increase in HAZV titre compared to the plaque assay. Using this displayed increase in titre CCHFV was estimated to have 1×10^4 pfu/ml.

To investigate the effect of the reduced CCHFV Pfu/ml titre on the susceptibility of ticks to the virus, *I. ricinus* nymphs were immersed in HAZV stock diluted to 1×10^4 Pfu/ml. Ticks were individually homogenised and tested via qRT-PCR. All ticks had detectable virus 13 days post-immersion (Figure 4.18).

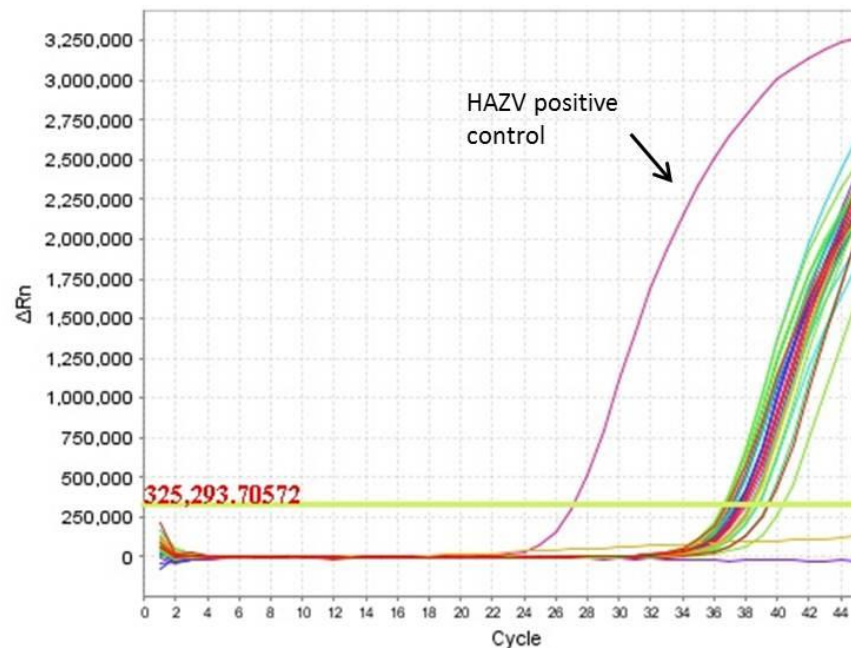


Figure 4.18: Taqman real-time RT-PCR amplification plot targeting HAZV mRNA transcripts from individual *I. ricinus* nymph (n=20) homogenates DAY 13 post-immersion. All samples produced detectable amplification and negative controls did not produce detectable amplification.

4.6 Discussion

The immersion technique is a successful technique for infecting *I. ricinus* nymphs with HAZV with ticks maintaining virus up to 97 days post-immersion. HAZV was able to establish itself, infect key target organs such as the midgut and salivary glands, replicate and produce infectious virus particles 63 days post-immersion.

The kinetics of HAZV within *I. ricinus* nymphs exhibited a cyclic pattern of detection, with three peaks observed on day 13, 40 and 63 post-immersion. This cycling pattern illustrates the survival and dissemination of the virus within the tick vector with the initial un-coating and establishment of virus particles within tick tissues over days 0-5 followed by replication of successfully established virus particles as seen on day 13. The drop in viral RNA detection over days 20 – 30 may be explained by the inability of virus particles to exit tissues in which they have replicated in. Using mutant clones of Venezuelan equine encephalitis virus the effect of viral bottlenecks during viral infection and dissemination within the mosquito vector was shown (Forrester *et al*, 2012). Infection was also influenced by viral titre with high doses of virus resulting in virus readily infecting the midgut but there was a sever reduction in the number of clones at the midgut escape barrier and lower titre viruses inducing a bottleneck at the initial midgut gut infection site. Hazara virus was shown here to increase within the

tick midgut on day 13 followed by an increase in both the midgut and salivary glands on day 63, the time point at which infectious virus was observed. This indicated that the secondary site of viral replication is within the tick salivary glands and this occurs prior to tick feeding unlike CCHFV which only undergoes replication within the salivary glands once feeding had commenced (Dickson *et al*, 1992). An interesting observation was the increase in intensity of virus staining within the tick cuticle. This may be enhancing the detection of viral RNA on day 13 but the reduction in viral RNA on proceeding days may be due to the virus unable to exit the subcuticular layer (SCL). The SCL is comprised of protein microfibrils and chitin (Hackman, 1982) and may be a barrier to infection in itself. The effect of chitin on pathogen establishment is evident during infection of *Ixodes dammini* with *Babesia microti* via the chitinous peritrophic membrane; an uneven layer of chitin and glycoprotein lattice structures (Hegedus *et al.*, 2009) which separates the gut lumen and the epithelial cells (Zhu *et al.*, 1991). In order for *Babesia microti* to replicate within the epithelial cells of the gut they must develop a specialised organelle, the arrowhead, during metamorphosis within the midgut lumen, allowing only those Babesia containing this organelle to pass through the PM (Rudzinska *et al.*, 1983). It has also been shown that some vectors susceptible to infection with viruses have a reduced level of chitin within the PM reducing the structural integrity of this layer allowing penetration of viruses into the epithelial cells of the gut (Levy *et al.*, 2011). Although infectious HAZV was detected in *I. ricinus* ticks this cuticular barrier may reduce the viral population size therefore reducing the RNA detection.

This cyclic pattern of viral detection is also seen in adult *I. ricinus* ticks immersed in tick-borne encephalitis virus (Belova *et al*, 2012) with an initial decrease in virus with viral replication occurring on day 7 and day 20 reaching a two log deficit compared to the virus inoculum titre 20 days post-immersion. This initial decrease followed by an increase in viral titre was also seen in ticks inoculated via the rectal method however, virus was able to exceed initial inoculum titres 20 days post-immersion. These studies have terminated after 20 days and as seen here the cyclic pattern occurs every 20 days, therefore a direct comparison cannot be made. Never the less this cyclic pattern does not appear to be specific to the route of inoculation.

To determine the ability of HAZV to overcome the cuticle barrier and therefore the success of the immersion technique to infect *I. ricinus* and determine the tissue tropism of HAZV, ticks were analysed using immunohistochemistry (IHC) targeting the HAZV NP. The use of IHC was able to clarify the route of inoculation via immersion. It was suggested by Mitzel *et al*, 2007 that the route of inoculation from the immersion technique was via the oral cavity simulating the natural route of infection via a blood meal. It was postulated that the incubation at 37°C dehydrated the ticks encouraging them to drink therefore imbibing the virus-media solution and transporting this along the pharynx and into the midgut. It would therefore be logical to assume that the virus would be present within the oral cavity immediately after immersion however, in this study no virus was detected within the oral cavity on day 0 post-immersion (Figures 4.14 and 4.15). *Ixodes ricinus* ticks are also known not to drink (Kahl *et al*, 1997) but absorb water from saturated air and via the cuticle (Lees *et al*, 1946). When dehydrated *I. ricinus* ticks, of all life stages, were presented with liquid bodies of water with active water uptake shown by an increase in body mass (Kahl *et al*, 1997). Ticks were never found to have their mouthparts inserted into the water nor did they have traces of an illuminant added to the water in their alimentary canals. In this study virus was readily detected within the sub-cuticular layer, within dermal gland cells immediately below the sub-cuticular layer and throughout the body cavity. Heating of the tick above 31°C has been shown to cause irreversible changes to the molecular structure of the wax lipid layer and is thought to increase permeability (Balashov, 1998; Beaumont 1959). Here, ticks were incubated at 37°C increasing the permeability of the tick cuticle. Furthermore, the ingestion of the virus – media suspension through the oral cavity following the route of a blood meal would then be guided directly to the midgut via the pharynx and oesophagus which is a closed system. The virus would then have to overcome the midgut infection and release barriers in order to disseminate throughout the tick. As shown here virus is detected within a number of organs immediately after immersion, this would not be visible if the virus was imbibed via the oral cavity. Mitzel only reports results from dissected midguts up to 15 days post-immersion, therefore a comparison of virus dissemination throughout the whole tick cannot be made.

This study has also shown that the immersion technique can be adapted to a high containment environment enabling investigation of the interaction between the tick vector and highly pathogenic zoonoses of public health importance to be undertaken. This method requires minimal direct contact with infected ticks and the viral inoculum providing a convenient and simple method of infecting ticks. *Ixodes ricinus* ticks were immersed with CCHFV but the virus was unable to establish an infection. During the first use of the immersion technique to infect larvae with Langkat virus, it was shown that the titre of the virus was a determining factor for viral infection (Mitzel *et al*, 2007). It was shown that Langkat virus at a titre lower than 10^5 Pfu/ml would not cause infection within *I. scapularis* larvae. It was shown here that CCHFV was immersed at an equivalent titre of 10^4 Pfu/ml however, a specific dose dependent study would need to be conducted to determine if this was an associated factor. Another possibility is the immersion method itself. When working at high containment it is generally slower than at the regular bench subsequently this resulted in ticks being maintained within 70% ethanol between 2 – 5 minutes which is essential for the surface sterilisation of the ticks. However ethanol has shown to inactivate CCHFV after 2 minutes of contact (Hardestam *et al*, 2007). This coupled with the increase in cuticle permeability may have provided enough time for the ethanol to penetrate down to the inner endocuticle and subcuticular layer inactivating the virus and the subsequent washes removing virus.

This study is the first to demonstrate the use of immersion with *I. ricinus* ticks and those at the nymph stage. Immersion is shown here to be an effective technique for simultaneously infecting a cohort of *I. ricinus* nymphs with a known titre of infectious material within both the CL2 and CL4 environment. These studies have shown that *I. ricinus* ticks are susceptible to infection with HAZV, the virus is able to disseminate to key target organs and produce infectious virus particles. However in order to accurately assess vector competence a virus must be able to overcome the internal infection barriers associated with blood meal digestion. In order to investigate this an *In vivo* feeding model is required.

Chapter 5. Development of a high containment tick *In vivo* transmission model

5.1. Introduction

In vivo models enable the natural interaction between the vector, pathogen and host to be observed and are essential in developing our understanding of medically important arboviruses. Investigation of vector competence of a tick species requires three key criteria to be investigated and met (Kahl et al., 2002); (i) a tick must acquire a pathogen via a natural blood meal when feeding on an infected host; (ii) the pathogen must replicate within the tick and be transmitted horizontally to a naive host and; (iii) vertically to one or more life stages. Investigation of vector competence therefore requires a natural feeding system.

Ticks transmit highly pathogenic viral agents causing severe human disease and as such require strict and stringent handling at the highest containment level; containment level 4 (CL4). Animal models of these tick-borne diseases have been established (Table 5.1) to study the clinical course of disease within the host as well as the suitability of anti-viral drugs (Oestereich et al., 2014) and the efficacy of vaccines (Buttigieg et al., 2014) however, currently in Europe, despite the existence of 8 facilities in 6 different countries and an additional 4 laboratories under construction (Carla Nisii, 2013), there are no small animal tick transmission models for use at CL4, hindering research into the intricate relationship of virus–vector–host. This may be due to the extensive work up required for establishing such models including; the establishment of protocols and procedures for working with vectors particularly at high containment, the training of staff in tick handling, the development of primary on-host tick containment, the development of secondary containment of the host within the high containment environment and optimisation of on-host tick attachment and feeding parameters. Establishing such a model must initially be conducted at lower levels of containment to ensure the robustness and functionality of all procedures and protocols. As a containment-level 2 (CL2) pathogen, HAZV is an ideal pathogen to utilise in the development and optimisation of a tick feeding and transmission model for use within the high containment setting. Using type I interferon deficient mice, the susceptible animal model for both Hazara (Dowall et al., 2012) and Crimean-Congo haemorrhagic

fever virus (Berezky et al., 2010) an *In vivo* tick feeding and transmission model was developed.

Table 5.1: Tick-borne haemorrhagic fever viruses causing human disease and classified for handling at containment level 4 (CL4) by the Centre for Disease Control (CDC, USA), and the Advisory Committee of Dangerous Pathogens (ACDP, UK). *Alkhumra virus is classified as a CL3 pathogen by the ACDP but is not listed within the CDC guidelines and is handled within the USA according to the classification of the closely related Kyasanur forest disease virus. The known or implicated main tick vector is listed as is the established animal model of disease for each virus.

Virus	BSL / ACDP classification	Tick vector	Animal model of disease	Reference
Crimean-congo haemorrhagic fever virus	4	<i>Hyalomma marginatum</i>	STAT1 KO Mouse model, IFN KO mouse model	(Bente et al., 2010; Berezky et al., 2010; Buttigieg et al., 2014)
Kyasanur Forest disease virus	4	<i>Haemaphysalis species</i>	None established	(Varma et al., 1960)
Alkhumra hemorrhagic fever virus	4 / 3	<i>Ornithodoros species</i>	Mouse	(Sawatsky et al., 2014)
Omsk hemorrhagic fever virus	4	<i>Dermacentor marginatus /D. reticulatus</i>	Mouse	(Holbrook et al., 2005)

Ixodes ricinus is a generalist feeder with over 300 host species recorded (Anderson, 1991). In Europe, rodents play an important role as hosts for *I. ricinus* immature stages; larvae and nymphs (Algimantas Paulauskas, 2009; Gray et al., 1999; Matuschka et al., 1991; Wodecka et al., 2014) and are reservoirs for tick-borne microorganisms such as *Borrelia Burgdorferi* (Humair et al., 1993) and tick-borne encephalitis virus (Randolph et al., 1999). In the laboratory mice have been used for the rearing of all life stages of ticks using a variety of primary containment devices including; adult females attached to mice via plastic cut-coned chambers which are shown to feed 1.5 times faster on mice than on guinea pigs, oviposit 1.6-1.7 days earlier and laid 1.5-1.6 times more eggs (Šimo, 2004); larvae and nymphs attached via gauze-covered neoprene chambers to mice infected with West Nile virus (Lawrie et al., 2004) and; BALB/c mice have been used to investigate the vector competence of *I. ricinus* to *Bartonella* species attached via a plastic cap (Reis et al., 2011). Ticks infested onto larger hosts such as rabbits and guinea pigs have used orthopaedic stockinette to contain ticks (Logan et al., 1990) and open ended aluminium tubes (Gonzalez et al., 1992) and a recent

publication has used plastic tubes to attach ticks to mice, rabbits and guinea pigs (Gargili et al., 2013). In this study, modified plastic tubes and neoprene patches will be trialled as primary on host containment devices for use with A129 IFN- α/β R-/- mice.

5.1.2. Chapter aims

The aim of this chapter is to develop an *In vivo* tick feeding model for use at high containment to investigate tick vector competence.

5.1.3. Chapter objectives

The above aims will be addressed by the following objectives;

- To develop & optimise on host tick primary containment
- To evaluate & optimise *I. ricinus* attachment and feeding rates on mice
- To determine the required viral titre of Hazara virus (HAZV) that will induce detectable viremia within host blood and ensure maximal survival of the host

5.2. Methods

5.2.1. Implementation of a tick *In vivo* feeding model

Inclusion of the use of ticks and laboratory animals as tick hosts was sought from the regulatory body within the UK; the Home Office under the Animals Scientific Procedures Act 1986. Amendments to current animal licences were made and all work was reviewed by an internal ethics board. All study plans were designed after consultation with those performing the animal work (the Biological Investigations Group) and authorised by the Health & Safety Executive.

5.2.2. *In vivo* study design and procedures

All *In vivo* studies were conducted by animal licence holders within the Biological Investigations Group (BIG) at Public Health England, Porton Down.

5.2.3. Maintenance and monitoring of mice

All experiments, unless stated otherwise, were conducted within a flexible film isolator. Animals were individually housed within cages with food and water *ad libitum*. Unless stated otherwise, mice were weighed and temperature checked once and health scored twice daily as per table 5.2.

Table 5.2: Health score criteria used throughout all animal studies.

Health Score	Criteria
0	Healthy
1	Ruffled fur
2	Lethargy
3	Rapid Breathing
4	Immobile
5	Dead

5.2.4. Assessment criteria for on-host primary containment

Throughout all studies devices were assessed via four criteria; durability, retention, functionality and on-host effects. Durability was determined via a visual scoring system as shown in Table 5.3. Retention capability was determined using 1.4mm ceramic beads in lieu of ticks and noting their presence within devices. Functionality for both the operator and the host was determined by handling of devices, observation of animal behaviour and health monitoring of animals.

Table 5.3: Durability scoring criteria for monitoring both tube and patch attachment devices.

Durability Score	Criteria
1	Substantial detachment and / or wear and tear of device
2	Moderate detachment and / or wear and tear of device
3	Slight detachment and / or wear and tear of device
4	Full attachment no wear and tear of device

5.2.5. Preliminary assessment of on-host tick primary containment

Two attachment devices; tubes and patches, were assessed for their suitability as on-host primary containment. Attachment tubes were made from 1.5ml screw top tubes (Sarstedt, Germany) cut to 2cm in length with latex (RS components, UK) attached to the base (Figure 5.1A). Patches 1cm X 2cm, were made from 5mm thick neoprene (Scandura UK) and sealed with 500 micron mesh (Clarcor UK) using Loctite superglue (Figure 5.1B). Attachment devices were fixed onto the mouse skin using Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate).

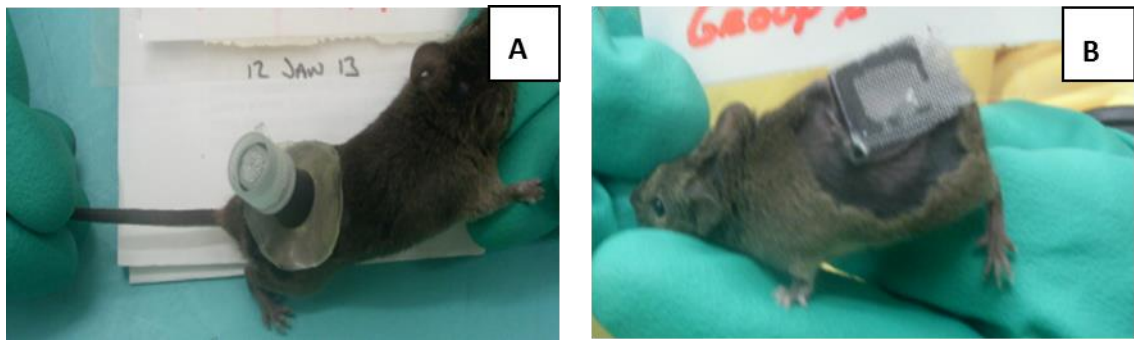


Figure 5.1: Attachment devices assessed for their suitability as on-host primary containment. A. Screw top 1.5ml tubes were attached to the mice via gluing the surrounding latex support directly to the dorsum. B The patches were glued to the dorsum of the mice and sealed with a mesh top using Loctite Superglue.

Nine adult (6-10 weeks) wild type 129 Sv/Ev mice (B&K Universals) were allocated into three groups: group 1, tube attachments (n=3); group 2, patch attachments (n=3); and group 3, negative controls comprising mice with no tick attachment (n=3). Animals were anaesthetised with 0.1ml Alfaxan via the intra peritoneal route, ID chipped (Identichip, Animal Care LTD, York), subdermal temperature transponders (Bio-Thermo) implanted and their dorsum shaved. Tubes and patches were attached (Figure 5.1) and once secured ten 1.4 mm ceramic beads (Roche UK) were added and animals weighed. Animals and attachment devices were monitored for a period of eight days.

