Zoonotic Pathogens of Peri-domestic Rodents

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Contents

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I. Abstract

Rodents are important vectors of disease as they have the potential, arguably more than any other wildlife species, to move pathogens across geographical distances. Although there is little known of the prevalence of zoonotic pathogens in UK rodents thus it is difficult to determine the public health risk. The aims of this project were to collect a large range of rodent sample from a variety of peri-domestic locations across the UK that could be used as a representation of the British rodent population and screen them for zoonotic pathogens that could be a potential risk to public health.

Rodent species were sampled from 2014 to 2016 from peri-domestic locations across Northern England, North Wales and Southern Scotland. A total of 333 rodent specimens were collected from this project which included; brown rats (*R. norvegicus*, n=68), house mice (*Mus musculus*, n=105), wood mice (*Apodemus sylvaticus*, n=48), bank voles (*Myodes glareolus*, n=56), field voles (*Microtus agrestis*, n=23), red squirrels (*Sciurus vulgaris*, n=21) and grey squirrels (*Sciurus carolinensis*, n=12). Each rodent carcass was examined post mortem and tissue samples were taken.

Viral zoonotic pathogens that were screened for in this project were Hantavirus (Seoul virus, SEOV, Puumala virus, PUUV and Tatenale virus, TATV), Lymphocytic choriomeningitis virus (LCMV) and Hepatitis E virus (HEV). RNA was extracted from kidney, lung and liver tissue. Each of the viruses were screened for using published pan RT-PCR assays specific to the viral genus. Positive PCR products were Sanger sequenced and phylogenetically analysed. Additional specific RT-qPCR assays were performed for SEOV and rat HEV. An LCMV ELISA was also performed on house mice serum samples. Histological examinations were performed on a subset of samples.

SEOV RNA was detected in 13/68 (19%, 95% CI 0-40%) brown rats and 4/47 brown rats in an RT-qPCR assay. TATV RNA was detected in 7/23 (30.4%, 95% CI, 11.6-49.2%) field voles. No PUUV RNA was detected in this study. The PCR screening results for LCMV revealed an overall prevalence of 8% (26/331, 95% CI 15-36) with LCMV RNA present in 3.2% brown rats, 17.5% house mice, 2% wood mice and 4% bank voles liver tissue. There was no LCMV RNA detected in field voles, red squirrels or grey squirrels. Seroprevalence in house mice was 7% (3/43). No histological changes were observed in the kidney tissue of LCMV infected house mice. In this study, 8/61(13%, 95% CI, 4.6-21.4) of brown rat livers were positive for rat HEV RNA. Lesions and necrosis were observed histologically in 2/3 samples examined, which appears to be indicative of HEV infection based on observations in other HEV infected animals. RT-qPCR results confirmed rat HEV. No HEV RNA of any variant was detected in any other rodent species. This is the first reported detection of rat HEV in a wild rat from the United Kingdom.

Bacterial zoonosis *Campylobacter* in rodents was also investigated in this study. *Campylobacter* from rodent faecal samples was cultured on *Campylobacter* specific media and DNA was extracted. An *lpx* gene PCR was performed to differentiate between *C. jejuni* and *C. coli*. In total, 28% (43/152) rodents were *Campylobacter* positive and of these, 86% (37/43) were shown to be either *C. jejuni* (20/43, 46%) or *C. coli* (17/43, 40%) and 14% (6/43) isolates that were *lpx* negative. House mice were shown to be most commonly infected with *C. coli* (8/10) and bank voles with *C. jejuni* (13/17). In brown rats, 50% (13/26) were positive in which 39% *C. jejuni* (5/13) and 61% *C. coli* (8/13) positive. Whole genome sequencing was also performed on a subset of isolates and sequence types ST-6561, ST-45 and ST-51were identified in brown rats and host-specific sequence type ST-3704 was present in bank voles.

This project has proved that there are multiple zoonotic pathogens circulating in the wild rodent population that could be hazardous to human health. It has also highlighted gaps in our current knowledge, such as the unknown zoonotic potential of some pathogens, such as TATV. In order to comment on the significance of a pathogen to public health the zoonotic potential must be known. The prevalence of known pathogens with known zoonotic potential, such as SEOV, LCMV and rat HEV in people remains unknown. This project has also indicated that there may be possible occupational risks and geographical hot spots for rodent zoonosis. Although further investigation including human surveillance, improved diagnostics and mathematical modeling could be used to determine the risks. This could aid in the prevention of possible outbreaks through improvement of biosecurity, pest control as well as raising public awareness, reduce the risk of exposure and be beneficial for public health in the future.

I. Abbreviations

AKI	Acute kidney injury
AMR	Antimicrobial resistance
ANDV	Andes virus
APHA	Animal and Plant Health Agency
AS	*Apodemus sylvaticus*
CAB	Columbia Agar Base
CCDA	*Campylobacter* Selective Agar
CDC	Centres for Disease Control and Prevention
CI	Confidence Interval
CPV	Cowpox virus
DC	Dendritic cells
DOBV	Dobrava-Belgrade virus
ELISA	Enzyme-linked immunosorbent assay
GBS	Guillain-Barré syndrome
GIT	Gastrointestinal Tract
H&E	Hematoxylin and Eosin
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HEV	Hepatitis E virus
HEV G1	Hepatitis E virus, Genotype 1
HEV G2	Hepatitis E virus, Genotype 2
HEV G3	Hepatitis E virus, Genotype 3
HEV G4	Hepatitis E virus, Genotype 4
HFRS	Hantavirus fever and renal syndrome
HNTV	Hantaan virus
HPS	Hantavirus pulmonary syndrome
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Histology and Immunohistochemistry
LASV	Lassa virus

LCMV	Lymphocytic choriomeningitis virus
MA	*Microtus agrestis*
MG	*Myodes glareolus*
$MgCl_2$	Magnesium Chloride
MM	*Mus musculus*
MPV	Monkeypox virus
NaCl	Sodium Chloride
NK	Natural killer cells
NTC	Negative control
OPD	o-phenylenediamine dihydrochloride
ORF	Open reading frame
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
PTC	Positive control
PUUV	Puumala virus
rat HEV	Rat Hepatitis E virus
RdRp	RNA-dependant RNA-polymerase
RN	*Rattus norvegicus*
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase PCR
RT-qPCR	Real-Time Quantitative PCR
SC	*Sciurus carolinensis*
SEOV	Seoul virus
SNV	Sin Nombre virus
ST	Sequence type
SV	*Sciurus vulgaris*
TATV	Tatenale virus
TULV	Tula virus
WHO	World Health Organisation

Chapter One: General Introduction:

Thesis introduction:

‘Zoonotic Pathogens of Peri-domestic Rodents’

1.1.1. Rodents

Rodents are mammalian species which belong to the order Rodentia which contains 2277 species (Han et al. 2015) that accounts for 41% of all mammalian species on Earth (Harris and Yalden 2008). A highly diverse group of mammals ranging from the 6 g harvest mouse (*Micromys minutus*) to the 60 kg capybara (*Hydrochoerus hydrochaeris*). They are also found in almost every type of habitat, from the Arctic to tropical rainforests to desserts to aquatic and even urban environments (Harris and Yalden 2008). Rodents are important vectors of disease as they have the potential, like many other wildlife species such as birds and bats, to move pathogens across great distances. In 1937, Frank G. Boudreau proclaimed, "*microbes know no frontiers*" in the title of an article promoting the League of Nations' international health week (Knab 2011). He included an illustration of a rat to highlight his point in that human borders (politically defined as well as physical) can easily be penetrated by pathogens, as non-human carriers can cross them with ease. Boudreau used an eye-catching illustration of a rat on a ship which symbolised how non-human carriers of disease can cross defined borders, the role animals have in the spread of disease and their interactions with people (Knab 2011). There have been several recorded incidents throughout human history of infected rats boarding ships and moving pathogens around the globe through international trade. This phenomenon has accounted for the global distribution of many diseases which have been detrimental to human health (Lenz and Hybel 2016).

Rodents have an interesting relationship with humans, as in one sense they can be seen as family pets such as rats, mice, hamsters and guinea pigs and the other as wild animals that are seen as vermin or pests. They are also used in research as laboratory subjects to further scientific knowledge or therapies that would be of benefit to mankind. Therefore, whether it is intentional or not, rodents have a high level of human interaction so there is an opportunity for the transfer of zoonotic infections from rodent to human at this human-animal interphase.

1.1.2. Rodents as carriers of zoonotic agents

The World Health Organisation (WHO) definition of zoonosis is "any disease or infection that is naturally transmissible from vertebrate animals to humans". Of the 1415 pathogens (viral, bacterial, fungal or parasitic) known to be pathogenic to humans, 868 (61%) are zoonotic (Taylor, Latham, and Woolhouse 2001) and it is estimated that zoonotic pathogens are responsible for a billion cases of human illness annually (Karesh et al. 2012).

Rodents are one of the most adaptable and abundant groups of mammals in the world today, and of

the 2277 known rodent species there are 217 which have been identified as reservoirs for 66 known zoonoses (viral, bacterial, fungal or parasitic) and 79 of those are thought to be hyper-reservoirs (being able to carry 2 or more zoonotic pathogens) (Han et al. 2015). Figure 1 shows the diversity of zoonotic pathogens carried by rodent species.

As peri-domestic rodents are wild animals, the control and eradication of disease becomes almost impossible, therefore an emerging zoonotic disease in wildlife is a threat to public health. For example, if a pathogen such *Mycobacterium bovis* (bovine TB), is found to be circulating in a cattle herd, steps can be taken, such as culling infected animals and close monitoring of entire herds eliminate the disease from the farm. However wild badgers have been shown to harbour *M. bovis* and are known to have a role in the transmission of *M. bovis* to British cattle herds (McCulloch and Reiss 2017), making eradication troublesome. Although this is not a rodent, in this case, the same principle can be applied, as when a pathogen is circulating in wildlife it becomes extremely difficult to eliminate it completely.

1.1.3. Rodent Zoonotic Disease Outbreaks; historical to modern day

Rodents have played a significant role in human history as historical pandemics have helped shape today's society. Historical and recent outbreaks of rodent-borne disease highlight how important the rodent reservoir in terms of public health.

1.1.3.1 Bubonic Plague (*Yersinia pestis*)

Throughout history, there have been three Bubonic plague pandemics resulting in catastrophic human loss due to infection with the a rodent zoonotic pathogen, the bacillus bacterium *Yersinia pestis* (Martin 2008). The first *Y. pestis* outbreak and one of the earliest recorded pandemics plague of Justinian (6^{th} to 8^{th} Century) which arrived in the Mediterranean Basin via the Red Sea and spread to throughout Byzantine empire and western provinces of the Roman empire (Green et al. 2014) before reaching Europe and killing an estimated 100 million people (Wagner et al. 2014). The second pandemic to cause devastation across Europe occurred in the 14th to 16th Century and is known as the 'Black Death'. It is thought that between a quarter to a third of Europe's population died with the population in England alone, falling from six million to just over three million (Martin 2008). The third pandemic occurred from 19^{th} to 20^{th} Century and re-emerged in the Chinese province of Yunnan, from which it spread to Hong Kong, Australia, India and several parts of Africa, with an estimated death toll of 15 million (Firth 2012).

It was in the third pandemic that the causative organism, *Y. pesits*, was identified in 1894 in Hong Kong. Four years after the organism was identified, in 1898, the Oriental rat flea (*Xenopsylla cheopis*) was shown to be the vector for *Y. pestis* and the sewer rats were shown to be the source (Firth 2012). An infected flea transfers the bacteria to a rat while taking a blood meal, then bacterium multiplies rapidly causing and extensive septicemia in the rat, thus any other fleas which feed on this rat would easily become infected with *Y. pestis*. When this flea then bites a human the bacteria is transmitted and the pathogenic symptoms of bubonic plague are observed 2-6 days after (Perry and Fetherston 1997). Retrospective studies have shown that rats may have played a significant role in dispersal and transmission of *Y. pestis* (Wagner et al. 2014). At the start of the third pandemic (1855) the disease followed the tin and opium trade, however by 1900 *Y. pestis* had reached ports on every continent due to infected rats boarding the new international trade steamships (Firth 2012). In the 'Black Death' the spread of the pandemic appeared to match the grain trade routes between countries in Europe (Lenz and Hybel 2016). Although recent studies suggest that human ectoparasites, such human fleas (*Pulex irritans*) or body lice (*Pediculus humanus humanus*), were more likely responsible for the second plague pandemic rather than the rats (Dean et al. 2018).

Plague is present in certain areas of the world today; such as Western Africa and it is endemic in California (Holt et al. 2009). There are still 1000 to 5000 cases globally reported and although it is treatable with antibiotics it is still responsible for 100-200 deaths annually (Stenseth et al. 2008; WHO 2004). Plague cannot be eradicated due to the fact that there are a number of wildlife reservoirs (Stenseth et al. 2008).

1.1.3.2. Monkeypox

An unprecedented outbreak of Monkeypox virus (MPV), linked to a rodent source, in the USA in May 2003 resulted in 72 confirmed cases across six states (Eurosurveillance Editorial Team 2004). The symptoms of monkeypox are similar to those of smallpox but significantly milder; they include fever, headaches, exhaustion and a pustular rash and illness typically lasts for 2-4 weeks (Ligon 2004). In this case, the vector for these infections was shown to be the pet prairie dog (*Cynomys* spp) which had been either transported or kept with imported African rodents which were confirmed to be infected with MPV (Eurosurveillance Editorial Team 2004). Of the 800 rodents imported to Texas from Ghana, West Africa, one Gambian pouched rat (*Cricetomys* spp), three dormice (*Graphiurus* spp) and two rope squirrels (*Funiscuirus* spp) were found to be infected with MPV (Ligon 2004). This is an example of how human activity can increase the distribution of zoonotic disease through the movement of the rodent host, in this case, the importation of infected rodents for the pet trade.

1.1.3.3. Lassa fever

Lassa fever is caused by a rodent-borne zoonotic Arenavirus, Lassa virus (LASV). A viral haemorrhagic disease with a fatality rate of 15-50% (Hallam et al. 2018). Transmission is thought to be through contact with rodents or their excretions and the main rodent reservoir is thought to be the multimammate rat (*Mastomys natalensis*), although other rodent species are also thought to be hosts for LASV, such as the African wood mouse (*Hylomyscus pamfi*) and the Guinea mouse (*Mastomys erythroleucus*) (Hallam et al. 2018). In early January 2018, Nigeria and several other West African countries reported an outbreak of Lassa fever. As of the 11th March 2018, there have been 365 cases and 114 deaths across 19 states in Nigeria (Roberts 2018). This highlights the devastation and seriousness of some rodent-borne viruses and why it is important to conduct surveillance in this area to protect public health.

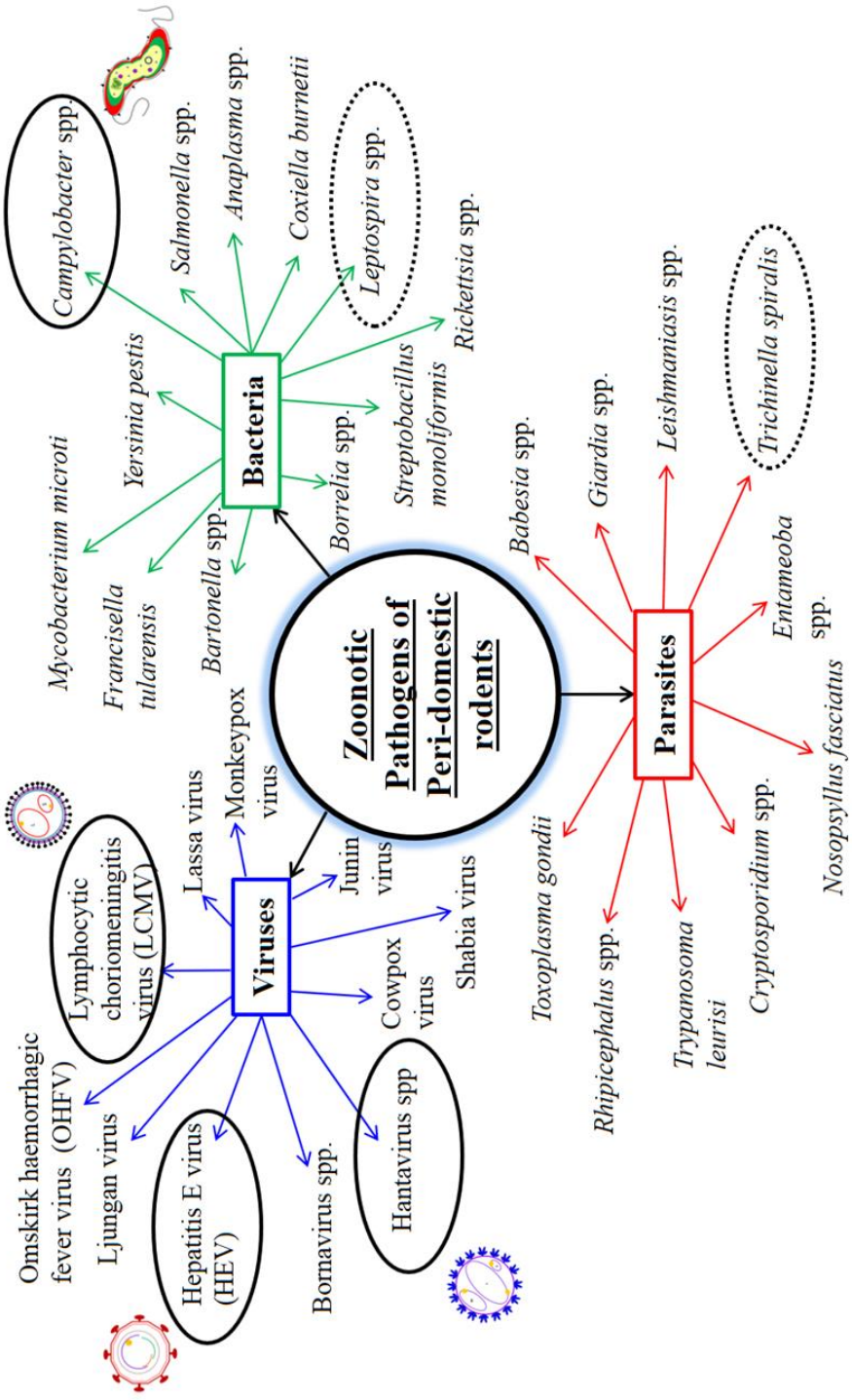

Figure 1.1: The variety of zoonotic pathogens (viral, bacterial and parasitic) known to be present in peri-domestic rodent species across the world. The solid circles represent pathogens investigated in this study and the dotted circles represent pathogens investigated in further studies using material from this project.

1.1.4. Peri-domestic rodent species of the United Kingdom

The English definition of the adjective of 'peri-domestic' is any animal species "*that is of or pertaining to live in and around human habitation*," this definition can, therefore, be applied to a variety of rodent species. There are fifteen different species of rodents (Table 1.1) in the United Kingdom according to the Mammal Society (The Mammal Society 2017) which range from rare species such as the Hazel dormouse (*Muscardinus avellanarius)* to the relatively common Grey squirrel (*Sciurus carolinensis*) to the recently reintroduced Eurasian beaver (*Castor fiber*).

1.1.4.1 Rats (*Rattus* genus)

The rodent species most commonly associated with transmitting infectious diseases to humans is the brown, Norway or common rat (*Rattus norvegicus*) (Figure 1.2a). Originally from Central Asia, it is thought that *R. norvegicus* spread across Europe and to Britain from Russian ships around 1720, largely replacing the ship rat (*Rattus rattus*), which was the dominant species since Roman times. (Harris and Yalden 2008). A highly adaptable and voracious species, the brown rat is able to successfully exploit most environments that it is found in, even in the harshest of conditions. Rats are found in a variety of habitats such as woodlands, grasslands, sewers, farms and even living parallel to humans in densely populated areas such as urban dwellings and cities. This species has a completely omnivorous diet but does prefer protein-rich foods, such as meat, fish, bones, root crops, rice grass and invertebrates, such as earthworms. Rats are also known to predate on smaller rodents such as wood mice or bank voles, due to their larger size and higher levels of aggression, smaller rodents will often inhabit different areas to this species to avoid them. The brown rat is one of the largest rodent species in the UK, weighing 40 g at weaning and growing to over 600 g in adulthood in some cases. They have a long pointed snout, a scaly tail which is almost body length and are usually brownish to grey with a cream or brown underbelly. It is estimated the UK pre-breeding population of brown rats is 6.79 million (Harris and Yalden 2008).

1.1.4.2. Mice (*Mus* and *Apodemus* genus)

House mice (*Mus musculus*) are a peri-domestic species which has the most interaction with humans due to the fact they mostly live in buildings such as houses, sheds and farm buildings. Older buildings with hollow walls or filled with insulation material, such as loft space, are frequent habitats for house mice. People with house mice infestations in their homes often notice the noise of mice living in the ceiling stating it was like 'someone tap-dancing on the ceiling'. R.J. Berry describes house mice as a 'weed' as the species is able to exploit its environment, withstand great adversity and in doing so reproduce rapidly. Females are sexually mature at 6 weeks and able to breed every 4 weeks producing

litters of 6-8 young, resulting in rapid population growth (Berry and Scriven 2005). Although usually found in buildings, house mice can live outdoors and in arable fields or on offshore islands. House mice that live indoors can have extremely small home ranges (<5 m^2) compared to mice which live in the outdoors which can have ranges of 100m^2 (Couzens et al. 2017). These attributes make house mice one of the most successful rodent pests and it is currently ranked the third most important rodent pest species in terms of its impact on humans across the world (Capizzi, Bertolino, and Mortelliti 2014). House mice are much smaller than rats, although young rats can be mistaken for adult mice, as house mice weigh from 12-22 g as adults, grey/brown in colour with small eyes and ears.

The UK has two species of mice that belong to the *Apodemus* genus, the wood mouse (*Apodemus sylvaticus*) (Figure 1.2b) and the yellow-necked mouse (*Apodemus flavicollis*). Both species look very similar, apart from the distinct yellow spot on the underside of the neck of the yellow-necked mouse, hence the name. They have dark to golden upper fur, white underbelly, large protruding eyes and ears and a long tail which is easily sloughed off in times of danger. Wood mice are often larger than yellow-necked mouse and adults weigh between 13-27g (Harris and Yalden 2008). Although both are present in the UK the yellow-necked mouse is only present in some parts of Southern England and Wales, whereas the wood mouse is distributed nationwide and in much greater numbers. Both are a promiscuous and prolific breeder and are able to breed from 7-8 weeks to produce a litter of 4-7 each time (Harris and Yalden 2008). Wood mice are interesting creatures as they are in the middle of the food chain as they predate many species of insects with their omnivorous diet while serving as a significant food source for much of the British wild carnivorous mammals and birds. They often live in grasslands and woodlands and are commonly found in arable fields, particularly in the weedy, food rich microhabitats of these fields (Tew, Todd, and Macdonald 2000). Wood mice may venture indoors in search of food, for example, they are often found in the grain or food stores, especially sugar beet, of farms although it is not thought that this is a source of large economic loss (Harris and Yalden 2008).

Species	Population	Location	Diet	Activity	Habitat
Brown rat (*Rattus norvegicus*)	6.79 million	UK and Ireland	Omnivore	Nocturnal to Diurnal	Farmland, urban dwellings, sewers, salt marshes, waterways, gardens and woodland
House mouse (*Mus musculus*)	5 million	UK and Ireland	Omnivore	Nocturnal	Indoor dwellings
Wood mouse (*Apodemus sylvacticus*)	38 million	UK and Ireland	Omnivore	Nocturnal	Grassland, woodland, moorlands, arable fields and hedgerows
Bank vole (*Myodes glareolus*)	23 million	UK wide and Southern Ireland	Omnivore	Diurnal and Nocturnal	Mature woodland, marshes and hedgerows
Skomer vole (*Myodes glareolus skomerensis*)	20,000	Skomer Island	Omnivore	Diurnal and Nocturnal	Long grass or bracken slopes
Field vole (*Microtus agrestis*)	75 million	UK wide	Herbivore	Nocturnal	Grassland, Young woodland, hedgerows
Orkney vole (*Microtus arvalis orcadensis*)	1 million	Orkney Islands and Channel Islands	Herbivore	Diurnal and Nocturnal	Grassland
Guernsey vole (*Microtus arvalis sarnius*)	150,000	Guernsey	Herbivore	Diurnal and Nocturnal	Grassland
Grey squirrel (*Sciurus carolinensis*)	2.52 million	UK and Ireland	Herbivore	Diurnal	Woodland, gardens
Red squirrel (*Sciurus vulgaris*)*	161,000	Northern England and Scotland, parts of Ireland	Herbivore	Diurnal	Pine and spruce dense woodland, gardens
Harvest mouse (*Micromys minutus*)	1,425,000	Middle and Southern England	Omnivore	Nocturnal (summer), Diurnal (winter)	Tall grassland, meadows, crops and salt marshes
Hazel dormouse (*Muscardinus avellanarius*)*	45,000	South West England and Wales	Omnivore	Nocturnal	Woodland, hedgerows and shrubbery
Yellow-necked mouse (*Apodemus flavicollis*)	750,000	Southern England	Omnivore	Nocturnal	Woodland, especially dead woodland, gardens, hedgerows, rural buildings
Water vole (*Arvicola amphibius*)*	875,000	UK wide	Herbivore	Diurnal	River banks, marshlands, upland streams
Eurasian beaver (*Castor fiber*)	150	Scottish highlands	Herbivore	Nocturnal	Aquatic environments, river banks, woodland rivers
Ship rat or Black rat (*Rattus rattus*)	1,300	Lambay island, Co Dublin	Omnivore	Nocturnal	Cliffs, buildings and ports
Edible dormouse (*Glis glis*)	10,000	South East England	Herbivore	Nocturnal	Beech woodland, rural buildings

Table 1.1: Summary of all the rodent species found in the United Kingdom and Ireland (Harris and Yalden 2008; Couzens et al. 2017; BBC 2011, 2018; The Mammal Society 2018).*Species which are classed as endangered and therefore given legally protected status.

1.1.4.3. Voles (*Myodes* and *Microtus* genus)

Voles are one of the most abundant land mammals in mainland Britain and although they are often mistaken for mice or small rats however they are distinctly different. Within the order of Rodentia, there is the family of *Cricetidae* and subfamily *Arvicolinae* to which voles belong (lemmings and muskrats also sit in this sub-family). The two most common species which are present in the UK are the field vole, or short-tailed vole (*Microtus agrestis*) and a red-backed vole, known as the bank vole (*Myodes glareolus*) (Figure 1.2c), both of which belong in the family *Microtidae*. Both species look fairly similar in appearance with both having a stouter body, rounder head, small ears and eyes and a hairy tail. They are of similar size with adult field voles 90-110 mm in length and weighing 20-40 g, whilst bank voles are between 80-120 mm in length and weigh between 15-40 g (Couzens et al. 2017). The field vole is often broader and has a distinctive short hairy tail, their coat is a greyish brown colour, where the bank vole is slimmer in shape, has a longer tail and its coat is reddish brown in colour (Harris and Yalden 2008). They both inhabit similar environments, however field voles are more common in grassland areas with a diet of mostly stems of grass, green leaves and bark, where bank voles are more often found in woodland habitat as they have a more varied diet than field voles living off grass, mast crops, flowers, berries, fungi and small insects and worms. Voles often live 3-6 months in the wild and it is rare for a vole to survive longer than 12 months due to the fact they are often prey for many other species and they do not hibernate over winter. The breeding season is from early spring to early autumn and in this time voles are capable of massive population growth, resulting in peak population numbers in the autumn months, like many other rodent species (Cooper, 2010).

1.1.4.4. Squirrels (*Sciurus* genus)

The UK has two species belonging to the *Sciurus* genus, the native red squirrel (*Sciurus vulgaris*) and the invasive grey squirrel (*Sciurus carolinensis*). The red squirrel weighs from 250-300g and can mostly be found in pine or spruce woodland. Once widespread throughout the UK, the red squirrel is now mostly restricted to Northern England and Scotland. Populations are in decline due, in part, to the outbreak of a fatal viral disease, Squirrel Pox, but mostly due to the out competition by the grey squirrel which was introduced from the USA between 1876-1929(Couzens et al. 2017). Not only is the grey squirrel more resistant to Squirrel Pox virus but is also much larger than the red squirrel, weighing between 400-600g. The grey squirrel also resides in woodland but is confident enough to spend time on the ground so can often be seen near human habitation such as in parks or gardens (Couzens et al. 2017).

Figure 1.2: Peri-domestic rodent species. (1.2a) A brown rat (*Rattus norvegicus*) photo by G. Kluiters, (1.2b) a wood mouse (A*podemus sylvaticus*) and (1.2c) a bank vole (*Myodes glareolus*) both photos courtesy of M. Bennett.

1.2. Rodent-borne zoonotic pathogens of significance in the United Kingdom

There are several rodent-borne zoonotic pathogens thought to be circulating in British rodents that could be a significant threat to public health. There have been reported cases of fatalities which have resulted from the infection with a rodent-borne pathogen, such as the fatal case of a male guest house and stable owner who contracted Weil's disease, which results from the infection with a member of the bacterial genus *Leptospira* (Forbes et al. 2012). There was a rat infestation at the patient's home and it was later determined that this was the likely source of the bacteria (Forbes et al. 2012). There have also been fatal cases of Pulmonary tuberculosis due to infection with *Mycobacterium microti*, of which the reservoir host is the field vole, where a 39-year old immunocompromised man who was HIV positive died despite medical treatment (Emmanuel et al. 2007).

There are some rodent zoonotic viruses in which human infection and disease can result despite there being no direct contact with rodents themselves, but from the pathogens shed in rodent secretions which people encounter in the environment. Hantaviruses and Lymphocytic Choriomeningitis virus (LCMV) are both examples, as both of these are known to cause infection and disease in people due to the inhalation of aerosolised virus in excretions produced by rodents (L. M. McElhinney et al. 2017; Lapošová, Pastoreková, and Tomášková 2013). The prevalence of both of these viruses in British rodents is not known. Contamination food chain could also be a source of human infections, as in the case of enteric bacterial pathogen *Campylobacter* spp (Humphrey, O'Brien, and Madsen 2007) and emerging viral pathogen Hepatitis E virus (HEV) (Berto et al. 2012). The extent to which rodents are maintaining or increasing the prevalence and transmission of these pathogen remains unclear. This project aims to investigate the prevalence of these four pathogens, with the background and significance explored in detail in the rest of this chapter.

1.3. Rodent Viral Zoonosis: Hantavirus

The *Orthohantavirus* genus belongs to the Family of *Hantaviridae* within the Order *Bunyavirales* contains at least 35 species (ICTV 2018) of hantaviruses which cause disease of varying degrees of severity in people (Cunze et al. 2018). The first human outbreak of hantavirus disease occurred during the Korean war (1950 to 1953), in which over 3000 American and Korean soldiers became infected with a then-unknown viral agent resulting in haemorrhagic fever (Mir 2010). It was not until 25 years later, in 1978, that the infectious viral agent was revealed to be a hantavirus, Hantaan virus (HTNV) (Lee, Lee, and Johnson 1978). Hantaviruses have since been shown to establish persistent infections in mammalian hosts, in particular, the species belonging to the order Rodentia (Meyer & Schmaljohn

2000). Although, other mammalian hosts such as bats and insectivores such as shrews and moles have since been identified as hosts for hantaviruses (Meyer and Schmaljohn 2000b; Zhang 2014). The involvement of a rodent host was shown when HNTV was detected in the lung tissue of the striped field mouse (*Apodemus agrarius*) (Lee et al. 2004) and then the successful growth of *A. agrarius* derived HNTV in A549 cell lines (adenocarcinomic human alveolar basal epithelial cells) in 1981 (Mir 2010). HNTV typifies the relationship between hantaviruses and their maintenance host. Generally, rodent host species are persistently infected with certain hantaviruses without succumbing to the pathogenic effects seen in humans and are therefore reservoir hosts for these viruses (McCaughey & Hart 2000).

Hantaviruses are single-stranded negative-sense RNA viruses (Figure 1.3), 70-350 nm in diameter/length, and have a roundish appearance when observed under electron microscopy (Hepojoki et al. 2012). The viral genome is tri-segmented with small (S) segment (1.8-2.1kb), medium (M) segment (3.7-3.8kb) and large (L) segment (6.5-6.8kb). The S segment is thought to encode the nucleocapsid (N) protein and the L segment which encodes the RdRp enzyme, which is essential for viral replication. The M segment encodes the glycoprotein spike complexes (Gn and Gc) which are impregnated in the viral envelope and it is likely that these play a role in the entry of the virus into host cells (Hepojoki et al. 2012).

The virions are transmitted via rodent excretions, most often urine, faeces and saliva (Muranyi et al. 2005). Person to person transmission of hantaviruses has not been reported, with the exception of Andes virus (ADNV) (Padula et al. 1998). Other rodents, of the same species, in the population that share the same environment, inhale the aerosolised virus and thus, the virus is then able to establish a persistent infection in a new rodent host of the same species. Humans are accidental hosts of hantaviruses, as spillover infection occurs when humans inhale aerosolised virus from the excretions of infected rodents (Jonsson et al. 2010). How hantaviruses travel throughout the human body after inhalation is not fully understood, although it has been suggested that infection of immature dendritic cells (DC) may have a significant role in virus transportation and immune system evasion (Mir 2010). Increased vascular permeability and decreased platelet count are classic indicators of hantavirus infection in humans. Hantaviruses are not cytopathogenic, so the clinical signs seen in humans are thought to be due to the immune system response to viral infection. The reason why pathology is observed in humans and not in rodents is remains unclear, although one hypothesis, proposed by Mir 2010, is that the signals produced from the infected rodent DC's stimulate the T-regulatory cells to down regulate the cytotoxic T lymphocytes, thus the immunopathogenic effects are not seen in the

rodent hosts (Mir 2010).

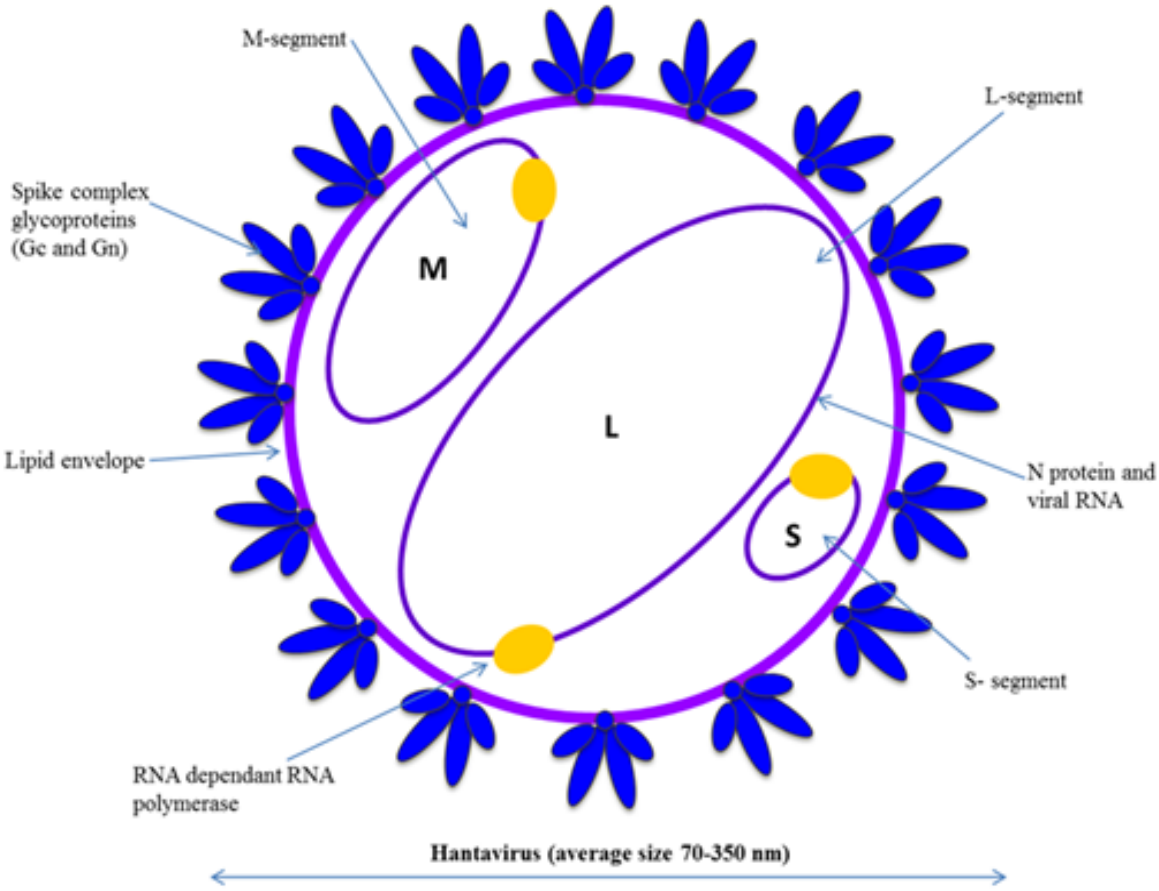

Figure 1.3: A schematic representation of the hantavirus virion adapted from the structure described in Hepojoki et al. 2012.

The severity of infection in humans can range from asymptomatic to severe and can even be fatal depending on a variety of factors, such as the immune status of the individual and which species of hantavirus is involved. Hantaviruses can be divided into two distinct groups based on the clinical manifestations in humans and the geographical locations of the rodent host (Jonsson, Figueiredo, and Vapalahti 2010). The New World hantaviruses include apparently non-pathogenic species but often cause more severe Hantavirus Pulmonary Syndrome (HPS), with a mortality rate of 35-50% (Kruger et al. 2015). In HPS the viral target is the microvascular endothelium, with initial 'flu-like' symptoms such as myalgia, headache and fever which can be followed by gastrointestinal distress such as abdominal pains, vomiting and diarrhoea. After the first five days, severe symptoms appear with pulmonary oedema, hypotension and shock which can rapidly become fatal (MacNeil, Ksiazek, and Rollin 2011). A survivor of HPS described the disease as a "tight band around my chest and a pillow

over my face" due to the accumulation of fluid in the lungs (CDC 2016). Rodent species of the *Sigmodontinae* are found in the Americas and include the deer mouse (*Peromyscus maniculatus*), which carries Sin Nombre virus (SNV) which was responsible for an outbreak in the Four Corners region of the US in 1993 where 50% (12/24) of infected people died (CDC 1993)and a recent outbreak in February 2017 in Washington State where there were five cases of HPS, three of which were fatal (Washington State Department of Health 2017). In South America, ANDV, carried by the pygmy rice rat (*Oligoryzomys longicaudatus*) has been responsible for several outbreaks of HPS (CDC 1993; Jonsson, Figueiredo, and Vapalahti 2010).

The Old World hantaviruses are mostly found in Europe and Asia and are often associated with the milder form of clinical disease, although degrees of severity vary depending on the species of virus, from asymptomatic to haemorrhagic fever and renal syndrome (HFRS), for which the mortality rate can range from 1-15% (CDC 2018). Symptoms can be mild, which can include fever, headache, back pain, abdominal pain and hypotension (Hansen et al. 2015). Due to a similarity in clinical signs and the link to rodents HFRS can be misidentified as a *Leptospira* infection (Izurieta, Galwankar, and Clem 2008). More serious symptoms are multisystemic haemorrhage and acute renal failure (Hansen et al. 2015). Some rodent species of the *Arvicolinae* subfamily are known to be reservoir hosts for HFRS hantavirus species, such as the bank vole (*Myodes glareolus*), which harbours Puumala virus (PUUV), and the common vole (*Microtus arvalis*), which carries Tula virus (TULV). On the whole, the more pathogenic (to humans) hantaviruses, causing HFRS, are those transmitted by the *Murinae*, while those carried by voles tend to induce only a very mild clinical HFRS or nephropathia epidemica (NE) with a mortality rate of less than 1% (Hjertqvist et al. 2010). Rodent hosts also include members of the *Murinae* subfamily such as the striped field mouse (*Apodemus agrarius*) as a carrier of HNTV in Korea, the yellow-necked mouse (*Apodemus flavicollis*) as a reservoir host for Dobrava-Belgrade virus (DOBV) in the Balkans and the brown rat (*Rattus norvegicus*) which carries Seoul virus (SEOV) worldwide. Recently it has been recommended to refer to human clinical disease as 'hantavirus fever' or 'hantavirus disease' instead of the previous HFRS or HPS clinical terms (Clement, Maes, and Van Ranst 2014; Clement et al. 2016). The two syndromes frequently overlap, with pulmonary involvement in HFRS cases (Connolly-Andersen et al. 2013) and renal impairment in HPS cases, therefore the presence, or absence, of particular clinical observations, can often lead to underreporting or misdiagnosis, particularly in the case of SEOV infections in the New World. Although both terms, HFRS and HPS, are still frequently referred to in the literature.

Retrospective studies have suggested that Hantaviruses could have been the etiological agent responsible for several epidemics with numerous human casualties. A hantavirus is suspected to be the agent responsible for five outbreaks of English Sweating sickness from 1485 to 1551, in which the mortality rates were between 30% and 50% (Heyman, Simons, and Cochez 2014). In times of conflict such as the American civil war (1862-1862) an outbreak of 'war nephritis', in which 14,000 individuals were affected, was thought to be due to infection with an unknown hantavirus. Another example the epidemic of 'trench nephritis' is in World War I in which the symptoms closely matched those of nephropathia epidemica (NE) which is associated with Puumala virus infection (Heyman, Simons, and Cochez 2014), and, now widespread in modern Europe (Vapalahti et al. 2003).

1.3.1. UK Hantaviruses: Seoul virus (SEOV)

In recent years there has been an increasing number of human HFRS cases in the United Kingdom (Table 1.2) which were suspected to be the result of locally acquired Seoul virus (SEOV) infection from an indigenous UK rodent, the brown rat (*Rattus norvegicus*) (McElhinney et al. 2017). Brown rats are widespread throughout the UK, with an estimated pre-breeding population of 6.79 million (Harris and Yalden 2008). The first reported HFRS cases in the UK occurred in 1977 when four laboratory workers became infected hantavirus, likely SEOV, after exposure to the virus from the laboratory animals they were working with. It is suspected that infection was due to exposure to aerosolised virus during the disposal of rodent material in a macerator, which was frequently opened mid-cycle (Lloyd and Jones 1986).

Year	Location	Likely exposure or source detection	Details	Reference
1977	Sutton, UK	Rat (Laboratory)	Four laboratory workers with clinical HFRS, Seropositivity in lab rats for hantavirus antibodies	(Lloyd and Jones 1986)
1983	Glasgow, UK	Unknown	21-year-old male seropositive and clinical HFRS	(Walker et al. 1985)
1988	Glasgow, UK	Boating pond attendant, possibly wild rats	18-year-old male seropositive and clinical HFRS	(Kudesia et al. 1988)
1983-89	UK	Cat (pet and feral)	9.6% (15/157) seropositive for hantavirus antibodies	(M. Bennett et al. 1990)
1991	Somerset, UK	Garden centre supervisor, possibly wild rats	21-year-old male seropositive and clinical HFRS	(Pether and Lloyd 1993)
1991	Somerset, UK	Unclear, possible exposure to wild rodents around local waterways	A 42-year-old male and 64-year-old female clinical HFRS	(Pether, Jones, and Lloyd 1991; Phillips et al. 1991)
1991	Sheffield, UK	Poultry farm visit and rat infestation	A 16-year-old female and 18-year-old female clinical HFRS	(Rice, Kudesia, and Leach 1993)
1991-93	Hereford/Preston, UK	Occupational exposure of farmers to wild rats	41 farmers seropositive for hantavirus antibodies	(Coleman 2000)
1992	Somerset, UK	Farm and sewage workers exposure to wild rats	26 clinical HFRS farmers and sewage workers	(Pether and Lloyd 1993)
1994	Nottingham, UK	Recent rat infestation	10-year-old male clinical HFRS	(Watson, Irving, and Ansell 1997)
1996	N. Ireland	Rat, wood mouse, house mouse, (wild)	11/51 rats, 1/31 wood mice and 17/59 house mice were seropositive for HTV	(McCaughey et al. 1996)
2011	Sweden	Rat (pet - export to Sweden from the UK)	1/20 rat SEOV RNA positive	(Lundkvist et al. 2013)

2011	Wrexham, UK	Rat (pet)	28-year old male clinical HFRS, pet rats SEOV RNA positive	(Jameson, Taori, et al. 2013)
2011	Yorkshire & the Humber, UK	Rat (wild)	1 59-year-old pig farmer clinical HFRS, 2/4 rats were SEOV RNA positive	(Jameson, Logue, et al. 2013)
2013	Gloucestershire, UK	Rat (pet, breeding colony)	2 females (mother and daughter) clinical HFRS, 20/21 Seropositive, 17/21 rats RNA SEOV positive	(McElhinney et al. 2017)
2013	Cheshire, UK	Field vole (wild)	1/8 TATV RNA positive	(Pounder et al. 2013)
2013-14	UK	Rats (pet and wild)	Seroprevalence study of UK residents, seroprevalence detected in 34.1% pet rat owners, 2.4% occupational exposure to wild rats and 3.3% random blood donors	(Duggan et al. 2017)
2015	Northumberland, UK	Field vole (wild)	8/48 TATV RNA positive	(Thomason et al. 2017)
2015	Glasgow, UK	Rat (pet)	Male rat owner clinical HFRS	(McElhinney et al. 2017)
2015	Cardiff, UK	Rat (pet and breeding rats)	3 Males (23, 26 and 56 years old) clinical HFRS	(McElhinney et al. 2017)

Table 1.2: Summary of hantavirus surveillance, detection and outbreak investigations in UK rodent species and humans from 1977 to the present day. Adapted from McElhinney et al 2017.

Since 1977, there have been several clinical cases of HFRS across the multiple regions of the UK, as well as multiple detections of hantavirus RNA or seropositivity in rats (McElhinney et al. 2017). Table 1.2 shows a summary of UK hantavirus reports from 1977 to the present day. On the whole, the species of hantavirus responsible was not identified, as this is difficult in serological assays, although in some of these cases it was suspected that infections were a result of exposure to SEOV from a wild rat source, although no conclusive source was found (J. V Pether and Lloyd 1993). In the case of the HFRS patients in Somerset in 1991 it was suggested that people likely became exposed

to the hantavirus due to living or working near to a rat-infested sewage works or engaging in hobbies which could bring them close to rat populations (J. V Pether and Lloyd 1993). The continual reporting of clinical HFRS cases, in which there was no pet or laboratory association, suggests that hantaviruses, possibly including SEOV, may be prevalent in the native UK wild rat population.

In 2012 the first UK SEOV strain derived from a wild rat source was identified after a reported clinical HFRS case in the Humber region (Jameson et al. 2013). In December 2011, a 59-year-old man presented at Hull Infirmary with a two-day history of fever, rigors, anorexia and a dry cough. He was admitted to hospital and blood results indicated acute kidney injury (AKI), elevated liver enzymes, lymphopenia and thrombocytopenia (K. Adams et al. 2014). He tested negative for leptospirosis, legionellosis and hepatitis. He lived and worked on a small pig farm in East Yorkshire and reported seeing high numbers of rats on this farm in the months before his illness. A serum sample was sent to Public Health England (PHE), which confirmed hantavirus infection (Adams et al. 2014) Following this, a PHE investigation was conducted where four rats from this farm were trapped and screened for hantavirus RNA. Two out of the four rats were positive for hantavirus RNA and the first UK wild rat SEOV strain (Humber strain) was reported (Jameson et al. 2013).

Human SEOV infection is not only derived from wild rats, but there also have been several reported HFRS cases in the UK in which SEOV exposure has been from pet rats (the domesticated form of *R. norvegicus*) (McElhinney et al. 2017). The domestication of rats first emerged during the early 19th Century, when rats were captured and bred for fighting in the rat 'pits', as a common form of entertainment. The owners of these establishments would keep any rats which were interesting colours and breed from them. These rats were then sold on as pets while their plain brown siblings were used in the pits, thus from the 19th Century rats became domesticated and, although the same species, a dual identity developed were rats were seen as either a family pet or dangerous vermin (Edelman 2002).

According to the Pet Food Manufacturers' Association (PFMA), there were 100,000 pet rats in 24,000 UK households (PFMA 2018). The first UK pet rat associated clinical HFRS case occurred in January 2013 in which a 28-year-old man from North Wales was diagnosed with acute kidney injury as a result of SEOV infection, due to exposure to the virus from his two agouti (breed) pet rats (Jameson, Taori, et al. 2013). It was likely he became infected through the handing of his rats or contact with infected bedding material when cleaning out the rat cages. Pet rats are also, as with wild rats, are unlikely to show any signs of disease while at the same time, intermittently shedding virus

throughout their life, so their owners would not have known their pet was infected (McElhinney et al. 2016). In a serological survey conducted by Public Health England in 2013-2014, 27/79 (34.1%) of pet rat owners were found to be seropositive for hantavirus antibodies, indicating that SEOV could be widely circulating in the pet rat community (Duggan et al. 2017).

Pet rat associated SEOV can be seen as an important public health issue due to the ease of horizontal transmission between rats in the pet rat colony, which can lead to most members of the colony becoming infected. For example, a high prevalence of SEOV was detected in a breeding colony in Cherwell in February 2013 (McElhinney et al. 2017). All the rats screened shown to be serologically (20/20) hantavirus positive and SEOV RNA was detected in 14/21 lung samples and 15/21 kidney samples, or in 17/20 rats in either organ (McElhinney et al. 2017). It is common practice for 'fancy' rat owners to take their pets to rat shows and 'swap meets' where there is frequent mixing and exchanging of different rat pet rats from different breeding colonies, which could increase the risk of transmission of SEOV within the pet rat community (McElhinney et al. 2017).

Thus lab, wild and pet rats can be seen as a potential risk to public health with regards to transmission of SEOV to humans. The strains detected in pet and wild rats in the UK belong to lineage 9 of phylogroup A of SEOV and have common ancestry. The brown rat or Norway rat (*R. norvegicus*) originated in south-east Asia, and only reached Europe, including the UK, relatively recently with the first reports in the UK date back only 300 years (Harris and Yalden 2008). Comparison of mitochondrial DNA sequences of rats suggests a wide diversity in mountainous regions of China, where the species is thought to have originated. However, there has been little diversity observed in rats from more anthropogenic environments across not just China, but the rest of the world suggesting that only one lineage of rat is responsible for its global spread. The recent evolutionary history of rats is mirrored in the phylogeny of SEOV, which is much more diverse in China, especially in mountain regions, with only one phylogroup (Group A) found worldwide (Lin et al. 2012).

Although there is common ancestry among SEOV strains, inferred from the small number of sequences available, there is sufficient genetic distance to be able to differentiate between pet, wild and lab strains, suggesting little transmission between these populations of rats. In terms of prevalence, there is very little information on SEOV in the British rat population to be able to accurately estimate whether there may be a significant public health risk. There may be more information available for pet rats as there is more traceability and in most cases, the source of human infection is easily identified, for example, the pet rat in the home. There is also increasing evidence

that there may be widespread dispersal of SEOV in UK pet rats (McElhinney et al. 2017; Duggan et al. 2017), this is not the case with wild rats, and therefore the true prevalence of SEOV in UK wild rats still remains unknown.

1.3.2. UK Hantaviruses: Tatenale virus (TATV)

SEOV is not the only hantavirus species known to be present in UK rodents. In 2013, a field vole (*Microtus agrestis*) from Cheshire was shown to be infected with a novel hantavirus species, Tatenale virus (TATV) (Kieran C. Pounder et al. 2013). Hantavirus RNA was detected in the kidney and lungs of the vole (B41) but not the liver. Comparative phylogenetic analysis of B41 to other vole (*Arvicolinae*) hantaviruses showed B41 to have 65.7%–78.8% similarity for S segment and 76.6%–77.5% similarity for L segment at the nucleotide level. This determined that TAV was distinct enough to be considered as a new species of hantavirus (Kieran C. Pounder et al. 2013).

Subsequently, TATV RNA was identified in a population of field voles in Northumberland, with hantavirus RNA being detected in 8/48 (16.7%) of field vole livers in that study (Thomason et al. 2017). Findings from the phylogenetic analysis revealed that although the viral sequences were more closely related to B41 (86.0-86.3% and 95.9-96.7% similarity at the nucleotide and amino acid levels, respectively) than other vole hantaviruses, there was still significant divergence. This together with the wide geographical distance between the two sites (230 km) could indicate there are multiple strains and lineages of TATV circulating in the British field vole population (Thomason et al. 2017). The field vole is the most abundant land mammal in the UK with an estimated pre-breeding population of 75 million (Harris and Yalden 2008), so there is a large potential rodent reservoir population to maintain TATV and allow the evolution of significant diversity. It is difficult to know what the public health implications are for TATV as there is very little known of the biology of TATV and whether or not TATV has zoonotic potential. The virus has yet to be isolated despite multiple attempts with different cell lines (personal communication with L. McElhinney). Further study is required to assess the prevalence and geographical distribution of this virus in field vole populations and gain a greater understanding of the virus itself. A TATV specific serological test, if developed, could be applied to cases of human acute kidney injury (AKI) where the cause is unexplained.

