Targeted surveillance for Ljungan virus and Hantaviruses in UK rodents

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Kieran Christopher Pounder

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Abstract

Zoonoses are a significant threat to public health and can also be a considerable economic burden. A large proportion of zoonotic pathogens have rodent hosts that provide important connections between wildlife communities and humans. This thesis aimed to better understand the risk to humans from hantaviruses and Ljungan virus in the UK by targeting rodents from urban and semi-rural environments, by sampling domesticated pet rats and also by studying brown rats in and around Lyon (France). Hantaviruses are zoonotic and cause haemorrhagic fever with renal syndrome in Europe, but their presence in the UK had never been confirmed. Ljungan virus (LV) has been associated with several human diseases in Europe, but has not been confirmed as zoonotic, although it has been detected in rural rodents in the UK. All samples were assayed for these viruses using PCR detection methods. Moreover, I used high throughput sequencing to quantify genomic variation in two new LV genomes to better understand the evolution of this group. I present molecular evidence of a novel hantavirus circulating in a rural rodent species within the UK, as well as a *Rattus*–associated hantavirus, Seoul virus, in pet rats in the UK and brown rats from Lyon. This study therefore not only adds confirmation of a novel hantavirus species circulating in the UK but also that Seoul virus might be more prevalent in European brown and pet rats than previously believed. Analyses of sequence variation (cytochrome *b*) of brown rats found few genetic differences, irrespective of infection status, country of origin and domestication, and thus could not be used to identify whether the introduction of non-indigenous rats into the UK is associated with Seoul virus. The prevalence of LV was much lower than that previously reported, possibly due to differences in habitat type and the virus' maintainability. I identified a lack of potential adaptive variation among LV genomes perhaps indicative of it being a slow evolving virus, a characteristic unlike other RNA viruses. This study has also shown that further surveillance should be conducted in the UK, targeting not only the two viruses described here, but also existing and novel zoonotic pathogens carried by rodents that have yet to be detected.

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Contents

Abbreviations

Cyt *b*	Cytochrome *b*
DOBV	Dobrava- Belgrade virus
EID	Emerging infectious disease
HCPS	Hantavirus cardiopulmonary syndrome
HFRS	Haemorrhagic fever with renal syndrome
HPeV	Human parechovirus
HTNV	Hantaan virus
HV	Hantavirus
LV	Ljungan virus
PUUV	Puumala virus
SAAV	Saaremaa virus
SEOV	Seoul virus
TATV	Tatenale virus

Chapter 1. General Introduction

1.1 Background

1.1.1 Zoonoses

Zoonoses as defined by the World Health Organisation (WHO) are diseases and infections transmitted naturally between vertebrate animals and man. It has been reported that approximately 61 % of all infectious organisms that are known to be pathogenic to humans are zoonotic (Taylor *et al.*, 2001). Emerging infectious diseases (EID) are defined as diseases that have either appeared for the first time, increased in incidence or been reported in new areas or hosts (Cleaveland *et al.*, 2001). Of 335 EIDs globally reported between 1940-2004, the majority were zoonotic and originated from wildlife species (Jones *et al.*, 2008). Examples of globally important EIDs include acquired immunodeficiency syndrome (AIDS), influenza A (H1N1; Swine flu) and severe acute respiratory syndrome (SARS). Zoonoses are not only a significant threat to public health but they can also incur considerable economic burden. An example is that of the economic impact of SARS in 2002 which cost an estimated US $90 billion worldwide (WHO, 2003).

Factors that have been proposed to explain the emergence of new diseases include pathogen evolution (e.g. mutations), host characteristics (e.g. immunosuppression), host population characteristics (e.g. size, behaviour, movement) and ecological (e.g. agriculture, climate domestication, land use, translocation, urbanisation), but by no means are these factors exclusive (Cleaveland *et al.*, 2001; Morris & Potter, 1997; Morse, 1995; Schrag & Wiener, 1995). Ecological factors are important, and are mostly associated with humans. As such they pose the greatest risk of disease emergence as a result of increased animal-human contact, and so the potential for increased transmission opportunities (Daszak, 2000; Jones *et al.*, 2008; Mahy & Murphy, 1998; Peters *et al.*, 1994). For example human outbreaks of two filoviruses, Ebola and Marburg are associated with human encroachment in Africa (Monath, 1999). Principally for a pathogen to successfully emerge it must first establish itself in a new host by jumping the species barrier, it then optimally persists and eventually spreads in that host (Morse, 1995).

Zoonotic diseases can result from infection with viruses (e.g. Rabies), bacterium (e.g. Q fever), macroparasites (e.g. Taeniasis), fungi (e.g. Sporotrichosis) or other unconventional agents (e.g. Bovine spongiform encephalopathy). Of these, viruses are the major cause of zoonotic infectious diseases and are highly likely to emerge (Cleaveland *et al.*, 2001). This is mainly because of the difficulty in treating viral diseases but also more importantly they exhibit greater mutation rates and shorter generation times than any other pathogen, and RNA viruses in particular have low fidelity polymerases (Domingo & Holland, 1997; Holmes, 2003). The transmission of such zoonotic organisms to humans can be directly through contact to the animal host (e.g. Nipah virus from fruit bats; Chua *et al.*, 2000) or indirectly via ticks (e.g. Tick-borne encephalitis virus; Dumpis *et al.*, 1999), mosquitoes (e.g. Chikungunya virus; Pialoux *et al.*, 2007), fleas (e.g. *Yersinia pestis*; Perry & Fetherston, 1997), food and water (e.g. *Giardia lamblia* and Salmonella; Adam, 2001; Newell *et al.*, 2010) or inhalation of excreta (e.g. Hantavirus; Schmaljohn & Hjelle, 1997).

1.1.2 Rodents as a source

A large proportion of zoonotic pathogens have rodents as hosts (22.5 %) and are only exceeded by pathogens which infect ungulates (cattle, goats, horses, pigs and sheep) (39.3 %) and carnivores (cats and dogs) (43 %) (Cleaveland *et al.*, 2001). A few important rodent-borne zoonotic pathogens found around the world include hantaviruses, *Leptospira*, lymphocytic choriomeningitis virus (LCMV), *Y. pestis* and *Spirillum minus*.

Since 2002, at least 33 notifiable or reportable zoonotic pathogens have been recorded in either animals or humans within the United Kingdom (UK) (Defra, 2012). As published in the latest annual report on zoonoses in the UK by the Department for Environment, Food & Rural Affairs (DEFRA), 28 of these 33 zoonotic pathogens were reported as circulating during 2011 (Defra, 2012). Organisms such as *Crytposporidium* sp. (3,600 laboratory-confirmed human cases), *Camplylobacter* sp. (72,000), *Borrelia burgdorferi* (1,200), *Salmonella* sp. (9,400) and verocytotoxin-producing *Escherichia coli* (VTEC) (1,400) are the cause of most human cases of zoonotic disease in the UK (Defra, 2012). Of these 28, at least five zoonoses are known to be associated with rodents including bovine tuberculosis (Cavanagh *et al.*, 2002), cryptosporidiosis (Chalmers & Giles, 2010), haemorrhagic

fever with renal syndrome (HFRS) (described below) (Jameson *et al.*, 2013a), leptospirosis (Ellis, 1999) and Lyme disease (O'Connell, 1995). Two other potential zoonoses that are associated with rodents in the UK but are not classified as notifiable or reportable include cowpox (Chantrey *et al.*, 1999) and *Capillaria hepatica* (McGarry et al, unpublished data).

A feature of many rodent populations is their fluctuating densities caused by a combination of factors; climate, competition, predation and food availability through mast years (the production of an exceptional amount of tree fruit in a given year) (Davis *et al.*, 2005). With the potential for rodent densities to increase in certain years and the already occurring human encroachment and habitat destruction, an increase in the relative contact between rodents and humans is expected, thus likely increasing the risk of zoonotic pathogen transmission and emergence.

Two important viruses that are of current interest include hantavirus, and Ljungan virus; a reported potential zoonosis (Niklasson *et al.*, 1999). Whilst both viruses have been reported in Europe there is insufficient evidence reported to also suggest their wide circulation within the UK. Within this thesis, using a systematic approach, we will investigate the presence and prevalence of these two viruses in UK wildlife.

1.1.3 Hantaviruses

Hantaviruses (Family *Bunyaviridae*, genus *Hantavirus*) are thought to be historically responsible for a variety of severe illness outbreaks spanning more than a century (Bradford, 1916; Bridson, 2001; Brown, 1916; McKee *et al.*, 1991). However the etiological agent had long eluded researchers until an outbreak of Korean haemorrhagic fever (KHF) occurred during the Korean War (1950-1953) and was accountable for the hospitalisation of more than 3,000 United Nations soldiers. An agent was isolated from the striped field mouse (*Apodemus agrarius coreae*) and given the name Hantaan virus (HTNV) (Lee *et al.*, 1978). Since then a further 23 hantavirus species have been classified by the International Committee on Taxonomy of Viruses (ICTV: www.ictvonline.org, date accessed 1/5/13) although many more have been proposed. The criteria that currently defines a new hantavirus species include having sufficient amino acid sequence diversity in the S and M segments (>10 %), detected in a rodent species not previously reported as a host of a

hantavirus, and can be differentiated from other hantaviruses by cross-neutralisation tests (Maes *et al.*, 2009; Nichol *et al.*, 2005). The genetic relationship of hantaviruses cluster according to relatedness of their carrier hosts (Figure 1.1) (Vapalahti *et al.*, 2003).

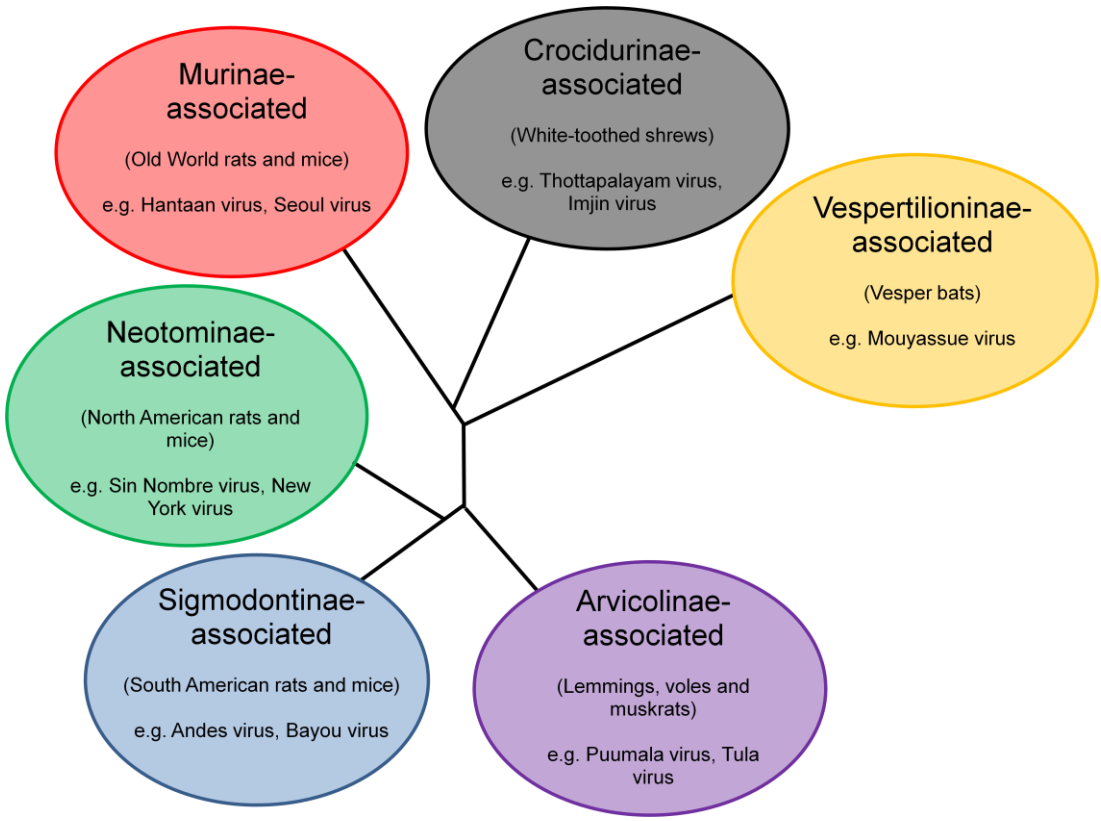


Figure 1.1. Schematic diagram adapted from Figure 5.3, illustrating the phylogenetic relationship of associated hantaviruses within subfamilies.

The hantaviruses are single stranded negative sense RNA viruses with a genome composed of three segments (Fig. 1.2), small (S) (1.7 kb) which encodes the nucleocapsid protein, medium (M) (3.5 kb) the glycoprotein and large (L) (6.5 kb) the RNA polymerase. Hantaviruses have been found circulating in rodents, insectivores and bats (Klempa *et al.*, 2007; Weiss *et al.*, 2012) and are primarily transmitted directly from the carrier via the inhalation of virus contaminated urine

and faeces. Rodents have been shown to have a chronic life-long infection however show no signs of disease. In contrast the virus in humans causes two severe clinical manifestations; 1) hantavirus cardiopulmonary syndrome (HCPS) mainly restricted to the Americas and responsible for 200 cases a year and causing mortality of up to 40 % (Bi *et al.*, 2008); 2) haemorrhagic fever with renal syndrome (HFRS) which is mainly restricted to Eurasia and responsible for 150,000-200,000 human cases each year and causing mortality of up to 12 % (Bi *et al.*, 2008; Vaheri *et al.*, 2012). Within Europe there are five established rodent-borne hantaviruses known to be circulating (Table 1.1).

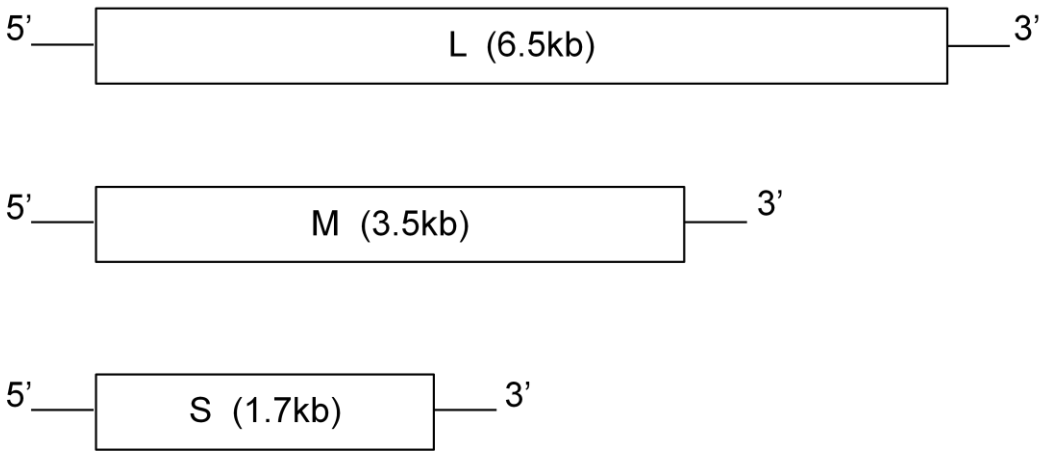


Figure 1.2. Schematic diagram of the tri-segmented hantavirus genome illustrating its organisation. The large (L) segment encodes the RNA polymerase, the medium (M) segment encodes the glycoproteins and the small (S) segment encodes the nucleocapsid protein. Approximate lengths (nucleotides) of each segment are shown.

Table 1.1. Hantavirus species known to be circulating in Europe, their rodent host, virus distribution and severity of HFRS disease caused.

Hantavirus	Rodent host species	Virus distribution[1]	HFRS severity[2]	References
Dobrava-Belgrade (DOBV)	Yellow-necked mouse (*Apodemus flavicollis)*	Central and eastern Europe	Severe	(Avsic-Zupanc *et al.*, 1992)
Puumala (PUUV)	Bank vole (*Myodes glareolus)*	Widespread	Mild	(Brummer-Korvenkontio *et al.*, 1980)
Saaremaa (SAAV)	Striped field mouse (*A. agrarius)*	Central and eastern Europe	Mild	(Plyusnin *et al.*, 1997b)
Seoul (SEOV)	Black rat (*Rattus rattus)*, brown rat (*Rattus norvegicus)*	Belgium, Ireland, France, Portugal, and UK.	Moderate	(Heyman *et al.*, 2004)
Tula (TULV)	Common vole (*Microtus arvalis)*	Central and eastern Europe	Unknown	(Plyusnin *et al.*, 1994)

[1]Virus distribution (Olsson *et al.*, 2010), [2]HFRS disease (Vaheri *et al.*, 2012)

Tropism of hantaviruses (pathogenic and non-pathogenic) in both humans and rodents is thought to be the same; primarily targeting the endothelial cells and macrophages of the lungs and kidneys (Green *et al.*, 1998; Lee *et al.*, 1981; Maes *et al.*, 2004; Yanagihara, 1990; Yanagihara *et al.*, 1985), although it remains unclear as to why rodents remain persistently infected yet do not generally exhibit any ill effect on health (Lee *et al.*, 1981; Meyer & Schmaljohn, 2000; Vaheri *et al.*, 2008; Yanagihara *et al.*, 1985). The severity of disease is generally dependent on the hantavirus involved, though variations in symptoms and severity within a species have been observed (Kanerva *et al.*, 1996; Mentel *et al.*, 1999; Peters *et al.*, 1999; Pilaski *et al.*, 1994; Vaheri *et al.*, 2012). The extent of endothelial permeability is thought to be a key factor in determining the severity of disease (Gavrilovskaya *et al.*, 2012; Maes *et al.*, 2004; Muranyi *et al.*, 2005; Vapalahti *et al.*, 2003). A genetic predisposition related to the type of human leukocyte antigen (HLA), a human equivalent to the major histocompatiability complex (MHC) has also been found to be a key factor in the severity of HV disease course (Kilpatrick *et al.*, 2004; Mustonen *et al.*, 1996). The full extent as to why some hantaviruses are pathogenic and others are not, is not fully understood however it is known that there are at least two requirements necessary for hantaviruses to be pathogenic; the ability to regulate an early interferon response and the use of specific integrins (Alff *et al.*, 2006; Alff *et al.*, 2008; Gavrilovskaya *et al.*, 2012; Gavrilovskaya *et al.*, 1999; Gavrilovskaya *et al.*, 1998; Geimonen *et al.*, 2002; Matthys *et al.*, 2011; Matthys *et al.*, 2010; Raymond *et al.*, 2005). Ultimately however the complete mechanisms behind why some hantavirus species are pathogenic and some are not, why some produce more severe disease than others, or why some exhibit primarily pulmonary rather than renal symptoms, remains to be determined and requires further research.

Dobrava-Belgrade virus (DOBV) and Puumala virus (PUUV), the causes of the most severe and mildest HFRS disease in Europe, respectively, are prevalent and thought to be responsible for the majority of human HV cases. Saaremaa virus (SAAV), Seoul virus (SEOV) and Tula virus (TULV) however show a low prevalence, and especially in the case of Seoul virus, sporadic, in Europe (Heyman *et al.*, 2011; Vaheri *et al.*, 2012). Numbers of human hantavirus infections in Europe are reported to be on the rise (Heyman *et al.*, 2011; Vaheri *et al.*, 2012). The recent increase in awareness and improvement in diagnostics goes some way to explaining this

(Heyman *et al.*, 2011; Reusken & Heyman, 2013). However other factors that could be the cause of the increased emergence of cases in Europe include aspects such as reservoir ecology (Tersago *et al.*, 2009), virus ecology (Hardestam *et al.*, 2007; Kallio *et al.*, 2006) and anthropogenic factors (Heyman *et al.*, 2012; Makary *et al.*, 2010; Mertens *et al.*, 2011; Reusken & Heyman, 2013; Vapalahti *et al.*, 2010).

Currently there are no reliable treatments available for hantavirus disease in humans however there have been trials using formalin–inactivated vaccines such as Hantavax® (Cho & Howard, 1999; Cho *et al.*, 2002; Hjelle, 2002; Johnson, 2001; Lee *et al.*, 1999), recombinant vaccines (Spik *et al.*, 2008) and antivirals such as Ribavirin (Bai *et al.*, 1997; Huggins *et al.*, 1991; Huggins *et al.*, 1986; Rusnak *et al.*, 2009; Severson *et al.*, 2003) with moderate success. At present the best course of treatment is supportive, specifically maintaining internal fluid balance (Vapalahti *et al.*, 2003). Once a patient is hospitalised, the need for treatment to reduce virus replication is too late and often no longer required, therefore breaking the transmission cycle is critical (Heyman *et al.*, 2009b). As such the best approach is preventing contact with rodents and their aerosolised excretions, and includes rodent control, rodent-proofing housing and food storage, and precautions to prevent the inhalation of aerosolised virus (Vapalahti *et al.*, 2003). Proposed risk factors for acquiring hantavirus infection include professions that may come into contact with rodents, cigarette smokers, and men more than women (Abu Sin *et al.*, 2007; Ahlm *et al.*, 1998; Makary *et al.*, 2010; Vapalahti *et al.*, 1999; Vapalahti *et al.*, 2010).

1.1.3.1 Hantavirus in humans in the UK

There have been very few human hantavirus cases reported in the UK. In the majority of instances the causative virus species was not confirmed in clinical specimens and the evidence is at best sporadic and highly insufficient in details (reviewed in Fhogartaigh *et al.*, 2011; McCaughey & Hart, 2000) (Table 1.2). The first recorded human case was in 1983 in a young male from Glasgow who was admitted with a clinical presentation suggestive of hantavirus disease and was found to have high titres to HTNV antibodies in his serum (Table 1.2) (Walker *et al.*, 1984). Between this first report and 2010 a further eight individual cases were detailed, some sharing symptoms as well as additional ones and even no renal

involvement in some cases (Kudesia *et al.*, 1988; Pether *et al.*, 1991; Pether *et al.*, 1993; Phillips *et al.*, 1991; Rice *et al.*, 1993; Watson *et al.*, 1997) (Table 1.2). These cases clearly show an inconsistent clinical picture for HFRS in the UK or possibly indicates the presence of more than one hantavirus species circulating. In several cases it was noted that rodent exposure coincided with the onset of symptoms and in only one case (2010) had a patient travelled abroad suggesting the circulation of an indigenous hantavirus within the UK.

Subsequent sero-epidemiological studies of potential at risk groups were conducted; involving those coming into close contact with rodents and their faeces/urine (e.g. farmers). Whilst many of the studies failed to identify the etiological agent, sero-prevalences of hantavirus specific antibodies of approximately 1-4.8 % of farmers (Coleman, 2000; Davies *et al.*, 1988; Stanford *et al.*, 1990; Thomas *et al.*, 1999) and 3.5-8.8 % in residents of Somerset (Pether & Lloyd, 1993) have been reported. Clinical cases include 2.1 % in patients presenting with HFRS symptoms reported from Northern Ireland (NI) (McKenna *et al.*, 1994), and higher seroprevalences have been found as a result of laboratory acquired infections in the UK (Lloyd *et al.*, 1984; Lloyd & Jones, 1986; Smith & Palmer, 1996). The majority of these studies included healthy individuals and as such suggests past exposure to hantaviruses and consequently a subclinical infection circulating.

Whilst these studies provide compelling evidence of the existence of hantaviruses in the UK, they did not confirm which hantavirus species were involved. In each case, hantaviral antibodies were detected by immunofluorescence assay (IFA) rather than actual virus and in several cases it was not specified exactly which hantavirus antigen was tested against. Reports that did describe a specific serotype in the UK suggested a Hantaan-like infection (Davies *et al.*, 1988; Walker *et al.*, 1984) and a Seoul-like infection (McKenna *et al.*, 1994) implicating *R. norvegicus* as likely sources, as cross-reactivity is common among *Murinae*-associated hantaviruses. Prior to 2012, in all but the lab acquired cases, a hantaviral agent responsible was not identified making these cases difficult to validate. This is most likely due to several factors, there was no rodent available to test, no molecular tests being employed,

difficulties in isolating the virus, difficulties in detecting virus in human cases and the cross reactivity between hantaviruses in serological testing.

In 2012, the first confirmation of SEOV RNA in UK brown rats was reported (Jameson *et al.*, 2013a). A patient from the Humber region with suspected hantavirus disease was confirmed to have high hantavirus antibodies (using HNTV and SEOV antigen). Hantavirus RNA was detected in the lungs of two of four brown rats trapped at the patient's residence. The hantavirus was confirmed as SEOV and designated strain Humber (Jameson *et al.*, 2013a). There are several ports situated along the Humber estuary that could have facilitated the introduction of SEOV infected rats into the UK. Interestingly however genetic analysis of this strain confirm it to be most similar to IR461, a strain that was previously responsible for UK human laboratory-acquired infections (Jameson *et al.*, 2013a; Shi *et al.*, 2003).

1.1.3.2 Hantavirus in UK rodents and domestic pets

The UK supports numerous rodent species including several species of mice, rats and voles. In 1993 in Somerset following a surveillance of human patients, hantavirus antibodies were detected in 4 % brown rats and ~1 % of 'mice' (Pether & Lloyd, 1993). Another study screened a cohort of 127 brown rats from 11 British farmsteads, and found five animals were positive for hantavirus antibodies and four were reactive to HTNV and one to SEOV (Lloyd, 1991; Webster & Macdonald, 1995). A study in Northern Ireland detected antibodies to HTNV and SEOV in brown rats (21.6 %), wood mice (*A. sylvaticus*) (3.2 %) and house mice (*Mus musculus*) (28.8 %) (McCaughey *et al.*, 1996). An earlier report had also found 7.4 % mice to be sero-positive in Northern Ireland (one house mouse and one wood mouse) (Davies *et al.*, 1988; Stanford *et al.*, 1990). Despite virus antibodies being detected in wild rodents no hantaviral agent had previously been isolated.

In addition, a serological survey of domestic cats in the UK reported the presence of hantavirus specific antibodies to HTNV in 15 % of domestic cats and 23 % of chronically ill cats (Bennett *et al.*, 1990). More recently human cases of suspected hantavirus disease have led to the detection and subsequent isolation of SEOV from pet rats in North Wales and Oxfordshire (Jameson *et al.*, 2013b; Taori *et al.*, 2013). Both of these studies highlight the public health concern that domestic pets could

pose, as closer contact with humans will inevitability result in a greater risk of zoonotic disease.

Table 1.2. Summary of the human cases of suspected hantavirus disease in the UK.

Year	Location	Age	Gender	Symptoms	Diagnostic Test	Antigen	Antibodies present	Ref.
1983	Glasgow	21	Male	Fever, submandibular swelling, conjunctivitis, erythematous rash.	IFA	HTNV	IgM & IgG	(Walker *et al.*, 1984)
1988	Glasgow	18	Male	Fever, abdominal pain, headache, haematuria.	IFA	-	IgG & IgM (?)	(Kudesia *et al.*, 1988)
1991	Somerset	42	Male	Abdominal pain, enlarged spleen and liver, rash, arthralgia.	IFA	-	IgM & IgG	(Pether *et al.*, 1991)
1991	Somerset	64	Female	Fever, rash, conjunctivitis, submandibular swelling, enlarged spleen & liver.	IFA	-	IgM & IgG	(Phillips *et al.*, 1991)
1991	Somerset	21	Male	Arthropathy, vasculitic rash, abdominal pain.	IFA	-	IgM & IgG	(Pether *et al.*, 1993)
1991	Sheffield	16	Female	Arthralgia, erythematous rash, abdominal pain.	IFA	-	IgG	(Rice *et al.*, 1993)
1991	Sheffield	18	Female	Abdominal pain, lethargy, arthralgia.	IFA	-	IgG	(Rice *et al.*, 1993)

Table 1.2. continued.