5.2.6. Optimisation of on-host tick primary containment

To modify attachment devices for further use in experimental studies in-house modifications involved; incorporating a magnifying insert into the lid of the tube to increase bead visualisation (Figure 5.2 A) and adding air holes 1mm in diameter, drilled into the rim of the lid to reduce condensation formation (Figure 5.2 B). Changes to the *In vivo* study design included removal of ID and temperature chips as animals were housed individually and temperature is not affected by attachment device. The attachment location was altered to the upper back / scruff of the neck to replicate tick attachment and feeding in nature and a different adhesive was used (Copydex instead of Vetbond).

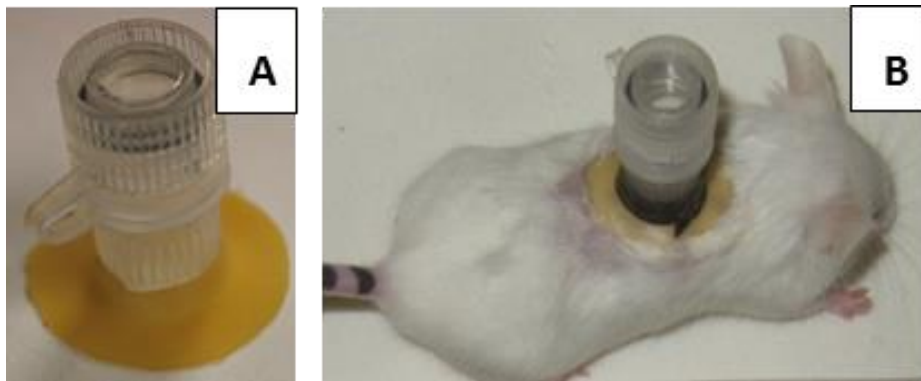


Figure 5.2: A. modified tube with magnification insert in lid and multiple air holes around the rim. B. attachment of tube to the upper dorsum of mouse

The evaluation of modified tubes was performed outside of a flexible-film isolator. Five adult AJ mice (Harlan, UK) were allocated into two groups; group one, tube attachments (n=3) and group 2, no attachment devices with animals acting as weight controls (n=2). To allow habituation of mice to the devices, ceramic beads were added two days post tube attachment. Animals and devices were monitored over a period of nine days.

5.2.7. Optimisation of adhesives

A visit to the Institute of Zoology, Slovak Academy of Sciences (Bratislava), was undertaken to observe tick handling and application onto BALB/c mice where all developmental stages of *I. ricinus* ticks were applied into neoprene patches. A distinct difference, however, was use of closed cell flexible neoprene foam in comparison to the industrial grade neoprene previously used. The patch was attached using copydex glue and the surrounding fur used to anchor the patch to the mouse. Once back in the UK a supplier was found and patches produced from this flexible foam. The ability of Vetbond™ tissue adhesive currently used for device attachment and the newly acquired Copydex was assessed in their ability to attach devices to the host skin as was Copydex and Loctite super glue in their ability to secure mesh to the patch.

The dorsum area of two culled AJ mice were shaved. One patch was attached using Vetbond™ and the other using Copydex. The patches were allowed to dry for approximately 10 minutes and assessed for security and flexibility by manual manipulation of the mouse skin. The mesh was then glued to each patch using either

Copydex or Loctite Superglue, allowed to dry and removed and replaced assessing security.

5.2.8. Optimisation of on-host tick primary containment: patches

Newly acquired neoprene foam was assessed for use as on host primary containment. Patches of approximately 1cm² were cut using a surgical scalpel blade (N^o 11, Swann Moiton, UK) and uniform 6mm diameter tick attachment holes were made using a leather puncher (Amtech UK). Patches were attached to the upper back / scruff of the neck using copydex glue with only the area directly under the patch shaved. The patch was anchored in place by gluing the surrounding fur to the side of the patch (Figure 5.3).

To account for the small size of transgenic mice to be used in future tick attachment studies, six baby wildtype 129 Sv/Ev mice were used and divided into two groups; group 1 patch attachments (n=3) and group 2 control animals with no attachment device (n=3). Ceramic beads were added to each patch one day post-attachment and assessed regularly for their retention capability.

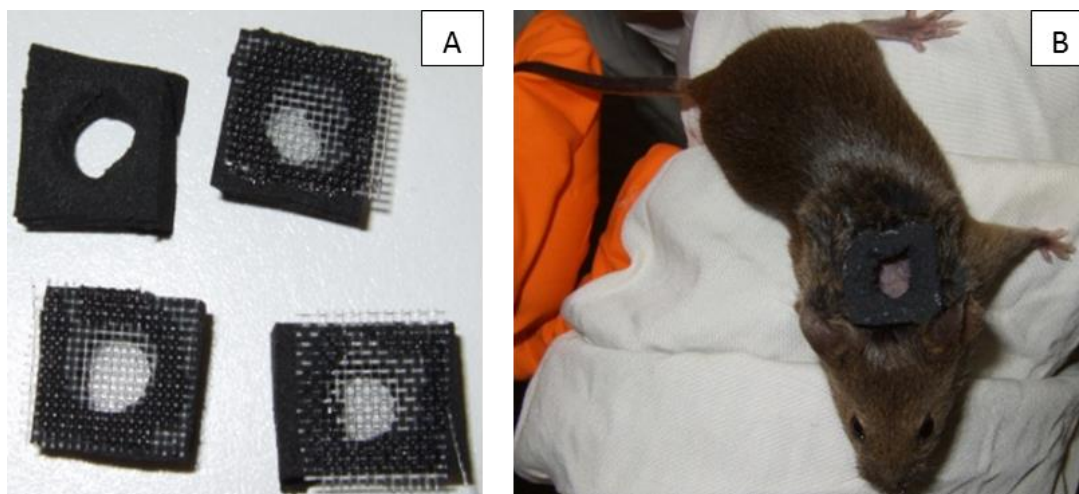


Figure 5.3: A Neoprene patches with a 6mm diameter attachment site. B Patches glued to the upper back of mouse using Copydex glue and anchored by surrounding fur.

5.2.9. Attachment of *I. ricinus* nymphs to A129 IFN- α/β R^{-/-} mice

To determine if A129 IFN- α/β R^{-/-} mice, the susceptible model for my arboviral infections, were suitable hosts for *I. ricinus* nymphs a preliminary tick attachment study was undertaken. In addition, the logistical handling of ticks within a flexible-film isolator was assessed.

Accountability and visualisation are essential requirements when handling ticks and preparation of the isolator and mouse housing was required. The working area of the isolator was covered in white paper with all host and tick manipulations conducted in this area, double sided tape was placed around the top rim of mouse boxes and over all openings with boxes placed into 1cm of vegetable oil, and housed within a flexible film half suit isolator providing a layered barrier effect (Figures 5.4 A, B, C and D).

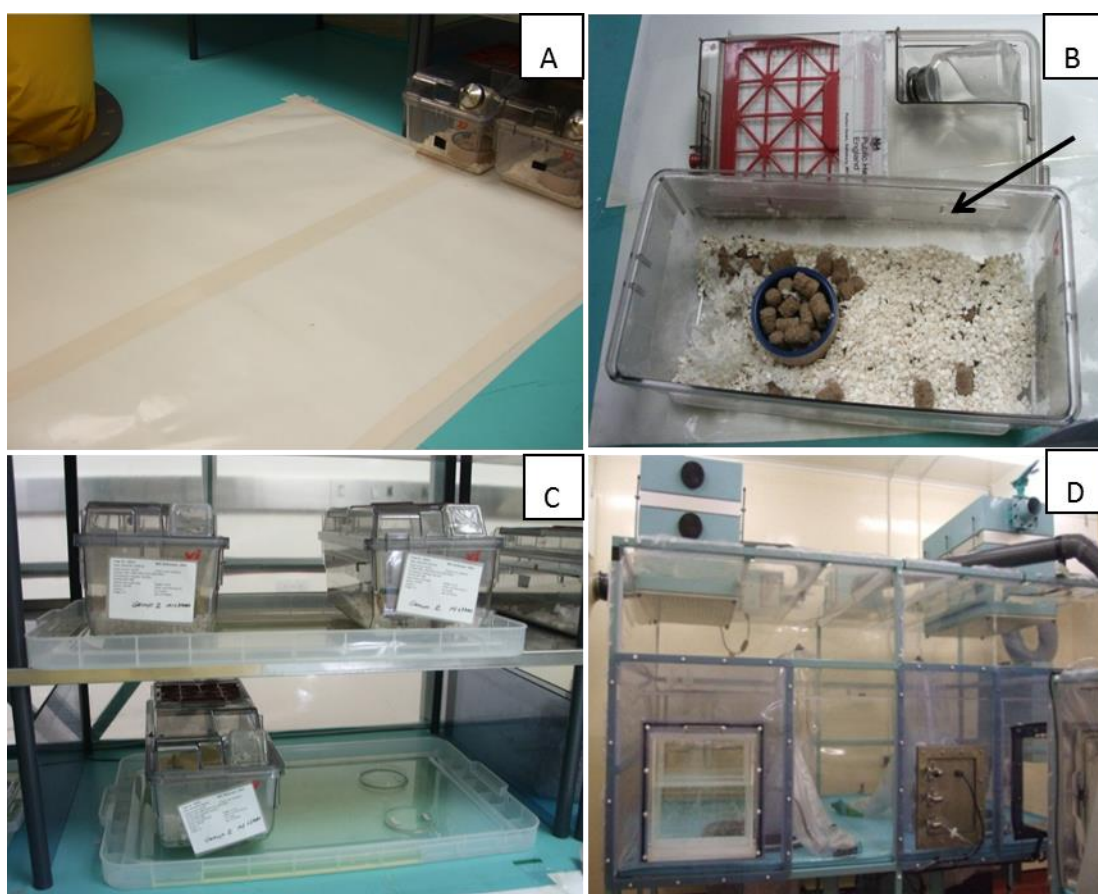


Figure 5.4: Arrangement of apparatus for *In vivo* tick attachment studies; A White paper covering the surface within the flexible-film isolator; B Mouse box containing double sided sticky tape along the inside top rim (arrow); C Mouse boxes suspended within 1cm vegetable oil and; D housed within a flexible-film isolator.

Twelve 6-8 week old A129 IFN- α/β R^{-/-} mice were divided into 2 groups both were anaesthetised with 0.1ml Alfaxan; group 1 (n=8) mice were infested with 3 *I. ricinus* nymphs and group 2 mice (n=4) acted as negative controls with no infestation of ticks and only patches added.

Ticks were aliquoted into tubes for each mouse resulting in the operator only coming into contact with a maximum of three ticks at any one time. Tick tubes were maintained on ice within the isolator prior to infestation to reduce their motility. Ticks

were applied using tweezers and patches sealed with 500micron mesh using loctite super glue and ticks observed for 7 days.

The attachment and feeding success was determined by daily observation and the removal of mesh on days 3 and 7 post tick infestation. Ticks were either recorded as unfed & detached, attached, fed & detached or dead. On days 3 and 7 fed & detached and dead ticks were placed into a cryovial, flash frozen and stored at -80°C. Patches were resealed on day 3 and discarded on day 7. Mouse weight was observed once daily and health status scored twice daily.

5.2.10. Optimisation of tick application methodology

Four approaches were trialled to apply a higher number of ticks (up to 10) simultaneously onto a host; (i) syringes: the plunger was removed and ten ticks placed into the barrel and the plunger returned utilising the air pressure to push the ticks through the syringe, (ii) utilisation of water surface tension: ticks were placed into water, rendering the ticks immobile, and applied to the patch, (iii) tick slide: a drinking straw was adapted by firstly cutting the straw in half, the end was then cut at an angle and rounded at one end. A water droplet was placed into the lumen of the straw and ticks suspended within it. Utilising the waxy surface of the straw, the water droplet was encouraged to slide down and deposit the water and the ticks into the patch, (iv) water droplet within the patch: a 20-30µl water droplet was placed into the patch attached to the host. Ticks were added one by one into the water droplet, again utilising the surface tension to render the ticks immobile. The patch was sealed with 500micron mesh and the water droplet absorbed out using tissue (Figure 5.5).

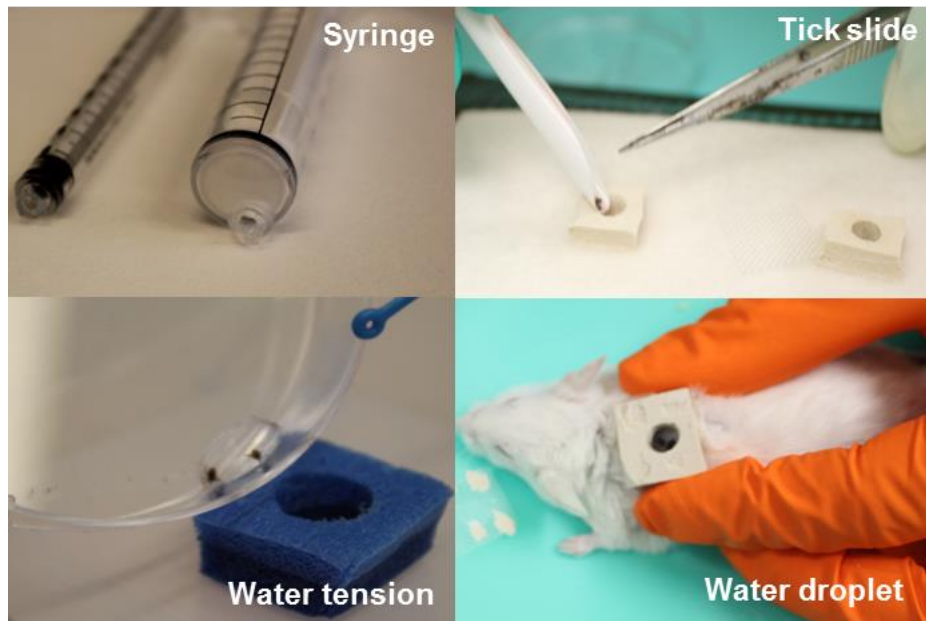


Figure 5.5: Tick attachment methods

5.2.11. Determination of infestation density on the attachment and feeding success of *I. ricinus* nymphs on A129 IFN- α/β R^{-/-} mice

The attachment and feeding rates of *I. ricinus* nymphs were assessed at varying infestation densities and measured in terms of (i) the number of ticks successfully attached to mice, (ii) the number of ticks successfully fed to repletion, (iii) the average weight of engorged ticks and (iv) the number of engorged nymphs moulting to the adult stage.

Nine wildtype 129 Sv/Ev mice were randomly allocated into three groups (n=3 per group) according to tick infestation density; group 1, 5 ticks per mouse: group 2, 8 ticks per mouse and group 3, 10 ticks per mouse. Mice were anaesthetised with 0.1ml of Alfaxan via the intraperitoneal route. The upper back of each mouse was shaved and a neoprene patch 1cm² was attached using Copydex glue. Ticks were attached by applying 20-30 μ l of water into the patch and ticks added using tweezers, utilising the surface tension to immobilise the ticks. A 500micron piece of mesh was glued to the patch using Loctite super glue and the water absorbed out using a piece of tissue.

The attachment and feeding success was determined by opening the patch on days 3 and 5 post infestation and on day 8 at the end of the study. Ticks were either recorded as attached, fed & detached or dead. Fed and detached ticks were removed and placed into individual 2ml screw top tubes (Sarstedt) with modified lids and maintained as

described in section (2.3.4). Dead ticks were placed into a cryovial and snap frozen on dry ice and stored at -80°C. The patch was re-sealed with a new piece of mesh.

The weight of engorged ticks and moulting success was determined by individually weighing detached ticks to the nearest 0.1mg. Ticks were individually placed into modified tubes, maintained as previously described (2.3.4), and allowed to moult to the adult stage. The time taken to moult, the resulting weight and gender were recorded.

5.2.12. Investigation of HAZV viremia levels in A129 IFN- α/β R^{-/-} mice

To provide ticks with the optimal chance of acquiring virus from an infected host it was essential to ensure that there was circulating virus within host blood. Fifty-one 6-10 week old adult A129 IFN- α/β R^{-/-} mice were fitted with ID chips (Identichip, Animal Care Ltd, York) and sub-dermal temperature transponders (Bio-Thermo) three days pre infection. Mice were divided into three groups and intradermally inoculated with 100 μ l of the challenge material; group one mice inoculated with 20 pfu/mouse (n=21), group two mice inoculated with 2 pfu/mouse (n=21) and group 3 mice inoculated with 100 μ l of phosphate buffered saline (PBS) solution (n=9). On days 1, 3, 5, 7 and 9 three mice from group 1 and group 2 were euthanased with blood and serum samples obtained for molecular and virological analysis. To determine viremia levels 100 μ l of whole blood was placed into a dry tube for plaque analysis, 200 μ l of whole blood was placed into 520 μ l of RNA later for future molecular processing and excess blood was placed into a serum separation tube for plaque assay as detailed in sections 2.6.4, 2.9.1 and 2.9.2. Samples were frozen at -80°C to ensure consistency of sample treatment and processed together at the end of the study.

5.3. Results

5.3.1. Comparison of tube and patch containment devices

Tubes and patch devices were assessed for their suitability as on-host primary containment of ticks with the durability, retention, functionality and on-host effects determined. Of particular importance was the weight of the host harbouring these devices; as *I. ricinus* nymphs will be engorging on mice it was essential to ensure that the attachment devices did not have an adverse effect themselves. Specific attention was paid from day 3 to day 5 post-attachment, the feeding range of *I. ricinus* nymphs.

Weight change in all mice was observed throughout the study (Figure 5.6) with a significant difference observed between each device and the control (Kruskall wallis, $P=0.001$) but not between the devices themselves (Kruskall wallis & Dunns multiple comparison test). An initial mean decrease in weight was observed between days 1 and 3PA for both tube (0.97%) and patch (2.15%) attachment devices (Figure 5.6) with a further decrease over the feeding period of *I. ricinus* nymphs for both tube (1.4%) and patch (5.8%) attachments. Throughout the study patch devices had a more marked decrease in weight notably over the known feeding period of *I. ricinus* nymphs.

A distinct difference in durability and retention between both devices was observed. All tubes remained attached to mice and retained all ceramic beads for the duration of the study. Slight detachment of the latex was observed on day 1 post-attachment (Figure 5.7) but all beads were retained. Tubes were able to withstand the normal behaviour of the mouse and interaction with the surrounding environment with no damage to the tube observed. Patches were less durable with only 1 out of 3 remaining moderately attached at the end of the study. Slight detachment was seen from day 0 post-attachment (Figure 5.7) with substantial detachments observed on day 2. It became apparent, from the partly eaten mesh, that the mice could reach the devices (Figure 5.7) and days 4 and 5 saw the complete detachment of two out of three patches with the loss of all beads, coinciding with the mean increase in weight.

Patches, with their large surface area, did provide good visualisation of contained beads. Due to their large surface area patches were also easy to handle and manipulate within the half-suit isolator. Tubes, however, resulted in impaired visualisation due to their depth and lack of light and condensation was noted within each tube. The height of tubes did enable a secure grip during opening and closing, which was necessary to ensure the latex did not twist with the opening of the tube and cause dislodgement.

Animal observations identified a clear difference in host behaviour between mice harbouring tubes and those with patches. Mice harbouring tubes exhibited normal movement and exploratory behaviour with flexibility and movement of the skin. Mice with patch attachments however, showed little movement and exploratory behaviour and were observed resting within their cages. At the end of the study it was observed

that the patches induced poor flexibility and restricted movement of the skin.

Increased vocalisations in both groups were noted after initial device attachment but these reduced throughout the study and during twice daily observations all animals scored normal for the study duration.

Due to their reduced effect on host weight, high durability and retention and ability to withstand the natural behaviour of the host, tubes were carried forward and modified to address the functionality issues described.