1.3.3. UK Hantaviruses: Puumala virus (PUUV) and Dobrava-Belgrade hantavirus (DOBV)

Another *Arvicolinae* hantavirus species which could be of interest in the UK rodents is Puumala hantavirus (PUUV) as the reservoir species, the bank vole (*Myodes glareolus*) is widely distributed through the UK, excluding N. Ireland, with a pre-breeding season population of 23 million (Couzens

et al. 2017). PUUV is the dominant hantavirus species in mainland Europe, with >2000 human cases reported annually in Germany alone, since the disease became notifiable in 2001, although Finland and Sweden have reported >3000 cases of infection in peak years (Reil et al. 2017). Infection with PUUV can result in a mild to moderate clinical nephropathia epidemica (NE) with a mortality rate of 0.1%. Clinical signs and symptoms include back pain, headache, fever, gastrointestinal and kidney problems (Reil et al. 2017; Fhogartaigh et al. 2011).

To date, there has been no recorded detection of PUUV infection in UK people or rodents. Surveys of bank voles in 1993 (n= 76) and 2013 n (= 35) were negative for hantavirus antibody and RNA, respectively (Pether and Lloyd 1993; Pounder 2013). Other, unpublished surveys of Cheshire and Northumberland rodents (not all of which were bank voles) in the 1990s also found no seropositive animals (personal communication with M. Bennett). However, this does not completely eliminate the idea that PUUV could be present in the UK, merely that it has not been detected yet. One study used mathematical modeling to demonstrate that bank vole ecology and the UK environment could support PUUV bank vole populations (E. Bennett et al. 2010). PUUV specific antibody has been detected in people in the UK, which could indicate people may have been exposed to this virus. For example, in one study a single farmer was serologically positive for PUUV antibody giving rise to an estimated prevalence of 0.8% (Duggan et al. 2017). However, following the subsequent discovery of TATV crossreactivity with PUUV, the detection of PUUV antibodies in the seropositivity study could be a false positive and could actually be due to exposure to TATV (Duggan et al. 2017; Pounder 2013).

In the same serological study by Duggan et al (2017), one pest control worker and one blood donor were seropositive to Dobrava-Belgrade hantavirus (DOBV) carried by the yellow-necked mouse (*Apodemus flavicollis*) of the *Murinae* subfamily. This hantavirus causes more severe disease in humans and has been detected in South Eastern Europe, including countries such as Greece, Slovenia, Albania and Croatia (Vapalahti et al. 2003). Although the reservoir is present in the UK, as for PUUV, there have not been any recorded DOBV detections in UK yellow-necked mice. This could be due to the fact that this species is fairly uncommon and geographically localised in Southern England and Wales with a rodent population of 750,000 before the breeding season (Couzens et al. 2017). However, cross-reactivity in serological assays is a common occurrence with hantavirus (Vilibić-Čavlek et al. 2015) so this could be a false positive for DOBV and might result from cross-reaction with another *Murinae*-carried hantavirus such as SEOV.

There is limited data available for the true prevalence and diversity of hantaviruses circulating in wild

rodents in the UK. The surveillance of hantaviruses conducted in this study through molecular methods and phylogenetic analysis is outlined in Chapter 3.

1.4. Rodent Viral Zoonosis: Lymphocytic choriomeningitis virus (LCMV)

Lymphocytic choriomeningitis virus (LCMV) is a negative sense enveloped RNA virus which has a bi-segmented genome and is a member of the *Arenaviridae* family. This is a circularly shaped virus has glycoprotein complexes protruding from the viral envelope and is 90-100 nm in diameter (Figure 1.4). Arenaviruses are able to cause persistent infections in rodent species, resulting in chronic viremia and are continually shed in rodent excretions such as urine, faeces and saliva (Zapata and Salvato 2013). LCMV is unique among Arenaviruses, as unlike other viruses such as Lassa virus (LASV) which are largely restricted to Western Africa (Olayemi et al. 2016), LCMV has a worldwide distribution. This is thought to be due to the global dispersal of the reservoir host, the house mouse (*Mus musculus*). The house mouse is one of the most abundant and adaptable rodent species, and is present in many countries (Zapata and Salvato 2013). In the UK alone, it is estimated that there is a pre-breeding house mouse population of 5 million, with extremely high-density populations associated with indoor human dwellings such a pig farms, chicken barns and both commercial and domestic premises (Harris and Yalden 2008).

The role of the house mouse as the reservoir species for LCMV was first described after the initial discovery of the virus in 1933 after an encephalitis outbreak in St Louis, USA (Armstrong and Lillie 1934). Since then, there have been multiple reports of human infections which have been attributed to LCMV exposure from house mice. In 1935-36 Traub studied LCMV infected laboratory mice colonies and found that house mice were viraemic throughout their life, with high levels of LCMV were shed in saliva, urine and faeces without showing any of the pathological signs observed in human infection (Traub 1939). Infected mice would produce infected offspring that would be persistently infected, and thus become carrier mice. This was demonstrated when the F1 generation of laboratory mice from infected wild-caught mice had these characteristics (Skinner and Knight 1979).

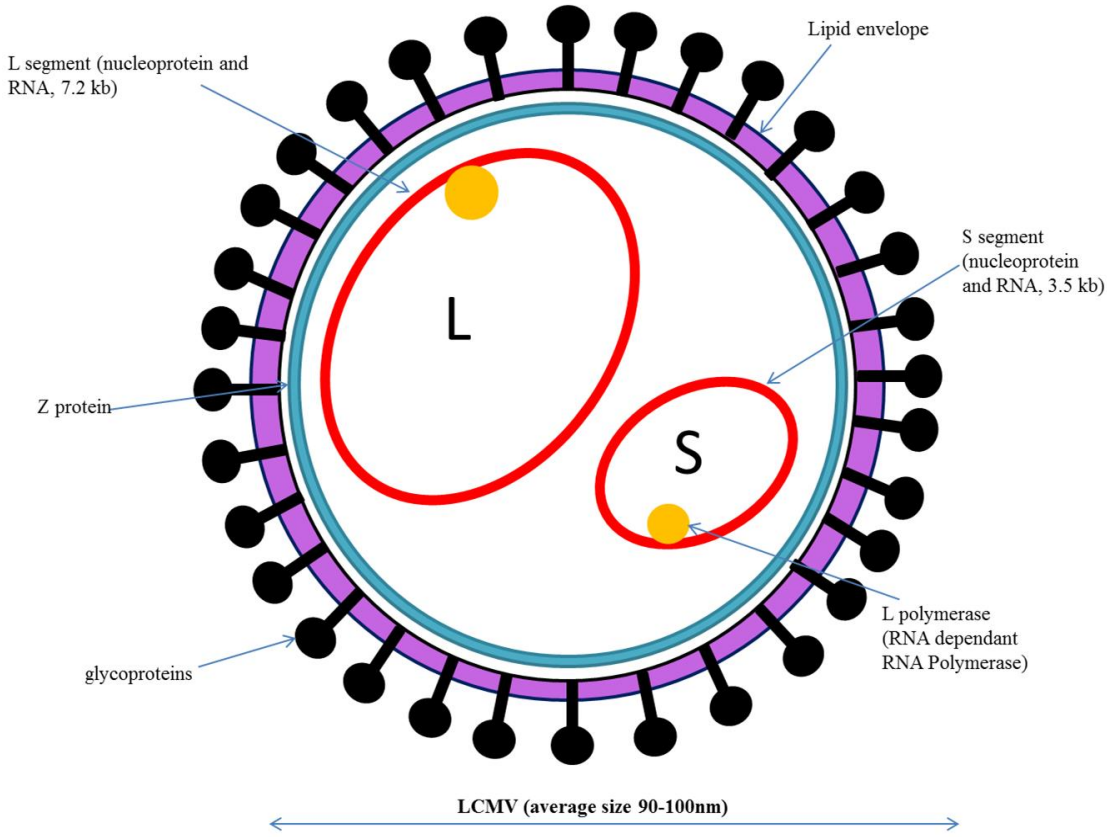

Figure 1.4: LCMV virus particle based on descriptions by Lapošová et al (2013).

LCMV is able to establish persistent infections in house mice due to the fact that this is a non-cytolytic virus and the rodent host does not suffer the detrimental effects of infection, thus they are able to shed virus throughout their lifetime (Figure 1.5). One reason for this is that mice are often transplacentally infected in utero and develop a state of intolerance so their immune system fails to mount a response to the virus (Bonthius 2012). Some studies have suggested that persistent infection is exclusively due to vertical transmission (Duh, Hasic, and Buzan 2017), however, there are other studies that have contradicted this. House mice can become infected as adults through horizontal transmission from other infected individuals in the population, however, there can be different outcomes with horizontal transmission as infection may only be temporary and may be cleared the mouse's immune system (Lapošová, Pastoreková, and Tomášková 2013). However, if a larger viral dose is received this can lead to persistent infection as the immune system fails to clear the infection. Newborn mice can become persistently infected even with a very low dose of LCMV virus (Lapošová, Pastoreková, and Tomášková 2013). LCMV can rapidly spread through a house mouse population reaching high levels quickly, making transmission to other individuals, other species and

people more likely.

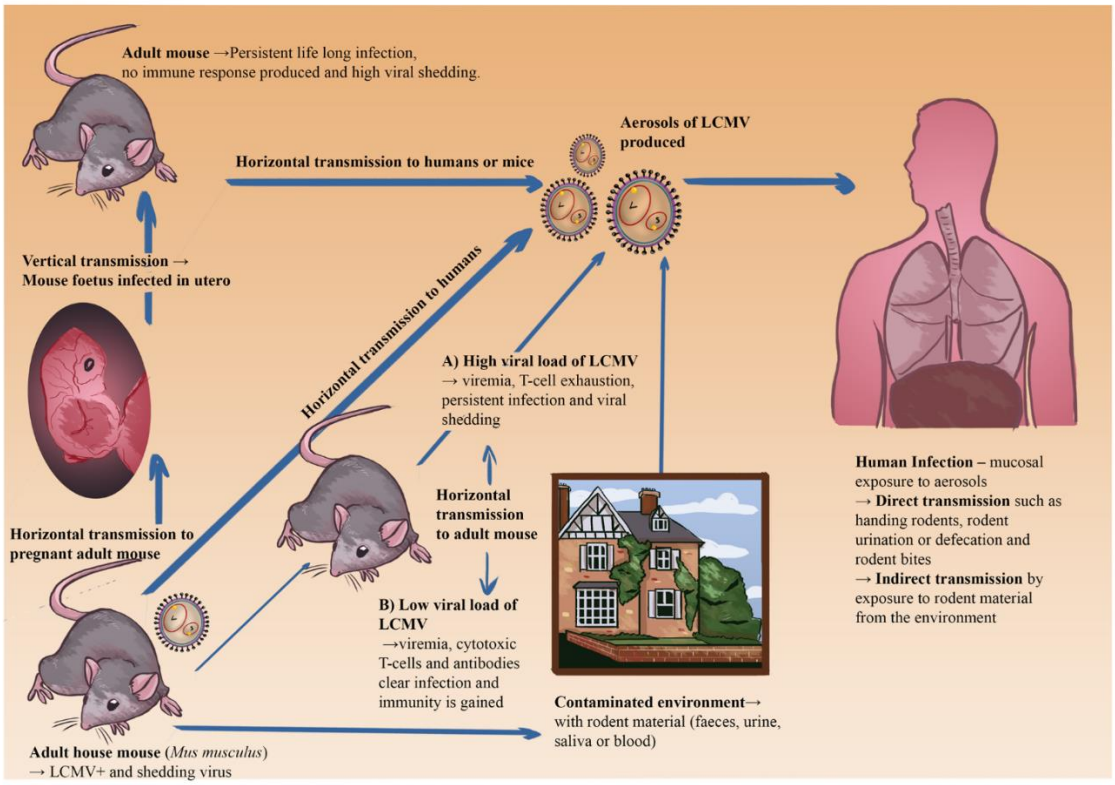

Figure 1.5: Horizontal and vertical transmission cycle within a house mouse population and how this can lead to viral transmission to humans (Lapošová et al. 2013). Original schematic diagram design by E. Murphy and illustration by E. Friel.

Humans can become infected with LCMV through direct contact, such as bites or handling infected rodents (Lapošová, Pastoreková, and Tomášková 2013). However, humans are more commonly infected indirectly through the inhalation of aerosolised virus that is shed in the excretions (urine, faeces, blood or saliva) that are produced by infected rodents. Infected rodent fomites, such as bedding, nest material or droppings can also be a source of human infection (Sosa et al. 2009). Such as in the case of a college student in Connecticut, USA in 2007 who was infected with LCMV and there was evidence of mice infestation, due to the presence of mice droppings in her dormitory(Sosa et al. 2009). In healthy individuals infection commonly goes undiagnosed due to the fact that infection with this virus is often asymptomatic to mild flu-like symptoms, which are mostly self-limiting (Lapošová et al. 2013).

LCMV has a mortality rate of less than 1%, also making LCMV different to other *Arenaviridae* family members (such as Lassa and Junin) have been shown to cause fatal hemorrhagic disease with a mortality rate of 15-30% (Cassady 2006). However, in rare cases, LCMV infection can result in serious illness in the form of aseptic meningitis. Such a case was reported in 2009 when a 49-year-old New York City (NYC) taxi driver presented with a seven-day history of fever, chills, neck rigidity and general weakness. He was diagnosed with acute LCMV infection and meningitis, although he reported no rodent exposure and there were no apparent risk factors which would have made him more susceptible to LCMV infection or severe illness (Asnis et al. 2010). Therefore, although rare, LCMV is capable of causing serious illness in healthy individuals. There is no vaccine for LCMV, and no specific antiviral therapies have been developed (Bonthius 2012), although antiviral drugs such as ribavirin and favipiravir have shown some promise (Cohen, Durstenfeld, and Roehm 2014), treatment largely consists of supportive care.

Human to human transmission of LCMV has yet to be reported, with the exception of vertical transmission from mother to fetus or horizontal transmission through solid organ transplantation (Sosa et al. 2009), in both of which infection can have serious consequences. Congenital infection with LCMV can lead to spontaneous abortion of the foetus, and if the foetus survives to infancy there can be further severe complications (Bonthius 2012). One of the most common signs of LCMV congenital infection is visual impairment due to the formation of bilateral chorioretinal scars, which in most cases can lead to visual loss. Microcephaly and macrocephaly have also been observed in congenital LCMV infection. Although degrees of severity can vary between individual cases the children who were infected in utero often have mental retardation, spastic quadriparesis and epilepsy. Prognosis in children diagnosed with congenital LCMV infect is generally poor and the mortality rate can rise to 35% by 21 months of age (Bonthius 2012). Hearing loss, although less common than visual problems, is also associated with congenital LCMV infection, which occurs in 7.4% of patients (Cohen, Durstenfeld, and Roehm 2014).

Immunosuppressed people are also a risk of the severe consequences of LCMV infection, such as solid organ transplant patients, in which LCMV infection can be fatal. There have been reported fatal cases (2003 and 2005) in the USA of the recipients of organs (lung, liver and kidney) from LCMV infected donors (Fischer et al. 2006; Amman et al. 2007). Epidemiological investigations into these cases revealed that it was the pet hamster (*Mesocricetus auratus*) of the organ donor in the 2005 cluster which was the source of the LCMV infection, no such source was identified, however, in the 2003 cluster (Amman et al. 2007). A similar case was reported in 2008 in Australia, of which an

LCMV-related virus was implicated in the deaths of three organ transplant patients who received organs from one donor (Palacios et al. 2008). As infection in healthy individuals is often asymptomatic and LCMV is not routinely screened for, thus the infection would not be diagnosed until the recipients of these organs started showing clinical signs of disease.

There have been several reported cases of LCMV transmission through laboratory accidents. LCMV is a pathogen which has been applied to many laboratory research studies and has been crucial in the development of modern immunology. Many key concepts in immunology have emerged through the study of LCMV such as the Major Histocompatibility Complex (MHC) restriction, T cell memory and exhaustion, persistent infections and the role of immune system in the pathology in disease (Zhou et al. 2012). However, although LCMV may be an advantageous pathogen to use in laboratory studies, there have been several reported cases of human illness due to exposure to LCMV positive lab animals. Such as in 1975 in the USA when 48 medical center personnel became infected with LCMV from lab hamsters (Hinman et al. 1975) or in 1992, also in the USA, in which an employee at a cancer research unit became infected from LCMV positive lab mice (Dykewicz et al. 1992). There has been a reported case of percutaneous infection as a result of a needle stick injury with LCMV lab strain ARM53b (Aebischer et al. 2016). Although, infection through laboratory accidents is not the natural route of transmission of LCMV and these incidents are rare, this highlights how important containment and safety protocols are in the handling of this virus.

Unlike some other rodent-borne viruses where there is strict host specificity, such as SEOV in which the reservoir host is almost exclusively the brown rat (*R. norvegicus*), LCMV has been detected in multiple rodent species other than the main reservoir, the house mouse. Hamsters, both pet and laboratory, have been shown to be a source of LCMV in which infection has resulted in human illness. For example, one distributor of pet hamsters was responsible for 54 cases of LCMV infection in NYC USA in 1974 (Biggar et al. 1975). Although hamsters may be competent hosts for LCMV they are not the reservoir host species, so infection is likely due to contact with LCMV from infected wild house mice. This was the case in the 2005 USA organ transplant cases in which a pet hamster was the source of infection (Fischer et al. 2006). In the epidemiological investigations that followed it was shown that the distribution center, which originally bred the hamster, in Ohio lacked effective biosecurity protocols as escaped rodents were able to run free and there was evidence of wild rodent infestation, so unsurprisingly, LCMV positive rodents were detected at this site (Amman et al. 2007).

LCMV carriage in brown rats, both wild and pet, is a controversial issue and is not fully understood.

It has been suggested that rats may be naturally immune to infection with LCMV (Charles River 2009) and cases of LCMV outbreaks among pet rodents would certainly support this idea. Such as in the case of an LCMV outbreak at a rodent breeding facility in Indiana and Kentucky USA where two staff members were hospitalised, a high prevalence of 21% (382/1820) in house mice was reported. However, there was no LCMV antibodies or RNA detected in the 399 rats screened from the same facility (Knust et al. 2014). Other studies, however, contradict this idea, as LCMV antibodies (Blasdell et al. 2008) and LCMV RNA (Stuart et al. 2011) have been detected in wild rats from UK studies and in rats from Spain through an Immunofluorescence assay (IFA) (Ledesma et al. 2009). The extent in which rats are able to shed and therefore transmit LCMV to other rodent species or humans still remains unclear.

Several species of wild rodents, other than brown rats, have been shown through the detection of RNA or antibodies, to be susceptible to LCMV infection, although their roles in the virus ecology are still unknown. LCMV infected field voles (*Microtus agrestis*) have been reported in the UK (Kaplan et al. 1980; Blasdell et al. 2008) and Finland (Forbes et al. 2014). Other vole species have also been shown to be infected with LCMV such as the common vole (*M. arvalis*) and the bank vole (*Myodes glareolus*) which in one Italian study were shown to have 20% and 7.4% seroprevalence to LCMV IgG antibodies, respectively (Tagliapietra et al. 2009). In Turkey, Robert's snow vole (*Microtus roberti*), and the sibling vole (*Microtus rossiaemeridionalis*) have also been identified as seropositive for LCMV (Laakkonen et al. 2006). Members of the *Apodemus* genus, including the yellow-necked mouse (*Apodemus flavicollis*), broad-toothed field mouse (*Apodemus mystacinus*) and the wood mouse (*Apodemus sylvaticus*) have been identified as possible hosts through seropositivity studies conducted across Europe (Laakkonen et al. 2006). LCMV seropositivity has also been detected in the black-tailed prairie dog (*Cynomys ludovicianus*) in a UK zoo (Blasdell et al. 2008).

Date	Rodent	Details	Reference
1980	Skomer vole, field vole and wood mouse	4/10 skomer voles, 1/2 field voles and 2/13 wood mice LCMV seropositive. 8 bank voles seronegative for LCMV.	(Kaplan et al. 1980)
2002	Grey squirrel	4/19 (21%) LCMV seropositive	(Greenwood and Sanchez 2002)
2007	House mouse (wild and laboratory)	1/24 (4%) recently caught wild mice, 18/38 (47%) captive wild-caught mice and 12/50 (24%) F1 captive bred mice LCMV seropositive	(Becker et al. 2007)
2008	house mice (wild and captive), brown rats, field voles, wood mice, harvest mice, red squirrel	House mice 2/89(wild) 30/87 (captive), brown rats 1/50, harvest mouse 1/3 wood mouse 2/315 field vole 2/114 red squirrel 4/65 seropositive for LCMV LCMV RNA detected in woodmice1/2, 1/4 red squirrel, captive mouse colony 122/403	(Blasdell et al. 2008)
2016	Brown rats	9/35 (25%) brown rats LCMV RNA positive.	(Stuart et al. 2011)

Table 1.3: LCMV rodent host diversity based on the results from studies conducted around the UK.

The overall global human exposure is still unknown with some studies conducted in the USA suggesting that it could be between 3-5% (Knust et al. 2011), although this has been shown to be extremely varied between countries. For example seropositivity to LCMV has been reported in Santa Fe province, Argentina of 2.38% (Ambrosio et al. 1994), 4% in Nova Scotia, Canada (Marrie and Saron 1998), 13-36% in Vin, Croatia (Dobec et al. 2006), 3.5% in Birmingham Alabama, USA (Park et al. 1997) and Madrid, Spain reporting 1.7% seropositivity to LCMV (Lledó et al. 2003). In the Spanish study, Lledó et al (2003), rodents trapped and sampled in the same region were also shown to be infected with LCMV, with seroprevalence in *M. musculus* (11.3%) and *M. spretus* (8%)

recorded (Lledó et al. 2003), indicating that infection in people may be correlated with infected rodents in the same area. LCMV has also be reported as the causative agent in two acute meningitis patients in Southern Spain (Pérez-Ruiz et al. 2012).

In the United Kingdom, historical data indicates that there may have been some recorded incidences of human infection such as 13 human cases previously reported between 1949-55 (Skinner and Knight 1979). The incidence rate of human infection reported by PHE laboratories in the years up to 1979 was thought to be less than 5 annually (Skinner and Knight 1979). The first case of congenital LCMV infection was reported in England in 1955 in which the infant died 12 days after birth, as a result of maternal infection (Barton and Mets 2001). However, due to the asymptomatic nature of LCMV infection and lack of routine screening, there is no current prevalence data available for LCMV infection in people in the UK.

In terms of prevalence in British rodents, there is slightly more information in the form of surveillance studies and reported detections of LCMV. Infected house mice have been reported in Manchester, Lewisham and London and in a study conducted around the surrounding area of the Animal Virus Research Unit, Pirbright, Surrey. Of the 84 wild house mice captured, there was an infection rate of 69% (Skinner and Knight 1979). In a study of wild-caught mice, a seropositivity rate of 4% (1/24) was detected, although this increased to 47% in the offspring of these mice which now formed a captive colony (Becker et al. 2007). Another study found 66 of 1,147 rodents had antibody to LCMV and 127/482 were PCR positive (Blasdell et al. 2008). Antibody was shown to be present in multiple rodent species, as well as house mice which had a seroprevalence of 17.5%, such as brown rats, wood mice, field voles and red squirrels (Blasdell et al. 2008). Another UK study has also detected LCMV RNA in 25% of brown rats across four field sites screened in 2011 (Stuart et al. 2011). A summary of UK rodent LCMV studies is shown in Table 1.3. Although, given that these studies were small in scale and conducted 10 years ago, there is little known about the current prevalence in UK rodents, in particular, the prevalence of LCMV in house mice.

Chapter 4 details the screening that was undertaken to investigate the prevalence and distribution of LCMV around the country. The prevalence data produced from this study to could be used to justify whether or not if it would be beneficial to conduct surveillance studies to investigate LCMV carriage in people. This could, in turn, be used to determine if LCMV is an important pathogen of significance to public health.

1.5. Rodent viral Zoonosis: Hepatitis E Virus (HEV)

Hepatitis E virus (HEV) infection is one of the leading causes of acute viral hepatitis and viral hepatitis (including infection with Hepatitis A, B and C virus) is the eighth leading cause of death globally, with an estimated 1.4 million deaths annually (Kokki et al. 2016). There are conflicting reports of who actually discovered HEV. In 1978 an epidemic of viral hepatitis occurred in the Kashmir valley in India in which Dr. Muhammad S. Khroo, though 14 years of investigative work, discovered the agent responsible was non-A and non-B hepatitis (NANBH) virus (Khuroo, Khuroo, and Khuroo 2016). In 1983, another research scientist, Mikhail S. Balayan, was investigating an outbreak of NANBH in Russian military personnel from Tashkent (now Uzbekistan) in 1983 (Khuroo, Khuroo, and Khuroo 2016). Unable to bring these samples back to Moscow with him, he decided to ingest a filtrate of pooled hepatitis patient stool samples mixed with yogurt. He subsequently developed viral hepatitis and HEV was isolated from his stool samples (Norkin 2010; Balayan 1993).

HEV is a 7.2kb positive sense non-enveloped, icosahedral RNA virus, that is 27-34 nm in diameter (Liu, Jane, and Zhang 2011) in the family Hepeviridae. Figure 1.6 shows a schematic representation of the HEV virion. Transmission among people is largely faeco-oral via contaminated food or water sources (Teshale, Hu, and Holmberg 2010), although there have also been reported cases of transmission through blood transfusion (Arankalle and Chobe 2000). Recently there has been a documented case in Australia of a six-year-old boy becoming infected after receiving a plasma donation during a liver transplant operation (Hoad et al. 2017). In addition, some transmission is zoonotic, as the infection is acquired through eating contaminated meat (Khuroo, Khuroo, and Khuroo 2016).

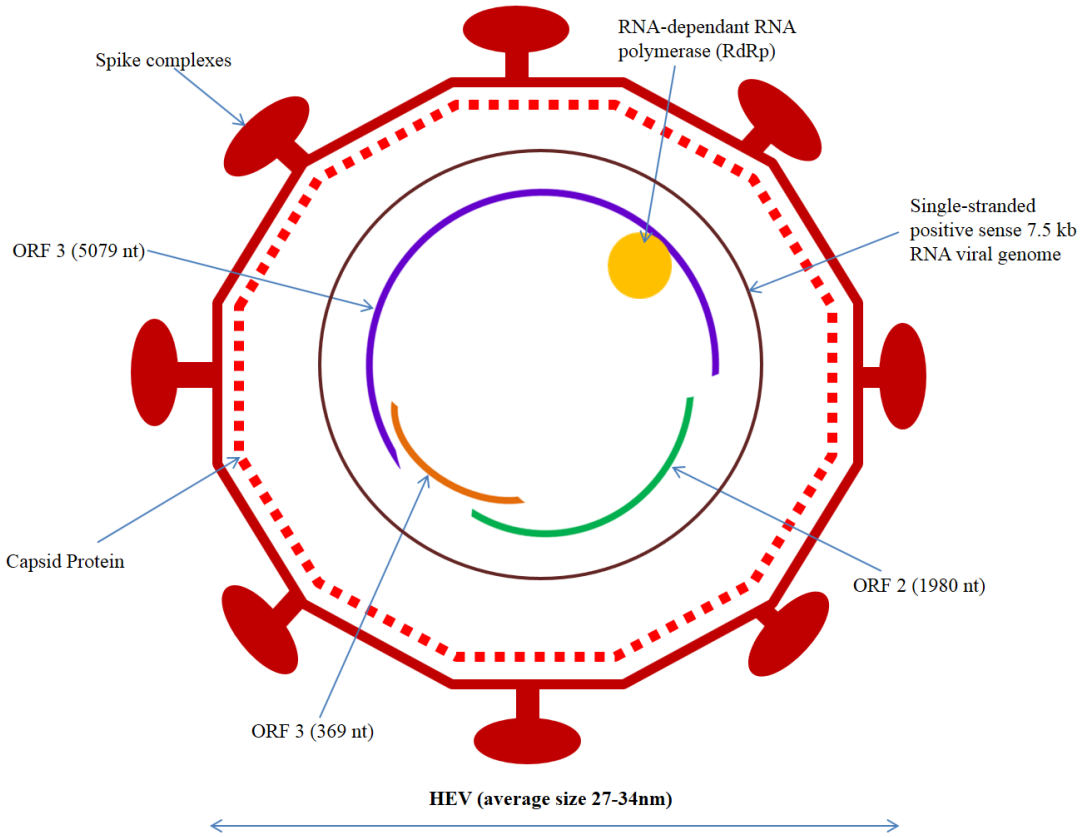

Figure 1.6: Schematic diagram of the hepatitis E virus particle (Liu et al. 2011; Reyes et al. 1993)

The pathogenesis of HEV is poorly understood. Once ingested, the virus may replicate in the intestine before traveling to the liver via the hepatic portal vein, most likely in the plasma, and replicates in the cytoplasm of hepatocytes. Virus replication is non-cytopathic, and liver damage appears to be immune-mediated through the activation of cytotoxic T-lymphocytes and Natural Killer (NK) cells that kill HEV-infected hepatocytes (Cook, Agostino, and Clarke 2016). Severe hepatitis has been linked to further escalation of the immune response with fulminant hepatitis E associated with high levels of both antibody and a range of cytokines. The virus is released into the blood and bile, and subsequently in the faeces (Lhomme et al. 2016). Extra-hepatic disease associated with HEV infection, such as neurological conditions such as Guillain–Barré syndrome (a neurological condition) and renal injury or impaired function, have been reported (Lhomme et al. 2016), but there is little known about where in the body the virus might replicate.

The acute viral hepatitis that results from HEV infection has very similar clinical signs of infection with other hepatotropic viruses. Signs include anorexia, malaise, fever, hepatomegaly, pruritus

(Teshale, Hu, and Holmberg 2010) and jaundice, which occurs in 75% of cases (Kamar et al. 2014). In most instances, HEV infection is asymptomatic and self-limiting, with a low mortality rate of 1% that rarely requires medical intervention. However, HEV infection can result in serious complications in certain individuals such as the immunosuppressed (transfusion patients, organ transplantation recipients and HIV positive individuals) and pregnant women, for whom the mortality rate is thought to be 20-25% (Echevarría 2014).

The taxonomy of the Hepeviridae family, and in particular the Orthohepevirus genus, has undergone much recent revision (Figure 1.7). Currently, human HEV (*Orthohepevirus A*) is divided into seven distinct genotypes G1-7, and of those G1-4 are thought to be responsible for the majority of reported human HEV infections (Doceul et al. 2016b). The epidemiology of human HEV is highly complex and still not fully understood. Human HEV G1-4 appear to have two distinct epidemiological patterns that differ in both transmission dynamics and geographical locations (Clemente-Casares et al. 2016). HEV infection in the developing world is often associated with contamination of the water supply due to poor sanitation and is mainly associated with HEV G1 (Asia) and HEV G2 (Mexico and Africa) (Clemente-Casares et al. 2016). The epidemiology of HEV in developed countries is different, as infection is mainly sporadic and often has a foodborne source. In addition, where HEV G1 and HEV G2 are thought to be exclusively found in humans, HEV G3 and HEV G4 have a wider zoonotic host range (Pavio, Meng, and Renou 2010).

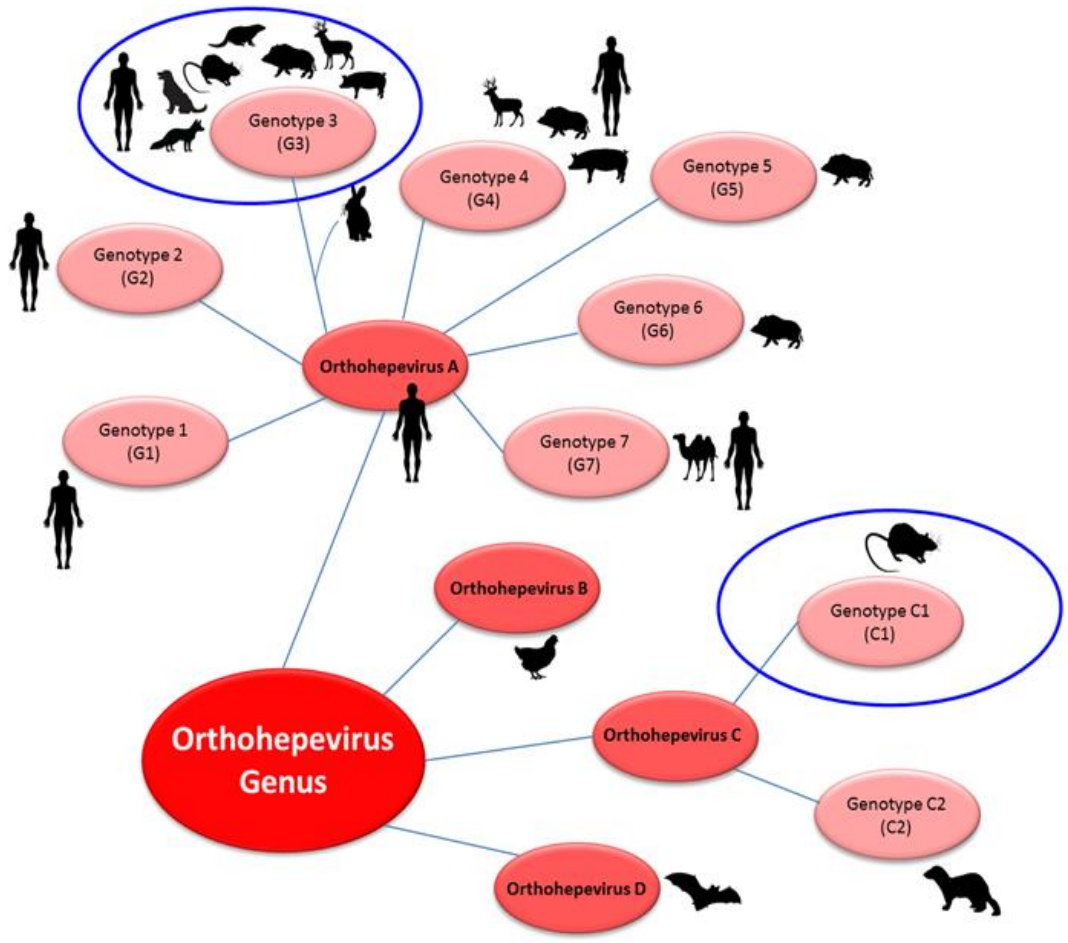

Figure 1.7: Schematic representation of the organisation of the reclassified *Orthohepevirus* genus showing all 4 species (A, B, C and D), the genotypes and predominant host species of each (D. B. Smith et al. 2014; Doceul et al. 2016b). The genotypes of significance for this study (due to their association with rodent hosts) are circled in blue. Branch lengths are not representative of genetic distance.

In the United Kingdom, the epidemiology of acute viral hepatitis is changing. The number of reported human cases as a result of infection with either Hepatitis A virus (HAV) or Hepatitis B virus (HBV) has declined, not least because of the use of safe and effective vaccines since the 1980s (Harvala et al. 2014). However, the incidence of acute viral hepatitis due to HEV infection has been increasing annually, for example in England and Wales the number of reported cases, shown in Figure 1.8, increased from 368 in 2010 to 1243 in 2016 (PHE 2018a). Travel associated HEV G1 and the indigenous HEV G3 have both been attributed as a cause of these infections. Males account for the majority of reported cases with 70% of HEV G3 and 67% of HEV G1, with a median of 62 and 37 respectively. Older men appear to be at greater risk in the case of HEV G3 infections with 76% being

greater than 50 years old compared to HEV G1 cases where only 30% were men 50 years or older (S. Ijaz et al. 2014). Prior to this increase reported by Public Health England, there have been reports from Cornwall and Devon in South West England of patients with unexplained hepatitis between 1998-2004 and in hospitals in the region to set up rapid access jaundice clinics to promptly manage patients with jaundice. Of the 333 patients with unexplained hepatitis, 21 were confirmed to have autochthonous HEV infection and most of the cases were middle-aged and male, which is indicative of the epidemiology of HEV G3 (Dalton et al. 2007). In the same region, a study of 225,000 blood donors showed an estimated prevalence of HEV to be one in 2848 with 79 (0.035%) samples with detectable HEV RNA (Hewitt et al. 2014). Studies have suggested that HEV G3 could be endemic throughout the country (Ijaz et al. 2009) and it has been proposed that there are 60,000 human HEV infections per year (Ijaz et al. 2014), although this is likely to be a gross underestimate.

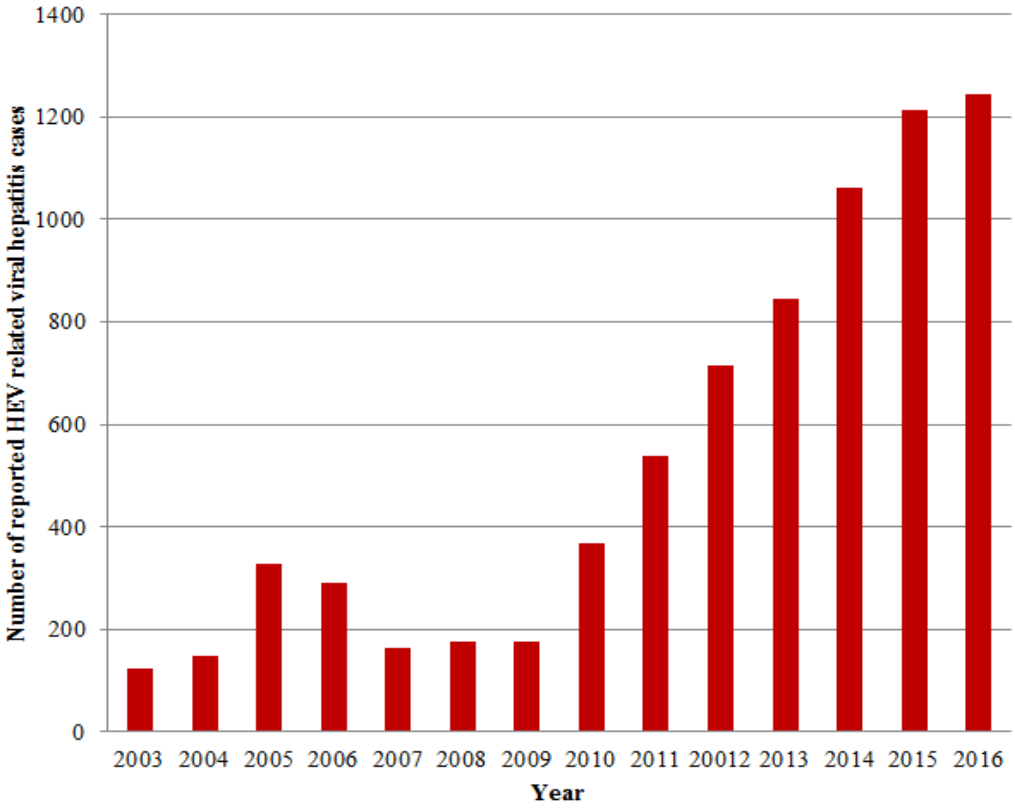

Figure 1.8: Number of human reported cases of HEV (2003 - 2016) in the UK from data provided by Public Health England. From 2010 onwards data shows the combined total between the reference lab data and notifications through the Second Generation Surveillance System (SGSS) which requires local diagnostic services to report evidence of HEV infection since 2010 (PHE 2018a).

Phylogenetic analysis of HEV RNA from hepatitis patients over a ten year period, excluding associated cases (HEV G1), revealed two distinct groups (group 1 and group 2) of HEV G3 sequences. From 2003 to 2009 the majority (94%) of human HEV G3 sequences clustered within the group 1. However between 2010 and 2012 group 2 became the dominant group, accounting for 58% of sequences (Ijaz et al. 2014). HEV G3 infections are often zoonotic, so the increasing incidence of infection in the UK, along with the change in genotype subgroup since around 2010, begs the questions: what are the non-human sources of infection, and have they changed? In 1997, Meng reported the first detection of a, then novel, strain of HEV in a pig from the USA which had high nucleotide identity with human HEV strains (Meng et al. 1997). Since then the association between HEV G3 and pigs or pig production has been well documented, with many industrialised countries reporting the detection of HEV G3 in pigs and pork products (Salines, Andraud, and Rose 2017). In the UK, a slaughterhouse study found 584/629 (92.8%) seropositivity in pigs associated with 93/629 (15%) detectable HEV G3 RNA in caecal contents (Grierson et al. 2015). Other UK studies have found HEV RNA in pigs on farms (Banks et al. 2010), in slurry lagoons (McCreary et al. 2008) and pork products, including the detection of HEV G3 RNA in 6/63 (10%) supermarket sausages (Berto et al. 2012) and pig liver (Banks et al. 2010). Epidemiological studies have also suggested that there is an association between the consumption of processed pork products, such as pork pies and ham, and HEV G3 infection (Said et al. 2014). Table 1.4 shows a summary of published UK and Ireland HEV studies and outbreaks, in which either seropositivity or RNA or both have been reported.

However, studies in the UK have found the dominant group to which HEV G3 strains cluster is group 1 which is no longer the most frequently detected group in human HEV infections, which mainly cluster in group 2 since 2010 (Grierson et al. 2015; Ijaz et al. 2014). Although HEV G3 subgroup 2 has been detected in pigs in other countries, so infection could be associated with imported meat products (Said et al. 2017). Therefore it remains possible that there may be another zoonotic source of HEV in the UK, either directly responsible for human infection or, indeed, as a further reservoir of HEV G3 group 2.

Year	Location	Source of HEV	Result	Reference
2004	England, UK	Pigs	11/42 faecal and 16/21 tissue samples HEV RNA positive 219/256 (85.5%) seropositive for HEV antibodies	(Banks et al. 2004)
2007	Yorkshire, UK	Pig slurry lagoons	2/9 slurry lagoons HEV RNA positive	(McCreary et al. 2008)
2007	Yorkshire, UK	Pigs (on farm)	21.5% pig faecal samples HEV RNA positive	(McCreary et al. 2008)
2008	Southampton, UK (Cruise ship outbreak)	Undetermined, possibly shellfish	4 passengers with acute HEV infection, 195/789 (25%) seropositive	(Said et al. 2009)
2009-10	UK	Porcine abattoir and sausages	5/40 (13%) Slaughterhouse pig faecal samples, 1/40 (3%) livers and 1/10 (10%) abattoir surface HEV RNA positive. 6/63 (9.5%) point of sale sausages HEV RNA positive	(Berto et al. 2012)
2012-13	South East England, UK	Blood donors	79/225,000 blood donors viraemic (positive for HEV G3 RNA)	(Hewitt et al. 2014)
2013	UK	Pigs (at slaughter)	584/629 (92.8%) pigs seropositive, 93/629 (15%) caecal contents HEV RNA positive, 22/629 (3%) of plasma samples,	(Grierson et al. 2015)
2014-2015	Edinburgh, UK	Sewage	14/15 (93%) pre-treated sewage HEV RNA positive	(Donald B Smith, Paddy, and Simmonds 2016)
2015	Ireland	Pigs (on farm)	89/330 (27%) individual pigs seropositive for HEV antibodies 13/16 (81%) herds seropositive for HEV antibodies	(O'Connor, Roche, and Sammin 2015)
2016	England, UK	House mice (from pig farms)	4/63 house mice with Swine HEV RNA present in the GIT tract	(Grierson et al. 2017)

Table 1.4: Zoonotic and other potential sources of HEV G3 in which either HEV antibodies or HEV RNA have been detected in studies or outbreaks across the UK and Ireland.

The role rodents play in HEV transmission is not understood and there is little known of the prevalence of HEV in the British rodent population. In previous studies rodents have been shown to carry to HEV G3, both human and swine variants (Lack, Volk, and Van Den Bussche 2012; Grierson et al. 2018). In terms of transmission of the swine variant of HEV G3 rodents can be seen as potential hosts as they are often present, sometimes in large numbers, on pig farming establishments. It is through this close contact with the livestock that rodents have the potential to become infected with HEV G3.

It has been reported that some pigs may be able to shed HEV in faeces for up to 12 weeks post-infection (Sanford et al. 2011). High levels of HEV have also been detected in urine as well as in faeces thus infected pigs that are shedding virus are highly likely to contaminate the local environment, such as pig pens, food and water troughs (Salines, Andraud, and Rose 2017). The rodents which live in these environments, particularly brown rats (*Rattus norvegicus*) and house mice (*Mus musculus*), are likely to become exposed to any excreted virus and ingest it through the faeco-oral route. They then have the potential to transfer, and possibly shed, the virus to other parts of the farm or between neighbouring farms.

In a study conducted in Japan in 2011, rats were trapped on and around a pig farm, on which the pigs were confirmed to be HEV positive. It was shown that 10/56 of these rats had detectable HEV RNA in their spleens and intestines which had high nucleotide homology (95.2-100%) the swine HEV G3 strain circulating in that region (Kanai et al. 2012). A recent study of rodents from English pig farms detected HEV RNA in the gastrointestinal tract of 4/63 house mice screened and this was shown to cluster with the G3 Swine HEV previously detected in pigs in 2013. No HEV RNA was found in any liver tissue of any house mice and not in any of the 15 brown rats screened (Grierson et al. 2018). This could indicate that rodents are simply ingesting the virus rather than becoming infected, however, to what extent they are able to transfer HEV to pigs, the environment or humans remains unclear. Therefore rodents could still pose a public health risk as rodents if they are infected with HEV as they could potentially increase the geographical spread and transmission of zoonotic G3 swine HEV.

However, another species of HEV, *Orthohepevirus C*, genotype C1 (rat HEV) has been detected in wild rats (both *Rattus norvegicus* and *Rattus rattus*) in Europe (Ryll et al. 2017). Rat HEV was first identified in Germany in 2010 (Reimar Johne et al. 2010) when a novel HEV-like virus was detected in the faeces and the liver of brown rats. This strain was shown to be genetically distinct from other

mammalian and avian HEV variants and has since been classified (Johne et al. 2010) and as a separate species (Doceul et al. 2016). Rat HEV has subsequently been identified in other countries such as the USA (Purcell et al. 2011), Vietnam (Li et al. 2013), China (Li et al. 2013) and Indonesia (Mulyanto et al. 2014). In a recently published study, rat HEV has been identified in 11 countries in Europe. This study screened 508 rats (both *R. norvegicus* and *R. rattus*) between 2005-2016, and at least one rat HEV RNA positive rat was detected in Germany, Denmark, Austria, Switzerland, Czech Republic, Belgium, France, Greece, Italy and Spain. Multiple detections of rat HEV across many countries could indicate that rat HEV may be endemic across mainland Europe (Ryll et al. 2017). However, the UK was not included in this study and to date, there has not been a recorded detection of rat HEV in the UK (Grierson et al. 2018).

Unlike swine HEV and other zoonotic HEV G3 strains where the zoonotic potential is well documented there is very little known about rat HEV and its transmission. There have been attempts to experimentally model the zoonotic potential of rat HEV by using animals such as non-human primates. One study conducted by Purcell et al in (2011) involved inoculating rhesus monkeys with a high dose (>100,000 times the infectious dose) of the USA rat HEV strain. No viral replication or seroconversion was observed in these monkeys throughout the entirety of the experiment (Purcell et al. 2011). Previous studies have suggested that rat HEV may have zoonotic potential such as a study of forestry workers in Germany, where rat HEV is known to be prevalent, who were strongly seropositive to rat HEV (Dremsek et al. 2012) and rat HEV has also been shown to successfully replicate in human hepatoma cell lines (Jirintai et al. 2014). However, only recently on the 28th September 2018 the first human case of rat HEV infection was reported in a 56-year-old man from Hong Kong (HKU Med 2018; Sridhar et al. 2018) and thus confirming that this virus is a zoonotic pathogen. Another two human cases of rat HEV infection was reported retrospectively in Hong Kong (Fleming 2018) and has been linked to a case of severe acute hepatitis in a Candian UN who had recently travelled to Africa (Andonov et al. 2019). Further study is therefore required to deepen the current understanding of the viral biology, pathogenicity and prevalence of rat HEV to be able to determine if there is a significant risk to public health.

Chapter 5 details the part of this study which investigated the prevalence of HEV in British rodents from both pig and non-pig farming establishments through molecular screening methods. The results from this study could be used to build on the current knowledge of possible zoonotic reservoirs of HEV in UK and highlight areas for future work on the transmission of HEV and risk assessment with regards to public health.

1.6. Bacterial Rodent Zoonosis: *Campylobacter* spp.

Members of the *Campylobacter* genus of bacteria are microaerophilic, Gram-negative bacilli (Figure 1.9), they have relatively small genomes (1.6 megabases) and under microscopy appear as curved rods. These may be pleomorphic and have flagella to aid motility (Jeon, Muraoka, and Zhang 2010). *Campylobacter* was initially described by two British veterinarians in 1906 when "large numbers of a particular organism" were observed in the mucus of a pregnant sheep, although a nonculturable spiral-shaped bacteria had been observed in 1886 (Silva et al. 2011). The genus *Campylobacter* was proposed in 1963, and interest in the genus increased when it was found to be present in human diarrhoea in 1973, although it was not thought of as a direct cause of human disease until the 1980s (Silva et al. 2011). To date, there are 39 recognised species and 16 sub-species in the *Campylobacter* genus (LPSN 2018). A subset of these species with their potential sources, incidence and significance in terms of public health are shown in Table 1.5.

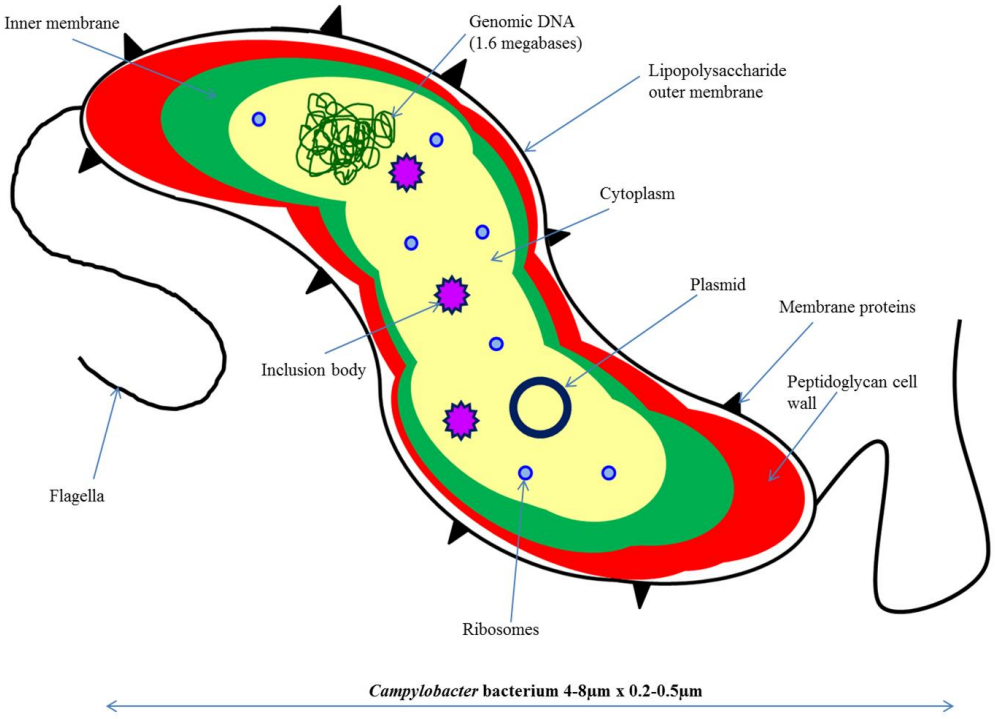

Figure 1.9: Schematic diagram of the *Campylobacter* bacterium.

Campylobacter species	Possible sources of human infection	Incidence and significance to public health	Reference
C. jejuni	Chickens, pigs, cattle, wild birds, rodents, domestic pets, unpasteurised milk and environmental sources	Very common. Bacterial gastroenteritis, diarrhea and has been linked to Guillain-Barré Syndrome.	(Rodrigues et al. 2001; Nyati and Nyati 2013)
C. coli	Pigs, bottled water, wildlife and environmental sources	Common. Bacterial gastroenteritis and diarrheal disease.	(Tam et al. 2003; Gillespie et al. 2002)
C. fetus	Gastrointestinal tract of cattle and sheep, mature from infected livestock	Uncommon. Often occupational exposure such as farming or abattoir work, 2.4% of intestinal campylobacteriosis.	(Wagenaar et al. 2014)
C. lari	Water, Gulls, shellfish, chickens, other wild birds and some domestic mammals	Rare. Causes enteritis, bacteraemia, bloodstream infection (BSI) and UTI although infection is infrequent and only severe in the immunocompromised. Post-surgical infections also reported.	(Werno et al. 2002; Morishita et al. 2013)
C. upsaliensis	Domestic dogs and cats	Rare. Causes enteritis, often acute but self-limiting, bacteraemia and weight loss and severe in the immunocompromised.	(Bourke, Chan, and Sherman 1998)
C. helveticus	Domestic cats	Unclear, not reported in humans or food, or implicated in human disease.	(Adams and Motarjemi 2006; Dep et al. 2001)
C. hyoilei	Pigs	Unknown	(Dep et al. 2001)
C. showae	Humans	Unclear, but has been linked to gingivitis and periodontal disease.	(Macuch and Tanner 2000)

C. curvus	Unclear, human, animal or environmental	Rare but has caused a small outbreak. Bloody or watery diarrhea and Brainerd's diarrhea (acute, lasting for >4 weeks)	(Abbott et al. 2005)
C. gracilis	Unclear, possible human	Rare. Periodontal disease and pleuropulmonary infections. One fatal case complicated by pneumonia recorded.	(Shinha 2015)
C. sputorum	Cattle, sheep, feral pigs and dogs	Rare. Human diarrhoeal disease	(Miller et al. 2017; On et al. 1998)
C. hominis	Unclear, possibly human.	Unclear, may be a commensal of the human GIT	(Lawson et al. 2001)
C. mucosalis	Pigs with proliferative enteropathies, not seen in un-diseased pigs	Unclear. Gastroenteritis cases in children and septicemia in rare cases.	(Lastovica et al. 1993; Söderström, Schalén, and Walder 1991)
C. hyointestinalis	Pigs, pigs with proliferative ileitis, cattle and hamsters	Rare. Gastroenteritis and human diarrheal disease that is often watery and/or bloody.	(Edmonds et al. 1987; Gorkiewicz et al. 2002)
C. rectus	Unclear, possibly human.	Rare. Periodontal disease and pregnancy complications	(Arce et al. 2010)
C. lanienae	Unclear, possibly farm animals	Unknown. Asymptomatic infection reported in abattoir workers.	(Logan et al. 2000)

Table 1.5: A subset of 16 species of the *Campylobacter* genus with the most likely source and significance to public health.