Year	Location	Age	Gender	Symptoms	Diagnostic Test	Antigen	Antibodies present	Ref.
1994	Nottingham	10	Male	Nausea, abdominal pain, haematuria.	IFA	-	IgM & IgG	(Watson *et al.*, 1997)
2010	London[a]	35	Male	Headache, backache, fever, myalgia.	IFA	PUUV	IgG	(Fhogartaigh *et al.*, 2011)
2011	Oxfordshire[b]	-	Male	Acute renal impairment, fever, splenomegaly, thrombocytopenia.	IFA	HTNV & SEOV	IgG	(Taori *et al.*, 2013)
2012	Yorkshire & Humber	-	-	Acute kidney injury	IFA	HTNV & SEOV	IgG	(Jameson *et al.*, 2013a)
2012	North Wales[b]	28	Male	Acute kidney injury,fever, shivers, sweating, vomiting,	IFA	HTNV & SEOV	IgG	(Taori *et al.*, 2013)
2013	Oxfordshire[b]	-	Female	-	IFA	HTNV & SEOV	IgG	(Taori *et al.*, 2013)

[a]Recent travel to Estonia
[b]Exposure to same population of pet rats

1.1.4 Ljungan virus

Ljungan virus (LV) (Family *Picornaviridae*, genus *Parechovirus*) was the second viral species within the parechovirus genus to be discovered and unlike Human parechoviruses (HPeV), LV is thought to be zoonotic (Niklasson *et al.*, 1999). Two initial lines of evidence led to the discovery of Ljungan virus and its potential impact on human health. Firstly, a group of orienteers contracted lethal cases of myocarditis in Sweden between 1989-1992, where it was assumed that contact must have been made with an etiological agent during path finding competitions (Wesslen *et al.*, 1992); secondly, human incidences of myocarditis, insulin-dependent diabetes mellitus (Type 1) and Guillain-Barré syndrome (GBS) followed the 3- to 4- year population fluctuation cycles of bank voles (*M. glareolus*) in northern Sweden (Hansson & Henttonen, 1985; Niklasson *et al.*, 1998). It was hypothesised that these diseases could be caused or triggered by an infectious agent carried by bank voles. Subsequently, three new parechoviruses were isolated from Swedish bank voles, and were named Ljungan virus (referring to the site of isolation). ICTV classify the *Parechovirus* genus in to two distinct species HPeV and LV, LV is then further classified into four genotypes, LV87-012, LV174F (gt1) and LV145SL (gt2), LVM1146 (gt3) and LV64-7855 (gt4) (Table 1.3). They are single stranded positive sense RNA viruses with a genome approximately 7.5 kb and composed of eleven proteins (Figure 1.3).

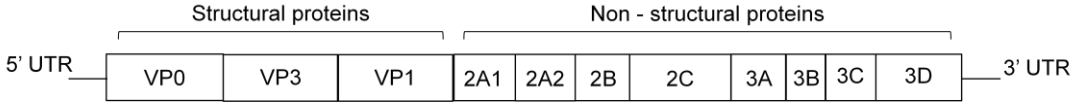


Figure 1.3. Schematic diagram illustrating the genomic organisation of Ljungan virus. The open reading frame encodes a polyprotein approximately 2,256 amino acids (aa) in length and is flanked by a 5'-untranslated region (UTR) and a 3'-UTR. The polyprotein is made up of 11 individual proteins: VP0 (259 aa), VP3 (244 aa), VP1 (297 aa), 2A1 (20 aa), 2A2 (135 aa), 2B (138 aa), 2C (333 aa), 3A (130 aa), 3B (29 aa), 3C (198 aa) and 3D (470 aa). Protein lengths are according to prototype strain LV87-012 (Accession number AF327920).

1.1.4.1 LV in wild animals

In general, LV appears to have a relatively wide geographical range, having been detected in both Europe and America although surveillance is limited. Within Europe, LV antibodies and antigens have been detected in Scandinavia in a number of rodents including *M. glareolus* (bank vole) (Niklasson *et al.*, 1998; Niklasson *et al.*, 2007a; Niklasson *et al.*, 1999), *Myodes rufocanus* (grey-sided vole), *Microtus agrestis* (field vole), *Lemmus lemmus* (Norway lemming), *Myopus schistocolor* (wood lemming) (Niklasson *et al.*, 2006a) and in northern Italy LV RNA has been detected in *A. flavicollis* (yellow-necked mouse) (Hauffe *et al.*, 2010). In 2013, LV RNA was also reported in a proportion of rural rodent species, *M. glareolus*, *M. agrestis*, *M. musculus* (house mouse) and *A. Sylvaticus* (wood mouse) in Kielder forest, Northumberland UK (Salisbury *et al.*, 2013). In North America, *Myodes gapperi* (southern red-backed vole) and *Microtus montanus* (montane vole) have been reported as hosts for LV (Johansson *et al.*, 2003; Johnson, 1965; Main *et al.*, 1976; Tolf *et al.*, 2009; Whitney *et al.*, 1970). LV does not appear to be restricted to rodent hosts, as it has been found (diagnostic not specified) in the arctic fox (*Vulpes lagopus*) (Niklasson *et al.*, 2007b) and LV-specific antigens have been found in foxes with hydrocephaly (*Vulpes vulpes*) from the UK (Niklasson unpublished). It remains to be determined whether all these potential hosts are capable of acting as reservoirs for Ljungan virus. In addition it is possible that additional surveillance for LV, which is a relatively recently-described virus, may expand the known distribution of this virus and increase the diversity of host species. There are currently no data to determine the route of transmission for Ljungan virus, though it is proposed to be like that of related parechoviruses (HPeV) and other picornaviruses, via the faecal-oral route (McDonald, 2009). However the varied host species range exhibited could give support to alternative routes (Niklasson *et al.*, 2007b).

Table 1.3. Isolation details for the five currently characterised Ljungan virus strains.

Genotype	Strain	Isolation			Ref.
		Year	Location	Species	
1	LV87-012	1994	Medelpad County, Sweden	*Myodes glareolus*	(Niklasson *et al.*, 1999)
	LV174F	1994	Medelpad County, Sweden	*Myodes glareolus*	(Niklasson *et al.*, 1999)
2	LV145SL	1994	Vasterbotten County, Sweden	*Myodes glareolus*	(Niklasson *et al.*, 1999)
3	LVM1146	1962	Oregon, USA	*Microtus montanus*	(Johansson *et al.*, 2003)
4	LV64-7855	1964/1965	St. Lawrence County, New York, USA	*Myodes gapperi*	(Tolf *et al.*, 2009)

1.1.4.2 LV and disease

All current evidence for LV and its pathogenesis disease comes from rodent models. Experimental infection of LV in suckling mice produced fatal outcomes (Johansson *et al.*, 2003; Niklasson *et al.*, 1999; Niklasson *et al.*, 2006a) and in CD-1 mice LV can induce diabetes (Type 1 and Type 2), myocarditis, foetal malformations and reproductive problems (Niklasson *et al.*, 2006a; Niklasson *et al.*, 2006b; Samsioe *et al.*, 2006). Additionally it is possible that stress plays an important role in the development of disease in laboratory rodent models, whereby a combination of virus insult and stress induces disease, whilst either stress or virus alone produces little or no disease pathology (Niklasson *et al.*, 2003b; Niklasson *et al.*, 2006b; Samsioe *et al.*, 2006). Attempts to confirm these results in wild rodents, however, have failed as efforts to establish and maintain a pathogen free bank vole colony have been unsuccessful (Niklasson *et al.*, 2003b; Niklasson *et al.*, 2006b). Nonetheless, a diabetes-like disease similar to that observed in laboratory mice and consistent with human type 1 diabetes (Niklasson *et al.*, 2003a) has been reported in several wild rodent species (*M. rufocanus, M. glareolus, M. agrestis* and *L. lemmus*), both directly at capture and after a duration in captivity and this was shown to be associated with the presence of LV antigen (Freimanis *et al.*, 2003; Niklasson *et al.*, 2003a; Niklasson *et al.*, 2003b; Niklasson *et al.*, 2006a; Schoenecker *et al.*, 2000). However, to date an unequivocal connection between LV and disease in wild rodents has not been confirmed.

1.1.4.3 LV in humans

While there is presently no evidence confirming Ljungan virus as an etiological agent for human diseases, there are strong statistical associations between the bank vole population fluctuations (3-4 yr cycles) (Hansson & Henttonen, 1985; Niklasson *et al.*, 1998) and the incidences of several human diseases in Sweden - insulin-dependent diabetes, myocarditis, GBS (Niklasson *et al.*, 1998), intrauterine foetal death (IUFD) (Niklasson *et al.*, 2007b), sudden infant death syndrome (SIDS) (Niklasson *et al.*, 2009a). There is also evidence of LV antigens and viral RNA being detected in specific human disease cases (Niklasson *et al.*, 2009a; Niklasson *et al.*, 2003a; Niklasson *et al.*, 1999; Niklasson *et al.*, 2009b; Niklasson *et al.*, 2007b). Whilst such evidence presents a compelling argument for the involvement of LV in a range of human diseases, the validity of some of these reports has been questioned

(Kinney & Thach, 2009; Krous & Langlois, 2009; 2010). The evidence for the association of LV with SIDs in particular is unconvincing at present due to the small number of human cases included in the study, no pathological changes observed in the SIDS cases despite identifying the virus, and virus was not found in every SIDS case (Kinney & Thach, 2009; Krous & Langlois, 2009). As for other LV human and rodent studies, attempts were not made to exclude other potential etiological agents, and once again small sample sizes continue to be a concern (Krous & Langlois, 2010). Nonetheless, due to potential diseases, screening for LV is essential to adequately assess the disease associations.

1.2 Methods

1.2.1 Screening method used in surveillances

There are two principal methods that can be used in diagnostic virology which have already been successfully implemented for both HV and LV detection, nucleic acid amplification and serology. The choice of which depends very much on the biological question being asked but the merits of each are discussed.

Serological assays involve testing for the presence of virus specific antibodies in samples such as serum, cerebrospinal fluid (CSF) and oral fluids, and include methods such as enzyme-linked immunosorbant assays (ELISA), virus neutralisation assays (vNA) and immunofluorescence assays (IFA). These assays have the advantage of being rapid which is of particular use for clinical diagnostic purposes, can be used to detect prior (IgG) and recent (IgM) viral exposure, used in place of traditional culturing techniques if the virus is difficult to cultivate, and is useful for non-invasive sampling i.e. oral fluids (Jeffery & Aarons, 2009). Disadvantages include cross-reactivity, poor sensitivity to some viruses, inherent potential for false positives, non-specificity of assays meaning the causative virus species cannot be distinguished, and these methods are measuring immune response rather than actual virus (Jeffery & Aarons, 2009).

Nucleic acid amplification techniques offer an alternative approach to screening and in many ways they improve the diagnostic capability compared to that of serological tests. Nucleic acid assays such as polymerase chain reaction (PCR) involve the amplification of a specific target. They can be used qualitatively and quantitatively

in diagnostics, the sensitivity can exceed other diagnostic systems, they are rapid, and typing of the target enables the differentiation of virus species (Jeffery & Aarons, 2009). These assays can be used on any type of tissue samples unlike serology which are restricted to bodily fluids. The main disadvantages to nucleic acid methods are that due to the increased sensitivity, there is an inherent risk of contamination. Also to design such assays it is essential to have knowledge of the target sequence (Jeffery & Aarons, 2009).

Within this thesis rodents will be screened using nucleic acid methods rather than serological because of the increased sensitivity, it will enable typing of any etiological agent we find but also we are testing multiple tissues which are more suitable for the specific detection of each virus species (e.g. kidney, liver or lung). The use of tissues rather than blood or urine is more advantageous in this study because in terms of hantaviruses the animal may not be shedding the virus or be viraemic. With regards to Ljungan virus since we are uncertain of the mode of transmission, urine or faeces may not be suitable.

1.2.2 Next Generation Sequencing

Advances in molecular technologies have led to the development of next generation sequencing (NGS) techniques. These have enabled millions of nucleotides to be sequenced in a very short time. There are several different chemistries, however, the one that will be employed in this thesis is pyrosequencing. Pyrosequencing involves a method of 'sequencing by synthesis' which consists of the release of pyrophosphate (PPi) following every nucleotide incorporation, and the subsequent conversion via ATP to generate light that is proportional to the number of incorporated bases (Radford *et al.*, 2012). There are many applications that NGS brings to virology including full genome sequencing, viral quasispecies that could lead to resistance and evolution, genome characterisation, and pathogen discovery (Radford *et al.*, 2012). NGS enables the rapid collection of genetic information and will be used within this thesis to provide a better understanding of both viruses.

1.3 Aim

This thesis is concerned with the targeted surveillance for hantaviruses and Ljungan virus in rodent species from a range of habitats.

1.4 Study species

1.4.1 Rodent species

Within this study three rodent species were sampled from urban areas, brown rats (*R. norvegicus*), house mice (*M. musculus*) and wood mice (*A. sylvaticus*) and three from semi-rural environments, bank voles (*M. glareolus*), field voles (*M. agrestis*) and wood mice (Table 1.4 & Figure 1.4).

Bank voles (*M. glareolus*) (Figure 1.4a) are found throughout mainland Britain but are absent from many offshore islands, and there are an estimated 23 million individuals (Harris & Yalden, 2008). These are primarily a diurnal species that inhabit woodland, scrubland and hedgerows and their diet mainly consists of grass, fruit, seeds and insects. Bank voles have been reported to be infected with PUUV and LV on continental Europe (Niklasson *et al.*, 1999; Vaheri *et al.*, 2012) and the UK (Salisbury *et al.*, 2013). Whilst they are primarily a rural species, human activity may bring them into closer contact and increase the risk from either of these viruses.

Field voles (*M. agrestis*) (Figure 1.4b) are one of the most numerous mammals of mainland Britain, with estimated populations exceeding 75 million (Harris & Yalden, 2008). They mainly inhabit ungrazed grassland and are primarily herbivorous in their diet. During the summer they tend to be more active during the night however in the winter they appear to change to a diurnal lifestyle. Whilst LV antibodies and antigens have been detected in field voles in Scandinavia (Niklasson *et al.*, 2006a) and LV RNA in the UK (Salisbury *et al.*, 2013), they have not been reported as a primary reservoir for hantaviruses, although they have been associated with the maintenance of TULV in Germany (Schmidt-Chanasit *et al.*, 2010).

The wood mouse (*A. sylvaticus*) (Figure 1.4c) can be found in both urban and semi-rural environments across Britain and Ireland. They are a common and widespread species with an estimated population of 38 million (Harris & Yalden, 2008), and inhabit a range of habitats. They are generally nocturnal species and have an omnivorous diet. The wood mouse has not been reported as a carrier for a hantavirus however other members of the *Apodemus* genus have been associated with DOBV, HTNV and SAAV (Avsic-Zupanc *et al.*, 1992; Lee *et al.*, 1978; Plyusnin *et al.*,

1997b). Salisbury *et al.,* (2013) published the detection of LV RNA for the first time in wood mice and in the UK.

The Norwegian/brown rat (*R. norvegicus*) (Figure 1.4d) represents a more cosmopolitan species having been found on every continent except for Antarctica. Its widespread distribution can be attributed to the extensive human movements over the past few centuries. Rats are mainly nocturnal and are highly variable in both habitat and diet preference and so can be found living in close proximity to humans. The brown rat population is estimated at, at least 6 million (Harris & Yalden, 2008). Whilst wild brown rats are associated with the distribution of SEOV around the world, they have not been reported to be infected with LV.

House mice (*M. musculus*) (Figure 1.4e) are thought to be the most widely distributed mammal after humans, originating from south western Asia, as a result of human movements, and as such this species is heavily associated with humans and buildings (Cucchi *et al.,* 2005). There are an estimated 5 million house mice in the UK (Harris & Yalden, 2008). They tend to be a nocturnal species and have an omnivorous diet. Currently hantaviruses have not been detected in house mice although their close proximity to humans puts them as a risk. LV RNA has however been detected in house mice in the UK (Salisbury *et al.,* 2013).

Table 1.4. Summary of rodent species included within this study.

Common name	Species	Food preference	Diurnal/ Nocturnal	Estimated UK populations[1]	Rural/ Urban	Previous LV infection	Previous HV infection
Bank vole	*M. glareolus*	Omnivorous	Diurnal	23,000,000	Rural	Yes[2,3]	PUUV[4]
Field vole	*M. agrestis*	Herbivorous	Annual rhythm: nocturnal (summer), diurnal (winter)	75,000,000	Rural	Yes[3,5]	Associated with TULV[6]
Wood mouse	*A. sylvaticus*	Omnivorous	Nocturnal	38,000,000	Both	Yes[3]	No
Norway (brown) rat	*R. norvegicus*	Omnivorous	Nocturnal	6,790,000	Both	No	SEOV[7]
House mouse	*M. musculus*	Omnivorous	Nocturnal	5,192,000	Urban	Yes[3]	No

[1](Harris & Yalden, 2008), [2](Niklasson *et al.*, 1999), [3](Salisbury *et al.*, 2013), [4](Brummer-Korvenkontio *et al.*, 1980), [5](Niklasson *et al.*, 2006a), [6](Schmidt-Chanasit *et al.*, 2010), [7](Heyman *et al.*, 2004).

a) 

b) 

c) 

d) 

e) 

Figure 1.4. Rodent study species within this investigation, a) bank vole *M. glareolus*, b) field vole *M. agrestis* courtesy of E. Oksanen, c) wood mouse *A. sylvaticus*, d) brown rat *R. norvegicus* courtesy of Ernie Janese, and e) the house mouse *M musculus* courtesy of Stephen Dalton.

1.5 Chapter outlines

Chapter 2: Field work

A large part of this project required the capture of rodents from around urban areas of the North West UK. The aim of Chapter 2 is to provide a record of the trapping method used, capture success and problems encountered throughout the trapping period.

Chapter 3: Targeted surveillance for Ljungan virus in UK wildlife.

LV is associated with human diseases and has been detected in several rodent species in Sweden, Denmark, Italy, America and the UK. There is also evidence of LV circulating in foxes in the UK. The aim of Chapter 3 is to determine if LV is also circulating in rodents from the North West UK.

Chapter 4: Molecular characterisation of two new Swedish Ljungan virus isolates, LV340 and LV342.

There are currently five full LV genomes published and yet there remain many unanswered questions regarding the virus' biology and evolution, by using next-generation sequencing technology we hope to get a better understanding of this virus and to add to the limited genome data currently available. In Chapter 4 using 454 pyrosequencing we characterise a further two LV isolates.

Chapter 5: Targeted surveillance for hantaviruses in UK wildlife.

Circulation of a hantavirus species in UK wildlife has only recently been confirmed in rats. However it remains to be answered if other rodents are carriers of hantaviruses. Chapter 5 screens rodents from semi-rural and urban environments of the North West UK for hantaviruses. Based on the evidence we hypothesise to find SEOV circulating in rats around the North West. The main reasons for this are that Liverpool is a major port and since rat distributions are facilitated by sea travel, SEOV may be introduced from endemic countries. SEOV is the cause of a relatively moderate form of HFRS which is frequently underreported. The previous UK human case reports suggest the potential for SEOV to be circulating throughout the country. We might also expect to find PUUV circulating in the UK however previous surveillances have not found evidence to suggest so (Henttonen & Bennett, pers. comms).

Chapter 6: Seoul virus in rats in Rhône-Alpes.

In France the situation is somewhat different with currently three of the five European hantaviruses being confirmed circulating: PUUV, SEOV and TULV (Heyman *et al.*, 2011; Heyman *et al.*, 2004; Plyusnina *et al.*, 2007). Prevalence of SEOV in rats in France has so far been based on antibody detection (Heyman *et al.*, 2004) however a prevalence study using a direct detection method (PCR) is required to assess the true prevalence in the rodent species to thereby understand the risk of transmission to humans. In Chapter 6 in collaboration with the FP7 WildTech project we screened brown rats from urban and rural areas of Rhône-Alpes for hantaviruses.

Chapter 7: Pet rats

Recent reported human hantavirus cases have been associated with pet rats. In this chapter we outline the distribution of SEOV RNA in the organs of an infected brown rat and the viral distribution of viral RNA in the lung and kidneys of a closed colony of 21 breeding rats.

Chapter 2. General Methods

2.1 Study sites

2.1.1 North West, UK

Animals were caught around the regions of Liverpool, Wirral and Cheshire. Trapping in these areas enabled the capture of several rodent species (Figure 1.1), a number of which have been reported as carriers for hantaviruses and Ljungan virus across Europe. The North West is a particularly good study area due to the multiple habitat types found in close proximity including urban (Liverpool), port (Liverpool), and semi-rural areas (Cheshire and the Wirral). Liverpool port is situated in the North West of England and is the third busiest in the country, and SEOV has been detected in several port cities around the world (e.g. (Cueto *et al.*, 2008; Glass *et al.*, 1994; Ibrahim *et al.*, 1996; Iversson *et al.*, 1994; Reynes *et al.*, 2003; Wu *et al.*, 2007). It could also facilitate the transportation of other infected non-indigenous rodents into the UK. Urban areas are important study locations due to the increased human-rodent interactions and subsequent risk.

2.1.2 Rhône-Alpes, France

A second study area included sites situated in the Rhône-Alpes region of France. This was specifically for trapping brown rats (*R. norvegicus*) as part of the FP7 WildTech project – Development of a microarray to detect a wide range of rodent-borne pathogens. This study area was particularly good due to the presence of both urban (Lyon) and semi-rural areas just outside of Lyon.

2.2 Field work

2.2.1 Pest control data

Prior to commencing field work the Liverpool Local pest control were approached to advise on ideal trapping sites around urban areas of Liverpool. They provided rodent "call-out" data for internal rats, external rats and mice for each electoral ward. Internal rats refer to animals reported within dwellings, external rats outside, and mice reports include both internal and external animals.

Analysing this data highlighted several hotspots around Liverpool that exhibited relatively large proportions of rodent call-outs (Figure 2.1). Some of the wards had

consistently high call-outs for all three types of rodents. It was also the perception of allotment holders and the local pest control that rodent populations have been increasing. However, this was not observed for either internal or external rats over seven years of data, rather numbers seem to be declining. Potential population fluctuation cycles of mice may be present as seen in the mice call-out data (Table 2.1). Absence of cycles may be as a result of animals being in a fragmented urban environment. This general lack of cycles meant we could not make associations with the limited human disease data that we had. It did however draw attention to seasonal changes suggesting that call-outs did appear to reduce between October and December suggesting them as potentially sub-optimal trapping times of the year.

Table 2.1. Total rodent call-outs for the Liverpool area, over seven years (April 2006-March 2013).

	Total call-outs		
Year (April-March)	**Rats Internal**	**Rats External**	**Mice**
2006-2007	2495	4420	3042
2007-2008	2583	4183	3157
2008-2009	2600	4403	3426
2009-2010	2432	3785	3291
2010-2011	1948	3592	3053
2011-2012	1970	3252	3194
2012-2013	1860	3195	3368

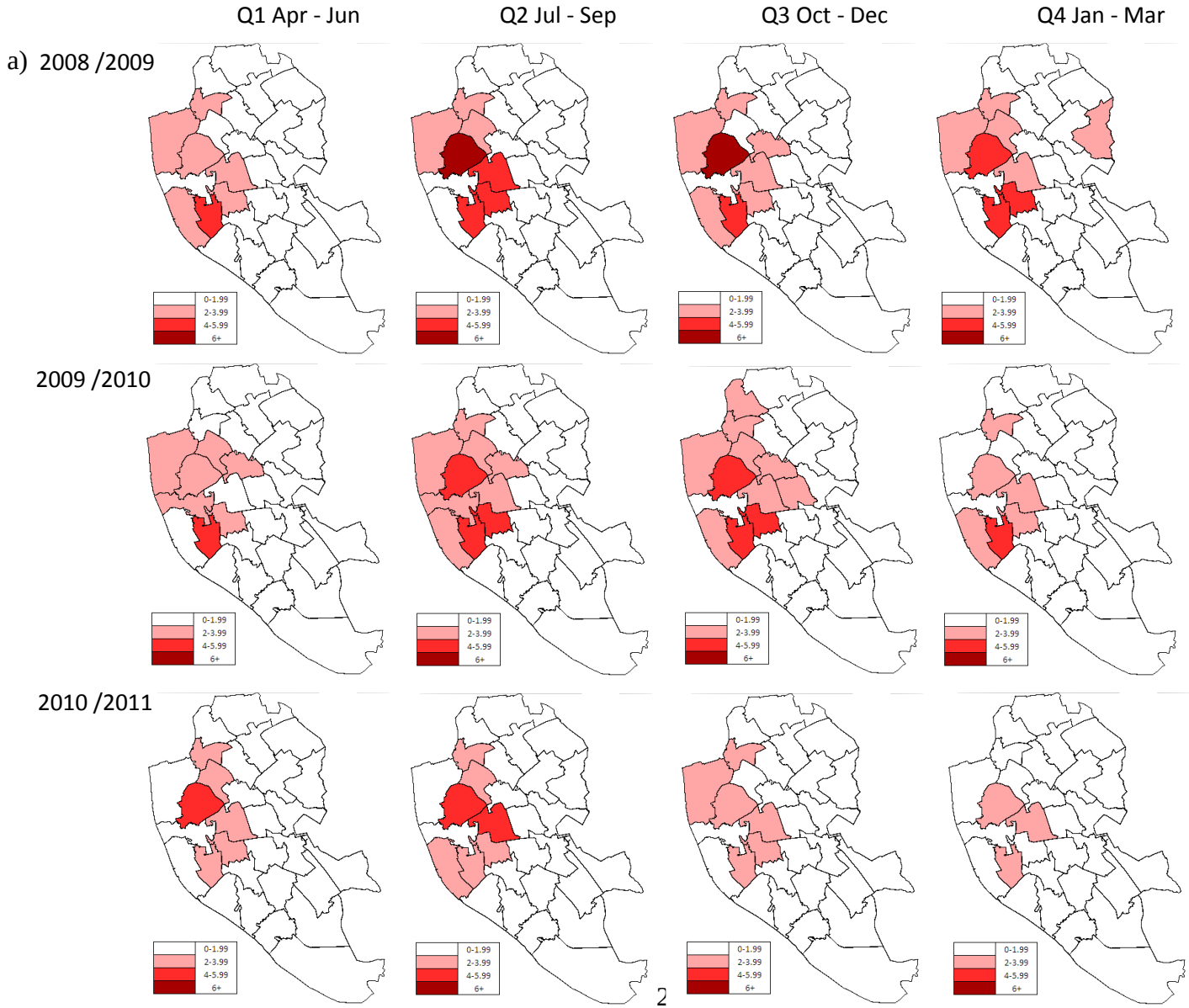


Figure 2.1. Spatial change in frequency of rodent call-outs reported to the Local pest control over the time period April 2008-March 2010, a) internal rats, b) external rats and c) mice. Colour charts represent the frequency of rodents per 1000 people.

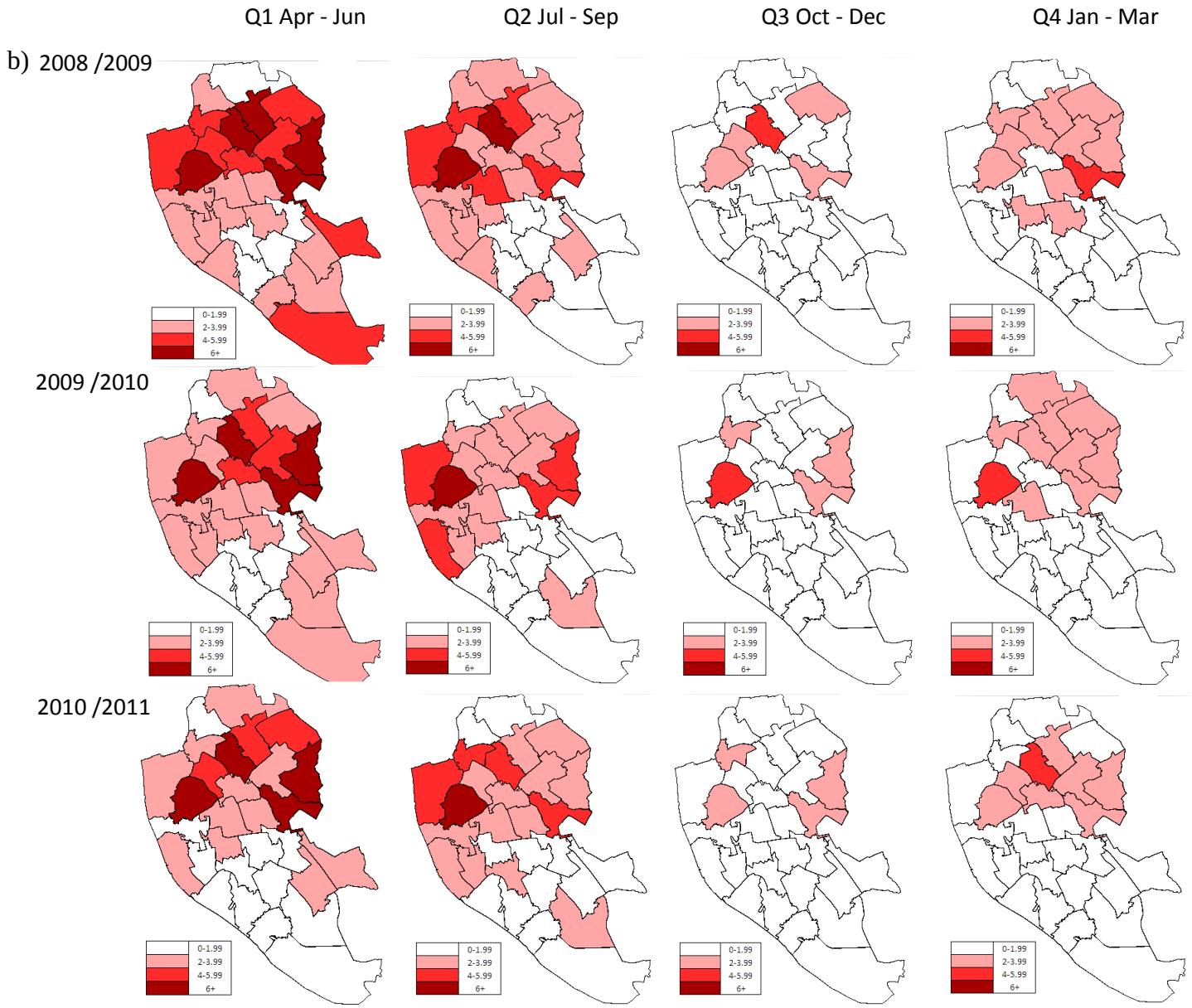


Figure 2.1. continued.