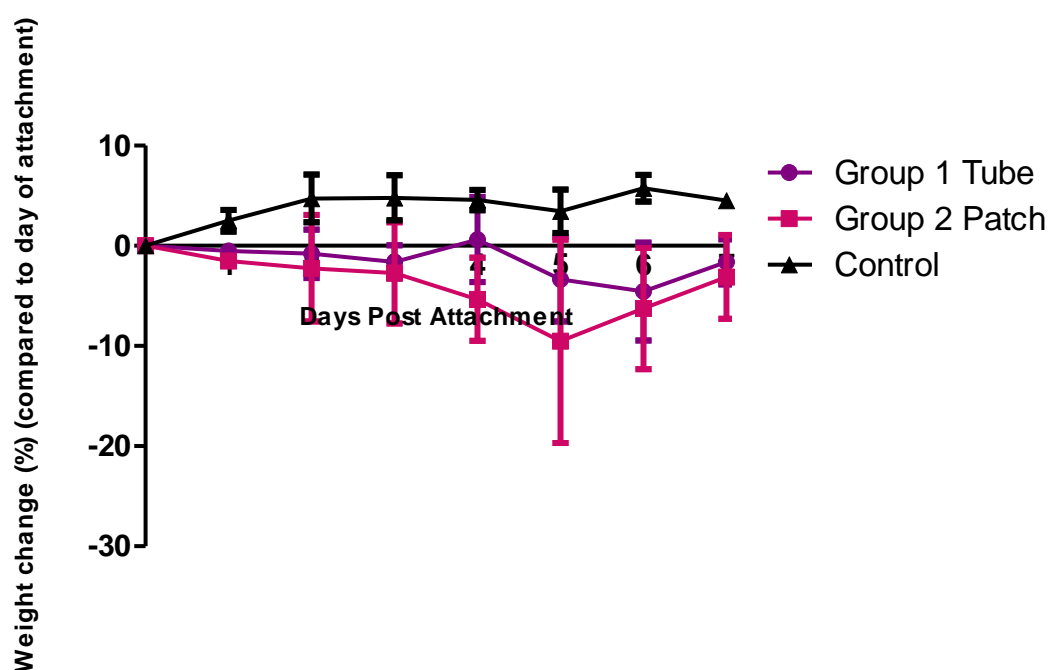


Figure 5.6: Percent weight change compared to the day of attachment. Mean weight change is shown with error bars denoting standard deviation.

Table 5.4: Mean weight change (%) compared to day of device attachment.

Days post-attachment	Tube mean weight decrease (%)	Patch mean weight decrease (%)
D1 – DAY 3	0.97	2.15
DAY 3 – DAY 5	1.4	5.8
DAY 5	3.3	9.5
D6	4.5	6.2
D7	1.6	3.1

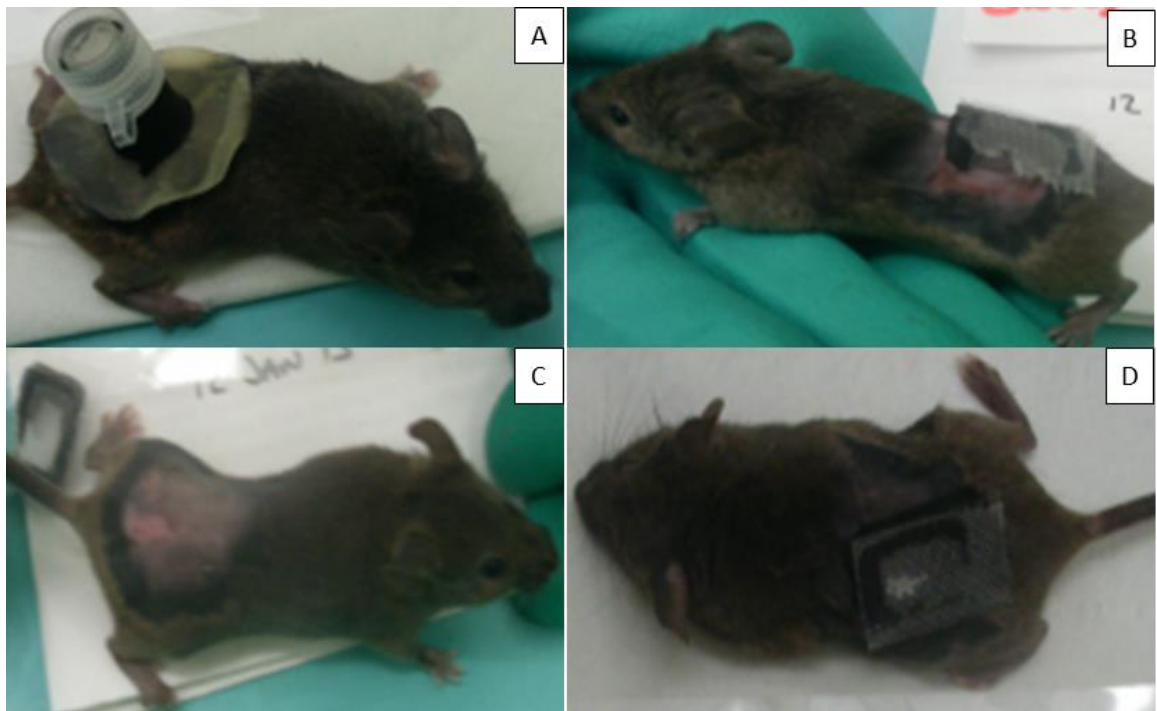


Figure 5.7: Durability and retention capability of attached tube and patch devices

5.3.2. Modification of tube containment devices

Modifications were made to the tube devices to address the functional issues of condensation build up and poor visualisation within the tubes. The addition of air holes to the side of the tube rim eliminated the formation of condensation and the magnification insert into the top of the tube improved the visualisation of the ceramic beads.

No significant difference in weight change ($P=0.2290$ Mann Whitney U test) was observed between tube attachments and the control group throughout the study. Tubes were attached two days prior to ceramic bead addition using copydex glue, no increase in vocalisations was observed and the skin exhibited good flexibility and movement. Animals exhibited normal exploratory and feeding behaviour throughout the study.

The modified tubes exhibited a reduction in their durability and retention capability with only two out of three tubes remaining attached, compared to all tubes in study one. Day 4 saw moderate detachment of one tube resulting in total loss of beads and on day 5 the tube was removed due to substantial detachment. Increased movement of the tube was also observed, coinciding with a change in tube location to the upper back over the shoulder blades possibly increasing the movement and stress on the

latex resulting in moderate detachment. A bowing effect was observed with all tubes and may be explained by the tube height coupled with the increased movement. Devices were designed to minimise the height as much as possible without compromising dexterity of the operator. Further reduction would minimise the handling area on the tubes, inhibiting the opening and closing of the lid and the addition and removal of ticks.

In this experiment AJ white colony mice were used. Their intact immune status facilitated the optimisation of on host containment outside of an isolator, eliminating the problem of isolator scheduling. A129 type 1 interferon receptor knockout mice to be used for tick-virus transmission studies require handling within containment and are smaller than AJ mice. Considering this and the observed bowing effect, and the inability to reduce the tube height further, the tubes are not a viable option for use with A129 mice.

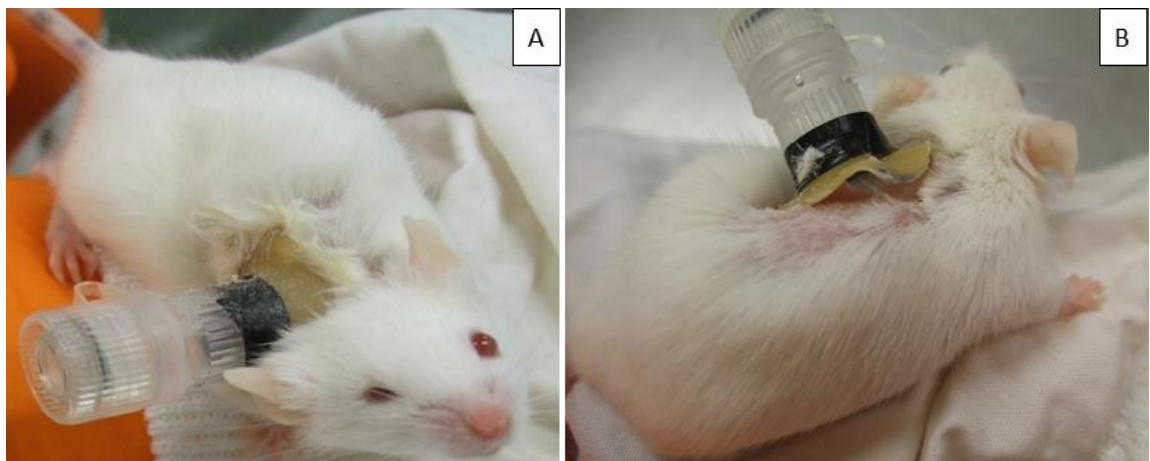


Figure 5.8: A; Photos of modified tubes attached to AJ mice, moderate detachment of tube 4 days post-attachment and B; tubes bowed to one side

5.3.3. Adhesive optimisation of patch attachment devices

Vetbond™ glue is a wound glue used in veterinary practices and polymerises within seconds of application to tissue and body fluids. The use of this adhesive with the foam patches resulted in rapid drying and secure attachment of the patch to the mouse skin. However, the glue caused hardening and notable reduction in flexibility of mouse skin. The glue was also absorbed by the neoprene foam and caused hardening of the patch itself. Copydex, a commercially available adhesive provided secure attachment of the patch to the skin and allowed flexible movement of the skin when manipulated.

Mesh was attached to the secured patch using Loctite superglue by applying the glue through the mesh and onto the patch. This provided a fast, drying, secure application with easy removal. Tweezers were used to remove the mesh top and a new piece glued onto the same patch. Copydex required a longer drying time and the fluidity of the glue caused it to become absorbed into the patch reducing the adhesion of the mesh to the patch.

Copydex, due to its secure attachment and flexibility whilst on the host skin, was deemed the optimal adhesive for attachment of patches to the mouse. Loctite super glue, due to its fast drying secure application was deemed the optimal adhesive for the attachment of mesh to the patch. These will be used in future studies.

5.3.4. Flexible neoprene patches for on-host primary containment

Flexible neoprene foam patches were assessed for their suitability as on host primary containment. Patches exhibited 100% durability and retention capability. Good visibility of ceramic beads was observed as was good flexibility of the mouse skin. No sign of damage to the patch from either the mouse or the surrounding environment was seen.

No behavioural observations were noted and all animals scored healthy throughout the study and mouse weight remained consistent. The use of a flexible neoprene foam patch has resulted in a primary containment device with 100% durability and retention capability and will be used in all future *In vivo* studies.

5.3.5. Attachment of *I. ricinus* nymphs to A129 IFN- α/β R^{-/-} mice

To determine if A129 IFN- α/β R^{-/-} mice were suitable hosts for *I. ricinus* nymphs a preliminary tick attachment study was undertaken. The logistics of tick handling and manipulating within a flexible-film half suit isolator was also observed.

Ixodes ricinus nymphs, infested at a density of three ticks per mouse, successfully attached to mice three days post-infestation, albeit at a low rate (9.5% (2/21)). All groups had a mean increase in weight throughout the course of the study. The handling of ticks within a high containment setting in a flexible film isolator was also assessed. The optimised neoprene patches worked well as primary tick containment

with all ticks remaining within the patches for the duration of the study and all were accounted for at the end of the study.

5.3.6. Optimisation of tick application methodology

To determine if higher tick densities resulted in increased attachment rates of *I. ricinus* ticks, four methods were assessed for their ability to simultaneously deposit 10 ticks into a neoprene patch.

Initial trials utilising water surface tension involved suspending ticks in water within a petri dish and trying to manipulate the water and ticks into the patch. This method was hindered once the surface tension was broken by the uneven edge of the petri dish. A straw was then cut in half and the end rounded to fit inside the patch with the plastic coating providing an ideal surface for the water and ticks to 'slide' down. However the water tension was again broken once the water reached the brittle end of the straw. The use of syringes to expel ticks using air pressure have been used to infest *I. ricinus* larvae onto a mouse host (Personal communication, Maria Kazimirova) however it was not very successful using nymphs. Due to the size of the nymphs the end of the syringe had to be cut to allow nymphs to exit the syringe but this reduced the air pressure and eliminated the desired effect. The ticks also attached themselves firmly to the inside of the syringe and the bottom of the internal plunger.

The utilisation of water surface tension to render the ticks immobile was deemed the most effective method. This requires a 20-30 μ l water droplet to be added to the patch once it has been secured onto the host. Ticks are then added to the water droplet, one by one using tweezers. Once all ticks have been added, the mesh covering is glued in place and the water droplet absorbed out using white tissue leaving only the ticks in the patch.

5.3.7. Determination of infestation density on the attachment and feeding success of *I. ricinus* nymphs on mice

To ensure that in future *In vivo* vector competency studies infected ticks are able to attach and feed to repletion thereby giving the best chance of viral transmission, the attachment and feeding success of *I. ricinus* ticks infested on mice at varying densities was assessed. Mice were infested with densities of either 5, 8 or 10 ticks and tick attachment success assessed via four criteria; the number of ticks successfully

attached to mice, the number of ticks successfully fed to repletion, the average weight of engorged ticks and the number of engorged ticks moulting to the adult stage. The effect of infestation density on mouse weight was also determined.

The attachment and feeding success of all groups was determined by recording how many ticks were attached and fed & detached on both days 3 and 5 post-attachment and at the end of the study (day 8). There was a density dependent trend in overall percentage of tick attachment and feeding rates between all groups (Table 5.5) with an infestation density of 10 ticks per mouse producing the highest overall attachment rate of 66.6% (n=20/30). All ticks that attached successfully engorged (Table 5.5) and by day 3 ticks were either attached, fed and detached or dead. Engorgement weight was not significantly different between infestation groups of 8 and 10 ticks (Unpaired t-test $P=0.6898$) (Table 5.6) and did not appear to be affected by the time taken to complete feeding between these two groups (Unpaired t-test $P=0.2174$) with a mean weight of 3.45 ± 0.86 SD for those completing feeding on day 3 and a mean weight of 3.92 ± 1.22 SD for those completing feeding on day 5. A single tick from group 1 was shown to take a longer time of 8 days to fully engorge and obtained a weight within the range of those ticks fed at higher densities.

Of all engorged ticks, an overall moulting rate of 50.72% (35/69) was achieved with a 75% (15/20) moulting rate achieved from those infested at a density of 10 ticks per mouse (Table 5.7). No difference in moulting time was observed between infestation groups of 8 and 10 ticks (Unpaired T-test $P=0.0903$). Nymph engorgement weight was shown to be a good predictor of adult weight (Figure 5.9) and resulting adult gender (Figure 5.10). Moulded adults weighed a mean $0.49\text{mg} \pm 0.06$ SD less than the nymph engorgement weight and those ticks engorging up to a weight of 3.6mg moulted as male and females resulted from an engorgement weight of 3.9mg.

A difference in mean weight of mice was observed between Infestation groups ($P=0.0003$) (Figure 5.11). There was a significant difference (One way ANOVA Turkey multiple comparison test) between mice infested with 5 ticks, of which only 1 tick successfully attached and engorged, with a mean percent weight loss of -3.24 ± 4.09 SD, and mice infested with 8 ticks which had a mean increase of 0.08 ± 2.99 SD. There was an extremely significant difference (One way ANOVA Turkeys multiple comparison

test) between an infestation of 5 ticks and 10 ticks per mouse which had the highest mean increase of 1.76 ± 2.94 SD though out the study. There was no significant difference in mouse weight between infestation densities of 5 and 8 ticks per mouse.

Table 5.5: Tick Infestation density is shown along with the total number of ticks per group, the percentage attachment, engorgement success and mortality rate per group and the number of ticks fed and detached at key time points; day 3, day 5 and day 8.

Tick Infestation Density	No of ticks observed	Attachment success (%)	Engorgement success (%)	Mortality rates (%)	Engorgement time		
					DAY 3	DAY 5	D8
5 (G1)	15	6.67	6.67 (1/15)	93.33 (14/15)	0	0	1
8 (G2)	24	58.33	58.33 (14/24)	41.66 (10/24)	6 42%	7 50%	1 7%
10 (G3)	30	66.66	66.66 (20/30)	33.34 (10/30)	9 45%	11 55%	0

Table 5.6: Tick engorgement weight in relation to infestation density is show. The mean weight (mg) and standard deviation per infestation group, and per group on days 3, 5 and 8 post-infestation is shown.

Tick density per mouse	Mean weight per group (mg) \pm SD	DAY 3	DAY 5	D8
5 (G1)	4.7			4.7
8 (G2)	3.77 ± 1.31	3.03 ± 0.58	4.54 ± 1.44	
10 (G3)	3.62 ± 0.91	3.73 ± 0.93	3.53 ± 0.92	
Overall		3.45 ± 0.86	3.92 ± 1.22	

Table 5.7: Moulting success per infestation group (%) and the time taken (range in days) for *I. ricinus* nymphs to molt to adults is shown.

Infestation Density	Engorgement success (%)	Moulting success (nymph – adult %)	Moulting time (range in days)
5	6.67 (1/15)	100 (1/1)	99
8	58.33 (14/24)	93 (13/14)	70-107
10	66.66 (20/30)	75 (15/20)	68-106

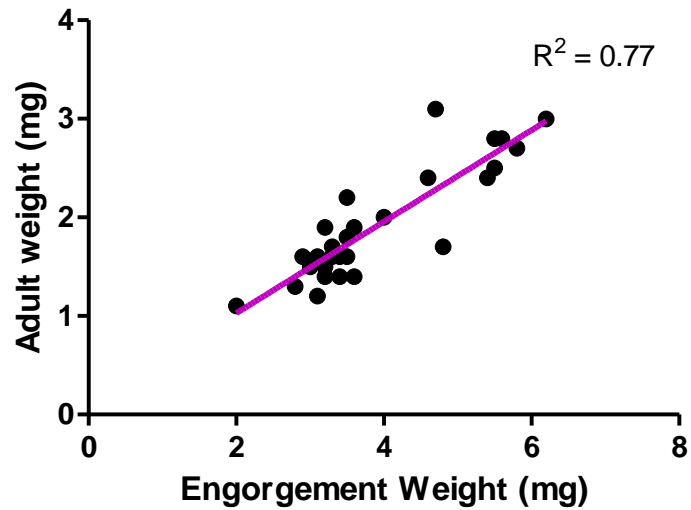


Figure 5.9: Linear regression of the engagement weight (mg) of *I. ricinus* nymphs and the resulting post-moult adult weight (mg) is shown. 77% of the variance in adult weight is attributed to the engagement weight as shown by the R^2 value.

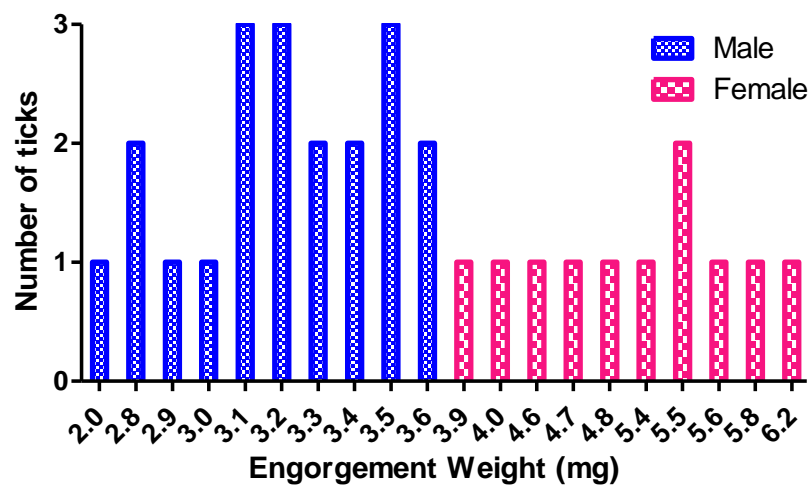


Figure 5.10: Adult post-moult gender in relation to nymph engagement weight (mg). Nymphs reaching an engagement weight of up to 3.6mg resulted in male adults and those obtaining a weight of 3.9mg upwards resulted in female adults.