Campylobacter infection is the most common cause of bacterial gastroenteritis (campylobacteriosis) worldwide with an estimated 400-500 million cases annually (Jeon, Muraoka, and Zhang 2010). In the United Kingdom between 2008-9, there were over half a million cases of campylobacteriosis with around 80,000 GP consultations (Nichols et al. 2012). The incidence of foodborne illnesses are thought to cost £1.5 billion per year in England and Wales (National Research Council 2015; FSA 2017). Furthermore, the incidence is thought to be rising across EU/EEA countries as in 2014 there

were 240 379 confirmed cases, and the UK had the highest number of cases with 66 790 reported and a notification rate of 104 per 100,000 people (ECDC 2016).

Infection, with as little as 500 bacterial cells, can cause disease, which has an incubation period of 2-5 days. Common signs include abdominal pain due to the inflammation of the gut (gastroenteritis), fever and acute diarrhea which is often watery and bloody in 75% of cases although this usually resolves within one week and does not routinely require medical therapeutic intervention (Young, Davis, and DiRita 2007; Allos 2001). If medical intervention is required antibiotics commonly used include erythromycin (a macrolide), or broad-spectrum antibiotics such as ciprofloxacin (a fluoroquinolone, FQ), and in systemic infections, a tetracycline, such as doxycycline may be used. However, antimicrobial resistance rates are increasing (Luangtongkum et al. 2009). For example, FQ resistance in Hong Kong and Thailand is thought to have reached 80% and a high prevalence of AMR in *C. coli* (15-80%) has been observed in chickens and pigs in European countries (Luangtongkum et al. 2009). This is a public health concern as it could lead to untreatable infections with higher morbidity and mortality.

More serious disease is rare but can include peritonitis, cholecystitis, pancreatitis and gastrointestinal haemorrhage. Bacteraemia can occur in up to 1% of gastroenteritis cases and in rare cases may lead to sepsis and even death, as campylobacteriosis has a mortality rate of 0.05 per 1000 infections (Allos 2001). In England and Wales in 2008 alone, there were 76 *Campylobacter* infection-related deaths (FSA 2017). However serious complications are most often only seen in immunocompromised patients, or in the very young or very old (Allos 2001). Infection with *C. jejuni* has been shown to be a risk factor for Guillain-Barré syndrome (GBS), an autoimmune disorder of the peripheral nervous system (PNS) and is a common cause of flaccid paralysis. Symptoms include weakness in the limbs and respiratory muscles as well as loss of reflexes and although often self-limiting, 15-20% of patients can be left with irreversible permanent neurological defects. It is estimated that 1 in 1,058 cases of *C. jejuni* infection leads to GBS. Indeed the GBS associated with campylobacteriosis can be severe (Nachamkin, Allos, and Ho 1998); in one UK study conducted in 1992-4 GBS patients who were infected with *C. jejuni* took significantly longer to walk unaided (median 89 days) than uninfected GBS patients (median 45 days) (Rees et al. 1995)).

C. jejuni and *C.coli* which combined account for 97% of all gastroenteritis cases in the UK. *C. jejuni* accounts for 94% of cases, is largely acquired through the consumption of undercooked or contaminated chicken meat (Humphrey, O'Brien, and Madsen 2007; Madden et al. 2011). Infection

is often associated with the handling of, or cross-contamination, between raw and cooked chicken meat. Farmland environments, unpasteurised raw milk, contaminated water and wild birds have also been identified as potential sources of *C. jejuni* (Humphrey, O'Brien, and Madsen 2007). In a surveillance study, it was found that in England and Wales there were 12 times as many *C. jejuni* infections for every *C. coli*, although *C. coli* infection is still a significant cause of gastroenteritis, as is responsible for 6% of gastroenteritis cases (Silva et al. 2011). Pork is thought to be the main source of *C.coli* in people and is thought to enter the food chain through contamination of the meat with the faecel matter at the time of slaughter. It was shown to be the dominant species in pigs from Ontario with an infection rate of 99% and so is often considered a normal component of the porcine gastrointestinal system (Varela, Friendship, and Dewey 2007). Another study in Germany has also identified *C. coli* as the main *Campylobacter* type in pigs, as all the isolates grown from slaughterhouse faeces were found to be *C. coli* (Alter et al. 2005). There is a further potential risk as when the meat progresses further down the food chain as infected meat increases the risk of cross-contamination through the handling of products. One study found that 11% of British butchers handled raw meat with bare hands and alarmingly 29% did not wash their hands between the handling of raw and cooked meat that is ready to eat (Little and de Louvois 1998).

Rodents are of interest in terms of *Campylobacter* transmission as they have been shown in previous studies to be a carrier of the bacterium, both species *C. jejuni* and *C. coli*, and are a very common feature on most farms. In Denmark, 69% of farmers reported regularly observing mice on their farms and 39% regularly observed rats (Meerburg and Kijlstra 2007). One hypothesis is that rodents may be a source of contamination and therefore be a risk factor for the introduction of *Campylobacter* spp into the food chain when *Campylobacter* is shed in the faeces. It is also extremely difficult to completely exclude rodents from animal housing as they are highly adaptable, especially brown rats (*Rattus norvegicus*) and house mice (*Mus musculus*). Studies have shown that mice which are infected with *C. jejuni* subsequently shed the bacterium in their droppings for several weeks (Backhans and Fellström 2012). Rodents may also have the potential to transmit *Campylobacter* from other sources to the livestock. In a Swedish study in 1996 rodents were identified as a risk factor for high *Campylobacter* prevalence in broiler flocks (Backhans and Fellström 2012). There have been many studies which have shown that the natural environment and farmland could act as a natural source for *Campylobacter*, for example, infected environmental water have been shown to be responsible for several human outbreaks of campylobacteriosis (Bronowski, James, and Winstanley 2014). Therefore rodents, such as brown rats which are known to have large home ranges, may be a source of *Campylobacter* and therefore have the potential for them to introduce the bacterium from

external sources to the livestock on farms. In the case of New Zealand where the incidence of campylobacteriosis was the highest of any developed country (396 cases per 100,000 people) in 2003. This has since dropped significantly (159 cases per 100,000 people) in 2012 is due, in part, to the Campylobacter Strategy introduced by the New Zealand Food Safety Agency (NZFSA) in 2006 in the poultry industry (Lane & Briggs 2014). One of the main interventions was to improve biosecurity which included rodent control at the farm level. The aim of this was to prevent the livestock becoming exposed to Campylobacter and reducing the entry of this bacterium into the food chain, which along with many other control strategies, has been credited with reducing the incidence of human illness (Sears et al. 2011).

Another hypothesis is that rodents are becoming infected from the livestock themselves and although they are not the original source of *Campylobacter*, they may have a role in maintaining high prevalence in livestock. For example, *Campylobacter* (*C. fetus*) has been isolated from rats on a farm in 1967 where there had been an outbreak of Vibriosis, in the pigs which indicates that rodents could have a role in the transmission of this organism (Pejtschev 2010). This idea that rodents could be spreading a bacterial zoonosis in the food chain has previously been demonstrated with other bacterial pathogens such as *Salmonella*, another cause of human gastrointestinal disease. A study of Spanish pig farms found that there was a high prevalence (46.2%), regardless of the number of rodents present, of *Salmonella* in these rodents and that the most common serotype (*S. enterica*, serovar Typhimurium) showed a high level of identity with the same strains detected in pigs on the same farms. This could suggest that rodents may play a significant role in the maintenance of *Salmonella* infection in pigs (Andrés-Barranco et al. 2014). This principle has also been shown with viral zoonosis Hepatitis E (HEV) were rats from HEV positive farms were shown to be infected with the same pathogen residing in the pigs (Kanai et al. 2012). If a similar phenomenon is occurring where rodents are becoming infected with *Campylobacter* species directly from the livestock then they could have a role in maintaining high bacterial prevalence in the farm animals. This could have implications for public health as if rodents remain infected then it can make eradication of the pathogen from the farm extremely difficult.

The aim of this study, outlined in Chapter 6, was to investigate the carriage of *Campylobacter* species in rodents and how the results could be used to determine to what extent that rodents could be seen to be contaminating or maintaining *Campylobacter* prevalence on pork food chain.

1.7. Aims

- To collect a large range rodent samples from a variety of peri-domestic locations across the UK that could be used as a representation of the British rodents.

- Optimise molecular screening methods to conduct zoonotic pathogen surveillance.

- Screen the rodents for Hantavirus species to explore the diversity and prevalence of hantaviruses circulating within British rodents.

- Screen rodents for LCMV to expand on current understanding of the prevalence of this virus in rodents.

- Screen rodents for HEV. Determine the type of HEV, if any, is carried by rodents and if rodents may have a role in the maintenance and transmission of HEV.

- Screen rodents for *Campylobacter* spp using molecular and microbiological methods to determine carriage and maintenance of this pathogen that could have implications for the food chain and public health.

- Identify areas which may require further investigation that could be beneficial for public health.

- Create a biobank of rodent material to be used in future studies and provide positive controls to be used for diagnostic purposes in future outbreaks.

Chapter 2: Rodent fieldwork

A review of the fieldwork methodology conducted throughout this PhD project and applications for further studies.

2.0. Abstract

To investigate the prevalence of zoonotic pathogens in peri-domestic rodent species of the United Kingdom and an adequate sample size of rodents from a wide geographical range was required. This chapter outlines the methods used to complete this aim and what has been learned from this project that may be useful in future rodent studies.

Sample sites were required through various methods such as contacts through the university, public engagement and voluntary recruitment through written communication. Field sites on University-owned land were also used in this study. A variety of trapping equipment was used to trap a range of rodent species. Specialist training was received in order to perform humane killing in accordance with ethical guidelines and successfully perform cardiac puncture. A total of 333 rodent specimens were collected from this project which included; brown rats (*R. norvegicus*, n=68), house mice (*Mus musculus*, n=105), wood mice (*Apodemus sylvaticus*, n=48), bank voles (*Myodes glareolus*, n=56), field voles (*Microtus agrestis*, n=23), red squirrels (*Sciurus vulgaris*, n=21) and grey squirrels (*Sciurus carolinensis*, n=12). Post-mortem examination of these rodents was performed, in the case of rats, a post-mortem examination was performed in a CL3 facility due to the risk of CL3 aerosolized Seoul virus (SEOV).

There were several elements that affected trapping success, such as bait type, trapping session length and trap placement. There were several strategies which we applied to overcome the level of neophobia in certain rodents, especially brown rats. The results from the fieldwork study could be used to optimise the trapping success of further rodent studies.

2.1. Introduction

Rodents are important to public health, not just because of the zoonotic pathogens they carry but also due to the interactions they have with people which presents an opportunity for transmission of infectious diseases. In order to successfully investigate the current infection status and prevalence of diseases rodent samples are required. There is also a requirement for these rodents to be live trapped to ensure the samples are as fresh as possible to ensure that viral RNA can be extracted from rodent tissue.

The rodent species that were of interest to this project brown rats (*R. norvegicus*), house mice (*Mus musculus*), wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), red squirrels (*Sciurus vulgaris*) and grey squirrels (*Sciurus carolinensis*). As red squirrel are a protected species they were not trapped and had died of natural causes or were road kill. Multiple locations were chosen to sample rodents to reflect the different environments peri-domestic rodents inhabit and may come into contact with humans. A special focus of this study was concentrated on pig farms and there were several reasons for this. There has been a reported clinical HFRS human case which involved human infection from a wild rat on a Yorkshire pig farm (Jameson et al. 2013). Also, HEV has been linked to the British pig farming industry, therefore, rodents could be used to assess whether a wildlife reservoir for this pathogen. Also, pig farms have been shown to be environments that could support large rodent populations and there is the additional connection to the food chain, which provides an additional entry point for transmission to humans.

This chapter outlines the fieldwork that was conducted as part of this project, what samples were collected and the sites included. Also detailed protocols on how to conduct this type of research and overcome several difficulties to maximize rodent sample collection.

2.2. Materials and Methods

2.2.1. Recruitment

There were several methods used to recruit sites for this project. These included trapping on University owned farms and receiving samples from other University-led projects. Other sites and samples were received through collaboration with other organisations such as the Lancaster Forestry commission, Animal Health and Laboratories Agency (APHA), Cheshire Badger society, Agriculture and Horticulture Development Board Pork (AHDB Pork) and Welsh Water. Pig farms were recruited through advertising the study and voluntary participation. The flyer advert for the study is shown in Appendix I. Informed consent from the landowners was received (please see Appendix I) and a detailed explanation of the project by Ellen Murphy was given before the start of any work at the site.

2.2.2. Sampling

2.2.2.1 Trapping

A trapping period from September 2014 to January 2016, during which 22 sites were sampled from across Northern England, North Wales and Scotland. A site assessment was carried out to determine the correct type of traps to set, the level of rodent activity and the optimal place for trap placement. Equipment was set up and left for a period of five days and four nights, with checks conducted and rodents removed every 24 hours. On the fifth day, all equipment was collected and transported back to the university to be thoroughly cleaned and sterilised before proceeding to the next site. To prevent the transfer of any pathogens between sites several biosecurity protocols were put in places, such as autoclaving of equipment and clothing, disinfection of footwear and leaving at least two days in between sites. This was particularly important when travelling between pig farms that there is at least 48 hours ‘pig free’ between farms to prevent the spread of haemorrhagic viruses and other pathogens between pig herds. Appendix II contains a trapping protocol that was implemented at each site. Samples were frozen down at -20°C in a mobile freezer for tempory storage for no longer than five days if off-site and transferred to Leahurst campus for processing and long term storage at -80°C.

2.2.2.2. Humane euthanasia and training

Rodents were euthanised in the most humane way possible to minimize the stress to the animal in question. Personal training in rodent handling and Schedule one humane killing practices (Home Office 1997) was given to Ellen Murphy by John Waters (Evolution, Ecology and Behaviour department, Leahurst Campus, University of Liverpool) before the commencement of fieldwork.

All rodents that were given an overdose of the anesthetic isoflurane (Merial Animal Health Ltd, Woking, UK) by which was placed on cotton wool and inhaled. Neck dislocation followed this to confirm death. This follows the ethical parameters stated in the ethics application of the University (please Appendix I for the ethical approval confirmation).

2.2.2.3. Cardiac puncture

Training in cardiac puncture was also given to Ellen Murphy by John Waters. Blood samples were taken via cardiac puncture shortly after the rodent was deceased. For rats a 5ml and a grey 27 gauge needle was used and for smaller rodents, a 1ml syringe and an orange 23 gauge or blue 22 gauge needle was used to take cardiac puncture blood samples. It was not always possible to take a cardiac blood sample from already deceased rodents due to the clotting process.

2.2.2.4 Faecal swabs

For *Campylobacter* screening, faecal swabs were taken, a charcoal agar was used for transport. For small rodents, the trap was emptied and the rodent species and the individual number was recorded then faeces were removed from the trap using the swab. The trap was cleaned with ethanol and reset for the next day. For brown rats, it was not possible to take faecal samples from the cage traps a swab was taken from the rectum after the rodent was deceased. Charcoal swabs were refrigerated until bacterial culture could commence.

2.2.3. Trapping rodents

Rat carcasses were collected from pest control programmes, as road kill or trapped. There were many types of bait used during this project for both live and kill traps. For rats, successful baits were chocolate spread, peanut butter, biscuit spread and chicken korma curry. There was some success with bait which the rats at a particular site were already familiar with, such as chicken or pig feed, which could help overcome neophobic behaviour. Baits that were unsuccessful in the trapping of rats in this study were coconut, cat food and Christmas pudding. Chocolate hazelnut spread was the most successful for house mice and if it was spread underneath the trip lever gave the greatest chance of the mouse entering the trap far enough to trigger that the door to close. Wood mice and voles were also attracted to chocolate spread but entered the traps with the grain and hay alone.

All small rodents, such as mice and voles, were trapped in Longworth small mammal traps (NHBS, Totnes, UK). These were live catch aluminum traps (Figure 2.1) that also contained a

shrew hole which would allow shrews to escape from the traps as they are a non-target small mammal. These traps had a large compartment, which was filled with food and hay bedding for warmth. Small rodents would be attracted to this and enter the trap through the smaller compartment and when they pressed the trigger the door at the entrance would close. The rodent would remain in the trap until it was emptied the following day.

Figure 2.1: Longworth traps used for trapping small rodents.

A: Longworth trap placed in a rural location on a transect.

B: Nest chamber (14 x 6.5 x 8.5cm) for food and bedding, each trap had a shrew hole to allow shrews to escape.

C: Entrance of the trap, full trap length when set is 25cm.

D: the tunnel showing the trigger (blue arrow) and the optimal area to place the bait (red arrow).

E: trap placed on a rodent run.

Rats were live-trapped in squirrel cage traps (Figure 1.2) such as the Easipet Humane Squirrel Trap (Easi Pet Shop, Devon, UK). This model was highly sensitive so small rats with a lower body mass could activate the trigger platform and the solid door to close. The trigger platform was solid which forced the rats to enter the trap, as other models with a wire mesh platform were not used

as the rats tunneled under the trap and consumed the bait through the mesh without entering the trap. The traps were checked every 24 hours, emptied and reset.

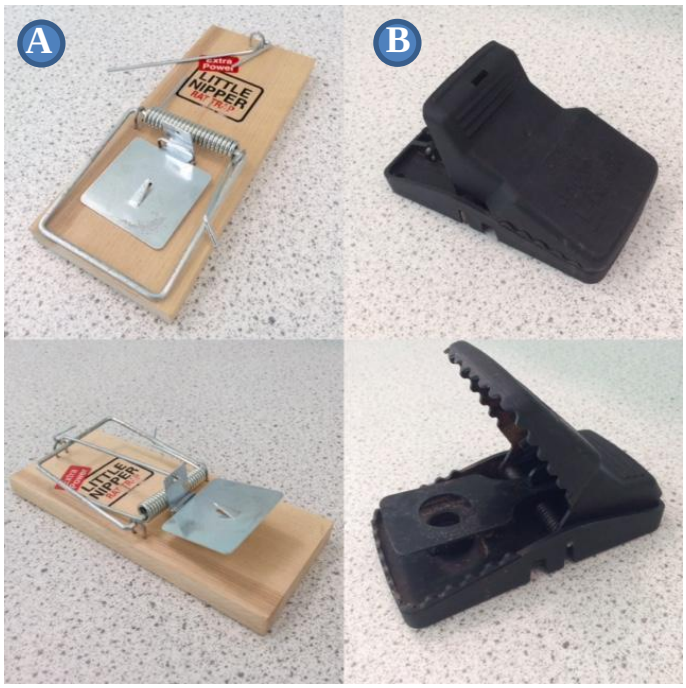

Figure 2.2: Spring snap traps for brown rats. 2.2a: Little Nipper, (peg spring) rat trap, unset and set. 2.2b: Trapper T-Rex snap (pull spring) rat trap, unset and set.

There were two varieties of snap trap (Figure 2.2) used in this study which included the Pest Stop, Little Nipper rat trap (Procter Bros, Leeds UK) which contained a peg spring mechanism and the Trapper T-Rex rat trap (Bell Laboratories Ltd, WI, USA) which operated by a pull spring mechanism. Both traps were purchased from www.amazon.co.uk. Both traps worked on the same premise as the bait is loaded onto a platform, which has a spring-based mechanism, and when the rat attempts to eat the bait the trap is activated. These traps provide an instant kill. The Little Nipper rat trap is highly has a peg spring or double peg spring has been demonstrated to have the greatest impact force when the trap is triggered. It is thought that the force generated is due to the 180° angle that the trap is set (Baker et al. 2012). The Trapper T-Rex rat trap with a pull spring and the opening of this trap is smaller than the Little Nipper rat traps (45-70°) so this trap is thought to have a weaker impact force, however, the smaller angle is thought to produce a higher clamping force (Baker et al. 2012). Both traps provide an instant kill and were used to increase the number

of rat samples from a site as they could be placed in smaller areas or on uneven ground, when it was not possible to place live traps, such as along rat runs or at the entrance to rat burrows.

The location where the traps were placed had an influence on the success of trapping rodents throughout this study. Certain signs can be useful in determining the best location to place rodents traps such as droppings, damage to property or seeing the rodents themselves when inspecting a site (Figure 2.4). Identifying areas with evidence of high rodent activity helped optimise trap placement. Knowledge from the people who work or live at these sites is also incredibly useful in finding the best place to set rodent traps. Infrared cameras were used to monitor rodent activity (Figure 2.3) to identify the suitable areas on a site to set traps. It was not possible to use these on every site due to financial and time constraints.

Figure 2.3. A brown rat (*R. norvegicus*), circled, captured by a motion-triggered the infrared camera.


A


B


C

Figure 2.4: Evidence of rodents and their activity at a site. 2.4a: Rat nest located under a farm trailer identified due to the high density of rat footprints in the mud.2.4b: Rat holes in the roof and window covers of a pig shed indicated by arrows. 2.4c: House mouse holes made through the concrete floor of pig stalls indicated by arrows. Mice were also observed foraging in the feed troughs.

2.2.3. Species identification

When rodent carcasses were collected the species was recorded. Table 2.1 was used as a guide to identify species by their physical appearance. Age, gender and weight were also recorded.

Species	Dimensions	Morphological description
Brown rat (*Rattus norvegicus*)	HB: 21-29 cm T: 17-23 cm Wt: 200-600 g	Much larger than mouse and vole rodent species. A long scaly broad tail that is almost body length. Greyish brown coat with a light grey to white under-belly. Pointed muzzle with small eyes and ears.
House mouse (*Mus musculus*)	HB: 6-10 cm T: 6-10 cm Wt: 12-22 g	Grey to greyish-brown coat with a light grey to brown under-belly. Smaller eyes, ears and hind feet, and less pointed muzzle compared to the wood mouse.
Wood mouse *(Apodemus sylvaticus*)	HB: 6.1-10.3 cm T: 7.1-9.5 cm Wt: 13-27 g	Golden to yellowish-brown coat with a white under-belly. Large predominant eyes and ears. Long tail and long pointed muzzle. Large hind feet and does not have a musky smell.
Bank vole (*Myodes glareolus*)	HB: 8-12 cm T: 3.3-4.8 cm Wt: 14-40 g	Warm brown to reddish brown coat with a light grey to cream under-belly. Small eyes and hair covered ears. The tail is two-thirds of the body length and is slightly smaller than the field vole.
Field vole (*Microtus agrestis*)	HB: 9-11 cm T: 2-5 cm Wt: 20-40 g	Similar to the bank vole. Greyish –brown coat with a light grey under-belly. Adults are slightly larger than the bank vole with a shorter tail that is half the body length.

Red Squirrel (*Sciurus vulgaris*)	HB: 18-24 cm T: 14-20 cm Wt: 250-300 g	Chesnut reddish-brown coat with a white under-belly. Smaller and slimmer than the grey squirrel, with tufts on the top of ears.
Grey Squirrel (*Sciurus carolinensis*)	HB: 24-28 cm T: 19-24 cm Wt: 400-600 g	Rounded ears with no tufts with a short muzzle and a long bushy tail. A predominantly grey coat that is peppered with white and a white under-belly. Can have reddish ginger patched on the flanks and legs.

Table 2.1: Morphological descriptions of the species collected in this study according to Couzans et al 2017. HB (head and body length), T (tail length) and Wt (Weight)

2.2.4. Postmortem examination

Each collected rodent was examined postmortem (Figure 2.5). All materials and instruments (rat tooth forceps, forceps, scissors, scalpels, etc) needed for the post-mortem were sterilised by autoclaving before the post-mortem examination began. Table 2.2 details the samples which were used for this project, although other samples were taken from other organs were also taken at the same time to be stored at -80°C for future work. Six 2 ml O-ring tubes or 1.5 ml eppendorfs were required, labelled heart, lungs, liver, spleen, kidney, faeces and a 5 ml Bijou labelled GI tract. Small rodents, such as mice and voles, were pinned to a corkboard to prevent movement during processing. The carcasses were sprayed down with disinfectant (1-10% distel) before the first incision was made, which is just above the urethral opening. Closed scissors were inserted in this incision and opened gently inside the body to separate the skin layer from the body cavity. The skin was cut away from this incision and care was taken not to pierce the body cavity.

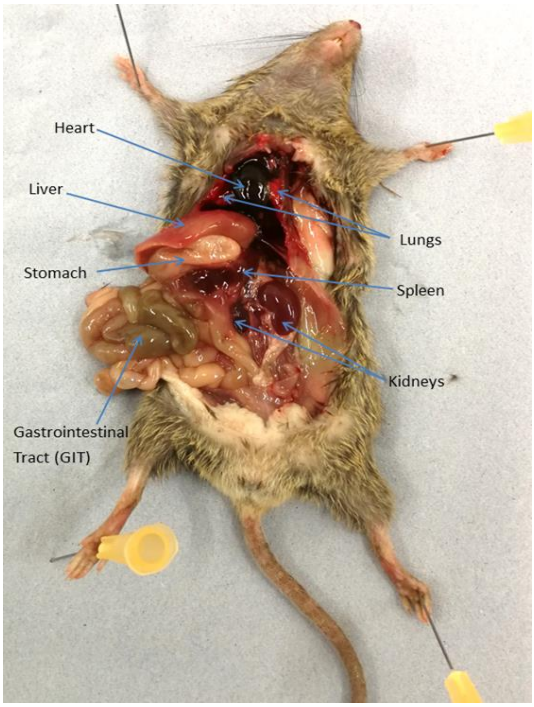

Figure 2.5: Postmortem examination of an adult female house mouse (*Mus musculus*) in which the tissue samples which were taken are labelled.

A horizontal incision was made below the rib cage and the ribs were removed to expose the chest cavity. The heart and lungs were removed and placed in their respective tubes. The rest of the body cavity was exposed by cutting through the muscle body wall, care was taken not to pierce the gastrointestinal tract (GIT). The liver, spleen and kidneys were then removed. A faecal sample was taken from the by piercing a small part of the lower GIT and removing some of the contents. The rest of the GIT was removed and placed in the 2 ml eppendorf. The remains of the carcass were disposed of in the clinical waste. All brown rat carcasses were processed at containment level 3 (CL3) due to the risk of possible SEOV carriage and the generation of aerosols during the post-mortem examination. A biobank of rodent tissue has been created at the Leahurst Campus, University of Liverpool from these samples.

Sample	When taken	Pathogen and target	Storage
Kideny	Post-mortem examination	Hantavirus (RNA) LCMV (histopathology only)	-80°C Room temperature (slides)
Lung	Post-mortem examination	Hantavirus (RNA)	-80°C
Liver	Post-mortem examination	Hepatitis E virus (RNA and histopathology) LCMV (RNA)	-80°C Room temperature (slides) -80°C
Serum	In the field	LCMV (antibodies)	-20°C
Faeces	Post-mortem examination and in the field	Campylobacter	-4°C (before culture) -80°C

Table 2.2: A list of the samples used in this study and storage details.

2.3. Results

2.3.1. Samples collected

A total of 333 rodent specimens were collected from this project which included; brown rats (*R. norvegicus*, n=68), house mice (*Mus musculus*, n=105), wood mice (*Apodemus sylvaticus*, n=48), bank voles (*Myodes glareolus*, n=56), field voles (*Microtus agrestis*, n=23), red squirrels (*Sciurus vulgaris*, n=21) and grey squirrels (*Sciurus carolinensis*, n=12).

Code	Location	Map number	Species Collected	Site description
Pig farm locations				
Pig Farm 1	Ripon, Yorkshire	A	RN, MM, AS, MG	Indoor pig farming unit with 3000 pigs. Current rat infestation.
Pig Farm 2	Cheshire	B	RN	Outdoor pig unit.
Pig Farm 3	Morpeth, Northumberland	C	MM	Indoor pig unit with 1500 pigs. Current house mouse infestation.
Pig Farm 4	Kingston Upon Hull	D	RN, MM, MG	Indoor pig unit with a chicken farm attached.
Pig Farm 5	Berwick, Northumberland	E	MM, MG	Small rare breed outdoor pig farm with holiday cottages on site.
Pig Farm 6	Driffield, Yorkshire	F	RN, MM, AS, MG	Split into 3 sites. Two indoor units and one outdoor breeding unit.
Pig Farm 7	Edinburgh	G	MM, AS	Indoor pig farm. Evidence of a high level of house mouse activity observed.
Pig Farm 8	Malton, Yorkshire	H	RN, MM, MA, MG	Outdoor pig farm of high rat activity around the site.
Pig Farm 9	Wetherby, Yorkshire	I	RN, AS, MG	Outdoor pig farm with evidence of high rat activity around the site.
Pig Farm 10	Morpeth, Northumberland	J	AS, MG	Outdoor pig unit.

Pig Farm 11	Telford, Shrewsbury	K	MM	Indoor farm. Evidence of a high level of house mouse activity observed.
Pig Farm 12	York, Yorkshire	L	MM	Indoor pig farm. Evidence of a high level of house mouse activity observed.
Other locations				
Farm 1	Cheshire	M	RN, MG, AS	Indoor dairy unit with a large area of surrounding farmland.
Farm 2	Derby, Derbyshire	N	RN	Beef and agricultural farm.
Farm 3	Cheshire	O	MA, MG	Outdoor beef farm with on-site butchers and surrounding farmland.
Rural 1	Llyn Cowyld, North Wales	P	MA, MG	Countryside surrounding a reservoir used in the water supply.
Rural 2	Ruthin, North Wales	Q	RN	Smallholding with sheep, dogs, horses, chickens and ducks. Evidence of rats.
Forest 1	North Wales (various)	n/a	SV	Range of locations around this area including parks, gardens and forest woodland.
Forest 2	Formby, Merseyside (various)	n/a	SC, SV	Range of locations around this area including parks, gardens and forest woodland.
Urban 1	Liverpool, Merseyside	T	RN	Commercial premises with an enclosed yard with a high level of rat activity.
Urban 2	Ellesmere Port, Cheshire	S	RN	Residential garden backing onto woods with backyard chickens.

Table 2.3: List of sites, rodent species collected and corresponding map numbers (Figure 2.6)

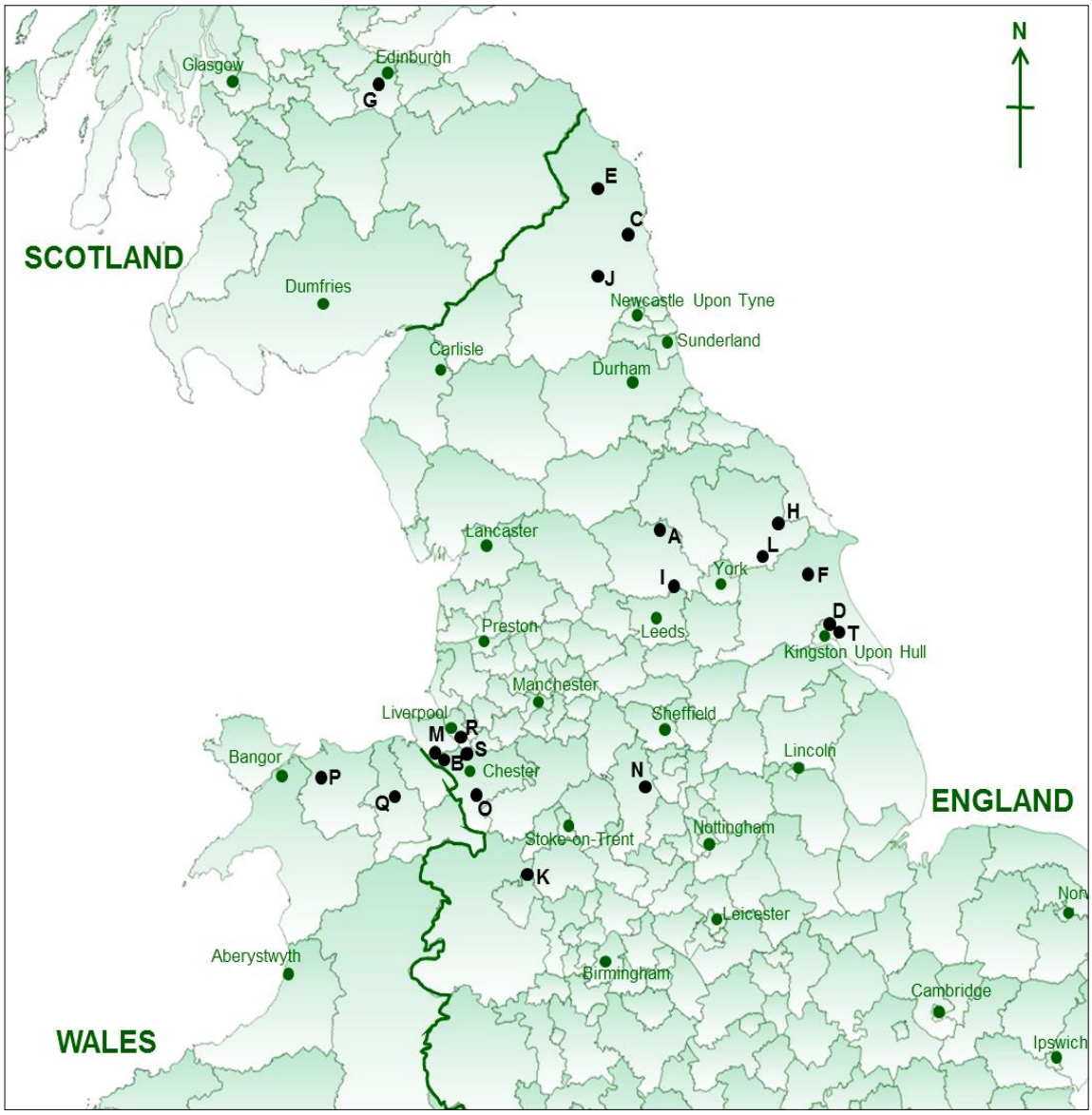

Figure 2.6: Map to show the location of the trapping sites in which rodents were collected from for this study. This map was created using GQIS Desktop 3.2.3 software.

2.3.2. Length of trapping session

The length of a trapping session was shown to influence trapping success. Figure 2.7 shows the percentage of successful catches for house mice and bank voles during the pig farm rodent field work. This data shows that the most successful night for trapping voles was the third consecutive night and for mice, it was the fourth. There could be several reasons for this, such as after the first night it is easier to identify areas of a site with higher levels of rodent activity so traps can be moved. For example sow and piglet enclosures where shown to be high-density areas for house mice due to the large amounts of food available, straw bedding and heat lamps. In addition, the longer time the trap remains in the environment the more the rodents become habituated to its presence, therefore they are more likely to venture in. This suggests that in order to conduct successful rodent fieldwork more than two consecutive nights of trapping may be required.

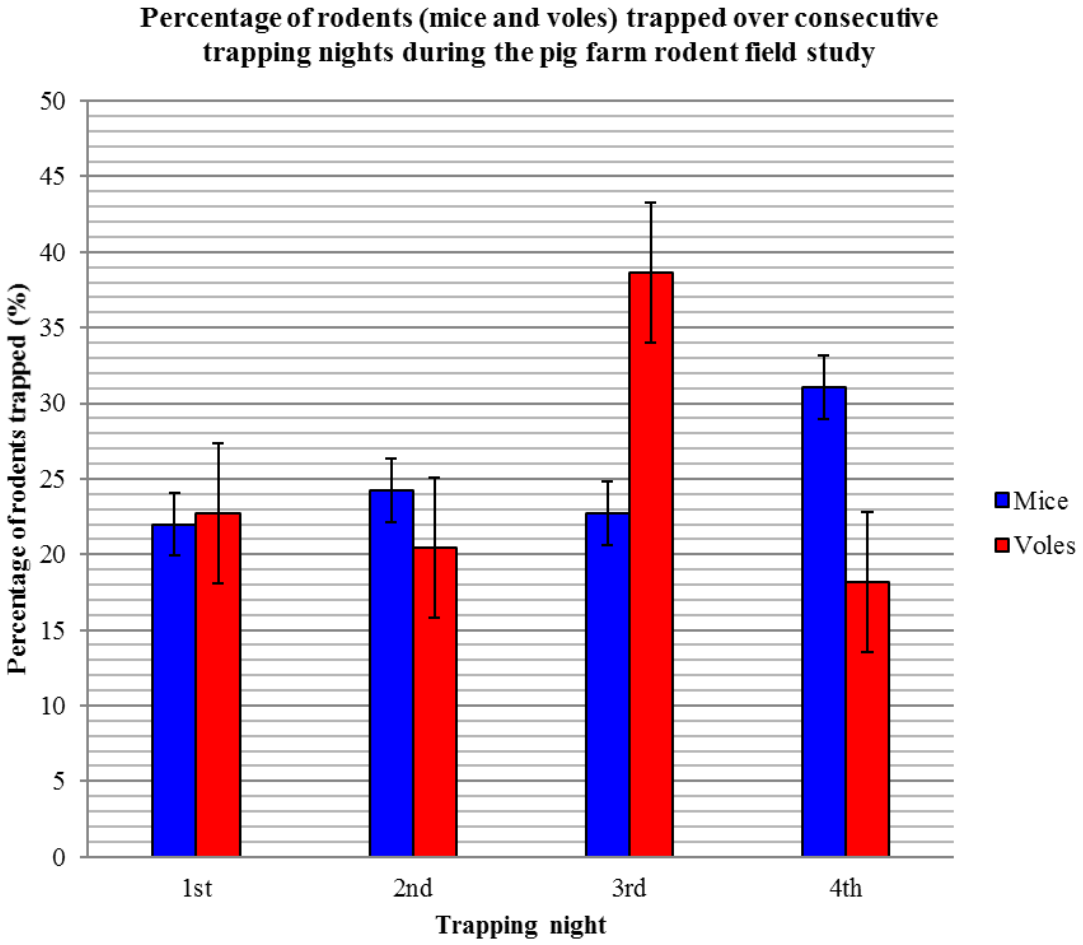

Figure 2.7: Graph showing the percentage of successful rodent catches of mice and voles over a four consecutive night period during the pig farm fieldwork.

Chapter Three: Hantavirus

Prevalence and Diversity of Hantavirus species circulating in British rodents

3.0. Abstract

Orthohantaviruses are members of the *Hantaviridae* family in the order *Bunyvirales* which are known to cause mild to severe disease in humans. Hantaviruses are known to be maintained in nature by mammalian hosts such as rodents. These viruses are able to replicate and be shed in rodent excretions (urine, faeces and saliva) without the host succumbing to the pathogenic aspects of infection. When spillover infection occurs in humans, usually by inhalation of aerosolised viral particles in these excretions, the consequences of infection can result in either haemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) depending on the species of hantaviruses involved. The UK is known to have at least two hantavirus species, Seoul virus (SEOV) in brown rats (*Rattus norvegicus*) and Tatenale virus (TATV) in field voles (*Microtus agrestis*). Puumala virus (PUUV) in bank voles (*Myodes glareolus*) has not been detected in the UK although the reservoir rodent species is present.

This study aimed to investigate the prevalence and diversity of hantaviruses circulating in a selected sample of rodent species. From 2014-16 rodent species that are known to carry hantaviruses were collected, these included brown rats (n=68), field voles (n=23) and bank voles (n=56). During post mortem examination kidney and lung tissue were taken and RNA extracted from these tissues using a guanidinium thiocyanate-phenol-chloroform extraction method. Hantavirus screening was then conducted in duplicate using a pan-hantavirus nested PCR, targeting the RNA dependant RNA polymerase gene located on the L-segment of the viral genome. PCR products were Sanger sequenced and phylogenetic analysis was performed. A Real-time SEOV/HNTV PCR was also performed on all the rat samples in duplicate.

SEOV RNA was detected in 13/68 (19%%, 95% CI 0-40%) rats and 12/13 strains, were shown to be the Humber strain already known to be circulating in UK wild rats, within SEOV lineage 9. A different strain was identified in one rat from Hull. TATV RNA was detected in 7/23 (30.4%, 95% CI, 11.6-49.2%) of field voles from one location in North Wales. No PUUV RNA was detected in this study. The results from this study show there are two different hantavirus species circulating in the British rodent population. In terms of zoonotic risk to public health concerning SEOV, it can be seen that there may a risk if there is frequent interaction with populations of infected wild rats. The results for this study, along with other studies, indicate that TATV could be endemic in the UK, however, the zoonotic potential is still unknown and further research and genome analysis may be required to understand the viral biology.

3.1 Introduction

Hantaviruses are tri-segmented RNA viruses that belong to the genus *Orthohantavirus*, which contains 35 recognised species (ICTV 2018). Hantaviruses establish persistent infections in their reservoir mammalian hosts, such as rodents, bats, and insectivores (Meyer and Schmaljohn 2000a; Zhang 2014). These reservoir hosts are capable of maintaining the infection without developing clinical signs or the immune-mediated pathology sometimes seen when these viruses infect humans (McCaughey and Hart 2000). Hantaviruses replicate in the reservoir hosts' cells and are subsequently shed in urine, faeces, and saliva. It is through the inhalation of aerosolised viruses from these excretions, that humans become infected (Hansen et al. 2015). The severity of the hantavirus infection in humans can vary from asymptomatic to fatal, largely dependent on the virus species. Hantavirus infections were differentiated clinically and geographically into two syndromes, Hantavirus pulmonary syndrome (HPS) and Haemorrhagic fever and renal syndrome (HFRS) (Kruger et al. 2015; CDC 2018). However, it has more recently been recommended to describe the clinical syndromes as 'hantavirus fever' or 'hantavirus disease' to avoid due to overlap between syndromes, global dispersal of Seoul virus (SEOV) and to avoid misdiagnosis (Clement, Maes, and Van Ranst 2014; Clement et al. 2016).

HFRS due to Seoul virus infection (SEOV-HFRS) was first reported in Great Britain (GB) in 1977(Lloyd and Jones 1986), and there have been several reports of human clinical disease. Seropositivity or viral RNA detection have also been reported in the reservoir host, the brown rat rats (*Rattus norvegicus*, including wild, pet and lab rats (McElhinney et al. 2017). In the case of pet or lab rats, it is easier to establish the source of human infection, i.e. the rat in the home or infected lab rat, however, this trace data is not always available for wild rats. There have been several cases of HFRS where a wild rat source was suspected, although not conclusively proven (McElhinney et al. 2017). In 2011, a 59-year-old man who worked on a rat-infested pig farm in Yorkshire, GB, was diagnosed with SEOV-HFRS, and SEOV RNA was detected in wild rats from the same pig farm (Adams et al. 2014).

There is very little known about the prevalence and dispersal of SEOV in wild rats in the UK. As SEOV has been shown to cause disease in people, which can be severe in some cases (Adams et al. 2014), the presence of this virus in peri-domestic wildlife could present a potential public health concern. To investigate SEOV prevalence in wild rats a molecular approach was taken due to the fact that hantaviruses are notoriously difficult to define through serological diagnostic methods due to the cross-reactivity with related hantavirus species (Burton et al. 1998). The tissues which

were chosen for this were lung and kidney as these are the main sites of viral replication, and have been previously used as the target organ in other hantavirus studies (McElhinney et al. 2017). Given that these were wild rat samples, and therefore the viral loads were unknown, an assay with a high degree of sensitivity was required to conduct the molecular screening to detect low-level infections. A nested pan-hantavirus reverse transcriptase (RT) PCR assay (Klempa et al. 2006) was chosen to conduct the molecular screening as it has been validated and has been shown to be highly sensitive as the assay is able to detect viral RNA at a dilution of 1:1000 (Pounder 2013). This pan-hantavirus RT-PCR is also used by the APHA virology diagnostic laboratories to detect SEOV infection in rats (personal communication with L. McElhinney). Sanger sequencing of the PCR amplicons produced in this assay was used to determine the hantavirus species and specify which viral lineage of SEOV they belong to. A Real-Time PCR assay, specific for SEOV and HNTV, was used to confirm and support the results of the pan-hantavirus RT PCR.

Another reason for choosing the pan-hantavirus RT-PCR assay was that it is able to detect multiple species of hantavirus (Klempa et al. 2006; Pounder 2013), therefore the same methodology can be applied to screen multiple rodents species for other hantaviruses, such as *Arvicolinae* hantaviruses Puumala virus (PUUV) and Tatenale virus (TATV). This assay has also been used to detect novel hantavirus species and was used in the initial detection of TATV in a field vole (*Microtus agrestis*) from Cheshire in 2013 (Pounder et al. 2013). TATV has since been identified in an additional site in Northumberland (Thomason et al. 2017) which is geographically distant from the original detection. This could suggest that TATV is widely dispersed among British field voles, however, there is limited information on the prevalence of TATV in the rodent host. TATV is of interest to this study as there is very little known about this hantavirus species due to its novel nature, including whether or not this species has zoonotic potential.

PUUV is also of interest in this study as although the virus has not yet been detected in GB the reservoir host, the bank vole (*Myodes glareolus*) is present and PUUV have been shown to cause clinical disease in humans in continental Europe (Vapalahti et al. 2003). There has also been recorded seropositivity detected in people in GB (Duggan et al. 2017), although PUUV antibodies have been shown to cross-react with TATV (Pounder et al. 2013) so from this study, it is not possible to determine which species is responsible for this seropositive reaction. Molecular screening could remove this problem as, like with SEOV, the Sanger sequence data can be used to differentiate between TATV/PUUV and comment on viral lineage.

This study aims to investigate the prevalence and dispersal of hantaviruses (SEOV, TATV and PUUV) that may be important to human health through the molecular screening of wild rodent tissue samples (*R. norvegicus*, *M. agrestis* and *M. glareolus*). The results from this study could be used to begin to better understand the epidemiology of this infection, and therefore its public health risk.

3.2. Materials and Methods

3.2.1. Fieldwork

Rodents were collected from a range of peri-domestic locations from around Northern England, North Wales and Southern Scotland between 2014 and 2016. For full details on the fieldwork part of this study see Chapter 2. The species collected were *R. norvegicus* (n=68), *M. agrestis* (n=23) and *M. glareolus* (n=56) There were no *A. flavicollis* collected in this study as this species is not present in the regions sampled.

3.2.2. Sample preparation

Rodent carcasses were examined as outlined in Chapter 2. Kidney and lung tissue were removed for hantavirus screening and stored at -80°C until required. Age, weight, reproductive status and any abnormalities seen were also recorded at the time of the examination. All rat carcasses were processed at containment level 3.

3.2.3. RNA extraction

RNA extraction was performed using a guanidinium thiocyanate-phenol-chloroform extraction method. A 50-100 mg sample of tissue from the kidney and lung of each rodent (brown rat, bank vole and field vole) was homogenised using a motor pestle (Sigma-Aldrich Company Ltd, Dorset, UK) with 1ml of TRIzol® Reagent (Thermofisher Scientific, Leicestershire, UK) in a 2 ml microcentrifuge tube (Sigma-Aldrich Company Ltd, Dorset, UK) and then incubated at room temperature for a minimum of 15 minutes. After this, 200 µl of chloroform (VWR International) was added and the microcentrifuge tube was inverted several times and left to incubate for 2-3 minutes, then centrifuged at 4°C at 10,000 x g for 15 minutes. The mixture separated into three layers (Figure 3.1); an organic phase (pink in colour) containing the phenol, an interphase (white in colour) and an aqueous phase (clear in colour) which contained the RNA. The aqueous phase (500 µl) was removed, with care not to disturb the interphase, by pipetting and transferred to a new 2 ml microcentrifuge tube and 500 µl of isopropanol (Sigma Aldrich) was added, then incubated at room temperature for 10 minutes to allow the RNA to precipitate. This was then centrifuged at 4°C at the same speed for a further 10 minutes. All of the supernatant was removed by pipetting leaving the RNA pellet in the microcentrifuge tube. The pellet was then washed with 75% ethanol and allowed to air dry for 4-5 minutes, resuspended in 10 µl of RNA free water and stored at -80°C if not used straight away.

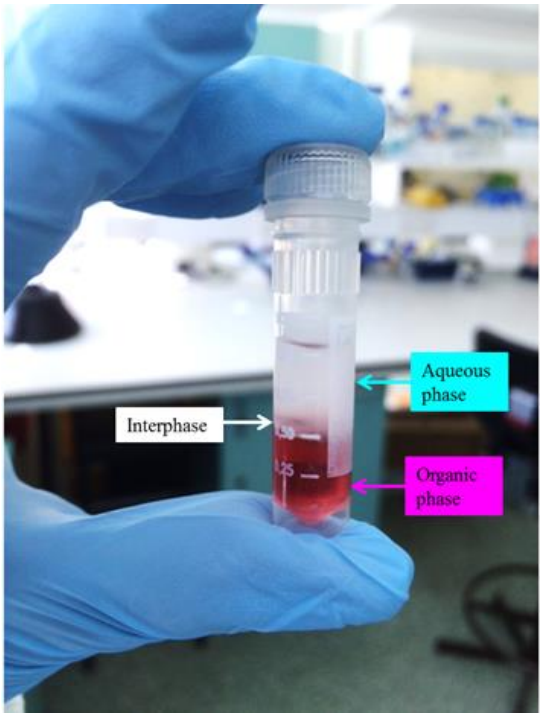

Figure 3.1: RNA extraction using Trizol reagent showing the phase separation part of the extraction. The aqueous phase (containing the RNA), the interphase (containing lipids and protein) and the organic phase (containing phenol) are labelled.

3.2.4. Pan-Hantavirus Nested RT-PCR

For the Pan Hantavirus nested PCR a One-step RT-PCR kit (Qiagen, UK) was used for the first round of the PCR. The kit contained an RT Enzyme mix (consisting of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq® DNA Polymerase), x5 concentrated buffer with 12.5mM MgCl and 10 pmol dNTPs. These reagents were combined with first-round primers HAN-L-F1 and HAN-L-R1 at 10 pmol concentration (Table 3.1) and 1 µl of extracted RNA (Klempa, et al., 2006). In a thermocycler (BioRad, Hertfordshire, UK) a reverse transcriptase step was performed at 50°C for 30 minutes followed by an initial denaturation at 95°C for 15 minutes then 45 cycles of 94°C for 30 seconds then 53°C for 30 seconds and 72°C for 1 minute with a final elongation step at 72°C for 7 minutes. The second round using the Pan-hanta Klempa primers, with HAN-L-F2 and HAN-L-R2 at 10 pmol concentration and the HotStarTaq *Plus* Master Mix Kit (Qiagen, UK) with 1 µl of the first-round product as template. Cycling parameters were the first hold at 95°C for five minutes followed by 40 cycles at 94°C for 30 seconds, 53°C for 30 seconds then 72°C for one minute, then a final elongation step at 72°C for 7 minutes (Klempa et al. 2006). PCR products from both rounds were visualised under UV

light after gel electrophoresis on a 1.8% agarose pegGREEN gel at 120V for 75 minutes. Each sample was screened in duplicate.

Primer name	**Sequence (5'-3')**	**Product size (bp)**
HAN-L-F1	ATGTAYGTBAGTGCWGATGC	452
HAN-L-R1	ACCADTCWGTYCCRTCATC	
HAN-L-F2	TGCWGATGCHACIAARTGGTC	390
HAN-L-R2	GCRTCRTCWGARTGRTGDGCAA	

Table 3.1: Primer sequences from the pan-hantavirus nested RT-PCR assay as published by Klempa et al. (2006) and the expected product size of each band after gel electrophoresis.

3.2.5. Verification of RT-PCR

To prove that this assay is able to detect multiple species of hantaviruses RNA from five different species of hantaviruses (HNTV, PUUV, TULA, SEOV and TATV) were used in the pan-hantavirus RT-PCR assay and the PCR products were visualised under UV light after 70 minutes of gel electrophoresis on a 1.8% agarose gel (Figure 3.2).