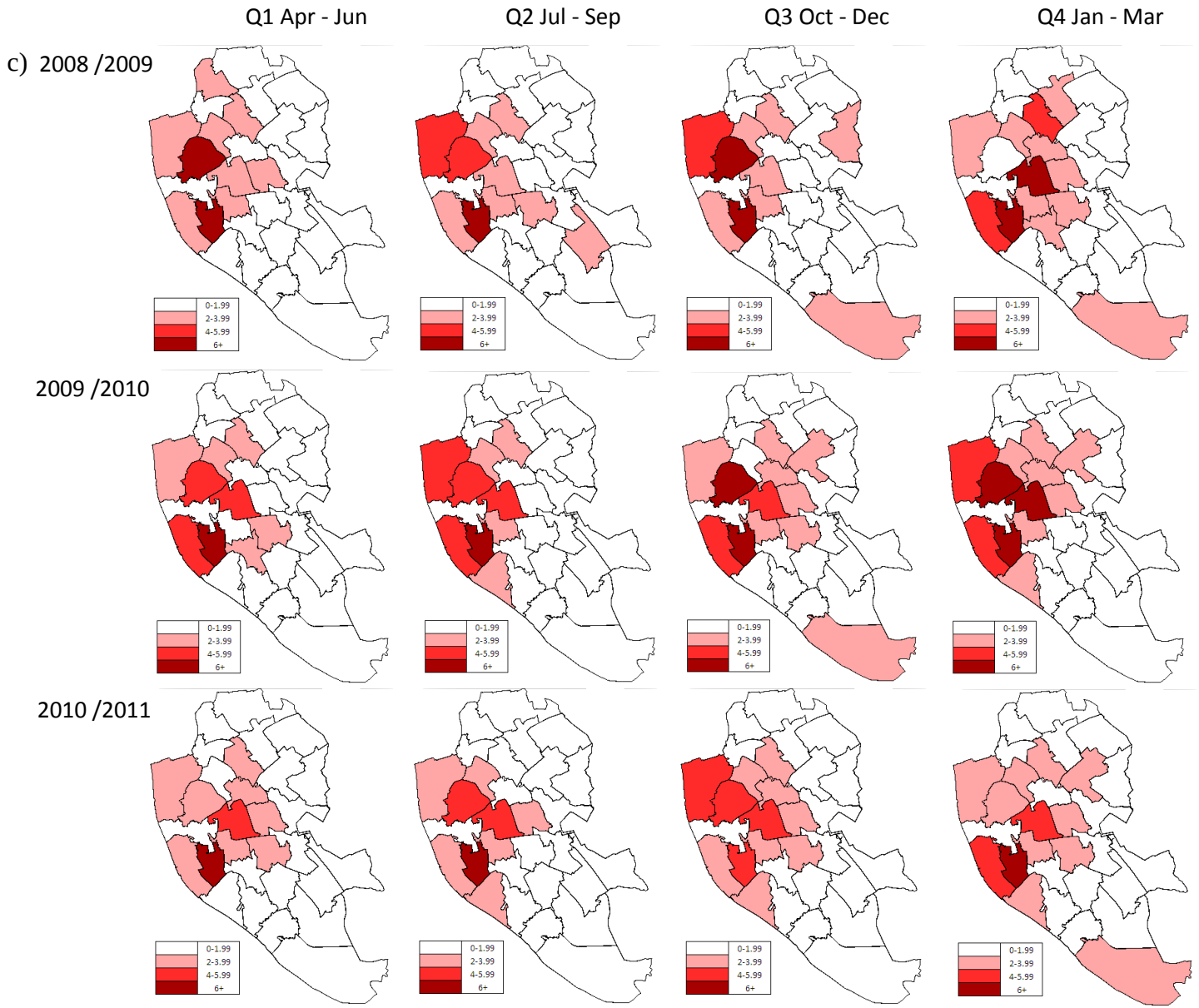


Figure 2.1. continued.

The rodent call-out data is the only indicator of rodent abundance fluctuations within an urban environment. However the data is subjective; there is a different response from the local pest control depending on whether the animal reported is indoors or outdoors and whether it is a rat or a mouse (level of importance: internal rats > external rats > mice), and errors could arise in mis-identification or public awareness of the preferential treatment. The data does not take into account numbers, as a call-out just refers to a single data point. Electoral wards with low rodent call-outs could be a result of an underreporting of such rodent problems due to the bad social connotations that come with it rather than there actually being less rodents there.

This evidence was useful in highlighting that previous word of mouth indications of the numbers of rodents increasing over the past few years does not seem to be the case. The Local pest control were also approached to assist in providing animals for the project however since their strategy is to poison rather than trap they were unable to provide us with any samples. Also their customers are mainly business contracts and domestic homes who want the animals regarded as pests, removed quickly rather than trapped, which would take longer.

2.2.2 Rodent trapping

Field work was carried out between October 2009 - August 2011. Field sites were identified on the criteria of being within an urban environment; having easy access; out of direct public attention; and having experienced recent or current rodent activity. The majority of sites included allotments as they are good small sites surrounded by urbanised areas that are out of the way of the majority of the public. Animals were caught using 14" wire cages (Figure 2.2a) for the rats and Longworth small mammal traps (Figure 2.2b) for the mice. Nine sites were located around urban areas of Merseyside (Figure 2.3 & Table 2.2). In total 329 animals were caught consisting of brown rats (*Rattus norvegicus*), house mice (*Mus musculus*) and wood mice (*Apodemus sylvaticus*) (Table 2.3). Once caught recordings were taken for weight (g), gender, age estimate (Juvenile, sub-adult, adult) and any significant observations. Animals were humanely euthanised in the field by the inhalation of the anaesthetic, Isoflurane, following UK Home Office Guidelines.

a) b)

Figure 2.2. *In situ* images of the two traps that were used within this study, a) 14" wire cages, and b) Longworth small mammal traps.

Trapping was generally carried out Monday to Friday with a total of four trapping nights a week, and 156 trapping nights for the entire study period. With a total of 329 animals, the trapping rate was approximately 2.1 animals a day.

Table 2.2. Locations of each of the nine field sites.

Site	Longitude	Latitude	Description
Greenbank	53°23'10.12"N	2°55'47.9"W	Urban allotment
Lister drive	53°25'14.4"N	2°55'56.6"W	Urban allotment
Liverpool port	53°26'27.0"N	3°00'36.7"W	Port
Seeds lane	53°28'13.3"N	2°56'32.2"W	Urban allotment
Sefton	53°23'11.18"N	2°55'57.14"W	Urban allotment
Southport	53°39'15.9"N	3°00'42.9"W	Recreational area
Sudley Drive	53°22'13.81"N	2°55'19.90"W	Urban allotment
Thingwall	53°23'58.0"N	2°54'29.3"W	Urban allotment
University of Liverpool	53°24'24.7"N	2°57'44.2"W	Campus

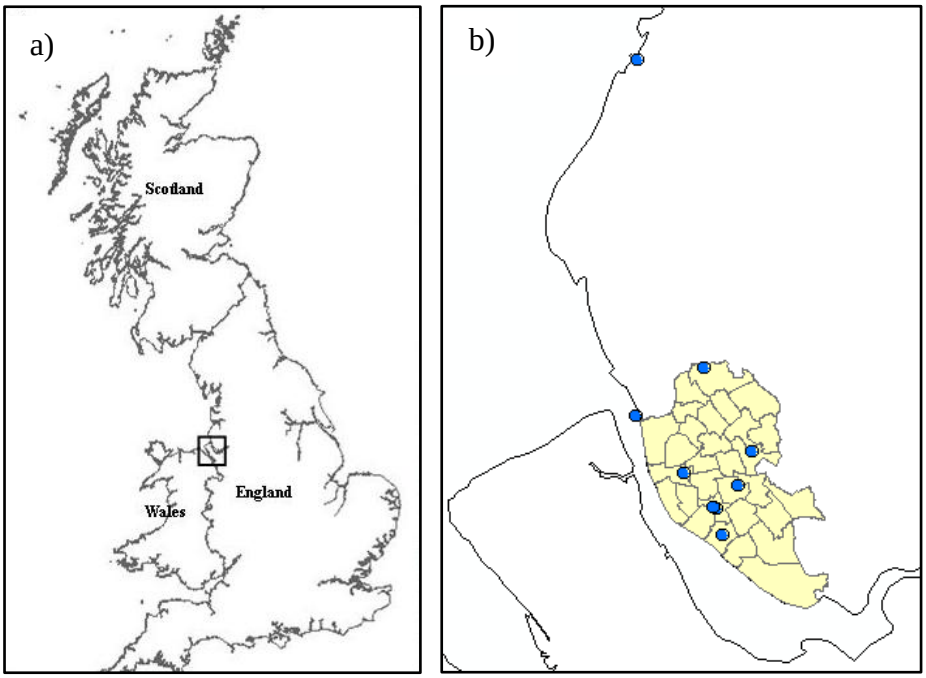


Figure 2.3. The location of the trapping sites within a) United Kingdom and b) North West England (sites marked by spots).

Table 2.3. Number of each rodent species caught at each of the nine field sites.

Site	**Brown rat (*R. norvegicus*)**	**House mouse (*M. musculus*)**	**Wood mouse (*A. sylvaticus*)**	**Total**
Greenbank	30	3	34	67
Lister drive	15	12	37	66
Liverpool port	15	0	5	20
Seeds lane	44	10	38	92
Sefton	4	0	28	32
Southport	2	0	0	2
Sudley Drive	5	0	16	21
Thingwall	4	5	7	16
University of Liverpool	10	5	0	15
Total	129	35	165	329

Several problems were encountered with the trapping of small mammals in urban areas.

1) Often there were sites which were recommended as potential trapping locations which had in the past experienced rodent activity. After further investigation and unsuccessful preliminary trapping nights, it was determined that for the benefits of trapping to exceed the time and financial costs, the site had to either be experiencing rodent activity at that time or had clear evidence of very recent activity e.g. clear runs and/or nests (Figure 2.4).
2) The accidental catching of other animals in traps also occurred on several occasions. This was most likely due to the types of bait being used that enticed other animals such as hedgehogs, magpies and brown birds, and the easy food source it provided. Bait and trap locations had to therefore be optimised to reduce by-catch.
3) Rats, in particular adults, are neophobic which means that they are fearful of new objects, this often placed delays on trapping as traps ideally had to be left open for a week prior to trapping and could not be moved to a new location without having to incur further delays.
4) A variety of baits had to be trialled that would encourage the attraction of rats. The bait had to be stable for as long as possible under different weather conditions, trigger the trap when taken and still be enticing to rats. Trialled baits included corn, grain, peanut butter, chocolate, smoked sausage, spam and tuna. The optimum bait was a slice of smoked sausage, this not only could be hooked on the trap to trigger it once the bait had been taken, but also it did not dry out too quickly and did not wash away following rain, it produced a strong aroma and it resulted in less by-catch compared to other baits.

2.2.3 Semi-rural rodents

To enhance sample sizes, additional rodents (n = 166) consisting of brown rats (*Rattus norvegicus*) (n = 4), wood mice (*A. sylvaticus*) (n = 104), bank voles (*Myodes glareolus*) (n = 50) and field voles (*Microtus agrestis*) (n = 8) were caught around semi-rural areas (Chester and Wirral). These samples were provided through collaborations with Chris Ball, Dr. Nicola Williams, Susan Withenshaw, Becci Barber, Dr. Niamh Quinn and Giovanni Pellegrini

2.3 Post mortems

Carcases from 23 of 129 (17.8 %) brown rats caught around urban areas of Liverpool were provided to Dr Udo Hetzel and Dr John McGarry (University of Liverpool) for the purpose of performing post mortems to find signs of pathological disease that could indicate to hantavirus or Ljungan virus infections (Appendix 1). The cohort was comprised of 11 males and 12 females. Due to financial constraints more rats were not included.

On post mortem analysis all 23 animals appeared to be 'healthy' with no histological abnormalities to suggest either hantavirus or Ljungan virus infections. However, helminths were observed in 15 animals (65.2 %). Identified helminths include *Trichosomoides crassicauda* in six animals (26.1 %), *Mastophorus muris* in three animals (13.0 %) and the zoonotic nematode *Capillaria hepatica* in at least two animals (8.7 %).

a) b)

Figure 2.4. Examples illustrating signs of rodent activity, a) a run and b) a nest.

Chapter 3. Ljungan virus surveillance

3.1 Abstract

Ljungan virus (LV) (family *Picornaviridae,* genus *Parechovirus*) are single-stranded RNA viruses. LV has been detected in several rodent species and has been shown to induce disease in laboratory rodent models. In humans however there are currently only associations between LV and human incidences of several diseases in Sweden. Previous surveillance has demonstrated the presence of LV RNA in rural rodents in Northumberland, North East UK. Between September 2009 and November 2011, wild rodents consisting of brown rats (*Rattus norvegicus*), wood mice (*A. sylvaticus*), house mice (*Mus musculus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*) were live caught across North West England (Cheshire, Liverpool and Wirral). Animals were screened using an in house optimised hemi-nested RT-PCR targeting the 5' UTR region. LV RNA was not detected in any of the 495 rodents tested. Validation and quality assurance panels confirmed the sensitivity and specificity of this assay for the purpose of rodent surveillances. Our findings and the detection of LV in rural rodents from a previous study suggest that LV may not be a major human health concern in urban areas. However with further human encroachment this situation might change.

3.2 Introduction

Ljungan virus (LV) (family *Picornaviridae*, genus *Parechovirus*) is a potentially serious zoonosis that has associations with several human diseases (McDonald, 2009). While a direct causative link between Ljungan virus and human disease has not yet been established, continued surveillance for this virus remains important.

As previously outlined, two initial lines of evidence led to the discovery of Ljungan virus and its potential impact on human health. Firstly, a group of orienteers contracted lethal cases of myocarditis in Sweden between 1989-1992, where it was assumed that contact must have been made with an etiological agent during path finding competitions (Wesslen *et al.*, 1992); secondly, human incidences of myocarditis, insulin-dependent diabetes mellitus (Type 1) and Guillain-Barré syndrome (GBS) followed the 3- to 4- year population fluctuation cycles of bank voles (*Myodes glareolus*) in northern Sweden (Hansson & Henttonen, 1985;

Niklasson *et al.*, 1998). It was hypothesised that these diseases could be caused or triggered by an infectious agent carried by bank voles. Subsequently, three new parechoviruses were isolated from Swedish bank voles, and were named Ljungan virus (referring to the site of isolation) strain 87-012, 174F and 145SL (Niklasson *et al.*, 1999). A further four LV isolates have been found, two from North America LVM1146 (Johansson *et al.*, 2003) and LV64-7855 (Tolf *et al.*, 2009), and two from Sweden, LV340 and LV342 (Chapter 4).

Ljungan virus is a single stranded positive sense RNA virus, whose genome is approximately 7.5 kb nucleotides (nt). The seven whole LV genomes so far described cluster into four "genotypes" (Tolf *et al.*, 2009): LV87-012 and LV174F (gt 1), LV145SL, LV340 and LV342 (gt 2) (Johansson et al., 2002) (Chapter 3), LVM1146 (gt 3) (Johansson et al., 2003) and LV64-7855 (gt 4) (Tolf et al., 2009). Presently gt 1 and 2 have been confirmed in bank voles in Europe whilst gt 3 and 4 have been found in two different vole species in America. Ultimately, however, little is known as to the host specificity and distributions of each LV.

In general, LV appears to have a relatively wide geographical range, having been detected in both Europe and America although surveillance is limited. Within Europe, LV antibodies and antigens have been detected in Scandinavia in a number of rodents including *M. glareolus* (bank vole) (Niklasson *et al.*, 1998; Niklasson *et al.*, 2007a; Niklasson *et al.*, 1999), *Myodes rufocanus* (grey-sided vole), *Microtus agrestis* (field vole), *Lemmus lemmus* (Norway lemming), *Myopus schistocolor* (wood lemming) (Niklasson *et al.*, 2006a) and in northern Italy LV RNA has been detected in *Apodemus flavicollis* (yellow-necked mouse) (Hauffe *et al.*, 2010). In 2013, LV RNA was also reported in a proportion of rural rodent species, *M. glareolus*, *M. agrestis*, *Mus musculus* (house mouse) and *Apodemus Sylvaticus* (wood mouse) in Kielder forest, Northumberland UK (Salisbury *et al.*, 2013). In North America, *Myodes gapperi* (southern red-backed vole) and *Microtus montanus* (montane vole) have been reported as hosts for LV (Johansson *et al.*, 2003; Johnson, 1965; Main *et al.*, 1976; Tolf *et al.*, 2009; Whitney *et al.*, 1970). LV does not appear to be restricted to rodent hosts, as it has been found (diagnostic not specified) in the arctic fox (*Vulpes lagopus*) (Niklasson *et al.*, 2007b) and LV-specific antigens have been found in foxes with hydrocephaly (*Vulpes vulpes*) from the UK (Niklasson

unpublished). It remains to be determined whether all these potential hosts are capable of acting as reservoirs for Ljungan virus. In addition it is possible that additional surveillance for LV, which is a relatively recently-described virus, may expand the known distribution of this virus and increase the diversity of host species. There are currently no data to determine the route of transmission for Ljungan virus, though it is proposed to be like that of related parechoviruses (HPeV) and other picornaviruses, via the faecal-oral route (McDonald, 2009). However the varied host species range exhibited could give support to alternative routes (Niklasson *et al.*, 2007b).

All current evidence for LV and its pathogenesis disease comes from rodent models. Experimental infection of LV in suckling mice produced fatal outcomes (Johansson *et al.*, 2003; Niklasson *et al.*, 1999; Niklasson *et al.*, 2006a) and in CD-1 mice LV can induce diabetes (Type 1 and Type 2), myocarditis, foetal malformations and reproductive problems (Niklasson *et al.*, 2006a; Niklasson *et al.*, 2006b; Samsioe *et al.*, 2006). Additionally, it is possible that stress plays an important role in the development of disease in laboratory rodent models, whereby a combination of virus insult and stress induces disease, whilst either stress or virus alone produces little or no disease pathology (Niklasson *et al.*, 2003b; Niklasson *et al.*, 2006b; Samsioe *et al.*, 2006). Attempts to confirm these results in wild rodents, however, have failed as efforts to establish and maintain a pathogen free bank vole colony have been unsuccessful (Niklasson *et al.*, 2003b; Niklasson *et al.*, 2006b). Nonetheless, a diabetes-like disease similar to that observed in laboratory mice and consistent with human type 1 diabetes (Niklasson *et al.*, 2003a) has been reported in several wild rodent species (*M. rufocanus, M. glareolus, M. agrestis* and *L. lemmus*), both directly at capture and after a duration in captivity and this was shown to be associated with the presence of LV antigen (Freimanis *et al.*, 2003; Niklasson *et al.*, 2003a; Niklasson *et al.*, 2003b; Niklasson *et al.*, 2006a; Schoenecker *et al.*, 2000). However, to date an unequivocal connection between LV and disease in wild rodents has not been confirmed.

While there is presently no evidence confirming Ljungan virus as an etiological agent for human diseases, there are strong statistical associations between the bank vole population fluctuations (3-4 yr cycles) in Sweden (Hansson & Henttonen, 1985;

Niklasson *et al.*, 1998) and the incidences of several human diseases in Sweden - insulin-dependent diabetes, myocarditis, GBS (Niklasson *et al.*, 1998), intrauterine foetal death (IUFD) (Niklasson *et al.*, 2007b), sudden infant death syndrome (SIDS) (Niklasson *et al.*, 2009a). There is also evidence of LV antigens and viral RNA being detected in specific human disease cases (Niklasson *et al.*, 2009a; Niklasson *et al.*, 2003a; Niklasson *et al.*, 1999; Niklasson *et al.*, 2009b; Niklasson *et al.*, 2007b). Whilst such evidence presents a compelling argument for the involvement of LV and a range of human diseases, the validity of some of these reports has been questioned (Kinney & Thach, 2009; Krous & Langlois, 2009; 2010). The evidence for the association of LV with SIDs in particular is unconvincing at present due to the small number of human cases included in the study, no pathological changes observed in the SIDS cases despite identifying the virus, and virus was not found in every SIDS case (Kinney & Thach, 2009; Krous & Langlois, 2009). As for other LV human and rodent studies, attempts were not made to exclude other potential etiological agents, and once again small sample sizes may not be statistically valid (Krous & Langlois, 2010). Nonetheless, due to potential diseases, screening for LV is essential to adequately assess the diseases associations.

Serological and molecular detection methods have been used for LV diagnosis including polymerase chain reaction (PCR), indirect immunofluorescence assays (IFA) and immunohistochemistry (IHC) (Mantke *et al.*, 2007; Niklasson *et al.*, 1999; Niklasson *et al.*, 2007b). PCR-based methods of detection are frequently used for diagnosis, primarily due to the advantage of speed, but also as they can be inherently more sensitive than other diagnostic methods (Johansson *et al.*, 2004; Nix *et al.*, 2008). This sensitivity might be particularly important for LV where low viral copy numbers might be characteristic of LV infections (Hauffe *et al.*, 2010; Samsioe *et al.*, 2009) (Niklasson unpublished data). PCR-based methods also enabled LV to be detected more than 6 months post viral exposure, whilst the antibody response to the virus can be inconsistent, weak or even absent in chronically-infected animals (Niklasson *et al.*, 1999; Samsioe *et al.*, 2008). Furthermore PCR provides a useful tool for investigating LV infection in detail, particularly tissue tropism and pathogenesis (Mantke *et al.*, 2007).

PCR-based diagnostic methods for LV currently include two published assays that amplify the 5' untranslated region (UTR), a parechovirus 5'UTR real-time assay (Nix *et al.*, 2008) and an LV specific real-time RT-PCR (Mantke *et al.*, 2007). The 5'UTR is a section of the genome situated at the 5' end of the mRNA that is not translated into protein but is involved in the initiation of transcription. A third hemi-/nested parechovirus-specific VP1 RT-PCR has recently been published (Nix *et al.*, 2010) targeting the VP1 gene. Both the VP1 and 5'UTR regions show some degree of sequence conservation making them suitable targets for primers. Both real-time assays incorporate Taqman chemistries which utilise a probe along with the primer pair to successfully amplify target sequences, this has the benefit of increased specificity. The hemi-/nested approach improves the sensitivity of assays by having two rounds of amplification. All three assays have been shown to detect all four LV genotypes with viral copy detection limits of 100, 60, and 10 for the parechovirus 5'UTR real-time assay (Nix *et al.*, 2008), hemi-/nested parechovirus-specific VP1 RT-PCR (Nix *et al.*, 2010) and the LV specific assays (Mantke *et al.*, 2007), respectively. LV has previously been detected in the UK (Salisbury *et al.*, 2013) however there is currently little sequence information available and as such it cannot be concluded exactly how different indigenous LV strains are to the others. Furthermore the two current assays designed to detect all parechoviruses as a consequence require a considerable amount of primer degeneracy that could affect LV detection. To this end I modified the two assays targeting the 5'UTR (Mantke *et al.*, 2007; Nix *et al.*, 2008), by removing the Taqman probe of the LV specific assay to reduce the specificity. With the aim of increasing the sensitivity of the assay, primers were combined from both to create a hemi-nested approach. First round primer set followed by a second round set using one new internal primer and one from the first round PCR. The reverse primer (AN344) (Nix *et al.*, 2008) was combined with the forward primer (LVF) (Mantke *et al.*, 2007) in the first round PCR, followed by the nesting of the forward and reverse primers (LVF and LVR) (Mantke *et al.*, 2007) in the second round.

Given the importance of LV as a potentially important zoonosis it is crucial to expand the scope of LV screening. At present, urban rodents have never been assessed for LV and it is here where the close proximity to humans will present a higher risk. This chapter aims therefore to provide an initial indicator of whether

Ljungan virus is circulating in wild rodents around the North West UK, both from urban and semi-rural environments. I also introduce a hemi-nested RT-PCR that combines the primers of two published real-time PCRs, targeting the 5'UTR of parechoviruses (Nix *et al.*, 2008) and Ljungan virus (Mantke *et al.*, 2007) in the aim of successfully identifying Ljungan virus in rodents from the North West UK.

3.3 Methods

Field Work

Rodents (n = 495) consisting of brown rats (*R. norvegicus*) (n = 133), wood mice (*A. sylvaticus)* (n = 269), bank voles (*M. glareolus*) (n = 50), house mice (*M. musculus*) (n = 35) and field voles (*M. agrestis*) (n = 8) were live-caught across North West England (particularly in and around Liverpool, Chester and Wirral, see Chapter 2). Animals were humanely euthanised in the field by the inhalation of the anaesthetic, Isoflurane, following UK Home Office Guidelines. Most liver tissues were removed within 2 hours of euthanasia, but where this was not possible the carcasses were stored at -80 °C until processing. All liver samples were stored at -80 °C.

Brain material from one field vole (*M. agrestis*) (F174) from Kielder forest, Northumberland was available for screening using this assay. This sample was previously found positive in a separate study (Salisbury *et al.*, 2013). This sample was treated in the same way as all the others.

A small cohort (n = 50) of wild fox (*V. vulpes*) livers were provided by The Food and Environment Research Agency (FERA).

RNA extraction

50-100 mg of liver tissue was homogenised in 1 ml TRIzol® Reagent (Invitrogen, Life Technologies, Paisley, UK) with QIAGEN Stainless steel beads (5mm) using a QIAGEN TissueLyser (Qiagen, UK) for 2 mins at 30 Hz. RNA was extracted from the homogenate as described in the Invitrogen TRIzol® Reagent instructions for animal tissues (Invitrogen, Life Technologies, Paisley, UK). The RNA was diluted 1:10 in sterile distilled water and quantified using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) and stored at -80 °C until reverse transcription.

Reverse Transcription

RNA was reverse transcribed following a standard first strand cDNA synthesis protocol (Invitrogen, Life Technologies, Paisley, UK). Briefly, RNA (>1 µg) was reverse transcribed in a 20 µl reaction containing 50 ng/µl random hexamers, 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH), 5X First strand buffer, 0.1 M DTT and 200 U SuperScript™ III Reverse Transcriptase (Invitrogen, Life Technologies, Paisley, UK). Cycling parameters were 65 °C for 5 mins, 50 °C for 60 mins and 70 °C for 15 mins in a Techne TC-5000 Thermal Cycler. The cDNA was stored at -20 °C.

PCR detection

Two µl of cDNA was PCR-amplified in a 14.5 µl reaction volume containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pM (each) of the forward and reverse primers (Eurofins MWG Operon, Germany) (Table 3.1). Cycling parameters were 95 °C for 10 mins, 20 three step cycles (30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C) and 72 °C for 5 mins.

Table 3.1. Forward and reverse primers used in each step of the nested PCR.

	Primers	
	Forward	Reverse
First round	LV Forward[a] (239-257[c]) (5'-GCGGTCCCACTCTTCACAG- 3')	AN344[b] (590-611[c]) (5'-GGCCCCWGRTCAGATCCAYAGT-3')[d]
Second round	LV Forward (5'- GCGGTCCCACTCTTCACAG –3')	LV Reverse[a] (405-425[c]) (5'- GCCCAGAGGCTAGTGTTACCA-3')

a. Primers from (Mantke *et al.*, 2007)

b. Primers from (Nix *et al*., 2008)

c. Positions are relative to the genome of LV87-012 complete genome (GenBank accession number AF327920).

d. Ambiguity codes: R, A or G; Y, C or T and W, A or T.

The second round PCR mix was prepared as before except first round PCR product was used in place of the cDNA template, and the hemi-nested forward and reverse primers were added (Table 3.1). Thermal cycling conditions are as described above, except that 35 amplification cycles were used. Five µL of PCR product was analysed by 1.5 % agarose gel electrophoresis (120 V for 70 mins). Ljungan virus positive samples gave a band at approximately 373 bp after the first round PCR and 187 bp after the second round (Mantke *et al.*, 2007).