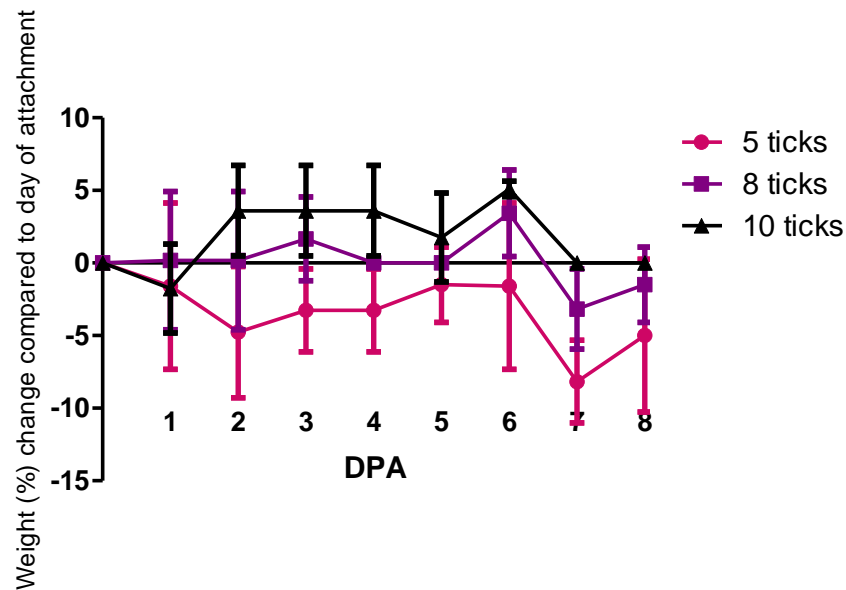


Figure 5.11: Percent weight change of mice infested with ticks compared to the day post attachment (DPA). Mean weight change is shown with error bars denoting standard deviation.

5.3.8. Investigation of HAZV Viremia Levels in A129 IFN- α/β R^{-/-} mice

A129 IFN- α/β R^{-/-} mice are an animal model for lethal HAZV infection (Dowall *et al*, 2012). The aim of this study was to determine the dose of HAZV that allows detectable viremia within host blood, specifically over days 3 and 5, the feeding time of *I. ricinus* nymphs and survival of the host to ensure complete engorgement of nymphs. Two doses were compared; 20pfu/mouse and 2pfu/mouse.

Whole blood was obtained from over half of all animals (30/51) with 100% (9/9) from control animals. The remaining 21 animals succumbed to disease before samples could be obtained. Viral RNA was detected in both groups on days 3, 5 and 7 post-inoculation (Figure 5.12). Ct values were inverted so that an increase in Ct value represents an increase in viral detection. There was a clear trend with an increase in Ct value as days PI increase, indicative of viral replication. Viral detection on day 3 was more pronounced in animals inoculated with 2pfu/mouse with a mean inverted Ct of 15.83 ± 1.39 SD compared to a Ct of 8.24 ± 0.33 in those inoculated with 20pfu/mouse. Detection of HAZV S segment increased in both groups on day 5 PI with a mean Ct of 19.39 ± 1.47 SD in group 2 and 17.94 ± 0.42 SD in group 1. Similar Ct values were reached on day 7 PI in both groups; 24.45 ± 3.17 SD in group 2 and 25.31 ± 3.87 SD in group 1. No significant difference in Ct value between the two groups was detected (P=1 Mann Whitney test). Two-way ANOVA was performed on qRT-PCT Ct values

analysing the effect of dose and time on viral detection. Despite early differences in Ct on Day 3PI there was no significant difference on overall effect between doses ($P=0.0562$) but there was a significant difference at each time point within each dose group ($P=0.0002$) meaning that as days post-inoculation increased so did the effect of each dose.

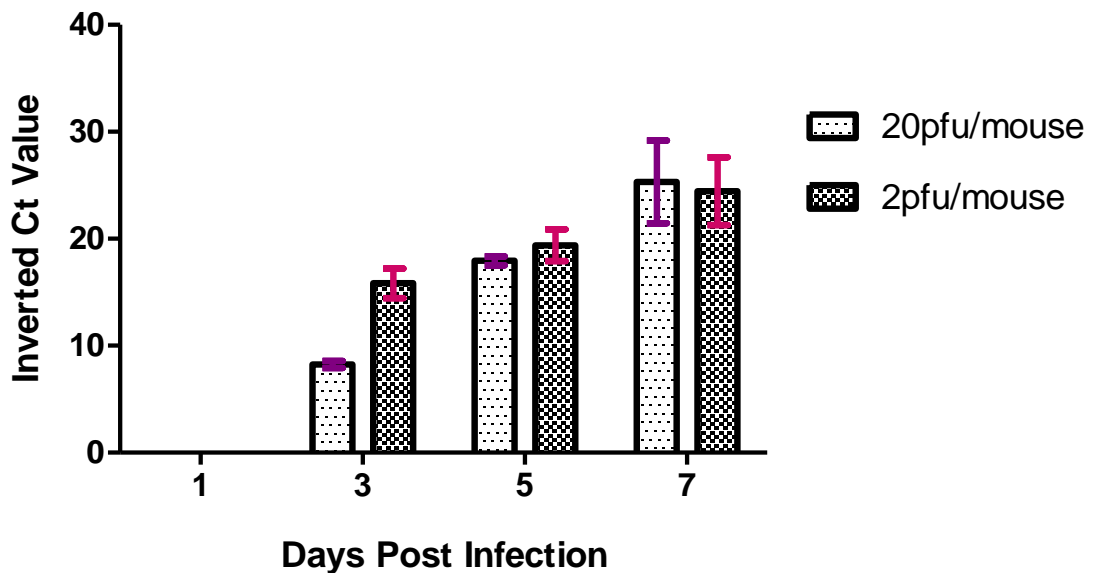


Figure 5.12: Amplification of HAZV nucleoprotein (NP) in mouse whole blood. Mean inverted Ct values; day 3 and day 7 (G1 and G2) $n=2$, day 5 (G1) $n=2$ and day 7 (G2) $n=3$ with error bars denoting standard deviation are shown. Increasing viral detection can be seen as days post-inoculation increase with no significant difference observed between dose groups ($P=1.000$) (two tailed T test).

Presence of infectious virus in blood samples was assessed via plaque assay. Only 5/21 animals inoculated with HAZV produced detectable plaques; 60% (3/5) were from the 20pfu/mouse group and 40% (2/5) from the 2pfu/mouse group with no detectable plaques from the negative control group (Table 5.8). Animals inoculated with 20pfu/mouse produced infectious virus on day 3PI at a titre 1.66×10^2 pfu/ml increasing to 3×10^3 pfu/ml and 1.61×10^6 pfu/ml on day 7. Infectious virus was not detected until day 7 PI in animals inoculated with 2pfu/mouse with titres of 3.1×10^4 pfu/ml and 1.1×10^5 pfu/ml.

Table 5.8: Samples obtained from all dose groups and tested via qRT-PCR and plaque assay. The overall number of positive qRT-PCR and PFU samples are shown and for days 3, 5 and 7 post-inoculation. The number of total samples tested per day are shown with () denoting how many of these samples tested positive by plaque assay. ** denotes both PCR and plaque assay data obtained from the same mouse.

	Blood samples obtained	PCR positive	PFU positive	PCR and PFU samples according to DPI		
				DAY 3	DAY 5	D7
Group 1 20pfu/ml	10/21 (47.6%)	6/10	3/10	2 (1)	2 (0)	2 (2)**
Group 2 2pfu/ml	11/21 (52.4%)	7/11	2/11	2 (0)	3 (0)	2 (2)**
Group 3 PBS	9/9 (100%)	0/9	0/9	0 (0)	0 (0)	0 (0)
Total	30/51 (58.8%)	13/30	5/30	4	5	4

The effect of HAZV dose on mouse survival was also observed. In order for *I. ricinus* nymphs to complete feeding the host must survive for at least 5 days. Animals in both dose groups had a median survival time of 7 days; however, the number of animals surviving to day 7 PI varied according to the dose group. Animals that received the lowest dose of HAZV at 2pfu/mouse, had a survival rate of 75% with the onset of death one day later (day 5 PI) than those inoculated with 20 pfu/mouse, with a survival rate of 70% and the onset of death on day 6 PI, however no significant difference in overall survival between dose groups (P=0.6752) was observed (Figure 5.13).

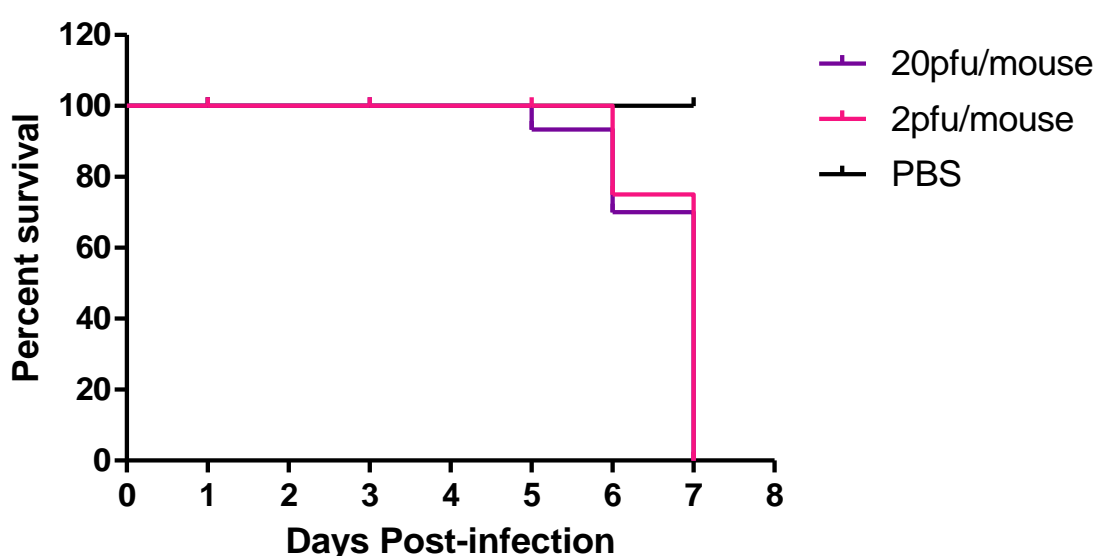


Figure 5.13: Kaplan Meyer survival analysis of HAZV inoculation groups. A significant difference

between all survival curves was shown ($P=0.0211$) but no significant difference in survival curves between dose groups was seen ($P=0.6752$),

5.4. Discussion

The use of HAZV and A129 IFN- α/β R^{-/-} mice in the development, establishment and optimisation of an *In vivo* tick feeding model has been successful. The development of primary on-host containment of ticks has resulted in the secure attachment of *I. ricinus* nymphs on mice using a flexible neoprene patch utilising a novel approach of water surface tension in the addition of ticks. A129 IFN- α/β R^{-/-} mice are suitable hosts for maintaining *I. ricinus* nymphs with attachment and feeding rates of up to 66.66% resulting in a 75% moulting rate confirming the full engorgement of ticks. Inoculation of mice with 20pfu of HAZV results in circulating viremia within host blood on day 3 at a titre of 1.66×10^2 pfu/ml rising to 1.6×10^7 pfu/ml on day 7 and results in host survival for up to 7 days. This ensures nymphs are feeding on a viremic host and allows nymphs to complete feeding before the mice succumb to disease.

5.4.1. Development of on-host primary tick containment

When handling vectors such as ticks, especially when infected with pathogens, accountability and visualisation are extremely important. These requirements are addressed by developing durable, retentive, functional primary and secondary containment of ticks and their hosts preventing escape of confined ticks and removal of the attachment device by the host. Devices must also be easy to manipulate when working in a half-suit flexible film isolator and not compromise user dexterity. Two attachment devices were chosen for evaluation; tick tubes and patches. Tick tubes have been used to contain *Hyalomma marginatum* nymphs and adults on mice, rabbits and guinea pigs (Gargili et al., 2013). These were documented to be very beneficial for use at high containment increasing the distance between the operator and infected ticks. However it is shown here that tubes are not suitable for the use with A129 IFN- α/β R^{-/-} mice. The uses of plastic chambers have primarily been used on larger hosts such as guinea pigs (Shepherd et al., 1991) and rabbits (Goddard, 2005) and it is shown here that tubes exhibited a bowing effect. Neoprene patches have been used for a number of host species including mice (Lawrie et al., 2004; Ogden et al., 1998), rabbits (Slovak, M et al., 2002) and guinea pigs (Kaufman and Nuttall, 2003) and proved very beneficial for use within a half-suit flexible film isolator. These provided improved

visualisation enabling accurate accountability of the ticks and flexibility of the mouse skin.

Prior to infestation onto mice, tick tubes were placed on ice. This successfully reduced their movement and eased handling within the isolator. Upon application to the mouse ticks became extremely active resulting in some ticks climbing out of the patch whilst others were still being added. The increase in tick activity is an expected behaviour and is in response to a variety of stimuli produced by the mouse including body heat, CO₂ and ammonia, all of which ticks use as detection for a suitable host in nature. Whilst this behaviour was expected and was manageable when handling 3 ticks, the short time required for ticks to become stimulated and reactive to the host may prove problematic when handling higher tick densities. As a result a method using the surface tension of water droplet was used to simultaneously add up to ten ticks per patch. Ticks are known to survive submersion in water due to the use of their spiracular plates allowing oxygen absorption from the water (Giannelli et al., 2012).

4.7 Density dependent attachment and feeding rates of *I. ricinus* nymphs

Initial tick attachment and feeding rates of *I. ricinus* nymphs were low, therefore a density dependent attachment study was conducted. A density dependent effect on tick attachment and feeding was observed; with an attachment rate of 6.67% from an infestation of 5 ticks up to 66.66% with an infestation of 10 ticks. It has also been shown that an increase in density increases the size of *I. dammini* larvae feeding on white footed mice (Davidar et al., 1989) and it is thought that this increased density enhances the cumulative effect of tick saliva compounds injected into the host which are known to contain a variety of bioactive compounds facilitating the generation of a feeding pool thereby increasing the amount of blood available for ingestion (Wang et al., 2001).

The data present in this study supports pheromone-mediated attachment and feeding behaviour of ticks. Tick feeding relates to a complex sequence of events and the way in which ticks carry out this sequence and their response to certain stimuli can vary depending on the tick species and the host itself. Tick feeding can be separated into nine stages: appetence, engagement, exploration, penetration, attachment and ingestion of host blood, finishing with; engorgement; detachment and disengagement

(Waladde, M.S, 1982). All of these stages require information to be relayed from host to tick, and from attached ticks to host seeking ticks. This is done via chemical mediators known as semio-chemicals (Sonenshine, 2004) which are secreted outside of the body and direct a specific behavioural response. Pheromones are a type of semiochemical which are secreted by one species of ticks to direct a behavioural response of the same species. These include attachment-aggregation-attachment pheromones which promote attachment and clustering on hosts (Norval et al., 1989). Norval 1989 illustrated that unfed *Amblyomma* nymphs had a 60% response to pheromones emitted by fed male ticks. It was also shown that unfed nymphs had a 70% response to pheromones released by fed nymphs and has also been shown for *I. ricinus* nymphs (Hajkova and Leahy, 1982).

Kairomones, compounds secreted from prospective hosts, are also important for initial tick attachment. These allow ticks to determine suitable locations on a host to feed (Sonenshine, 2004) and include compounds such as CO₂, ammonia and squalene which is a lipid on the surface of mammalian skin and acts as an attractant for hungry ticks (Yoder et al., 1999) and can vary according to hosts (Osterkamp et al., 1999). It is evident that the process of tick attachment and feeding is a complex feedback loop between host, fed ticks and unfed ticks. It could therefore be assumed that the maintenance and feeding of ticks within a laboratory setting, especially those requiring primary containment of ticks on hosts, could hinder some of these behaviours. Ticks are unable to locate the optimal site of attachment and feeding and are confined to a designated area on a host by a neoprene patch. However the opposite was shown when *Hyalomma* ticks were confined onto a mouse hosts using a plastic tube with an attachment rate of 40-60% compared to 1.55% on freely attached larvae (Gargili et al., 2013). It has also been shown that ticks allowed to freely infest anaesthetised mice compared to non-anaesthetised mice had a higher attachment rate of 73.67% compared to 12.67% (Levin and Fish, 1998). It was suggested that the anaesthetisation of mice allowed the ticks to attach before mice could groom therefore suggesting that confinement of ticks would protect them from host grooming increasing the attachment rate. If the attachment rate was due to the confinement of the ticks within a certain area, conferring protection to removal from host grooming as suggested by Levin and Fish (1998), it would be expected that an attachment of 3-5 ticks, as in the

preliminary attachment study performed here, would have produced a higher attachment rate than 6.6% as demonstrated here. This study has shown the same density dependent attachment pattern on anaesthetised mice however nymphs only require an infestation density of 8 to achieve an attachment rate of 58.33% and a rate of 10 ticks per mouse for a 66.66% attachment rate.

5.4.2. Host viremia and survival

With the purpose of assessing vector competence within the laboratory it is important to ensure firstly; that there is detectable viremia within the host blood and secondly; the survival of the host is adequate for the complete feeding of the tick vector. This will ensure that a tick species has the best possible chance of acquiring the virus via a blood meal if it is able to do so. Previous studies have shown that host survival does exhibit a dose response effect (Dowall et al., 2012). Mice survived for 7 days when challenged with 10pfu/mouse compared to 5 days when challenged with 4×10^4 pfu/mouse and 10^3 pfu/ mouse. In this study a comparison of 20 pfu/mouse and 2pfu/mouse was conducted. Both doses had a median survival of day 7 but the onset of death was dose dependent. Animals inoculated with 20pfu/mouse experienced a loss of one animal on day 5, 3 animals on day 6 with the remainder on day 7. Group 2 mice inoculated with 2pfu/mouse experienced a loss of 3 animals on day 6 and the remainder on day 7. Detection of viral titre from both dose groups exhibited high variation and the titre of 1.1×10^5 pfu/ml on day 7 from group 2 indicates viral replication is most likely occurring at earlier time points but is undetected. The limit of detection of the plaque assay is 1pfu/ml, the assay is therefore able to detect low levels of infectious virus and is evident by titres obtained in group one. Therefore the lack of detection may be due to the sample type. Infectious virus has previously been obtained from serum, the non-cellular component of blood, as early as day 1 PI during HAZV infection. To ensure accurate titre whole blood was analysed as HAZV and CCHFV have been shown to hemagglutinate (Casals and Tignor, 1974). Samples were difficult to process and many had coagulated hindering the ability to acquire a true representation of infectious virus particles in a sample. This also contributed to the high variation observed in both groups. Despite this it is shown that both viral titres cause clinical disease in animals, with mortality observed from day 5PI. Survival was greatest within mice inoculated with 2pfu/mouse and this translates to an additional

animal surviving until day 7PI. However, the median time to death in both groups was the same at day 7. As the median time to death was the same in both groups and the viral titres shown in both groups exhibit the same level of variability, a dose of 20pfu/mouse will be used.

The development of this tick feeding model within a high containment half-suit flexible film isolator is the first of its kind in Europe. Combined with the ability to infect ticks with high containment pathogens at CL4, as described in the previous chapter, this model allows investigation into the vector-pathogen-host interactions of some of the most fatal human zoonotic pathogens.