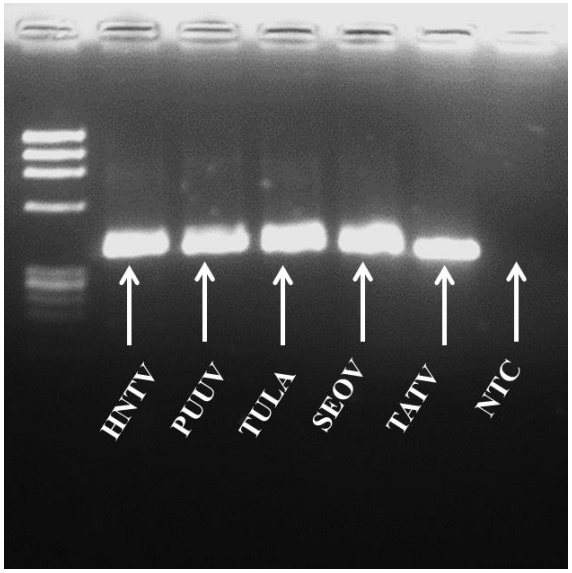

Figure 3.2: Gel photograph of the 2nd round pan-hantavirus nested PCR (390 bp) to confirm that the assay is able to detect a range of species of hantavirus such as Hantaan virus (HNTV), Puumala virus (PUUV), Tula virus (TULA), Seoul virus (SEOV) and Tatenale hantavirus (TATV). The negative control (NTC) is included in the gel photograph. RNA was kindly provided by K. Pounder and supplied to the University of Liverpool by the European Virus Archive.

There were steps included to avoid contamination in this assay, such as changing rooms between the preparation of the PCR mastermix and the addition of viral RNA, frequently changing of gloves and additional negative control in the 2nd round of the PCR. In the initial transfer of the assay from the APHA to Leahurst there was a contamination issue with the 2nd round product as the negative control from the 1st round was positive. To avoid this the addition of the 1st round as a DNA template in the 2nd round was prepared in a different room and gel electrophoresis was conducted only upon completion of both PCR rounds of the assay. Sequence data was used to confirm that the positive results were not a result of contamination with the positive control which was a pet rat SEOV strain (GenBank KM948594) provided by the APHA.

3.2.6. Sanger Sequencing and phylogenetic analysis

The positive 2nd round PCR product from the nested pan-hantavirus RT-PCR was sent to the central sequencing unit (CSU) at APHA Weybridge for PCR product clean-up and Sanger sequencing. Sequence data were analysed using a DNAStar Lasergene software package and using SeqMan Pro to assemble contiguous sequences with the forward and reverse data. Chromatogram profiles of each contiguous sequence were compared and trimmed to remove primer sequences and produce a consensus sequence. Consensus sequences were uploaded into Megalign (DNAStar Lasergene software) and aligned using the 'ClustalW' method (Y. Li 2017; Thompson, Higgins, and Gibson 1994). Any differences were examined and compared with the raw chromatogram data. This part of the study was performed by Daisy Jennings. The consensus sequences were sent to Ellen Murphy and compared with published sequences using the nucleotide Basic Local Alignment Search Tool (BLAST) programme produced by Nation Center for Biotechnology Information (NCBI). Phylogenetic analysis was conducted in MEGA 7 and the sequences from this study were aligned with other related hantavirus sequences. A phylogenetic tree of maximum likelihood was constructed using a best fit model with the highest BIC score and bootstrap analysis was performed with 1000 repeats.

3.2.7. RT Real-Time Q-PCR for S-segment of SEOV

This TaqMan Real Time-PCR targets the 144 base pair region of the S-segment of both SEOV and HTNV. A 19 µl RT-PCR master mix was made with 6.2 µl HPLC water, 10 µl 2x iTaq universal reaction mix (Bio-Rad, Hertfordshire UK), 0.5 µl iScript advanced Reverse Transcriptase (Bio-Rad, Hertfordshire UK) 1 µl HTN/SEOV forward primer 5'CATGGCWTCHAAGACWGTGGG3', 1 µl HTNV/SEOV reverse primer 5'TTKCCCCAGGCAACCAT3', both primers at 20 pmol/ul and 0.3 µl HTNV/SEOV TMGB X probe 5'FAM-TCAATGGGRATACAACT3'. A housekeeping gene (β-actin) Real-Time PCR reaction was also used with the same master mix components, apart from the primers and probe. For the β-actin PCR assay 1 µl forward intronic primer CGATGAAGATCAAGATCATTG, 1 µl reverse primer AAGCATTTGCGGTGGAC and 0.3 µl probe ROX-TCCACCTTCCAGATGTGGATCAGAAG were used. In each reaction, 1 µl of RNA (ideally at a concentration of 1 µg/µl) to the master mix and loaded into the Mx3000 for cycling. Cycle parameters included a reverse transcription step at 50°C for 15 minutes followed by inactivation of the reverse transcriptase at 95°C for 5 minutes followed by 45 cycles at 95°C for 10 seconds and 60°C for 30 seconds (Kramski et al. 2007; Jameson et al. 2013).

3.3. Results

3.3.1. Brown rat screening results

3.3.1.1. Nested Pan-Hanta PCR results

From the RT pan-hantavirus assay 19% (13/68, 95% CI 0-40%) brown rats were positive for hantavirus RNA which after sequence analysis was found to be SEOV. Multiple locations around the Yorkshire and Humber regions (Ripon, Hull, Malton and Wetherby) were shown to contain SEOV infected rats and 4/6 Yorkshire based sites contained at least one brown rat which was infected with SEOV. There was also another site in Cheshire of which 30% (3/10) of brown rats surveyed were SEOV positive.

Year	**Site ID**	**Map I.D.**	**Location**	***R. Norvegicus* collected**	**Hantavirus positive (%)**
2014	Farm 1	A	Cheshire	5	0/5 (0)*
2015	Farm 2	B	Derby	1	0/1 (0)*
2015	Farm 4	C	Middlewich, Cheshire	6	0/6 (0)*
2015	Rural 2	D	Ruthin, North Wales	1	0/1 (0)*
2015	Urban 1	E	Liverpool, Merseyside	4	0/4 (0)*
2015	Urban 2	F	Ellesmere Port, Cheshire	4	0/4 (0)*
2015	Pig Farm 1	G	Ripon, Yorkshire	16	2/16 (12.5)

2015-16	Pig Farm 2	H	Cheshire	10	3/10 (30)
2015	Pig Farm 4	I	Hull, Yorkshire	1	0/1 (0)
2015	Roadkill A165	J	Hull, Yorkshire	1	1/1 (100)
2015	Pig Farm 6	K	Driffield, Yorkshire	2	0/2 (0)
2015	Pig Farm 8	L	Malton, Yorkshire	1	1/1 (100)
2015	Pig Farm 9	M	Wetherby, Yorkshire	16	6/16 (37.5)
			Total	**68**	**13/68 (19)**

Table 3.2: Indicates the number of rats collected from each farm in this study and the number of rats which are hantavirus positive. Only sites which brown rats were collected are shown in this table. Map I.D. correspond to Figure 3.3.

*Rats only screened using kidney tissue due to financial constraints at the end of the project

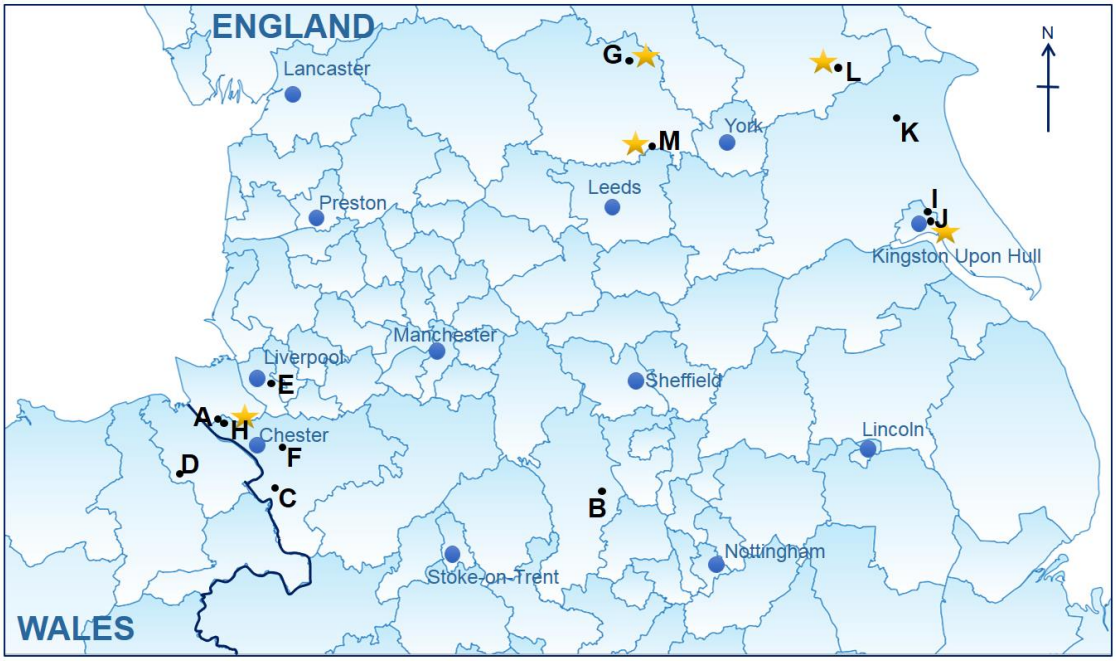

Figure 3.3: Locations of sites at which rat (*R. norvegicus*) samples were collected and correspond to Table 1. Letters correspond to the sites which rats were collected and stars indicate the sites where hantavirus SEOV positive rats were detected. This map was created using GQIS Desktop 3.2.3 software.

There were more males (45/68, 66%) collected in this study than females (23/68, 34%) and of the hantavirus positive rats, 11/13 (85%) rats were male and of those 10/13 were adult males (Table 3.3a). However, any association of the sex of rats and hantavirus positivity was shown not to be statistically significant (95% CI 0.63-34, P=0.193) according to Fisher's test.

a)

	Males	**Females**	**Total**
Rats tested	45	23	68
Rats +ve	11	2	13
% rats +ve	24	8.7	19

b)

	Adults	**Juveniles**	**Total**
Rats tested	50	18	68
Rats +ve	11	2	13
% rats +ve	22	11	19

Table 3.3. Sex (a) and Age (b) differences in SEOV positive and negative rats in this study.

A higher proportion of adult rats (>4 months of age) (11/50, 22%) were infected with hantavirus than juveniles (2/18, 11%) (Figure 3.3b). However, age was shown, like sex, in Fisher's test not to be a statistically significant finding (95% CI 0.41-22.98, P=0.49).

3.3.1.2 Phylogenetic analysis of the 329 nt sequence

		Sequence Similarity %	
		SEOV Yorkshire and Cheshire cluster	**SEOV R62**
UK SEOV, lineage 9	**SEOV Yorkshire and Cheshire cluster**	99.4-100	97-97.6
	SEOV R62	97-97.6	100
	SEOV UK wild rat (Humber)	97.6-98.2	96.7
	SEOV UK pet rat	96.4-96.7	96.1
	SEOV UK lab rat	96.4-96.7	97.3
	SEOV UK export to Sweden (lineage 7)	93.1-93.7	93.4

Table 3.4. Sequence similarity between SEOV strains that have been detected in rats from the UK. All strains are within lineage 9, apart from the rat export from the UK to Sweden, which is Lineage 7. Sequence similarity percentages were generated in DNASTAR MegAlign.

Analysis of the partial 333 nt L-segment fragments showed that all the SEOV sequences, apart from R62, were closely related to the Humber strain of SEOV, which was previously identified in a wild rat from Yorkshire in 2013 with 97.6-98.2% sequence similarity at a nucleotide level (Table 3.4). The strains that have been detected in wild rats differ from those detected in pet rats and the lab rat strain identified in 1984. One sequence, R62 from a rat in Hull, has been shown to be slightly divergent from other wild rats with 97-97.6% sequence similarity observed between R62 and the SEOV Yorkshire/Cheshire cluster. All the SEOV sequences detected in this study are within lineage 9.

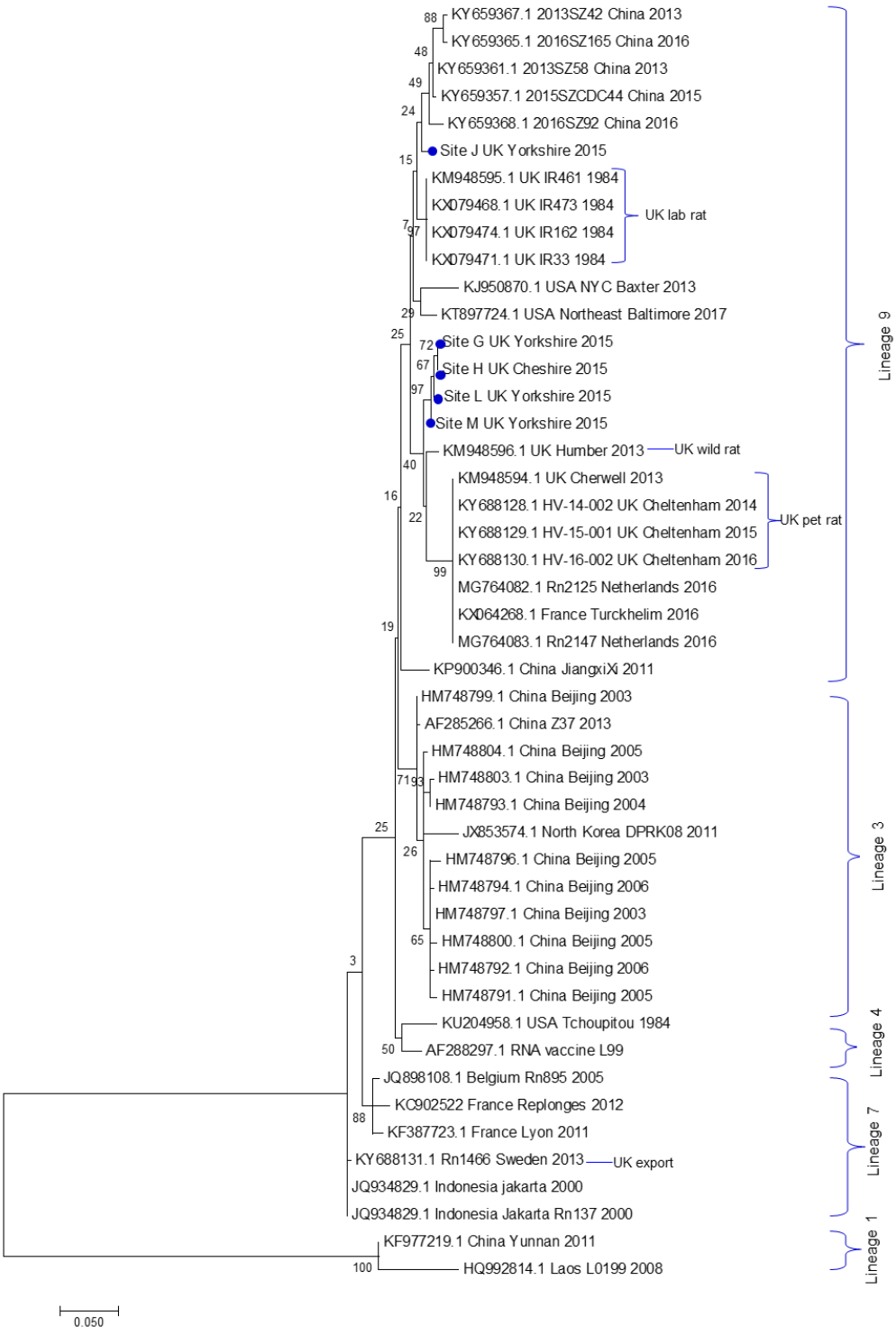

Figure 3.4: Phylogenetic tree of SEOV sequences including those detected in this study and other strains detected in GB. The evolutionary history was inferred by using the Maximum Likelihood

method based on the Tamura 3-parameter model plus gamma (Tamura, Nei, and Kumar 2004). The analysis involved 47 nucleotide sequences (published and sequences from this study) of a partial 329 nt fragment on the L-segment. Sequences from this study are shown with a blue dot. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, and Tamura 2016). Lineages were previously defined by McElhinney et al (2017). Full sequences, including Genbank accession numbers, are available in Appendix VIII.

A species is the lowest taxonomic level in the hierarchy approved by the ICTV. A species is a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria (Adams et al. 2013). These species can then be further classified into viral lineages. The sequence data derived from the PCR amplicons confirms that the hantavirus species in these brown rats are SEOV and that all the UK sequences in this study reside in viral lineage 9 of SEOV along with published other UK rat sequences (McElhinney et al. 2017). However, there are several branches with low bootstrap values which reduces the confidence of the positions in this tree so definitive phylogenetic conclusions cannot be made based on Figure 3.4.

3.3.1.3a. Real-time SEOV PCR

The results from the Real Time SEOV PCR assay (Table 3.5) detected hantavirus RNA in 4/47 brown rats in both the lung and kidney tissue of the same rats (R62, R71, R73 and R74). This corresponds with the results of the nested pan hantavirus PCR. The Ct values of the Real-Time SEOV ranged from 33.07 to 39.58 in the kidney and 32.53 to 38.51 in the lung. The results from the Real Time match what was seen in the Pan-hantavirus PCR assay as Hantavirus RNA was detected in all of the 4 rats by each assay in both organs. However, the real-time assay did not detect SEOV RNA in a rat which the RT-PCR detected in a single organ. For example, RNA and sequence data was obtained from R65 kidney tissue only via the RT-PCR assay but was not detected in the Real Time assay. All rats were positive for the β-actin housekeeping gene in the lung tissue and 45/47 rats were β-actin positive in the kidney. Rats R46 and R55 were negative for β-actin indicating either an extraction failure or that there was no amplifiable RNA in the kidney tissue of those rats. All other rats in the study were negative for SEOV RNA in both the Real-Time SEOV assay and the nested pan-hantavirus assay.

Sample ID	Nested Pan Hanta PCR (Kidney)	(Lung)	Real-Time SEOV PCR (Kidney)		Real-Time SEOV PCR (Lung)		Real-Time β-actin housekeeping PCR (Kidney)		Real-Time β-actin housekeeping PCR (Lung)	
R39	-	-	-	n/a	-	n/a	+	29.43	+	30.08
R40	-	-	-	n/a	-	n/a	+	30.70	+	27.50
R41	-	-	-	n/a	-	n/a	+	31.13	+	25.11
R42	-	-	-	n/a	-	n/a	+	38.82	+	30.52
R43	-	-	-	n/a	-	n/a	+	26.66	+	29.33
R44	-	-	-	n/a	-	n/a	+	31.06	+	28.10
R45	-	-	-	n/a	-	n/a	+	30.35	+	29.23
R46	-	-	-	n/a	-	n/a	-	n/a	+	31.87
R47	-	+	-	n/a	-	n/a	+	31.02	+	33.53
R48	-	-	-	n/a	-	n/a	+	28.10	+	27.90
R49	-	-	-	n/a	-	n/a	+	31.67	+	28.50
R50	-	-	-	n/a	-	n/a	+	28.86	+	24.93
R51	+	-	-	n/a	-	n/a	+	30.67	+	41.75
R52	-	-	-	n/a	-	n/a	+	30.12	+	33.33
R53	-	-	-	n/a	-	n/a	+	30.64	+	30.04
R54	-	-	-	n/a	-	n/a	+	28.45	+	26.59
R55	-	-	-	n/a	-	n/a	-	n/a	+	27.95
R56	+	-	-	n/a	-	n/a	+	30.56	+	28.75
R57	-	-	-	n/a	-	n/a	+	32.17	+	27.98
R58	-	-	-	n/a	-	n/a	+	30.01	+	27.91
R59	-	-	-	n/a	-	n/a	+	29.17	+	28.53
R60	+	-	-	n/a	-	n/a	+	29.83	+	27.48
R61	-	-	-	n/a	-	n/a	+	38.18	+	27.34
R62	+	+	+	39.58	+	38.51	+	31.05	+	27.49
R63	-	-	-	n/a	-	n/a	+	30.09	+	25.95
R64	-	-	-	n/a	-	n/a	+	31.46	+	27.38
R65	+	-	-	n/a	-	n/a	+	29.16	+	28.51
R66	-	+	-	n/a	-	n/a	+	24.97	+	32.81

R67	-	-	-	n/a	-	n/a	+	28.42	+	31.90
R68	-	-	-	n/a	-	n/a	+	30.37	+	30.59
R69	+	-	-	n/a	-	n/a	+	30.73	+	32.33
R70	+	-	-	n/a	-	n/a	+	33.65	+	28.17
R71	+	-	+	33.07	+	32.78	+	30.22	+	28.96
R72	-	-	-	n/a	-	n/a	+	29.00	+	26.92
R73	+	-	+	35.30	+	32.97	+	31.78	+	29.53
R74	+	-	+	36.87	+	32.53	+	31.86	+	27.79
R75	-	-	-	n/a	-	n/a	+	29.48	+	31.28
R76	-	-	-	n/a	-	n/a	+	29.87	+	29.18
R77	-	-	-	n/a	-	n/a	+	29.50	+	29.35
R78	-	-	-	n/a	-	n/a	+	31.51	+	32.54
R79	-	-	-	n/a	-	n/a	+	25.82	+	23.75
R80	-	-	-	n/a	-	n/a	+	30.19	+	30.27
R81	-	-	-	n/a	-	n/a	+	29.17	+	29.33
R86	+	-	-	n/a	-	n/a	+	26.71	+	26.62
R87	-	-	-	n/a	-	n/a	+	24.13	+	28.58
R88	-	-	-	n/a	-	n/a	+	28.51	+	26.20
R89	-	-	-	n/a	-	n/a	+	25.50	+	28.54
	11/47	**6/47**		**4/47**		**4/47**		**45/47**		**47/47**

Table 3.5: Results from the Real-time SEOV PCR compared with the results from the nested pan-hantavirus PCR on a subset of rat kidney and lung tissue. The housekeeping Real Time β-actin PCR results are also shown. For negative samples, no Ct values were obtained (n/a).

3.3.2 Field vole screening results

This study has detected the presence of TATV in 7/20 field voles from Llyn Cowlyd in North Wales based on the results from the pan-hanta RT-PCR assay. No TATV RNA was detected in field voles from Malton Yorkshire or Tattenhall, Cheshire, therefore the overall detection for this study was 7/23 (30.4%, 95% CI, 11.6-49.2%) (Table 3.6).

Year	**Site ID**	**Location**	***M. agrestis* collected**	**Hantavirus positive (%)**
2015	Farm 3	Tattenhall, Cheshire	2	0/2 (0)
2015	Rural 1	Llyn Cowyld, North Wales	20	7/20 (35)
2015	Pig Farm 8	Malton, Yorkshire	1	0/1 (0)
		Total	**23**	**7/23 (30.4)**

Table 3.6: Summary of the field vole screening results. Only sites which field voles were collected from are shown in this table.

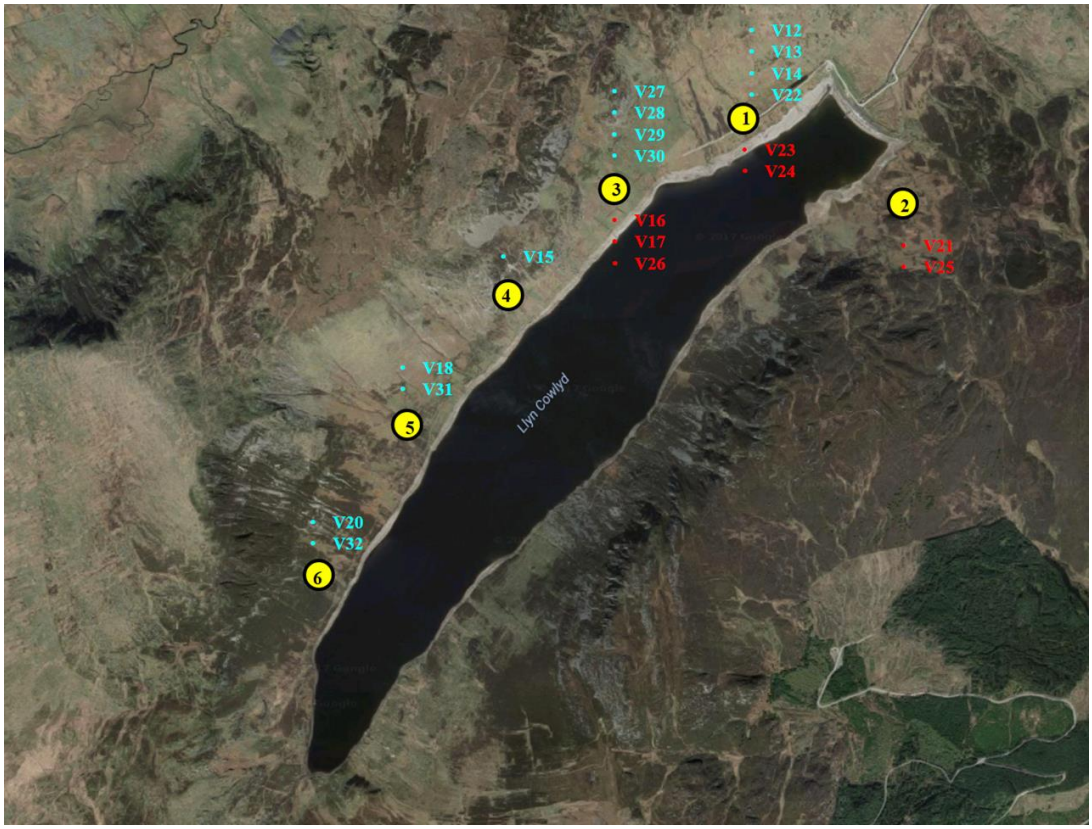

Figure 3.5: Sample sites around Llyn Cowlyd, North Wales were TATV positive field voles (red) were identified. TATV negative field voles are also shown (turquoise). This satellite map was created using Google maps.

There were six sites covered in Llyn Cowlyd, which were clustered around the water reservoir and the sites of infected field voles are shown in figure 5. TATV infected field voles were only detected in sites 1, 2 and 3, which were clustered around the one area of the reservoir, the sites 4, 5 and 6 did not contain field voles that were infected with TATV.

There were 3/13 (23%) male voles and 4/9 (44%) female voles which had detectable TATV RNA (Table 3.7a). Although after Fisher's test this was shown to be not significant (95% CI, 0.08-5.34, P=1). There was, however, a difference observed in the age of the field voles as it was predominantly adult field voles which were infected with TATV (n=6) with only one juvenile female vole containing detectable TATV RNA (Table 3.7b), although there were only two juvenile voles collected in the entire study. This was also shown to be non-significant (95% CI, 0.004-36.6, P=0.5257).

a)

	Males	**Females**	**Total**
Voles tested	13	9	22*
Voles +ve	3	4	7
% voles +ve	23	44	32

b)

	Adults	**Juveniles**	**Total**
Voles tested	21	2	23
Voles +ve	6	1	7
% voles +ve	29	50	30.4

Table 3.7: Sex (a) and Age (b) differences in TATV positive and negative field voles in this study. A gender was unable to be assigned to V27 due to a lower abdominal injury to the vole before capture (*).

The phylogenetic analysis shows that the TATV sequences from this study, based on a 291 nt partial sequence, cluster with the other TATV sequences already detected in the UK in Cheshire (B41, Genbank accession number JX316008) and Northumberland (Genbank accession numbers KY751731 and KY751732). However, there was genetic divergence observed in the UK TATV sequences, with 89-89.3% and 87.3-88% genetic similarity B41 and Northumberland vole sequences, respectively (Table 3.8). This may indicate that there are multiple TATV strains circulating in the UK. There was also a greater genetic diversity observed between TATV sequences in this study from other *Arvicolinae* vole hantavirus species such as PUUV (75.9-76.3%

genetic similarity), Khabarovsk virus (78.4-79% genetic similarity) and Tula virus (77.3-78% genetic similarity).

		Sequence Similarity %		
		TATV (this study)	**TATV (B41)**	**TATV (Northumberland)**
Vole hantaviruses	**TATV (this study)**	99.3-100	89-89.3	87.3-88
	TATV (B41)	89-89.3	100	87.3-87.6
	TATV (Kielder)	87.3-88	87.3-87.6	99.7
	Puumala virus	75.9-76.3	78.4-78.7	79.4-80.1
	Khabarovsk virus	78.4-79	78-78.4	76.6-77
	Tula virus	77.3-78	79.4-79.7	76.6-78.7

Table 3.8: Estimates of evolutionary divergence between partial L-segment sequences of *Arvicolinae* subfamily hantaviruses, shown as percentages of genetic similarity which were generated in DNASTAR MegAlign.

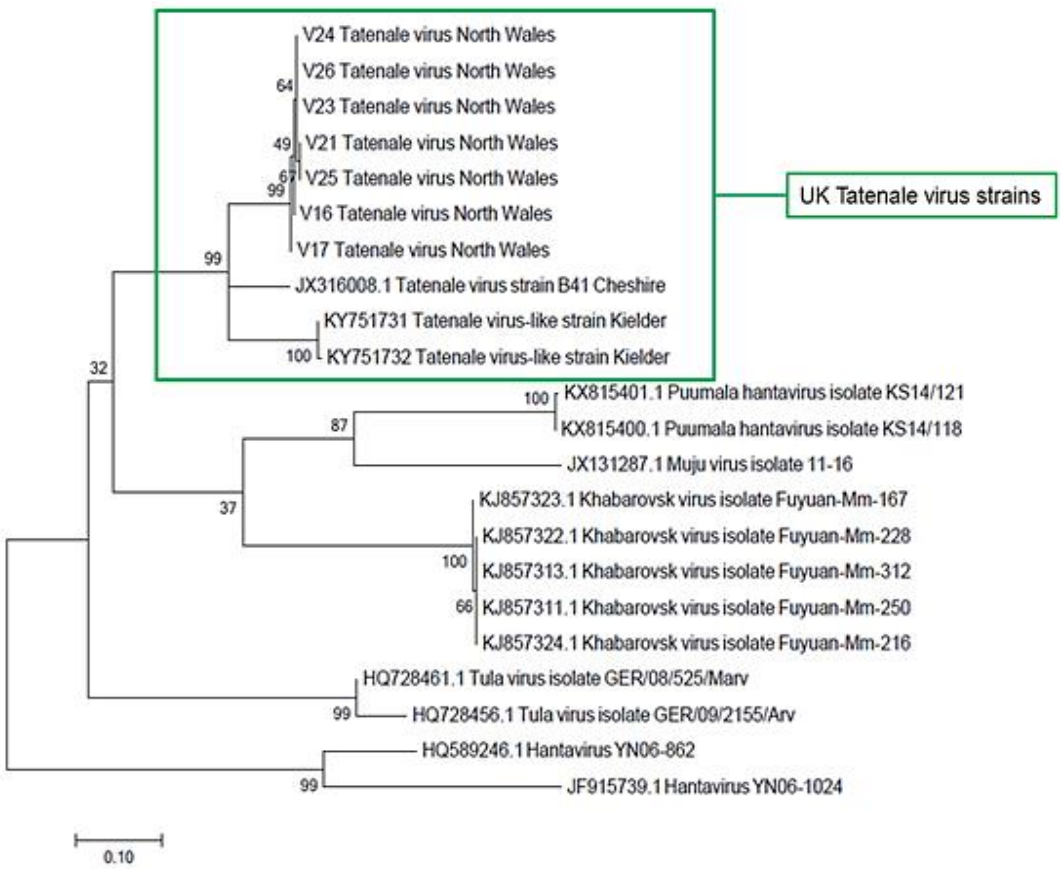

Figure 3.6: Phylogenetic tree of the evolutionary history of *Arvicolinae* subfamily hantaviruses using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992). All UK strains, including those in this study (V16, V17, V21, V23, V24, V25 and V26) are highlighted. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences and was based on a 291 nt partial sequence of the L polymerase gene. All positions with less than 95% site coverage were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

3.3.3. Bank vole screening results

There was no PUUV RNA detected in the 56 bank voles at 10 different peri-domestic locations screened by the pan-hantavirus nested PCR assay in this study (Table 3.9).

Year	Site ID	Location	*M. glareolus* collected	Hantavirus positive (%)
2014	Farm 1	Wirral, Merseyside	3	0/3 (0)
2015	Farm 3	Tattenhall, Cheshire	6	0/6 (0)
2015	Rural 2	Llyn Cowyld, North Wales	2	0/2 (0)
2015	Pig Farm 1	Ripon, Yorkshire	8	0/8 (0)
2015	Pig Farm 4	Hull, Yorkshire	1	0/1 (0)
2015	Pig Farm 6	Driffield, Yorkshire	8	0/8 (0)
2015	Pig Farm 8	Malton, Yorkshire	9	0/9 (0)
2015	Pig Farm 9	Wetherby, Yorkshire	4	0/4 (0)
2015	Pig farm 10	Ponteland, Northumberland	3	0/3 (0)
		Total	**56**	**0/56 (0)**

Table 3.9: Summary of the bank vole screening results. Only sites which bank voles were collected are shown in this table.

3.4. Discussion and conclusion

3.4.1. Hantaviruses in British rodents

There have been few studies of hantaviruses in wild rodents in the UK. In this study a pan-hantavirus PCR was used in order to determine which hantaviruses were present in the most common and likely hosts of hantaviruses: rats (Seoul virus, SEOV), bank voles (Puumala virus, PUUV) and field voles (Tatenale virus, TATV). No viral RNA was detected in the bank voles, but two distinct species of hantavirus were detected in rats (SEOV) and field voles (TATV).

3.4.2. Detection of SEOV in brown rat populations

The results from this study indicate that SEOV is circulating in the British wild rat population although not uniformly distributed. The prevalence of SEOV was shown to be 13/68 (19%, 95% CI 0-40%). All of the SEOV infected wild rats were either trapped from pig farms (12/13) or collected as road kill (1/13) from a road near multiple pig farms. SEOV RNA was detected on four pig farms of the six sampled. A previous study by Pounder (2013) reported no SEOV RNA in 133 rats sampled in semi-rural and urban areas around Liverpool, Merseyside. This suggests that SEOV is likely to be present in localised foci of infection. Pig farms may provide may suitable environments capable of sustaining large populations of brown rats. However, this is applicable to a variety of habitats such as agricultural farms, sewers or rubbish dumps which also provide suitable habitats for large rat populations, including some of those sampled by Pounder (2013).

Pig farm 1 (Ripon) and Pig 9 (Wetherby) were both shown to contain SEOV infected rats and there was evidence at both locations that there was a high level of rat activity that indicated there were large numbers of rats present. This may suggest that transmission and maintenance of SEOV in a population of brown rats could be density dependent. In the SEOV-HRFS case in a Yorkshire pig farmer, it was reported that the farm in question had a current rat infestation and 50% rats trapped had detectable SEOV RNA, although this was based on a small sample size (n=4) (Jameson et al. 2013). When there is a higher number in a population there will be more interactions between infected and non-infected individuals as the population expands. As population pressure increases so does the aggressive behaviour towards members of the established colonies due to the increased competition for food and territory (Harris and Yalden 2008). Higher numbers of rats that could lead to more frequent aggressive encounters and this contact could lead to an increase of SEOV transmission in a population via infected saliva in bites (Hinson et al. 2004). This has been proposed in infected pet rats that were housed with non-

infected rats in close proximity, SEOV easily spreads throughout the entire colony (McElhinney et al 2017). However, the results from this study alone are not enough to verify this. Comparative studies of multiple rat populations and mathematical risk modelling would be required to determine if whether large populations of rats present a greater risk of SEOV transmission.

A sex bias has been observed in several published studies, with male rats thought to be more likely to be infected with SEOV than females. This has been demonstrated in other studies, as experimentally infected male rats were more likely to shed hantavirus in their saliva than their female counterparts, as well as shedding the virus in faeces and saliva for a longer period of time (Escutenaire et al. 2002). Wounding from aggressive encounters, with the most frequently bitten rump of the rat (Harris and Yalden 2008), is thought to be correlated with SEOV infection (Hinson et al. 2004). Males are also known to be more aggressive making fighting contact between male individuals more likely, thus increasing transmission (Hinson et al. 2006). Males with more severe wounds have been shown to be more likely to be SEOV seropositive and shed SEOV virus in their excretions than females or adult males with less severe injuries (Hinson et al. 2004). Higher concentrations of testosterone have been observed in severely wounded males than those male rats with less severe or no wounds, which could suggest that SEOV infection of the testes could alter behaviour by increasing testosterone (Hinson et al. 2004). This may increase aggression in males and make them more likely to engage in aggressive behaviour for a longer duration. Dominant aggressive males also appear to have higher viral loads in kidney, lung and testis tissue than less aggressive males (Klein, Zink, and Glass 2004). This notion is supported in this study as there appears to be a sex bias as more males (11/13, 85%) were infected with SEOV than females (2/13, 15%), and of these male rats, 9/11 were also sexually mature. Although more males (45/68, 66%) were sampled in this study than females (23/68, 34%), which may bias the results and in this incidence sex bias was shown not to be statistically significant (P= 0.193), likely due to the small sample size. Therefore it is not possible to conclusively comment, based on the results of this study alone, whether males are more likely to be infected than females.

There has also been an age bias reported, as older animals are more likely to be SEOV positive than younger members of the population. There may be a lower rate of infection with SEOV in young rats due to the presence of maternal antibodies, which can be passed in utero or through breast milk from an infected dam (Dohmae, Koshimizu, and Nishimune 1993). This could provide a protective immune response which prevents SEOV infection while maternal antibodies remain. Also, as with sex bias, age bias could likewise be linked to aggressive behaviours with a rat population as younger rats are less likely to engage in aggressive behaviour than older rats (Hinson

et al. 2004). Thus are less likely to sustain fighting wounds which have been shown to be linked to SEOV transmission in rats. Additionally, older females are also more likely to be seropositive for SEOV and have viral RNA in their tissue than younger females (Hinson et al. 2004). In this study, there also appears to be an age bias, as 15% (2/13) SEOV positive rats were juveniles (<4 months old yet to reach sexual maturity). Unfortunately, the sample size was insufficient (18/68 juveniles sampled) to prove any age bias, with no statistical significance (P= 0.49) demonstrated.

There were six sites that had SEOV infected rats, five of which, were in the Yorkshire region. This raises the question of whether there might be a risk of SEOV infection in Yorkshire due to a larger proportion of the wild rats being SEOV positive in that region. Comparative surveillance studies on wild rats from other geographical regions of the UK have shown other rat populations to be negative for SEOV RNA, such as in the Liverpool region where 133 brown rats from urban areas and allotments were screened (Pounder 2013) and rural regions of Gloucester where 27 rats were negative (APHA unpublished data, personal communication with L. McElhinney). The first UK wild rat SEOV strain to be reported, the Humber strain, that also was responsible for a clinical HFRS case, originated from the Yorkshire region (Jameson et al. 2013). The results from our study show the 2013 HFRS case was not an isolated incident, as multiple pig farms of the same region have been shown to contain SEOV positive rats. However, the results from this study are not enough to make conclusions about the risk of SEOV infection from wild rats for the entire region. The sample size (n=37) may be too small and not sufficient to represent the whole rat population or make any predictions about increased risks to public health. Also, a large proportion of samples in this study (37/68, 54%) were derived from this region, which could bias the results. Further surveillance in a wider study of multiple rat populations across the UK would be needed to compare to statistically prove whether or not there is a greater public health risk of SEOV infection in the Yorkshire region.

This study has also identified two different SEOV strains circulating in British wild rats. In this study 12/13 positive rats representing both Yorkshire and Cheshire cluster with the Humber strain (97.6-98.2% genetic similarity), which had previously been identified in wild rats in Yorkshire (Jameson et al. 2013). However, a second novel strain (97-97% genetic similarity to Yorkshire/Cheshire cluster) has been identified in one rat from Hull (R62) and the sequence was most closely related to a US wild rat SEOV strain (Baxter strain) identified in rats from New York City USA (Firth et al. 2014). All of these UK SEOV rat strains clustered within lineage 9 (Figure 3.3 and 3.4), which includes pets rats (apart from one rat export from the UK to Sweden, which was lineage 7) (McElhinney et al. 2017). The diversity observed in the closely related strains

within lineage 9, as well as the distribution of SEOV to multiple regions, is due to the movement of the reservoir host. Brown rats have been known to commonly travel 0.5km, with the longest overnight single journey recorded being 3.3 km, though when a stable food source is available movement is likely to be less than 65 m (Harris and Yalden 2008). This movement could allow for the dispersal of SEOV between different populations of rats.

All of the strains of SEOV that have been identified have been proposed to have Chinese ancestry, including the Baxter strain and the Humber strain (Firth et al. 2014). SEOV is believed to have originated from China, with all non-Chinese variants of SEOV clustering within phylogroup A and this dispersal is due to human activity, predominantly the shipping trade (Lin et al. 2012), whereby infected rats board ships and travel to new areas and mix with susceptible indigenous port rats. Although there may be rodent control measures in place it is possible for these to fail, as in Hokkaido in Japan and Philadelphia, Houston in the USA (Bi et al. 2005). The main UK lineage of SEOV, lineage 9, has also been identified in the Netherlands in farmed rats and pets rats purchased from the same facility (Swanink et al. 2018), as well as in French pet and farmed rats which have also been shown to be infected with SEOV lineage 9 strains (Reynes et al. 2017). This highlights that the distribution, even across countries, of SEOV, could be wide due to the movement of rats. This also raises the question of whether the increased detection of infected brown rats is due to the emergence of SEOV due to a recent introduction or enhanced awareness and surveillance. Brown rats are thought to have been present in the UK for nearly 300 years (Harris & Yalden 2008), in which there could have been numerous opportunities for SEOV introductions, therefore, it is not possible to state when this occurred. It is likely that SEOV is not an emerging pathogen but these detections are due to increased interest, more surveillance studies and improved diagnostics.

There were two types of PCR diagnostic tests used in the project to screen rat kidney and lung tissue for SEOV RNA, the nested RT pan-hanta PCR assay and the Real-Time SEOV/HNTV assay. The RT pan-hanta assay detected 13/68 rats positive for SEOV RNA and the real-time SEOV/HTNV assay 4/47 only in the same rats. Only rats which had RNA in both organs, according to the RT pan-hanta assay were shown to be positive in the real-time assay. This could indicate that the real-time may be less sensitive than the RT pan-hanta assay, as the presence of RNA in both organs could indicate a higher viral load in that animal, which may be required for the real-time assay to detect SEOV RNA. Even then, Ct values were high, with RNA detected in the kidney at cycles 33.07-39.58 and cycles 32.58-38.51 in the lung in a 45 cycle reaction, suggesting low viral loads. Therefore in screening rats in the future, in the case of an outbreak, for

example, it may be beneficial to use the RT pan-hanta assay rather than the real-time assay. Although there are limitations to using the RT pan-hanta assay, such as the introduction of a second round which can increase the risk of contamination so multiple repeats, in triplicate, are required and which can increase costs. Sequencing is also required to confirm the hantavirus species due to the pan-nature of the assay, although this can be advantageous if using the pan-assay to screening multiple rodent species for multiple hantaviruses. Sequencing may not be required in the real-time assay as it is highly specific and will only detect SEOV or HNTV RNA that is present.

In this study, 4/47 rats were positive in both kidney and lung tissue, excluding the 21 rats in which the kidney was the only organ tested. Kidney could be seen as a more reliable organ to test for hantavirus, as in this study where a single organ was positive for SEOV RNA, there were 7/47 rats positive in only the kidney compared to 2/47 in lungs only. A study by McElhinney et al. (2017) also showed that hantavirus was more reliably detected in the replicate testing of kidney tissue compared to lung tissue. However, the real-time assay showed that there was no amplifiable RNA, by the lack of β-actin housekeeping gene, in the kidney of two rats (R46 and R55), which could indicate that the RNA extraction had been unsuccessful. β-actin however, was detected in the lung tissue of these rats, therefore it could be possible, as these rats were donated from pest control programmes and had been deceased for an unknown period, that the kidney tissue quality may have decreased or inhibitors (such as poison) present in the kidney. The kidney tissue is in closer proximity to invasive bacteria migrating from the autolysing GIT, therefore the kidney is more likely to degrade quicker than the lung, and lose its RNA viability and histological architecture (personal communication with J. Chantrey). Every attempt was made to preserve the tissue integrity however variability may have been introduced through temporary storage at -20°C and freeze-thawing of carcasses. Thus, in future studies, it might be beneficial to screen both tissues when the rodent has been deceased for an unknown, potentially prolonged, amount of time before screening.

3.4.3. Detection of TATV in field voles

Tatenale virus (TATV) RNA was detected in 7/23 (30.4%, 95% CI, 11.6-49.2%) field voles (*M. agrestis*). All of the positive samples (n=7) came from one location in North Wales. This is the third detection of TATV in a British field vole in recent years with the initial discovery of TATV in Cheshire in 2013 (Pounder et al. 2013) and the identification of 8/48 TATV infected field voles in 2015 in Kielder forest, Northumberland (Thomason et al. 2017). The TATV sequences from this study cluster with the sequences published in the two previous studies. The 291 nt sequences

in this study derived from a conserved region of the L-segment were shown to be highly similar to TATV sequences from Cheshire (89-89.3% genetic similarity) and Northumberland (87.3-88% genetic similarity). This further supports the idea, originally proposed by Pounder et al (2013), that this could be a new hantavirus species, although there is still genetic diversity observed between the UK TATV sequences. This could indicate that there are multiple strains of TATV circulating within British field voles.

There was a greater amount of genetic diversity observed of between the TATV sequences and other vole sequences such as TULV (77.3-78% genetic similarity), PUUV (75.9-76.3% genetic similarity) and Khabarovsk virus (KBR) isolated from the reed vole (*Microtus fortis*) in Russia (78.4-79% genetic similarity) (Horling et al. 1996). Therefore the referral of TATV and Tula-like (Clement & Van Ranst 2016) or a strain of TULV is incorrect as TATV is clearly a distinct hantavirus species. Although the full genome of TATV has not been published and would be required to apply for species status from ICTV. Vole material generated in this study could be applied to future studies to accomplish this.

As TATV was only first detected in 2013, there is very little known about its transmission dynamics within field vole populations. Comparisons can be drawn with other vole hantavirus hosts which share similar behavioural ecologies, such as bank voles (PUUV) and common voles (TULV), for which there is more understood about the transmission and viral maintenance of these viruses in continental Europe (Deter et al. 2008). There has been recorded horizontal transmission of PUUV within bank vole populations through a contaminated shared environment, such as shared burrows and runways, which may be more common in high-density colonies (Deter et al. 2008). Voles are territorial animals and mark their territories by daily scent marking which could aid the dispersal of the virus. Indeed a new uninfected vole could take ownership of a territory and become infected within several days after the disappearance of the previously infected vole, thus the virus is continually maintained (Sauvage et al. 2003). Field voles have been shown to scent mark and use specific latrines (Couzens et al. 2017), so transmission of TATV through a contaminated shared environment is highly likely. This could provide an explanation for the TATV distribution at the North Wales site around Llyn Cowlyd all of the TATV infected field voles were clustered around one area of the lake (Figure 3.4).

Field vole home ranges could also be used in the approximation of dispersal of TATV within a vole population. Home ranges can vary due to a number of factors such as sex, age, season and population density. In Sweden greater home ranges have been recorded during the breeding season, a male in the breeding season has been thought to move 1434-910 m^2 compared to a non-

breeding season breeding male which is thought to have a home range of 600-447 m^2 (Harris and Yalden 2008). As voles are a polyamorous species, so breeding males visit multiple female territories and visit excretory points more frequently, therefore are more likely to become infected. How widespread TATV is may be difficult to determine as the populations where the virus has been detected are geographically isolated from each other, although in rare incidences field voles have been shown to travel >1 km over land or through water that could lead to colonisation of new habitats (Harris and Yalden 2008). The site in North Wales is 75 km from Cheshire and approximately 250 km for Kielder forest, therefore it is very unlikely that there will have been an interaction between these populations. In terms of the movement of TAV by the rodent host, the home ranges of field voles are not large enough to account for detection in multiple geographical locations. The detection of this hantavirus in three geographically separate locations could be an indication that TATV is endemic to the UK. Although further surveillance on other parts of the UK, such as S. England, S. Wales, Scotland and N. Ireland, would be required to confirm this, as at present, there is no TATV prevalence data from field voles in those areas.

There has been an age bias observed in previous studies for other vole associated hantaviruses, that older voles are more likely to be infected than their younger counterparts (Deter et al. 2008). It has been observed in older common voles that they are more likely to be seropositive for TULV than younger ones (Deter et al. 2008) and with PUUV the greatest seropositivity has been observed in overwintered male bank voles (Bernshtein et al. 1999). As for SEOV infected rats, juveniles are thought to be less likely to be infected than adults as they retain maternal antibodies for 2-3 months if the dam is infected, and vertical transmission has not been recorded between voles. If they remain at the nesting site, infection from the dam may be possible once the maternal antibodies have decreased. Young mice and voles also may disperse from the nesting site while still containing maternal antibodies (Deter et al. 2008). In this study, there was no statistically significant age bias (P=0.5257) recorded, possibly due to the small sample size. Only two juvenile voles were collected, one of which was TATV positive, compared to 18 adult voles, which would bias the results. This is likely due to the time of year as most voles were collected mid to late summer, which was at the height of the breeding season and there is the greatest amount of movement from breeding adults, therefore adult voles are more likely to be trapped. There were a greater number of TATV infected females (44%) than infected males (23%), contrasting to what was observed for SEOV in this study, however, it is not possible to comment further as this was also shown to not statistically be significant (P=1).

Infectivity and pathogenicity of TATV to humans is unknown. Comparisons can be made to other

Arvicolinae species hantaviruses, such as related viruses PUUV and TULV, to estimate what would be the potential consequences of human infection would be. The closest related hantavirus is Tula virus (TULV) and is mainly found in the common vole (*Microtus arvalis*) which is a species present widely across Europe, in which there are several documented cases of human TULV cases. TULV has also been shown to be a highly promiscuous virus that is able to infect many species from the *Microtus* genus as including *M. rossiaemeridionalis*, *M. gregalis*, *M. subterraneus* and the European water vole (*Arvicola amphibius*) in Germany and in Switzerland (Schlegel et al. 2012). TULV is thought to only result in very mild HFRS symptoms and in some cases, seropositivity has been detected in individuals who have no symptoms at all (Mertens et al. 2011). However, there have been rare cases were infection with TULV has resulted in hospitalisation, such as a case in France where a man in his mid-thirties became infected while handling voles his cat had caught (Reynes et al. 2015) and a serious case of an immunosuppressed 14-year-old boy from the Czech Republic (Zelená, Mrázek, and Kuhn 2013). Human TATV infection may exhibit a similar clinical disease as TULV human infection in a sense that infection may only result in asymptomatic to mild disease. To date, there have been no human infections in the UK attributed to TATV infection recorded. In that sense the public health risk is likely to be low, however, at this stage, it is purely an assumption as there is not enough known about this virus to accurately estimate the zoonotic risk. TATV antibodies have been shown to cross-react with PUUV antibodies (Pounder et al. 2013) in current diagnostic serological tests for human HFRS. Therefore a more specific diagnostic assay would be required to determine if TATV is present and could be used to determine the zoonotic potential of this virus.

3.4.4. Detection of PUUV in Bank voles

There was no PUUV RNA detected in the kidney or lung tissue of the 56 bank voles screened in this study. This concurs what has been observed in other studies by Pether and Lloyd et al in 1993 and K Pounder in 2013, were no antibodies or PUUV RNA were found in any of the bank voles that were screened in either study. These results could be a true reflection of the infection status in a sense that British bank voles are not infected with PUUV. This is in contrast to what is seen in mainland Europe where PUUV is the dominant and most clinically relevant hantavirus in people. Bennett et al (2010), has suggested that a reason for this could be the differences in woodland coverage, as in the UK the forests are more fragmented and do not cover large land areas. This could lead to populations of voles becoming isolated and so there is less likely to be mixing of infected and naive populations making the transmission and maintenance of PUUV less likely (Bennett et al. 2010). In Germany where there are vast areas of forest there have been

multiple outbreaks of PUUV infection in people recorded since 2001 and it is the most clinically significant hantavirus in that country (Hofmann et al. 2008). One study conducted in 1996-1999 in Belgium, in which the behaviour of bank voles was examined, it was found that during nephropathia epidemica (NE) epidemic years there was low rodent mobility but high population densities which could indicate that there is a greater level of infection between rodents host due to the overlapping of home ranges (Escutenaire et al. 2002).

There are some studies which have argued the case that there could be PUUV circulating in British bank voles, as Bennett et al 2010 argues that the host ecology and UK environment would be able to maintain the presence of this hantavirus. There is a resident pre-breeding population of 23 million bank voles (Harris and Yalden 2008) and there is some inclination that certain aspects of the UK climate make the maintenance of such a large population possible. Bank vole population increases in Europe are thought to be linked to the 'mast phenomenon' of broadleaf trees, mostly beech trees, in which the seeds (mast) are continually produced providing a stable food source for the voles (Clement et al. 2010) . Outbreaks of nephropathia epidemica (NE) in Belgium, France and Germany often follow good mast years, as good masting has been shown to prolong the breeding season making conditions more favourable for overwintering voles, thus increasing the transmission of the virus (Bennett et al. 2010). Although the UK does not have extensive forested areas the masting of the beech trees has been observed and certain aspects have been shown to increase masting. For example, climate change in the UK has had an impact on bank vole habitat and a prolonged drought is thought to increase beech masting (Bennett et al. 2010). This would allow the UK to support and maintain a large PUUV infected bank vole population. The sample size in this study of 56 was too small to represent the whole population, therefore, the possibility of PUUV presence in British bank voles cannot be ruled out completely based on the findings of this study alone.