PCR product purification and DNA Sequencing

Primers and unincorporated nucleotides were removed from PCR products using an ExoSAP digest. Five µl PCR product was added to 2 µl mix containing 10X RX buffer, 0.2 U Shrimp Alkaline phosphatase (USB, UK) and 1 U Exonuclease I (New England BioLabs, UK). Cycling parameters were 37 °C for 45 mins, 80 °C for 15 mins. The cycle sequencing reaction was set up using the BigDye® Terminator v3.1 Cycle Sequencing kit. One µl of ExoSAP product was added to a 9 µl reaction mix containing 5X sequencing buffer, 0.75 µl BigDye 3.1 (Applied Biosystems, Life Technologies, Paisley, UK) and 1.6 pM of LV forward or LV reverse primer. Cycling parameters were 25 three step cycles (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 mins). The sequencing product was then precipitated using 3 M sodium acetate prior to resuspension in HiDi™ formamide (Applied Biosystems, Life Technologies, Paisley, UK), and then run on an ABI3130xl.

PCR – 18S rRNA Housekeeping gene

A housekeeping control was implemented to confirm successful reverse transcription of samples and so the presence of amplifiable DNA. cDNA from each sample was amplified in a separate PCR reaction using specific primers targeting the 18S ribosomal RNA; a gene present in all eukaryotic cells. Briefly, 1 µl of cDNA was PCR-amplified in a 14.5 µl reaction containing containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene), and 5 pM of the 18S rRNA primers and 18S rRNA competimers (Ambion, UK). Cycling parameters were 95 °C for 10 mins, 24 three step cycles (30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C) and 72 °C for 6 mins. PCR product was analysed by 1.4 % agarose gel electrophoresis (120 V for 60 mins). Evidence of amplifiable cDNA gave a band at approximately 489 bp.

3.4 Results

PCR design and validation

Many attempts were made to design superior primers targeting the 5'UTR and VP1 regions of the LV genome, as well as experimenting with different combinations of currently published primers to improve assays and provide better opportunity to detect UK LV strains. All were unsuccessful apart from a combination of primers from (Mantke *et al.*, 2007) and (Nix *et al.*, 2008), into a hemi-nested RT-PCR approach.

Genome alignments confirmed the conservation of primer sites amongst the five published genomes and the two new strains described in Chapter 4 (Figure 3.1). Round one and two of the LV hemi-nested RT-PCR described here were both optimised for $MgCl_2$ concentrations between 1.5 mM and 2.5 mM, annealing temperature ranging between 55℃ and 65℃ and cycle number ranging between 20 and 40 increasing in five increments. Optimum conditions were 1.5 mM $MgCl_2$ and 60 °C for both rounds and to minimise non-specific binding, 20 cycles for round one and 35 cycles for round two. In comparison the published LV-specific real-time assay (Mantke *et al.*, 2007) used 4 mM $MgCl_2$, a single round of 40 cycles, and an annealing temperature of 60 °C.

The optimised Ljungan assay was tested using positive Ljungan strains received from Bo Niklasson (AB Apodemus). Samples included four of the seven Ljungan virus strains (Johansson *et al.*, 2002) (Chapter 4). The new assay was able to detect all of the Ljungan positive controls (Figure 3.2). When comparing the sensitivity of the standard assay minus the probe (Mantke *et al.*, 2007) against our hemi-nested assay, we were able to show that our assay has a sensitivity 10-fold greater than that of the published method (Figure 3.3). Actual sensitivity limits were estimated using standards LV340 and LV342 (Chapter 4) with known viral copy numbers received from Bo Niklasson (AB Apodemus). Assuming RNA extraction and reverse transcription were 100 % efficient, this hemi-nested assay had a sensitivity threshold of at least $1x10^3$ viral copies.

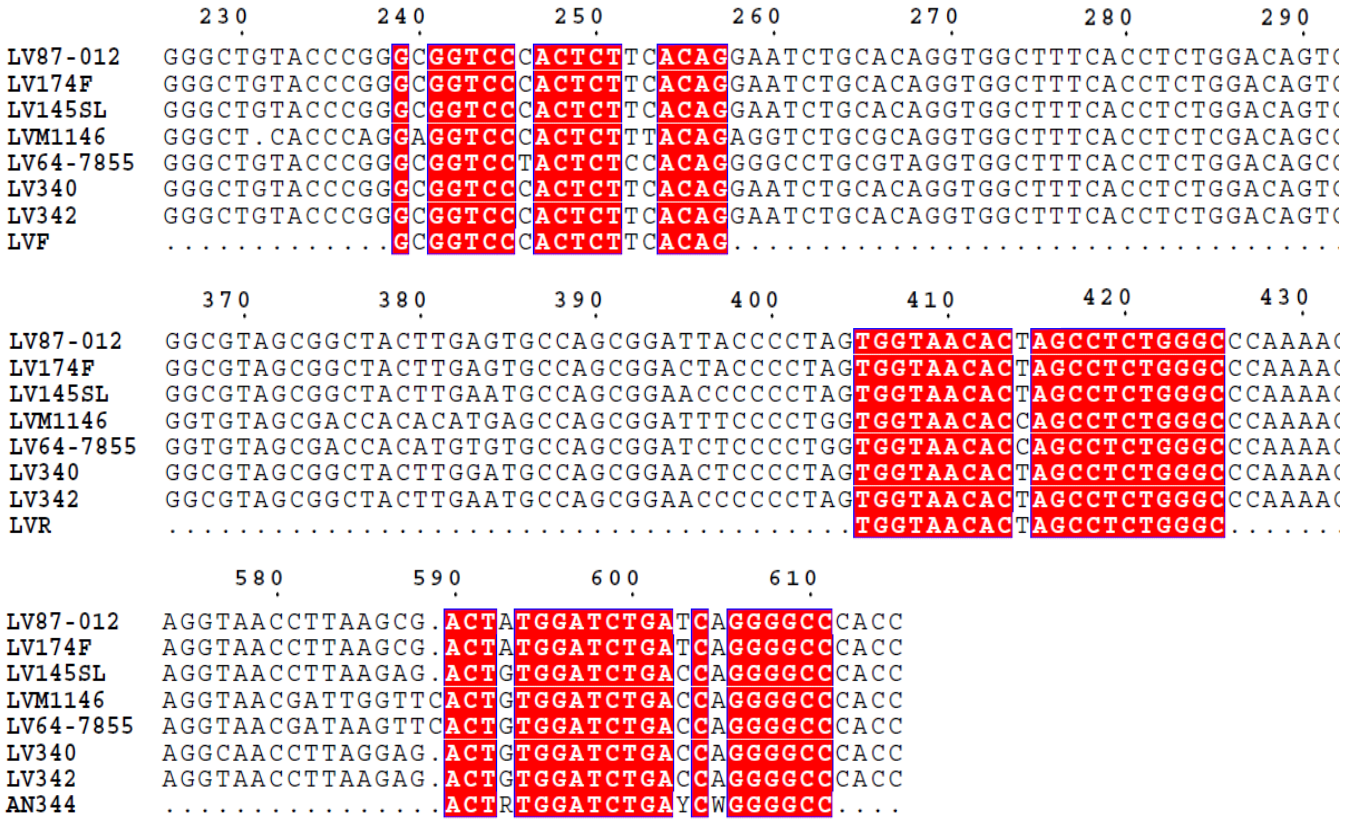


Figure 3.1. Sequence alignments of the seven Ljungan virus 5'UTR sequences and the published primers. Primers were obtained from (Mantke *et al.*, 2007) (LVF and LVR) and (Nix *et al.*, 2008) (AN344). The nucleotide sequences were retrieved from GenBank (http://blast.ncbi.nlm.nih.gov/) and were aligned in MEGA5 (Tamura *et al.*, 2011). Ljungan virus accession numbers: LV87-012, AF327920; LV174F, AF327921; LV145SL, AF327922; LVM1146, AF538689; LV64-7855, EU854568. The numbering follows the LV87-012 sequence.

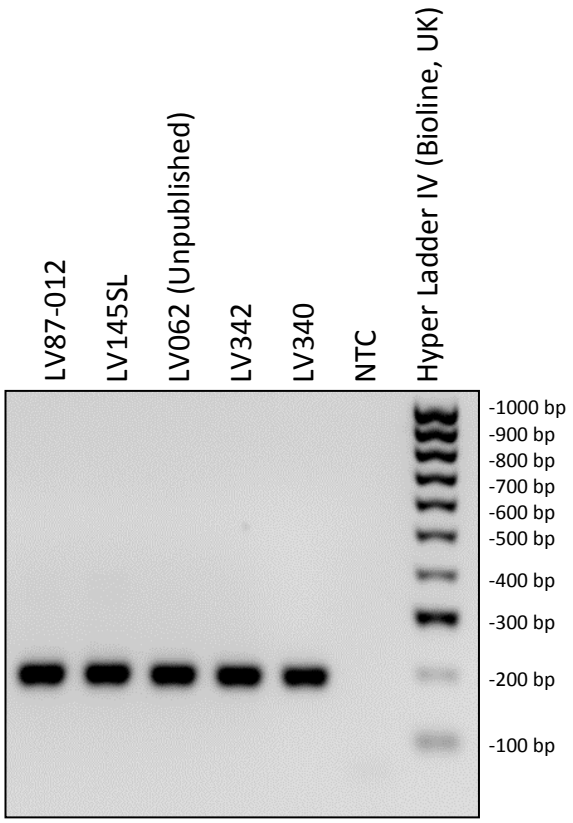


Figure 3.2. Ljungan virus validation panel tested using the hemi-nested RT-PCR assay, bands (187 bp) were observed on an agarose gel (1.5 %).

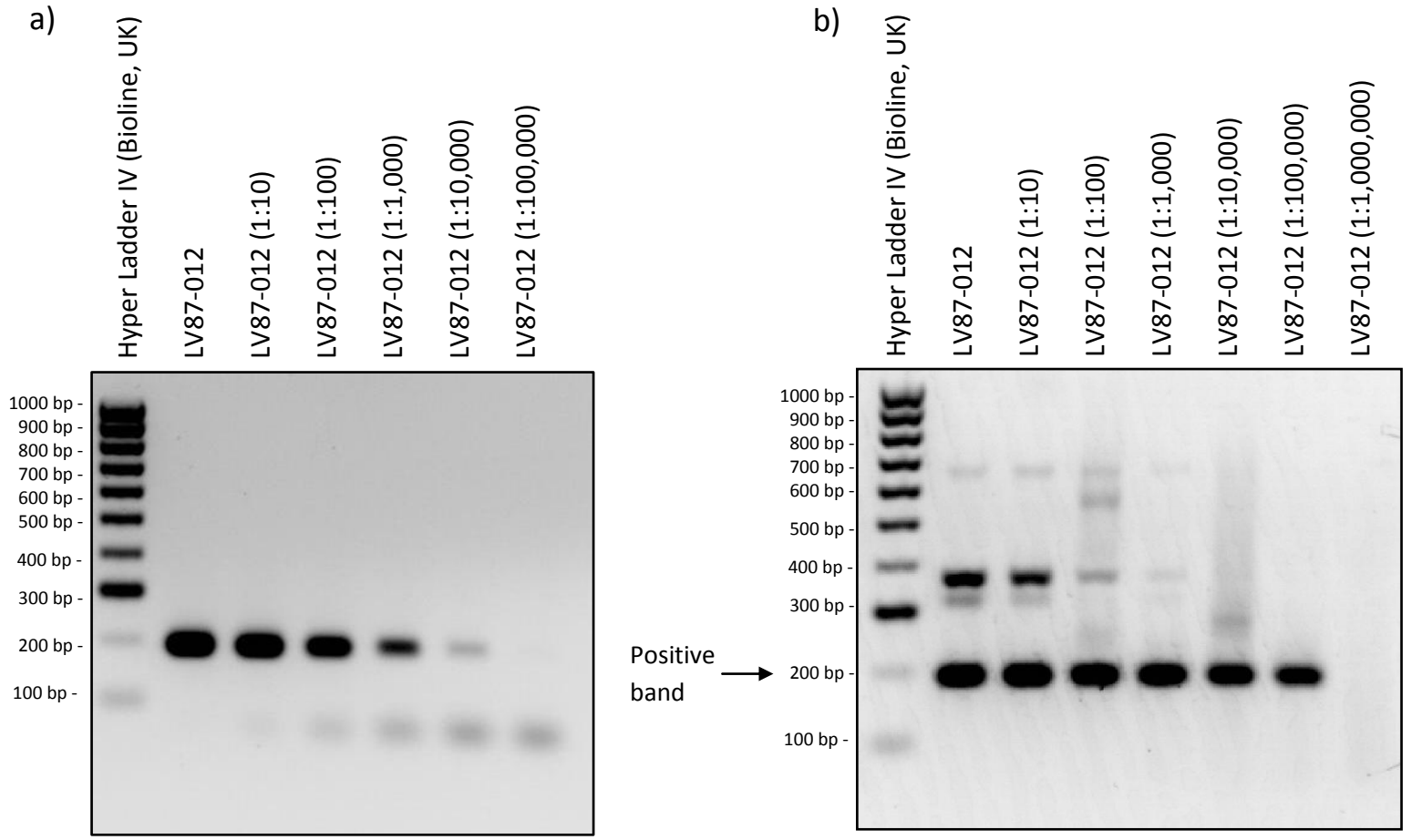


Figure 3.3. LV87-012 positive control dilution series run on an agarose gel (1.5 %) comparing second round band sizes using the a) standard Ljungan virus assay minus Taqman probe (Mantke *et al.*, 2007), and b) the new optimised hemi-nested assay described here.

PCR quality assurance (QA)

To validate the assay on actual rodent tissues, bank vole (*M. glareolus*) liver samples were provided in a quality assurance field panel (Bo Niklasson, AB Apodemus). 4/7 positives and 5/5 negatives were in agreement however our hemi-nested assay failed to detect RNA in three positives previously detected using an unpublished LV specific real-time PCR used by the reference lab (AB Apodemus) (Table 3.2). Our results were also in accordance to the results obtained using the published LV real-time PCR method minus Taqman probe (Mantke *et al.*, 2007).

Table 3.2. Comparison of LV diagnostic results of the field panel between the reference lab and the University of Liverpool.

	Diagnostic result	
Sample	**UoL**	**AB Apodemus**
152	Neg.	Neg.
153	Neg.	Neg.
154	Neg.	Neg.
155	**Pos.**	**Pos.**
160	Neg.	**Pos.**
161	Neg.	Neg.
162	Neg.	**Pos.**
163	Neg.	**Pos.**
170	Neg.	Neg.
171	**Pos.**	**Pos.**
172	**Pos.**	**Pos.**
173	**Pos.**	**Pos.**

Neg. = Negative, **Pos. = Positive**

An *in vitro* transcribed RNA sample (LV87-012) as published in (Mantke *et al.*, 2007) was provided by Bo Niklasson (AB Apodemus), however on testing, plasmid DNA was found to still be present.

Screen results

RNA from the livers of all animals sampled (n = 495) were negative for Ljungan viral RNA using this hemi-nested RT-PCR directed at the 5'UTR. Given that we only caught a finite number of each rodent species and that none were found positive for LV RNA, from this data we cannot rule out prevalences less than approximately 1.1 % for wood mice, 2.3 % for brown rats, 5.8 % for bank voles, 8.2 % for house mice and 32 % for field voles. The 18S housekeeping gene confirmed successful reverse transcription and the presence of amplifiable cDNA in all samples. The small cohort of fox livers were screened however the housekeeping gene failed suggesting the PCR was being inhibited, most likely as a consequence of the unclean nature of the liver and due to limited funds and time they could not be purified.

A field vole (F174) trapped in Kielder forest previously believed to be infected with LV (97 % identical to LV87-012) (nucleotide sequence alignments created in Gblocks were provided by James Stewart, University of Liverpool) (Salisbury *et al.*, 2013) was also found positive for Ljungan viral RNA in this study, and on sequencing was 100 % identical to reference strain LV87-012. Exhaustive efforts were also made to replicate other positive results from tissue and RNA from Salisbury *et al.*, 2013, however unfortunately they could not be repeated. A subset of liver samples was also sent to the reference laboratory in Sweden (AB Apodemus) for confirmation using the LV specific real-time assay (Mantke *et al.*, 2007) and only one out of a previously suspected 12 reported by Salisbury *et al.*, 2013 was positive for LV (unpublished data).

3.5 Discussion

Assay

In this study we developed a new hemi-nested RT-PCR assay that combined primers from two published assays (Mantke *et al.*, 2007; Nix *et al.*, 2008). Only LV RNA from four isolates were available to test the specificity of the assay, representing

genotypes 1 and 2. Requests for the US strains (genotypes 3 and 4) were unsuccessful. However, sequence identity at the primer binding sites and confirmation from previous publications of the detection of all genotypes indicates there would be no issues in detecting the American strains also. This optimised hemi-nested assay detected all four strains available but also more importantly we were able to show a minimum 10-fold increase in the sensitivity (Figure 3.2 & 3.3) and crude estimations of the sensitivity limit using two standards with known viral copy numbers, LV340 and LV342 provided by Bo Niklasson found it to be at least $1x10^3$ (1,000 viral copies).

We were unable however to detect three positives in the field panel provided. Since this was the case with the LV real-time assay as well, it is thought that the likely cause of failure to detect RNA lies with the degradation of the panel rather than our assay. Regardless, to increase the sensitivity of our assay we first require sufficient sequence information specifically for indigenous UK strains. This can only be achieved by first performing a broad screen that ensures the best opportunity of detecting 'all' LV variations; we are confident that the hemi-nested RT-PCR assay reported here will accomplish this. Once we have indigenous UK strain information, more sensitive methods can be designed.

Screening

Using this assay we were able to show that all animals collected for this study panel were negative for LV RNA. The only positive result we did find was from a field vole caught from a separate study in Kielder forest, Northumberland (Salisbury *et al.*, 2013). Interestingly, this animal had also been found positive for LV RNA in a previous independent extraction although difference in sequence identity was observed.

Data from this study suggests LV is not circulating in rodents around North West UK, particularly urban areas where our animals were primarily sourced from. Also it shows a large difference in the prevalence compared to the previous UK study (Salisbury *et al.*, 2013) but perhaps this reflects the difference in habitat types, rural (Kielder forest) vs. urban (Liverpool). However we were able to confirm a single

positive animal from the previous study (Salisbury *et al.*, 2013) and is suggestive that LV is in fact circulating elsewhere in the UK, perhaps in more rural areas. An explanation for finding no LV positive animals in urban environments could be that LV might not be maintainable in these fragmented urban rodent populations who potentially lack sufficient connectivity for the effective spread of the virus (Hess, 1996; McCallum & Dobson, 2002). We might expect then to find positive animals just outside of these urban areas in the semi-rural environment, this was not the case. We did not find any LV infected animals in fresh rural rodent material collected for this study but perhaps the sample size was too small since rural rodents were not the primary target for this study. If the virus was limited to rural areas it would most likely result in fewer human-rodent interactions and so the risk to humans would be lower.

A possible explanation as to why we did not find any positive animals could be attributed to the testing approach. The screening process is reliant on the quality of diagnostic test and whilst we were able to show that our optimised assay has increased sensitivity over other published tests it does not necessarily mean that we will therefore detect all LVs. Firstly, it might be characteristic of LV to be present in very low copy numbers (Hauffe *et al.*, 2010; Samsioe *et al.*, 2009) (Niklasson unpublished data), perhaps sometimes even beyond that which our assay can detect. Secondly, UK LV could be quite distinct from American and Swedish isolates although the single sequence we obtained from a field vole suggests this was not the case. RNA viruses generally possess exceptionally high mutation rates (Domingo & Holland, 1997; Stanway, 1990), however they do show considerable nucleotide conservation in the 5'UTR; PCR target region (Hyypiä *et al.*, 1992; Stanway, 1990)(data not shown). Thirdly, the Italian LV surveillance report suggested testing the liver for LV RNA (Hauffe *et al.*, 2010), at the conception of this survey the reference lab (Niklasson pers. comms.) also advised that if an animal was positive for LV RNA it could always be detected in the liver; but not always in other tissues. However, the liver may in fact not be a primary target in LV pathogenesis. Previous studies have alluded to LV being possibly more neurotropic, targeting the brain and CNS (Mantke *et al.*, 2007; Niklasson *et al.*, 2009a) and greatest virus loads have previously been found in the brain of experimentally infected lab mice (Mantke *et*

al., 2007). Brain material has been archived from this survey however due to limited resources and advice from the reference lab it was not tested, perhaps in future it could also be screened for LV. If liver material is the optimal choice for LV detection, then there is still an issue of tissue tropism, in particular will LV be distributed throughout the tissue or be localised? Thus suggesting not all portions of a specific tissue might show a positive detection for LV RNA and so produce false negative results. Immunohistochemical staining of LV viral antigens have been observed in discrete areas of certain human tissues such as the chorionic plate of the placenta (Samsioe *et al.*, 2009), beta cells of the pancreas (Niklasson *et al.*, 2003a; Niklasson *et al.*, 2003b) and the muscle fibres of the heart tissue (Niklasson *et al.*, 2009a; Tolf *et al.*, 2008). These findings illustrate that LV might in fact be localised within tissues. In an attempt to minimise a potential effect of localisation within the liver, a 50-100 mg piece was homogenised and the whole homogenate was carried forward for RNA isolation.

As was previously mentioned LV has been found in a number of rodent species, of these we only included four in this study, *M. glareolus*, *M. agrestis*, *M. musculus* and *A. sylvaticus* (Niklasson *et al.*, 1999; Niklasson *et al.*, 2006a; Salisbury *et al.*, 2013). This study was largely focused on urban animals where the human zoonotic risk would be magnified, and so the two primarily rural rodent species, *M. glareolus* and *M. agrestis* were heavily under represented n = 50 and n = 8, respectively. Perhaps focus needs to be directed towards screening a larger number of these hosts, for confirmation. Whilst the brown rat has not previously been reported to have LV infection, it does not necessarily mean they cannot act as hosts, especially considering the current species range.

This study has found no evidence of LV in rodents in the North West UK, however a sequence from a single positive field vole in Northumberland suggests this virus may be circulating elsewhere in the UK.

Chapter 4. Molecular characterisation of two new Ljungan virus isolates LV340 and LV342

4.1 Abstract

Ljungan virus (LV) (family *Picornaviridae*, genus *Parechovirus*) is a suspected zoonosis with associations to human incidence of several disease in Sweden. LV is a single-stranded RNA virus with a positive sense genome. There are currently five published Ljungan virus strains, three Swedish and two American, and are classified into four genotypes. A further two strains described here were isolated from wild bank voles (*Myodes glareolus*) caught in Västmanlands county, Sweden in 1994. They were sequenced using next generation pyrosequencing technology on the GS454flx. Genetic and phylogenetic analysis of the obtained genomes confirm isolates LV340 and LV342 as two new members of genotype 2 along with LV145SL, with 92 % and 99 % identities respectively. Only two codon sites throughout the entire genome were identified as undergoing positive selection, both situated within the VP3 structural region, in or near to major antigenic sites. Whilst these two strains do not constitute new genotypes they have provided evidence suggesting the evolution of Ljungan virus to be markedly slow, a characteristic unlike other picornaviruses. Ultimately genomic information is required from different species as well as geographical locations to further understand the potential of this virus.

4.2 Introduction

Picornaviridae, a family of diverse viruses, are responsible for some common and serious diseases affecting humans and animals, examples being polio and foot-and-mouth disease. According to the International Committee on Taxonomy of Viruses (ICTV), this family is currently divided into 17 confirmed genera: Aphthovirus, Avihepatovirus, Aquamavirus, Cardiovirus, Cosavirus, Dicipivirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Megrivirus, Parechovirus, Salivirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus, although more have been proposed (Sauvage *et al.*, 2012). The majority are comprised of pathogens of either humans or other animals however a few include both. Prior to 1998, the *Parechovirus* genus was thought to contain only human pathogens (Human

parechoviruses; HPeV) but several isolates of a novel rodent pathogen, Ljungan virus (LV) have since been discovered and proposed to be zoonotic (Niklasson *et al.*, 2003a; Niklasson *et al.*, 2003b; Niklasson *et al.*, 1999; Niklasson *et al.*, 2006b; Samsioe *et al.*, 2006; Samsioe *et al.*, 2008) .

Ljungan virus, originally isolated from bank voles (*Myodes glareolus*) in Sweden, has proposed associations with several diseases in humans including myocarditis, diabetes, Guillain-Barré syndrome (GBS) (Niklasson *et al.*, 1998), intrauterine fetal death (IUFD) (Niklasson *et al.*, 2007b), foetal malformations (Niklasson *et al.*, 2009b) and sudden infant death syndrome (SIDS) (Niklasson *et al.*, 2009a). Whilst there is substantial evidence demonstrating the detection of both LV antigens and viral RNA in certain human disease cases, LV has not yet been confirmed as the cause (Niklasson *et al.*, 2009a; Niklasson *et al.*, 2003a; Niklasson *et al.*, 1999; Niklasson *et al.*, 2009b; Niklasson *et al.*, 2007b). Very little is known as to the pathogenesis of LV but suggested target tissues in humans include the heart and brain, basically the areas where most research has so far been conducted (Niklasson *et al.*, 2009a; Niklasson *et al.*, 2009b). LV is thought to be transmitted via the faecal-oral route, like other picornaviruses, with rodents as suggested sources (McDonald, 2009). However, LV RNA has been detected in several rodent species and LV specific antigens have been detected in foxes, the full extent of carrier hosts is still unclear as the nature of infection is unknown (Hauffe *et al.*, 2010; Johansson *et al.*, 2003; Johnson, 1965; Main *et al.*, 1976; Niklasson *et al.*, 2003a; Niklasson *et al.*, 1998; Niklasson *et al.*, 2007a; Niklasson *et al.*, 1999; Niklasson *et al.*, 2006a; Tolf *et al.*, 2009; Whitney *et al.*, 1970).

Parechoviruses are single stranded RNA viruses, with a positive sense genome approximately 7.5-8 kb long. The RNA genome contains one open reading frame that encodes a single polyprotein, human parechoviruses are made up of 10 individual proteins and Ljungan virus has 11: VP0-VP3-VP1-(2A1-LV only)-2A2-2B-2C-3A-3B-3C-3D (Chapter 1: Figure 1.3), each responsible for specific functions in virus replication and survival (Johansson *et al.*, 2002; Racaniello, 2001).

Single stranded RNA viruses evolve exceptionally fast, mainly as a result of high mutation rates accrued because RNA polymerases lack any proofreading mechanisms, resulting in the accumulation of multiple mutations (Domingo & Holland, 1997; Holmes, 2003; Stanway, 1990). This provides a large 'pool' of genetic diversity that under the right selective pressures is a strong driving force for virus evolution – quasispecies. Despite this increased error potential there remain areas of the genomes that have important roles in successful replication and are thus highly conserved, in particular those coding for non-structural proteins as presumably mutations in these regions would be costly for the virus' viability. The structural proteins in contrast are responsible for encoding the capsid proteins and tend to show a higher degree of variability mainly due to the immune pressure imposed by the host and the requirement to avoid recognition (Stanway, 1990).

Currently there are five published LV genomes that cluster into four "genotypes" according to genetic and phylogenetic analysis: LV87-012 and LV174F (gt 1), LV145SL (gt 2) (Johansson *et al.*, 2002), LVM1146 (gt 3) (Johansson *et al.*, 2003) and LV64-7855 (gt 4) (Tolf *et al.*, 2009). Genotypes 1 and 2 have been confirmed in bank voles in Europe whilst gt 3 and 4 have been found in two different vole species in America. LV has also been detected in wild rodents from Denmark, Italy and UK (Hauffe *et al.*, 2010; Niklasson *et al.*, 2007a; Salisbury *et al.*, 2013). However there is no sequence information available for these.

The more genetic information we can obtain for LV the closer we will be to understanding this virus. In this study we obtain a further two LV genome sequences from two recent isolates and assign them to genotype 2 using genome analysis. We review key motifs and structures within the available LV genomes. With access to more recent tools we analysed the evidence for positively selected sites on LV.

4.3 Methods

Virus strains

Ljungan virus (LV) strains 340 and 342 were isolates extracted from wild caught bank voles (*M. glareolus*) in Västmanlands county, Sweden in 1994. They were grown in baby hamster kidney cells (BHK-21) for five passages before being passed

once through suckling mouse brain (SMB) in the reference laboratory in Sweden (AB Apodemus).

RNA extraction, cDNA synthesis and PCR amplification

Total RNA was extracted from whole brains using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's standard protocol.