Chapter 6. *In vivo* transmission of Hazara virus by *Ixodes ricinus* nymphs

6.1. Introduction

Using the developed *In vivo* tick feeding model, the ability of *I. ricinus* nymphs to transmit and acquire HAZV was assessed. In Europe Ixodid ticks are predominantly three host species (Labuda and Nuttall, 2004) feeding once per life stage and in order for an arbovirus to survive within nature it must be transmitted to a vertebrate host and naive ticks (Nonaka et al., 2010). Arboviruses are maintained in nature in enzootic cycles between an arthropod vector and a vertebrate host via three main transmission routes; (i) horizontal transmission between the vector and vertebrate host; (ii) vertical transmission from one life stage to another and; (iii) co-feeding transmission between different life stages on the same vertebrate host (Figure 6.1).

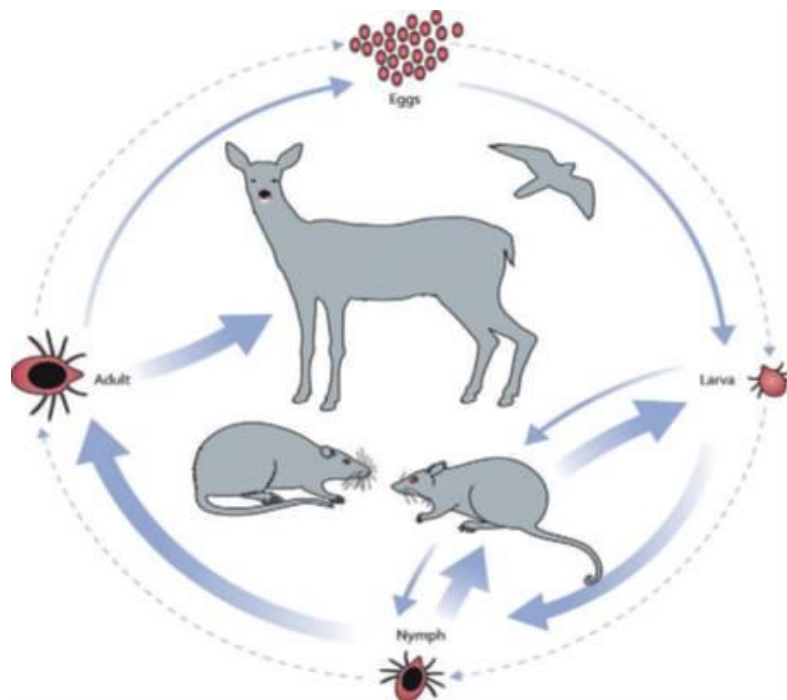


Figure 6.1: Arboviral transmission routes in nature illustrating horizontal, vertical, and co-feeding transmission. Figure obtained from Nuttall and Labuda 2004 with permission.

Horizontal transmission between an infected tick vector to an uninfected host and from an infected host to an uninfected tick was originally thought to be the primary route of vector inoculation (Nuttall and Labuda, 2003). In mosquito-virus cycles a threshold level of viremia within host blood was defined as a critical requirement for

infection of feeding vectors (Lord et al., 2006) and any host not meeting this threshold was discounted as a viable contributor in the ecology of the disease. This was disproved for hosts of tick-borne viruses when the role of guinea pigs, who exhibit no clinical signs and no viremia when infected with Thogoto virus, and hamsters who develop a high level of viremia when infected with Thogoto virus was assessed (Jones et al., 1987). It was shown that infected adult ticks co-feeding alongside non-infected nymphs resulted in more infected nymphs when feeding on a non-viremic guinea pig host compared to the high viremic hamsters (Jones et al., 1987). This study used non-natural hosts of ticks but other studies using the natural hosts of tick species also support non-viremic co-feeding transmission. Wild caught hares, a natural host for *I. ricinus* nymphs and adult ticks, were shown to support non-viremic transmission of Louping Ill virus between co-feeding infected and non-infected *I. ricinus* nymphs (Jones et al., 1997). This method of virus transmission has also been shown for CCHFV in *Hyalomma marginatum* ticks from infected adults to larvae and nymphs (Gordon et al., 1993) and Palma and Banja bunyavirus transmitted to co-feeding ticks on mice (Labuda et al., 1997a). Non-viremic transmission occurs in the absence of systemic disease, utilising the host as a vessel for tick feeding and virus transmission between co-feeding ticks (Jones, 1989) and is considered particularly important for viruses such as tick-borne encephalitis virus which have short infectious periods within their hosts (Labuda et al., 1993; Nonaka et al., 2010). Ticks are known to feed in aggregates on hosts with 20% of the host population harbouring 80% of ticks (1.1.2) often with immature tick stages from various species (Nuttall and Labuda, 2004) facilitating NVT in nature. This method of virus transmission has highlighted the importance of the localised bite site at the vector – host interface. The skin site is an important area for virus replication after initial transmission (Labuda et al., 1996). Skin explants taken from the bite site of infected and co-feeding non-infected *I. ricinus* ticks showed viral replication within monocytes, macrophages with the virus taken up and transported to the bite site of non-infected ticks via Langerhans cells and neutrophils. NVT also occurs in the presence of neutralising anti-bodies where by 89% of animals immunized against TBEV supported transmission to feeding ticks (Labuda et al., 1997b).

In order for viruses to perpetuate in nature they must be transmitted to a vertebrate host and as Ixodid ticks only feed once per life stage viruses must persist from one

instar to the next. Ticks exhibit a behaviour known as diapause, whereby ticks can have a period of inactivity and over winter if a suitable host is not found (Labuda and Nuttall, 2004) and this can lead to an increase in the tick life cycle as seen with *I. ricinus* ticks which can take up to three years to complete one generation (Hillyard, 1996). Trans-stadial transmission of virus between life stages of infected ticks is therefore an important requirement for the long term maintenance of virus in nature (Randolph et al., 1999). Trans-stadial transmission has been shown for a number of viruses including CCHFV (1.3.4) and Powassan virus (Costero and Grayson, 1996) where nymphs are shown to be more efficient in transmitting this virus to subsequent newly moulted adults (54%) compared from larvae to nymphs (9.5%) and larvae through to adults (10%). Transovarial transmission has been shown for both CCHFV (Gonzalez et al., 1992) and TBEV (Danielová et al., 2002) and for TBEV it occurs at low levels (0.1%) and on its own is not a very effective method of virus propagation. However, when taking into consideration the large amount of eggs produced from females, the large numbers of these resulting infected larvae feeding on the same host, amplification of low levels of virus may occur (Nonaka et al., 2010). This can then be transmitted to co-feeding ticks, of different developmental stages. Although indirect, this vertical transmission between life stages may enhance the prevalence of virus in nature.

This chapter will investigate the ability of infected *I. ricinus* nymphs to horizontally and vertically transmit HAZV.

6.1.1. Chapter aims

The aim of this chapter is to determine whether artificially infected *I. ricinus* nymphs are able to transmit virus to a susceptible host, and whether they can acquire and maintain virus from an infected blood meal.

6.1.2. Chapter objectives

This will be achieved by evaluating the ability of *I. ricinus* nymphs to;

- Transmit HAZV to A129 interferon receptor knockout mice via a natural blood meal,
- Acquire the virus from viremic A129 IFN- α/β R^{-/-} mice via a natural blood meal and
- Maintain virus trans-stadially to the next developmental stage.

6.2. Methods

6.2.1. Maintenance of mice

All experiments were conducted within a flexible film isolator. Animals were individually housed within cages with food and water *ab libitum*. Animals were weighed and temperature checked once and health scored twice daily. Animals were health scored on a scale from 1 – 5 as follows: healthy, 0; ruffled fur, 1; lethargy, 2; rapid breathing, 3; inactive, 4 and death, 5. Mice exhibiting severe signs of disease including a 20% reduction in weight and a temperature of more than 39°C for two consecutive days were euthanased.

6.2.2. Mouse sampling

Upon reaching clinical end point or completion of the study, sera, liver, spleen and skin biopsies were obtained. To investigate localised viral transmission, a skin biopsy from the tick attachment site from each mouse was taken. This, along with 1/3 of the liver and 1/3 of the spleen were placed into pre-weighed 2ml eppendorf tubes containing 5 volumes of RNAlater. Tubes were stored at -80°C for future molecular processing. A 1/3 of the liver and spleen was also placed into a 2ml eppendorf tube containing PBS and frozen at -80°C for future plaque analysis. The remaining liver and spleen tissue was placed into 10% non-buffered formalin for histological examination. Serum samples were tested via plaque assay for circulating infectious virus.

6.2.3. Application of ticks

Ticks were applied to mice on the days of patch attachment utilising the surface tension of a water droplet as detailed in section 2.8.3.

6.2.4. Tick sampling

Unless stated otherwise on days 3, 5 and at the end of all studies patches were opened and the feeding status of ticks assessed. Ticks were recorded as either; unfed and detached, attached, fed and detached or dead. Ticks that were fed and detached were placed into labelled cryovials and either flash frozen on dry ice and stored at -80°C or collected alive. Dead ticks were flash frozen on dry ice and stored at -80°C.

6.2.5. Horizontal transmission of HAZV from *I. ricinus* nymphs to A129 IFN- α/β R^{-/-} mice a preliminary study

A preliminary study was conducted to assess the ability of *I. ricinus* nymphs to transmit HAZV to a naive susceptible host and was conducted within a CL4 flexible film isolator. Eight A129 IFN- α/β R^{-/-} mice were randomly divided into two groups (n=4); group 1, mice were infested with HAZV immersed ticks and; group 2 mice infested with non-infected ticks. Patches and ticks were applied on day 0 as previously described (2.8.2 and 2.8.3) and mice monitored over ten days. Ticks were collected on the day of detachment, weighed and maintained individually within modified tubes within a plastic desiccator and allowed to moult to adults.

Six engorged nymphs were immediately processed and assayed for virus; ticks were pooled and homogenised in 500 μ l of serum free MEM and extracted using the Qlamp Viral RNA kit (2.4.3). Moulded adult ticks were pooled in a group of six, homogenised and RNA extracted using the Qlamp viral RNA kit (2.4.3). Viral RNA was detected via qRT-PCR, normalised to the tick β -actin endogenous control and calibrated to day 63 post-immersion ticks and HAZV expressed as relative fold change.

6.2.6. Horizontal transmission of HAZV from *I. ricinus* nymphs to A129 IFN- α/β R^{-/-} mice a twenty one day study

To investigate the changes observed in the liver of mice infested with HAZV infected ticks, mice were again infested with infected ticks and observed over twenty one days. A129 IFN- α/β R^{-/-} mice (n=21) were randomly divided into three groups; group 1(n=13), mice were infested with ten HAZV infected ticks; group 2(n=5), mice were infested with ten non-infected ticks and; group 3 (n=3), mice were intradermally inoculated with 20pfu of HAZV. On day 0 animals were anaesthetised with 0.1ml Alfaxan via the intra peritoneal route, ID and temperature chipped with patches and ticks attached as previously described (2.8.2. and 2.8.3).

On day 12 post-infestation three mice from group 1 and group 2 were euthanized. All surviving mice were euthanized on day 21 at the end of the study. Tissue samples were obtained from all euthanased animals and those succumbing to disease; to determine localised viral transmission a skin biopsy under the patch attachment was taken for molecular analysis; liver and spleen samples were taken for molecular,

virological and histological examination and serum was taken for molecular, virological and immunological testing as detailed in sections 2.6.4, 2.9.1 and 2.9.2.

6.2.7. Horizontal transmission of HAZV from a viremic host to *I. ricinus* ticks

To investigate the ability of *I. ricinus* ticks to acquire HAZV naturally via a blood meal, ten unfed un-infected *I. ricinus* nymphs were allowed to feed on six virally infected A129 IFN- α/β R^{-/-} mice. On day 0 animals were anaesthetised with 0.1ml Alfaxan via the intra peritoneal route, their dorsum shaved, patches attached and ticks applied as described previously (2.7.2. and 2.7.3). Once ticks and patches were applied, mice were inoculated via intradermal injection with 100 μ l of 20pfu/mouse of HAZV.

At the end of the study six engorged nymphs were immediately processed and assayed for virus; ticks were pooled and homogenised in 500 μ l of serum free MEM and extracted using the Qlamp Viral RNA kit (2.3.3). Viral RNA was detected via qRT-PCR, normalised to the tick β -actin endogenous control.

6.3. Results

6.3.1. Horizontal transmission of HAZV from *I. ricinus* nymphs to A129 IFN- α/β R^{-/-} mice a preliminary study

A preliminary transmission study was undertaken to determine if immersed infected HAZV ticks could transmit virus to a naive host and if virus could be transmitted to the next developmental stage. *Ixodes ricinus* ticks successfully engorged with an attachment and engorgement rate of 77.5% (Table 6.1). Throughout the study all mice were scored healthy with no significant difference in temperature between mice infested with HAZV infected ticks or non-infected ticks (Unpaired t-test, P=0.4410) with an increase in weight seen in mice infested with HAZV infected ticks. Mouse serum, spleen and liver samples were negative via qRT-PCR however two skin biopsies were positive via qRT-PCR for HAZV (Figure 6.2). Histological examination of liver from these two animals showed moderate numbers of multifocal, mixed inflammatory cell infiltrates were observed scattered randomly within the parenchyma (Figures 6.3 and 6.4) indicative of an inflammatory reaction. This study was successful in illustrating low level viral transmission and to further investigate this a longer term study was performed.

Table 6.1: Tick Infestation density is shown along with the total number of ticks per group, the percentage attachment and engorgement success with the number of ticks fed and detached on days 3 and 5 post-infestation.

Tick Infestation Density	No of ticks observed	Attachment success (%)	Engorgement success (%)	Engorgement time	
				DAY 3	DAY 5
10 (G1)	40	77.5% (31/40)	77.5% (31/40)	12.9% (4/31)	87.1% (27/31)
10 (G2)	40	47.5% (19/40)	47.5% (19/40)	0%	100% (19/40)

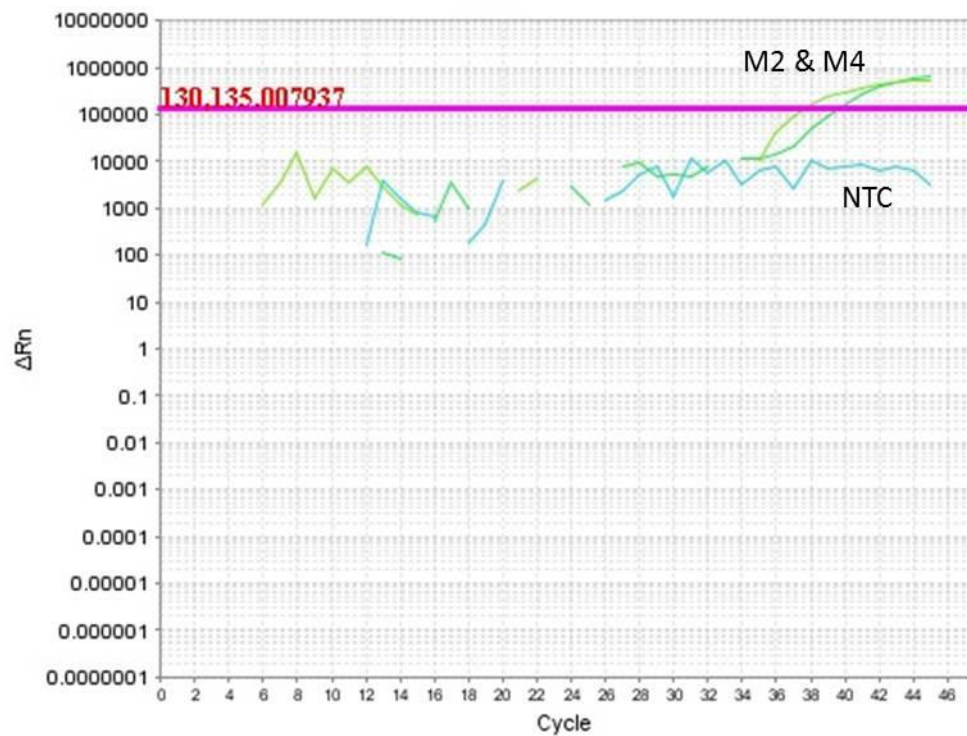


Figure 6.2: Real-time Amplification of skin biopsies obtained from two mice; mouse 2 (M2) and mouse 4 (M4) ten days post-infestation with HAZV infected *I. ricinus* nymphs. NTC= no template control.

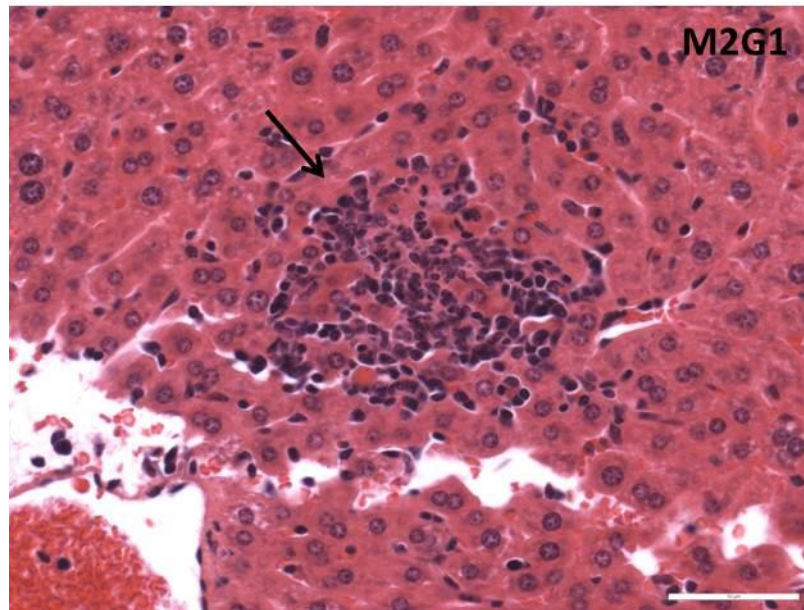


Figure 6.3: Liver obtained from M2G1 infested with HAZV infected ticks. Focal infiltrate of scattered neutrophils and lymphocytes can be seen. Scale bar denotes 50 μ m.

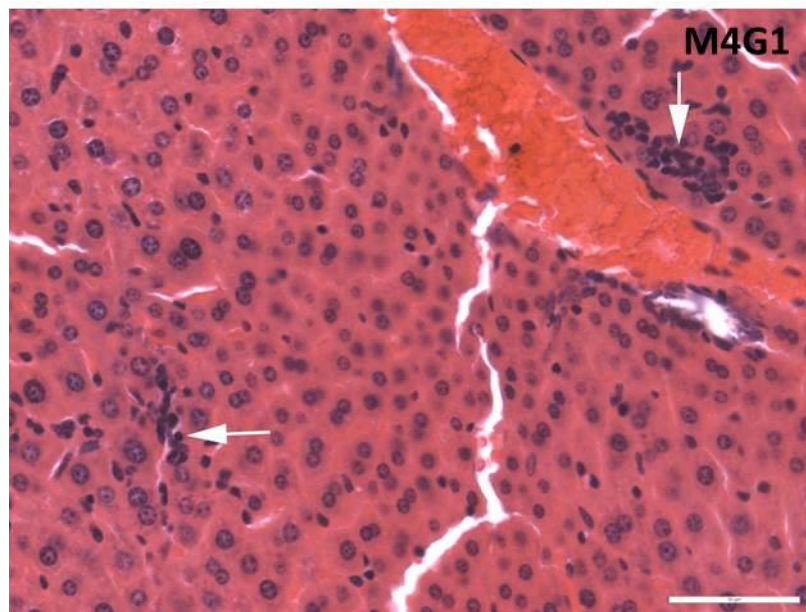


Figure 6.4: Liver obtained from M4G1 infested with HAZV infected ticks. Minimal infiltrates of similar scattered neutrophils and lymphocytes to that of mouse 2 can be seen. Scale bar denotes 50 μ m.