There has also been seropositivity to PUUV detected in one farmer during a serology screening study in 2015 (Duggan et al. 2017), which could indicate that there has been exposure PUUV from bank voles, although no RNA was retrieved from this sample to confirm hantavirus species. However, PUUV antibodies have been shown to react positively to TATV antigens, as demonstrated by Pounder et al. (2013) with TATV positive field vole serum from B41. Therefore it is possible that the positive serology result could indicate that the farmer may have been exposed to TATV instead of PUUV, although this cannot be conclusively proven. Furthermore, there is no significant evidence for NE cases in the UK due to PUUV infection, this could be due to misdiagnosis or lack of reporting due to the mild clinical symptoms or reduced awareness.

However, it is unlikely that if there were NE outbreaks occurring in the UK, similar to those seen in Germany, that they would go unrecognised (Bennett et al. 2010). In conclusion, this study cannot conclusively dismiss the presence of PUUV in bank voles but can only state that in this instance this hantavirus was not detected.

3.4.5. Diversity of Hantaviruses in British rodent populations and public health risk

This study has confirmed the presence of two genetically distinct hantavirus species in the British rodent population, SEOV in brown rats and TATV in field voles. While the zoonotic potential of TATV remains unknown, it does not appear to be of huge public health significance: either it does not infect people or it causes a very mild HFRS or is asymptomatic, perhaps like Tula virus. Further investigation of TATV in humans is important, however, if only to rule out misdiagnosis should TATV antibody be detected in people and cause confusion with, for example, Seoul virus infection. It does appear, however, that TATV may be endemic in field vole populations due to the detection in disparate regions. If it is non-zoonotic, it might provide a useful model system for studying hantavirus ecology in rodent populations.

Much more is known about human infection with SEOV as there have been many documented cases and the relatively high SEOV prevalence found suggests that this could be a public health concern. Rats may have more opportunity to interact with humans than other rodent species. For example, field voles mainly inhabit grasslands and undergrowth in rural settings, in the case of Llyn Cowlyd this was a very isolated location where there was little human activity present, apart from reservoir workers or recreational visitors. Therefore there is a reduced chance of humans coming into contact with infected field voles or their excretions, thus making the transmission of TATV unlikely which could be an explanation for the lack of disease or prevalence data for this virus. This is a sharp contrast to the reservoir species of SEOV as rats are known to be able to exist near to human dwellings so there is a greater risk of infection with SEOV through contact with the rat or their excretions. Therefore it can be argued that there are more public health concerns with SEOV than with TATV.

The presence of SEOV in rat populations in different geographical locations of the UK does raise the question of why has there have not been more reported human cases linked to exposure to SEOV from wild rats as the majority of cases of UK HFRS are as a result of exposure to SEOV from pet rats. There has also been a higher seroprevalence (34.1%) reported in pet rat owners compared to those with occupational exposure (1.7%) (Duggan et al. 2017). This could be due to

the different relationship people have with pet and wild rats. Pet rat owners often have a close relationship with their pets and there is often close contact through handling and shared living quarters as well as frequent exposure to rat excretions through the cleaning of rat cages. There is also a high rate of transmission between rats which are kept in colonies in confined spaces, as it has been shown that once the virus has been introduced it can easily spread throughout the entire colony (McElhinney et al. 2017). This could explain why there are more cases due to exposure to SEOV relating to pet rats rather than wild rats. Although this study has detected multiple locations with SEOV positive rats the risk of exposure of SEOV from wild rats may be less than in pets rats as there is less direct interaction and exposure is likely to be coincidental when living, working or visiting an area with an infected rat population.

The question then arises why there aren't more cases of SEOV from wild rats, such as in the individuals at these locations were SEOV was present? One of the reasons that there may be so few clinical cases attributed to wild rats is that a low level of exposure to SEOV could provoke a subclinical infection where an efficient immune response cleared the viral infection without any obvious symptoms. Not all cases of SEOV infection result in severe disease and individuals can become seropositive without showing any symptoms. The humoral immune response is thought to be long-lasting and repeated infections with the same hantavirus have not been reported thus far (Krüger, Schönrich, and Klempa 2011). In the case of PUUV, high titres of IgG neutralizing antibody have been detected in patients decades after the initial PUUV infection (Krüger, Schönrich, and Klempa 2011).

At many of the SEOV locations several other rodent species were captured as well as brown rats but no hantavirus cross-species infection was observed. For example in Wetherby where there was a high prevalence of SEOV in the rat population, but no SEOV RNA was detected in the four bank voles from this location. This fits with the concept of the co-evolution of hantaviruses and their rodent hosts. SEOV has been identified in many *Rattus* species (*Rattus norvegicus, Rattus flavipectus, Rattus losea, and Rattus nitidus*) in other countries, which indicates that multiple species infection is possible (Holmes and Zhang 2015), no other rodent species from other genera beyond of *Rattus* have been found to be infected with SEOV. Similarly, Tula virus seems able to infect many species within the *Microtus* genus and Puumala virus other species within the *Myodes* genus. This does raise the question, however, of why hantaviruses should be so host restricted among rodents, yet so many are infectious (and pathogenic) to the more phylogenetically different *Homo sapiens*.

3.4.6. Conclusion

In conclusion, this study has identified two hantavirus species circulating in the British wild rodent population. The identification of SEOV in five independent wild rat populations indicates that this virus is circulating in more than one region and could potentially be widespread throughout the UK. Furthermore, while most strains were closely related to the Humber sequence, a novel strain was detected indicating that there may be multiple SEOV strains circulating in British wild rats. Phylogenetic analysis of the wild SEOV sequences shows that these strains, although closely related, are different from pet or lab rat strains. However, all UK SEOV strains cluster in lineage 9. Further analysis of the full genome of these viruses would provide a greater understanding of their common ancestry and co-evolution. TATV was identified in field voles from one site in North Wales and this, put together with the TATV in field voles in other studies, make it likely that TATV is endemic in British field voles and could be widespread throughout mainland UK. Isolation of this virus and full genome sequencing would be required to fully understand the viral biology and specific serological assays would be required to assess the potential zoonotic risk, which currently is unknown. No PUUV RNA was detected in this study, although this study does not provide enough evidence to completely exclude the possibility that PUUV could be present in British bank voles.

Chapter 4. Lymphocytic choriomeningitis virus (LCMV):

Prevalence of LCMV in British rodents

4.0. Abstract

Lymphocytic choriomeningitis virus (LCMV) is an Arenavirus that is widespread across the world due to the abundance and global distribution of its reservoir host, the house mouse (*Mus musculus*). There have also been several reports of LCMV in other rodent species, however, this probably represents spillover infection from the reservoir host. Human infection is often asymptomatic with mild-flu like symptoms, although in rare cases, acute LCMV infection can lead to aseptic meningitis and other complications. The prevalence of this LCMV in both people and rodents in the UK is poorly documented, therefore this study aimed to investigate its carriage by wild rodents and understand further its prevalence and distribution. Wild caught rodents (n=331) were collected from a variety of locations from Northern England, Wales and Scotland. Liver and kidney tissue were removed during post-mortem examination. Serum samples were also collected at the time of death by cardiac puncture. RNA was extracted, cDNA was generated and a Pan-arenavirus PCR was performed. Positive PCR products were Sanger sequenced and phylogenetically analyzed in MEGA. Serology was performed using a commercially available ELISA. A section of a kidney was examined histologically. PCR screening revealed an overall prevalence of 8% (26/331, 95% CI 15-36) in British rodents, which included 3.2% (2/61) brown rats, 17.5% (21/120) in house mice, 2% (1/49) wood mice and 4% (2/50) bank voles. There was no LCMV RNA detected in field voles (0/19), red squirrels (0/21) or grey squirrels (0/12). Seroprevalence in house mice was 7% (3/43). No histological changes were observed in the LCMV infected house mice renal tissue. The PCR prevalence described in rodents, especially in house mice, was higher than has been previously reported, as was the wider geographic distribution. These results imply that LCMV is likely to be endemic and widespread in British rodents and this study provides justification to monitor human infection of LCMV more widely as a potential public health concern.

4.1. Introduction

Lymphocytic choriomeningitis virus (LCMV) is a negative sense enveloped RNA virus with a bi-segmented genome member of the *Arenaviridae* family and is globally distributed (Zapata and Salvato 2013). Arenaviruses are able to cause persistent infections in rodent species, in which they cause chronic viraemia and are shed continually in secretions such as urine, faeces and saliva (Zapata and Salvato 2013). The reservoir host for LCMV is the house mouse (*Mus musclus*) and like other Arenaviruses in their rodent hosts, LCMV is able to establish persistent and lifelong infections, without causing detrimental damage to the reservoir host, thus allowing the continual shedding of virus (Lapošová, Pastoreková, and Tomášková 2013). Zoonotic transmission usually occurs through bites from handling infected rodents or inhalation of aerosols from contaminated fomites, such as bedding, nest material or droppings (Sosa et al. 2009). In healthy people, individual infections are often asymptomatic but can cause mild flu-like symptoms which are mostly self-limiting (Lapošová et al. 2013). The mortality rate of LCMV is 1% (Cassady 2006), although in rare cases LCMV infection can result in serious illness in the form of aseptic meningitis (Asnis et al. 2010) and even death (Cassady 2006). Immunosuppressed people, such as organ transplant patients, are at risk of the severe, sometimes fatal, consequences of LCMV infection (Fischer et al. 2006; Amman et al. 2007; Palacios et al. 2008). As infection in healthy individuals is often asymptomatic and LCMV is not routinely screened for, it would therefore not be diagnosed until the recipients of donated organs started showing clinical signs of disease.

In the UK, historical data indicates that there may have been some recorded reports of human infection, such as 13 cases reported between 1949-55. The incidence rate of human infection reported by PHE laboratories in the years up to 1979 was thought to be less than 5 annually (Skinner & Knight 1979). House mice infected with LCMV have been identified in Manchester, Lewisham and London. In a study conducted around the Animal Virus Research Unit, Pirbright, Surrey, of the 84 captured wild house mice there was an infection rate of 69% (Skinner & Knight 1979). In a study of wild-caught mice, a seropositivity rate of 4% (1/24) was detected, although this increased to 47% in the offspring of these mice which formed a captive colony (Becker et al. 2007). Another study found 66 of 1,147 rodents had antibody to LCMV and 127/482 were PCR positive (Blasdell et al. 2008). Antibody was shown to be present in multiple rodent species, as well as house mice (Blasdell et al. 2008). Another UK study has also detected LCMV RNA in 25% of brown rats in 2011 (Stuart et al. 2011). Although, given that most of these studies were small in scale and conducted 10 years ago, there is little knowledge about the current prevalence of UK rodents.

Liver tissue was taken from these rodents to screen for LCMV as this organ has been previously been

used to detect viral RNA in infected animals (N'Dilimabaka et al. 2014; Vieth et al. 2007) and LCMV has been shown to replicate in hepatocytes in laboratory mice (Beier et al. 2015). This also presents and opportunity to screen for other viral pathogens using the same extracted RNA, such as Hepatitis E virus (HEV). To screen the liver tissue of these rodents for LCMV RNA a published pan-Arenavirus RT-PCR (Vieth et al. 2007) was used. Validation data has been published in the development of this assay to show that it is highly sensitive assay and has been optimised so is able detect LCMV RNA at very low concentrations (20-100 PFU/ml) (Vieth et al. 2007). This assay is also able to detect novel and variant strains of LCMV, which would be useful as divergent strains of LCMV have been reported in non-reservoir host rodent species (Ledesma et al. 2009). Sanger sequencing of the PCR amplicons will allow the Arenavirus species to be confirmed.

Serum samples of house mice were also screened for LCMV antibodies (IgG) with an ELISA LCMV. Infection with LCMV in mice is highly complex as there can be different infection states, for example, if house mice are infected in utero then they will not produce an immunological response to the virus and persistently shed LCMV. However if they are exposed to LCMV as an adult they may produce an immune response and clear the infection resulting in antibody product, however, there may not be any detectable RNA in their livers (Lapošová, Pastoreková, and Tomášková 2013). Screening house mice by molecular and serological methods will provide the most accurate assessment of LCMV infection at these locations. Histological examination of the kidney tissue of LCMV infected house mice was conducted as pathology has been reported in this tissue due to LCMV infection in neonatal mice (Oldstone and Dixon 1969).

The aims of this study were to screen UK rodents for LCMV to determine the viral prevalence and geographic distribution. This information can be used to help understand the epidemiology of LCMV. This study's prevalence data could be used to assess whether it would be beneficial to conduct wider, more systematic surveillance studies to investigate LCMV carriage in people and so determine how important the pathogen to public health.

4.2. Materials and Methods

4.2.1 Sample collection

A range of peridomestic rodents (n=331) were collected from 21 geographical locations. The full details of the fieldwork and sampling of this project can be found in chapter 2. Serum was collected from rodents by cardiac puncture immediately after death and these blood samples were centrifuged at top speed (17x g) for 1 minute then frozen at -20°C until required. Serum collection from some rodents was not possible as they were already deceased when collected, so blood clotting prevented a cardiac sample being obtained. Rodent carcasses were examined post-mortem and the liver tissue was removed and stored at -80°C until required. In addition 22 house mouse liver samples from mice caught around the Merseyside region and were donated to this project by Dr K. Pounder (University of Liverpool).

4.2.2. RNA extraction from liver tissue

RNA was extracted from a 50-100mg section of rodent liver tissue using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions and extracted RNA was quantified using a Nanodrop.

4.2.3. cDNA synthesis

The concentration of RNA was diluted to 500 ng/µl in 10 µl, before cDNA synthesis. Random hexamers were added to the mixture, which incubated for 10 minutes at 25℃ then placed on ice. Buffer x5, 10 mmol of dTNP and reverse transcriptase were added and incubated for 60 minutes at 42℃ followed by 10 minutes at 70℃, with a 20µl final volume of cDNA produced.

4.2.4. Pan Arenavirus PCR

This method was originally described in Veith et al 2007, as a pan-arena RT-PCR that targets the L-segment of arenaviruses, including Lassa virus and LCMV. The positive LCMV control for this assay was provided by Amanda Davidson from the Mammalian Biology and Evolution department, Leahurst campus. This protocol was optimised using the HotTaqStar DNA polymerase kit (Qiagen, Manchester) according to the manufacturer's instructions. A master mix was prepared which consisted of 5 µl of Buffer containing 15 mM $MgCl_2$, Veith et al's four primers (Table 4.1) at 10 pmol each (1 µl of each primer), 10 pmol dNTP's (1 µl per reaction), HotTaqStar DNA polymerase (0.25 µl per reaction) and 1 µl of cDNA sample was made at room temperature. The thermal profile for this PCR was 95°C for 15 minutes followed by forty-five cycles at 94°C for 30 seconds then 55°C

for 1 minute and 72°C for 1 minute with a final elongation step of 72°C for 10 minutes. Assays were repeated in duplicate. In a dilution series, this PCR was able to detect LCMV RNA to 10^{-5} (Figure 4.1). Gel electrophoresis was performed at 120 V for 65 minutes on a 2% agarose peg green gel then PCR products were visualised under UV light (Figure 4.2).

Primer	Primer sequence (5' to 3')
LVL 3359D plus	AGAATCAGTGAAAGGGAAAGCAATTC
LVL 3754D minus	CACATCATTGGTCCCCATTTACTGTGATC
LVL 3359G plus	AGAATTAGTGAAAGGGAGAGTAACTC
LVL 3754A minus	CACATCATTGGTCCCCATTTACTATGATC

Table 4.1: Primer sequences used in the pan-arenavirus PCR as designed by Veith et al 2007.

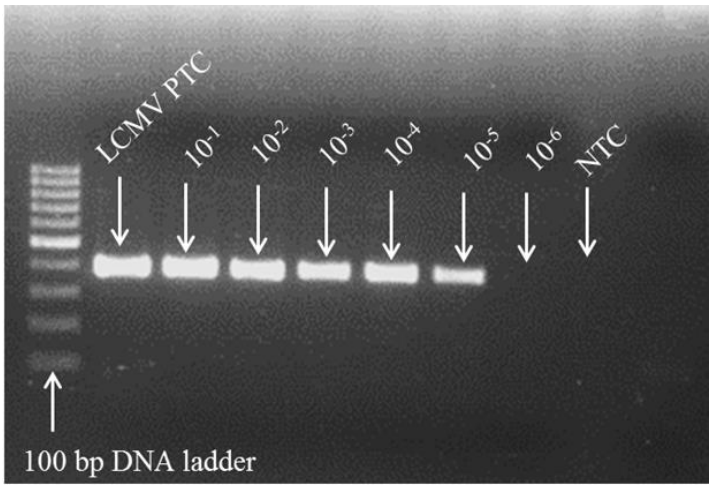

Figure 4.1: Estimation of sensitivity for the Pan-arenavirus PCR using a dilution series of 10 to 10^{-6} using the positive control kindly provided by Amanda Davidson from Mammalian Biology and Evolution, Leahurst campus. This helped determine the concentration at which the positive control could be used in this assay.

4.2.5. Sanger sequencing and phylogenetic analysis

The positive PCR products from the nested pan-arena PCR were sequenced by the Central Sequencing Unit (CSU) at APHA Weybridge. Sequencing data were analysed using a DNAStar Lasergene software package and using SeqMan Pro to assemble contiguous sequences of the forward and reverse data. Chromatogram profiles of each contiguous sequence were compared and trimmed

to remove primer sequences and produce a consensus sequence of the 259 nt fragments. Consensus sequences were uploaded into Megalign (DNAStar Lasergene software) and aligned using the 'clusterW' method, any differences were examined and compared with the raw chromatogram data. This part of the analysis was completed by Daisy Jennings.

The LCMV complete consensus sequences then sent to Ellen Murphy and then compared with other published Arenavirus sequences using the nucleotide Basic Local Alignment Search Tool (BLAST) programme produced by the National Center for Biotechnology Information (NCBI). An alignment was created in MEG7 using the 'clustal W' method (Figure 5.2a and 5.2b).

4.2.6. Phylogenetic analysis

Phylogenetic analysis was conducted in MEGA 7 (Kumar, Stecher, and Tamura 2016) and the sequences from this study were aligned with other LCMV sequences. A phylogenetic tree was constructed using a best fit model of Kimura 2-parameter plus gamma and bootstrap analysis was performed with 1000 repeats.

4.2.7. Antibody IgG ELISA

A commercial LCMV ELISA kit was used (Charles River, Margate UK). Table 4.2 describes the solutions and reagents used in the antibody ELISA. Serum samples were defrosted at 4°C and diluted to a working concentration of 1/60 for the ELISA. To do this a dilution of 1:5 (10 µl serum to 40 µl sterile PBS, pH 7.0-7.4) was made in a 96 well sterile plate, then 40 µl of this solution was added to 220 µl of Blotto serum dilute solution and thoroughly mixed by pipetting. ELISA plates, previously stored at -80°C were defrosted at room temperature. Then 50 µl of the diluted serum was added to each well in duplicate. The controls were supplied at x2 concentration and were added at 25 µl control sera to 25 µl Blotto serum dilute solution. The plate was covered and incubated for 40 minutes at 35-40^{o}C. The first wash was completed by adding approximately 300 µl of wash solution to each well by the plate washer. The plate was then inverted and tapped on white roll on the side of the bench three times to expel any remaining liquid from the well. This process was repeated five times.

Then 50 µl of the diluted conjugate, at 1/450 concentration, was added to all wells and the plate was incubated at 35-40°C for a further 40 minutes. The wash procedure was then repeated. Then 200 µl Horseradish peroxidase (HRP) substrate O-phenylenediamine dihydrochloride (OPD) solution was added to each well and incubated at room temperature in the dark for 30 minutes. Plates were immediately read at 450 nm in the plate reader. Interpretation of the results followed the manufacturer's instructions (Charles River, Margate UK). Conversion of the absorbance readings

was converted to scores by dividing each reading by 0.13. The score for the negative control (if the net score was below 2) was subtracted from each value to account for background signal in the ELISA. If the net score was greater than or equal to 2.5 it was interpreted as seropositive, if between 2.5 and 1.5 the result was borderline or equivocal and if it was below 1.5 the result was interpreted as seronegative.

Solution	Reagents required	Instructions	Storage
1.0 M Tris-HCl	- 12g Tris base - 100ml distilled H_20	Dissolve Tris base in the first 80ml of H_20 and adjust pH to 8.7 using HCl. Makeup to 100ml	6 months at 4°C
Blotto serum dilute	-6.05g Tris-buffered saline (TBS), 50mM - 8.76g NaCl -1L of distilled H_20 (adjust - pH to 7.4 with HCl if required) -5g non-fat milk powder	Mix all regents in 1L of distilled H_20	2 weeks at 4°C
Conjugate dilatant	- 1ml of 1.0 M Tris-HCL - 0.9g NaCl - 0.1ml Gentamicin (10mg/ml) -15ml FBS - 100ml distilled H_20 - Conjugate (Charles River)	Used at 1/450 in the ELISA. Add 50 µl Conjugate to 2275 µl Conjugate dilatant (1/45) Add 1.2 ml this solution to 10.8 ml Conjugate dilatant (1/450)	6 months at 4°C
Wash solution	- 5ml 10% Tween 20 solution - 9g NaCl - 1L of distilled H_20 - Autoclaved before use	Mix all regents in 1L of distilled H_20 Autoclaved before use	1 week at 4°C
OPD Peroxidase Substrate	-1 SIGMAFAST™ OPD tablet - 20ml distilled H_20	Use immediately for best results.Makeup in a light-tight container as OPD is light sensitive.	4°C in tablet form

Table 4.2: Reagents and instructions on how to prepare solutions required for the LCMV ELISA. All reagents, apart from FBS (provided in-house by S. Bonner) and the Conjugate (Charles River), were purchased from Sigma Aldrich, Ltd.

4.2.8. Histopathology

Kidney tissue samples, from five LCMV positive house mice, were fixed in 10% formalin for a minimum of 16 hours then histologically processed with 5 µm sections taken from the formalin fixed paraffin embedded tissue block which were stained by Haematoxylin and Eosin (H&E) before being examined microscopically. This staining was conducted by Elena Fitzpatrick, please see Appendix VII for protocols.

4.3. Results

4.3.1. PCR screening results

The overall PCR prevalence of LCMV RNA detected in all rodents sampled was 8% (26/331, 95% CI, 15-36). The gel photograph can be seen in Figure 4.2 and PCR results are summarised in Tables 4.3 and 4.4. LCMV positive rodents were detected at seven locations including Edinburgh, Telford, Merseyside and several locations in Yorkshire. The rodent species with the highest prevalence was the house mouse 21/120 (17.5% 95%CI 12-26). For locations, apart from donated samples from Merseyside, house mice had detectable LCMV RNA in their liver, LCMV prevalence in those individual populations of house mice ranged from 50-100% of the mice screened. A lower prevalence of LCMV was detected in brown rats (2/61, 3.3%, CI0.1-11), wood mice (1/49 2% CI 0.3-11) and bank voles (2/50 4% CI 1-13). No LCMV RNA was detected in field voles, red squirrels or grey squirrels.

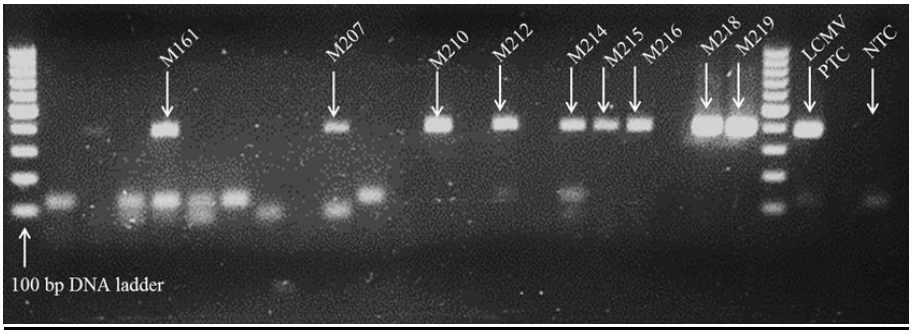

Figure 4.2: Gel picture of a 2% agarose peq green gel, visualised under UV light, showing LCMV positive products of RNA extracted from liver tissue. LCMV amplicons were 400 bp and are indicated with their sample numbers. The LCMV positive control (PTC) and negative control (NTC) are also shown.

Site name	Location	Species and Result	Total
Pig 1	Ripon, Yorkshire	AS (0/1), MM (0/1), MG (1/8), RN (2/16)	3/26
Pig 2	Cheshire	RN (0/10)	0/10
Pig 3	Morpeth, Northumberland	MM (0/38)	0/38
Pig 4	Hull	MM (0/9), MG (0/1), RN (0/2)	0/12
Pig 5	Berwick, Northumberland	MG (0/8), MM (0/16)	0/24
Pig 6	Driffield, Yorkshire	AS (1/10), MG (1/12), MM (0/3) RN (0/2)	2/27
Pig 7	Edinburgh	AS (0/9) MM (3/6)	3/15
Pig 8	Malton, Yorkshire	MA (0/1) MG (0/9) MM (2/3) RN (0/1)	2/14
Pig 9	Wetherby, Yorkshire	AS (0/22) MG (0/4) RN (0/16)	0/22
Pig 10	Ponteland, Northumberland	AS (0/20) MG (0/3)	0/23
Pig 11	Telford, Shrewsbury	MM (6/6)	6/6
Pig 12	York, Yorkshire	MM (9/16)	9/16
Farm 1	Cheshire	RN (0/5), MG (0/1) AS (0/6)	0/12
Farm 2	Derby	RN (0/1)	0/1
Farm 3	Cheshire	MA (0/2), MG (0/3)	0/5
Rural 1	Llyn Cowyld, North Wales	MA (0/16), MG (0/1)	0/17
Rural 2	Ruthin, North Wales	RN (0/1)	0/1
Forest 1	North Wales	SV (0/21)	0/21
Forest 2	Formby	SC (0/12)	0/12
Urban 1	Liverpool, Merseyside	RN (0/3)	0/3
Urban 2	Ellesmere Port, Chehsire	RN (0/4)	0/4
Urban 3	Merseyside (various)	MM (1/22)	1/22
		Total	**26/331**

Table 4.3: Results of the Pan-Arenavirus PCR by site and species.

RN (*Rattus norvegicus*), MM (*Mus musculus*), AS (*Apodemus sylvaticus*) MA (*Microtus agrestis*), MG (*Myodes glareolus*), SV (*Sciurus vulgaris*) and SC (*Sciurus carolinensis*)

Species	LCMV PCR positive	Species prevalence (%)
R. norvegicus	2/61	3.2 (95% CI, 1-11)
M. musculus	21/120	17.5 (95% CI, 12-26)
A. sylvaticus	1/49	2 (95% CI, 0.3-11)
M. agrestis	0/19	0
M. glareolus	2/50	4 (95% CI, 1-13)
S. vulgaris	0/21	0
S. carolinensis	0/12	0

Table 4.4: Aggregate LCMV PCR prevalence by rodent species

4.3.2. ELISA serology results

Overall 43 house mice were tested for LCMV antibody, of which 3/43, 7% (95% CI, 2-12%) were seropositive. A further eight mice were borderline seropositive according to the manufacturer's instructions for interpretation of the ELISA results (if the score calculated from the absorbance reading was 1.5 to 2.5). If these were interpreted as positive, then the seroprevalence would be 11/43, 25.6% (95%CI, 16-36%).

The results of each mouse serum sample tested, and that individual's PCR results, are shown in Table 4.5 and summarised in Tables 4.6 and Table 4.7. Of the two locations at which seropositive house mice were detected, one also harboured mice which had detectable LCMV RNA in their livers (Table 4.6). If borderline seropositive sites were counted as seropositive, then of five seropositive sites, three also had PCR positive mice. Of those four PCR positive sites at, which serology was done, one had seropositive mice and two borderline seropositive mice.

Only one site had both seropositive and borderline seropositive mice, and that site had no PCR positive mice. At the individual mouse level, (Table 4.7), only 1/43 mice were both PCR and seropositive, and 1/43 PCR positive and borderline seropositive. Of the PCR positive mice tested serologically, 10/12 were seronegative. Of the PCR negative mice tested serologically, 2/31 were seropositive and a further 7/31 borderline seropositive.

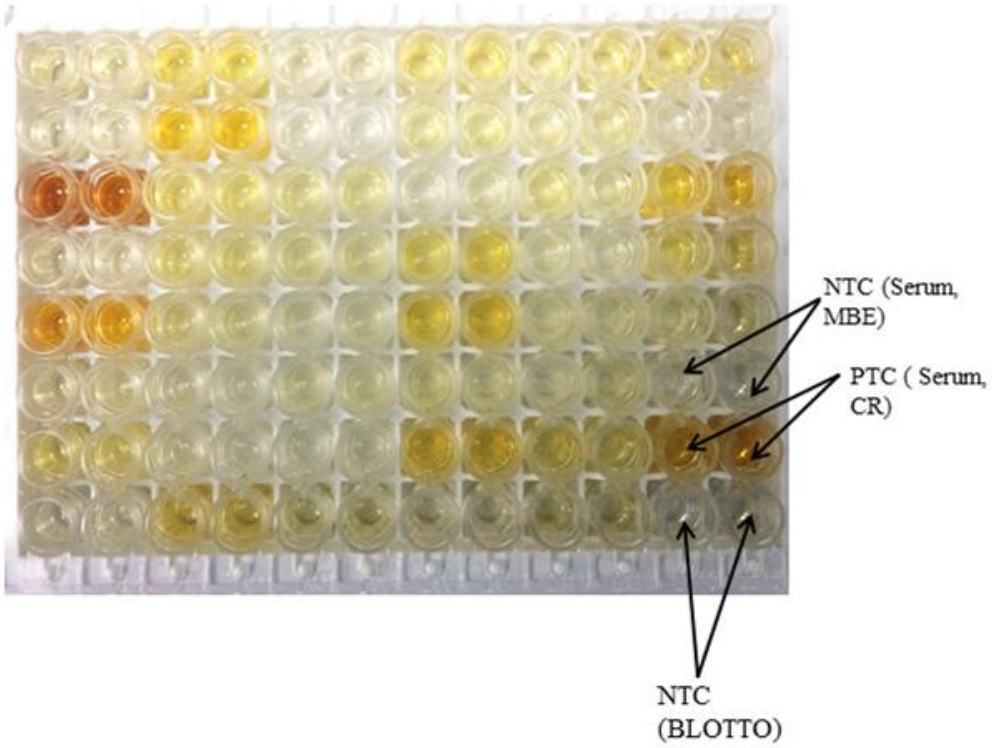

Figure 4.3: ELISA plate with house mice serum and OPD as the HPR substrate, in which a seropositive reaction ranges from yellow to brown in colour. Positive controls (PTC) and negative controls (NTC), both the negative serum (kindly provided by A. Davidson from the Mammalian Biology and Evolution Department, University of Liverpool) and blotto solution are shown.

Sample number	Location	Absorbance (450nm)	ELISA Result	PCR result
M61	Pig Farm 3 (Northumberland)	0.149	-	-
M63	Pig Farm 3 (Northumberland)	0.436	borderline	-
M64	Pig Farm 3 (Northumberland)	0.174	-	-
M68	Pig Farm 3 (Northumberland)	0.374	borderline	-
M69	Pig Farm 3 (Northumberland)	0.423	borderline	-
M72	Pig Farm 3 (Northumberland)	0.259	-	-
M73	Pig Farm 3 (Northumberland)	0.195	-	-
M74	Pig Farm 3 (Northumberland)	0.192	-	-
M75	Pig Farm 3 (Northumberland)	0.134	-	-
M76	Pig Farm 3 (Northumberland)	0.140	-	-
M85	Pig Farm 3 (Northumberland)	0.147	-	-
M96	Pig Farm 3 (Northumberland)	0.271	-	-
M98	Pig Farm 3 (Northumberland)	0.199	-	-
M118	Pig Farm 5 (Northumberland)	0.345	borderline	-
M120	Pig Farm 5 (Northumberland)	0.118	-	-
M121	Pig Farm 5 (Northumberland)	0.724	+	-
M122	Pig Farm 5 (Northumberland)	0.324	borderline	-
M123	Pig Farm 5 (Northumberland)	0.191	-	-
M136	Pig Farm 6 (Yorkshire)	0.159	-	-
M144	Pig Farm 7 (Edinburgh)	0.116	-	+
M145	Pig Farm 7 (Edinburgh)	1.982	+	-
M155	Pig Farm 7 (Edinburgh)	0.132	-	-
M156	Pig Farm 7 (Edinburgh)	1.010	+	+
M157	Pig Farm 7 (Edinburgh)	0.155	-	+
M158	Pig Farm 8 (Yorkshire)	0.260	-	-
M159	Pig Farm 8 (Yorkshire)	0.126	-	-
M161	Pig Farm 8 (Yorkshire)	0.127	-	-
M194	Pig Farm 11 (Telford)	0.347	borderline	+
M195	Pig Farm 11 (Telford)	0.147	-	+
M198	Pig Farm 11 (Telford)	0.215	-	+
M199	Pig Farm 11 (Telford)	0.181	-	+

M200	Pig Farm 11 (Telford)	0.114	-	+
M201	Pig Farm 11 (Telford)	0.121	-	+
M202	Pig Farm 12 (Yorkshire)	0.321	borderline	-
M204	Pig Farm 12 (Yorkshire)	0.106	-	-
M208	Pig Farm 12 (Yorkshire)	0.221	-	-
M209	Pig Farm 12 (Yorkshire)	0.162	-	-
M210	Pig Farm 12 (Yorkshire)	0.127	-	-
M212	Pig Farm 12 (Yorkshire)	0.136	-	+
M213	Pig Farm 12 (Yorkshire)	0.129	-	-
M215	Pig Farm 12 (Yorkshire)	0.175	-	+
M216	Pig Farm 12 (Yorkshire)	0.229	-	+
M217	Pig Farm 12 (Yorkshire)	0.336	borderline	-

Table 4.5: ELISA results for the screening of house mouse serum using the commercial kit. Scores were calculated per the manufacturer's instructions and the net score by the removal of the negative control (NC) serum score (0.905) from each individual to account for background noise. Comparison with the PCR screening for each mouse and the locations are also shown.

Site	PCR prevalence	Seropositive prevalence	Borderline prevalence	Summary
Pig Farm 3	0/38	0/13	3/13	-/-/+
Pig Farm 5	0/16	1/5	2/5	-/+/+
Pig Farm 6	0/3	0/1	0/1	-/-/-
Pig Farm 7	3/6	2/5	0/5	+/+/-
Pig Farm 8	2/3	0/3	0/3	+/-/-
Pig Farm 11	6/6	0/6	1/6	+/-/+
Pig Farm 12	9/16	0/10	2/10	+/-/+

Table 4.6: A comparison of PCR and serology tests for LCMV by location

Serology results	**PCR results**	
	+	-
+	1	2
borderline	1	7
-	10	22

Table 4.7 A comparison of PCR and serology tests for LCMV in individual mice

4.3.2. Sequence analysis

LCMV sequence(s)	**LCMV UK Yorkshire % Sequence similairy**	**LCMV UK Edinburgh % Sequence similarity**
LCMV UK Yorkshire (this study)	86-100	82-85
LCMV UK Edinburgh (this study)	82-85	100
LCMV (J04331 Armstrong reference strain)	83-89	85
LCMV (Europe)	84-89	80-86
LCMV (USA and South American strains)	82-83	84-85

Table 4.8. Sequence similarities between the LCMV sequence obtained in this study and other published LCMV sequences.

Most of the sequences of the 259 nt partial L-segment from UK rodents cluster together although in a single clade, however, house mice derived sequences from Pig farm 7, in Edinburgh, appear different and cluster with an *M. mus* isolate from French Guiana, South America (Genbank accession number KT731537). There was 81.9-85.3% genetic similarity between the Yorkshire sequences and those from house mice in Edinburgh.

There was a degree of diversity observed in the Yorkshire sequences (86-100% genetic similarity) and also genetic diversity observed within house mice from Pig farm 12 as M207 and M210 appear different to the other sequences from this location. Genetic differences can be seen between different rodent species with V62 and V41, however, in R42, R43 and M135, this was not observed.

The UK LCMV sequences of this study had 80-89.1% genetic similarity at the nucleotide level with other published LCMV sequences, confirming that the sequences in this study are LCMV. The LCMV sequences of this study were also shown to be more distant from to other published Arenavirus sequences such as Lassa virus (71.7-74%), Kodoko virus (61.5-69.4%), Luna virus (68.8-74.5) and Cardamones virus (69.3-75%).

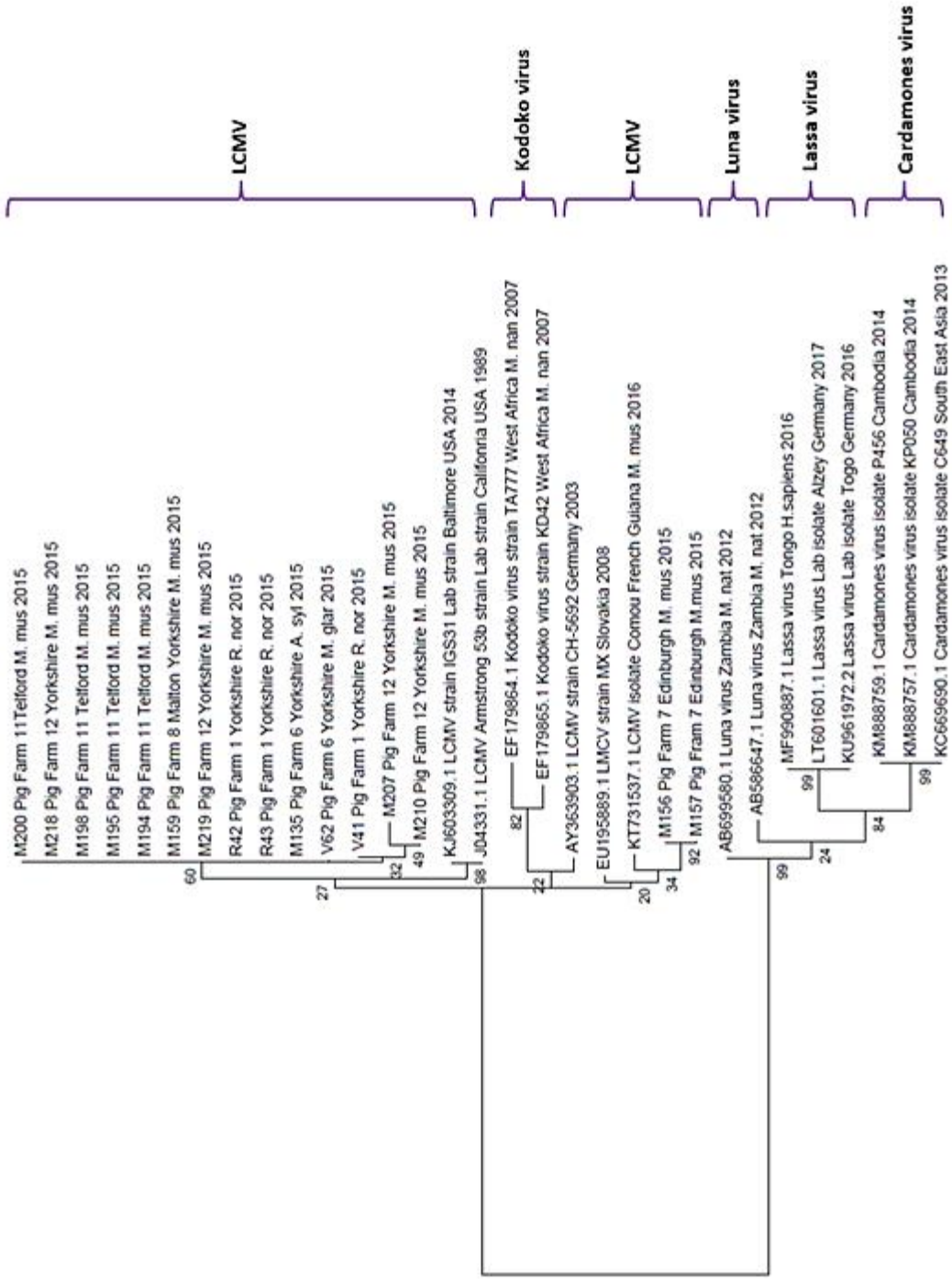

Figure 4.4a: Molecular phylogenetic analysis by Maximum Likelihood method of the partial L-segment of LCMV from rodents in this study. The evolutionary history was inferred using the

Maximum Likelihood method based on the Tamura 3-parameter model plus gamma (Tamura 1992). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis of a 259 nt sequence of the L-segment and involved 31 nucleotide sequences. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Sequences not generated in this study were obtained from Genbank.

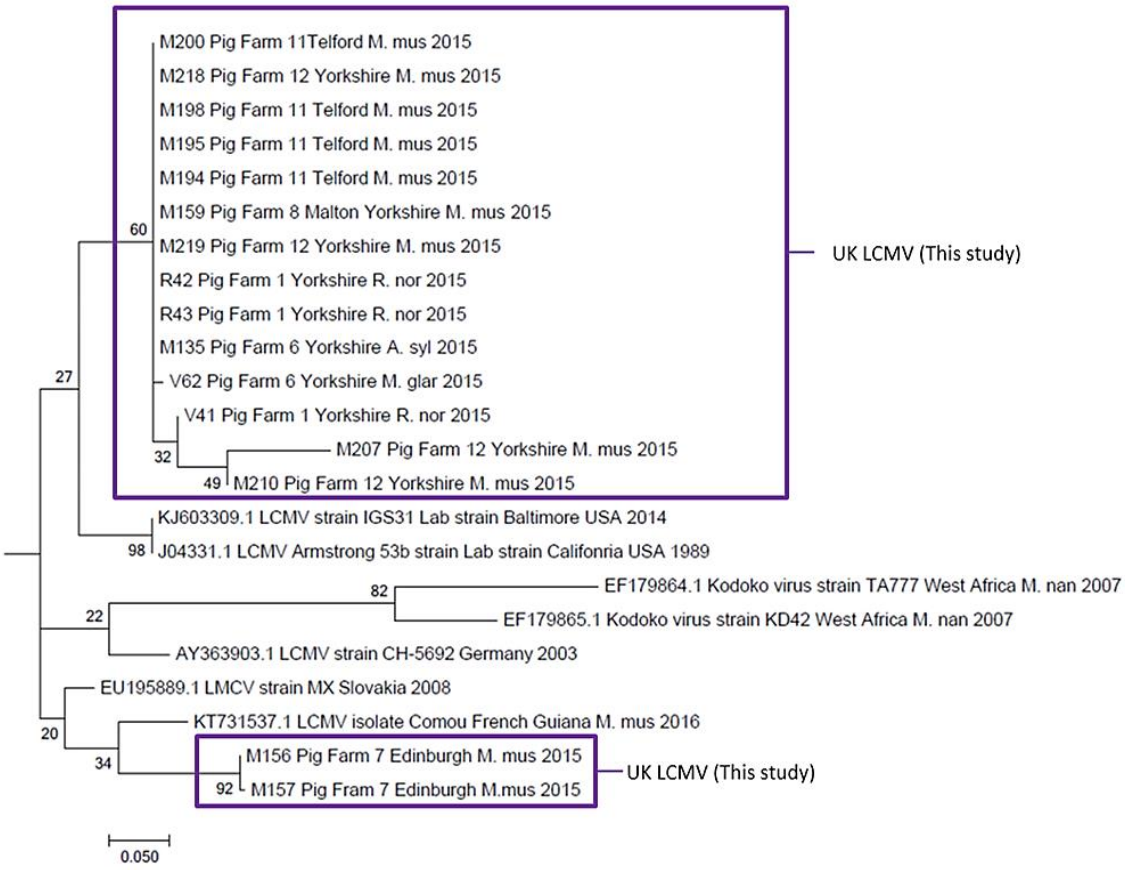

Figure 4.4b: Subtree (derived from Figure 4.4a) showing the LCMV clade (including Kodoko virus sequences). This tree contains 23 nucleotide sequences with 259 nucleotides in each. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

4.3.4. Histopathology

No abnormal histological changes were observed in any of the H+E sections examined reflecting minimal host inflammatory or degenerative reaction to LCMV infection.

4.4. Discussion and conclusion

LCMV was found to be widespread geographically, with 7/22 sites sampled having one or more positive animals by PCR. As in previous studies, infection was mainly associated with house mice. However small numbers of other host species were also found to be positive by PCR, including rats, bank voles and wood mice, all of which have also been previously reported as spillover, or accidental, hosts (Stuart et al. 2011; Blasdell et al. 2008). If only sites at which house mice were sampled are counted then 7/10 sites had one or more positive animals. Two sites contained no LCMV infected house mice but did contain other species with were LCMV RNA positive. It is perhaps also relevant that while house mice and rats share similar habitats, both are peri-domestic, preferring built environments. Rats will also predate upon mice, providing opportunities for direct and indirect cross-species transmission.

The other species sampled tend to have a preference for field, hedgerow and woodland habitats and might have less contact with house mice. Furthermore, other species, including bank voles, might also have contact with house mouse populations distant from those sampled at the main site. This could be due to the size of the species home ranges, as house mice often have home ranges between 5-100 m^2 (Couzens et al. 2017), which is much smaller than most rodents. Bank voles in Germany have been shown to have large home ranges from 150-4850 m^2 in females to 1200-11000 m^2 in males, depending on age and time of year (Korn 1986). Overall, these results support the general view that house mice are the main reservoir and maintenance host of LCMV, and that LCMV is geographically widespread. The prevalence of LCMV detected by PCR in this study (17.5%, 95% CI 12-26) was similar to that detected in house mice in England by Blasdell et al 2008 who found 127 of 482 mice to be PCR positive. However, that study only tested mice from populations already found to be antibody positive.

There was a lack of genetic diversity observed in the LCMV between some of the sequences detected in this study. There was a slight divergence observed between some sequences, such as those in house mice from Pig Farm 12 and bank voles, however, most of the sequences were very similar with between 86-100% genetic similarity between the UK strains in this study. Identical sequences from different animals could suggest a contamination issue, however, steps were taken to avoid this, such as the sterilisation of instruments and gloves were worn at all times. Another reason for identical sequences from different animals could be that the targeted L-segment region of this PCR is conserved and is likely to be a region, so there may be less genetic diversity seen in that region. This supports the idea that the identical sequences in UK LCMV sequences are not due to contamination

but due to the sequencing of a highly conserved region. The pan-arenavirus nature of this PCR is advantageous in a sense it can detect multiple arenaviruses, including known and novel species (Vieth et al. 2007). The sequence data from this PCR assay was enough to confirm that the virus in these rodents was LCMV (80-89.1% genetic similarity) as there was greater genetic distance from other Arenaviruses (61.5-75% genetic similarity). However, the amplification of a conserved region may not be as useful when determining phylogenies. Therefore, if conclusions are to be made about the genetic diversity and ancestry, sequencing of multiple regions which have been shown to have a higher level of genetic diversity such as S segment or glycoprotein (GPC) genes (Blasdell et al. 2008; Ledesma et al. 2009), may be required. This will also conclusively confirm that there has not been any contamination in the L-segment PCR.

There was genetic divergence observed, in the sequences derived from house mice from Pig Farm 7, in Edinburgh, which appears to cluster away from the main LCMV UK clade. These sequences cluster with an LCMV house mouse isolate from French Guiana, South America (Genbank accession number KT731537). A level of diversity within LCMV sequences is expected and could be due to the geographical separation between Edinburgh and Yorkshire, it is unlikely that there would be interactions between the two populations. Divergent strains have been reported across several countries, for example, highly divergent strains within the *Mus* derived LCMV group has been detected in Southern France with strain HP65-2009 (Yama et al. 2012). This genetic diversity could be due to the nature of the reservoir host which has a long and highly complicated evolutionary history and ecology, as the movement, and globalisation of this species, which has greatly increased in the last 500 years due to human activity (Yama et al. 2012). House mice are thought to have spread to the Mediterranean Basin around 8000 BC then to the rest of Europe around 1000 BC, however, they were not introduced to the America's until the 16th Century. Their longer presence in Europe could explain why there is a greater level of genetic diversity in European strains than those seen in the USA (Albariño et al. 2010).

Diversity within British house mice populations has been reported by Searle et al (2009) through the analysis of the mitochondrial (mtDNA) genome from UK house mice. They report that there are 40 haplotypes (a set of genetic determinants located on a single chromosome) that can be separated into two distinct lineages within house mice in the UK based on phylogenetic analysis of mtDNA (Searle et al. 2009). These two lineages are also thought to be geographically separated, with the Orkney mtDNA lineage detected in Northern Scotland (including Shetland and Orkney), Isle of Mann and Ireland. This may mirror the migration and settlements of Norwegian Vikings, as Orkney was central to Norwegian Viking kingdom, and the same haplotypes have been found in Norway, therefore it can

be assumed that these house mice arrived when Viking settlers used when they colonised these areas (Searle et al. 2009). There is evidence of house mice presence before this, which could explain the presence of different lineages (Searle et al. 2009). Arenaviruses are known to co-evolve with the host (Zapata and Salvato 2013), therefore this could explain why distinguishable stains of LCMV were detected in Scotland compared to the main UK clade detected in this study.

This study found 7% (3/43) seropositivity in house mice. This is a slightly higher seroprevalence than previously reported by Becker et al (2007), who reported a 4% (1/24) seroprevalence in wild-caught mice and Blasdell et al (2008) who reported a 2.2% (2/89) seropositivity to LCMV. Not all mouse sera from all sites were tested serologically, but one extra site (Pig Farm 5, Northumberland) was identified with seropositive mice (1/5 seropositive, 2/5 borderline seropositive), where none of the mice tested were PCR positive. Although the inclusion of borderline results could be problematic as this ELISA was designed for laboratory mice so it is possible that there may be more background interference detected with wild mice, producing a higher absorbance reading. The lack of detectable LCMV RNA in any of the mice from Pig farm 5 could be due to several reasons. It is possible that LCMV may have been recently introduced into the population but has yet to become established in the resident mouse population at this location or that it may be circulating at low levels. However, as these mice were only collected at one-time point during this study, therefore, it is not possible to conclusively state that LCMV is a recent occurrence from the seropositivity results.

Pig farm 7, Edinburgh, contained both seropositive mice and mice which had LCMV RNA in their livers. One mouse (M145) was strongly seropositive yet there was no detectable LCMV RNA its liver, which could indicate horizontal transmission to a naive mouse from other viraemic individuals in the population, either through direct contact or a shared contaminated environment. Thus M145 may have produced a strong immune response once exposed to LCMV. Mice horizontally infected with LCMV will have detectable levels of antibodies by the 7th day of infection (Lapošová, Pastoreková, and Tomášková 2013). It has been suggested that infection may not be permanent in these mice and could be completely cleared in 14 days especially if the mouse is infected with a relatively small dose of the virus infection (Lapošová, Pastoreková, and Tomášková 2013). Another mouse from this location, M156, was both seropositive and viraemic. This could indicate that this mouse, although was not infected in utero, received a large dose of the virus leading to an inadequate host immune response, resulting in the failure to clear the infection (Lapošová, Pastoreková, and Tomášková 2013) despite the production of antibodies or alternatively is in the process of currently clearing the virus.

Several house mice which were seronegative were shown to have detectable LCMV RNA in their livers. This is likely due to vertical transmission in which the mice were infected in utero and do not produce an immune response or antibodies to LCMV and remain viraemic persistent life-long shedders (Oldstone 2009). This could indicate that LCMV is likely to be endemic at a particular site and may have been circulating within the population for some time to facilitate vertical transmission. The results from this study highlight the complexity of LCMV transmission in house mice populations, however, it is difficult to compare both PCR and serology, is serology a measure of exposure while PCR measures active infection, therefore it is important to conduct both tests in order to get the most comprehensive information during the screening of rodents for LCMV.

There has been a suggestion that carrier mice who were infected shortly after birth do suffer the damaging effects of LCMV infection (Oldstone and Dixon 1969), which may induce a selection pressure against LCMV. Pathological LCMV lesions, including chronic glomerulonephritis and renal necrosis, can be associated with carrier mice which could be fatal (Oldstone and Dixon 1969). However, no renal lesions, necrosis or significant levels of inflammatory cells were observed in the histological examinations of the kidney tissue of LCMV infected mice (n=5) in this study. As there have been other studies reporting a lack of disease in LCMV infected house mice (Labudová, Pastorek, and Pastoreková 2016), it is likely there are a range of factors (host and viral genetics, host immunocompetence, infective dose, environment, etc) which impact the range of different pathological lesions which could develop. The high level of adaptation of the virus to its reservoir host (Labudová, Pastorek, and Pastoreková 2016; Lapošová, Pastoreková, and Tomášková 2013) means that LCMV induced glomerulonephritis in carrier mice is uncommon.