RNA was reverse transcribed following a standard first strand cDNA synthesis protocol (Invitrogen Life Sciences, UK). Briefly, approximately 5 µg of RNA was reverse transcribed in a 20 µl reaction volume containing FR26RV-N (20 pmol) and FR40RV-T (1 pmol) primers (Djikeng et al., 2008), 10 mM dNTP mix (10 mM each dNTP), 5X First strand buffer, 0.1 M DTT and SuperScript™ III Reverse Transcriptase (200U) (Invitrogen Life Sciences, UK). Cycling parameters were 65 °C for 5 min, 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min.

cDNA was made double stranded by incubation with Exo-Klenow fragment (5U) (Ambion, UK); 5 µl first strand cDNA sample was added to a 25 µl reaction containing 100 pmol FR26RV-N (Djikeng et al., 2008) and 4 pmol 5' Ljungan virus specific primer (5'-GCCGGAGCTCTGCAGATATCGGTGGGGTGG-3'), 500 mM Tris-HCl, 10 mM dNTP mix, 50 mM $MgCl_2$, 0.1 M DTT and 5 mg/ml BSA. The reaction was heated to 95 °C for 5 min and then maintained at 37 °C for 30 min, 1 µl of 0.5 M EDTA was added to stop the reaction. Primers were removed from the double stranded cDNA by incubating the reaction at 37 °C for 30 mins with shrimp alkaline phosphatase (ds cDNA, 0.2U Shrimp Alkaline Phosphatase (SAP) (USB, UK) and 1U Exonuclease I (New England BioLabs, UK).

The double stranded cDNA was then PCR-amplified using the Advantage 2 Polymerase protocol (Clontech, UK). Briefly, cDNA was amplified in a 50 µl final reaction volume containing 10X Advantage buffer, FR20V primer (10 pmol), 10 mM dNTP mix and 50X Advantage® 2 Polymerase mix (Clontech, UK). Thermal cycling parameters were 95 °C for 1 min, 15 two step cycles (30 s at 95 °C, 6 min at 68 °C) and at 68 °C for 6 minutes.

454 sequencing

Library construction and pyrosequencing was completed by the Centre for Genomic Research (CGR, http://www.liv.ac.uk/cgr/), University of Liverpool, UK on a 454 GS FLX system (Roche). Samples LV340 and LV342 were multiplex identified (MID-tagged) and sequenced using 1/16 GS454flx sequencing run. Viral genome sequencing was thus random with regard to the total RNA present. Sequencing reads were quality trimmed and adaptor and primer sequences were removed prior to assembly. Contig assembly was performed using Newbler (release 1.1.03.24.Roche) with overlap settings of 35 bp and 99 % identity and default value for the remaining parameters.

BLAST identity searches and sequence annotation

Sequence contigs were submitted to BLAST for identification. Briefly, contigs were compared against the Ljungan virus viral protein Uniprot database (accessed 5/2/2010) with an e-value cut off of $1e^{-5}$ (probability of the alignment occurring by chance), identity searches were conducted using BLASTX implemented in the standalone blast program (Altschul *et al.*, 1990). Apparent gaps in the Ljungan virus genomes were filled by designing primers that flanked the gap and then subsequent PCR and Sanger sequencing.

Phylogenetic trees

Representative $3D^{pol}$ and VP1 sequences for members of the picornavirus family were taken from UniProt (Consortium, 2012) and used for phylogenetic analysis (Table 4.1). Multiple amino acid sequence alignments were generated in MEGA5 (Tamura *et al.*, 2011). Sequence identities were compared using Geneious 5.6.5 (Biomatters: www.geneious.com, date accessed: 1/8/12). Maximum likelihood phylogenetic trees were produced in MEGA5 (Tamura *et al.*, 2011) with bootstrap replications of 1,000 (Felsenstein, 1985). Optimum substitution models were estimated in MEGA5 (Tamura *et al.*, 2011).

Positive selection

Sites specific positive selection (adaptive evolution) was identified by calculating the ratio of dN (Non-synonymous)-to-dS (Synonymous) substitutions. The analysis was performed using the Datamonkey HYPHY package web interface

(http://www.datamonkey.org/) (Delport *et al.*, 2010; Pond & Frost, 2005a; Pond *et al.*, 2005). All 11 viral genes were analysed separately for the seven genomes. Random effects likelihood (REL), fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) (Pond & Frost, 2005b) were employed to predict putative selection. Sites were considered under positive selection if significant support was found in two or more methods (SLAC/FEL: $p<0.1$; and REL: Bayes factor >100).

Recombination

Recombination events were analysed using the GARD and SBP models (http://www.datamonkey.org/) (Pond *et al.*, 2006).

RNA secondary model prediction

The predicted RNA secondary folding structure was modeled using the mfold software version 3.5 (http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003).

4.4 Results and Discussion

Sequence output and assembly statistics

Sequencing of LV340 sample yielded a total of 1,149 contigs (totaling 369,911 bp) and 53,047 singletons, with a mean length of 322 bp (ranging between 97-3841 bp) and an average read depth of 7. There were 53 contigs ≥500 bp. For sample LV342 sequencing yielded a total of 3,216 contigs (totaling 1,148,716 bp) and 73,030 singletons, with a mean length of 357 bp (93-4535 bp) and an average read depth of 4. There were 90 contigs ≥500 bp.

Table 4.1. Picornavirus representatives used in the $3D^{pol}$ and VP1 analysis.

Genus	Virus/ Strain	Abbreviations	Uniprot no.
Aphthovirus	Foot-and-mouth disease virus (strain O1)	FMDV1	P03305
	Foot-and-mouth disease virus (strain C1-SantaPau)	FMDV2	P03311
Aquamavirus	Seal picornavirus type 1	SePV1	A8D7N3
Avihepatovirus	Duck hepatitis A virus	DHV1	Q0GH72
Cardiovirus	Encephalomyocarditis virus	EMCV	P03304
	Mengo encephalomyocarditis virus	MENGO	P12296
	Saffold virus	SAFV	A4ZKN2
	Theiler's murine encephalomyelitis virus (strain GDVII)	TMEV	P08545
Cosavius	Human cosavirus A1	HCoSV	B8XTP8
Dicipivirus	Canine picodicistrovirus	CPDV	H6VBU5
Enterovirus	Bovine enterovirus (strainVG-5-27)	BEV	P12915
	Coxsackievirus B3 (strain Nancy)	CAB	P03313
	Human enterovirus 70 (strain J670/71)	HEV	P32537
	Human rhinovirus 2	HRV2	P04936
	Poliovirus type (strain Sabin)	PV	P03301
	Swine vesicular disease virus (strain UKG/27/72)	SVDVU	P13900
Erbovirus	Equine rhinitis B virus 1 (strain Equine/Switzerland/P1436/71/1971)	ERV	Q66776
Hepatovirus	Human hepatitis A virus genotype IA (isolate LA)	HAV1	P06441
	Human hepatitis A virus genotype IB (isolate MBB)	HAV2	P08617
Kobuvirus	Aichi virus	AIV	O91464

Table 4.1. continued.

Genus	Virus/ Strain	Abbreviations	Uniprot no.
Megrivirus	Turkey hepatitis virus	THV	E9L812
Parechovirus	Ljungan virus 87-012	LV87012	Q8JV21
	Ljungan virus 174F	LV174F	Q8JV20
	Ljungan virus 145SL	LV145SL	Q8JV19
	Ljungan virus M1146	LVM1146	Q80N16
	Ljungan virus 64-7855	LV647855	C0J6D4
	Human parechovirus 1 (Harris)	HPeV1	Q66578
	Human parechovirus 2 (Williamson)	HPeV2	O73556
	Human parechovirus 3	HPeV3	G1UJH6
	Human parechovirus 4	HPeV4	Q00MX7
	Human parechovirus 6	HPeV6	A7LIU8
	Human parechovirus 7	HPeV7	B9UD49
	Human parechovirus 8	HPeV8	B9UK62
	Human parechovirus 11	HPeV11	ADV16096
Salivirus	Salivirus NG-J1	SVNGJ1	C5MSH2
Sapelovirus	Porcine sapelovirus (strain V13)	PSV13	O91257
Senecavirus	Seneca Valley virus (isolate United States/SVV-001/2002)	SVV	Q155Z9
Teschovirus	Porcine teschovirus 1 (isolate Pig/United Kingdom/F65/1967)	PTV	Q9WJ28
Tremovirus	Avian encephalomyelitis virus (strain Calnekvaccine)	AEV	Q9YLS4
Pasivirus[a]	Swine pasivirus 1	SPaV1	I6YQK4
Picorna-related insect virus	Infectious flacherie virus	IFV	O70710
	Sacbrood virus	SBV	Q9WCE9

[a]Proposed genus (Sauvage *et al.*, 2012)

Based on BLASTX identity searches against a Uniprot Ljungan virus database, we identified four LV specific contigs (0.35 %), contig31 (3,840 bp), contig57 (3,027 bp), contig208 (477 bp), and contig977 (123 bp) for sample LV340 and three gaps of approximately 118, 40 and 86 bp. For sample LV342 two LV specific contigs were identified (0.06 %), contig14 (2,571 bp) and contig237 (3,724 bp) with three gaps of approximately 143, 98 and 1048 bp. Gaps were filled as described in the methods.

Identity

Alignment of protein sequences of the LV340 and LV342 isolates with the five published LV genome sequences and a closely related Human parechovirus (HPeV1-Harris prototype serotype) shows clear similarity between our isolates and the Swedish LV145SL genotype, with at least 96.6 % identity across the whole genome (Table 4.2). It is easiest to observe conserved proteins across all LVs by scanning the more distantly related strains from America e.g. LVM1146 and LV64-7855, since all Swedish strains are relatively similar making it difficult to distinguish overall patterns. Whilst the non-structural proteins (2A1-2A2-2B-2C-3A-3B-3C-3D) share between 75.0-93.6 % aa sequence identity, structural proteins (VP0-VP3-VP1) exhibit greater variability 70.1-78.3 % aa,. Interestingly, the LV340 and LV342 membrane associated proteins 2B, $2C^{ATPase}$ and 3A, whilst these regions are similar to the genotype 2 (LV145SL), show a higher degree of identity with the American genotypes rather than the other Swedish genotype. This is particularly interesting as these isolates are geographically isolated, and as such provide evidence of a cross over event or questions the virus' origin.

Both isolates also exhibit single nucleotide polymorphisms (SNPs) that are different to their consensus genome sequences. LV340 had three SNPs all located within the structural proteins: G740A (VP0), T2152C (VP3) and A2801G (VP1), and two of these resulted in non-synonymous changes G740A (Valine to Isoleucine) and A2801G (Lysine to Glutamate). LV342 also had three SNPs, however two were located in the non-structural proteins: T2145C (VP3), G3455A (2A2) and T4014C (2C). G3455A was the only non-synonymous mutation (Glutamate to Glycine).

Genome analysis

The individual protein cleavage sites previously predicted for Ljungan virus genomes (Johansson *et al.*, 2003; Johansson *et al.*, 2002) are consistently found in these two isolates providing further support for these sites.

5'UTR

It has been reported that the 5'UTR shows considerable similarity amongst closely related viruses (Stanway, 1990). This is presumably due to the specific function that it facilitates in RNA replication (Ekström *et al.*, 2007; Tolf *et al.*, 2009). Sequence identity comparisons between LV340, LV342, published LVs and HPeV 5'UTR nucleotide sequences are displayed in Table 3. Several studies have looked at the precise secondary folding of this region and found that it encodes for an internal ribosome entry site (IRES), a structure vital for cap-independent initiation of translation (Belsham, 2009; Racaniello, 2001). This IRES, previously predicted in published Swedish and American Ljungan viruses (Johansson *et al.*, 2002; Tolf *et al.*, 2009) can now also been found within these two sequences in this study, exhibiting high similarity, in particular in the two motifs reported to be important for functionality, a GNRA and a pyrimidine rich region (Johansson *et al.*, 2003). The specific folding corresponds to a type II IRES, and is the most common amongst members of the picornaviridae including closely related parecho- and cardioviruses (Ghazi *et al.*, 1998; Racaniello, 2001). The IRES which is situated at the 3' end of the 5'UTR lies short of an initiator codon, a motif located in an optimal Kozak context ANNAUGG (Hyypiä *et al.*, 1992; Johansson *et al.*, 2002; Kozak, 1986) and indicates the beginning of the coding polypeptide. From our analysis our two genomes conform to the predicted initiation codons for all published LV genotypes.

Table 4.2. Comparison of the percentage protein identities between the two new Ljungan isolates, LV340 (non-bold) and LV342 (bold) with all LV strains and HPeV1-Harris prototype.

Region	Genotype 1 LV87-012		Genotype 1 LV174F		Genotype 2 LV145SL		Genotype 3 LVM1146		Genotype 4 LV64-7855		HPeV1 (Harris)	
VP0	90.0	**90.0**	90.0	**90.0**	99.2	**99.6**	74.5	**74.9**	76.4	**76.4**	42.9	**43.2**
VP3	84.4	**84.4**	84.4	**84.4**	99.6	**99.6**	74.2	**74.6**	77.9	**78.3**	54.6	**54.6**
VP1	79.1	**78.8**	79.5	**79.1**	98.0	**99.3**	70.0	**70.0**	72.1	**72.1**	37.5	**36.6**
2A1	85.0	**85.0**	85.0	**85.0**	100.0	**100.0**	90.0	**90.0**	75.0	**75.0**	NA	
2A2	94.8	**94.8**	94.8	**94.8**	98.5	99.3	84.4	**82.2**	85.9	**85.2**	44.2	**42.8**
2B	89.3	**89.3**	90.0	**90.0**	100.0	**100.0**	92.9	**92.9**	93.6	**93.6**	52.0	**52.0**
2C	80.8	80.8	80.8	**80.8**	99.4	**100.0**	88.0	**87.4**	89.2	**88.6**	51.7	**51.4**
3A	77.7	**77.7**	77.7	**77.7**	100.0	**100.0**	80.0	**80.0**	80.8	**80.8**	30.5	30.5
3B	89.7	**89.7**	89.7	**89.7**	96.6	**96.6**	79.3	**79.3**	89.7	**89.7**	31.0	**31.0**
3C	98.0	**98.0**	97.0	**97.0**	100.0	**100.0**	87.4	**87.4**	86.4	**86.4**	48.0	**48.0**
3D	97.0	97.9	96.4	97.0	98.7	99.6	83.4	83.4	84.3	83.8	48.9	49.1

Table 4.3. Nucleotide comparison of three important genomic regions in LV340 (non-bold) and LV342 (bold).

Region	LV87-012		LV174F		LV145SL		LVM1146		LV64-7855		HPeV1 (Harris)	
5'UTR	85.0	**85.2**	86.1	**85.8**	94.8	**99.9**	68.0	**66.6**	71.6	**72.4**	46.0	**44.9**
3'UTR	87.7	**90.4**	86.8	**88.6**	92.7	**99.1**	61.0	**61.9**	65.7	**65.7**	42.4	**45.7**
VP1	72.8	**71.7**	71.8	**70.9**	89.8	**99.8**	64.4	**65.2**	67.2	**67.3**	47.3	**48.3**

Polyprotein

Key features of the polyprotein previously identified in reference Ljungan virus genomes and other parechoviruses (Ghazi *et al.*, 1998; Hyypiä *et al.*, 1992; Oberste *et al.*, 1998) are also conserved in the two genomes presented here. These include the BC loops and β-barrels (Johansson *et al.*, 2003; Racaniello, 2001; Rossmann & Johnson, 1989; Tolf *et al.*, 2009) that make up the conserved eight-stranded antiparallel beta-barrel structure found across picornaviruses (Niklasson *et al.*, 1999) and hold the major neutralizing antigenic sites, structures involved in immune selection (Mateu, 1995; Racaniello, 2001). The 2A1 DvExNPgIP motif found in several picornaviruses and suggested to be involved in promoting the separation of the 2A│2B proteins (Johansson *et al.*, 2003). The $2C^{ATPase}$ involved in RNA synthesis (Gorbalenya & Koonin, 1993). The 3B protein which is highly conserved amongst LVs and responsible for encoding a viral VPg peptide, essential for successful polymerase activity (Johansson *et al.*, 2002; Paul *et al.*, 1998; Racaniello, 2001; Tolf *et al.*, 2009). In addition, the 3B is also the site of the *cre* (cis-acting replication element); a structure found in many picornaviruses (Goodfellow *et al.*, 2000), and is important in the initiation of virus replication by the uridylation of the VPg peptide (Paul *et al.*, 2000; Rieder *et al.*, 2000; Tolf *et al.*, 2009). $3C^{pro}$ involved in the processing of the individual proteins at the inter domain junctions (Johansson *et al.*, 2002).

3-UTR

The 3'UTR region is important in the replication and translation of the picornavirus genome (Dobrikova *et al.*, 2003; Rohll *et al.*, 1995), although its definitive function is still unknown. A comparison of the predicted secondary folding of the 3'UTR regions of LV340 and LV342 show a similar stable folding structure to LV87-012 (Figure 4.1a) (Johansson *et al.*, 2003), however energy optimal folds are lower for LV340 and LV342 with ΔG = -19.60 and -23.30 Kcal mol^{-1}, respectively. This suggests the stem loop structure of these new isolates are less stable than all the Swedish genotypes LV87-012, LV174F and LV145SL but less open and more stable than the American genotypes (LVM1146: ΔG = -17.8 Kcal mol^{-1} and LV647855: ΔG = -17.2 Kcal mol^{-1}) (Figure 4.1a - g) (Johansson *et al.*, 2003). Domain II of the 3'UTR folding structure is highly conserved amongst LV and remains to be the case with the

addition of these two new genomes therefore suggesting their significant role in viral replication.

Classification

Whilst the exact criteria for classifying new LV strains is undetermined, a method employed for enteroviruses and that is supported by the current genotype placings, is that a new isolate is considered to be homologous to current genotypes providing that nucleotide and amino acid sequence identities of the VP1 region are >75 and >88 %, respectively (Oberste *et al.*, 1999; Tolf *et al.*, 2009). If we apply these criteria to LV340 and LV342 then they clearly join LV145SL as two new members of genotype 2 (Table 4.2 & 4.3). The addition of these genomes further supports the inclusion of LV within the parechovirus genus.

Phylogenetic tree

Further support for LV placing can be deemed from phylogenetic analysis. LV has been proposed to be situated with the parechoviruses yet still be sufficiently different to be potentially classed as a separate genus (Johansson *et al.*, 2002; Lindberg & Johansson, 2002; Tolf *et al.*, 2009). With the addition of the two new isolates, phylogenetic analysis of the $3D^{pol}$ region confirms the classification of LV as a species within the parechovirus genus (Figure 4.2a). Phylogenetic analysis of the VP1 protein (Oberste *et al.*, 1999) further supports the presence of LV and human parechoviruses as two distinct species within the genus (Figure 4.2b).

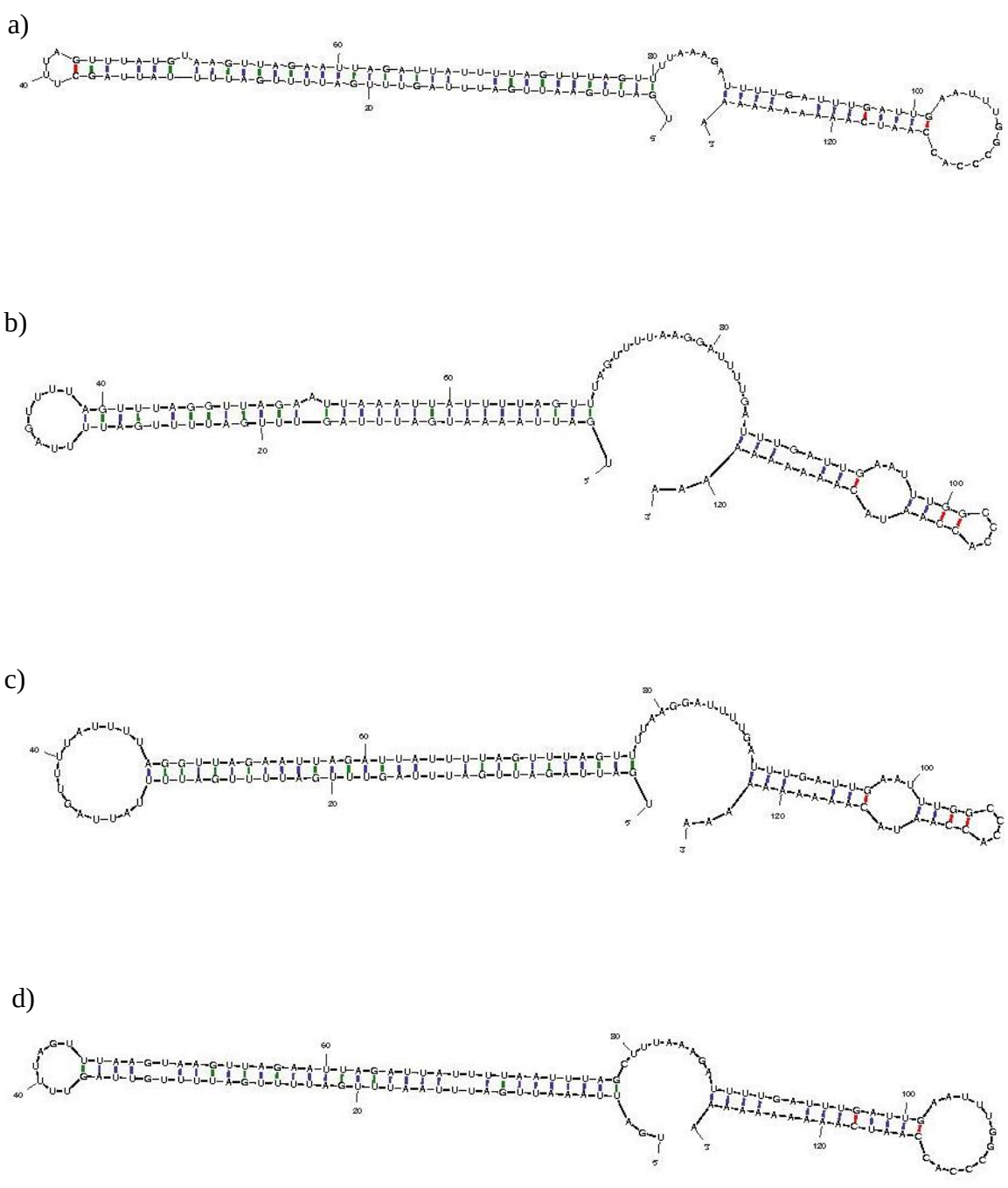
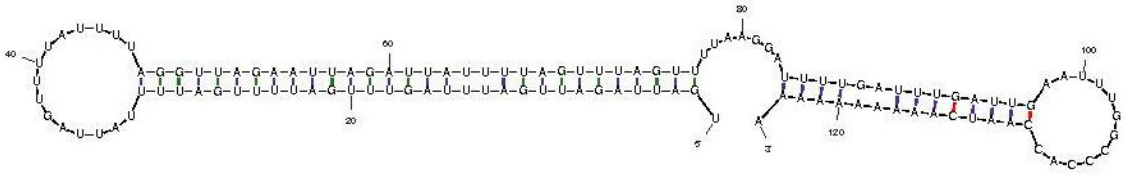
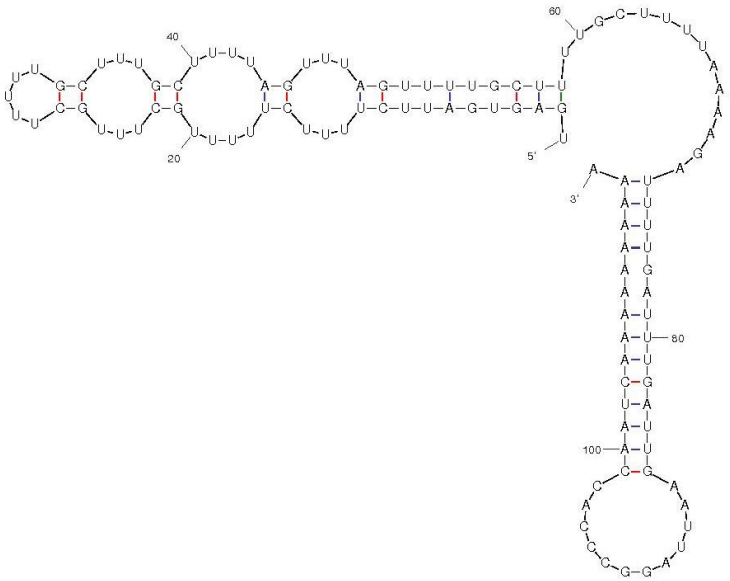


Figure 4.1. Predicted secondary structures from mfold version 3.5 for the 3'UTR of LV87-012: ΔG = -30.4 Kcal mol^{-1} a), LV174F: ΔG = -26.9 Kcal mol^{-1} b), LV145SL: ΔG = -26.9 Kcal mol^{-1} c), LV340: ΔG = -19.6 Kcal mol^{-1} d), LV342: ΔG = -23.3 Kcal mol^{-1} e), LVM1146: ΔG = -17.8 Kcal mol^{-1} f), and LV64-7855: ΔG = -17.2 Kcal mol^{-1} g) (Johansson *et al.*, 2003).

e)



f)



g)

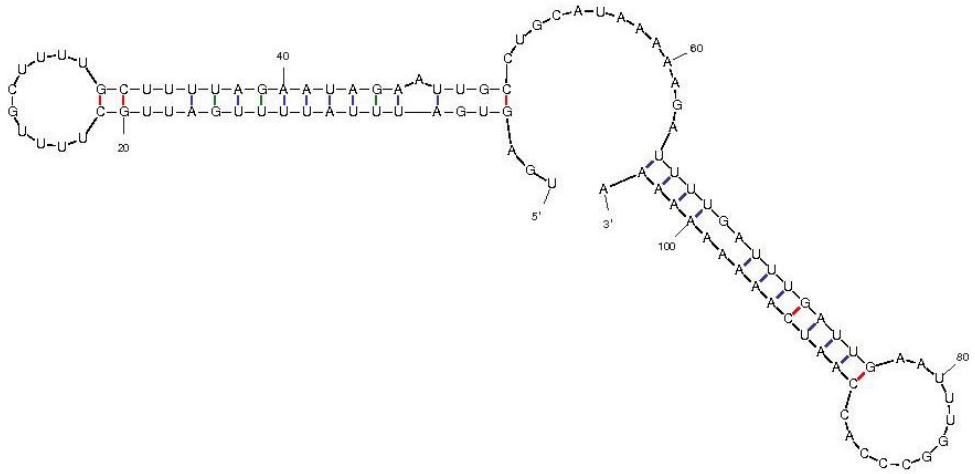


Figure 4.1. continued.

a)

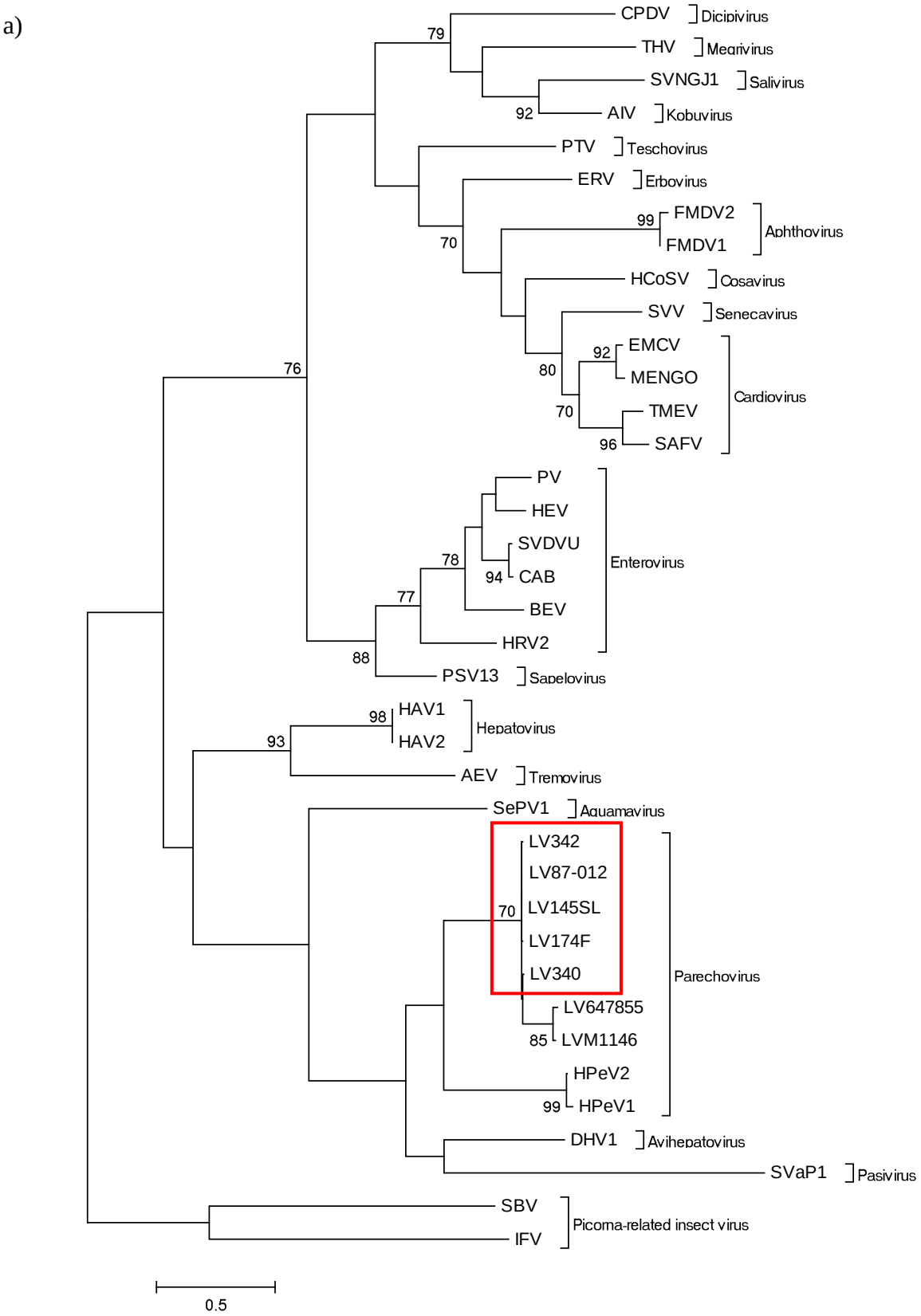


Figure 4.2. Maximum likelihood trees using the models rtREV+G (Dimmic *et al.*, 2002) for $3D^{pol}$ sequences n = 38 (a) and WAG+G (continued overleaf).