HAZV RNA was detected in both engorged and moulted adult ticks. Engorged ticks were shown to have 0.36-0.56 fold less HAZV-NP compared to unfed immersed ticks 63 days post-immersion however, HAZV replicated 96-149 fold in moulted adult ticks (Figure 6.5).

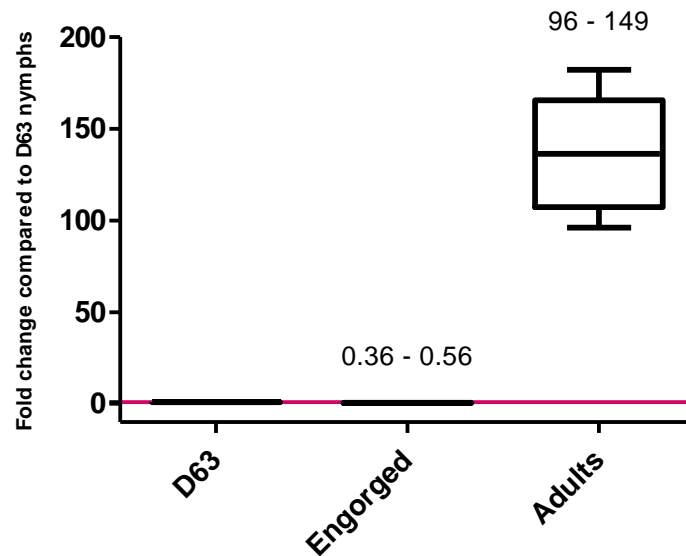


Figure 6.5: Relative fold change of HAZV normalised to tick- β actin endogenous control and calibrated to day 63 post-immersion ticks. Minimum to maximum values are shown. Day 63 represents a pool of ten unfed nymphs, engorged ticks sampled in a pool of 6 and adult ticks sampled in a pool of 4 with 5 replicates performed via real-time PCR. Viral RNA was detected in all groups. The solid pink line indicates a fold change of 1, values above this indicate a fold increase in viral RNA detection relative to day 63 unfed nymphs and values below this indicate a fold decrease in viral RNA detection relative to day 63 unfed nymphs.

6.3.2. Horizontal transmission of HAZV from *I. ricinus* nymphs to A129 IFN- α/β *R*^{-/-} mice a twenty one day study

The capability of *I. ricinus* nymphs to transmit HAZV was determined by infestation of ticks onto naive interferon type I deficient mice with mice observed over 21 days. An attachment rate of 75.38% was achieved for HAZV infected ticks with the majority of ticks completing feeding on day 5 post-infestation (Table 6.2).

Ixodes ricinus nymphs were successful in transmitting HAZV to 38% (5/13) of mice with viral RNA detected in the liver, spleen and skin via qRT-PCR both 12 and 21 days post-infestation (Figure 6.6). The highest levels were observed in the liver, followed by the spleen and then the skin. This indicates a dissemination pattern within the mice from the skin, to the spleen and then the liver where the virus appears to replicate. Virus is not detected within the liver without prior detection in the spleen. In mouse four, virus was not detectable within the skin indicating total virus dissemination to the spleen and liver.

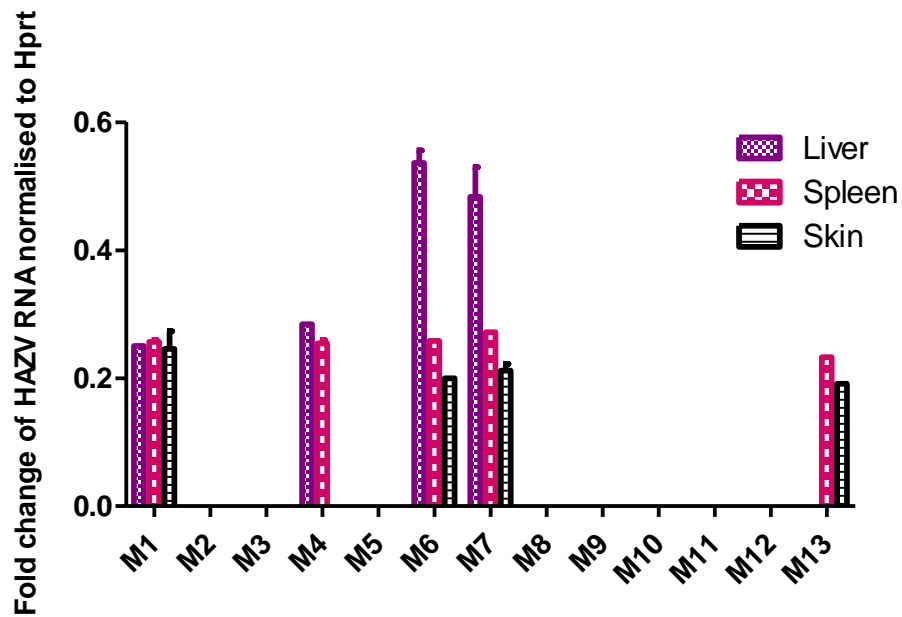


Figure 6.6: RNA obtained from mouse liver, spleen and skin samples was amplified via qRT-PCR. Levels of HAZV RNA was normalised to that of the endogenous control hypoxanthine guanine phosphoribosyl transferase (HPRT). Samples were performed in triplicate and error bars denote standard deviation. HAZV RNA was detected in 38% of mice (5/13). The lowest levels of virus are detected within the skin followed by the spleen and then the liver.

All qRT-PCR positive animals had detectable infectious virus in the liver and all mounted an immune response with detectable IgG in the serum (Table 6.3 and Figure 6.7). Histological analysis was performed on both liver and spleen samples with changes consistent with Hazara virus observed in the liver of 3/5 mice. All spleen samples were negative for HAZV via qRT-PCR, plaque assay and histological analysis.

Table 6.2: Tick Infestation density is shown along with the total number of ticks per group; the percentage attachment and engorgement rate per group and; the number of ticks fed and detached on day 3 and day 5.

Tick Infestation Density	No of ticks observed	Attachment success (%)	Engorgement success (%)	Engorgement time	
				DAY 3	DAY 5
10 (G1)	130	75.38 (98/130)	75.38 (98/130)	11 (11%)	87 (89%)
10 (G2)	50	54 (27/50)	54 (27/50)	5 (18.5%)	22 (81.5%)

Table 6.3: Five A129 IFN- α/β R^{-/-} mice were positive for HAZV. Positive (+) and negative (-) detection of infectious virus in the liver and spleen are shown. The intensity of the IgG response is indicated as strong (++) representing numerous fluorescent foci or weak (+) represented by single foci of fluorescent cells.

Mouse	Day sampled	Pfu Liver	Pfu Spleen	IgG response	Histology
M1	12	+	-	++	Necrosis of the liver
M7	21	+	-	+	Necrosis of the liver
M13	21	+	-	+	Necrosis of the liver
M4	21	+	-	+	ND
M6	21	+	-	+	ND

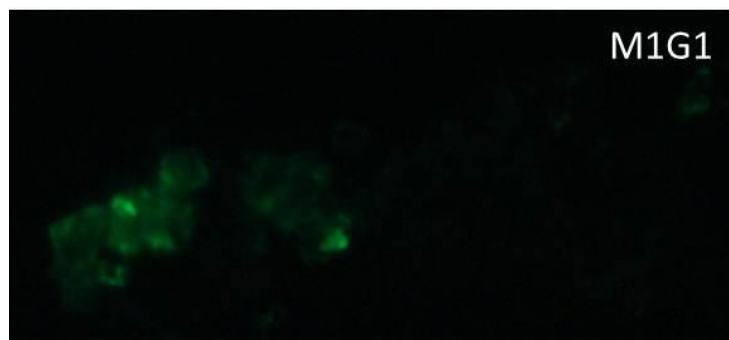


Figure 6.7: Positive fluorescent foci resulting from IgG antibody responses in mouse serum obtained from M1 infested with ten HAZV infected ticks. Mouse serum was absorbed onto HAZV infected SW13 cells on multispot slides and detected using anti-mouse IgG FITC conjugated antibody and cells counterstained with Evans blue.

Histopathological changes included those observed in the preliminary study with the addition of liver necrosis. A negative liver sample obtained from mice inoculated with PBS shows normal lobules with central veins and portal triads (Figure 6.8). Mice infested with non-infected ticks showed occasional, minimal background changes in the liver comprising of small foci of inflammatory cells (Figure 6.9). This is considered an incidental inflammatory response to antigens drained from the gastro-intestinal tract via the portal system.

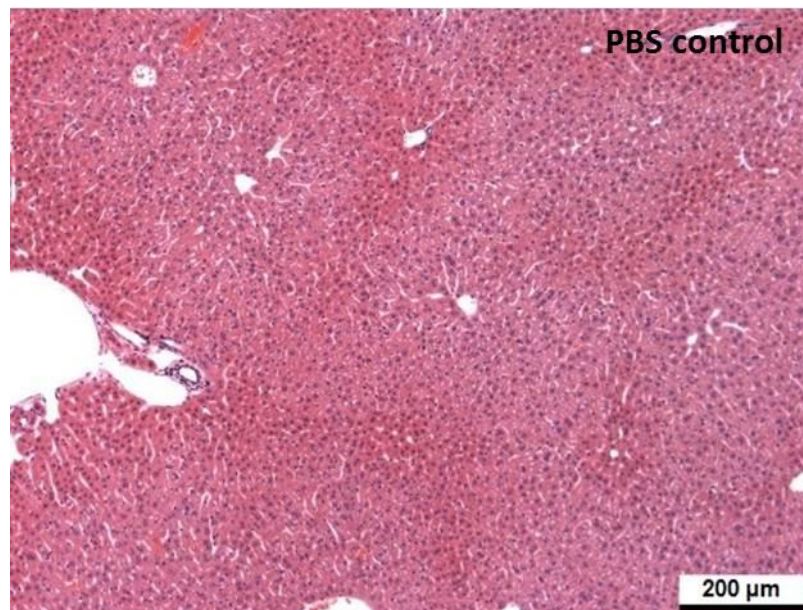


Figure 6.8: Image shows normal liver, obtained from mice inoculated with a PBS control, comprising lobules with central veins and portal triads

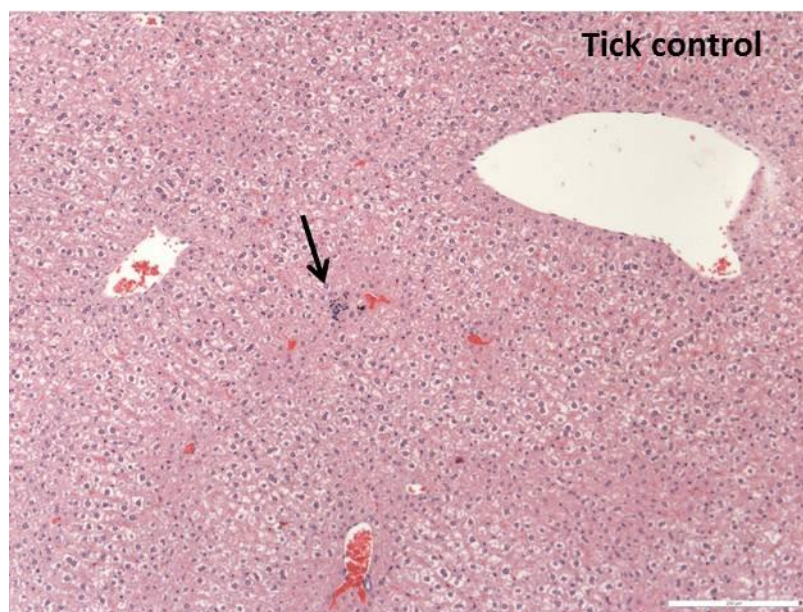


Figure 6.9: Liver obtained from mice infested within non-infected ticks. Occasional, minimal, background changes are shown comprising of small foci of inflammatory cells mainly mononuclear with occasional polymorphonuclear leukocytes (indicated by the arrow). This is considered an incidental, inflammatory cell response to antigens drained from the gastrointestinal tract via the portal system. Scale bar denotes 200μm.

Liver necrosis was observed in 3 out of 5 HAZV positive mice (Figures 6.11 – 6.13) and is consistent with that seen in HAZV infected mice (Figure 6.10). Areas of hepatocyte degeneration and necrosis can be seen with an infiltrate of inflammatory cells.

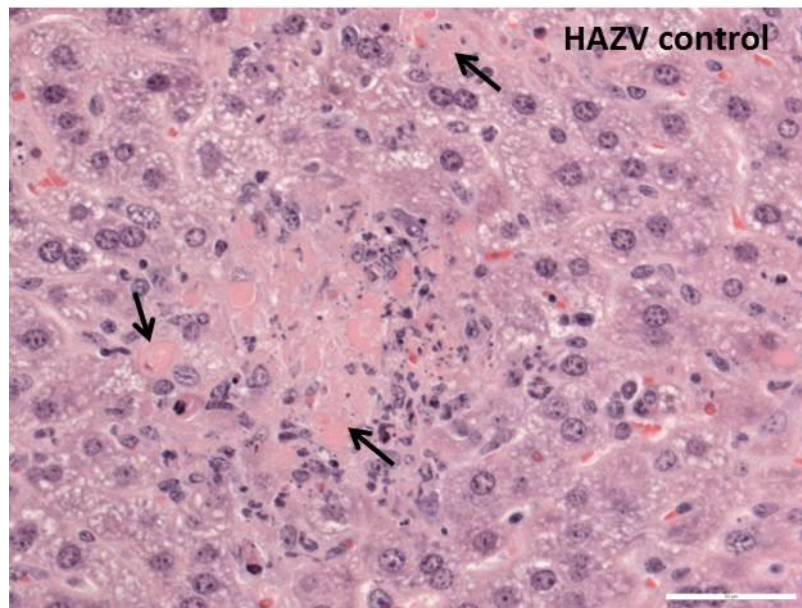


Figure 6.10: Liver obtained from mouse intradermally inoculated with 20pfu of HAZV. Lesions comprising multiple foci of hepatocellular degeneration and necrosis were seen scattered randomly throughout the parenchyma (arrows), characterised by cytoplasmic hyper-eosinophilia and nuclear pyknosis/karyorrhexis. Associated with these lesions was a mixed, inflammatory cell infiltrate, mainly neutrophils, with scattered mononuclear cells. Scale bar denotes 50µm.

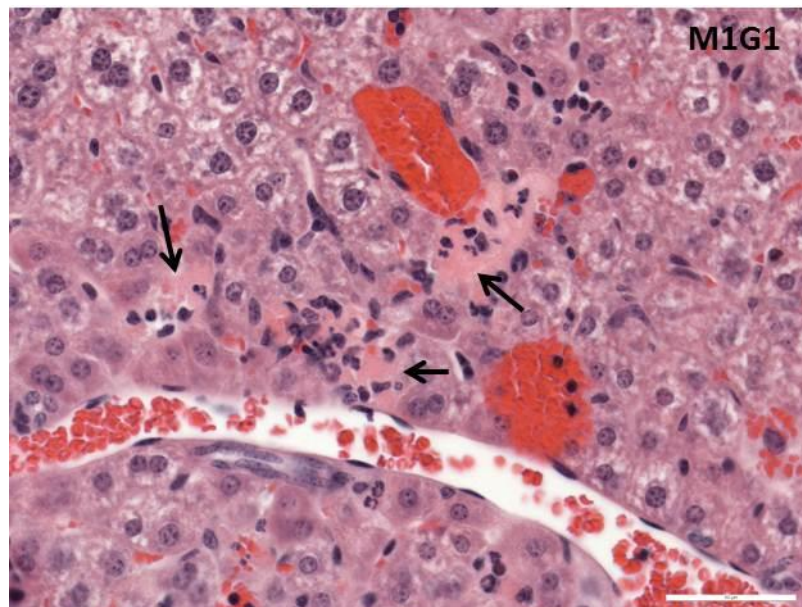


Figure 6.11: Liver of M1G1 infested with HAZV infected ticks. Lesions comprising multiple foci of hepatocellular degeneration and necrosis were seen. Scale bar denotes 50µm.

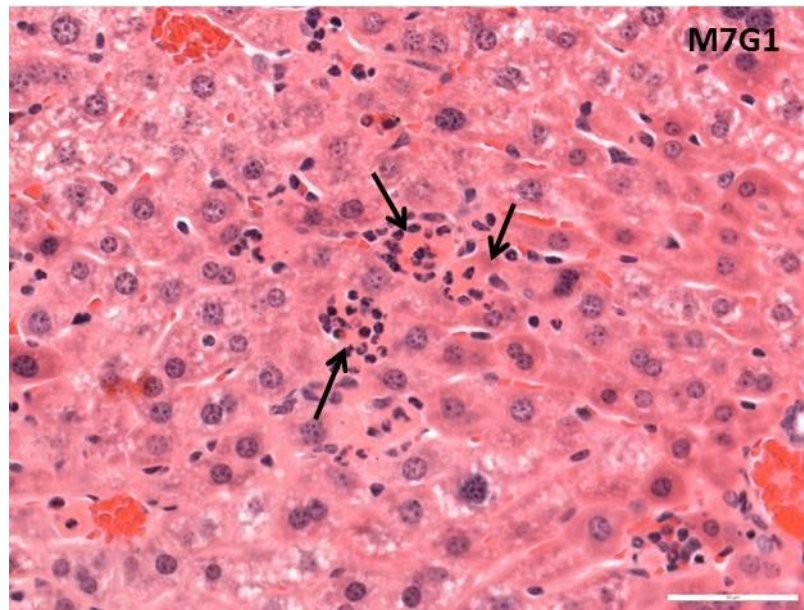


Figure 6.12: Liver of M7G1 infested with HAZV infected ticks. Degeneration and necrosis of hepatocytes with a neutrophil cell infiltrate is seen (arrows). Scale bar denotes 50µm.

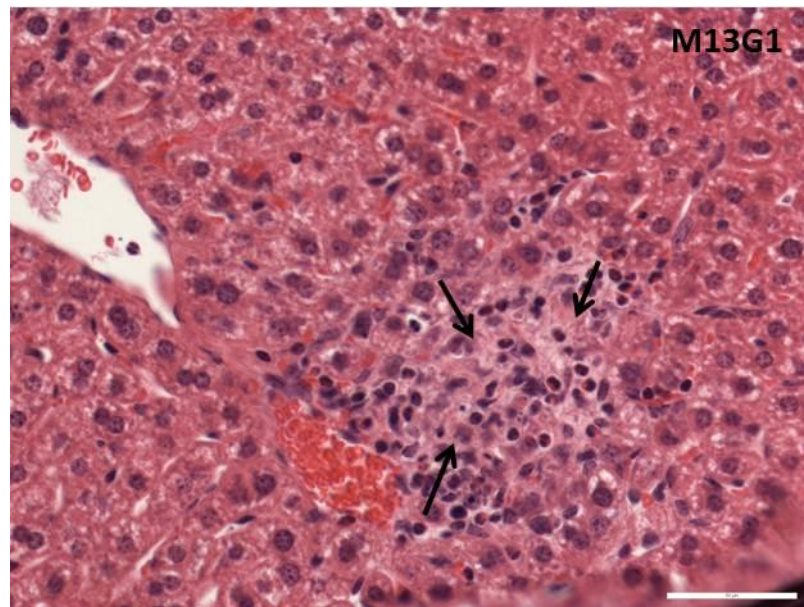


Figure 6.13: Liver of M13G1 infested with HAZV infected ticks. Hepatocyte degeneration and necrosis is seen with infiltration of neutrophils and mononuclear cells mainly lymphocytes (arrows). Scale bar denotes 50µm.