Both previous UK studies (Becker et al. 2007; Blasdell et al. 2008) investigated captive rodent colonies as well as wild ones and reported higher seroprevalence in comparative captive house mice populations with 24-47% and 35% seropositivity respectively. LCMV RNA was also detected in 30% of captive mice in Blasdell et al 2008. High prevalences of other aerosol transmitted rodent viral zoonoses, such as Seoul virus (SEOV), have also been observed in captive populations (McElhinney et al. 2017). LCMV prevalence may be higher in captive house mice populations than their wild counterparts due to the close proximity of the environment in which they live. Captive mice often live in cages or enclosures in which there are frequent interactions, therefore transmission of LCMV may be more likely. With wild house mice, the environment will be more varied, so there may be fewer interactions between individuals resulting in less frequent transmission of LCMV through a population.

In addition, captive mice are more likely to live longer than their wild counterparts, with lab mice commonly living for longer than two years whereas the mean life expectancy for wild mice may be as short as 100 days (Harris and Yalden 2008). This will be due to a combination of increased predation, a less stable environment, and inconsistent food sources. LCMV infected mice can shed virus throughout their lifetime, therefore captive mice with greater longevity will have more opportunity to be exposed or shed this virus, hence LCMV has been shown to persist in captive populations (Becker et al. 2007; JAMA 2014). This will also increase the chance of LCMV remaining in a population, as other studies have demonstrated the resilience of LCMV to persist in a captive population through generations (Becker et al. 2007). Therefore, it could be argued that exposure to an infected captive population will pose a greater zoonotic risk of LCMV transmission due to the increased prevalence and more frequent rodent contact, such as handling rodents or the cleaning of enclosures. There have been several reported cases of human illness attributed to LCMV exposure from infected lab animals (Hinman et al. 1975; Dykewicz et al. 1992). However, wild mice cannot be discounted as some farms had LCMV positive individuals, as well as a high prevalence within populations (50 to 100%), indicating that there was a high level of endemic infection. There is still a high level of interaction between people and mice at these locations, due to the frequent contact with rodent excretions in the environment or by the emptying of rodent traps in pest control programmes.

Certain human activities could be increasing the house mouse population and therefore also the level of LCMV transmission. For example, as many features of pig farms provide a suitable environment for mice, such as hollow walls, large amounts of easily accessible food and straw bedding. A Croatian study found that at illegal waste sites near human habitation, classified as having a medium to high level of anthropogenic impact had higher (37%) seroprevalence of LCMV infection compared to sites which had no or low anthropogenic impact or natural habitat (17%). This suggests that the presence of these waste sites could increase the mouse population and as there are more rodent host interactions when the population increases (so more opportunity to transmit LCMV to others), thus the distribution of LCMV in the rodent population becomes more widespread. This, in turn, increases the risk of transmission to humans (Duh, Hasic, and Buzan 2017). However, while human activity can increase house mice populations this does not necessarily mean that LCMV prevalence will also increase. In this study, the location with the largest number of house mice (Pig farm 3, n=38) due to a present ongoing infestation did not have any LCMV seropositive, (three mice were borderline), nor RNA positive house mice.

The highest LCMV prevalence was detected in house mice, as was this is the reservoir host species, although LCMV RNA also was detected in 2/61 brown rats, 2/50 bank voles and 1/49 wood mice.

LCMV infection in other rodent host has been frequently documented in other studies (Ledesma et al. 2009). An Italian study detected seropositivity in 205 (7.5%) of 2,732 rodent serum samples over a four year period, in the following species; *Microtus arvalis* (prevalence 20%), *A. flavicollis* (8.9%) and *M. glareolus* (7.4%) (Tagliapietra et al. 2009). In the UK, seropositivity in low levels has been reported in brown rats, red squirrels, wood mice, field voles and even the black-tailed prairie dog (*Cynomys ludovicianus*) (Blasdell et al. 2008). Therefore, if other rodent species are capable of becoming infected with LCMV as well as the reservoir host, it raises the question whether there would be a greater risk of transmission to people if there is a greater number of rodent maintenance hosts. However, it is not clear whether additional rodent hosts would be able to increase the transmission of LCMV or they are merely accidental dead-end hosts.

The detection of LCMV RNA in the liver of rats in this study could indicate viral replication and questions the notion that rats cannot be maintenance hosts for LCMV. Although the low prevalence in other rodent species compared to house mice does suggest that this could again be spillover infection due to exposure to LCMV infected house mice. For example, wood mice are known to enter buildings in search of food and therefore could encounter with house mice or their excretions in the process. In the case of the two rats and one bank vole (V41), all came from the same location (Pig Farm 1), indicating LCMV could be circulating in multiple rodent species in and around that farm. Only one house mouse was received from this farm, which was negative for LCMV RNA, however, this data is not sufficient to comment on the LCMV status of the house mice population at this location or conclusively show the occurrence of spillover infection.

There were three rodent species, the field vole, red squirrel and grey squirrel, in this study that were negative for LCMV RNA. One explanation for this could be that these species occupy separate ecological niches so it very unlikely that these rodents would be able to interact or even enter the same environment as the house mouse reservoir, therefore it is unlikely that spillover infection would occur. In this study, most of the field vole samples (n=16) collected were from an extremely isolated rural location, Llyn Cowlyd, where it would be highly unlikely that contact with house mice would occur. One study in Finland found that certain areas contained LCMV seropositive field voles, and in some cases, although prevalence was low, it was within the range reported in house mice (Forbes et al. 2014). It is unclear in the Finnish study whether there was any interaction with house mice populations, therefore it is merely an observation that there were similar prevalences in house mice and vole populations. Other studies have reported the detection of seropositivity in other vole species, such as the common vole (*M. arvalis*), in which there was a 14.3% prevalence of LCMV in Northern Italy (Kallio-Kokko et al. 2006). Therefore, it is possible for voles to be seropositive for LCMV

without the interaction of a resident infected house mice population, however, the extent that these voles are able to maintain and shed LCMV is still unclear. As no field voles were positive in this study, this would suggest the importance of field voles in the maintenance of LCMV is small. Although field vole sample numbers were low (n=19) so more sampling would help clarify the situation. Similar can be stated for both squirrel species as no LCMV RNA was detected in squirrel, red (n=21) or grey (n=12), samples in this study.

The overall prevalence of LCMV by PCR in British rodents is 8% (26/331, 95% CI 15-36) according to this study with a range of 0-17.5% depending on the rodent species in question. The finding of a higher than previously reported level across a larger geographical range does raise the question of whether there is an increasing risk to public health if there is a greater chance of zoonotic LCMV transmission. In the UK, there have been few incidents of reported human clinical disease as a result of LCMV infection, for which there could be several reasons. One that is most likely is the fact that infection with LCMV is most often asymptomatic therefore the person infected would not necessarily realise, nor would they report this to a medical professional for diagnostic testing. In 60% of suspected viral encephalitis cases in the UK (Kennedy, Quan, and Lipkin 2017), the infectious agent is not identified due to a failure of conventional laboratory techniques. There are multiple differential diagnoses that can be made, with Herpes Simplex virus (HSV)-1 as the most common cause of fatal encephalitis (Kennedy, Quan, and Lipkin 2017), therefore it may be problematic to make a definitive LCMV diagnosis. There also is no routine surveillance conducted for LCMV infection in people, as there is with other zoonotic pathogens, such as routine screening for Hepatitis E virus in the NHS blood transfusion service (NHS 2016). Another reason why there may be LCMV circulating in rodents but not reported in people could be due to the difference in pathogenicity in LCMV strains. It has been shown in lab mice that LCMV strains which cluster together can result in different pathological effects (Takagi et al. 2012). Therefore it is unclear whether the strains detected in this study or other British rodent studies, could cause clinically significant disease in humans.

It could be argued that conducting LCMV surveillance in humans may be a waste of resources due to the lack of clinical cases and the resulting mild symptoms of infection. However, this means that there is no prevalence data available for human exposure or infection with LCMV. There is a danger that this pathogen is present in healthy individuals where it may go unrecognised and undiagnosed until there is a case of serious human illness. For example, in the USA, there was a case of LCMV transmission through a solid organ transplant, as there was no routine LCMV screening conducted before donation, leading to fatal consequences for the recipients (Fischer et al. 2006). Does the lack of LCMV surveillance mean that we are playing a waiting game until someone succumbs to the

serious consequences of LCMV infection? Therefore investigation into human LCMV prevalence may be beneficial to prevent transmission to those individuals in which infection can have serious implications.

4.4.2. Conclusion

The results from this study indicate that LCMV is probably endemic and widespread in rodents throughout the UK, which supports the findings of other UK LCMV rodent studies. The highest prevalence was detected in house mice, which was expected as this is the reservoir species. There was very little genetic diversity observed in the sequences derived from the mice, and other rodent species, in this study. However, it is likely this is due to the highly conserved region targeted by the PCR, as there was a high level of similarity observed between other published LCMV sequences and other Arenaviruses. Therefore, more sequence data from other regions should be obtained to draw more comprehensive phylogenetic conclusions. The serology results support the findings of the molecular screening and identified further LCMV, detecting LCMV antibody, in mice that were virus negative by PCR. The 8% PCR prevalence of LCMV in rodents does present a potential health risk, particularly to certain groups of people such as pregnant women or immunosuppressed people e.g. organ transplant patients. However, the degree to how widespread LCMV infection is in people is unknown as there no prevalence data available in the UK, so it is unknown if this prevalence in rodents has resulted in zoonotic infection. Therefore, further investigation into LCMV infection in people is needed to accurately assess the risk to public health and determine whether pre-treatment screening would be justified.

Chapter 5: Hepatitis E virus:

First detection of Rat Hepatitis E virus (*Orthohepevirus C*) in wild brown rats in the United Kingdom

5.0. Abstract

Hepatitis E virus (HEV) is currently one of the leading causes of acute viral hepatitis infections in humans and is an important public health concern across the globe. In the United Kingdom, there has been a steady annual increase of reported HEV infections in people and most of these are thought to be indigenously acquired *Orthohepevirus A* genotype 3 (HEV G3) which has been linked to pork production and consumption. HEV G3 RNA has also been detected in British pigs. However, the dominant subgroup circulating in pigs differs from that which is found in people in the UK. Therefore an alternative, potentially zoonotic, source is suspected as a possible cause of these infections. Rodents, brown rats (*Rattus norvegicus*) in particular, have been shown to carry HEV, both the swine HEV G3 and *Orthohepevirus C*, genotype C1 (rat HEV). To investigate the prevalence of HEV in British rodents liver tissue was taken from 307 rodents collected from pig farms and other locations. These included samples from brown rats (*R. norvegicus*, n=61), house mice (*Mus musculus*, n=97), wood mice (*Apodemus sylvaticus*, n=48), bank voles (*Myodes glareolus*, n=49), field voles (*Microtus agrestis*, n=19), red squirrels (*Sciurus vulgaris*, n=21) and grey squirrels (*Sciurus carolinensis*, n=12). The RNA from these samples was extracted and screened using a pan-HEV nested RT-PCR assay. Histopathology was also performed on three positive liver samples. In this study, 8/61(13%) of brown rat livers were positive for HEV RNA. All of the positives were found to be rat HEV with 89-92% nucleotide identity to other rat HEV sequences circulating within Europe. Lesions and necrosis were observed histologically in 2/3 samples examined, which appears to be indicative of HEV infection based on observations in other HEV infected animals. No HEV RNA other rodent species or HEV G3 (Swine or human variant) was detected in any rodent species in this study. This is the first reported detection of rat HEV in a wild rat from the United Kingdom. Rat HEV has recently been identified as a zoonotic pathogen although further study would be required to assess the implications and risk to public health.

5.1. Introduction

Hepatitis E virus (HEV) is a leading cause of viral hepatitis globally (Guerra et al. 2017). While most infections are probably self-limiting and subclinical, the infection can lead to severe complications such as liver failure, chronic hepatitis, and cirrhosis (Guerra et al. 2017). The number of reported human HEV infection cases in the UK had been increasing annually since 2010, although a decreasing trend has been observed since 2016, although recent data (2016 to 2017) show a decreasing trend (PHE 2018a). Most of these reported infections were with *Orthohepevirus A* genotype 3 (HEV G3) and likely zoonotic in origin given that HEV G3 has a wide host range and there is a well-established link between HEV infection and pig meat products in industrialised countries (Pavio, Meng, and Renou 2010). In the UK, HEV has been found in the pork food chain (Grierson et al. 2015; McCreary et al. 2008) including point-of-sale pork products, such as British pork sausages (Berto et al. 2012). However, while the genotype of HEV circulating in people in the UK is HEV G3 subgroup 2, HEV in British pigs is mainly HEV G3 subgroup 1 (S. Ijaz et al. 2014). Thus most human infections in the UK appear to be either from imported pork products (Said et al. 2017; Salines, Andraud, and Rose 2017), or there may be an alternative zoonotic source.

Rodents have been shown to be susceptible to infection with a diverse range of HEV species and could, therefore, be potential sources of human and livestock infections (Takahashi et al. 2014; Ryll et al. 2017). Large populations of some rodent species are a common sight on and around farms due to the availability of food and shelter. In a Japanese study, 10/56 brown rats (*Rattus norvegicus*) were positive for swine HEV G3 (Kanai et al. 2012). In the UK, HEV G3 RNA subgroup 1 has been reported in the intestines (not livers) of house mice (*Mus musculus*) on one pig farm, but this is believed to be due to ingestion of virus from pig excreta rather than the mice being infected (Grierson et al. 2018). Furthermore, brown rats have been shown to have their own distinct species of HEV, *Orthohepevirus C*, genotype C1 (rat HEV). Rat HEV was first detected in Germany in 2010 (Reimar Johne et al. 2010) and since then has been identified in the USA, China, Vietnam and continental Europe (Ryll et al. 2017). Prior to this study rat HEV had not been detected in the UK (Grierson et al. 2018) and was previously not thought to be zoonotic, however, the first case of human infection in a 56-year-old man was recently reported in Hong Kong (HKU Med 2018; Sridhar et al. 2018).

There are several diagnostic approaches that could be used to investigate the prevalence of HEV in rodents. For this study, a molecular approach was taken to determine HEV infection through the detection of viral RNA in rodent tissue. Liver tissue was used as this is the organ most associated with HEV infection and pathogenesis (Lhomme et al. 2016), liver cells have been shown as a site of

viral replication (Jirintai et al. 2014; Lhomme et al. 2016), and liver tissue has been used in previous rodent studies to detect HEV RNA (Lack, Volk, and Van Den Bussche 2012; Johne et al. 2010a). In a comparison of viral loads in the tissues of rat HEV infected rats the highest Ct values were recorded in the liver (Johne et al. 2010a). The use of feacel samples was discussed but faeces were not used as the presence of HEV RNA in the faeces does not always indicate infection (Grierson et al. 2018) and those which are infected are likely to have RNA in both liver tissue and faeces (Widén et al. 2014). A pan-HEV RT-PCR assay, which was originally developed and published by Johne et al, (2010), was chosen to conduct the molecular screening in this study. This assay was developed through the alignment of 22 full-length HEV sequences of genotypes 1–4 derived from humans, pigs and wild boars plus one from an avian HEV isolate and amplifies a highly conserved region of ORF-1 (Johne et al. 2010b). The authors demonstrate that is this a highly sensitive assay and that it is able to detect many HEV species, novel HEV-like viruses and rat HEV (Johne et al. 2010). The primary reason this PCR assay was chosen for the molecular screening in this study is the fact that it is able to detect multiple HEV species, therefore this assay would allow each rodent to be screened for both *Orthohepevirus A* (Genotype 3) and *Orthohepevirus C* (rat HEV, genotype C1). As with the other viral pathogens investigated in the project, Sanger sequencing of the PCR amplicons produced was used to determine the HEV species and viral lineage.

Screening of samples using serological methods was considered for this study but not used for several reasons. HEV ELISA's can be useful to show possible subclinical infection in people (Shimizu et al. 2016; Dremsek et al. 2012) although the interpretation of these may be difficult due to the cross-reactivity between HEV species (Sridhar et al. 2018). The Wantaii ELISA has been used in previous HEV studies and as a diagnostic tool, however, this assay has been shown only to detect HEV G1-G4 (Trémeaux et al. 2016), therefore this serological screening method could not be used to investigate rat HEV seroprevalence. Furthermore, these assays are primarily used to investigate HEV seroprevalence in cases of human HEV infection, so it is unclear whether they could be used to screen rodents, therefore a molecular-based screening methodology is favourable. Immunohistochemistry was used to investigate the rodent host response and view any immune-mediated pathology that may be present in the liver tissue of infected rodents.

The aim of this study was to investigate the prevalence of HEV in British rodents from both pig and non-pig farming establishments. The results from this study could be used to build on the current knowledge of possible zoonotic reservoirs of HEV in UK and highlight areas for future work on the transmission of HEV and risk assessment concerning public health.

5.2. Materials and Methods

5.2.1 Sample collection

Rodents were collected and sampled from a range of sites across northern England, Wales and Scotland between September 2014 and January 2016 (Table 5.3). Study sites included 12 pig farms, four other farms, and a small number of other rural and urban locations (Table 5.4). Rodents were live-trapped and humanely euthanised on site. For full details on the fieldwork aspect and ethics for this study, please refer to Chapter 2. In addition, some animals were donated from pest control programs already established at these sites or collected as roadkill. Carcasses were cooled and then stored frozen at -80°C until post-mortem examination could be performed. Liver tissue was removed for RNA extraction.

5.2.2 RNA extraction and cDNA synthesis

RNA was extracted from liver tissue using a GenElute Mammalian Total RNA Mini-prep kit (Sigma-Aldrich Ltd, Dorset UK) according to the manufacturer's instructions. The 50 µl RNA elute produced was assessed using a Nanodrop (Labtech International Ltd, Sussex). RNA samples were then stored at -80°C until required. Generation of cDNA was performed using RevertAid RT Reverse Transcription Kit (Thermofisher Scientific, Loughborough, UK). Using the data from the Nanodrop the concentration of RNA was adjusted (with RNAse free water) to 500 ng/µl per 10 µl. Part I of the cDNA synthesis involved the addition of 2 µl per reaction of RNAse free water and 0.5µl per reaction of random hexamer primer to the RNA. This was then incubated at 25°C for 10 minutes then the reaction was halted on ice. Part II of the cDNA synthesis involved the addition of 4.5 µl 5x Reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2 µl dNTP Mix (10mM) and 1 µl RevertAid Reverse Transcriptase per reaction to each tube. This was incubated at 42°C for 60 minutes then 70°C for 10 minutes. The 20 µl of cDNA that was generated was stored at -20°C until required.

5.2.3. PCR assays

5.2.3.1. Pan-HEV nested RT-PCR

A pan-HEV nested RT-PCR originally developed by Johne et al (2010) was used to screen the rodent liver samples for HEV RNA. This PCR targets a conserved region on ORF-1 of the viral genome which was discovered by the alignment of 22 full-length genomes of HEV derived from human, pig, wild boar and chicken. This assay was chosen as it is able to detect multiple HEV species, including that outside of *Orthohepevirus A*, which will allow this assay to detect HEV G3 and rat HEV (Johne

et al. 2010). The master mix used in both rounds of this PCR was 5x HOT FIREPol® Blend Master Mix with 15 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia) according to the manufacturer's instructions.

The first round reaction mix included, per reaction, 4 µl Hot Firepol Master Mix, 13 µl RNAse free water, 1 µl (10 pmol) of forward primer HEV-cs, 1 µl (10 pmol) reverse primer HEV-cas and 1µl of cDNA. For primer sequences please refer to table 5.2. The cycling conditions were; 95°C for 15 minutes followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 74°C for 45 seconds with a final elongation of 74°C for 5 minutes. The second round used the same PCR reagents as the first, but with 1 µl forward primer HEV-csn (10 pmol) and 1 µl reverse primer HEV-casn (10 pmol) and 1 µl of 1st round PCR product. The cycling conditions were 95°C for 15 minutes followed by 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 45 seconds and a final elongation of 72°C for 7 minutes. Products were run on a 2% peq green agarose gel at 120 V for 65 minutes then visualised under UV light.

Primer name	**Sequence (5' to 3')**	**Annealing temperature (ºC)**	**Product size (bp)**
1st round:			
HEV-cs	CGCGCATCACMTTYTTCCARAA	55	469-472
HEV-cas	GCCATGTTCCAGACDGTRTTCCA		
2nd round:			
HEV-csn	TGTGCTCTGTTTGGCCCNTGGTTYCG	62	331-334
HEV-casn	CCAGGCTCACCRGARTGYTTCTTCCA		

Table 5.1: Primer sequences for the Pan-HEV nested RT-PCR. Modified annealing temperatures and expectant product sizes are also shown (Johne et al. 2010)

5.2.3.2. Real-Time qPCR

RNA from HEV positive samples (detected in the Johne et al. 2010 PCR) were screened by S. Grierson at APHA to confirm C1 genotype using the two published real-time qPCR assays (Mulyanto et al. 2014; Johne et al. 2012) specific to this genotype of HEV. The Ct values gained from these assays can be used to compare the viral load and effectiveness of the PCR assays.

5.2.4.1. Nucleotide sequencing

PCR products from the nested ORF1-HEV PCR and the second round primers (see table 5.1) were sent to the Central Sequencing Unit at APHA Weybridge for Sanger sequencing. Sequencing data were analysed using a DNAStar Lasergene software package and using SeqMan Pro to assemble contiguous sequences of the forward and reverse data. Chromatogram profiles of each contiguous sequence were compared and trimmed to remove primer sequences and produce a consensus sequence of the 224 nt fragments. Consensus sequences were uploaded into Megalign (DNAStar Lasergene software) and aligned using the 'clustal W' method, any differences were examined and compared with the raw chromatogram data. The raw sequence data analysis was performed by Daisy Jennings.

The seven complete consensus sequences then sent to Ellen Murphy and then compared with 48 published sequences using the nucleotide Basic Local Alignment Search Tool (BLAST) programme produced by the National Center for Biotechnology Information (NCBI). An alignment was created in MEG7 using the 'clustal W' method (Figure 5.2).

5.2.4.2. Analysis of the ORF-1 224 nt sequences

Phylogenetic analysis was conducted in MEGA 7 and the sequences from this study were compared with 37 others published 224 nucleotide sequences in the C1 cluster of rat HEV obtained from GenBank. A phylogenetic tree was constructed using the Maximum Composite Likelihood (MCL) approach and model Kimura 2-parameter plus gamma. Bootstrap analysis was performed with 1000 replications.

5.2.5. Histopathology

Liver tissue from three HEV positive rats (R5, R43 and R76) were fixed in 10% formalin for a minimum of 16 hours then processed by Elena Fitzpatrick with Hematoxylin and Eosin (H&E) staining. Protocols used by the histology department were provided in Appendix VII. Any samples with distinctive lesions related to viral infection were processed for immunochemistry (IHC) by Elena

Fitzpatrick using a manual staining technique with stains CD3, CD79a and PAX-5. Assistance with the interpretation of these results was provided by veterinary pathologists Raneri Verin and Julian Chantrey.

5.3. Results

5.3.1. Rodent samples screened for HEV RNA

In total 307 rodent liver samples, from seven species (see Table 3) were screened for HEV RNA.

Species (common name)	Species (Latin name)	Number sampled
Brown rat	*Rattus norvegicus*	61
House mouse	*Mus musculus*	97
Wood mouse	*Apodemus sylvaticus*	48
Bank vole	*Myodes glareolus*	49
Field vole	*Microtus agrestis*	19
Red squirrel	*Sciurus vulgaris*	21
Grey squirrel	*Sciurus carolinensis*	12

Table 5.2: Species of rodents sampled for this study. Liver tissue was removed from each rodent and used in PCR assays to screen for HEV RNA.

5.3.2. PCR

HEV RNA from liver tissue was detected in the liver tissue by fully nested ORF-1 PCR in 13% (8/61) of brown rats and not in any other rodent species. HEV RNA was detected on three pig farms (Cheshire, Wetherby and Ripon), as well as a dairy farm in the Cheshire, but which was in close proximity to the other Cheshire farm which also kept pigs.

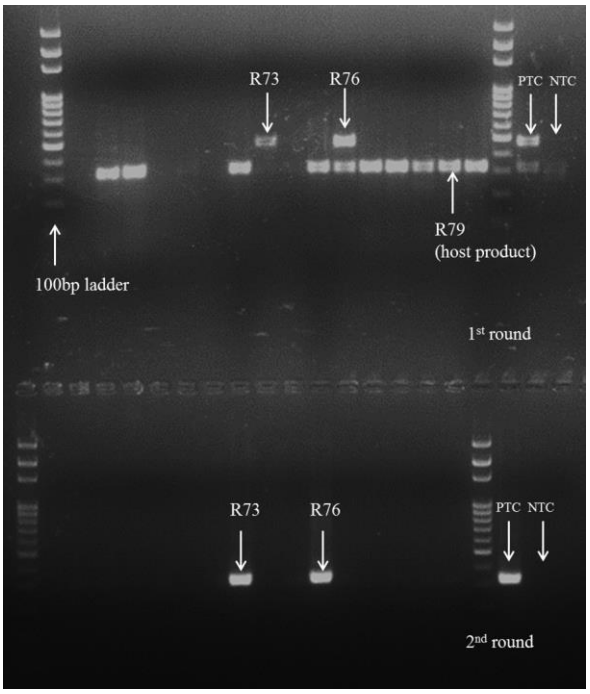

Figure 5.1: Gel photo displaying the first and second round PCR products. PCR products can be seen at 470bp in the first round (R73 and R76). R79 was HEV RNA negative but a band can still be observed at 300-320 bp on the first round, (this is a host RNA). The second round HEV positive PCR product is 330 bp in size. No band was observed for R79 in the second round. The positive control (PTC) and negative (NTC) can also be seen.

Figure 5.1 shows the amplicons generated by the first and second rounds of the PCR. The first round sometimes produced a product of 300-320 bp, as shown in figure 2 with sample R79. This, however, had 86-88% identity with *Mus musculus* chromosome 17 (GenBank accession number AC102769), was only observed in brown rat samples so is likely to be an unreported host gene. The presence of HEV often caused a double band to be observed. This host gene, which cross-reacted with the HEV first round primers, was observed in 40/61 rats screened. The second round and the higher annealing temperatures than those previously published (Johne et al 2010) increased the specificity of the assay, thus the host product was removed. To verify that this PCR, with the adjusted annealing temperatures and reagents, was capable of detecting the desired HEV species HEV G3 and rat HEV controls was used in the assay. The HEV G3 control was derived from the GIT of an HEV G3 positive house mouse in another study (control provided by S. Grierson, APHA) and sequenced rat HEV control from this from a postive rat (R43) in this study. A positive band was observed in both rounds of the

PCR which confirms that this assay can detect rat HEV and HEV G3. Details of the PCR results for each rodent species and each location are presented in table 5.3.

Location name	Location	Species and Result	Total
Pig farms:			
Pig Farm 1	Ripon, Yorkshire	RN (2/16), MM (0/1), AS (0/1), MG (0/7)	2/25
Pig Farm 2	Cheshire	RN (1/10)	1/10
Pig Farm 3	Morpeth, Northumberland	MM (0/38)	0/38
Pig Farm 4	Hull	RN (0/2), MM (0/9), MG (0/1)	0/12
Pig Farm 5	Berwick, Northumberland	MM (0/16), MG (0/8)	0/24
Pig Farm 6	Driffield, Yorkshire	RN (0/2), MM (0/3), AS (0/10) MG (0/12)	0/27
Pig Farm 7	Edinburgh	MM (0/6), AS (0/9)	0/15
Pig Farm 8	Malton, Yorkshire	RN (0/1), MM (0/3), MA (0/1), MG (0/9)	0/14
Pig Farm 9	Wetherby, Yorkshire	RN (2/16), AS (0/2), MG (0/4)	2/22
Pig Farm 10	Morpeth, Northumberland	AS (0/20), MG (0/3)	0/23
Pig Farm 11	Telford	MM (0/6)	0/6
Pig Farm 12	York, Yorkshire	MM (0/15)	0/15
			5/231
Location name	**Location**	**Species and Result**	**Total**
Other sites:			
Farm 1	Cheshire	RN (3/5), MG (0/1) AS (0/6)	3/12
Farm 2	Derby	RN (0/1)	0/1
Farm 3	Tattenhall, Cheshire	MA (0/2), MG (0/3)	0/5
Rural 1	Llyn Cowlyd, North Wales	MA (0/16), MG (0/1)	0/17
Rural 2	Ruthin, North Wales	RN (0/1)	0/1
Forest 1	North Wales (various)	SV (0/21)	0/21
Forest 2	Formby (various)	SC (0/12)	0/12
Urban 1	Liverpool, Merseyside	RN (0/3)	0/3
Urban 2	Ellesmere Port, Cheshire	RN (0/4)	0/4
			3/76

Table 5.3: Results from the HEV surveillance by pan-HEV RT-PCR of rodents from UK pig farms and other locations. RN (*Rattus norvegicus*), MM (*Mus musculus*), AS (*Apodemus sylvaticus*), MG (*Myodes glareolus*), MA (*Microtus agrestis*), SC (*Sciurus carolinensis*) and SV (*Sciurus vulgaris*)

In this study, only brown rats (*R. norvegicus*) were shown to be positive for HEV RNA in the pan-HEV RT-PCR and no other rodent species had detectable HEV RNA in their liver tissue. Of the HEV positive rats, there were 8/61 (13%, 95% CI, 5-21) and they were from two regions of the UK (Yorkshire and Merseyside) across four different sites. Sanger sequencing revealed that all positive rats detected, apart from R1 which was not able to be sequenced, were infected with rat HEV (*Orthohepevirus C*, C1 genotype).

	Sex	**Age**	**Total**
R. norvegicus HEV+	M: 7/8 F: 1/8	A: 6/8 J: 2/8	8/61
R. norvegicus HEV-	M:32/53 F:21/53	A: 37/53 J: 16/53	53/61
Total	M:39/61 F:22/61	A:43/61 J:18/61	

Table 5.4: Sex and Age data for the *R. norvegicus* of this study. With the HEV positive and HEV negative groups compared.

There were more males (n=7) than females (n=1) infected with rat HEV. However, in the study, there were more males (n=39) than females (n=22) screened. There were more adults (n=6) than juveniles (n=2) positive for rat HEV. However, in this study, there was a greater number of adults (n=43) than juveniles (n=18) screened. Fisher's exact test was performed and showed that both sex (p= 0.2387, 95% CI 0.5- 216.5) and age (p= 1, 95%CI 0.2-14.4) biases were not statistically significant.

Rat I.D.	Age	Sex	Real Time: Mulyanto et al 2014	Ct value	Real Time: Johne et al 2012	Ct value
R1	A	M	+	33.29	+	34.12
R3	A	M	+	21.22	+	23.07
R5	A	F	+	26.16	+	27.26
R43	A	M	+	23.29	-	No Ct
R45	J	M	-	No Ct	-	No Ct
R58	J	M	+	33.99	+	38.00
R73	A	M	+	26.66	+	30.13
R76	A	M	+	20.41	+	30.65

Table 5.5: Results for the rat HEV positive samples (previously screened in the pan-HEV RT-PCR) screened with two C1 genotype-specific real-time PCR assays. The results and Ct values generated from the Mulyanto et al (2014) and Johne et al (2012) real-time PCR assays can be seen. All rat HEV positives belong to C1 genotype of *Orthohepevirus C*. These results were provided by S. Grierson at APHA.

RNA from rat HEV positive rats, identified in the pan-HEV RT-PCR assay were sent to S. Grierson at the APHA, Weybridge. Table 5.5 shows the results from the two HEV C1 real-time qPCR assays performed by S. Grierson to confirm the presence of rat HEV RNA in the liver tissue of the previously identified positive rats. Rat HEV RNA was not detected in all of the samples which were shown to be positive in the pan-HEV RT-PCR assay, such as R45 which was negative in both real-time assays and R43 which was negative in Johne et al (2012) real-time assay. This could indicate that the real-time assays may be less sensitive than the pan-HEV RT-PCR, therefore, less likely to detect rat HEV RNA when infection is at a low level.

The results from two positive juvenile rats indicated a low level of infection, as R45 was negative and R58 had high Ct values compared to the adults in the study with 33.99 in Mulyanto et al (2014) and 38.00 in Johne et al (2012). Higher Ct values were also observed in R1. The positive PCR product from R1 in the pan-HEV RT-PCR assay was not able to be sequenced, however, this sample was positive in both C1 real-time PCR assays which confirms that it is also rat HEV.

5.3.3. Sequence analysis of the ORF-1 RT-PCR product

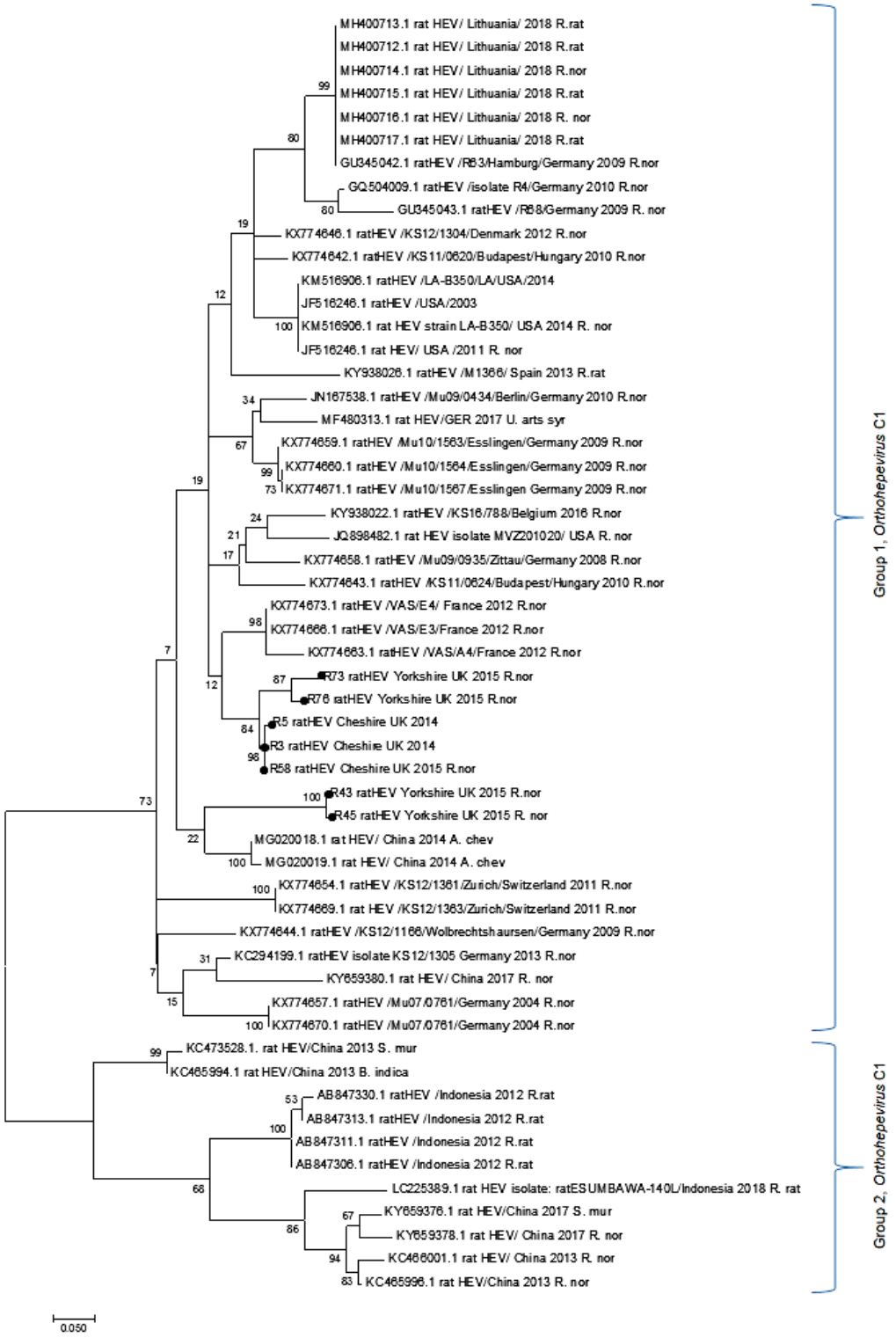

Figure 5.2: Phylogenetic tree was inferred using the Maximum likelihood method using Kimura 2-parameter model (Kimura 1980) constructed from of 55 nucleotide sequences with 224 positions in

the final data set of a partial ORF-1 fragment of *Orthohepevirus C*, genotype C1. These included 48 published sequences and seven sequences from this study (highlighted by the ● icon). Phylogenetic analyses were conducted in MEGA7 (Kumar, Stecher, and Tamura 2016). Species abbreviations; R. nor (*Rattus norvegicus*), R. rat (*Rattus rattus*), A. chev (*Apodemus chevrieri*), B. indica (*Bandicota indica*), S. mur (*Suncus murinus*) and U. arts syr (*Ursus arctos syriacus*).

From an initial BLAST search, it can be seen that there is a high level of genetic identity (89-92% nt identity) with other published mainland Europe strains of rats. Such as those detected in Belgium (91% nt identity to R73 and R76), Germany (89% nt identity to R43 and R45) and France (92% identity to R58, R3 and R5). They all cluster within the Group 1 of the C1 genotype, although there is still divergence between the British strains, as there are two different clusters seen on the phylogenetic tree. Bootstrap values indicate that there is a high likelihood that these sequences are in Group 1 (73), although it is difficult to have a high level of confidence in the individual positions on the tree with respect to other Group 1 sequences as the bootstrap values are lower (22 and 19 respectively).

5.3.3. Histopathology

Lesions and immunopathology in the cross-section of liver tissue rat R5 and to a lesser extent in R76 typical of HEV were observed. These were not observed in R43.

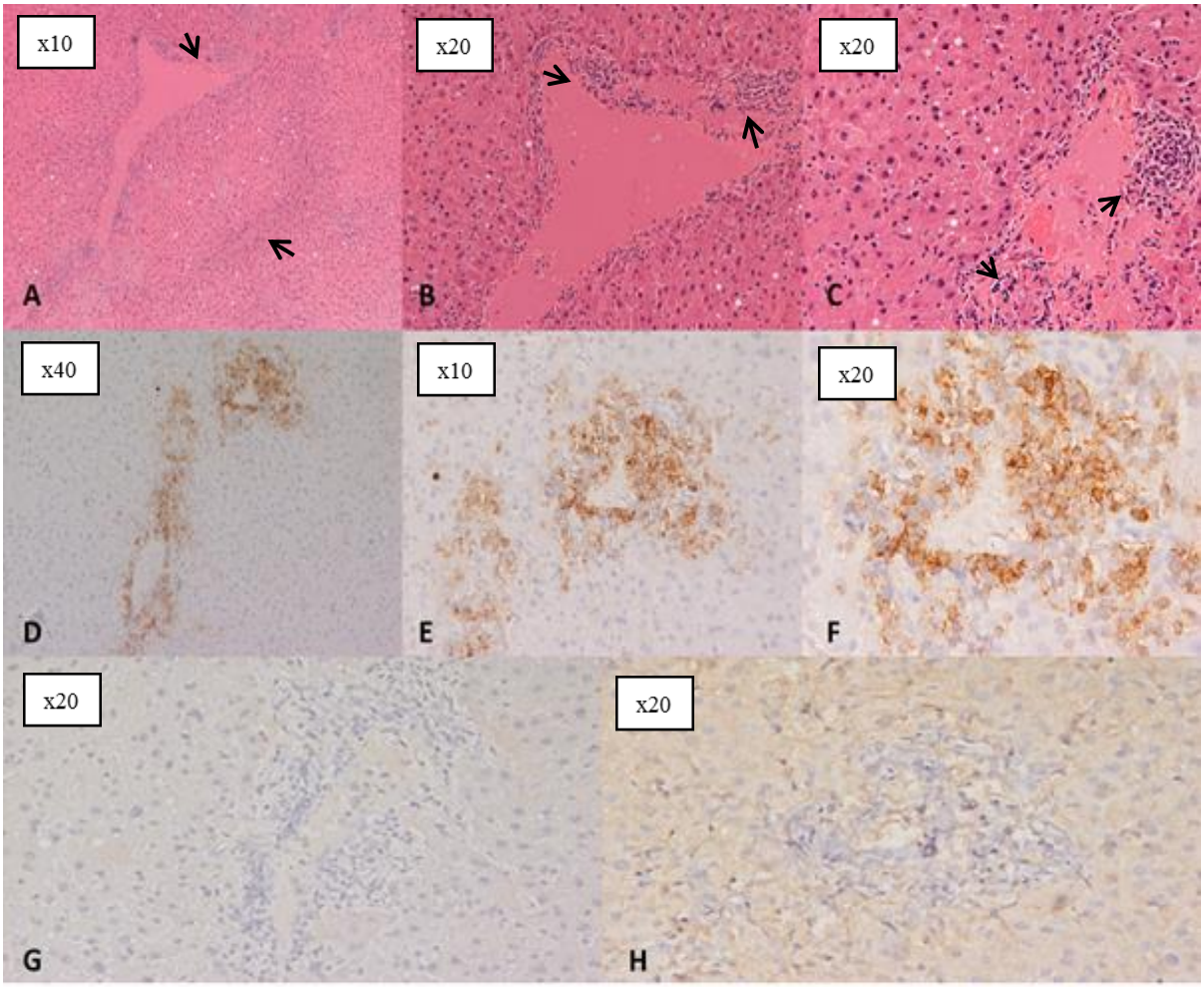

Figure 5.3: Histology and Immunohistochemistry (IHC) results for Rat R5. **A, B, C:** Rat R5, liver, multifocal dense lymphocytic periportal infiltrates (see arrowheads), H&E. **D, E, F:** Rat R5, liver, different magnifications of the same portal area showing dense lymphocytic infiltrates composed of CD3 positive small round cells (T lymphocytes), CD3 I HC. **G:** Rat R5, liver, same microscopic area presented in D, E and F showing the absence of B lymphocytes in the periportal infiltrates, CD79a IHC.**H:** Rat R5, liver, Same microscopic area presented in D, E and F showing the absence of B lymphocytes in the periportal infiltrates, PAX-5 IHC. Microscope magnification objectives are shown for each image.

The lymphocytic infiltrates which were observed in R5 were T-lymphocytes which were positive for CD3. This has also been observed in human liver biopsies from patients with acute viral hepatitis due

to HEV G3 infection, in which T-lymphocytes that were positive for CD3 were the majority of infiltrating cells (Drebber et al. 2013). Therefore, infection with rat HEV may induce a similar inflammatory response in rats as HEV G3 does in people. However, without specific viral antibody immunohistochemistry, it is difficult to conclude that this is a direct result of rat HEV infection as it is possible that, as this was a wild rat, could have been infected with multiple pathogens.

5.4.1. Discussion

No HEV G3 RNA (swine or human strain) was detected in the livers of any of the rodents tested in this study. This may indicate that only rare transmission of HEV G3 occurs between pigs and rodents. Although pigs on these farms were not directly tested, so this result may be due to the fact that the pigs themselves were not infected with HEV G3. The latter is unlikely, however, as a high prevalence of HEV G3 has previously been found in British pigs (Grierson et al. 2015), pig farms and in slurry lagoons (McCreary et al. 2008). In a study conducted in Japan, HEV G3 RNA was detected in the livers and spleen of rats from a pig farm in which the pigs were already known to be HEV G3 positive (Kanai et al. 2012). This could indicate that if the pigs are positive then it is highly likely that the rodents on the same farm would be, which strengthens the argument that the lack of HEV G3 RNA detected is due to the HEV negative status of the pigs.

It may also be that different subgroups of genotypes have different host and geographic ranges. Studies have observed that South West England could be an area in which HEV may be highly prevalent in people, with seropositivity in donated blood products (Hewitt et al. 2014) and frequent reported cases of jaundice in people reported in the same region (Dalton et al. 2007). This study only sampled rodents from pig farms from Northern England and one area in Scotland, so this could be an area of the country where there is a lower HEV G3 prevalence in pigs or people. However, if pigs and pork meat are a source of HEV G3 it is difficult to narrow the origins to a particular region due to the highly distributed pork food chain. Therefore, it is more likely that HEV G3 could be present in pigs throughout the country. Although currently, there is also not enough data available on all regions of the UK to highlight regional prevalence or to accurately map the emergence of HEV in pigs or people.

A similar study found HEV G3 (swine strain) RNA in the GIT of four house mice from one pig farm in England but not in the livers (Grierson et al. 2018). In our study, only the liver was screened and therefore HEV G3 may have been ingested by these rodents and present in the GIT of the rodents but was not detected. Another reason for the lack of HEV G3 RNA in liver tissue could be that rodents may not be susceptible to HEV G3 infection. This was observed in laboratory nude rats, which had already been shown to be susceptible to rat HEV infection, as antibodies and rat HEV RNA were found in stools of rat HEV inoculated rats (Li et al. 2013). However, when these rats were inoculated with HEV G3, no antibodies or RNA was detected in the liver nor the faeces of these rats (Li et al. 2013). Therefore, even if pigs on the farms were positive for HEV G3, the rodents may not be as they are not susceptible to this genotype of HEV. The study by Grierson et al (2017) supports this idea as

although ingested virus was detected in house mice there was no active infection or viral RNA in the liver observed. This, however, is contradictory to the findings in the Japanese study (Kanai et al. 2012), and findings in a US study in which HEV G3 RNA was isolated from the liver tissue of 34/446 rats (*R. norvegicus* and *R. rattus*) (Lack, Volk, and Van Den Bussche 2012). The infectivity of HEV G3 in rodents is still not fully understood and remains a controversial topic. The results from our study alone are not enough to discount rodents as possible carriers of HEV G3 in the UK and all that can be interpreted in this instance, is HEV G3 RNA was not detected in the liver tissue any rodents screened.

The prevalence of rat HEV (8/61, 13%, 95% CI, 5-21) detected in this study is similar to that found in French rats from Lyon where 15% (12/81) of rat livers and 13.6% (11/81) of faecal samples were positive for rat HEV RNA. These French rat HEV strains had an 87% nucleotide similarity to other rat HEV strains in mainland Europe (Widén et al. 2014). The British rat HEV sequences detected in this study had 87-92% nucleotide similarity with those in mainland Europe as they all cluster in Group 1 of *Orthohepevirus* C1. Phylogenetic analysis suggested that sequences from the same site clustered together, but there is no clear clade associated with British rat HEV. Indeed, there is no clear geographic clustering among other European sequences (Figure 5.2), however, there are genetic differences between European and Asian sequences. One explanation for the high levels of similarity may be the movement of the rats, both in the past and more recently. Brown rats arrived in Europe (probably from Central Asia) around the 18th Century and were reported in Great Britain around 1720 (Harris and Yalden 2008), doubtless, there have been many introductions since then. Thus it would be expected that any virus endemic in rats would also be closely related those across Europe and, given numerous opportunities for introduction and movement, that there would not be clades associated with any particular area of Europe. Equally expected would be significant differences between European and Asian HEV strains. However, it would be interesting to compare whole genome sequences of HEV globally and compare these with host genetics as the two might provide insight into the co-evolution of the host and virus as well as the migratory history and globalisation of the brown rat.

In this study, it is not possible to define the detection of rat HEV as a recent introduction or as a result of increased screening due to a recent interest in the HEV in the UK. There has also not been any screening specifically reported for rat HEV before this study. The movement of rats is a credible argument for explaining pathogen dispersal, however, in this study rat HEV was detected in two regions which are geographically isolated therefore it is unlikely that there would have been mixing

of the separate rat populations. This supports the argument further that rat HEV could be endemic and widespread in the UK rat population. Although, the sample size in this study is relatively small (n=61) and samples were collected from Northern England and Scotland so it is difficult to view this as a representation of the whole UK rat population. In a recent published English pig farm rodent study 15 brown rats were screened but no C1 HEV RNA was detected (Grierson et al. 2018). However in the UK, as this study is the first reported detection of rat HEV, there is very little prevalence data available therefore further study on a wider sample base would be required to accurately estimate the true prevalence.

In this study, all rats which were positive for rat HEV RNA, apart from one, were male (n=7), although this was not shown to be significant, likely due to the small sample size, so, therefore, is merely an observation. This could be due to the social behaviour of rats in colonies, for example with SEOV there is a higher rate of transmission between male rats as there is often increased contact through aggressive encounters. These encounters are thought to increase viral transmission as there is more chance of contact with SEOV aerosols produced in saliva through bites or present any urine or faeces on the rodent fur (Hinson et al. 2004). It is not clear if aggressive encounters would contribute to a greater rate of transmission as, unlike SEOV, rat HEV is transmitted via the faeco-oral route. Therefore contact with a contaminated shared environment is more likely, as the three sites in which HEV was detected contained multiple rats with rat HEV RNA in their livers. Rat HEV shed in faeces has been shown to easily infect other rats, both in the wild (Johne et al. 2010) and under experimental conditions (Purcell et al. 2011). For example, when infected laboratory rats were co-housed with other sentinel rats after 21 days all the sentinel rats were shown to be infected with rat HEV and had higher faecal HEV RNA viral titers than the seeder rats (Debing et al. 2016).

It is not known how long rat HEV would be able to survive in faeces or what the required infective dose would be. It is also unclear how long the infected rat will shed virus as it is unknown how long rats remain infected. One study in Germany detected rat HEV antibodies, but no rat HEV RNA in the same rats which may indicate that infection may not be persistent in rats (Johne et al. 2012). The lesions and immunohistopathology observed in one rat infected with rat HEV could indicate that infection induces a similar immune response to that seen in human cases of HEV G3 infection. Whether rat HEV is capable of inducing acute viral hepatitis in rats or if it is a mild self-limiting infection remains unknown.

There also appears to be an age bias with most (n=6) infected rats being adults, although, like the gender observation, there are not sufficient numbers to prove statistical significance. A similar observation was made in another study in French rats, as all rat HEV positive brown rats were adults, although there was also a bias, as 88% of rats in the study were adults (Widén et al. 2014). One possible explanation for a higher detection rate in adults could be a result of the lasting protective effects of the maternal antibodies in juveniles, thus the virus is less able to establish infection. In this study, the two juvenile rats which were positive for rat HEV were either negative (R45) or had high Ct values in both real-time PCR, assays which could indicate a low viral load. In pigs, it is generally understood that HEV G3 infection usually occurs at 8-12 weeks which would coincide with the drop in maternal antibodies, followed by viraemia for 1-2 weeks after and a period of shedding that may last 3-7 weeks (Crossan et al. 2015). However, it is still unclear if this would be the case for rat HEV as the infection and maintenance of rat HEV in this reservoir host species not fully understood. Also, the length of time and degree to which maternal antibodies would persist throughout the juvenile stage to provide protection against rat HEV infection is not known. No serological analysis was conducted in this study so it is difficult to comment on the infection history of the other, juvenile or adult, HEV negative rats.

The transmission routes of rat HEV between rats have been observed, however, the transmission ability of rat HEV from rats to other species is not clear. Rat HEV has been detected in several species of the *Rattus* genus, including the black rat (*R. rattus*) (Ryll et al. 2017), Tanezumi rat (*R. tanezumi*) and *R. rattoides losea* (Li et al. 2013). In addition, strains of the C1 genotype have also been detected outside the *Rattus* genus such as in greater bandicoot rats (*Bandicota indica*) (Li et al. 2013), the Asian musk shrew (*Suncus murinus*) in China (Guan et al. 2013) and the Syrian Brown bear (*Ursus arctos syriacus*) from a zoo in Germany (Spahr et al. 2017). The Asian musk shrew was shown to have a 77.4%–99.6% nucleotide sequence identity to other rat HEV strains, which indicates that the Asian musk shrew could be a reservoir for rat HEV (Guan et al. 2013). Transmission to other rodent species has not been observed and in this study, no other rodent species were positive for rat HEV RNA, including those which were obtained from the same location as positive rats. One possible explanation for this could be the lack of interaction between rodent species, as smaller rodents, such as mice and voles, occupy different ecological niches to rats (Couzens et al. 2017). Therefore, there is less opportunity for other rodent species to become infected with rat HEV through contamination of a shared environment. However, this is not always the case, as there is often overlap, particularly between rats and house mice, especially if there is a stable food source available, such as a grain store on a pig farm. A more likely reason could be that these other rodent species are not susceptible to rat

HEV infection. This has been observed in some experimental studies where cross-species infection has been attempted with rat HEV but has been unsuccessful. In one experimental study, no rat HEV RNA was detected in the livers or faeces of lab mice after they were intravenously injected with LA-B350 rat HEV strain (Debing et al. 2016). Cross-species infection with intravenous inoculation of rat HEV has also been attempted in pigs, but no evidence of infection was observed (Cossaboom et al. 2012). This could indicate that rat HEV may have a narrow host range that could potentially limit the infectivity of the virus.