b)

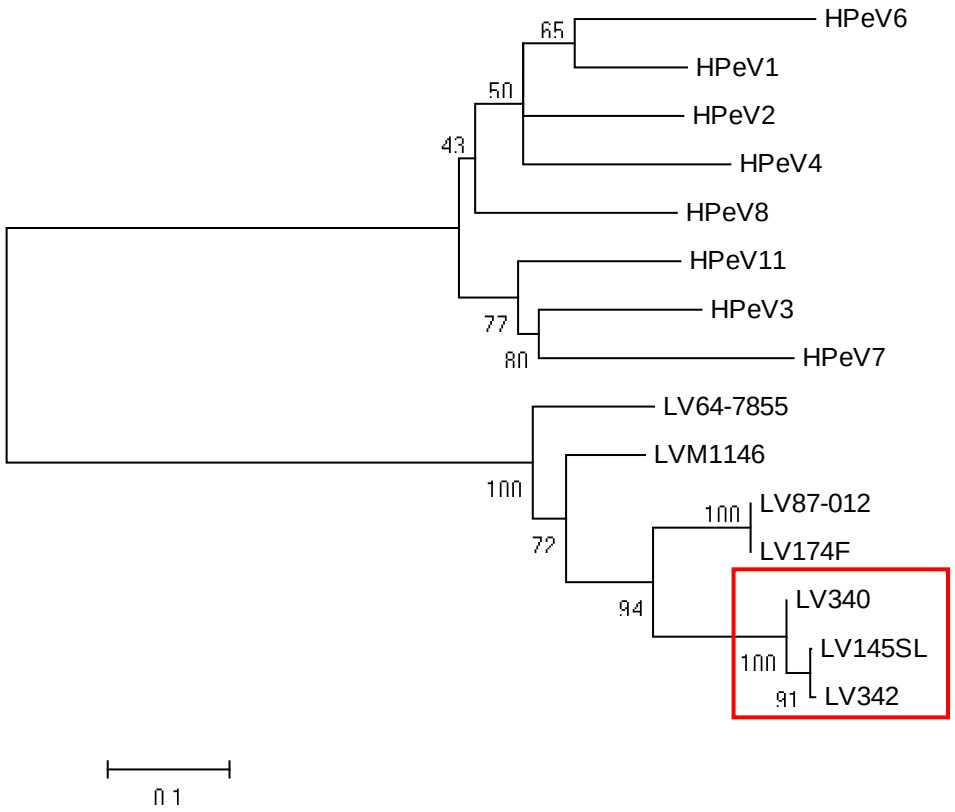


Figure 4.2 continued. (Whelan & Goldman, 2001) for VP1 sequences n = 15 (b) in MEGA5 (Tamura *et al.*, 2011). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates amino acid substitutions per site. Only bootstrap support of >70 % are shown. Positions with less than 95 % site coverage were eliminated. There were a total of 239 positions for the $3D^{pol}$ and 211 for the VP1 in the final datasets. The phylogenetic positions of LV340 and LV342 are shown in relation to representative picornaviruses a) and more closely related parechoviruses b). Uniprot accession numbers as shown in Table 4.1

Positive selection

Many picornaviruses have been reported to undergo positive selection including the closely related human parechoviruses (Benschop et al., 2008; Fares et al., 2001; Faria et al., 2009; Simmonds, 2006). In each case immune-mediated selection is proposed to be acting on the structural proteins of the virus. When investigating selection amongst the seven LV genomes it was found that most regions were predominantly evolving neutrally with a good proportion also showing signs of being under purifying selection. There were however two sites in the VP3 protein that were being positively selected for, codons 96 and 197 (Table 4.4), suggesting mutations at these sites are being fixed due to an inferred biological advantage. Site 96 is located in the predicted BC-loop of the VP3 capsid protein, a major antigenic site in picornaviruses, a site implicated in the virus-immune system interaction and shown to be involved in the viruses evasion from neutralization by monoclonal antibodies (Mateu, 1995; Niklasson et al., 1999). Site 197 is located within the suggested beta strand G2 of the VP3 beta-barrel complex (Niklasson et al., 1999; Stanway et al., 1994). Throughout the whole coding region there was a considerable amount of sites evolving neutrally, coupling this with the limited positive selection observed and the fact that the two American genotypes were isolated between 1962-1965 implies these geographically isolated viruses are not evolving very fast, a characteristic unusual for RNA viruses. Picornaviruses are typically found to have extremely large mutation rates of almost one mutation per replication, close to the viability threshold (Agol, 2002; Drake & Holland, 1999). The 'slow' evolution may suggest that LV could potentially be 'avoiding' immune pressure in some other way. Human parechoviruses, the other species within the genus exhibit high substitution rates (Faria et al., 2009), although these are reported to be solely human pathogens and as a result may not be directly comparable to Ljungan virus. When broadening the category to include RNA viruses found in a variety of mammal hosts, typical substitution rates have been reported within the range of 10^{-2} – 10^{-4} nucleotide substitutions per site per year (Hanada *et al.*, 2004; Jenkins *et al.*, 2002). Variations in these substitution rates could be attributed to differences in the infection e.g. long-term (chronic) infections may prevent the necessity to evolve fast, and the mode of transmission, for example multi-host viruses will require increased substitution rates to successfully transmit (Hanada *et al.*, 2004). Further evolutionary analysis is required to determine the substitution rate for LV.

Table 4.4. Identified positively selected sites within the VP3 protein.

		FEL[a]		REL[b]	
Region	**Codon**	**dN-dS**	**p value**	**dN/dS**	**Bayes factor**
VP3	96	0.92	0.05	0.64	170.62
VP3	197	0.75	0.06	0.86	331.74

[a] $p < 0.1$
[b] Bayes factor > 100

A factor that needs to be taken into consideration is the potential of recombination within the genomes as this could result in false positives (Pond *et al.*, 2006) when looking for sites under selection. We already know that the LV genome has a recombination point in the 2C3A region (Tolf *et al.*, 2009), however on testing the VP3 region no breakpoints were found with significant support to suggest a recombination event that could explain the selection seen and there is also support from the congruence of tree topologies (data not shown). Recombination analysis throughout the genome found only one site with significant support to suggest a recombination event located within the 3A protein. This would suggest the positive selection observed in the VP3 region is genuine and not an artifact of recombination.

Conclusion

As expected LV340 and LV342 exhibit greater sequence divergence in the structural rather than non-structural regions, this is most likely due to the immune pressure imposed on the virus to avoid recognition. A surprisingly large amount of neutral selection was observed across the LV genomes, with positive selection only being seen at two codons within the VP3 gene, one of which is located in a suggested antigenic site. These two isolates contain all the key motifs and structures of the five previously published isolates of Ljungan virus. Phylogenetic analysis confirms the placing of LV amongst the picornaviruses and within the parechovirus genus. All evidence supports the inclusion of LV340 and LV342 not only within the Ljungan virus clade but more specifically within genotype 2 alongside LV145SL. The more sequence information we can collect for LV especially from different locations, the better we are in understanding it, and should it be confirmed as a zoonosis then this information will inevitably benefit future research. All researchers publishing LV

prevalence data should be obliged to publish their sequence data to expand the understanding of LV epidemiology in different hosts and countries.

Chapter 5. Hantavirus surveillance in the United Kingdom

Adapted from Pounder et al. (2013) *Emerging Infectious Diseases* 19: 673-675 (Appendix 3)

5.1 Abstract

Serological studies and sporadic human cases have previously suggested the presence of hantavirus in the UK. However, until recently the species of hantavirus present in UK wildlife has never been confirmed. Between September 2009 and November 2011, wild rodents consisting of brown rats (*Rattus norvegicus*), wood mice (*Apodemus sylvaticus*), house mice (*Mus musculus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*) were live caught across North West England (Cheshire, Liverpool and Wirral). With the exception of a single field vole, the lungs from all rodents sampled were negative for hantaviral RNA using a pan-hantavirus RT-PCR. However, partial sequences for small (S) and large (L) genome segments were recovered from the lung of the field vole and confirmed the presence of a novel hantavirus (Tatenale Virus) in the United Kingdom. Coincidently in 2012, HPA investigations following a case of haemorrhagic fever with renal syndrome in Northern England, led to the subsequent isolation of Seoul hantavirus from a wild brown rat. The prevalence and public health impact of the two hantavirus species in the UK are not yet known.

5.2 Introduction

Hantaviruses are single-stranded RNA viruses that form their own distinct genus within the *Bunyaviridae*. Unlike other members of the *Bunyaviridae*, hantaviruses are not transmitted by arthropods but primarily by rodents of the families *Cricetidae* and *Muridae*, although insectivore and bat hosts have recently been reported (Klempa *et al.*, 2007; Weiss *et al.*, 2012). Each hantavirus appears to be adapted and largely restricted to an individual reservoir host species, implying that they have co-evolved, although phylogenetic analyses suggest that this apparent co-evolution may be more attributed to recent preferential host switching and local adaptation (Ramsden *et al.*, 2009).

Transmission to humans is primarily via inhalation of aerosolised virus in contaminated rodent urine and faeces. Whilst infected reservoir hosts are

asymptomatic, human infections can lead to two clinical manifestations, haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), with varying degrees of morbidity, mortality and burden (Vaheri *et al.*, 2012).

Zoonotic surveillance projects throughout Europe have detected five rodent-borne hantaviruses; Dobrava-Belgrade (DOBV), Saaremaa (SAAV), Seoul (SEOV), Puumala (PUUV) and Tula (TULV) plus two insectivore-borne hantaviruses; Seewis (SWSV) and Nova (NVAV) (Olsson *et al.*, 2010; Vaheri *et al.*, 2012). The relative geographic distribution of each hantavirus is defined by their reservoir host (Olsson *et al.*, 2010). All mainland European countries neighbouring the UK have reported hantavirus infections. The most common and widespread hantavirus across northern, central and eastern Europe is PUUV, which is associated with the mildest form of HFRS (Vaheri *et al.*, 2012).

There have been very few human hantavirus cases reported in the UK and in all instances the causative virus species was not confirmed in clinical specimens (reviewed in (Fhogartaigh *et al.*, 2011; McCaughey & Hart, 2000) (Table 1.1: Chapter 1). If these were all caused by the same hantaviral agent then there does not appear to be a consistent clinical picture for HFRS in the UK and do not match entirely to the guidelines outlined by WHO (Gajdusek *et al.*, 1983). In several cases it was noted that rodent exposure coincided with the onset of symptoms whilst no patients had been abroad, suggesting the acquisition of a strain circulating in the UK. Further evidence of hantaviruses circulating naturally in the UK comes from longitudinal studies which reported considerable hantavirus seropositivity in human cohorts. The majority of these studies included healthy individuals suggesting past exposure to hantaviruses or subclinical infection (seroprevalence 1-4.8 %) (Coleman, 2000; Davies *et al.*, 1988; McCaughey & Hart, 2000; McKenna *et al.*, 1994; Pether & Lloyd, 1993; Stanford *et al.*, 1990; Thomas *et al.*, 1999). In addition, serological surveys of rodents (rats and mice) and cats in the UK have reported the presence of hantavirus specific antibodies but not the species of hantavirus (Bennett *et al.*, 1990; Davies *et al.*, 1988; Lloyd, 1991; McCaughey & Hart, 2000; McCaughey *et al.*, 1996; Pether & Lloyd, 1993; Stanford *et al.*, 1990; Webster & Macdonald, 1995).

It was only in the later stages of 2012 that the first confirmation of SEOV RNA was found in UK brown rats (*R. norvegicus*) (Jameson *et al.*, 2013a). A patient from the Humber region with suspected hantavirus disease was confirmed to have high hantavirus antibodies to HNTV and SEOV, and this led to the subsequent trapping of rodents at their residence. Two of four brown rats tested were found positive for hantavirus RNA, this indigenous UK SEOV has been designated strain Humber (Jameson *et al.*, 2013a). There are several ports situated along the Humber estuary that could have facilitated the introduction of SEOV infected rats into the UK, interestingly however genetic analysis of this strain confirm it to be most similar to IR461, a strain that was previously responsible for UK human laboratory-acquired infections (Jameson *et al.*, 2013a; Shi *et al.*, 2003). Further work is required to confirm the Humber strains pathogenicity to humans.

At the commencement of this project there had been no confirmation of hantaviruses circulating in the UK, however since then Seoul virus RNA has been found in the brown rat (Jameson *et al.*, 2013a). Questions that remain to be answered are whether SEOV can be found in rats throughout the UK and is this the only hantavirus species circulating? This study aimed to determine, using a molecular approach, if hantaviruses are circulating in rodents in the North West UK. It is hypothesised that we would find Seoul virus due to the ubiquitous presence of its host the brown rat and the North West's strong links with sea travel that has facilitated rat distributions and with it possibly the introduction of Seoul virus. To test this we screened five rodent species using a published Pan-hantavirus PCR assay (Klempa *et al.*, 2006).

5.3 Methods

Field work

Rodents (n = 495) consisting of brown rats (*R. norvegicus*) (n = 133), wood mice (*Apodemus sylvaticus*) (n = 269), bank voles (*Myodes glareolus*) (n = 50), house mice (*Mus musculus*) (n = 35) and field voles (*Microtus agrestis*) (n = 8) were live caught across North West England (particularly in and around Liverpool, Chester and Wirral, see Chapter 2). Animals were sacrificed in the field by inhalation of Isoflurane, following UK Home Office Guidelines. Where possible, kidney, liver and lung tissues, were removed within 1 hour of euthanasia. Blood samples were collected when field conditions allowed, otherwise heart tissue was retained. The

samples, and if necessary the unprocessed carcases, were stored at -80 °C until required.

RNA extraction and reverse transcription

50-100 mg of tissue was homogenised in 1 ml TRIzol® Reagent (Invitrogen, Life Technologies, Paisley, UK) with QIAGEN Stainless steel beads (5 mm) using a QIAGEN TissueLyser (Qiagen, UK) for 2 mins at 30 Hz. RNA was extracted from the homogenate as described in the Invitrogen TRIzol® Reagent instructions for animal tissues (Invitrogen, Life Technologies, Paisley, UK). RNA was purified using Ambion® PureLink™ RNA Mini Kit (Life Technologies, Invitrogen, Paisley, UK) and quantified using the Qubit® Fluorometer (Invitrogen, Life Technologies, Paisley, UK), as described in the manufacturer's protocol, and stored at -80 °C until reverse transcription.

RNA was reverse transcribed following a standard first strand cDNA synthesis protocol (Invitrogen, Life Technologies, Paisley, UK). Briefly, RNA (>1 µg) was reverse transcribed in a 20 µl reaction containing 50 ng/µl random hexamers, 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH), 5X First strand buffer, 0.1 M DTT and 200 U SuperScript™ III Reverse Transcriptase (Invitrogen, Life Technologies, Paisley, UK). Cycling parameters were 65 °C for 5 mins, 50 °C for 60 mins and 70 °C for 15 mins in a Techne TC-5000 Thermal Cycler. The cDNA was stored at -20 °C.

Pan-hantavirus RT-PCR

Two µl of cDNA was PCR-amplified in a 14.5 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pM (each) of the first round forward and reverse primers (Eurofins MWG Operon, Germany) (Klempa *et al.*, 2006). Cycling parameters were 95 °C for 15 mins, 40 three step cycles (30 s at 95 °C, 45 s at 53 °C, 30 s at 72 °C) and 72 °C for 6 mins.

The second round PCR mix was made up as before except first round PCR product was used in place of the cDNA template, and the nested forward and reverse primers were added. Thermal cycling conditions are as described above. Five µL of PCR

product were analysed by 1.5 % agarose gel electrophoresis (120 V for 70 mins). Hantavirus positive samples gave a band at approximately 452 bp after the first round and 390 bp after the second round (Klempa *et al.*, 2006).

PCR product purification and DNA Sequencing

Primers and unincorporated nucleotides were removed from PCR products using an ExoSAP digest. Five µl PCR product was added to 2 µl mix containing 10X RX buffer, 0.2 U Shrimp Alkaline phosphatase (USB, UK) and 1 U Exonuclease I (New England BioLabs, UK). Cycling parameters were 37 °C for 45 mins, 80 °C for 15 mins. The cycle sequencing reaction was set up using the BigDye® Terminator v3.1 Cycle Sequencing kit. One µl of ExoSAP product was added to a 9 µl reaction mix containing 5X sequencing buffer, 0.75 µl BigDye 3.1 (Applied Biosystems, Life Technologies, Paisley, UK) and 1.6 pM of the second round forward or reverse primer. Cycling parameters were 25 three step cycles (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 mins). The sequencing product was then precipitated using 3 M sodium acetate prior to resuspension in HiDi™ formamide (Applied Biosystems, Life Technologies, Paisley, UK), and then run on an ABI3130xl.

PCR – 18S rRNA Housekeeping gene

A housekeeping control was implemented to confirm successful reverse transcription of samples and so the presence of amplifiable DNA. cDNA from each sample was amplified in a separate PCR reaction using specific primers targeting the 18S ribosomal RNA; a gene present in all eukaryotic cells. Briefly, 1 µl of cDNA was PCR-amplified in a 14.5 µl reaction containing containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene), and 5 pM of the 18S rRNA primers and 18S rRNA competimers (Ambion, UK). Cycling parameters were 95 °C for 10 mins, 24 three step cycles (30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C) and 72 °C for 6 mins. PCR product was analysed by 1.4 % agarose gel electrophoresis (120 V for 60 mins). Evidence of amplifiable cDNA gave a band at approximately 489 bp.

PCR – cytochrome b

Morphological species determination of small mammals was confirmed by molecular identification using degenerate cytochrome *b* (cyt *b*) primers (Schlegel *et*

al., 2011). Briefly, 1 µl cDNA was PCR-amplified in a 14.5 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pM of the CytB Uni fw primer and CytB Uni rev primer (Eurofins MWG Operon, Germany) (Schlegel *et al.*, 2011). Cycling parameters were 94 °C for 3 mins, 40 three step cycles (30 s at 94 °C, 30 s at 47 °C, 1 min at 72 °C) and 72 °C for 10 mins. PCR product was analysed by 1.3 % agarose gel electrophoresis (120 V for 60 mins). Successful amplification of the cyt *b* gene gave a band at approximately 947 bp. Amplicons were sequenced as described above except using the CytB Uni fw or CytB Uni rev primer in the cycle sequencing reaction.

Partial S segment PCR and indirect fluorescent antibody tests (IFAT) were carried out by collaborators in Finland (Tarja Sironen: Finnish Forest Research Institute). Partial S segment was recovered using the following primers in the reverse transcription and the first round of PCR: forward (SF490) AARGANAAYAARGGNACN and reverse (SR1157) YTGDATHCCCATNGAYTG. Nested PCR followed with primers: forward (SF604) ATGAARGCNGADGARHTNACN, and reverse (SR1061) CATDATNGTRTTHCTCATRTC.

Phylogenetic analysis

Multiple nucleotide and amino acid sequence alignments were generated in MEGA5 (Tamura *et al.*, 2011). Sequence identities were compared using MegAlign (Lasergene DNAStar). Bayesian phylogenetic trees were produced in the BEAST package of software (Drummond & Rambaut, 2007) with MCMC chain lengths of 10 million and strict clock. Optimum substitution models were estimated in MEGA5 (Tamura *et al.*, 2011).

5.4 Results

PCR validation and quality assurance (QA)

The optimised Pan-hantavirus assay was tested using panels of positive material requested from the European Virus Archive (EVA). Hantavirus species cDNA (DOBV, HTNV and PUUV) were received from Dr Boris Klempa (Slovak Academy of Sciences) and positive hantavirus species RNA (HTNV, PUUV, SEOV, TULV)

were received from Dr Mark Outlaw (National Collection of Pathogenic Viruses: NCPV) (Figure 5.1). Samples included four of the five hantavirus species reported to be circulating in Europe and the Hantaan virus prototype species.

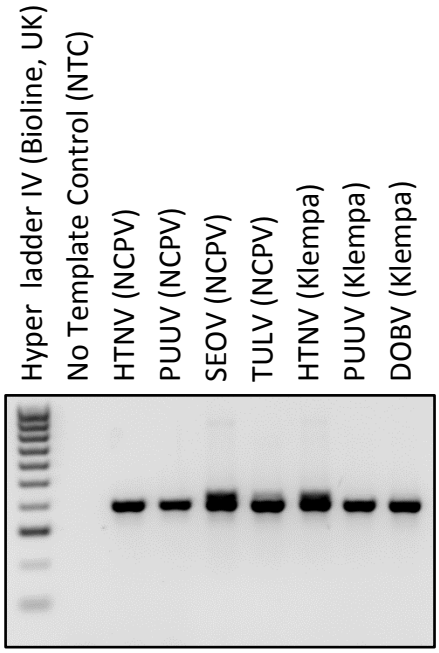


Figure 5.1. Positive control panel, cDNA Hantaan Virus (HTNV) (Strain 76-118), Puumala Virus (PUUV) (Strain Sotkamo) and Dobrava-Belgrade Virus (DOBV) (Strain Slovakia) (Dr Boris Klempa), RNA for Hantaan Virus (Strain 76-118), Puumala Virus, Seoul Virus (SEOV) (Strain R22), Tula Virus (TULV) (NCPV) tested using the Pan-hantavirus RT-PCR assay.

The sensitivity of this published pan-hantavirus assay was tested using the Hantaan virus (HTNV) (Strain 76-118) provided by Dr Boris Klempa. HTNV viral RNA was detected at a dilution of 1:1000 which matched that detected in Dr Boris Klempa's laboratory.

To validate the assay on actual rodent tissues, bank vole (*M. glareolus*) lung samples were provided in a blind panel that previously had been tested by serology and RT-

PCR (Professor Heikki Henttonen; Finnish Forest Research Institute). 15/15 positives and 11/13 negatives were in agreement, however two of the 13 negatives were positive in triplicate by our assay suggesting potential increased sensitivity.

Screen results

With the exception of a single male field vole (B41) collected near the village of Tattenhall, Cheshire (Figure 5.2), the lungs from all rodents sampled were negative for hantaviral RNA using a nested pan-hantavirus RT-PCR, directed against partial polymerase (L) gene sequences. Based on the finite number of each rodent species caught and that none were found positive for HV RNA, from this data we cannot rule out prevalences less than approximately 1.1 % for wood mice, 2.3 % for brown rats, 5.8 % for bank voles and 8.2 % for house mice. With regards to the field voles with one out of a total eight individuals infected with HV RNA we could expect the true prevalence of this virus to be anywhere between 0.3 and 52.7 % based on the 95 % confident intervals (Exact binomial test).

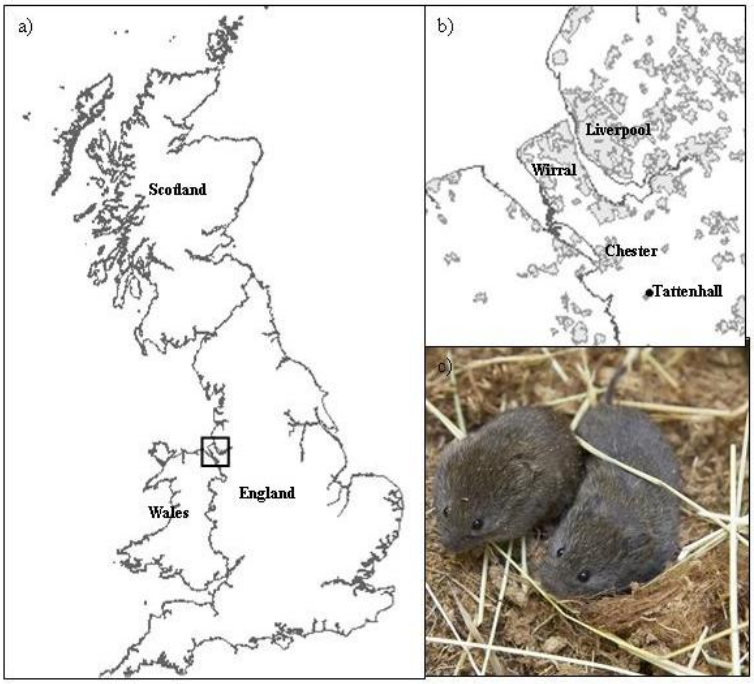


Figure 5.2. The location of the trapping site of B41 within a) United Kingdom and b) North West England (urban areas shown in grey). An image of a Field vole (*M. agrestis*) courtesy of E. Oksanen (c).

Partial L (371 nucleotides) and S segment (396 nucleotides) sequences were retrieved. Established M segment RT-PCR assays were unsuccessful. L and S sequences have been deposited with NCBI (Genbank Accession numbers JX316008 and JX316009 respectively). Table 3 shows nucleotide and amino acid sequence identity comparisons between B41 and other members of the Hantavirus genus. The Arvicolinae-associated hantaviruses showed the highest similarity to the UK sequence at both the nucleotide (65.7 %–78.8 % for S and 76.6 %–77.5 % for L) and the amino acid level (66.4 %–86.3 % for S and 80 %-88 % for L) (Table 5.1).

In the partial L tree (Figure 5.3a), B41 viral sequence clusters with Prospect Hill virus (PHV) and Tula virus (TULV) with good support, whilst in the partial S tree (Figure 5.3b), B41 appears more closely related to the Asian *Microtus*-associated hantaviruses albeit with low posterior probability values.

Table 5.1. Similarity (% identity) of B41 partial S and L segment sequences with those of other hantaviruses*†

	S segment		L segment	
Hantavirus	**nt**	**aa**	**Nt**	**aa**
TOPV	78.8	86.3	n/a	n/a
Fusong	75.0	80.9	77.2	80.0
KHAV	74.7	85.5	n/a	n/a
PUUV	73.7	80.2	76.6	84.8
HOKV	73.5	79.4	76.9	84.8
PHV	68.7	73.3	77.5	88.0
TULV	65.7	66.4	77.5	86.4
SNV	58.1	53.4	71.9	78.4
SWSV	56.3	48.1	68.0	70.4
ANDV	55.8	51.9	72.2	81.6
DOBV	52.8	42.7	63.3	66.4
SEOV	52.8	42.3	63.6	68.8
SAAV	51.8	43.5	63.9	65.6
HTNV	51.5	41.2	66.9	69.6
TPMV	49.2	41.2	63.9	63.2
MGB/1209	n/a	n/a	65.0	62.4

*S, small; L, large; TOPV, Topografov virus (AJ011646); Fusong (EU072481 and FJ170807); KHAV, Khabarovsk (U35255); PUUV, Puumala virus (M32750 and M63194); HOKV, Hokkaido virus (AB675463 and AB675455); PHV, Prospect Hill virus (M34011 and EF646763); TULV, Tula virus (NC005227 and NC005226); SNV, Sin Nombre virus (NC005216 and L37901); SWSV, Seewis virus (GQ293136 and EF636026); ANDV, Andes virus (AF291702 and AF291704); DOBV, Dobrava-Belgrade (AY961615 and GU904039); SEOV, Seoul virus (AY273791 and X56492); SAAV, Saaremaa virus (AJ616854 and AJ410618); HTNV, Hantaan virus (NC005218 and NC005222); TPMV, Thottapalayam virus (AY526097 and NC010707); MGB/1209, Magboi/1209 virus (JN037851); n/a, sequence not available.