Animals were health scored throughout the study and apart from one animal in group 1 showing lethargy on day 8 and an additional animal showing rapid breathing on day 16 post-infestation all mice in groups 1 and 2 scored healthy. Mice in group 3 exhibited clinical signs consistent with mice inoculated with 20pfu of HAZV; ruffled fur, lethargy and an arched back were seen on day 5 before succumbing to disease on day 6 post-

inoculation; preceded by temperature and weight increase on day 4 followed by a rapid decline on day 5.

6.3.3. Horizontal transmission of HAZV from a viremic host to *I. ricinus* ticks

Ixodes ricinus nymphs were infested on six HAZV infected mice. All mice exhibited a pathological response indicative of Hazara infection. Mice decreased in weight on day 4 with a mean decrease of 0.25% progressing to -5.26% on day 5, -10.03% on day 6 and -13.63% on day 7 (Figure 6.14). Mice became febrile on day 4 and 5 post-inoculation with a mean temperature of 38.15°C across the two days compared to a mean of 36.5°C in the control group (Figure 6.15). All mice showed clinical signs of disease from day 4 with ruffled fur and lethargy, progressing to rapid breathing on day 5 followed by inactiveness on day 6 and 7 (Figure 6.16). Two animals reached humane clinical endpoint on day 6 and the remaining four on day 7 post-inoculation (Figure 6.17). Mice were viremic during the feeding period of *I. ricinus* nymphs with a titre of 3.5×10^3 pfu/ml and 2.3×10^4 pfu/ml obtained from mouse serum on day 3 and 5 post-inoculation. Histological examination of the liver and spleen from each animal was also consistent with HAZV infection with both the liver and spleen having moderate to marked pathological changes (Figures 6.18 – 6.20).

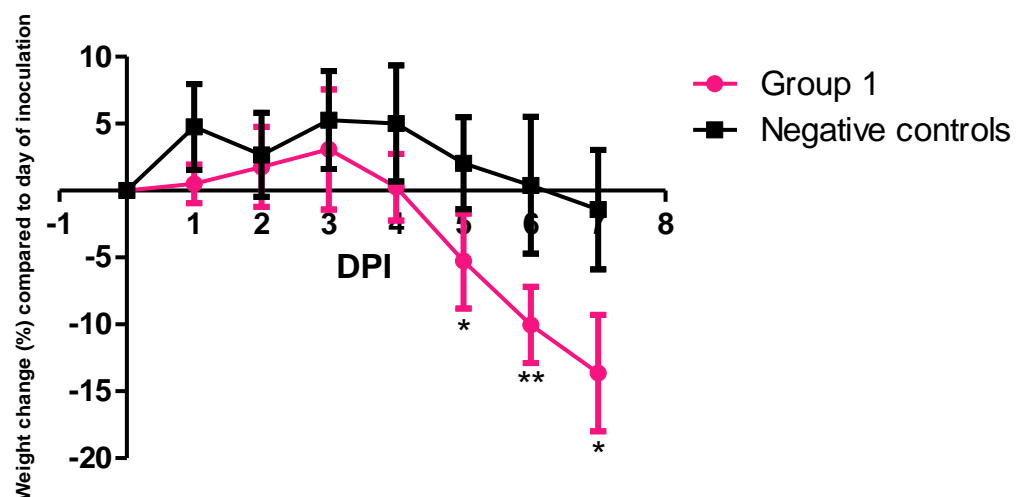


Figure 6.14: Percent weight change of mice intradermally inoculated with 20pfu HAZV (Group 1) compared to the day of inoculation. Mean weight change is shown with error bars denoting standard deviation. Asterisks are illustrated at time point 5, 6 and 7 where P=0.05 is denoted by *, P=0.01 denoted by ** and P=0.001 denoted by ***.

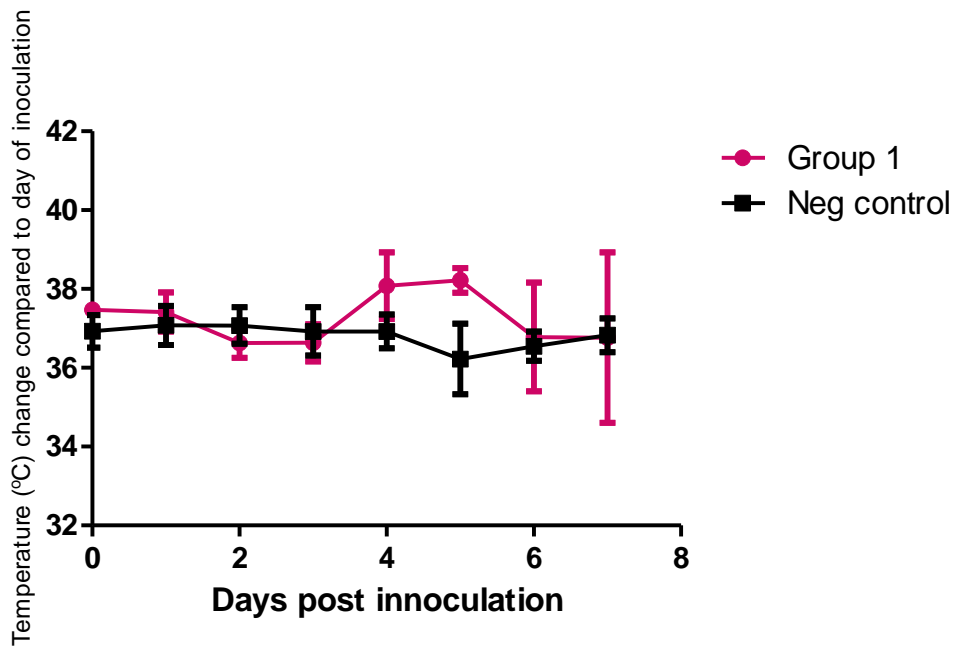


Figure 6.15: Temperature change (°C) of mice compared to the day of inoculation is shown. Mean temperature change is shown with error bars denoting standard deviation.

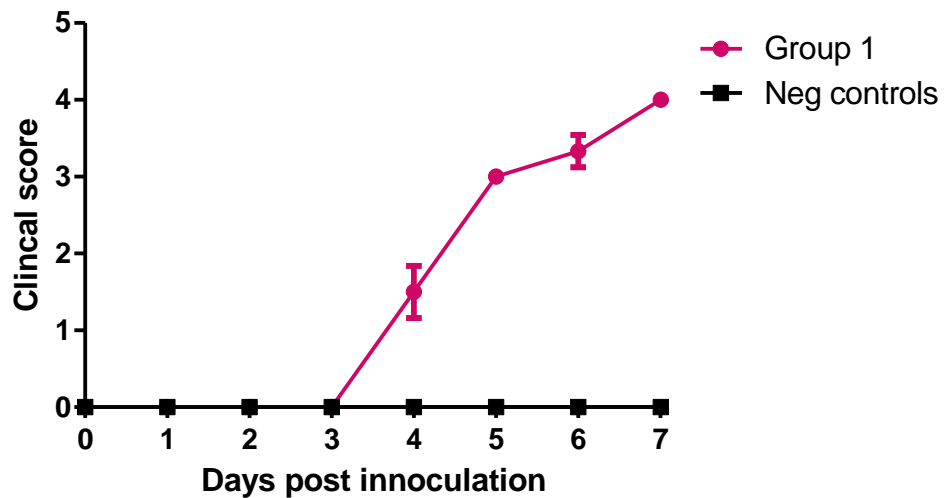


Figure 6.16: Clinical score of mice intradermally inoculated with 20pfu of HAZV and infested with ten *I. ricinus* nymphs and negative controls infested within non-infected ticks. Animals were scored from 1-5 as follows: healthy, 0; ruffled fur, 1; lethargy, 2; rapid breathing, 3; inactive, 4 and death, 5. Mice exhibiting severe signs of disease were euthanased. Mean clinical score is shown with error bars denoting standard error.

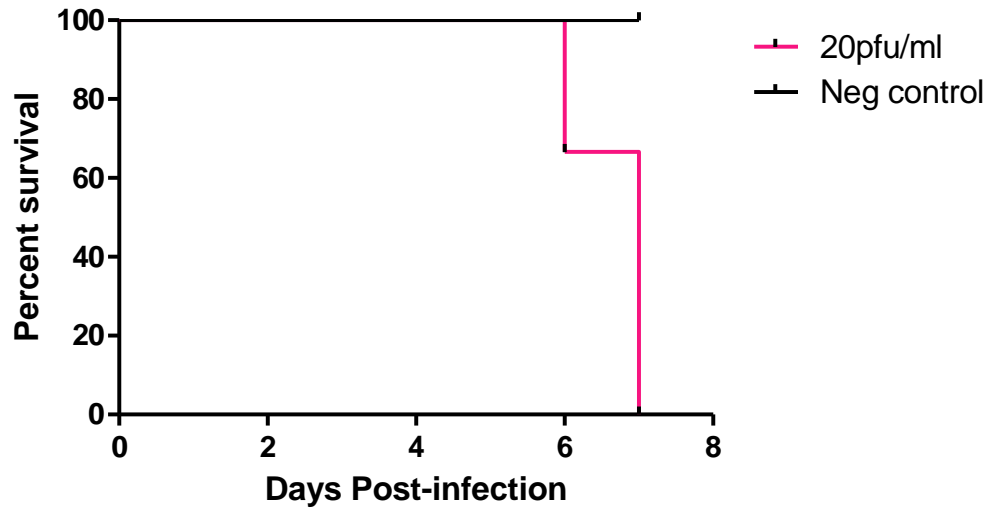


Figure 6.17: Survival of mice inoculated with 20pfu HAZV and negative controls. Two mice reached humane clinical endpoint on day 6 and the remaining four on day 7. All negative control mice survived and were euthanased at the end of the study on day 7.

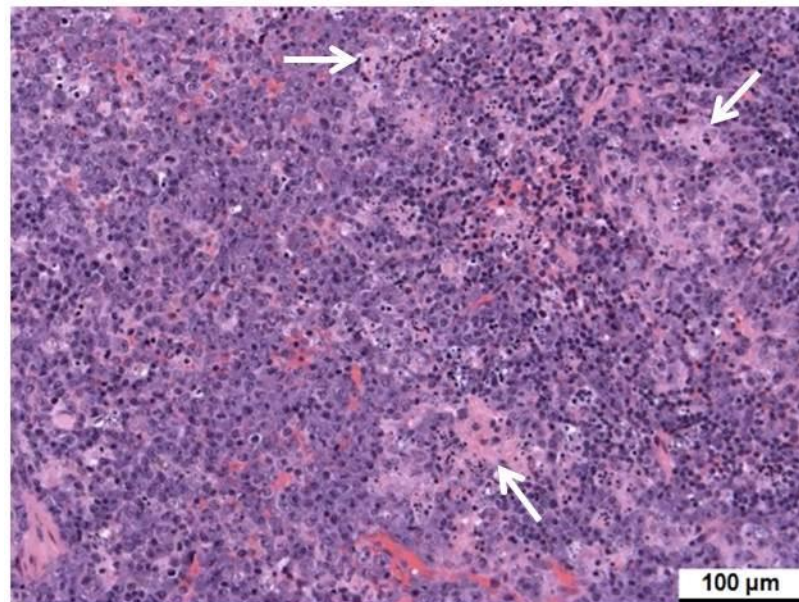


Figure 6.18: In the liver, lesions were observed which comprised multiple foci of hepatocellular degeneration and necrosis scattered randomly throughout the parenchyma, characterised by cytoplasmic hyper-eosinophilia and nuclear pyknosis/karyorrhexis. Associated with these lesions was a mixed, inflammatory cell infiltrate with scattered mononuclear cells.

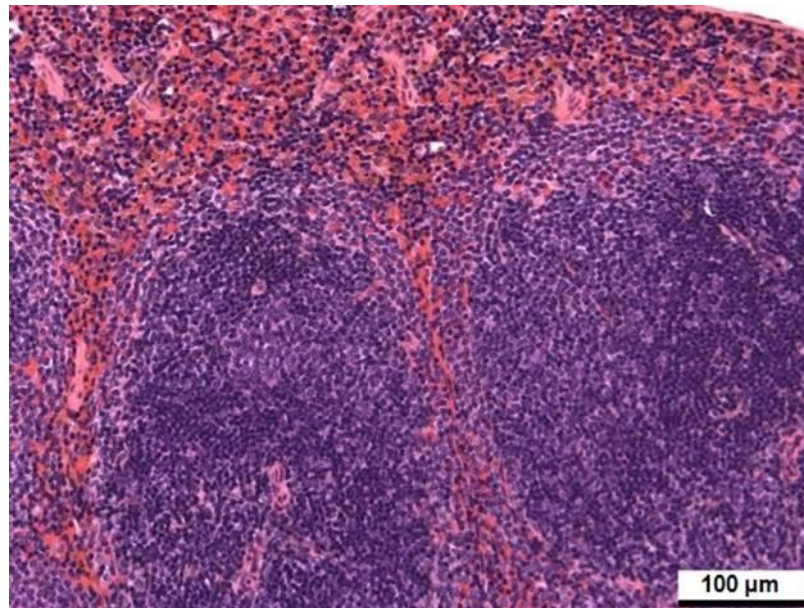


Figure 6.19: Photomicrograph of splenic parenchyma showing normal architecture of the red and white pulp.

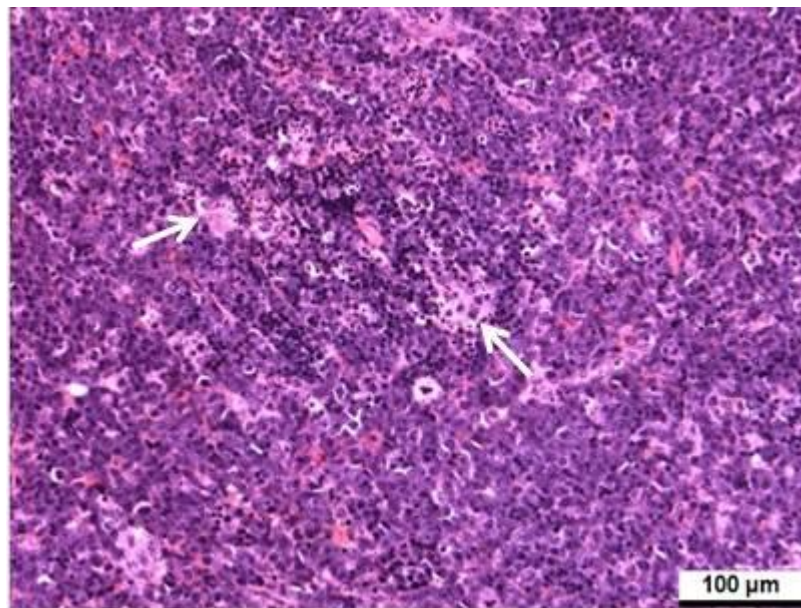


Figure 6.20: In the spleen, white pulp appeared irregular due to multifocally extensive loss of lymphocytes. Associated with these areas were scattered, tinged body macrophages and a variable infiltrate of histiocytes and polymorphonuclear leukocytes (PMNs).

Ixodes ricinus nymphs successfully fed to repletion on HAZV inoculated mice with an attachment and engorgement rate of 73.3% (Table 6.4). This study was performed at the same time as the preliminary tick-host transmission study in section 6.3.1 therefore the same control animals were used.

Table 6.4: Tick Infestation density is shown along with the total number of ticks per group; the percentage attachment and engorgement rate per group and; the number of ticks fed and detached on day 3 and day 5.

Mouse	N° of ticks observed	Attachment success (%)	Mortality rates (%)	Engorgement time	
				DAY 3	DAY 5
G1	60	73.3 (44/60)	26.6 (16/60)	11.66% (7)	61.66% (37)
Control	40	47.5% (19/40)	47.5% (19/40)	0%	100% (19/40)

Engorged nymphs were able to acquire virus via a natural blood meal from a viremic host with a 6.4 – 9.6 fold increase of HAZV compared to the endogenous control tick β -actin. Virus obtained from nymphs feeding on a viremic host was compared to the level of viral RNA obtained from engorged nymphs infected via immersion. Compared to immersed ticks, nymphs feeding on a viremic host acquired 12.4 – 18.6 fold more HAZV (Figure 6.21).

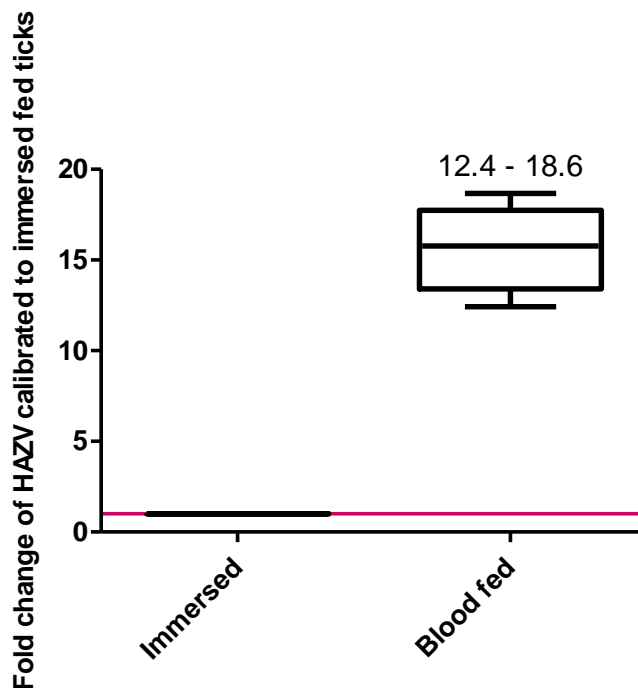


Figure 6.21: Relative fold change of HAZV within immersed infected nymphs engorged on a mouse host and *I. ricinus* nymphs acquiring HAZV via a blood meal from a viremic host. HAZV was normalised to tick- β actin endogenous control and calibrated to day immersed fed ticks. Minimum to maximum values are shown. Each group represents a pool of six engorged nymphs. The solid pink line indicates a fold change of 1, values above this indicate a fold increase in viral RNA detection relative to immersed fed nymphs and values below this indicate a fold decrease in viral RNA detection relative to immersed fed nymphs.

A moulting rate of 9% was achieved with all four adults sent for histological examination (Laudier Histology, USA). All adult ticks were positive for HAZV (Figure 6.22).

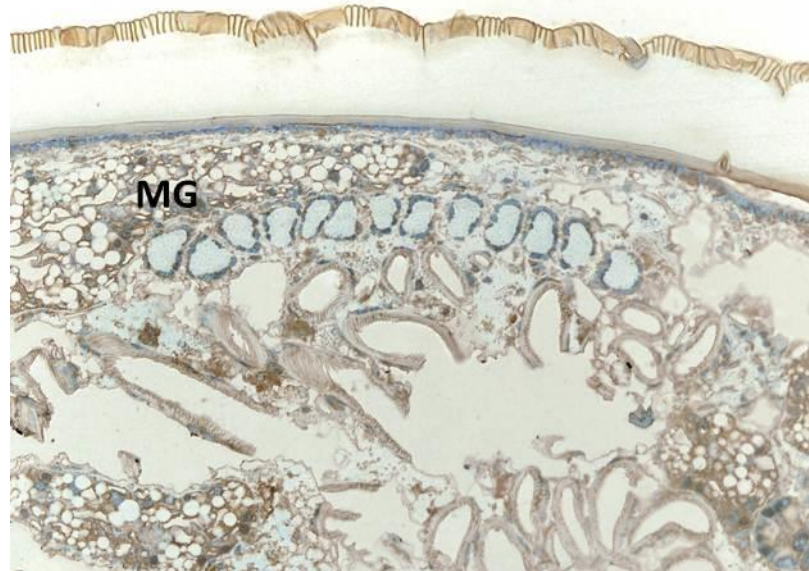


Figure 6.22: A sagittal section of an *I. ricinus* adult tick moulted from nymphs engorging on a viremic host. HAZV can be seen associated with numerous structures including the mid-gut (MG). Positive viral detection is shown by staining with a polyclonal antibody targeting the HAZV nucleoprotein and detected using an anti-sheep horseradish peroxidase secondary antibody. Image was visualised under standard bright-field light under 200 x magnification.