Rat HEV has only recently been shown to be a zoonotic pathogen, with the first case of human infection and clinical disease being recognised in Hong Kong in September 2018 (Sridhar et al. 2018). There have been subsequent cases of human rat HEV infection retrospectively recognised, in Hong Kong in November 2018 and a clincial case of acute viral hepatitis in a Canadian UN worker in 2019 (Andonov et al. 2019). Before these cases there were no reports of rat HEV infecting people (Nan et al. 2017) and rat HEV was thought to be a non-zoonotic variant. Like HEV G3 infections, rat HEV could also be asymptomatic or mild therefore an infected individual would likely not be screened for HEV, although advice from PHE encourages any unexplained hepatitis cases to be screened for HEV (PHE, 2018). There is currently no prevalence data available with regards to human infection with rat HEV in the UK. One of the reasons for this could be that the current PCR and serological diagnostic tests used would not detect rat HEV even if a person was infected. The Wantaï HEV-Ag detection ELISA assay, which is commonly used in the UK for diagnosing HEV G3, only detects antibody responses to HEV G1-4 (Trémeaux et al. 2016) and would, therefore, fail to detect rat HEV.

However, in Germany, where multiple detections of rat HEV in brown rats have been recorded, a serological survey of blood donors and forestry workers found several serum samples from forestry workers reacted strongly to rat HEV (Dremsek et al. 2012). Following this detection, another study found that rat HEV (homogenates originally from wild *R. rattus* from Indonesia) were able to successfully replicate in human hepatoma cell lines which could indicate that rat HEV may indeed have zoonotic potential (Jirintai et al. 2014). If zoonotic transmission and infection in humans with rat HEV is possible then could a potential zoonotic risk to public health. Therefore it could be possible that people in the UK are infected but there is no prevalence data available and current diagnostic tests used for HEV surveillance would not detect rat HEV. There is also not enough knowledge of the viral biology, pathology and transmission dynamics of rat HEV to conclude that rat HEV is a potential emerging zoonotic pathogen. The results from this study have shown that rat HEV is present

in the UK resident rat population and have highlighted an area of research that may require further investigation.

5.4.2. Conclusion

This is the first recorded detection of *Orthohepevirus C,* C1 (rat HEV) present in a wild brown rat in the United Kingdom. No *Orthohepevirus A* G3 was detected in any rodents in this study, although other studies have demonstrated that some rodents can be hosts for some strains of *Orthohepevirus A* G3. In this study, although rodent sample numbers were not huge, the results indicate that while rodents may be occasional hosts for zoonotic HEV in the UK, it is unlikely that they are reservoir hosts.

The detection of *Orthohepevirus C* in rats from multiple locations (Yorkshire and Cheshire) indicates that this virus could be indigenous and widespread in the UK rat population, although more data would need to be collected to this investigate further. As rat HEV has recently been reported as a zoonotic agent there may be public health implications. Although currently not enough is known about the virus or prevalence in people to ascertain whether rat HEV may pose a significant risk to public health, so further study is required.

Chapter Six: *Campylobacter* spp:

Prevalence of *Campylobacter* species (*C. jejuni* and *C. coli*) in the microflora of British rodents

6.0 Abstract

Campylobacter infection is the most common cause of bacterial gastroenteritis in the United Kingdom and it is estimated that there are over half a million cases annually resulting in 80,000 GP visits and a cost of £500 million to the British economy (Nichols et al. 2012). There are 16 species and 6 subspecies in the *Campylobacter* genus, however, the most common are *C. jejuni* and *C. coli* accounting for 97% of clinical isolates from human cases in the UK. The most common source of this bacterium is thought to be the food chain, chicken meat in particular, although swine, cattle and environmental sources have been implicated as possible reservoirs (Humphrey et al. 2007). Wild rodents have been shown to be carriers of *Campylobacter* spp and could be a source, however, there is little known about the carriage and prevalence of *Campylobacter* in this group of mammals. Rodents are often present on farms so could also be aiding in the transmission of this bacterium to livestock and could potentially increase the contamination risk of the food chain.

Rodent fecal samples (n= 152) were collected from pig farms in Northern England and Scotland as part of a surveillance survey of zoonotic pathogens in pig farm rodents between 2014-16. Faecal samples were cultured using published microbiological techniques designed to aid the growth of *Campylobacter*. An *lpx* gene PCR was performed to differentiate between *C. jejuni* and *C. coli* species. Through microbiological culture, 28% (43/152) rodents were *Campylobacter*-positive and of these 86% (37/43) were shown to be either *C. jejuni* (20/43, 46%) or *C. coli* (17/43, 40%) and 14% (6/43) isolates that were negative. Off these, 50% (13/26) of faecal samples from brown rats (*R. norvegicus*) were positive in which 39% *C. jejuni* (5/13) and 61% *C. coli* (8/13) positive. 41% (17/41) of faecal samples from bank voles (*M. glareolus*) were positive, in which *C. jejuni* was the most common (13/17). In house mice (*M. musculus*) 16.6% (10/60) were positive although *C. coli* (8/10) was the most common. In wood mice (*A. sylvaticus*) 12.5% (3/24) of faecal samples were positive and only one *C. jejuni* sample was identified. There was only one Field vole faecal sample collected and this was negative for *Campylobacter spp*. Full genome sequencing of *C. jejuni* isolates revealed that 4/5 bank voles were infected with ST-3704, which is a bank vole specific sequence type. There were multiple STs detected in brown rats (*R. norvegicus*) with ST-6561, ST-45 and ST-51. Novel sequence types were detected in one brown rat (R79) and in one wood mouse (M56). This study has confirmed the presence of both *C. jejuni* and *C. coli* in the faecal matter produced by a variety of rodent species that dwell on and around pig farms in the UK. House mice, which reside almost exclusively in pig farm buildings, could have a role in the maintenance and transmission of *C. coli* in pig herds due to the bias toward *C. coli* in pigs. Brown rats may have the most important contribution to *Campylobacter* carriage due to the identification of both *C. jejuni* and *C. coli*, which could indicate

that they have a role in the maintenance of *Campylobacter* prevalence in pig herds and contamination introduced from other external sources. The findings have highlighted the importance of biosecurity and the need for efficient rodent control, which if successful may help reduce the amount of *Campylobacter* introduced into the food chain by rodents.

6.1. Introduction

Members of the *Campylobacter* genus of bacteria are microaerophilic, Gram-negative bacilli (Figure 6.1), with relatively small genomes (1.6 megabases) and under microscopy appear as curved rods (Jeon et al. 2010). *Campylobacter* infection is the most common cause of bacterial gastroenteritis (campylobacteriosis) with an estimated 400-500 million cases annually worldwide (Jeon et al. 2010). Infection, with as little as 500 bacterial cells, can cause disease with common signs including abdominal pain due to the inflammation of the gut (gastroenteritis), fever and acute diarrhea, which is often watery and bloody in 75% of cases (Young et al. 2007; Allos 2001). More serious disease is rare but can include peritonitis, cholecystitis, pancreatitis and gastrointestinal hemorrhage. Bacteraemia can occur in up to 1% of gastroenteritis cases and in rare cases may lead to sepsis and even death, as campylobacteriosis has a mortality rate of 0.05 per 1000 infections, although serious complications are only usually seen in those with compromised immunity (Allos 2001). Infection with *C. jejuni* has also been associated with Guillain-Barré syndrome (GBS) (Nyati & Nyati 2013).

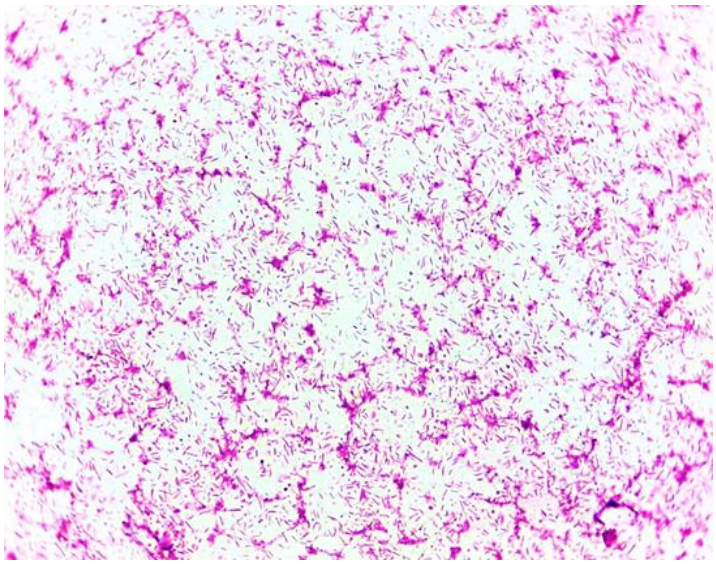

Figure 6.1: Gram stain of *C. jejuni* (pleomorphic curved Gram-negative rods) from a brown rat (*R. norvegicus*) taken from blood agar after 48 hours culture and visualised under x100 oil immersion.

Rodents are of interest in terms of *Campylobacter* transmission as they have been shown in previous studies to be a carrier of both *C. jejuni* and *C. coli*. Rodents are a very common feature on most farms as in Denmark 69% of farmers reported regularly observing mice on their farms and 39% regularly observed rats (Meerburg & Kijlstra 2007). They are also extremely difficult to completely exclude from farm buildings. Rodents could, therefore, be a source of contamination, in a sense, they could transmit *Campylobacter* from external sources. These sources include the surrounding farmland or outdoor livestock like cattle and sheep, to other livestock such as pigs or chickens. A 1996 Swedish

study identified the presence of rodents as a risk factor for *Campylobacter* high prevalence in broiler flocks (Backhans & Fellström 2012). Rodents may also have a maintenance role in high *Campylobacter* prevalence on farms due to if they become infected from the livestock themselves. For example, *Campylobacter* (*C. fetus*) has been isolated from rats on a farm in 1967 where there had been an outbreak of Vibriosis in the pigs, which indicates that rodents could have a role in the transmission of this organism (Pejtschev 1969). This could have implications for public health as if rodents remain constantly infected, although they are not the original source of *Campylobacter*, then it can make eradication of the pathogen from the farm extremely difficult.

To study the prevalence of *Campylobacter* in rodents faecal samples were taken from either traps or from the rectum of the deceased rodents. *Campylobacter* is a fastidious organism and difficult to grow so the microbiological culture (Lynch et al. 2011; Davis and DiRita 2008), there this study included an enrichment step and growth on two specific mediums. The cultured bacteria were screened using an *lpx* gene PCR assay (Klena et al. 2004) which would confirm and differentiate between the two species, *C. jejuni* and *C. coli*. These species were screened for as they are the most clinically important species, accounting for 97% of human infections (Humphrey, O'Brien, and Madsen 2007). From the *C. jejuni* that was isolated a limited number were selected for whole-genome sequencing to extract multi-locus sequence typing (MLST) data to explore the diversity of *Campylobacter* carried by rodents. Antimicrobial resistance (AMR) is an important issue in veterinary and public health. Some of the *Campylobacter* strains could be a source of human infection that may require antibiotic treatment. Given the bacteria is in a wildlife host, it may indicate if some strains have been transmitted from other animal hosts, such as livestock species which are given antibiotics more routinely. Furthermore, antibiograms can be used as a crude method to distinguish between isolates.

The aim of this study was to investigate the carriage of *Campylobacter* species in rodents and determine to what extent the rodents on these farms could be contaminating the pork food chain. Results from this study could be used to inform on whether rodent control strategies could have an impact on reducing *Campylobacter* levels on farms. Reducing the introduction of *Campylobacter* at a farm level by reducing sources of livestock contamination, such as rodents, has been shown, together with other control measures, to have a positive effect on reducing the incidence of human *Campylobacter* infections.

6.2. Materials and Methods

6.2.1. Sample collection

Rodents were sampled between September 2014 and January 2016 in Northern England and Scotland (see Chapter 2). A total of 152 faecal samples from 5 different rodent species were collected. Small rodent traps were emptied every 24 hours and faecal samples were collected on charcoal swabs and refrigerated until they could be transported back to the research laboratory for microbiological culture. Once the rodent was removed from the trap, species, age and sex were noted, the trap was then cleaned with ethanol and cotton wool and reset. For brown rats, rectal swabs were taken after the animal was deceased, as the traps were made from wire mesh so it was not possible to take samples directly from the trap. Chapter 2 contains extensive details of this fieldwork and sample collection.

6.2.2. Microbiological growth

A 5% Exeter broth was made with 1100 ml distilled water and 27.5 g of Nutrient broth (Lab M, Lancashire, UK), 55 ml (5%) of lysed horse blood (Southern Group Laboratory, Corby, UK), SV59 (trimethoprim 10 mg/L, rifampicin 5 mg/L, polymyxin B 2500iu/L, cefoperazone 15 mg/L and amphotericin B 2 mg/L) and SV61 supplements (sodium pyruvate 250 mg/L, sodium metabisulphite 250 mg/L and ferrous sulphate 250 mg/L) (Mast Group Ltd, Bootle, UK). Swabs were transferred to 3ml aliquots of Exeter broth and incubated in microaerobic conditions (80% N_2, 12% CO_2, 5% O_2 and H_2 3%) for 48 hours (Davis and DiRita 2008). A 5µl loopful of the incubated broth was streaked onto *Campylobacter* Selective Agar (CCDA) LAB112 (Lab M, Lancashire, UK) that contained cefoperazone 32mg/L and amphotericin 10mg/L. The agar plate was incubated for a further 48 hours in microaerobic conditions and checked for growth of single colonies. Purified *Campylobacter* colonies were identified as silver with a metallic sheen and are often sticky in texture (Figure 6.2).

Figure 6.2: *C. jejuni* from a bank vole grown on CCDA over 48 hours in microaerobic conditions.

Single colonies were picked from the CCDA plate in quadruplicate and transferred to two agar plates comprising of Columbia Agar Base (CAB) LAB001 (Lab M, Lancashire, UK), with 5% defibrinated horse blood. Four picks were chosen as it is enough to represent the diversity of *Campylobacter* species that may be present and, from a practicality point of view, the most manageable when processing large numbers of samples for molecular screening. One plate was placed in microaerobic conditions and the other was placed in air at 37°C, both were incubated for 48 hours. *Campylobacter* spp on CAB are often grey and have a mucoid appearance on CAB plates (Figure 6.3). Colonies which grew on the CAB plates in aerobic conditions were discounted as likely to be an *Arcobacter* spp. Colonies which only grew in microaerobic conditions were used in the study or frozen down in cryovials at -80°C until required.

Figure 6.3: Four single colonies picked from the *C. jejuni* from a bank vole CAB agar plate and grown on blood agar after 48 hours in microaerobic conditions.

6.2.3. Chelex preparation

Bacterial DNA was prepared for PCR with Chelex 100 Molecular Grade Resin (BioRad, Deeside, UK). From the CAB agar plate, a 5 µl loopful was resuspended into an eppendorf containing 300 µl of Chelex and incubated for 10 minutes at 90°C in a heat block. This was then centrifuged at top speed (17 x *g*) for 2 minutes and 50 µl of the supernatant was diluted 1:10 450 µl of sterile water. This was stored at -20°C until required.

6.2.4. LPX PCR

Primer	Sequence (5' to 3')	Amplicon size (bp)	Reference
CjejlpxAF (Forward)	ACAACTTGGTGACGATGTTGTA	331	(Klena et al. 2004)
CcolilpxAF (Forward)	AGACAAATAAGAGAGAATCAG	391	
CjejlpxAR (Reverse)	CAATCATGDGCDATATGASAATAHGCCAT		

Table 6.1: Primer sequences for the LPX PCR (Klena et al. 2004)

This PCR targeted a fragment of the *lpxA* gene, which encodes the enzyme LpxA that catalyses the first step of lipid biosynthesis. The differences between these genes in *C. jejuni* and *C.coli* were exploited, as the PCR products from the LPX PCR are different sizes (331bp for *C. jejuni* and 391bp for *C.coli*), therefore it is possible to differentiate between the species (Klena et al. 2004). For the LPX PCR 4 µl per reaction of FIREPol 5x Master Mix with 12.5 $MgCl_2$ (Solis Biodyne, Tartu, Estonia), 16 µl per reaction of RNA free water and 1 µl of each forward and reverse primer at 10 pmol concentration (Table 6.1). For the *C. jejuni*, the forward primer CjejlpxAF and for *C. coli* forward primer CcolilpxAF were used. The same reverse primer was used for both species in each reaction (CjejlpxAR). Then 2 µl of DNA was added to each reaction to give a 25 µl reaction volume. The thermal profile for this PCR reaction was 94°C for 5 minutes then 30 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute followed by a final elongation at 72°C for 10 minutes. PCR products were electrophoresed on a 2% peq green agarose gel at 120V for 45 to 70 minutes depending on the size of the gel. Products were visualised under UV light and the differences in product size were used to determine species of *Campylobacter* (Figure 6.4).

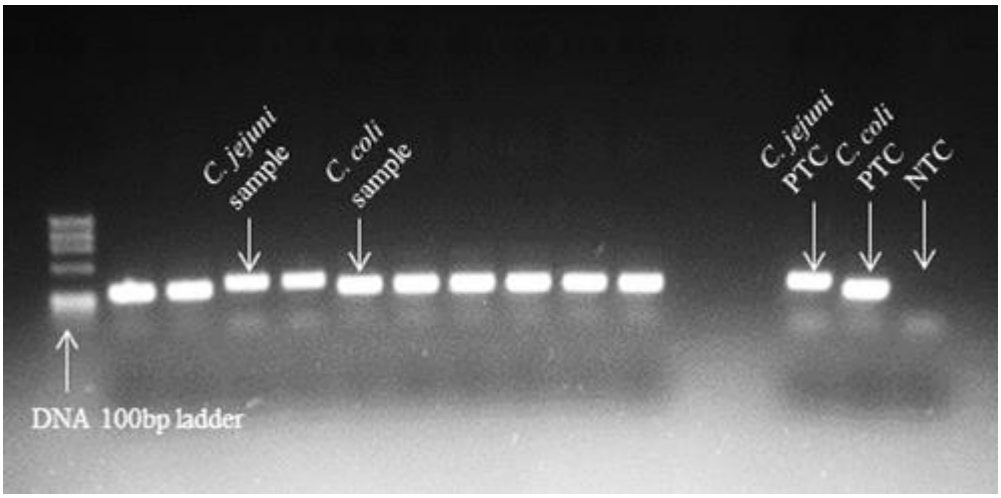

Figure 6.4: LPX PCR gel electrophoresis photograph showing the difference in product size between *C. jejuni* and *C. coli*. The positive controls (PTC) of both species and the negative control (NTC) are also shown.

6.2.5. Antimicrobial resistance (AMR) screening

A subset of *C. jejuni* isolates were screened for AMR susceptibility. A 5 µl loop full of pure *Campylobacter* colonies was taken from a CAB plate (figure 6.2) and used to make a suspension in 3 ml of sterile water (0.5 McFarland). A sterile cotton swab was soaked in this suspension and then evenly spread onto a Muellar-Hinton agar plate (Lab M, Lancashire, UK) containing 5% defibrilated horse blood and 1% Nicotinamide adenine dinucleotide (NAD). Antibiotic-impregnated discs (ciprofloxacin 10 µg, erythromycin 10 µg, doxycycline 30 µg and tetracycline 30 µg) were placed on the plates and incubated under microaerobic conditions for up to 48 hours. The diameter of the zones of inhibition was measured and assessed against the EUCAST clinical breakpoints to determine the AMR of the *C. jejuni* isolates.

6.2.6. DNA extraction and whole genome sequencing (WGS) of rodent *C. jejuni* isolates

A 5 µl loop full of *C. jejuni* 48 hour old colonies was taken from a CAB plate and resuspended in 1 ml of PBS solution. This was centrifuged at 3000 x g for 1 minute to pellet the bacterial cells and the supernatant was removed and the pellet was resuspended in ATL buffer by pulse vortexing. DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of the DNA was assessed by Nanodrop and Qubit, before being sent to the Centre for Genomic Research (CRG) at the University of Liverpool for whole genome sequencing (WGS).

Further analysis was undertaken on a subset of eleven rodent *C. jejuni* isolates for whole-genome sequencing using the method described by Jones et al 2017. Multilocus sequence typing (MLST) alleles were extracted from the WGS data and the sequence type for each rodent isolate inferred. The MLST allele sequences were obtained from the *Campylobacter* PubMLST website (https://pubmlst.org/campylobacter/) against each genome assembly using Bowtie2. For each locus, if the allele sequence aligned perfectly, then the sample was assigned to this allele (Jones et al. 2017). Sam Halenby conducted this analysis and provided the results.

6.3. Results

In total 152 rodent faecal samples were collected from brown rats (*R. norvegicus*, n= 26), house mice (*M. musculus*, n=60), wood mice (*A. sylvaticus*, n=24), bank voles (*M. glareolus*, n=41) and a field vole (*M. agrestis*, n=1).

6.3.1. Microbial culture and LPX PCR results

Species of rodent	Sample number	Positive Culture	Percentage positivity %	*C. jejuni*	*C. coli*	LPX negative
R. norvegicus	26	13	50 (95% CI 31-69%)	5	8	0
M. musculus	60	10	17 (95% CI 9-25%)	1	8	1
A. sylvaticus	24	3	12.5 (95% CI 11-37%)	1	0	2
M. glareolus	41	17	41 (95% CI 26-56%)	13	1	3
M. agrestis	1	0	0	n/a	n/a	n/a
Total	**152**	**43**	28 (95% CI 21-35%)	**20**	**17**	**6**

Table 6.2: Results of the microbiological culture and the LPX PCR (number of rodents which were positive for each Campylobacter species) screening of pig farm rodents.

The results from a microbiological culture and PCR confirmation are shown in Table 6.2 in which out of the total rodents sampled 28% (43/152, 95% CI 21-35%) were *Campylobacter* positive. This was determined initially by growth on CCDA and then subsequently CAB with typical *Campylobacter* morphology (Figures 1 and 2) as well as a lack of growth on CAB in aerobic conditions. Through LPX PCR 86% (37/43, 95% CI 76-96%) were shown to be either *C. jejuni* at 46% (20/43, 95% CI 31-61%) or *C. coli* at 40% (17/43, 95% CI 25-55%) and 14% (6/43, 95% CI 4-24%) of isolates were LPX negative. The rodent species with the highest percentage positivity, 50% (13/26, 95% CI 31-69%), was the brown rat (*R. norvegicus*) however there was almost an even split in the detection of *C. jejuni* (5/13) and *C. coli* (8/13). This was not observed in other species as in bank voles (*M. glareolus*), another species with a high prevalence of *Campylobacter* (41%, 17/41, 95% CI 26-56%), *C. jejuni* was found to be the most common species (13/17) and in house mice (*M. musculus*) the most common species was *C. coli* at 17% (8/10, 95% CI 9-25%). A low prevalence of Campylobacter was detected in wood mice (*A. sylvaticus*) with a prevalence of 12.5% (3/24, 95% CI 11-37%) and only one sample positive for *C. jejuni*. There was only one field vole faecal sample collected and this was negative for *Campylobacter* spp.

6.3.2. MLST of rodent *C. jejuni* isolates

Multilocus sequence typing (MLST) data was extracted from the whole genome sequencing (WGS) which was conducted on 11 rodent isolates. *C. jejuni* is the most clinically significant species in terms of human infections, therefore, the diversity of rodent *C. jejuni* strains detected in this study were explored through WGS MSLT.

ID	Species	Location	aspA	glnA	gltA	glyA	pgm	tkt	uncA	ST
R54A	*R. norvegicus*	Ripon	288	388	29	28	74	450	35	6561
R43A	*R. norvegicus*	Ripon	4	7	10	4	1	7	1	45
R79A	*R. norvegicus*	Wetherby	23	2	T3	91	T1	T2	51	P10
R91D	*R. norvegicus*	Cheshire	7	17	2	15	23	3	12	51
R92D	*R. norvegicus*	Cheshire	7	17	2	15	23	3	12	51
V34A	*M. glareolus*	Ripon	33	X	X	T14	X	X	X	Fail
V37A	*M. glareolus*	Ripon	227	297	253	338	424	337	250	3704
V50A	*M. glareolus*	Berwick	227	297	253	338	424	337	250	3704
V53	*M. glareolus*	Driffield	227	297	253	338	424	337	250	3704
V72B	*M. glareolus*	Malton	227	297	253	338	424	337	250	3704
M56	*A. sylvaticus*	Ripon	T16	186	2	62	257	223	6	P7

Table 6.3: Results of MLST screening showing the ST and alleles associated with results of 11 rodent *C. jejuni* isolates subject to whole genome sequencing. Novel alleles (T) and novel ST's (P) were identified.

MLST derived from WGS showed that all the isolates from bank voles (*M. glareolus*), apart from V34 where the sequence types could not be determined due to a lack of data for all alleles, were infected with *C. jejuni* sequence type (ST) 3704, a host-specific genotype of *C. jejuni* (Williams et al. 2010; Hepworth et al. 2011) . In contrast, multiple genotypes were detected in brown rats (*R. norvegicus*) with ST-6561, ST-45 and ST-51 found. Novel ST's were detected in one brown rat R79 and in one wood mouse M56 (*A. sylvaticus*), with new alleles also detected.

6.3.3. Antimicrobial resistance (AMR) screening

The same subset of *C. jejuni* rodent isolates subject to WGS MLST were also screened for AMR against the main antibiotic classes used in the therapeutic interventions for *Campylobacter* infection.

Isolate	Species	ST	Cip (10)	Doxy (30)	Tet (30)	Erythm (10)
R54	*R. norvegicus*	6561	S	S	S	S
R43	*R. norvegicus*	45	S	R	R	S
R79	*R. norvegicus*	P10	S	S	S	S
R91	*R. norvegicus*	51	S	R	R	S
R92	*R. norvegicus*	51	S	R	R	S
V34	*M. glareolus*	n/a	R	R	R	R
V37	*M. glareolus*	3704	S	S	S	S
V50	*M. glareolus*	3704	S	S	S	S
V53	*M. glareolus*	3704	S	S	S	S
V72	*M. glareolus*	3704	S	S	S	S
M56	*A. sylvaticus*	P7	S	S	S	S

Table 6.4: Antimicrobial resistance (AMR) data from the 11 *C. jejuni* rodent isolates. Whether an isolate is resistant was determined by the EUCAST breakpoints (EUCAST 2018) and shown as either susceptible (S) or resistant (R) in the results table.
Antibiotic codes; ciprofloxacin 10 mg (Cip 10), doxycycline 30 mg (Doxy 30), tetracycline 30 mg (Tet 30) and erythromycin 10 mg (Erythm 10)

Resistance to tetracyclines (tetracycline and doxycycline) was observed in isolates from 3/5 brown rats screened, which included ST-45 and ST-51. There was no resistance observed in strains ST-6561 and the novel strain form R79, as all isolates were susceptible to each class of antibiotic. There was also no AMR observed in the bank vole ST-3704 isolates, however multidrug resistance (MDR) (resistant ⩾3 classes of antibiotic) was observed in bank vole V34 for which no ST was assigned. No resistance was observed in the only wood mouse isolate, M56.

6.4 Discussion and Conclusion

6.4.1. Discussion

Overall *Campylobacter* spp was isolated from 43/152 (28%) of rodents, but the prevalence of *C. jejuni* and *C. coli* was different between rodent host species. The most frequently detected *Campylobacter* spp in house mice (*M. musculus*) was *C. coli*; 8/10 of isolates from mouse samples were *C. coli*. One possible reason could be that mice on these farms are becoming infected with *C. coli* by the pigs themselves. House mice reside almost exclusively indoors therefore, they have the most frequent interaction with the livestock, whether that be direct or indirect through contact with pig faeces. In this study, the sow and piglet area was often the part of the farms that had high levels of house mouse activity. The indoor habitation would also make it less likely that house mice would interact with external environmental sources of *Campylobacter*. Therefore, house mice could have a role of maintaining high levels of *C. coli* on pig farms by spreading the bacteria to other pigs or areas of the farm when the mice move between the buildings. For example another area of pig farms which there is high house mouse activity is the grain or pig food stores as another study of farm rodents (9 pig farms and 1 broiler farm) received 70% (58/83) of their house mice samples from the feed passage or storage area (Meerburg and Kijlstra 2007).

House mice which are infected and shedding *C. coli* in their faeces could contaminate the food which will, in turn, aid the transmission of *C. coli* to pigs and could maintain high levels in pork production. This could complicate measures to try and eradicate *C. coli* from the farm by disinfecting and restocking as if the mice still remain infected and shedding they could then reintroduce the bacterium to the new pigs in the herd. However, this study did not collect any isolates from the pigs so it cannot be stated for certain that the pigs are the source of *C. coli* in these mice, although the reported high prevalence in pigs in other studies (Varela, Friendship, and Dewey 2007) would make it highly likely that the pigs were the original source. A similar study found that there were no shared *Campylobacter* genotypes between the pigs and multiple rodent species, but it was not possible to exclude transmission between the two as pigs have been shown to carry multiple strains in a mixed infection (Meerburg and Kijlstra 2007).

The most common *Campylobacte*r species isolated from bank voles (*M. glareolus*), however, was *C. jejuni* (13/17). Bank voles rarely venture into the pig farm buildings and prefer hedgerow and woodland habitat, so are therefore less likely to have contact with the pigs or pig faeces than house mice, although one bank vole was infected with *C. coli*. The genotype of *C. jejuni* identified in all bank voles in this study (apart from V34) was ST-3704. This genotype was first identified in farm

and woodland study conducted in Cheshire in 2001 to 2005 and was shown to be common in bank voles, and only found rarely in sympatric rodents (1/655 wood mice) and cattle (1/497) (Williams et al. 2010). Furthermore, transmission of ST-3704 in a captive colony was also observed from ST-3704 positive parent bank voles to their offspring. As the F1 progeny remained ST-3704 positive, although these bank voles were fed an artificial diet and there was no environmental exposure to ST-3704. This could indicate that this genotype can be naturally maintained by the bank vole host (Williams et al. 2010). Thus, it appears that *C. jejuni* ST-3704, which in this study was detected over a wide geographical range (Northumberland, Ripon, Malton and Driffield), is the main genotype throughout the British bank vole population.

The wider host range and zoonotic potential of ST-3704 is unclear and there have not been any reported human infections due to this genotype. A Finnish study (Llarena et al. 2015) found that there several deletions in the genomic regions of ST-3704 which are usually present in *C. jejuni* genotypes isolated from human infections, along with several deleted regions in the genes required for colonization of the chicken gut and other virulence factors. Furthermore, ST-3704 was unable to colonize in the gut of 21-day-old chickens unlike other genotypes (Hepworth et al. 2011). The lack of AMR observed in ST-3704 might reflect less antibiotic exposure among wild bank voles, and therefore the AMR reported in other rodent GIT bacteria, such as *E. coli* in bank voles (Williams et al. 2011), may reflect selection elsewhere than in bank voles. Thus, the zoonotic potential of ST-3704 may be low and therefore although there was high prevalence in bank voles, there is unlikely to be a high public health risk.

Both *C. jejuni* (5/13) and *C. coli* (8/13) were detected in brown rats (*R. norvegicus*). While no further tying of *C. coli* was undertaken, the MLST of the *C. jejuni* isolates demonstrated a diversity of genotypes, even from the same site. This suggests that unlike bank voles, rats do not support an endemic host-adapted *Campylobacter*. Rather infection may reflect the ecology and behaviour of this rodent species. Rats are a highly adaptable and versatile species that have an extremely varied diet that allows them to exploit the environment they are in with a high level of success (Harris and Yalden 2008). Rats are a common sight on pig farms, probably because of the wide range of sites to establish nests and readily available food. For example, in this study, it was observed that pig farms that had a mill on site to produce their own pig food often had large populations of rats. Hence frequent contact (direct or indirect) between the rats and the livestock, including faecal contamination from both species that could facilitate the transmission of *Campylobacter* between the two. This could explain

why 8/13 of *Campylobacter* spp isolated from rats was *C. coli*, so like house mice they could have a role in the maintenance of *C. coli* levels in pigs.

However, unlike house mice, rats are have been known to venture greater distances into the surrounding the environment, although journeys are likely to be smaller if there is a stable food source present (Harris and Yalden 2008). This means that rats could potentially move *C. coli* from the original source to other locations, such as neighbouring pig farms or the surrounding environment. The roaming nature of rats could explain the presence of *C. jejuni* in 5/13 of *Campylobacter* isolates from brown rats. A Danish study found pigs are capable of carrying mixed infections of *C. coli* and *C. jejuni*, therefore pigs cannot be excluded as a possible source of *C. jejuni* in these rats. However, the same study detected that there was a higher *C. jejuni* prevalence in pigs when cattle were kept at the same location and it was implied that the open-air traditional method of cattle farming may contribute to transmission of the bacterium (Boes et al. 2005). Rats could be seen as an infection risk in contaminating the pigs with *C. jejuni* from other environmental sources, such as cattle. Therefore, rats could have a dual role in maintaining the *Campylobacter* in levels in herds as well as a source of possible contamination through the introduction of *Campylobacter* from external sources.

The sequence types of *C. jejuni* from brown rats have shown, unlike those isolated from bank voles, that there is a large degree of diversity of genotypes that can be carried by rats. This has also be shown in other studies as rats were shown to be capable of carrying multiple genotypes, including novel variants such as ST-5129, ST-5130 and those detected in this study in one rat (R79). Rats have also been shown to carry generalists strains, such as genotype ST-586 which has been associated with infections in cattle, chickens and humans (Stuart et al. 2011).

In this study, one pig farm in Ripon (Pig farm 1) contained rats that were carrying different genotypes of *Campylobacter*. One rat (R54) was shown to be carrying *C. jejuni* ST-6561 is thought to be genotype found only in rats and the zoonotic potential of this genotype is unclear. Another rat from the same location (R43) was infected with *C. jejuni* ST-45. This sequence type has previously been identified in human disease, chicken flocks, chicken meat, beef offal and in the environment (Dingle et al. 2002). A Finnish study found that ST-45 was the dominant genotype in chicken flocks as every third isolate in a 380 isolate study between 2004-2012 was shown to be this genotype (Llarena et al. 2015). ST-45 is also considered a generalist genotype and able to colonise the GIT of many different species leading to a high rate of transfer between species. The writers of this study speculated that it was likely that the introduction of this bacteria into Finnish chicken flocks, as it was not seen every

year, could be a result of environmental contamination by farm workers, insect vectors such as flies or rodents (Llarena et al. 2015; Dearlove et al. 2016). The generalist properties of ST-45 could explain why this genotype has been able to colonise in the gut of a brown rat. However, ST-45 has not been associated or detected in pigs or pig meat so pigs are unlikely to the source of this genotype. Two rats from a garden in Cheshire were found to carry *C. jejuni* ST-51, which has previously been isolated from patients, hospitalised with enteritis and commercial chicken flocks (Oh et al. 2017). The ability of many different strains of *C. jejuni* to colonize the rat gut suggests that rats are competent reservoir hosts for this bacterium and have the potential to become infected and transmit many strains, some of which have been shown to be causes of human illness.

Other rodent species, such as the wood mouse were shown to have a low prevalence of *Campylobacter* with 12.5% (3/24) positive for *Campylobacter* spp, with one *C. jejuni* identified. This is an interesting finding as wood mice in habitat similar surroundings to bank voles and venture into buildings so could have contact with livestock. The one field vole sample was negative for *Campylobacter* spp by LPX PCR, although one sample cannot be used as a representative for the *Campylobacter* carriage for this species. However, it can be argued that these species may be less significant in the transmission or maintenance of *Campylobacter* than other rodent species such as brown rats or house mice, therefore the zoonotic potential may be low.

6.4.2. Conclusion

This study has confirmed the presence of both *C. jejuni* and *C. coli* in the faecal matter produced by a variety of rodent species that dwell on and around pig farms in the UK. Different rodent species may be more important in terms of transmission or contamination of the food chain. Therefore, species such as wood mice, field voles and bank voles may have a low importance as *Campylobacter* prevalence may be low in these species or they may be infected with a strain that has low public health significance, such as ST-3704 in bank voles. House mice, which reside almost exclusively in pig farm buildings, could have a role in the maintenance of high *C. coli* prevalence in the pig herd due to the bias toward *C. coli* in this species. Brown rats may have the most important contribution to *Campylobacter* carriage due to the identification of both *C. jejuni* and *C. coli*, which could indicate that they have a role in the maintenance of *Campylobacter* prevalence in pig herds and contamination introduced from other external sources. Rats were also shown to be a reservoir host for a diverse range of *C. jejuni* strains (ST-45, ST-51 and ST-6561), some of which have been shown to cause human illness. The findings have highlighted the importance to biosecurity and the need for efficient

rodent control, which if successful may help reduce the amount of *Campylobacter* introduced into the food chain by rodents if applied in conjunction with other control measures.

Chapter Seven: General Discussion

7.1. General Discussion

The aim of this project was to investigate the prevalence and diversity of zoonotic pathogens that may be a potential risk to public health in the British pre-domestic rodent populations. Rodents are important animals in terms of public health, as they have more interaction with humans than many other mammals, therefore, transfer of zoonotic pathogens at this human-animal interface is possible. Rodents also have the potential to transport disease great geographical distances with no confinement to defined borders, as 1937 US epidemiologist Frank G. Boudreau proclaimed in his article "*microbes know no frontiers*" (Knab 2011), in which he used an eye-catching illustration of a rat. This rat symbolised several aspects of infectious diseases, such as the ease at which non-human carriers can cross borders (physical and political), the importance of animals in the spread of disease (plague-infected rats sailing on ships) and the interaction these animals have with people (Knab 2011).

This study focused on four viral and one bacterial infection, all of which are potentially zoonotic, in wild rodents. Rodent samples were collected from a wide range of peri-domestic locations across the UK, which included pig farms, rural and urban areas. Seoul virus (SEOV) was detected in 13% (13/68) rats, and rat hepatitis E virus (rat HEV) in 13% (8/61) rats from Yorkshire and Cheshire, but in no other species tested. Another hantavirus, Tatenale virus (TATV), was detected in 7/23 (30%) of field voles from one site in North Wales. LCMV was detected in a wider range of rodent species, largely in house mice 17.5% (21/120), but also in rats (3.2%, 2/61) wood mice (2%, 1/49) and bank voles (4%, 2/50). *Campylobacter* was detected in 28% (43/152) of rodents overall, of which 86% (37/43) isolates were shown to be either *C. jejuni* (20/43, 46%) or *C. coli* (17/43, 40%).

For several of the pathogens, observations of bias were made, that were shown to be statistically non-significant. The sex and age bias observed in the detection of rat HEV with both sex (P= 0.2387, 95% CI 0.5- 216.5) and age (P= 1, 95% CI 0.2-14.4) biases were not statistically significant. This may be due to the small sample size collected and highlights one of the problems in conducting wildlife surveillance studies as a large sample size is not guaranteed. Trapping rodents is expensive and often highly time-consuming work. It was also not possible to accurately determine the sample size required as there was no prevalence data available for these pathogens. Rat HEV had previously not been detected in the UK, therefore it was not possible to conduct sample size calculations before the fieldwork took place. The results indicate that the prevalence of SEOV in wild rats is 13% (8/61, 95% CI 5-23), therefore if this study was to be repeated a sample size of 174 rats would be required. However, the practicalities of obtaining such high numbers are extremely difficult.

The screening in this study was primarily conducted using molecular methods that were previously published and genus-specific pan-RT-PCR assays which could detect a variety of viral species within a particular genus. The pan-hanta RT-PCR could amplify a region on the L-segment of multiple hantavirus species or the pan-HEV PCR which could amplify the ORF-1 region of multiple species in the *Orthohepevirus* genus. The optimisation of these methods outlined in this project can be used to accurately and reliably classify pathogens in future outbreaks. Rodent tissue of known infection status from this project can be used as a control material in these assays. These molecular techniques would provide rapid and efficient diagnostic screening that could be also an effective strategy to prevent future emerging zoonotic infections through early identification (Morse et al. 2012). The advantage of using a previously published, and in some cases widely used, assays is that there is a large amount of sequencing data available on Genbank to make a comparison. This allows quick identification of an unknown agent through the sequencing of a PCR product in the case of an outbreak.

However, although pan-RT-PCR assays are extremely useful for rapid diagnostic purposes, they may not be as useful in evolutionary studies when commenting on phylogenetic ancestry. In the case of SEOV, a 329 nt partial L-segment amplicon was used to construct a Maximum Likelihood tree in MEGA (Kumar, Stecher, and Tamura 2016) with published sequences of the same conserved region (Figure 3.4). Therefore, there is a limited amount of conclusions can be made other than the confirmation of species and viral lineage. The SEOV tree, along with several other generated in this study, also contained branches with low bootstrap values (<70) which could reduce the confidence in the interpretation of the tree. The use of other models may address this, such as the Bayesian method and may be more appropriate to use if further extensive phylogenetic analysis was to be conducted. Further work, such as the amplification of other regions of the virus, would be needed to confidently comment on phylogenetic ancestry and could be useful in determining the genome. Material form this project is currently being analysed to produce the full genome of TATV to allow an application to be made to ICTV for species status, as this virus is not yet recognised as a hantavirus species.

There are also limitations to relying solely on molecular methods alone in human outbreaks, as they require the presence of viral RNA or DNA. In animal studies, this is less complicated, as organ tissue can be harvested and screened for viral RNA once the animal has been euthanised, which is not possible in cases of human disease. If a person has clinical HFRS suspected to be due to SEOV infection, for example, they would have to be in the viraemic phase of the disease and have viral

RNA in their blood for the pan-RT PCR to detect the infection. Therefore serology, such as an IgG or IgM ELISA, should be used in conjunction with the molecular methods to make an accurate diagnosis (Kruger et al. 2015). In the LCMV study of this project serological methods in addition to the molecular assay were used to screen house mice for LCMV, as there were some mice in which LCMV could not be detected by PCR alone.

Co-infection with multiple zoonotic viruses was observed in this study. In Pig Farm 1 three parasites (SEOV, LCMV and rat HEV) were in brown rats at this location, R43 was co-infected with both rat HEV and LCMV. Co-infections with other pathogens such as bacteria, protozoans, enteric macroparasites, etc that be likely present in wild rodents. For example, the non-zoonotic tapeworm (*Taenia polyacantha*) was found in two voles (see Appendix VI). Nevertheless, this is a rare investigation and co-infection was encountered. Infection with multiple agents, such as parasites has been shown to alter the susceptibility, both positively and negatively, of a host to new infections. Parasites, although they might cause mortality directly, may have a negative impact on the overall success of a population, for example, parasitic infection could impair body condition (such as reduction in growth rate) or exacerbate current threats to hosts (such as winter survival) (Stringer and Linklater 2014). In field voles, infection with *Babesia microti* decreased the susceptibility of the vole to *Bartonella spp*, whereas infection with cowpox virus (CPV) consistently increased host susceptibility to parasitic infections (Telfer et al. 2010).

Additional factors, as well as co-infection, could affect the susceptibility of a rodent host to infection with the zoonotic pathogens included in this study. In the case of SEOV in brown rats, older male rats are more likely to be infected with SEOV than their younger counterparts (Hinson et al. 2004). Age could also be a risk factor for acquiring parasitic co-infections that may increase the susceptibility to other infectious agents. In saiga antelopes (*Saiga tatarica*) the intensity of a parasitic nematode, *Marshallagia* spp, has been shown to increase with age (Coulson et al. 2018). In a male-biased polygamous mating systems, as many mammalian systems are, the males are often more heavily parasitised than females (Moore and Wilson 2002), which could reduce reproductive success and predispose males to infection with other agents. However, in this study, there was inevitability limited by the range of pathogens able to be screened, so others, including parasites, would have been missed, therefore it is not possible to comment on whether parasitic burden had a negative or positive effect on infection predisposition in these rodents.

Therefore, another question would be whether co-infection of rodents would present a greater public health risk to people? Simultaneous infection with Dobrava-Belgrade virus (DOBV), *Leptospira* spp and *Babesia* spp in 11% *A. flavicollis* in Croatia have been reported and it is thought that co-infected rodents present a greater possibility that more than one infection could be transmitted to humans resulting in disease (Tadin et al. 2012). Potentially, there is a higher shedding rate of one or both pathogens in co-infected animals so transmission is more probable. This may be more likely if the pathogens in question share the same transmission route, for example, one rat was infected with SEOV, *Campylobacter* spp and rat HEV, which can all be transmitted to humans through faeces. Therefore, contact with faeces from that rat could potentially result in an infection with three zoonotic pathogens instead of one. Several rodent zoonoses have similar clinical manifestations in humans, such as SEOV can often be misdiagnosed as an infection with the rodent zoonotic pathogen *Leptospira* spp (Izurieta, Galwankar, and Clem 2008). Multiple infections in humans can make reaching the correct diagnoses difficult, and misdiagnosis can result in delivery of the incorrect treatment and prolong illness (Tadin et al. 2012).

Two farms which were identified as containing multiple pathogens in the rats sampled (Pig farm 1 and Pig farm 9) were observed to have evidence of a large resident rat population. This raises the question as to whether large populations of rats are more capable of maintaining multiple zoonotic agents and is transmission to other rats more probable? This would be likely as SEOV, LCMV and rat HEV are transmitted to other members of the population through a shared contaminated environment. Mathematical modeling, such as those outlined by Allen et al (2011), may be required to comment further on whether greater population densities may make the distribution of zoonotic pathogens, and thus transmission to people, more likely. Although transmission dynamics of these pathogens in rodent populations were not investigated in this study, this would be an interesting area to pursue in the future.

It is also unclear whether there would be any fitness cost to the rodent host due to infection with zoonotic agents. Zoonotic pathogens are thought to have co-evolved with their natural reservoir host so are able to co-exist without disease occurrence (Bean et al. 2013). For example, there is little pathology observed in bats which are infected with Ebola virus compared to the severe disease seen in humans (Bean et al. 2013). When hantaviruses infect their rodent reservoir host species the virus interacts with the rodent host immune system to dampen down the proinflammatory response and thus the rodent does not succumb to immune-mediated pathology. The exact mechanism for this is still unclear (Mandl et al. 2015). Although in the case of rat HEV there have been adverse effects

reported in the host species, such as a parenchymal foci of necrosis associated mild hepatitis reported in laboratory rats infected with rat HEV (Purcell et al. 2011) and the inflammation and lesions observed in R5 in this study. This could infer that there may indeed be a cost to the host due to an infection, even if the rodent host is the natural reservoir for the virus. It is also unclear on whether a reduced host fitness would have any impact on pathogen transmission, to rodents or humans.

In this study LCMV was the only viral pathogen in which RNA was detected in multiple rodent species as no RNA was detected in non-host rodents for SEOV, TATV or HEV. In the case of rat HEV, there have been reports of infection outside the host genus (*Rattus*) (Spahr et al. 2017; Guan et al. 2013) although there is very little knowledge of other cross-species infections in other mammals. For example, could rat HEV infect pigs and would this cross-species infection be advantageous? Could exposure to rat HEV generate any protection against infection with HEV G3 in pigs? If so would this naturally acquired immunity be effective in reducing the rate of infection in the UK pig herd? However, there is not enough known about the cross-species transmission of rat HEV. One study suggests that pigs are not susceptible to rat HEV infection, as when 6-week old pigs were inoculated intravenously with the US rat HEV strain no seroconversion, viraemia or faecal shedding was observed (Cossaboom et al. 2012). Although in the Cossaboom et al (2008) study, infection was intravenously induced which differs from the faeco-oral route, which would occur naturally. Cossaboom et al 2012 and Purcell et al 2011, the latter who inoculated rhesus monkeys with rat HEV, both used the US rat HEV strain in both studies and neither recorded any response in a non-rat species. No cross-species infection has been attempted with European rat HEV strains, which are closely related to the strains detected in this study. Therefore different strains may have varying degrees of pathogenicity and ability to infect more than one species. If porcine infection is possible or if there is any rat HEV carriage currently circulating in the British pig herd remains unknown. Further study of British pigs would be required to determine whether this was possible and if rat HEV would provide any advantageous natural immunity.

The identification of zoonotic pathogens that could present a potential public health concern circulating in rodents has also highlighted there are several gaps in the knowledge in terms of human prevalence. For example, the detection of multiple pathogens on pig farms could indicate that there may be certain groups of people, in this case pig farmers, more likely to be at risk of infection than others. In the case of SEOV, there could be an occupational risk associated with living and working on pig farms. This study shows, through the detection of SEOV positive rats on multiple pig farms, the 2012 human clinical case of SEOV-HFRS (Jameson et al. 2013) was not an isolated incident. In

this study, there was also no investigation into the current human infection of the individuals who lived or worked at these establishments, as this was outside the remit of this study. Presently the results from this study are not enough to conclusively correlate human SEOV infection and pig farming or confirm if the zoonotic transmission had occurred at these locations.

To investigate if pig farm workers are more at risk of infection than other occupations a serosurvey study, like the study conducted by Duggan et al (2017), could be conducted. This study should also include other pathogens as well as SEOV, such as rat HEV or LCMV, that were detected on pig farms in this project. The pig farms in this study (77%) were mostly located in Yorkshire, therefore other regions of the UK with high numbers of pig farms, such as Norfolk or Whilture could be included and compared. Non-pig farming professions that may have exposure to wild rodents through their occupation could also be investigated such as sewage workers, forestry workers or pest controllers (as well as negative controls). The occupational risk in certain professions has already been reported, such as in Germany; forestry workers, muskrat hunters and horse farm employees have been identified as 2.2-6.2 times more at risk of hantavirus infection than the general population based on the seroprevalence in those groups (Zöller et al. 1995). German forestry workers have also been identified as being highly seropositive to rat HEV (Dremsek et al. 2012). Farming has been linked to an increased risk of PUUV infection in the Northern and central regions of Sweden (Ahlm et al. 1998). Further monitoring and risk analysis would also be required to confirm if there is an increased occupational risk associated with pig farming, or other professions, and human infection.

The findings from this project could be seen as justification to conduct surveillance and investigate human infection in the wider population, as currently, there is no prevalence data available for LCMV, TATV or rat HEV. One reason for the current lack of human prevalence data is that there is no routine screening conducted for these pathogens in the UK. In the case of LCMV, in healthy individual's infection is asymptomatic and therefore goes underreported or unrecognised, so it could be argued that LCMV is not a public health concern so routine screening is not required. Although, in the UK, 60% of viral encephalitis cases, of which LCMV can cause, remain unexplained (Kennedy et al. 2017). This study showed that there were several closely related LCMV strains circulating in British rodents, however, it is not clear if these strains would be all capable of infecting or causing disease in people. Without prevalence data for human LCMV infection in the UK it is not possible to comment further or make comparisons between human and rodent strains of LCMV. This makes the public health implications of LCMV difficult to determine. Screening for LCMV may be beneficial to public health in the prevention of future outbreaks, for example, if LCMV screening

was introduced before organ transplantation this would prevent further fatal consequences, like those seen in the USA (Fischer et al. 2006). A seropositivity study investigating human infection in non-clinical individuals may be useful in assessing human prevalence of these rodent zoonotic pathogens. A study was conducted in 2013 to assess the seroprevalence of SEOV in different groups of people who may have contact with rats (pet owners, farmers, pest control workers and a control group) (Duggan et al. 2017), therefore conducting a study, with a similar methodology, may be useful investigating human infection.

In the case of HEV, there is large scale screening conducted of blood products (PHE 2018b) however there is no available prevalence data for rat HEV in people. The current assays used for human HEV screening would not detect the presence of rat HEV as the Wantaii ELISA is only designed for *Orthohepevirus A* (Genotypes1-4) (Trémeaux et al. 2016). As rat HEV has only recently been identified as a zoonotic pathogen (HKU Med 2018) there is no prevalence data available for UK human infection. Therefore a review of uncharacterised viral hepatitis cases using tests capable of detecting rat HEV, such as the pan-HEV ORF 1 PCR assay used in this study, may be warranted to determine the true prevalence of this virus in humans. The application of specific serological assays, such as those used to detect rat HEV seroprevalence in forestry workers in Germany (Dremsek et al. 2012), could also be usual in determining the prevalence of rat HEV in UK people. Development of specific serological assays could also be useful in increasing the knowledge of the pathogen. For example in the case of hantavirus-specific serological assays, that are able to differentiate between vole hantavirus (TATV/PUUV), as there is cross-reactivity reported (Pounder et al. 2013; Duggan et al. 2017). If specific TATV assays were developed this could not only be used to assess human infection prevalence but also to determine whether this TATV is zoonotic, as currently, the zoonotic potential is unknown. If TATV produces similar mild HFRS, similar to other vole hantaviruses such as TULV or PUUV, then these assays could be applied to clinical cases of acute kidney injury where the cause is unexplained.

In the case of SEOV, there is no specific treatment, with several antivirals still being studied as possible candidates, supportive therapy such as continuous renal replacement therapy (CRRT) are used (Jiang et al. 2016). Therefore, development of a vaccine may be beneficial. A hantavirus vaccine, against non-UK hantaviruses HNTV and PUUV, has already entered Phase I clinical trials in the USA (Hooper et al. 2014). However, it can be argued that research and development of a SEOV vaccine may not be of significant benefit to public health in the UK due to the condition's rarity, although they can be serious to the individual, of human illness related to infection with a rodent

zoonosis. In the case of SEOV, there are have been 48 clinical HFRS reported cases since 1977 in the UK (McElhinney et al. 2017), compared to China, which accounts for 90% of the world's HFRS cases, there have been 1.4 million cases including 45,000 deaths between 1950 and 2010 (Tian et al. 2018). Therefore, the development of SEOV vaccine may be beneficial to public health in China but not as useful in the UK. Infectious diseases are still a significant burden to the health service and the economy with an estimated cost of 30 billion annually (which includes the cost to the health service, days lost in work and to the individual) (Parliamentary Office of Science 2017). Any wider scale surveillance work in people would incur a substantial financial cost, therefore, the pathogen in question must be of significance to public health. Other infectious diseases are much more common such as measles which there were 547 UK cases in 2016 (PHE 2017) and HIV of which there are 101,200 UK people are currently infected (PHE 2016). So it could be argued that it would be more beneficial to direct funds towards the screening and researching diseases which are more clinically and economically relevant to public health in the UK today.