†396 nucleotides (nt) of the S segment (positions 620-1015), and 371 nt of the L segment (positions 2962-3332) and the deduced amino acid (aa) sequences (131 aa, position 194-324 of the nucleocapsid protein; 123 aa, position 976-1098 of the viral RNA-dependent RNA polymerase) have been compared using MegAlign (Lasergene DNAStar). Fragment positions were defined according to complete sequences of PUUV strain CG1820.

a)

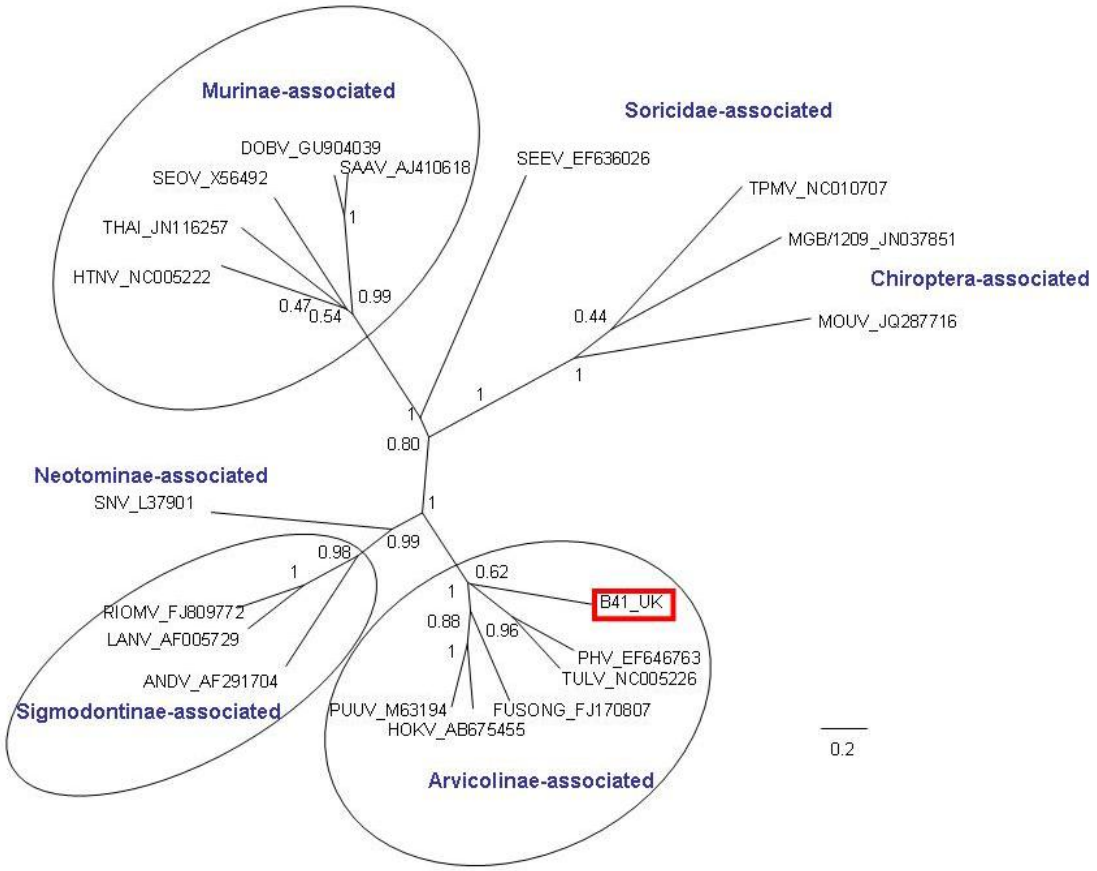


Figure 5.3. Bayesian phylogenetic trees using the models HKY+Gamma for partial L segment sequences n = 19 (a) and GTR+Gamma for partial S segment n = 39 (b, overleaf) within the BEAST package of software (Drummond & Rambaut, 2007). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The numbers at each node are posterior probabilities. All Effective Sample Size values exceeded 150 for partial L and 1600 for partial S. The scale bar indicates nucleotide substitutions per site. The phylogenetic position of B41 is shown in relation to representative hantaviruses a) and more closely related Arvicolinae-associated hantaviruses b). All positions containing gaps and missing data were eliminated. Genbank accession numbers are shown next to taxa names. B41; VLAV, Vladivostok virus; TOPV, Topografov virus; KHAV, Khabarovsk virus; PUUV, Puumala virus; HOKV, Hokkaido virus; MUJV, Muju virus; PHV, Prospect Hill virus; ISLAV, Isla Vista virus; TULV, Tula virus; LANV, Laguna Negra virus; ANDV, Andes virus; SNV, Sin Nombre virus; NYV, New York virus.

b)

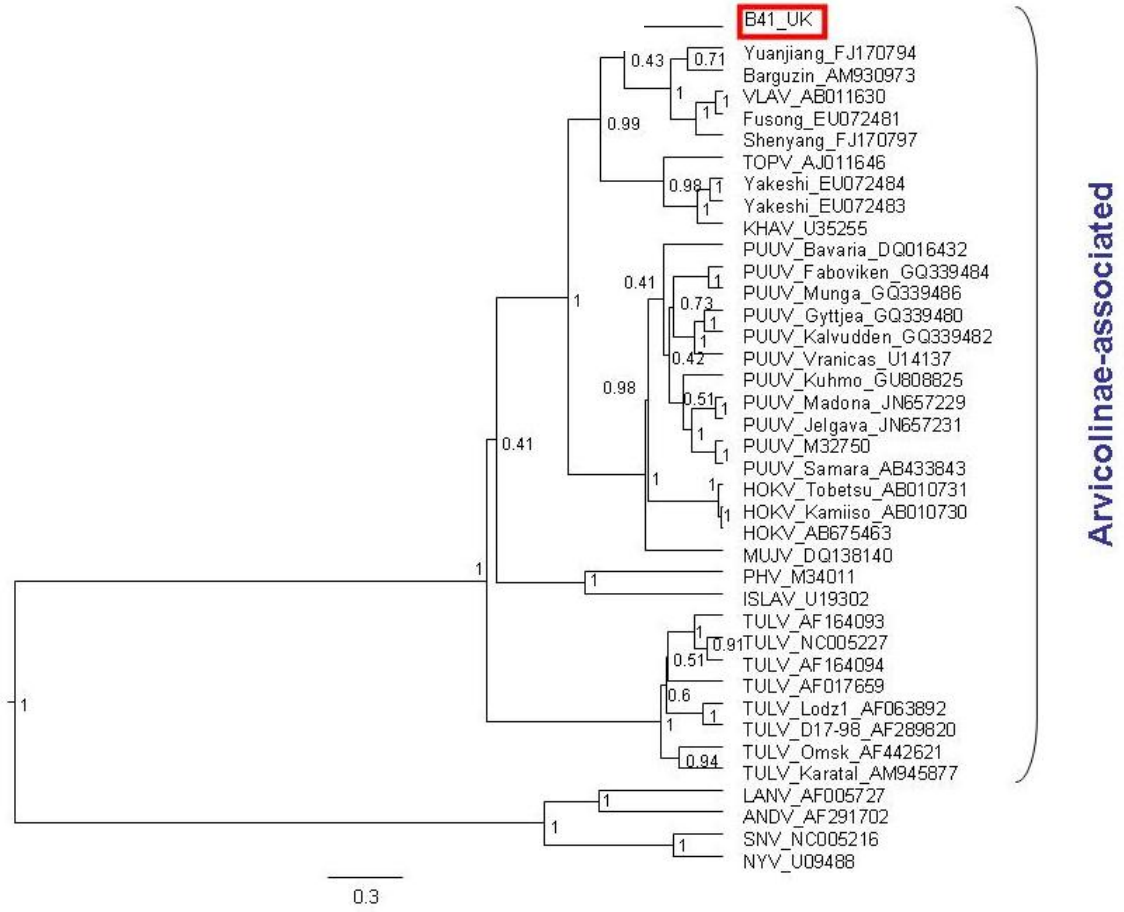


Figure 5.3. continued.

Blood collected from B41 was sent to the reference laboratory in Finland (Professor Heikki Henttonen's Research group). The blood was found positive for hantavirus-specific antibodies (indirect fluorescent antibody test using Puumala antigen (Vaheri *et al.*, 2008) suggesting that there is cross reactivity between the two viruses, well-known for hantaviruses within the same host subfamilies (Vaheri *et al.*, 2008). Hantavirus RNA was detected in the kidney but not the liver of B41 nor the lung, liver and kidney of the seven other field voles. Degenerate cytochrome *b* gene PCR and sequencing (Schlegel *et al.*, 2011) was employed to confirm the morphological identification of the field voles (B41 cyt *b* Genbank accession no. KC222031).

5.5 Discussion

Phylogenetic analyses of partial L sequences (Figure 5.3a) and partial S sequences (Figure 5.3b) confirm the inclusion of B41 as a distinct member of the Arvicolinae-associated hantaviruses. Although phylogenetic position cannot be fully resolved based on these partial sequences and the differences in tree topologies most likely reflect the different composition of the sequence datasets. The nucleotide and amino acid sequence divergence between B41 and the most related hantaviruses corresponds to that typically found between different hantavirus species (Klempa *et al.*, 2006). In addition, the phylogenetic analysis of the B41 S and L sequences further supports it as a distinct hantavirus. As such we propose to name this novel virus, Tatenale Virus (TATV), representing the name of the village in which it originated as it was first recorded in the Doomsday book of 1086. This was to avoid any negative connotations for the area.

Microtus agrestis has not previously been shown to be a primary carrier of a specific hantavirus although recent studies suggested an involvement in the maintenance of TULV infection in Germany (Schmidt-Chanasit *et al.*, 2010). *M. agrestis* is one of the most numerous mammals of mainland Britain, with estimated populations exceeding 75 million (Harris & Yalden, 2008), but it mainly inhabits ungrazed grassland and as such may pose a lower public health risk than other more synanthrophic hosts. Further investigations are required to determine if *M. agrestis* is the only or main reservoir host of this novel virus. More extensive targeted surveillance of field voles in the UK, using TATV specific RT-PCR and IFAT, is necessary to provide an estimate of virus prevalence, to determine the zoonotic

morbidity of TATV and to confirm if *M. agrestis* is the reservoir host of this novel virus. Such data are essential to inform policy and determine the relative risks to human and animal health. Current knowledge of other *Microtus*-borne hantaviruses suggests that while they may infect man, generally their pathogenic potential is low (Vaheri *et al.*, 2012). Future work will also involve attempts to isolate TATV and generate full genome sequence.

Despite the recent confirmation of SEOV in localised UK brown rats we did not find any evidence of SEOV in rodents caught around the North West. One explanation for this might be that if SEOV infected rats have only been introduced via the ports in the Humber region of the UK then it is possible that the virus has not had long enough to effectively spread to the North West. Although suggestive evidence from past UK human reports, if caused by SEOV give the impression of a wider distribution e.g. Glasgow (Walker *et al.*, 1984) and Nottingham (Watson *et al.*, 1997). There is also the possibility that these infected rats are in some way genetically different to non-infected rats making them more suitable carriers. The port of Liverpool is the largest on the west coast of Britain and is the third busiest port in the UK. Liverpool has strong import/export links with several countries with reported rodent and human SEOV cases including China and the USA (Steve Seddon, Mersey Port Health Authority, pers. comms.).

Due to the broad clinical features of hantavirus disease, it is likely that many UK human cases would be misdiagnosed. The confirmation of our novel hantavirus and the recently reported SEOV case in indigenous wildlife in the UK may promote the inclusion of a hantavirus differential when patients present with acute renal failure, undiagnosed febrile illness and have had previous exposure to rodents (Fhogartaigh *et al.*, 2011).

Chapter 6. Genetic comparison of Seoul hantavirus in *Rattus norvegicus* in Lyon, France

6.1 Abstract

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are single-stranded RNA viruses, which are transmitted to humans primarily via inhalation of aerosolised virus in contaminated rodent urine and faeces. Whilst infected reservoir hosts are asymptomatic, human infections can lead to two clinical manifestations, haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), with varying degrees of morbidity and mortality. Surveillance in Europe has detected six rodent-borne hantaviruses; Dobrava-Belgrade Virus (DOBV), Saaremaa virus (SAAV), Seoul virus (SEOV), Puumala virus (PUUV), Tatenale virus (TATV) and Tula virus (TULV). The prevalence of rodent and human cases of SEOV in Europe are considered to be low, and speculated to be driven by the sporadic introduction of infected brown rats (*Rattus norvegicus*) via ports. Between October 2010 and March 2012, 128 brown rats were caught at sites across the Lyon region in France. SEOV RNA was detected in the lungs of 14 % (95 % CI 8.55 – 21.31) of brown rats tested using a nested pan-hantavirus RT-PCR (polymerase gene). We did not detect any evidence of a genetic difference between infected and non-infected rats (cytochrome *b* gene). Our findings and the recent detection of SEOV in UK brown rats, suggest that SEOV is more prevalent in European brown rats and may contribute to a greater number of the reported HFRS cases in Europe than previously believed.

6.2 Introduction

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are single-stranded RNA viruses. Unlike other members of the *Bunyaviridae*, hantaviruses are not transmitted by arthropods but primarily by rodents of the families *Cricetidae* and *Muridae*, although insectivore and bat hosts have recently been reported (Klempa *et al.*, 2007; Weiss *et al.*, 2012). Each hantavirus appears to be adapted and largely restricted to an individual reservoir host species, implying that they have co-evolved, although phylogenetic analyses suggests that this apparent co-evolution may be more attributed to recent preferential host switching and local adaptation (Ramsden *et al.*, 2009).

Transmission to humans is primarily via inhalation of aerosolised virus in contaminated rodent urine and faeces. Whilst infected reservoir hosts are asymptomatic, human infections can lead to two clinical manifestations, haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), with varying degrees of morbidity and mortality (Vaheri *et al.*, 2012). Surveillance in Europe has detected six rodent-borne hantaviruses; Dobrava-Belgrade virus (DOBV), Saaremaa virus (SAAV), Seoul virus (SEOV), Puumala virus (PUUV), Tatenale virus (TATV) (Pounder *et al.*, 2013) and Tula virus (TULV) plus two insectivore-borne hantaviruses; Seewis virus (SWSV) and Nova virus (NVAV) (Heyman *et al.*, 2011; Olsson *et al.*, 2010; Vaheri *et al.*, 2012). The relative geographic distribution of each hantavirus is defined by their reservoir host (Olsson *et al.*, 2010). The most common and widespread hantavirus across northern, central and eastern Europe is PUUV, which is associated with the mildest form of HFRS (Vaheri *et al.*, 2012).

The Norwegian/brown rat (*Rattus norvegicus*) is a reservoir host for Seoul virus, a cause of mild to moderate HFRS disease in humans (Vaheri *et al.*, 2012). Brown rats are a cosmopolitan species and thus provide the potential to spread SEOV worldwide. They represent the predominant and widely distributed host of hantavirus in China, where, a total of 1,557,622 cases of HFRS were reported in humans between 1950–2007 with 46,427 deaths (3 %) (Lin *et al.*, 2012; Zhang *et al.*, 2009).

Recent data suggests that the brown rat originated from northern China and only migrated to the rest of the world within the last two centuries (Lin *et al.*, 2012). It is proposed that this recent radiation also brought with it the distribution of SEOV from China (Lin *et al.*, 2012). To date, confirmed human SEOV infections have been reported in Asia (Japan (Kariwa *et al.*, 2000), South Korea (Kim *et al.*, 1995), China (Song, 1999; Zhang *et al.*, 2009)) and the Americas (USA (Glass *et al.*, 1994), Brazil (Iversson *et al.*, 1994)). Within Europe however, beyond laboratory acquired infections (Shi *et al.*, 2003), there has been only been one SEOV confirmed HFRS case where SEOV RNA had been detected in a patients serum in Lyon, France (Macé *et al.*, 2013). There have also been a further three human cases in the UK which are presumed SEOV infections due to suspected or known exposure to SEOV infected rats (Jameson *et al.*, 2013a; Jameson *et al.*, 2013b; Taori *et al.*, 2013) and a

single case confirmed serologically by virus neutralisation assay (vNA) in Lyon (Lundkvist personal comment; (Heyman *et al.*, 2004)). However, rat seroprevalences of 10-78.9 % and 27.1 % to SEOV antibodies have been seen in France (Heyman *et al.*, 2004) and Belgium (Heyman *et al.*, 2009a), respectively. SEOV was confirmed as the etiological agent by RT-PCR in both studies.

This study aimed to determine the prevalence of SEOV in wild rats (*R. norvegicus*) trapped in and around Lyon, France and analyse any resulting molecular epidemiological data. We also assessed the usefulness of mitochondrial cytochrome *b* (cyt *b*) gene analysis to determine if the infected rats were non-indigenous to Lyon (a major port) and thereby support a relatively recent importation into France.

6.3 Methods

Field work

The survey was subdivided in two 6-month-periods. The first period aimed at rural rat sampling which was conducted from October 2010 to February 2011. All sites were located within 15 km of Rhône-Alpes (Figure 6.1a). The second aimed at urban rat sampling which was conducted from October 2011 to March 2012 in Lyon (Figure 6.1a). During the two periods, 184 free living Norway rats (*R. norvegicus*) were trapped from which 128 were screened for hantaviruses by RT-PCR. Rats were trapped using small (28 cm x 9 cm x 9 cm) or large (50 cm x 15 cm x 15 cm) single catch rat traps. Captured rats were transported to the laboratory where live rats were immediately anaesthetised using Isoflurane and sacrificed by cervical dislocation whereas dead rats were frozen (-20 °C) and thawed on the day of the dissection. Lungs were collected from different lobes. Samples were directly stored at -80 °C (rural sampling) or -20 °C (urban sampling) until their shipping to the University of Liverpool where they were stored at -80 °C.

Rats provided for this study were trapped for the purpose of pest control (agreement no. 69-1810). They were euthanized and used (agreement no. 69-020931) according to ethical rules supervised by the ethical committee of VetAgro Sup and European regulation (Directive EU 86/609).

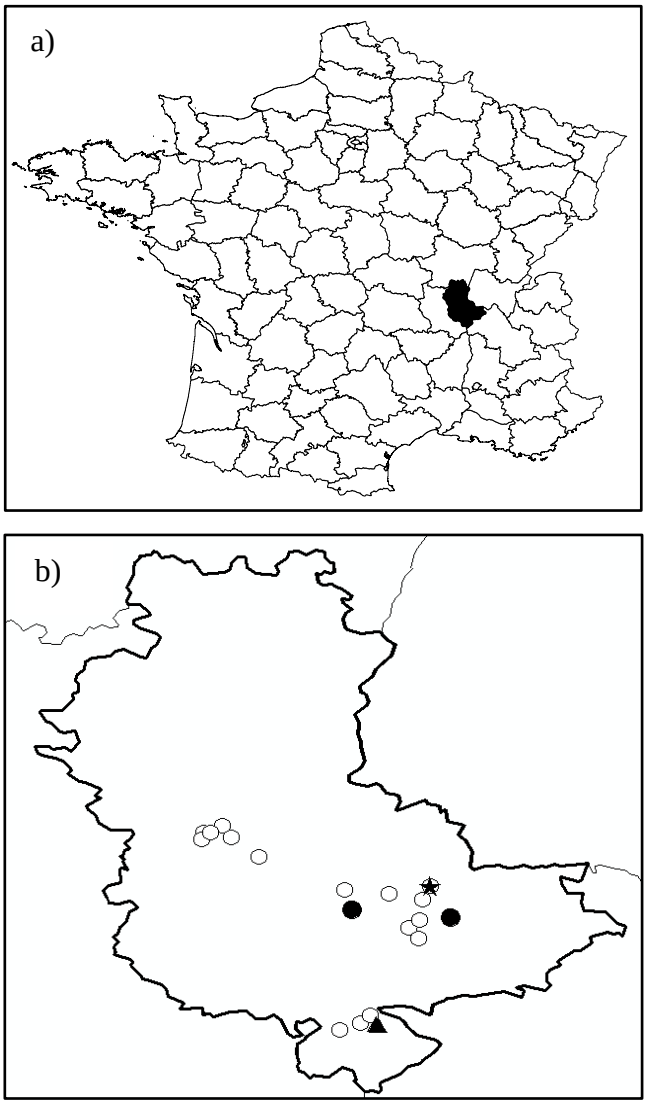


Figure 6.1. The locations of the trappings sites within a) France and b) Rhône department. SEOV infected groups 'Lyon I, II and III' are represented by a star, triangle and blocked out circles, respectively. Non-infected individuals are marked by open circles.

Screening

RNA extraction

50-100 mg of liver tissue was homogenised in 1 ml TRIzol® Reagent (Invitrogen, Life Technologies, Paisley, UK) with QIAGEN Stainless steel beads (5 mm) using a QIAGEN TissueLyser (Qiagen, UK) for 2 mins at 30 Hz. RNA was extracted from the homogenate as described in the Invitrogen TRIzol® Reagent instructions for

animal tissues (Invitrogen, Life Technologies, Paisley, UK). The RNA was diluted 1:10 in sterile distilled water and quantified using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) and stored at -80 °C until reverse transcription.

cDNA synthesis

RNA was reverse transcribed following a standard first strand cDNA synthesis protocol (Invitrogen, Life Technologies, Paisley, UK). Briefly, approximately 1 µg of RNA was reverse transcribed in a 20 µl reaction containing 50 ng/µl random hexamers, 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH), 5X First strand buffer, 0.1 M DTT and 200 U SuperScript™ III Reverse Transcriptase (Invitrogen, Life Technologies, Paisley, UK). Cycling parameters were 65 °C for 5 mins, 50 °C for 60 mins and 70 °C for 15 mins in a Techne TC-5000 Thermal Cycler. The cDNA was stored at -20 °C.

Hantavirus PCR

Screening of the rodent lung samples for hantaviral RNA was performed using a nested pan-hantavirus PCR directed against partial polymerase (L) gene sequences (Klempa *et al.*, 2006). Briefly, 2 µl cDNA was synthesised in a 14.5 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pmol (each) of HAN-L-F1 and HAN-L-R1 primers (Eurofins MWG Operon, Germany) (Klempa *et al.*, 2006). Cycling parameters were 95 °C for 15 mins, 40 three step cycles (30 s at 95 °C, 45 s at 53 °C, 30 s at 72 °C) and at 72 °C for 6 mins.

Primers HAN-L-F2 and HAN-L-R2 were used in the second round PCR (Eurofins MWG Operon, Germany) (Klempa *et al.*, 2006). Thermal cycling conditions are as described above. Five µl of PCR product were analysed by 1.5 % agarose gel electrophoresis (120 V for 70 mins). Hantavirus positive samples gave a band at approximately 452 bp after the first round and 390 bp after the second round.

cyt b PCR

Morphological species determination of small mammals was confirmed by molecular identification using degenerate cyt *b* primers (Schlegel *et al.*, 2011).

Briefly, 1 µl cDNA was PCR-amplified in a 14.5 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pM of the CytB Unifw primer and CytB Uni rev primer (Eurofins MWG Operon, Germany) (Schlegel *et al.*, 2011). Cycling parameters were 94 °C for 3mins, 40 three step cycles (30 s at 94 °C, 30 s at 47 °C, 1 min at 72 °C) and 72 °C for 10 mins. PCR product was analysed by 1.3 % agarose gel electrophoresis (120 V for 60 mins). Successful amplification of the cyt *b* gene gave a band at approximately 947 bp.

PCR product purification and DNA Sequencing

Primers and unincorporated nucleotides were removed from PCR products using an ExoSAP digest. Five µl PCR product was added to 2 µl mix containing 10X RX buffer, 0.2 U Shrimp Alkaline phosphatase (USB, UK) and 1 U Exonuclease I (New England BioLabs, UK). Cycling parameters were 37 °C for 45 mins, 80 °C for 15 mins. The cycle sequencing reaction was set up using the BigDye® Terminator v3.1 Cycle Sequencing kit. One µl of ExoSAP product was added to a 9 µl reaction mix containing 5X sequencing buffer, 0.75 µl BigDye 3.1 (Applied Biosystems, Life Technologies, Paisley, UK) and 1.6 pM of either LV forward, LV reverse, CytB Unifw or CytB Uni rev primer. Cycling parameters were 25 three step cycles (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 mins).The sequencing product was then precipitated using 3 M sodium acetate prior to resuspension in HiDi™ formamide (Applied Biosystems, Life Technologies, Paisley, UK), and then run on an ABI3130xl.

Phylogenetic analysis

Multiple nucleotide sequence alignments were generated in MEGA5 (Tamura *et al.*, 2011). Sequence identities were compared using Geneious 5.6.5 (Biomatters: www.geneious.com, date accessed: 1/8/12). Maximum likelihood phylogenetic trees were produced in MEGA5 (Tamura *et al.*, 2011) with bootstrap replications of 10,000 (Felsenstein, 1985). Optimum substitution models were estimated in MEGA5 (Tamura *et al.*, 2011).

454 method

Viral specific reads from one positive sample, *LYO852*, were obtained directly from lung tissue. Briefly, TRIzol® extracted viral RNA was depleted of host genomic DNA using RNase-free DNAse (Qiagen, UK) and host ribosomal RNA was depleted using Terminator™ 5'-Phosphate-Dependent Exonuclease (Epicentre Biotechnologies). The RNA was fragmented, a random-primed cDNA library was made and run using the Roche 454 GS FLX System. The sequencing data were initially assembled in the GS de novo assembly software (Roche). Subsequently, previously published SEOV sequences were used to map specific reads from the original raw data using GS Reference Mapper (Roche).

6.4 Results

Screening

A total of 84 brown rats were caught from urban areas and 44 from rural areas around Lyon (Table 6.1). Screening of rat lung samples tested in triplicate for the presence of Seoul hantavirus RNA showed an overall prevalence of 14.1 % (18/128) (Exact binomial test 95 % confidence intervals, 8.55 – 21.31). There was a male bias of 2:1 in the infected individuals (11 adult males, one juvenile male, one pregnant female and five adult females). The proportion of all males infected was larger than females, 16.4 % and 11.3 % respectively, but this was not significant (Pearson's Chi-squared test, $\chi^2 = 0.6568$, df = 1, P = 0.4177). On separating the infected adults according to habitat type, there was no significant difference found between males and females in urban areas (Pearson's Chi-squared test $\chi^2 = 0.0854$, df = 1, P = 0.77). However the proportion of males infected in rural areas was significantly larger than that of females (Fisher's Exact Test, $P < 0.05$) (Table 6.2).

All 18 RT-PCR positive rats were selected for genetic analysis and partial sequences of the L segment (317 bp) were recovered. Eight variable sites were located within this partial sequence. Phylogenetic analysis of the SEOV-positive brown rats shows that they divide into three clusters: Lyon I, II and III (Figure 6.1b & Figure 6.2). Analysis of all SEOV partial L segments shows highest identity to the Belgium SEO/Belgium/Rn895/2005 strain (JQ898108) (98.0-98.8 %) (Heyman *et al.*, 2009a). Lyon I sequences (LYO903 and LYO906) were somewhat more divergent from the other Lyon sequences (0.5-1.4 %). All 18 RT-PCR L sequences clustered with

previously described Lineage #7 (Plyusnina *et al.*, 2004), with moderate bootstrap support of at least 62 (Figure 6.2).

Table 6.1. Field sample break down of caught urban and rural brown rats (*R. norvegicus*) by gender and age.

	Male		Female		
Habitat	**Adult**	**Juvenile**	**Adult**	**Juvenile**	**Total**
Urban	35	22	22	5	84
Rural	11	5	19	7	42[a]
Total	46	27	41	12	126

[a]Two additional juvenile rats gender not known

Table 6.2. Gender comparison of SEOV prevalence in adult rats from urban and rural areas.

	Male		Female	
	Total	**Infected (%)**	**Total**	**Infected (%)**
Urban	35	20.0	22	27.3
Rural	11	36.4	19	0.0

cyt b

Partial sequences of the cyt *b* gene (833 bp) were recovered from the 18 infected samples and 15 non-infected samples. Eighteen variable sites were located within this partial sequence. Pairwise comparisons among all sequences (including out groups) ranged from 0 to 11.9 %. All Lyon cyt *b* sequences in this study had an average genetic distance of 0.3 % (ranging between 0 to 1.0 %). Compared to group

A (non-Chinese and Chinese non-mountainous Seoul virus variants, Lin *et al.*, 2012) members, Lyon sequences had an average distance of 0.4 % (range between 0 to 1.0 %) whereas to group B (Chinese mountainous Seoul virus variants, Lin *et al.*, 2012) there was an average of 5.5 % (range 5.4 to 5.8 %). The average genetic distance between Lyon *R. norvegicus* and the outgroup *R. tanezumi* and *R. rattus* was 11.3 % (ranging from 10.8 to 11.9 %). Infected rats (Figure 6.3: In red bold) formed two main clusters, one including Lyon III individuals and the other Lyon I and II. All partials regardless of clusters assembled with group A sequences (Figure 6.3). Partial cyt *b* sequences were also retrieved from n = 56 brown rats collected for Chapters 3 and 5, providing UK representatives for analysis.