6.4. Discussion

Using A129 IFN- α/β R^{-/-} mice, the animal model for HAZV, it is shown here that *Ixodes ricinus* ticks have the intrinsic ability to transmit HAZV to a susceptible vertebrate host during a blood meal infecting 5/13 mice. *I. ricinus* nymphs are able to acquire HAZV via a natural blood meal from a viremic host with a 6.4 - 9.6 fold increase in HAZV compared to the endogenous control, and maintain HAZV during moulting to the adult stage. This illustrates the ability of HAZV to overcome the infection and dissemination barriers of the midgut and salivary glands associated with blood feeding, a key determinant of vector competence.

HAZV delivered by a tick bite was shown to induce the same histological response as described with intradermal inoculation of HAZV (Dowall *et al.*, 2012) and that of CCHFV (Bente *et al.*, 2010; Bereczky *et al.*, 2010). Viral RNA was detected in both the spleen and liver on day 12 post-infestation of ticks and persisted in both organs to day 21 however virus levels within the liver were constantly higher than the spleen with

infectious virus only obtained from the liver supporting the notion that this is the target organ for HAZV replication (Figure 6.4). Unlike intradermal inoculation of HAZV where mice showed clinical disease and died on days 6 and 7 post-inoculation, mice inoculated via tick bite did not suffer mortality or show any overt clinical signs of disease. It is known that A129 IFN- α/β R^{-/-} mice die from HAZV when inoculated with 10pfu/mouse (Dowall *et al.*, 2012) and 2pfu/mouse (section 5.3.8). In this study mice survived infection from a tick bite and a detectable IgG anti-body response was detected. Ticks are known to contain important bioactive molecules within their saliva to mitigate the host response to tick infestation (Kazimírová and Štibrániová, 2013); vasodilation and platelet aggregation are initially targeted by secretion of IRS-2 at the bite site targeting thrombin disrupting the recruitment of platelets and allowing a feeding pool to be generated (Chmelar *et al.*, 2011); anti-IL-8 has been reported from a number of hard tick species and impairs the chemokine CxCL8 and their recruitment of neutrophils to the bite site and the molecule *Iris* within *I. ricinus* ticks suppresses the secretion of Th1 cytokines inhibiting activation of macrophages, dendritic cells and natural killer cells (Wikel, 2013). The suppression of cell migration to the bite site due to the attachment of the tick and delivery of tick saliva into the wound site may affect the dissemination of the virus and may explain the subsequent delay in infectious virus detection. In this study HAZV is first detected within the localised skin site of tick attachment from day 10 progressing to the liver and spleen day 12 post-infestation, 7 days post-detachment of ticks. This is a delay of 7-9 days compared to intradermal inoculation of mice with 10pfu (Dowall *et al.*, 2012) and corresponds to the pre-incubation period observed in tick bite cases of CCHFV (Hoogstraal, 1979; Swanepoel *et al.*, 1989). This period is characterised by a decrease in platelet and white blood cell counts (Ergönül, 2006) but with no overt clinical signs of disease. In the 2002 outbreak of CCHFV in Turkey the average time for presentation at hospital was 5.5 days after the onset of illness, possibly 8 – 12 days after a tick bite (Ergönül *et al.*, 2004). This delay in clinical presentation may be attributed to the suppression of macrophage and dendritic cell migration to the tick bite site by tick saliva. These cells are important for the replication and dissemination of viral haemorrhagic fevers (VHF) such as CCHFV (Bray, 2005) with virus infecting and replicating rapidly within macrophages releasing pro-inflammatory cytokines resulting in the onset of fever. Infection of dendritic cells is

also important as these cells are antigen presenting cells stimulating an adaptive immune response however, in VHF infections these cells do not mature to fulfil this role contributing to host death (Bray, 2005). The suppression of these cells may contribute to the reduced circulation of virus within the mouse host, initially allowing only localised virus replication within dendritic cells, macrophage and keratinocytes within the skin at the tick bite site (Merad *et al.*, 2008) but allowing enough viral replication and dissemination of cells to induce an immune response. This is particularly important for the non-viremic transmission of TBEV in co-feeding *I. ricinus* nymphs (Labuda *et al.*, 1996).

It is also shown that *I. ricinus* nymphs are competent in acquiring virus from an infective blood meal and maintaining this through to the adult stage. *I. ricinus* ticks replicate HAZV after moulting to adults as seen with *Dermacentor andersonii* ticks infected with Powassan virus (Chernesky, 1969). This is in contrast to TBEV where virus replicates after feeding as seen in partially fed adults collected in Germany which had 9 times higher virus than unfed adults (Süss *et al.*, 2004). It has been suggested that this is a virus survival strategy whereby virus localises with tissues that do not undergo histolysis and it was shown that Dugbe virus associated with hemocytes in *Amblyomma variegatum* ticks carried virus over to the adult stage. It was shown in chapter 4 that HAZV was associated with loose connective tissue and throughout the body cavity and in Figure 6.19 virus was again distributed throughout the body cavity.

Ixodes ricinus ticks exhibited a good attachment and feeding rate on both naive hosts and infected hosts with non-infected ticks exhibiting a reduced attachment and feeding rate on naive and infected hosts. It has been shown that tick behaviour is influenced by pathogen infection; *I. ricinus* adults artificially inoculated with TBEV had increased activity, were more aggressive in their pursuit of a food source and were more tolerant of the repellent DEET and were able to increase their questing height (Belova *et al.*, 2012). *I. scapularis* nymphs were also shown to increase their questing height and were able to overcome physical barriers when infected with *Borellia burgdorferi*, the causative agent of lyme disease (Lefcort and Durden, 1996). The effect of parasites on their vector is also evident with mosquitoes whereby the biting rates of

Anopheles species is increased when infected with malaria sporozoites (Anderson *et al.*, 1999).

I have shown here that *I. ricinus* nymphs are physiologically capable of acquiring and replicating HAZV from an infected host, maintaining this to the next developmental stage and transmitting HAZV to a susceptible naive vertebrate host. To complete the vector competence cycle, the resulting infected adult ticks would be infested onto mice to complete the cycle of TBD transmission. Data shown here supports *I. ricinus* nymphs as competent vector for Hazara virus.

Chapter 7. Discussion

7.1 Introduction

Investigation into the virus-vector interaction of CCHFV with tick species has been so far hindered due to the strict and stringent handling requirements within a CL4 facility and no method of infecting ticks without the use of sharp fine tipped needles (Gonzalez et al., 1991; Shepherd et al., 1989) or direct contact with the vector mouthparts and high titre inoculum (Burgdorfer, 1957). In this thesis I have addressed these two points firstly; by optimisation of the immersion method (Mitzel et al., 2007) for use at high containment providing a convenient and simple method of simultaneously infecting a cohort of ticks in a defined concentration of virus stock without the need for specialised equipment and secondly; I have developed, optimised and established an *In vivo* tick feeding model for use at high containment.

The immersion method has only previously been used with Adult *I. ricinus* and larval *I. scapularis* ticks with variable success (Belova et al., 2012; Mitzel et al., 2007). Here 100% infection of *I. ricinus* nymphs was achieved with ticks maintaining virus up to 97 days post-immersion. HAZV is able to establish itself within the tick, infect key target organs, replicate and produce infectious virus particles. I have successfully optimised and used this method to infect ticks within a CL4 setting, and I have provided evidence to strongly suggest the route of virus acquisition is via the cuticle not the oral cavity as previously suggested (Mitzel et al., 2007).

The use of HAZV as proof of principle has allowed the successful establishment of the first *In vivo* feeding model for use at CL4 in Europe allowing investigation into the vector-pathogen-host interactions of some of the most fatal human zoonotic pathogens. I have used this model to show that *I. ricinus* ticks have the intrinsic ability to transmit HAZV to a susceptible vertebrate host during a blood meal, they are able to acquire HAZV via a natural blood meal from a viremic host and maintain HAZV during moulting to the adult stage. This strongly implicates *I. ricinus* ticks as competence vectors of HAZV.

The animal model of disease for HAZV uses A129 IFN- α/β R^{-/-} mice which succumb to disease 7 days post-inoculation. During my research here, an interesting finding was

the survival of A129 IFN- α/β R^{-/-} mice 21 days after virus transmission via a tick bite. These are animals that succumb to disease 7 days post-inoculation with 2pfu/ml of HAZV (Chapter 5). This is in stark contrast to the published animal model (Dowall et al., 2012). The estimated titre of immersed ticks infested on mice was 20pfu obtained from a cohort of ten ticks which equates to 2pfu/tick. However, this assumes an equal distribution of virus within the tick population which may not be the case. The size of the ticks and the resulting surface to volume ratio may influence the amount of virus absorbed into each individual tick. The key difference between these two models is the route of inoculation. As discussed in Chapter 6 the immune-modulatory effects of tick saliva and the ability to suppress the migration of macrophage and dendritic cells (Wikel, 2013), key sites of replication for viral haemorrhagic fever viruses (Bray, 2005) to the bite site may reduce the dissemination of the virus to target organs. Here infectious virus within mice was not detected until day 12 post-infestation a similar incubation time of 10-14 days observed in mice infested with TBEV infected ticks (Kozuch and Nosek, 1971). This is unlikely to be due to a period of reactivation and replication prior to transmission to a vertebrate host as seen for *Borellia burgdorferi* transmission (Piesman et al., 1987) and *Anaplasma phagocytophilum* (Katavolos et al., 1998) as ticks were infested on day 63 post-immersion when infectious virus was present. Viral RNA was also detected in skin samples obtained from day 10 post-infestation, suggesting localised viral replication at the bite site from day 5 after ticks have detached. As skin biopsies are not sequential, to further investigate the importance of this localised virus and implications for virus maintenance in nature particularly in immune-compromised mice, co-feeding should be investigated *In vivo*.

Ixodes ricinus nymphs exhibit higher attachment and feeding rates on mice at higher infestation densities. Aggregation of ticks on small mammal hosts has been shown to be important for the persistence of TBEV increasing the reproductive number (R_0), which represents the average number of secondary infections arising from one infected tick with $R_0 > 1$ required for a pathogen to establish itself (Harrison and Bennett, 2012; Randolph et al., 1999). The ability of mice used in this PhD to firstly overcome infection by mounting an immune response but also producing infectious virus within the liver may suggest the future circulation of virus in the blood. This needs further investigation but would enable feeding ticks to acquire virus from the

host enabling horizontal transmission and also enable co-feeding transmission to naive ticks. It was also shown that infected nymphs had a higher attachment and feeding rate (75.38%) compared to non-infected nymphs (54%) supporting findings by Belova 2012 that infected TBEV adults were more aggressive in seeking a host and the biting rates of *Anopheles* species increased when infected with malaria sporozoites (Anderson et al., 1999). The implications of this for the maintenance of virus within host species and the subsequent effect of virus persistence and transmission within the UK rodent population should be investigated.

Ixodes ricinus nymphs were competent in acquiring virus from an infected host and maintained this to the next developmental stage, a requirement of vector competence (Kahl et al., 2002). Virus replication was not observed after engorgement of ticks already infected with HAZV but was shown to increase 96-149 fold after adult emergence as seen in CCHFV infected *Hyalomma* ticks (Shepherd et al., 1989) but in contrast to that of TBEV infected adults where virus replication increases prior to moulting (Belova et al., 2012).

Vector competence is a multifaceted relationship between pathogen-vector-host and is influenced by both intrinsic and extrinsic factors. This thesis addresses the intrinsic ability of *I. ricinus* nymphs to acquire, replicate and transmit HAZV. However, in order for a virus to persist and perpetuate within nature, factors such as the availability of hosts (Dobson, 2014), differences in habitat type (Hoch et al., 2010) and the influence of climate (Gilbert et al., 2014) all have an effect. To accurately determine whether a tick species is a competent vector both intrinsic and extrinsic factors should be considered.

In addition to establishing *in vitro* and *In vivo* techniques for tick transmission studies, this novel work required the optimisation of protocols for the handling of live ticks and the extraction of tick RNA. Prior studies have investigated the storage of ticks and other arachnids such as spiders and scorpions for morphological examination and DNA isolation (Corriveau et al., 2011; Mtambo et al., 2006; Vink et al., 2005) all of which recommend 70% ethanol. Whilst this is suitable for nucleic acid amplification it is unsuitable for isolation of infectious viable virus and data presented here is the first comparison of whole tick storage for viral RNA isolation and amplification. To ensure

isolation of quality RNA and accurate quantification of RNA, the optimal storage condition of *I. ricinus* nymphs prior to processing was determined by evaluating RNA yield and quality. Optimal RNA yield was obtained from immediately sampled ticks with no prior storage (14.88ng/ul), however this is not always possible and to ensure consistency in sample processing ticks were flash frozen on dry ice and frozen at -80°C producing a mean RNA yield of 11.38ng/ul. The use of RNeasy later recommended by Qiagen and Life technologies was unsuitable for use with ticks, resulting in ticks floating on top of the solution preventing complete permeation. To ensure efficient RNA lysis and extraction, the optimal homogenisation speed and duration was also determined. It was demonstrated that an increase in duration and speed of homogenisation produced a 2.3 fold increase in viral RNA yield as demonstrated using stock virus in the presence of a tick. It was shown by (Galasso and Sharp, 1965) that 79% of vaccinia virus particles form aggregates and disruption of these using sonic vibrations increases the viral titre and this may be occurring with HAZV particles causing them to separate therefore increasing the viral titre. An endogenous control was also developed for use with *I. ricinus* ticks. This is the first study to compare different genes for use with *I. ricinus* ticks. Tick β -actin was deemed the most suitable with GAPDH resulting in very low amplification at cycle 39 due to the low sequence homology between different tick species. This supported the findings by Browning that tick β -actin was the most suitable endogenous control for use with unfed and fed nymphs.

HAZV is serologically closely related to CCHFV (Casals and Tignor, 1974, 1980) however, there do appear to be differences in susceptibility of *I. ricinus* ticks to CCHFV with no detectable CCHFV within immersed *I. ricinus* nymphs. This does not discount them as a potential vector and the next stage would be to utilise the *In vivo* feeding model to investigate the ability of ticks to acquire CCHFV via a natural blood meal.

With the relaxation of importation requirements of animals entering the UK via the pets travel scheme enforced in December 2011 (Roberts, H, 2011), an increase in the number of ticks entering the UK has increased. The brown dog tick; *Rhipicephalus sanguineus*, confined to the Mediterranean basin and associated with CCHFV (Bursali et al., 2011) has been imported on ten separate cases into the UK (Hansford et al.,

2014) with high infestation levels found in domestic dwellings (Hansford et al., 2015). The importation of *Hyalomma marginatum*, the natural vector of CCHFV, has been detected from a horse imported from Portugal (Jameson and Medlock, 2011) as have *Hyalomma* species on tortoises (L Paul Phipps, 2014). The importation of the known CCHFV vector; *Hyalomma marginatum* ticks via migratory birds into the UK (Jameson et al., 2012) has also been detected as well as the CCHF virus itself by returning travellers from Afghanistan and Bulgaria (Atkinson et al., 2012b; Lumley et al., 2014). This in combination with the potential of *I. ricinus* as a vector for CCHFV, the UK can be considered at an increased risk to CCHFV infection.

Many risk assessments are concerned with the natural tick vector *Hyalomma marginatum* coming into the UK and establishing itself (EFSA, 2010; Gale et al., 2010; Hoek, M, 2012; Maltezou and Papa, 2010) however, the ability of native tick species already established in the UK climate and habitat to acquire and replicate the virus itself would enable an alternate route of entry. This research supports the intrinsic ability of *I. ricinus* to transmit HAZV and has developed the necessary tools to address the important question of whether the most widely distributed tick in Europe is susceptible to CCHFV infection.

This work has resulted in the establishment of a much needed tick *In vivo* transmission model whereby the vector-pathogen-host interaction can be investigated. The handling of ticks at high containment requires strict measures and the establishment and optimisation is a timely process. Here, a model has been developed within a half suit flexible film isolator and adapted to the high containment environment. Safe, durable, and retentive on-host confinement of ticks as well as a safe novel method of applying ticks to the patches have been developed. Using HAZV I have demonstrated the capability of Interferon knockout mice as hosts for *I. ricinus* nymphs and as many disease animal models (section) use immune-compromised mice this model can be used for a wide variety of pathogens.

Due to recent changes in legislation, the migration of host species and the movement of tick vectors via human and animal movements, it is essential to investigate the potential of tick species in becoming vectors for new emerging and re-emerging pathogens. Investigation into the transmission parameters can also be investigated such as the time

taken for CCHFV to be transmitted from an infected tick to a naive host which may provide timely public health information.

7.2 Further work

7.2.1 *Vector competence of I. ricinus ticks for CCHFV*

The immediate next step is to utilise this *In vivo* feeding model to investigate the vector competence of *I. ricinus* ticks to CCHFV as well as determining the transmission time from tick to a host. This may provide important information on the timely removal of ticks attached to humans and the delivery of therapeutic remedies.

7.2.2 *Susceptibility of field caught I. ricinus ticks*

The influence of the natural tick fauna on pathogen acquisition and replication also should be investigated. Laboratory reared nymphs were used throughout this thesis as they reduced the variation produced from using wild caught ticks; the number, developmental stage, gender and fed status could be stipulated.

In other vector species such as mosquitoes the presence of bacterial symbionts are been shown to have an effect on pathogen infection and transmission with differences between laboratory reared and field caught mosquitoes. Field caught *Anopheles* mosquitoes have distinct bacterial symbiotic communities based on their locality with only 20 genera of bacteria shared between 80% of mosquitoes (Boissière et al., 2012). The abundance of Enterobacteriaceae correlated with *Plasmodium* infection status with malaria positive mosquitoes harbouring more of these bacteria (Boissière et al., 2012). *Wolbachia* is shown to influence viral replication of West Nile virus in both mosquito cells and adults. *Wolbachia* infected cells had significantly reduced amounts of secreted virus compared to non-infected cells with virus infection and transmission completely in *wolbachia* infected adults (Hussain et al., 2013).

Bacterial symbionts are known in many tick species and are shown to vary in diversity according to life stage and gender (Moreno et al., 2006; Williams-Newkirk et al., 2014). *Wolbachia* has been detected in field populations of ticks and but the comparison to laboratory reared ticks and the influence on pathogen establishment, replication and dissemination has not yet been investigated. *Ixodes ricinus* show genetic differences

based on the hosts they parasitize and this may have an effect on the symbionts acquired during feeding and therefore pathogen susceptibility (Kempf et al., 2011).

7.2.3 Susceptibility of wild vertebrate hosts

Adequate density of reservoir hosts is necessary for the efficient transmission of tick borne diseases especially as ticks exhibit a pattern of aggregation known as the Pareto principle whereby 80% of the tick population feeds on 20% of the host population (Randolph et al., 1999), therefore adequate host density is required to maintain the tick feeding population. The susceptibility of wild vertebrate hosts and their ecological role in disease maintenance should be determined particularly if *I. ricinus* nymphs are able to transmit a low level of virus which can be maintained by hosts and infect co-feeding immature forms which may exhibit higher susceptibility to virus.

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