Reducing the number of rodents through effective rodent control may also reduce the risk of transmission of these pathogens from rodents to humans. This may be important in terms of public health as certain pathogens, such as SEOV and LCMV, are capable of resulting in human infection through the contamination of the environment as an aerosolised virus in rodent excretions are the most common route of human infection. Others could enter the food chain, such as *Campylobacter* or rat HEV and people potentially become infected through consumption of contaminated food. Increased rodent control at a farm level was one of the control measures introduced in the poultry industry by the New Zealand Food Safety Agency (NZFSA) in 2006 credited with reducing the incidence of campylobacteriosis in people (Lane and Briggs 2014). There were also several other control measures such as improved processing practices, the introduction of leak-proof packaging and greater consumer awareness were also used to reduce *Campylobacter* infection (Sears et al. 2011).

Although the practicalities of implementing rodent control are not always straightforward. This project has certainly demonstrated the difficulties in trapping rodents, such as neophobia, even in locations where there are large numbers, so removal of an established rodent colony could be problematic. Although the effectivity of pest control programs that rely solely on poisons may be compromised due to the rise of resistance to anticoagulants. Many UK house mice are now resistant to first generation anticoagulants such as warfarin and widespread VKORC1 mutations in brown rats which has lead to resistance to commonly used poisons (Buckle et al. 2018). In New York City a

successful pilot study was trialed by using birth control in bait stations to reduce rat populations as an alternative to poisoning (Filippino 2018). Improving biosecurity measures may also help to reduce the risk of infection. In the case of pig farm workers, for example, for aerosolised viruses, wearing appropriate Personal Protective Equipment (PPE) such as facemasks and gloves when cleaning out livestock areas or emptying rodent traps, could be recommended. As well as avoidance of stirring up dust and proper ventilation of rooms where there are rodents present (Krüger, Ulrich, and Lundkvist 2001). For faeco-oral transmitted pathogens effective handwashing stations should be available for all workers, and adequate disinfection practices. Keeping the farm clean and tidy with animal feed stored in vermin-proof containers may discourage rodents from venturing into buildings from the surrounding farmland. Improving public awareness and an understanding of what pathogens are carried by rodents and how they are transmitted to humans could also help prevent future outbreaks.

7.2. Conclusion and future work

This project has proved that there are zoonotic pathogens circulating in the wild rodent population that could be hazardous to human health. It has also highlighted gaps in our current knowledge, such as the unknown zoonotic potential of some pathogens such as rat HEV. In order to comment on the significance of a pathogen to public health, the zoonotic potential must be known. Specific assays, such as those used in Germany, which are able to detect rat HEV antibodies could be used to confirm this. Clinical human acute viral hepatitis, were they are HEV G1-4 negative, could also be screened for rat HEV. Specific assays for TATV antibodies could be developed and also be used to confirm the zoonotic potential of this virus. Prevalence of these viruses in people remains unknown. In the case of LCMV, there is no human prevalence data available for the UK. There may be differences in the pathogenicity of LCMV strains, therefore, a comparison should be made between people and rodents to investigate if the strains in this project are infectious to humans. This project has also indicated that there could be possible occupational risks and geographical hot spots for rodent zoonosis, with a SEOV, detected on multiple pig farms in Yorkshire, although further investigation would be needed to confirm this. Further rodent surveillance, using the trapping methods in this project, could be used to survey rodents from other regions of the UK then use mathematical modeling to determine the risks. This could aid in the prevention of possible outbreaks through improvement of biosecurity, pest control as well as raising public awareness could reduce the risk of exposure and be beneficial to public health in the future.

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Appendix I

Veterinary Research Ethics Committee

Committee Chairman
Carol Gray
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Dear Malcolm

I am pleased to inform you that the Veterinary Research Ethics Committee has approved your application for ethical approval. Details of the approval can be found below.

Ref:	VREC267
PI:	Malcolm Bennett
Title:	Zoonotic viruses of peridomestic rodents
School:	School of Veterinary Science
Department:	Epidemiology and Population Health
First Reviewer:	Diana Williams
Second Reviewer:	Dai Grove-White
Date of initial review:	27.11.14
Date of Approval:	29.11.14

This approval applies for the duration of the research. If it is proposed to extend the duration of the study as specified in the application form, the Veterinary Research Ethics Committee should be notified. If it is proposed to make an amendment to the research, you should notify the Veterinary Research Ethics Committee by following the Notice of Amendment procedure outlined at http://www.liv.ac.uk/researchethics/application/forms_and_templates/.

If the named PI / Supervisor leaves the employment of the University during the course of this approval, the approval will lapse. Therefore please contact the RGO at ethics@liverpool.ac.uk in order to notify them of a change in PI / Supervisor.

All serious adverse events must be reported to the Committee within 24 hours of their occurrence, via the Research Governance Office (ethics@liv.ac.uk)

With best wishes

Carol Gray, Chair, Veterinary Research Ethics Committee

A member of the
Russell Group

Figure I.1. Letter of ethical approval for this project by the Veterinary Research Ethics Committee.

Figure I.2. Advertising flyer sent to pig farmers to recruit them for the pig farm rodent study.

UNIVERSITY OF LIVERPOOL

Committee on Research Ethics

PARTICIPANT CONSENT FORM

Title of Research Project:
Zoonotic Pathogens of Peri-domestic rodents.

Researcher(s): Ellen Murphy, Nicola Williams, Malcolm Bennett, Julian Chantrey and Lorraine McElhinney.

Please initial box

1. I confirm that I have read and have understood the information sheet dated for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. In addition, should I not wish to answer any particular question or questions, I am free to decline. ☐

3. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. ☐

4. I agree to take part in the above study. ☐

Participant Name	Date	Signature
Ellen Murphy		
Name of Person taking consent	Date	Signature
Ellen Murphy		
Researcher	Date	Signature

Figure I.3. Consent form given to the participants of this study.

Appendix II Trapping itinerary

Day	
1 **(Sunday)**	Collect all traps and bedding material from the autoclave. Travel to the pig farm location. Obtain consent to contact the study (Appendix III) and discuss rodent activity with the establishment owner. Walk round the whole site to determine the wear traps can be placed . Set all traps if possible. **Biosecurity and safety:** Wellies and overalls to be worn when accessing the farm. Dip feet in disinfectant when arriving and leaving the farm. Gloves must be worn. It may not safe to set snap traps on all sites.
2-4 **(Monday, Tuesday and** **Wednesday)**	Check traps. Remove any rodents form traps and record the species, date, sex and age of each rodent as well as assigning a unique number. Schedule 1 cull rodents using isoflurane and cerebral dislocation. Take cardiac blood samples, centrifuge whole blood to collect serum then freeze down. Freeze rodent bodies in mobile freezer for temporary storage at -18°C. Charcoal swabs are used to collect faces from the traps for campylobacter screening. Clean each trap with ethanol on cotton wool then refill with fresh sterile hay and feed. Rebate rat traps if necessary. **Biosecurity and safety:** Wellies and overalls to be worn when accessing the farm. Dip feet in disinfectant when arriving and leaving the farm. Gloves must be worn when handling rodents or traps. Place needles in sharps bin. Isoflurane cotton wool should be placed in a sealed waste bag.
5 **(Thursday)**	Repeat the same procedure as previous days. Collect all traps and empty hay and feed into bio hazard bag and seal with a cable tie. Drive back to Leahurst campus. Store all rodents at -80°C ready for post mortem. Store serum at -20°C. Transfer faecal from swabs into Exeter broth and place microaerophilic conditions. **Biosecurity and safety:** Wellies and overalls to be worn when accessing the farm . Dip feet in disinfectant when arriving and leaving the farm. Gloves must be worn when handling rodents or traps. Place needles in sharps bin. Isoflurane cotton wool should be placed in a sealed waste bag.
6 **(Friday)**	**Biosecurity and safety:** Clean traps with 10% distel and prepare them for autoclaving with fresh bedding and feed. Wash overalls and trapping clothes. Autoclave overalls. Disinfect wellies with FAM. Clean car wheels with FAM. Repack the car to travel to the next pig farm.

Figure II.1. Trapping protocol used during the pig farm part of the study.

Appendix III results tables of rodent samples

Sample number	Site	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
R1	Farm 1	M	A	397	-	-	-	+	nt
R2	Farm 1	M	A	444	-	-	-	-	nt
R3	Farm 1	M	A	446	-	-	-	+	nt
R4	Farm 1	M	A	261	-	-	-	-	nt
R5	Farm 1	F	A	264	-	-	-	+	nt
R6	Rural 1	F	A	154	-	-	-	-	nt
R7	Farm 4	F	A	277	-	-	nt	nt	nt
R8	Farm 4	F	A	207	-	-	nt	nt	nt
R9	Farm 4	F	A	500	-	-	nt	nt	nt
R10	Farm 4	M	A	468	-	-	nt	nt	nt
R11	Farm 4	M	A	349	-	-	nt	nt	nt
R12	Farm 4	M	A	489	-	-	nt	nt	nt
R21	Urban 1	F	A	275	-	-	-	-	nt
R22	Urban 1	F	A	265	-	-	-	-	nt
R23	Urban 1	F	A	233	-	-	-	-	nt
R24	Urban 1	F	A	405	-	-	nt	nt	nt
R33	Farm 2	F	J	111	-	-	-	-	nt
R39	Pig Farm 1	F	A	303	-	-	-	-	nt
R40	Pig Farm 1	M	A	354	-	-	-	-	nt
R41	Pig Farm 1	M	A	369	-	-	-	-	nt
R42	Pig Farm 1	M	A	450	-	-	+	-	nt
R43	Pig Farm 1	F	A	532	-	-	+	+	C.J.
R44	Pig Farm 1	M	J	100	-	-	-	-	nt

R45	Pig Farm 1	M	J	102	-	-	-	+	nt
R46	Pig Farm 1	M	A	478	-	-	-	-	C.J.
R47	Pig Farm 1	M	A	285	+	-	-	-	nt
R48	Pig Farm 1	M	A	470	-	-	-	-	nt
R49	Pig Farm 1	M	A	455	-	-	-	-	nt
R50	Pig Farm 1	M	A	445	-	-	-	-	nt
R51	Pig Farm 1	M	A	336	+	-	-	-	nt
R52	Pig Farm 1	F	J	200	-	-	-	-	C.J.
R53	Pig Farm 1	F	A	350	-	-	-	-	-
R54	Pig Farm 1	M	A	383	-	-	-	-	C.J.
R55	Pig Farm 1	M	J	117	-	-	-	-	nt
R56	Pig Farm 2	F	J	118	+	-	-	-	nt
R57	Pig Farm 2	F	J	143	-	-	-	-	nt
R58	Pig Farm 2	M	J	103	-	-	-	+	nt
R59	Pig Farm 2	F	J	103	-	-	-	-	nt
R60	Pig Farm 2	M	J	102	+	-	-	-	nt
R61	Pig Farm 4	M	A	341	-	-	-	-	-
R62	Pig Farm 4	F	A	467	+	-	-	-	-
R63	Pig Farm 6	F	A	380	-	-	-	-	C.J.
R64	Pig Farm 6	M	J	92	-	-	-	-	-
R65	Pig Farm 8	M	A	483	+	-	-	-	nt
R66	Pig Farm 9	M	J	238	+	-	-	-	C.C.
R67	Pig Farm 9	M	J	180	-	-	-	-	C.C.
R68	Pig Farm 9	M	A	545	-	-	-	-	-
R69	Pig Farm 9	M	A	639	+	-	-	-	-

R70	Pig Farm 9	F	A	354	-	-	-	-	-
R71	Pig Farm 9	M	A	423	+	-	-	-	C.C.
R72	Pig Farm 9	M	A	442	-	-	-	-	-
R73	Pig Farm 9	M	A	562	+	-	-	+	C.C.
R74	Pig Farm 9	M	A	488	+	-	-	-	-
R75	Pig Farm 9	M	J	159	-	-	-	-	C.C.
R76	Pig Farm 9	M	J	269	-	-	-	+	-
R77	Pig Farm 9	M	J	230	-	-	-	-	C.C.
R78	Pig Farm 9	F	A	435	-	-	-	-	-
R79	Pig Farm 9	M	J	131	-	-	-	-	C.J.
R80	Pig Farm 9	F	A	304	-	-	-	-	C.C.
R81	Pig Farm 9	M	A	407	-	-	-	-	-
R86	Pig Farm 2	M	J	213	+	-	-	-	-
R87	Pig Farm 2	M	A	237	-	-	-	-	-
R88	Pig Farm 2	F	J	196	-	-	-	-	-
R89	Pig Farm 2	F	A	209	-	-	-	-	-
R91	Urban 2	M	J	42	-	-	-	-	C.J.
R92	Urban 2	M	J	40	-	-	-	-	C.J.
R93	Urban 2	F	J	132	-	-	-	-	-
R94	Urban 2	F	A	312	-	-	-	-	-

Table III.1 Brown rat (*Rattus norvegicus*) samples

nt indicates that this sample was not tested for a particular zoonotic pathogen.

Sample number	Location	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
V1	Farm 2	F	A	14	-	-	-	-	nt
V2	Farm 2	F	A	16	-	-	-	-	nt
V3	Farm 2	F	A	14	-	-	nt	nt	nt
V4	Farm 2	F	A	15	-	-	nt	nt	nt
V5	Farm 2	M	A	14	-	-	nt	nt	nt
V7	Farm 2	M	A	15	-	-	nt	nt	nt
V9	Farm 1	M	A	16	-	-	nt	nt	nt
V10	Farm 1	F	A	15	-	-	-	-	nt
V11	Farm 1	F	J	12	-	-	nt	nt	nt
V19	Llyn Cowlyd	F	A	20	-	-	nt	nt	nt
V33	Llyn Cowlyd	F	A	14	-	-	nt	nt	nt
V34	Pig Farm 1	M	A	13	-	-	-	-	C.J.
V35	Pig Farm 1	F	J	11	-	-	-	-	C.J.
V36	Pig Farm 1	F	A	15	-	-	-	-	-
V37	Pig Farm 1	F	A	14	-	-	-	-	C.J.
V38	Pig Farm 1	F	A	13	-	-	-	-	C.J.
V39	Pig Farm 1	M	A	18	-	-	-	-	C.J.
V40	Pig Farm 1	F	A	14	-	-	-	-	nt
V41	Pig Farm 1	F	A	19	-	-	+	-	nt
V42	Pig Farm 4	M	A	16	-	-	-	-	nt
V43	Pig Farm 5	M	A	14	-	-	-	-	-
V44	Pig Farm 5	M	J	13	-	-	-	-	-
V45	Pig Farm 5	M	J	13	-	-	-	-	-
V46	Pig Farm 5	M	A	15	-	-	-	-	-

V47	Pig Farm 5	A	F	20	-	-	-	-	-
V48	Pig Farm 5	A	M	14	-	-	-	-	nt
V49	Pig Farm 5	A	M	15	-	-	-	-	C.J.
V50	Pig Farm 5	A	F	16	-	-	-	-	C.J.
V51	Pig Farm 6	A	F	15	-	-	-	-	LPX neg
V52	Pig Farm 6	J	F	12	-	-	-	-	LPX neg
V53	Pig Farm 6	M	A	16	-	-	-	-	C.J.
V54	Pig Farm 6	M	A	20	-	-	-	-	C.J.
V55	Pig Farm 6	M	A	17	-	-	-	-	C.J.
V56	Pig Farm 6	M	J	12	-	-	-	-	-
V57	Pig Farm 6	F	A	19	-	-	-	-	-
V58	Pig Farm 6	M	A	17	-	-	-	-	-
V59	Pig Farm 6	M	A	20	-	-	-	-	-
V60	Pig Farm 6	M	A	17	-	-	-	-	-
V61	Pig Farm 6	F	A	23	-	-	-	-	-
V62	Pig Farm 8	F	A	15	-	-	+	-	-
V63	Pig Farm 8	F	A	16	-	-	-	-	-
V64	Pig Farm 8	F	A	15	-	-	-	-	-
V66	Pig Farm 8	M	A	15	-	-	-	-	-
V67	Pig Farm 8	M	A	14	-	-	-	-	C.J.
V68	Pig Farm 8	M	A	17	-	-	-	-	C.C.
V69	Pig Farm 8	M	J	11	-	-	-	-	-
V70	Pig Farm 8	M	A	16	-	-	-	-	C.J.
V71	Pig Farm 8	M	A	15	-	-	-	-	LPX neg
V72	Pig Farm 8	M	J	11	-	-	-	-	C.J.
V73	Pig Farm 9	M	A	17	-	-	-	-	-

V74	Pig Farm 9	M	A	16	-	-	-	-	-
V75	Pig Farm 9	F	A	19	-	-	-	-	-
V76	Pig Farm 9	M	A	16	-	-	-	-	-
V77	Pig Farm 10	M	J	14	-	-	-	-	-
V78	Pig Farm 10	M	J	12	-	-	-	-	-
V79	Pig Farm 10	M	J	14	-	-	-	-	-

Table III.2 Bank vole (*Myodes glareolus*) samples

Sample number	Location	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
V6	Farm 2	F	A	19	-	-	-	-	nt
V8	Farm 2	M	A	20	-	-	-	-	nt
V12	Llyn Cowlyd	M	A	18	-	-	-	-	nt
V13	Llyn Cowlyd	M	A	31	-	-	-	-	nt
V14	Llyn Cowlyd	M	A	21	-	-	-	-	nt
V15	Llyn Cowlyd	M	A	15	-	-	-	-	nt
V16	Llyn Cowlyd	M	A	28	-	+	-	-	nt
V17	Llyn Cowlyd	F	A	24	-	+	-	-	nt
V18	Llyn Cowlyd	M	A	16	-	-	-	-	nt
V20	Llyn Cowlyd	F	A	33	-	-	nt	nt	nt
V21	Llyn Cowlyd	M	A	26	-	+	-	-	nt
V22	Llyn Cowlyd	M	A	26	-	-	-	-	nt
V23	Llyn Cowlyd	F	A	22	-	+	-	-	nt
V24	Llyn Cowlyd	F	A	16	-	+	-	-	nt
V25	Llyn Cowlyd	F	A	13	-	+	-	-	nt
V26	Llyn Cowlyd	M	A	24	-	+	-	-	nt
V27	Llyn Cowlyd	N/A	A	14	-	-	-	-	nt
V28	Llyn Cowlyd	F	A	17	-	-	-	-	nt
V29	Llyn Cowlyd	M	A	15	-	-	-	-	nt
V30	Llyn Cowlyd	F	J	10	-	-	nt	nt	nt
V31	Llyn Cowlyd	M	A	24	-	-	nt	nt	nt
V32	Llyn Cowlyd	F	A	15	-	-	nt	nt	nt
V65	Pig Farm 8	M	A	15	-	-	-	-	-

Table III.3 Field vole (*Microtus agrestis*) samples

Sample number	Location	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
M57	Pig 1	M	A	17	nt	nt	-	-	C.J.
M61	Pig 3	M	A	16	nt	nt	B/-	-	nt
M63	Pig 3	M	A	14	nt	nt	-/-	-	nt
M64	Pig 3	F	A	17	nt	nt	-/-	-	nt
M68	Pig 3	F	A	20	nt	nt	B/-	-	-
M69	Pig 3	F	A	21	nt	nt	B/-	-	LPX neg
M71	Pig 3	M	A	19	nt	nt	-	-	-
M72	Pig 3	F	A	19	nt	nt	-/-	-	-
M73	Pig 3	M	A	18	nt	nt	-/-	-	-
M74	Pig 3	F	A	17	nt	nt	-/-	-	-
M75	Pig 3	F	A	16	nt	nt	-/-	-	-
M76	Pig 3	F	J	9	nt	nt	-/-	-	-
M77	Pig 3	F	A	19	nt	nt	-	-	-
M78	Pig 3	F	A	20	nt	nt	-	-	C.C.
M80	Pig 3	F	A	17	nt	nt	nt	-	nt
M81	Pig 3	F	A	23	nt	nt	nt	-	nt
M82	Pig 3	F	A	21	nt	nt	-	-	-
M83	Pig 3	M	A	18	nt	nt	-	-	nt
M84	Pig 3	F	A	19	nt	nt	-	-	nt
M85	Pig 3	F	A	17	nt	nt	-/-	-	nt
M86	Pig 3	F	A	18	nt	nt	-	-	nt
M87	Pig 3	F	A	22	nt	nt	-	-	nt
M88	Pig 3	M	J	6	nt	nt	-	-	nt
M89	Pig 3	F	A	22	nt	nt	-	-	nt
M90	Pig 3	F	A	13	nt	nt	-	-	nt
M91	Pig 3	M	A	19	nt	nt	nt	nt	nt
M92	Pig 3	M	A	20	nt	nt	-	-	nt
M93	Pig 3	M	J	11	nt	nt	-	-	nt
M94	Pig 3	M	A	19	nt	nt	-	-	nt
M95	Pig 3	M	A	16	nt	nt	-	-	nt

M96	Pig 3	F	A	17	nt	nt	-/-	-	nt
M97	Pig 3	M	A	21	nt	nt	-	-	nt
M98	Pig 3	F	A	21	nt	nt	-/-	-	nt
M99	Pig 3	F	A	13	nt	nt	-	-	nt
M100	Pig 3	F	J	6	nt	nt	-	-	nt
M101	Pig 3	M	A	21	nt	nt	-	-	nt
M102	Pig 3	F	A	18	nt	nt	-	-	nt
M103	Pig 3	M	A	20	nt	nt	-	-	nt
M104	Pig 3	M	A	18	nt	nt	-	-	nt
M105	Pig 3	M	A	19	nt	nt	-	-	-
M106	Pig 3	F	A	15	nt	nt	-	-	-
M107	Pig 3	M	J	8	nt	nt	-	-	nt
M108	Pig 3	F	A	15	nt	nt	-	-	nt
M109	Pig 3	F	A	17	nt	nt	-	-	nt
M110	Pig 4	M	A	17	nt	nt	-	-	nt
M111	Pig 4	M	A	17	nt	nt	-	-	nt
M112	Pig 4	F	A	21	nt	nt	-	-	nt
M113	Pig 4	M	A	20	nt	nt	-	-	nt
M114	Pig 5	F	A	24	nt	nt	-	-	-
M115	Pig 5	M	A	14	nt	nt	-	-	-
M116	Pig 5	M	A	22	nt	nt	-	-	-
M117	Pig 5	F	A	20	nt	nt	-	-	-
M118	Pig 5	F	A	17	nt	nt	B/-	-	-
M119	Pig 5	M	A	14	nt	nt	-	-	-
M120	Pig 5	M	A	20	nt	nt	-/-	-	-
M121	Pig 5	M	A	13	nt	nt	+/-	-	-
M122	Pig 5	F	A	15	nt	nt	B/-	-	-
M123	Pig 5	M	A	15	nt	nt	-/-	-	-
M124	Pig 5	F	J	13	nt	nt	-	-	nt
M125	Pig 5	M	J	12	nt	nt	-	-	nt
M126	Pig 5	FM	A	20	nt	nt	-	-	nt

M127	Pig 5	M	A	16	nt	nt	-	-	nt
M128	Pig 5	M	A	20	nt	nt	-	-	-
M129	Pig 5	F	A	23	nt	nt	-	-	-
M133	Pig 6	M	A	15	nt	nt	-	-	-
M134	Pig 6	F	J	10	nt	nt	-	-	nt
M136	Pig 6	M	A	18	nt	nt	-/-	-	-
M143	Pig 7	M	A	16	nt	nt	-	-	nt
M144	Pig 7	M	A	12	nt	nt	-/+	-	-
M145	Pig 7	M	A	15	nt	nt	+/-	-	-
M155	Pig 7	M	J	13	nt	nt	-/-	-	-
M156	Pig 7	F	J	10	nt	nt	+/+	-	-
M157	Pig 7	F	J	9	nt	nt	-/+	-	-
M158	Pig 8	F	A	22	nt	nt	-/-	-	-
M159	Pig 8	F	A	23	nt	nt	-/-	-	C.C.
M161	Pig 8	F	A	15	nt	nt	-/-	-	C.C.
M194	Pig 11	F	A	14	nt	nt	B/+	-	-
M195	Pig 11	M	A	21	nt	nt	-/+	-	-
M196	Pig 11	F	A	20	nt	nt	nt	nt	C.C
M197	Pig 11	M	A	21	nt	nt	nt	nt	-
M198	Pig 11	F	A	18	nt	nt	-/+	-	-
M199	Pig 11	M	A	19	nt	nt	-/+	-	C.C
M200	Pig 11	F	J	7	nt	nt	-/+	-	-
M201	Pig 11	F	J	11	nt	nt	-/+	-	-
M202	Pig 12	F	A	22	nt	nt	B/-	-	nt
M203	Pig 12	M	J	8	nt	nt	-	-	-
M204	Pig 12	F	J	10	nt	nt	-/-	-	-
M205	Pig 12	F	A	23	nt	nt	-	-	-
M206	Pig 12	M	A	22	nt	nt	-	-	-
M207	Pig 12	M	A	20	nt	nt	+	-	nt
M208	Pig 12	M	A	16	nt	nt	-/-	-	-
M209	Pig 12	F	A	12	nt	nt	-/-	-	-

M210	Pig 12	M	J	7	nt	nt	-/+	-	C.C
M211	Pig 12	M	J	7	nt	nt	-	-	-
M212	Pig 12	F	J	7	nt	nt	-/+	-	-
M213	Pig 12	M	J	7	nt	nt	-/-	-	-
M214	Pig 12	M	J	11	nt	nt	-	-	-
M215	Pig 12	M	J	10	nt	nt	-/+	-	C.C.
M216	Pig 12	F	A	19	nt	nt	-/+	-	C.C.
M217	Pig 12	F	A	15	nt	nt	B/-	-	-
M218	Pig 12	M	J	11	nt	nt	+	-	nt
M219	Pig 12	M	A	15	nt	nt	+	-	nt
M220	Pig 4	F	A	24	nt	nt	-	-	nt
M221	Pig 4	M	A	10	nt	nt	-	-	nt
M222	Pig 4	M	A	17	nt	nt	-	-	nt

Table III.4 Sample numbers and results for house mice samples (*Mus musculus*). Serology results are included with the PCR results for LCMV where both serum and tissue were screened. (B) indicates borderline serology.

Sample number	Location	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
M4	Farm 1	M	A	16	nt	nt	-	-	nt
M7	Farm 1	F	A	17	nt	nt	-	-	nt
M10	Farm 1	M	A	14	nt	nt	-	-	nt
M11	Farm 1	M	A	19	nt	nt	-	-	nt
M21	Farm 1	M	A	15	nt	nt	-	-	nt
M36	Farm 1	M	A	17	nt	nt	-	-	nt
M56	Pig 1	F	J	13	nt	nt	-	-	C.J.
M130	Pig 6	M	A	14	nt	nt	-	-	-
M131	Pig 6	M	A	20	nt	nt	-	-	-
M132	Pig 6	F	A	16	nt	nt	-	-	-
M135	Pig 6	M	A	17	nt	nt	+	-	-
M137	Pig 6	M	A	16	nt	nt	-	-	LPX neg
M138	Pig 6	F	A	18	nt	nt	-	-	-
M139	Pig 6	F	A	17	nt	nt	-	-	nt
M140	Pig 6	F	A	19	nt	nt	-	-	nt
M141	Pig 6	F	A	14	nt	nt	-	-	nt
M142	Pig 6	M	A	21	nt	nt	-	-	nt
M146	Pig 7	M	A	16	nt	nt	-	-	-
M147	Pig 7	M	A	17	nt	nt	-	-	-
M148	Pig 7	F	J	13	nt	nt	-	-	-
M149	Pig 7	F	J	13	nt	nt	-	-	-
M150	Pig 7	M	J	11	nt	nt	-	-	nt
M151	Pig 7	M	A	19	nt	nt	-	-	-
M152	Pig 7	M	A	16	nt	nt	-	-	nt
M153	Pig 7	F	A	20	nt	nt	-	-	nt
M154	Pig 7	M	A	16	nt	nt	-	-	nt
M160	Pig 8	M	J	13	nt	nt	-	-	-
M162	Pig 8	M	A	16	nt	nt	nt	nt	LPX neg
M163	Pig 9	F	A	18	nt	nt	-	-	nt

M173	Pig 9	M	J	12	nt	nt	-	-	nt
M174	Pig 10	F	A	16	nt	nt	-	-	-
M175	Pig 10	F	J	12	nt	nt	-	-	nt
M176	Pig 10	F	J	12	nt	nt	-	-	-
M177	Pig 10	M	A	15	nt	nt	-	-	nt
M178	Pig 10	F	A	16	nt	nt	-	-	-
M179	Pig 10	F	A	14	nt	nt	-	-	nt
M180	Pig 10	M	A	15	nt	nt	-	-	-
M181	Pig 10	M	A	17	nt	nt	-	-	-
M182	Pig 10	M	J	12	nt	nt	-	-	nt
M183	Pig 10	F	A	16	nt	nt	-	-	nt
M184	Pig 10	F	A	23	nt	nt	-	-	-
M185	Pig 10	F	A	18	nt	nt	-	-	nt
M186	Pig 10	F	J	13	nt	nt	-	-	-
M187	Pig 10	F	A	13	nt	nt	-	-	-
M188	Pig 10	F	A	24	nt	nt	-	-	nt
M189	Pig 10	M	J	13	nt	nt	-	-	nt
M190	Pig 10	F	A	14	nt	nt	-	-	nt
M191	Pig 10	F	A	20	nt	nt	-	-	nt
M192	Pig 10	F	J	10	nt	nt	-	-	-
M193	Pig 10	M	A	18	nt	nt	-	-	-
M223	Pig 4	F	A	17	nt	nt	-	-	-
M224	Pig 4	M	A	18	nt	nt	-	-	-

Table III.4 Sample numbers and results for woodmice samples (*Apodemus sylvaticus*).

Sample number	Location	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
SR1	Forest 1	F	A	220	nt	nt	-	-	nt
SR2	Forest 1	F	A	245	nt	nt	-	-	nt
SR3	Forest 1	F	A	290	nt	nt	-	-	nt
SR4	Forest 1	M	A	300	nt	nt	-	-	nt
SR5	Forest 1	F	A	320	nt	nt	-	-	nt
SR6	Forest 1	M	A	310	nt	nt	-	-	nt
SR7	Forest 1	M	A	340	nt	nt	-	-	nt
SR8	Forest 1	M	A	205	nt	nt	-	-	nt
SR9	Forest 1	F	A	380	nt	nt	-	-	nt
SR10	Forest 1	F	A	250	nt	nt	-	-	nt
SR11	Forest 1	M	A	300	nt	nt	-	-	nt
SR12	Forest 1	M	A	220	nt	nt	-	-	nt
SR13	Forest 1	F	A	355	nt	nt	-	-	nt
SR14	Forest 1	F	A	305	nt	nt	-	-	nt
SR15	Forest 1	M	A	340	nt	nt	-	-	nt
SR16	Forest 1	F	A	345	nt	nt	-	-	nt
SR17	Forest 1	M	A	290	nt	nt	-	-	nt
SR18	Forest 1	M	A	330	nt	nt	-	-	nt
SR19	Forest 1	F	A	235	nt	nt	-	-	nt
SR20	Forest 1	M	A	250	nt	nt	-	-	nt
SR21	Forest 1	F	A	320	nt	nt	-	-	nt

Table III.5 Sample numbers and results for red squirrel samples (*Sciurus vulgaris*).

Sample number	Location	Sex	Age	Weight (g)	SEOV	TATV	LCMV	HEV	*Campylobacter* spp.
S5	Forest 2	F	A	328	nt	nt	-	-	nt
S7	Forest 2	F	A	405	nt	nt	-	-	nt
S12	Forest 2	M	A	279	nt	nt	-	-	nt
S14	Forest 2	F	A	368	nt	nt	-	-	nt
S16	Forest 2	F	A	395	nt	nt	-	-	nt
S22	Forest 2	M	A	512	nt	nt	-	-	nt
S24	Forest 2	M	A	294	nt	nt	-	-	nt
S26	Forest 2	M	A	365	nt	nt	-	-	nt
S27	Forest 2	F	A	435	nt	nt	-	-	nt
S38	Forest 2	M	A	428	nt	nt	-	-	nt
S44	Forest 2	M	A	322	nt	nt	-	-	nt
S50	Forest 2	F	A	497	nt	nt	-	-	nt

Table III.6 Sample numbers and results for grey squirrel samples (*Sciurus carolinensis*).

Appendix IV Virus classification

Viral classifications of the viral zoonosis which have recently been updated by the International Committee on Taxonomy of Viruses (ICTV), however the older classifications are still widely used in the literature. The present and previous classifications of the viruses in this study are shown below.

IV.1. Hantavirus (Chapter 3)

ICTV Classification date	Order	Family	Genus	Species
Pre-2016	Unassigned	*Bunyaviridae*	*Hantavirus*	*Seoul hantavirus*
Post-2016	*Bunyavirales*	*Hantaviridae*	*Orthohantavirus*	*Seoul orthohantavirus*

Tatenale virus (TATV) has not yet been classed as an official hantavirus species.

IV.2. LCMV (Chapter 4)

ICTV Classification date	Order	Family	Genus	Species
Pre-2014	Unassigned	*Arenaviridae*	*Arenavirus*	*Lymphocytic choriomeningitis virus*
Post-2014	Unassigned	*Arenaviridae*	*Mammarenavirus*	*Lymphocytic choriomeningitis mammarenavirus*

IV.3. Hepatitis E virus (Chapter 5)

ICTV Classification date	Order	Family	Genus	Species
Pre-2014	Unassigned	*Hepeviridae*	*Hepevirus*	*Hepatitis E virus* • Genotype 1 (human only) • Genotype 2 (human only) • Genotype 3 (human and animals) • Genotype 4 (human and animals) Avian Hepatitis E virus Rat Hepatitis E virus Ferret Hepatitis E virus Bat Hepatitis E virus
Post-2014	Unassigned	Hepeviridae	Orthohepevirus	*Orthohepevirus A* • All variants that infect humans (previously known as genotypes 1-4) • Also infect animal hosts (deer, wild boar, pig, mongoose, rat and camel) *Orthohepevirus B* • Variants that infect avian species *Orthohepevirus C* • Variants that infect rats, ferrets and minks *Orthohepevirus D* • Variants that infect bats

Appendix V Fisher's test R code

R code for Fisher's test, in this example Table 3.3a is used to determine if there is a statistically significant sex bias in within the small sample size.

	Males	**Females**	**Total**
Rats +ve	11	2	13
Rats -ve	34	21	55
Total			68

```
>tableA <- matrix(c(11,34,2,21), nrow=2,
dimnames=list(Test=c("Pos", "Neg"),Sex=c("Male", "Female")))
fisher.test(tableA)
```

```
OR 3.44, CIs 0.63-34.00, P=0.193.
```

This code was used to test other potential associations in this project.

Appendix VI Vole tapeworm

Three vole samples were found to have been infected with the non-zoonotic fox tapeworm *Taenia polyacantha* (species identified by Dr John McGarry, University of Liverpool).

Figure VI.1. *Taenia polyacantha* in a field vole. This animal had 24 cysts inside its body cavity (see arrows).

Appendix VII – Veterinary Histology department, University of Liverpool protocols supplied by Miss Elena Fitzpatrick

Vet Histology Protocol 1: Haematoxylin and Eosin Staining

The staining method involves application of the basic dye haematoxylin (1g Haematoxylin (TCS Biosciences Ltd HD1475), 0.2g sodium iodate, 50g Potassium or ammonium alum, 20ml glacial acetic acid and 1litre distilled H_20) which colours basophilic structures with blue-purple hue, and alcohol-based acidic eosin Y (50ml 1% Eosin Y Stain (TCS Biosciences Ltd, HS250-1L), 390ml 95% Ethanol and 2ml glacial acetic acid), which colours eosinophilic structures bright pink. The structures do not have to be acidic or basic to be called basophilic and eosinophilic. The terminology is based on the affinity to the dyes.

1. Dewax samples in xylene 5mins
2. Transfer sections to another xylene and take sections down through descending grades of alcohol (100%, 96%, 85%, 70%) to distilled H_20
3. Stain sections in Haematoxylin 5mins
4. "Blue" sections in running tap water 6mins
5. Stain sections in Eosin for 2mins
6. Take sections through 3x 96% alcohol for 1min each (dip sections up and down to remove excess eosin)
7. Take sections through 3x 100% alcohol
8. Take sections through 2x xylene
9. Mount sections DPX & coverslip

Vet Histology Protocol 2: CD79a Protocol on Autostainer Link 48

Monoclonal mouse anti-human CD79a (AbD serotec, MCA2538H)

PT Link

Deparaffinization, rehydration and epitope retrieval using:

EnVision™ FLEX Target Retrieval Solution (TRS) Low pH (Citrate buffer, pH 6.1, K8005)

Dako Autostainer Link 48

Step	**Reagent**	**Volume**	**Time**
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	CD79a 1:300 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150ul	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1min 96% ethanol, 2x3min 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)

3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

Vet Histology Protocol 3: CD3 Protocol on Autostainer Link 48

Polyclonal Rabbit anti Human CD3 (Dako A0452)

PT Link

Deparaffinization, rehydration and epitope retrieval using:

EnVision™ FLEX Target Retrieval Solution (TRS) High pH (Citrate buffer, pH 9.0, K8004)

Dako Autostainer Link 48

Step	Reagent	Volume	Time
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	CD3 1:500 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150ul	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1min 96% ethanol, 2x3min 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)

4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

Vet Histology Protocol 4: Pax-5 Protocol on Autostainer Link 48

Monoclonal Purified Mouse anti-Pax-5 (DB 610863)

PT Link

Deparaffinization, rehydration and epitope retrieval using:

EnVision™ FLEX Target Retrieval Solution (TRS) High pH (Citrate buffer, pH 9.0, K8004)

Dako Autostainer Link 48

Step	**Reagent**	**Volume**	**Time**
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	PAX-5 1:100 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150ul	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1min 96% ethanol, 2x3min 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)

4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

Appendix VIII Sequence data

Sequence name	Sequence 329 nt of a partial L-segment fragment
R62 UK Yorkshire 2015/SEOV/*R. norvegicus* (MK492669)	CCAGGTGACAATTCAGCAAAATTCAGGCGATTCACTGCTGCCCTTCATAATG GATTACCTGATGACAGGTTAAAGAACTGTGTTATTGATGCCTTGCGCCATGT ATATAAGACTGATTTTTATATGTCTAGAAAACTTAGACACTATATTGATTCTA TGGATACTTATGAACCTCATGTTAGAGACTTCTTAAATTTCTTTCCAGATGGG CACCATGGAGAGGTACGAGGCAATTGGTTGCAGGGTAACTTGAACAAGTGC TCATCATTATTTGGTGTGGCAATGTCTTTACTATTTAAAGAAATCTGGACAAG GCTATTTCCAGAATT
Pig Farm 1 UK Yorkshire 2015/SEOV/*R. norvegicus* (MK492670)	CCAGGTGACAATTCAGCAAAATTCAGGCGATTCACTGCTGCCCTTCATAATG GATTACCTGATGACAGGTTAAAAAACTGTGTTATTGATGCTTTGCGCCATGT ATATAAGACTGATTTTTATATGTCTAGAAAACTTAGACACTATATTGATTCTA TGGACACTTATGAACCTCATGTCAGAGACTTCTTGAATTTCTTTCCAGATGGG CACCGTGGAGAGGTACGGGGCAATTGGTTGCAGGGTAACTTGAACAAGTGC TCATCATTATTTGGTGTGGCAATGTCTTTACTATTTAAGGAAATCTGGACTAG GTTATTTCCAGAATT
Pig Farm 2 UK Cheshire 2015/SEOV/*R. norvegicus* (MK492671)	CCAGGTGACAATTCAGCAAAATTCAGGCGATTCACTGCTGCCCTTCATAATG GATTACCTGATGACAGGTTAAAAAACTGTGTTATTGATGCTTTGCGCCATGT ATATAAGACTGATTTTTATATGTCTAGAAAACTTAGACACTATATTGATTCTA TGGACACTTATGAACCTCATGTCAGAGACTTCTTGAATTTCTTTCCAGATGGG CACCGTGGAGAGGTACGGGGCAATTGGTTGCAGGGTAACTTGAACAAGTGC TCATCATTATTTGGTGTGGCAATGTCTTTACTATTTAAGGAAATCTGGACTAG GTTATTTCCAGAATT
Pig Farm 8 UK Yorkshire 2015/SEOV/*R. norvegicus* (MK492672)	CCAGGTGACAATTCAGCAAAATTCAGGCGATTCACTGCTGCCCTTCATAATG GATTACCTGATGACAGGTTAAAAAACTGTGTTATTGATGCTTTGCGCCATGT ATATAAGACTGATTTTTATATGTCTAGAAAACTTAGACACTATATTGATTCTA TGGACACTTATGAACCTCATGTCAGAGACTTCTTGAATTTCTTTCCAGATGGG CACCGTGGAGAGGTACGAGGCAATTGGTTGCAGGGTAACTTGAACAAGTGC TCATCATTATTTGGTGTGGCAATGTCTTTACTATTTAAGGAAATCTGGACTAG GTTATTTCCAGAATT
Pig Farm 9 UK Yorkshire 2015/SEOV/*R. norvegicus* (MK492673)	CCAGGTGACAATTCAGCAAAATTCAGGCGATTCACTGCTGCCCTTCATAATG GATTACCTGATGACAGGTTAAAAAACTGTGTTATTGATGCTTTGCGCCATGT ATATAAGACTGATTTTTATATGTCTAGAAAACTTAGACACTATATTGATTCTA TGGACACTTATGAACCTCATGTCAGAGACTTCTTGAATTTCTTTCCAGATGGG CACCATGGAGAGGTACGAGGCAATTGGTTGCAGGGTAACTTGAACAAGTGC TCATCATTATTTGGTGTGGCAATGTCTTTACTATTTAAGGAAATCTGGACTAG GTTATTTCCAGAATT

Table VIII.1 SEOV 329 nt L-segment sequences from this study. GenBank accession numbers are included.

Sequence name	Sequence 291 nt of a partial L-segment fragment
V16/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAATTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAATTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATTTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG
V17/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAATTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAACTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATTTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG
V21/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAGTTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAATTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATCTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG
V23/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAGTTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAATTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATTTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG
V24/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAGTTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAATTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATTTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG
V25/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAGTTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAATTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATCTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG

V26/TATV/North Wales/2015 *M. agrestis*	GGGATGATAAGTTAAAAAATTGTGTAGTGGATGCATTGAGAAATA TCTATGAAACAGAATTTTTCATGTCCCGGAAATTACACCGGTACAT TGATGGAATGGAGGATCTGTCTGAGAATGTGGAAGATTTTTTGTCA TTCTTTCCCAACAATGTCTCTGCACTGATTAAAGGTAATTGGTTACA AGGTAATTTAAATAAGTGTTCATCCTTATTTGGTGCTGCAGTGTCCT TGTTATTTAAGAGGGTGTGGACAAATTTATTTCCTGAATTAGATTG TTTCTTTGAGTTTG

Table VIII.2 TATV 291 nt L-segment sequences from this study.

Sequence name	**Sequence 307 nt of a partial L-segment fragment**
M156/Pig farm 7/ Edinburgh/2015 *M. musculus*	CATTAACAAAATGCATGAGTGCTGCCTTAAAAAATTTATGTTTTTAT TCAGAAGAATCACCAACATCATACACCTCAGTTGGTCCTGACTCCG GAAGGTTGAAGTTTGCACTGTCCTATAAAGAGCAGGTCGGGGGAA ACAGAGAGCTGTATATTGGGGATTTAAGAACAAAAATGTTTACAAG GCTAATAGAGGATTATTTTGAATCCTTTTCTAGTTTCTTTTCAGGTT CATGTTTAAACAATGACAAAGAGTTTGAGAATGCCATCCTTTCAAT GACCATAAATGTGCGGGAAGGGTTTTTGAACTACAGCATG
M157/Pig farm 7/ Edinburgh/2015 *M. musculus*	CATTAACAAAATGCATGAGTGCTGCCTTAAAAAATTTATGTTTTTAT TCAGAAGAATCACCAACATCATACACCTCAGTTGGTCCTGACTCCG GAAGGTTGAAGTTTGCACTGTCCTATAAAGAGCAGGTCGGGGGAA ACAGAGAGCTGTATATTGGGGATTTAAGAACAAAAATGTTTACAAG GCTAATAGAGGATTATTTTGAATCCTTTTCTAGTTTCTTTTCAGGTT CATGTTTAAACAATGACAAAGAGTTTGAGAATGCCATCCTTTCAAT GACTATAAATGTGCGGGAAGGGTTTTTGAACTACAGCATG
M159/Pig farm 8/ Yorkshire/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
M194/Pig farm 11/ Telford/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG

M195/Pig farm 11/ Telford/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
M198/Pig farm 11/ Telford/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
M200/Pig farm 11/ Telford/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
M207/Pig farm 12/ Yorkshire/2015 *M. musculus*	CATTGACGAAATGTATGAGTGCTGCTTTGAAGAACCTGTGCTTCTA CTCGGAAGAATCACCAACATCATATACCTCAGTTGGGCCTGACTCT GGGAGATTGAAGTTCGCATTATCTTATAAGGAACAGGTTGGGGGGA ACAGAGAACTTTATATCGGGGACCTGAGAACAAAAATGTTTACAA GATTGATAGAAGATTATTTTGAGTCCTTTTCCAGTTTCTTTTCAGGT TCATGTTTGAATAACGACAAAGAGTTTGAGAATGCAATTTTGTCAA TGACCATCAATGTTCGAGAAGGATTTCTAAATTATAGCATG
M207/Pig farm 12/ Yorkshire/2015 *M. musculus*	CATTGACGAAATGTATGAGTGCTGCTTTGAAGAACCTGTGCTTCTA CTCGGAAGAATCACCAACATCATATACCTCAGTTGGGCCTGACTCT GGGAGATTGAAGTTCGCATTATCTTATAAGGAACAGGTTGGGGGGA ACAGAGAACTTTATATCGGGGACCTGAGAACAAAAATGTTTACAA GATTGATAGAAGATTATTTTGAGTCCTTTTCCAGTTTCTTTTCAGGT TCATGTTTGAATAACGACAAAGAGTTTGAGAATGCAATTTTGTCAA TGACCATCAATGTTCGAGAAGGATTTCTAAATTATAGCATG
M218/Pig farm 12/ Yorkshire/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
M219/Pig farm 12/ Yorkshire/2015 *M. musculus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT

	CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
R42/Pig farm 1/ Yorkshire/2015 *R. norvegicus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAATAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
R43/Pig farm 1/ Yorkshire/2015 *R. norvegicus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
V41/Pig farm 1/ Yorkshire/2015 *M. glareolus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGATTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ATTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGTT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACCATCAATGTTCGAGAAGGATTTCTAAATTATAGCATG
V62/Pig farm 6/ Yorkshire/2015 *M. glareolus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTCATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATTGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG
M135/Pig farm 6/ Yorkshire/2015 *A. sylvaticus*	CACTGACAAAATGCATGAGTGCTGCCTTGAAAAATCTGTGCTTTTA CTCAGAGGAATCACCAACATCATACACCTCAGTTGGTCCTGATTCT GGTAGACTAAAATTTGCACTATCTTATAAAGAGCAGGTTGGAGGGA ATAGAGAGCTTTATATTGGAGATTTAAGAACAAAGATGTTCACAAG ACTGATAGAAGATTATTTTGAATCCTTTTCCAGTTTCTTTTCAGGCT CGTGTTTGAACAATGATAAAGAGTTTGAGAATGCAGTCCTGTCAAT GACTATCAATGTGCGAGAAGGATTCTTGAACTACAGTATG

Table VIII.3 LCMV 307 nt L-segment sequences from the pan-Areanvirus RT-PCR used in this study.

Sequence name	Sequence 224nt fragment of HEV ORF-1
R3 ratHEV Cheshire UK 2014 *R. norvegicus*	CCTGAATGGTGCTTTTATGGTGATTGTTATGCCCAGGAGAAGCTAGAAGCT GCTGTGGCTGGAGCAAAAGCATGTCGTGTCTTTGAGAATGATTTCAGTGAG TTTGACAGCACACAGAATAATTATTCCTTGGGCCTTGAGTGTTTATTGATG AAGGAGGCCGGGGCACCCGAATGGATGTGGAGGTTGTACCACCTGCTCCG CTCTGCATGGGTGCTGCAGGC
R5 ratHEV Cheshire UK 2014 *R. norvegicus*	CCTGAATGGTGCTTTTATGGTGATTGTTATGCCCAGGAGAAGCTAGAAGCT GCTGTGGCTGGGGCAAAAGCATGTCGTGTCTTTGAGAATGATTTCAGTGAG TTTGACAGCACACAGAATAATTATTCCTTGGGCCTTGAGTGTTTATTGATG AAGGAGGCCGGGGCACCCGAATGGATGTGGAGGTTGTACCACCTGCTCCG CTCTGCATGGGTGCTGCAGGC
R58 ratHEV Cheshire UK 2015 *R. norvegicus*	CCTGAATGGTGCTTTTATGGTGATTGTTATGCCCAGGAGAAGCTAGAAGCT GCTGTGGCTGGAGCAAAAGCATGTCGTGTCTTTGAGAATGATTTCAGTGAG TTTGACAGCACACAGAATAATTATTCCTTGGGCCTTGAGTGTTTATTGATG AAGGAGGCCGGGGCACCCGAATGGATGTGGAGGTTGTACCACCTGCTCCG CTCTGCATGGGTGCTGCAGGC
R43 ratHEV Yorkshire UK 2015 *R. norvegicus*	CCTGAGTGGTGCTTTTATGGCGACTGCTATGTGCCGGAGAGGTTAGAGGCT GCTGTGGCTGGGGCGAAGGCATGCCGAGTTTTCGAGAATGACTTCAGTGA GTTTGATAGCACACAGAATAATTATTCCCTGGGCTTAGAATGTTTACTGAT GAAGGAGGCCGGAGTGCCCGAGTGGATGTGGAGGCTGTACCATCTGCTCC GCTCGGCGTGGGTGCTGCAGGC
R45 ratHEV Yorkshire UK 2015 *R. norvegicus*	CCTGAGTGGTGTTTTTATGGCGACTGCTATGTGCCGGAGAGGTTAGAGGCT GCTGTGGCTGGGGCGAAGGCATGCCGAGTTTTCGAGAATGACTTCAGTGA GTTTGATAGCACACAGAATAATTATTCCCTGGGCTTAGAATGTTTACTGAT GAAGGAGGCCGGAGTGCCCGAGTGGATGTGGAGGCTGTACCATCTGCTCC GCTCGGCGTGGGTGCTGCAGGC
R73 ratHEV Yorkshire UK 2015 *R. norvegicus*	CCTGAATGGTGCTTTTATGGTGATTGTTATGCCCAGGAGAAGCTAGAAGCC GCAGTGGCTGGAGCAAAGGCATGTCGTGTCTTTGAGAATGATTTTAGTGAG TTTGACAGCACACAGAACAACTACTCCTTGGGCCTTGAGTGTTTATTGATG AGGGAGGCTGGGGCACCTGAGTGGATGTGGAGGTTGTACCATTTGCTCCG CTCAGCATGGGTGCTGCAGGC
R76 ratHEV Yorkshire UK 2015 *R. norvegicus*	CCTGAATGGTGCTTTTATGGTGACTGTTATGCCCAGGAGAAGCTGGAAGCC GCTGTGGCTGGAGCAAAAGCATGTCGTGTCTTTGAGAATGATTTTAGTGAG TTTGATAGCACACAGAACAATTATTCCTTGGGCCTTGAGTGTTTATTGATG AGGGAGGCCGGGGCACCCGAGTGGATGTGGAGGTTGTACCATTTGCTCCG CTCAGCGTGGGTGCTGCAGGC

Table VII.4 SEOV 224 nt ORF-1 sequences from the pan-HEV RT-PCR used in this study.

Appendix VIIII Original Manscripts accepted for publication from this thesis

Murphy, Ellen G, Nicola J Williams, Malcolm Bennett, Daisy Jennings, Julian Chantrey and Lorraine M. McElhinney. "Detection of Seoul virus (SEOV) in wild brown rats (*Rattus norvegicus*) from pig farms in Northern England." *Veterinary Record* (2019) 184, doi: 10.1136/vr.105249

Murphy, Ellen G, Nicola J. Williams, Daisy Jennings, Julian Chantrey, Ranieri Verin, Sylvia Grierson, Lorraine M. McElhinney and Malcolm Bennett. "First detection of Hepatitis E virus (*Orthohepevirus C*) in wild brown rats (*Rattus norvegicus*) from Great Britain" *Zoonosis and Public Health* (2019*) In Press*.