454 S and M segment sequence output and assembly statistics

De Novo assembly of LYO852 reads yielded 59 contigs (consisting of 73,105 reads, totalling 24,730,464 bp) representing 82 % total reads, with a mean length of 702 bp (ranging between 105-2920 bp). There were 15 contigs ≥500 bp. Based on BLAST identity searches all contigs were host or mycoplasma sequences. Mapping of the reads using GS Reference Mapper (Roche) with published SEOV genome sequences identified 44 (0.03 %) SEOV specific reads yielding 9 contigs in total for LYO852. Two partial nucleocapsid (S) gene contigs were retrieved, of 715 and 786 bp, and showed greatest identity to Seoul strain Singapore/06(RN46) (98 %) and SEO/Belgium/Rn895/2005 (98 %), respectively. Three partial glycoprotein (M) gene contigs were retrieved, of 987, 1,735 and 612 bp and showed greatest identity to Seoul strain Singapore/06(RN46) (98 %), Seoul strain 5CSG (98 %) and 5CSG (98 %), respectively. However, contig 2 (1,735 bp) was 99 % identical over 283 nt to a previously published Lyon colony rat partial M sequence (Heyman *et al.*, 2004). Four partial polymerase (L) gene contigs were retrieved, of 459, 603 and 740 bp, with greatest identity to lineage #4 Seoul strain 80-39 (96 %) and one contig (1,564 bp) had greatest identity to lineage #3 Seoul strain DPRK08 (97 %) (Wang *et al.*, 2000). All S (Figure 6.4 & data not shown), M (data not shown) partials for LYO852 clustered within lineage #7 (Plyusnina et al., 2004). Total coverage for each of the three segments of LYO852 was 84.8 % (S), 91.3 % (M) and 51.5 % (L).

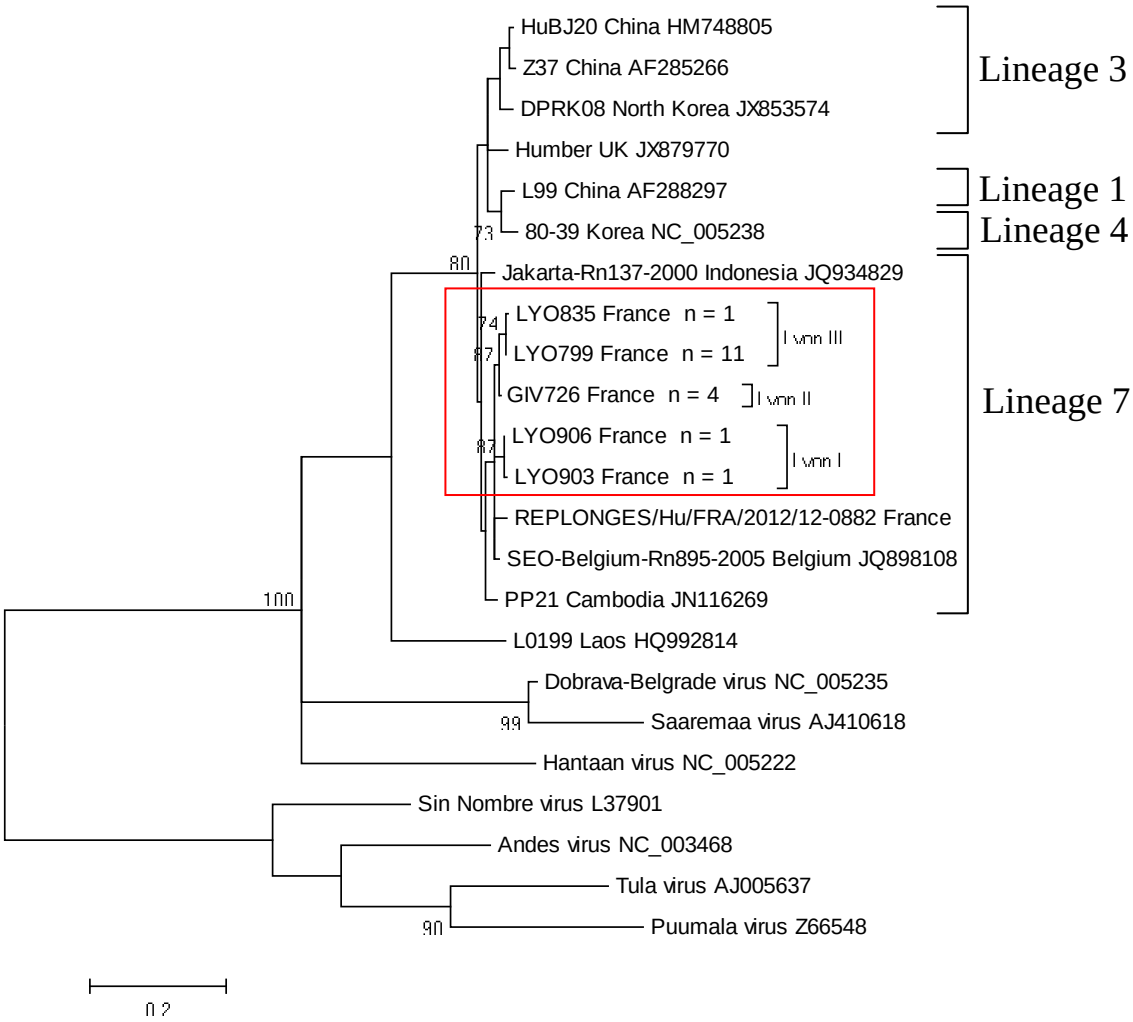


Figure 6.2. Maximum likelihood tree using the model T92+Gamma (Tamura, 1992) for SEOV partial L segment sequences n = 23 in MEGA5 (Tamura *et al.*, 2011). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates nucleotide substitutions per site. Only bootstrap support of >70 % are shown. Positions with less than 95 % site coverage were eliminated. There were a total of 317 positions in the final dataset. The phylogenetic positions of groups Lyon I, II and III are shown in relation to representative Seoul strains. LYO726 partial L sequence was identical to LYO733, 737 and 757. LYO799 partial L sequence was identical to LYO837, 838, 839, 843, 845, 848, 852, 853, 884 and 871. Genbank accession numbers are shown next to taxa names.

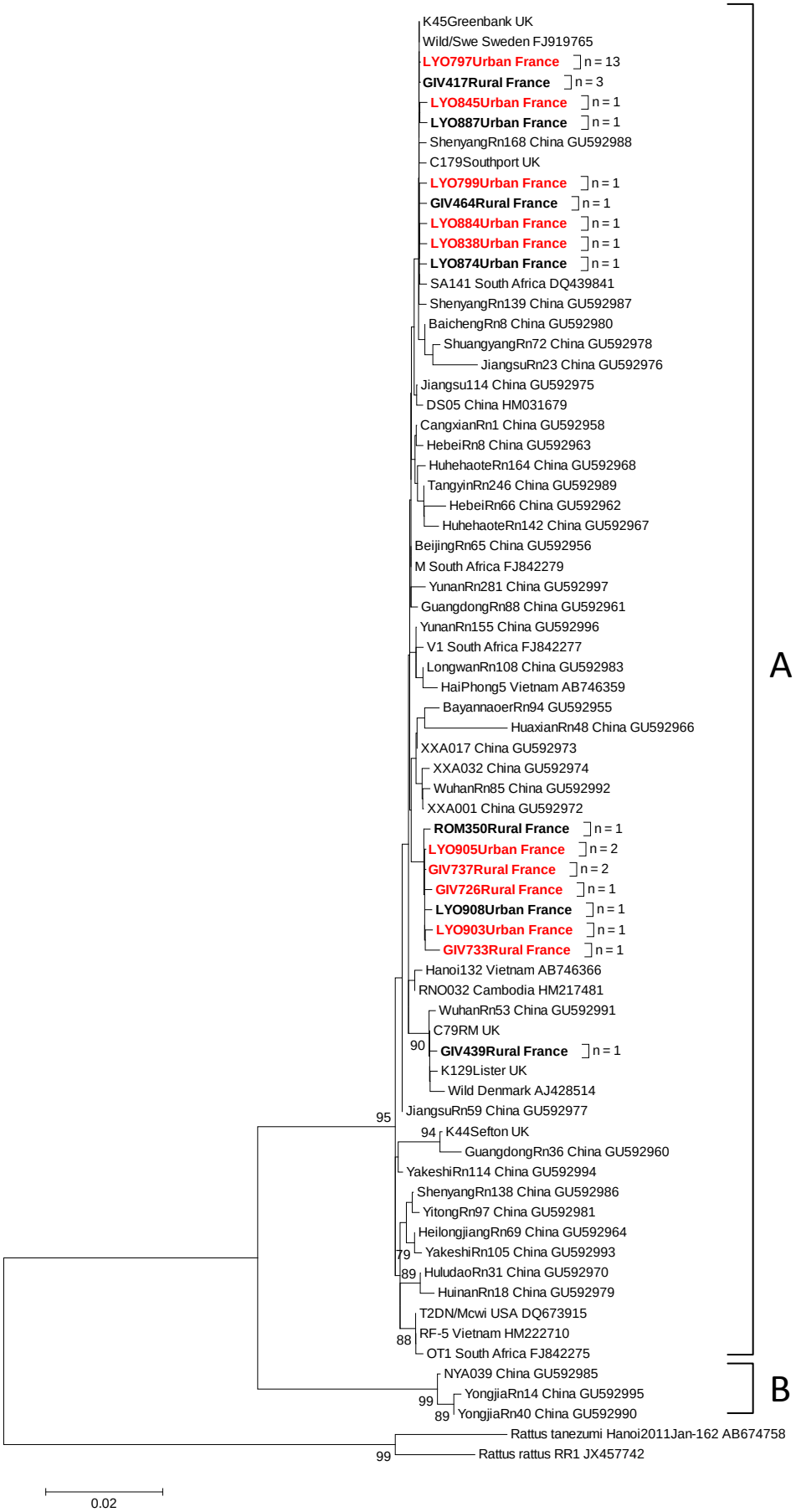


Figure 6.3. Maximum likelihood tree using the model (continued overleaf)

Figure 6.3. **continued**. HKY+Gamma (Hasegawa *et al.*, 1985) for partial cytochrome *b* sequences n = 72 in MEGA5 (Tamura *et al.*, 2011). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates nucleotide substitutions per site. Only bootstrap support of >70 % are shown. Positions with less than 95 % site coverage were eliminated. There were a total of 833 positions in the final dataset. The phylogenetic positions of Lyon *R. norvegicus* are shown in relation to representative *R. norvegicus* sequences. Representative Lyon rats are highlighted in bold, and those that include at least one infected individual are red bold. Genbank accession numbers are shown next to taxa names.

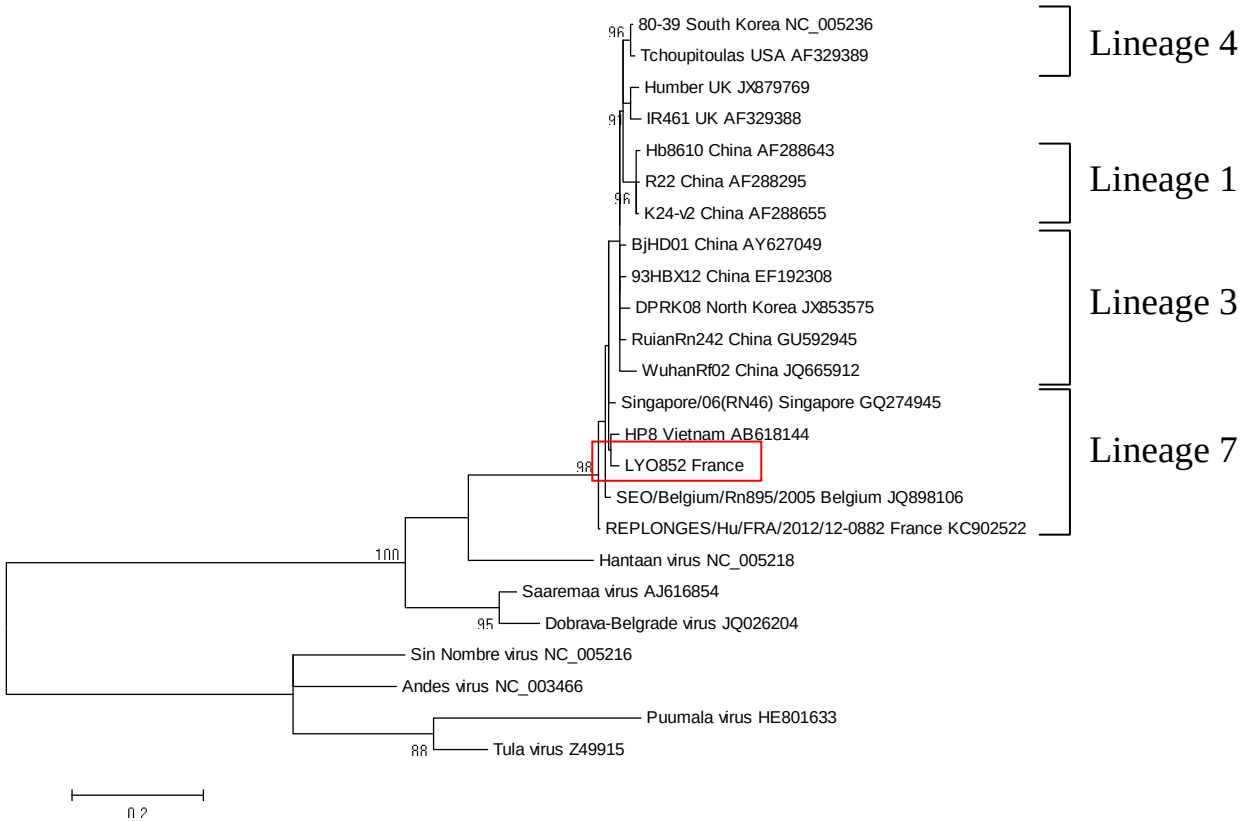


Figure 6.4. Maximum likelihood tree using the model TN93+Gamma (Tamura & Nei, 1993) for SEOV partial S segment sequences n = 24 in MEGA5 (Tamura *et al.*, 2011). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates nucleotide substitutions per site. Only bootstrap support of >70 % are shown. Positions with less than 95 % site coverage were eliminated. There were a total of 702 positions in the final dataset. The phylogenetic position of Lyon852 is shown in relation to representative Seoul strains. Genbank accession numbers are shown next to taxa names.

6.5 Discussion

The prevalence of SEOV hantaviral RNA in Lyon rats of 14.1 % shown in this study falls within the range of seroprevalences found in studies from Argentina (0 - 26 %) (Cueto *et al.*, 2008), Baltimore (52 %) (Easterbrook *et al.*, 2005), Belgium (7.6 - 50 %) (Heyman *et al.*, 2009a), Cambodia (20.9 %) (Reynes *et al.*, 2003), China (13.5 %) (Li *et al.*, 1995), Indonesia (10 %) (Ibrahim *et al.*, 1996), Northern Ireland (21.6 %) (McCaughey *et al.*, 1996), South Korea (9.6 %) (Kim *et al.*, 2007) and Vietnam (14 %) (Koma *et al.*, 2012). The previous French SEOV investigation reported a seroprevalence as large as 78.9 % (Heyman *et al.*, 2004). This figure however relates to a study with two subgroups each containing ten animals, whereas our study involved a much larger sample size (n = 128). Furthermore, we employed molecular methods to detect viral RNA rather than the more variable serological detection of antibodies.

Within the sample group of SEOV infected individuals, we only observed a male biased ratio amongst the adult rural samples. A male biased ratio amongst SEOV infected rats is not uncommon however, and has been reported on several occasions (Cueto *et al.*, 2008; Glass *et al.*, 1988; Klein *et al.*, 2000). Whilst neither male nor female rats are believed to be more susceptible to Seoul virus infection, males do shed the virus for a longer duration in their urine, faeces and saliva (Klein *et al.*, 2000) and so the viral RNA may be detectable for longer in the host tissues. In addition the primary route of transmission between adult males is thought to be through wounds (Hinson *et al.*, 2004), so it has been suggested that the likelihood of males acquiring the Seoul virus is greater due to them having more aggressive encounters (Klein *et al.*, 2000). Perhaps contact with con-specifics occurs less frequently in rural areas and when it does tends to be male-male interactions.

Despite their disparate isolation, most SEOV variants published to date are genetically homogenous with up to 95 % nucleotide sequence identity (Zhang *et al.*, 2009) making it difficult to determine source of introduction. Phylogenetic analysis of the SEOV partial L segment show Lyon sequences cluster by location. Lyon I sequences, were more divergent from the other two groups (Figure 6.2). S and M available partial segments of the Lyon samples supported their grouping within lineage #7 along with all other European strains except for the recently isolated

'Humber' strain in the UK (Jameson *et al.*, 2013a). Surprisingly, this strain is more similar to the UK laboratory-acquired strain IR461 despite the laboratory rats originating from Japan via Belgium (Jameson *et al.*, 2013a; Shi *et al.*, 2003). Interestingly, whilst Lyon I sequences were more divergent from both Lyon II and III in the partial L segment, in the cytochrome *b* gene, Lyon I rats were more similar and clustered with Lyon II rats than either were to Lyon III rats. Although 454 pyrosequencing did not deduce the complete genome for LYO852, we were able to obtain reads from all three segments, the S and M partials substantiate the RT-PCR L gene data as clustering with lineage #7 (Plyusnina *et al.*, 2004). However the L partial sequences obtained from the 454 cluster with lineage #3 and #4 (Wang *et al.*, 2000). Good coverage of sequence information was obtained for the S and M segments (>84 %), and whilst only 50 % was recovered for the L segment this actually corresponds to >3,000 bp. *The* low level of viral reads in the *RNA pool* (0.03 %) possibly reflects the low viral load in the organ at the time of sampling.

The cytochrome *b* gene is a useful target for species recognition as it has a tendency to be more reliable than morphological characteristics. Genetic divergences typically seen in the cytochrome *b* gene between individuals at the species level and indicative of species recognition is >11 % (Bradley & Baker, 2001), and we support this with a comparison of *R. norvegicus* against the outgroups (11.3 %) (*R. tanezumi* and *R. rattus*). Several studies have looked at the genetic variation expected in the cyt *b* gene within rodent species and found divergences of 0 – 8.1 % (Gering *et al.*, 2009; Myers *et al.*, 1995; Smith & Patton, 1991; Tiemann-Boege *et al.*, 2000; Van Daele *et al.*, 2007). The brown rat has been found worldwide however it only radiated in the last few centuries with the aid of human globalisation (Lin *et al.*, 2012). Very few rodent species exhibit such an extensive distribution so it makes sense to compare the divergences of the brown rat with a species that exhibits much greater mobility than rodents in general i.e. Chiroptera. Single bat species have been found widely segregated around the world and studies have investigated the genetic divergence between these and have found evidence of greater differences. For example, *Myotis muricola* populations in the Malay Archipelago have found divergences on average of 8 and 9.5 % (ranging between 0-16.4 %) (Wiantoro *et al.*, 2012), and *Miniopterus schreibersii* populations in Europe and South Africa were found to be divergent by 10.5 % (Stadelmann *et al.*, 2004). Lin *et al.*, (2012) previously described the

dispersal of *R. norvegicus* as two clusters, cluster A being composed of all non Chinese, and Chinese non-mountainous SEOV variants and cluster B composed of a cohort that originated from mountainous areas of China. Our data support the two lineages proposed with genetic divergences confirming the placing of Lyon brown rats within cluster A (divergence 0.4 %), rather than to cluster B (5.5 %). Considering the brown rat is a cosmopolitan species it has surprisingly lower genetic divergence than expected. It was also thought that infected rats may be genetically different to non-infected rats making them more likely hosts for SEOV. With evidence from our partial cyt *b* sequences we could not confidently support the theory that SEOV infected rats were continually being introduced via boats etc. Rather infected and non-infected rats were found in both clusters (Figure 6.3).

We present here not only further confirmation of the circulation of Seoul virus in and around Lyon, France but we also demonstrate a revised SEOV prevalence estimate in brown rats using molecular methods. All SEOV RT-PCR L partials and the available S and M partial segments were consistent in their support for being placed among lineage #7 along with European and Southeast Asian strains, although the four partial L sequences retrieved from *De Novo* sequencing did not show this, rather identities were closer to lineage 3 and 4 Seoul strains (Wang *et al.*, 2000). To confidently resolve the L segments lineage, more sequence data would be required. There is no evidence of SEOV infected rats being different to non-infected rats in the partial cytochrome *b* gene. SEOV has been found circulating in brown rats in France, Belgium and the UK and it is to be expected that it will also be circulating in other European countries. This is the first large scale molecular survey of SEOV in urban and rural rats in Europe. We have confirmed a greater prevalence in European brown rats than previously believed. Our data may suggest a greater role of SEOV in European HFRS cases than previously thought, warranting further surveillance.

Chapter 7. Seoul virus in Pet Rats

7.1 Abstract

Wild brown rats (*Rattus norvegicus*) are reservoirs for Seoul virus (SEOV), a cause of mild to moderate HFRS disease in humans. Currently the prevalence of rodent and human cases of SEOV in Europe are considered to be low. However, following two separate suspected human hantavirus cases, SEOV has been detected in wild brown rats (*Rattus norvegicus*) in the Humber region of the UK and a pet rat in North Wales. In 2012, 21 pet rats (*R. norvegicus*) from a breeding colony in Oxfordshire, the origin of the North Wales pet rat, were screened for hantaviral RNA using a pan-hantavirus RT-PCR. Multiple organs were screened from a single individual to investigate SEOV tropism. SEOV RNA was detected in the lungs and kidneys of 81 % of pet rats tested and all but one of the organs from the single individual (liver). Sequence analysis showed it to be most similar to the recently isolated SEOV Humber strain (97 %). No evidence was detected for a genetic difference between pet rats and wild UK rats (cytochrome *b* gene). Domesticated rats are not generally considered a public health problem, however these findings suggest that hantaviruses may pose a greater risk than previously believed.

7.2 Introduction

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are single-stranded RNA viruses. Unlike other members of the *Bunyaviridae*, hantaviruses are not transmitted by arthropods but primarily by rodents of the families *Cricetidae* and *Muridae*, although insectivore and bat hosts have recently been reported (Klempa *et al.*, 2007; Weiss *et al.*, 2012). Each hantavirus appears to be adapted and largely restricted to an individual reservoir host species, implying that they have co-evolved, although phylogenetic analyses suggests that this apparent co-evolution may be more attributed to recent preferential host switching and local adaptation (Ramsden *et al.*, 2009).

Transmission to humans is primarily via inhalation of aerosolised virus in contaminated rodent urine and faeces. Whilst infected reservoir hosts are asymptomatic, human infections can lead to two clinical manifestations, haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary

syndrome (HCPS), with varying degrees of morbidity and mortality (Vaheri *et al.*, 2012). Zoonotic surveillance projects throughout Europe have detected five rodent-borne hantaviruses; Dobrava-Belgrade (DOBV), Saaremaa (SAAV), Seoul (SEOV), Puumala (PUUV) and Tula (TULV) plus two insectivore-borne hantaviruses; Seewis (SWSV) and Nova (NVAV) (Olsson *et al.*, 2010; Vaheri *et al.*, 2012). The relative geographic distribution of each hantavirus is defined by their reservoir host (Olsson *et al.*, 2010). The most common and widespread hantavirus across northern, central and eastern Europe is PUUV, which is associated with the mildest form of HFRS (Vaheri *et al.*, 2012). As described in Chapter 4, a new hantavirus, Tatenale virus (TATV) has now been added to this list.

The Norwegian/brown rat (*Rattus norvegicus*) is the principal reservoir host for Seoul virus, a cause of mild to moderate HFRS disease in humans (Vaheri *et al.*, 2012). Brown rats are a cosmopolitan species and thus provide the potential to spread SEOV worldwide. SEOV specific antibodies have been detected in wild rats in Belgium (Heyman *et al.*, 2009a), France (Heyman *et al.*, 2004), Northern Ireland (McCaughey *et al.*, 1996), Portugal (Filipe *et al.*, 1991) and most recently the UK (Humber region) (Jameson *et al.*, 2013a). Within Europe, human SEOV cases have mainly been associated with laboratory acquired infections (Shi *et al.*, 2003), however there has been one confirmed HFRS case where SEOV RNA had been detected in a patients serum in Lyon, France (Macé *et al.*, 2013). There have also been a further three presumed SEOV human cases in the UK with either suspected or known exposure to SEOV infected rats (Humber, North Wales and Oxfordshire) (Jameson *et al.*, 2013a; Jameson *et al.*, 2013b) and a single case confirmed serologically by virus neutralisation assay (vNA) in Lyon, France (Lundkvist personal comment; (Heyman *et al.*, 2004)). Since 1983 there have been a number of unconfirmed suspected hantavirus reports in the UK where either SEOV or HTNV antibodies were found in both humans and rodents or if antibodies were not specified then exposure to rats was reported, all proposing the presence of SEOV in UK brown rats (Coleman, 2000; Davies *et al.*, 1988; Lloyd, 1991; McCaughey *et al.*, 1996; McKenna *et al.*, 1994; Pether & Lloyd, 1993; Rice *et al.*, 1993; Stanford *et al.*, 1990; Thomas *et al.*, 1999; Walker *et al.*, 1984; Watson *et al.*, 1997; Webster & Macdonald, 1995). Unfortunately no virus was isolated from these cases, making

these reports difficult to validate. This evidence suggests SEOV introduction into the UK may not have been a recent event.

The presence of zoonoses in domestic pets is a cause for great concern as the risk to humans would be significantly greater. In China and the USA there have been several reports of hantavirus specific antibodies being detected in domestic pets (Childs *et al.*, 1987; Leighton *et al.*, 2001; Malecki *et al.*, 1998). In Europe, PUUV antibodies have been found in domestic cats (16.9 %) and dogs (4.9 %) in Belgium (Dobly *et al.*, 2012) and cats (5 %) in Austria (Nowotny, 1994; Nowotny *et al.*, 1994). HTNV antibodies have been detected in UK healthy (9.6 %) and chronically ill cats (23 %) (Bennett *et al.*, 1990), but due to cross-reactivity amongst *Murinae*-associated hantaviruses, this may indicate to the circulation of SEOV rather than HTNV. However it is thought that cats and dogs do not play a role in the maintenance and transmission of hantaviruses and most likely represent dead end hosts and spill over events (Dobly *et al.*, 2012). The only instance where they may pose a risk to humans is in the exception of domestic pets bringing infected rodents into homes into closer proximity to humans. Pet rats on the other hand are a more likely route of transmission. In 2013, following a suspected human case in North Wales, a strain of SEOV designated 'Cherwell' was isolated from a pet rat of the patient (Jameson *et al.*, 2013b). This is the first confirmation of hantaviral RNA in a domestic pet and led to the subsequent investigation into the breeding colony where that pet rat had originated

Fancy (pet) rats (Figure 7.1) originated in Europe in the 19th century from the domestication of wild *Rattus* species in particular brown rats (*R. norvegicus*). Whilst fancy rats are not genetically different enough to be classified as a separate species, since domestication they have changed considerably in terms of their behaviour and physiology compared to wild counterparts. A 2012 survey by the Pet Food Manufacturers Association (PFMA) estimated there to be at least 200,000 pet rats within the UK. In general, pet rats are not thought to pose any more of a health risk than other common pets. However this recent finding suggests otherwise. Detection of SEOV in a pet rat raises the question of its origin and whether it has been naturally maintained in pet rats or recently introduced.

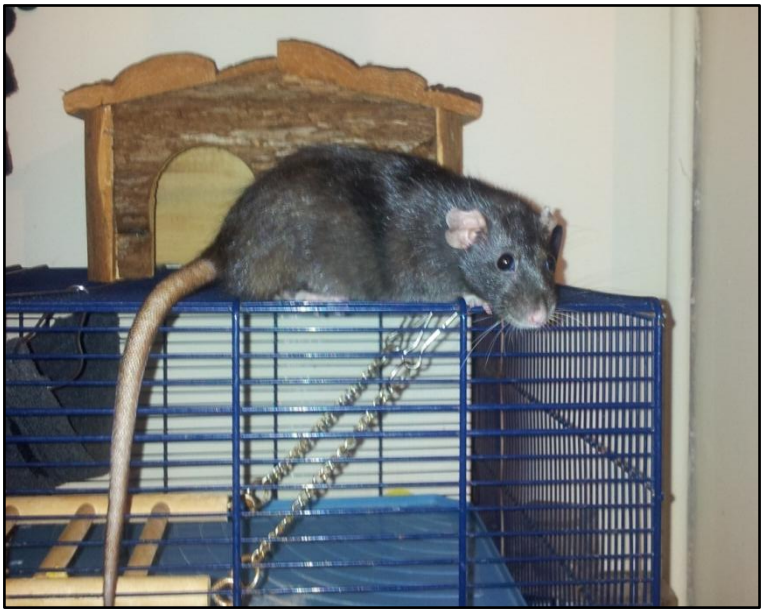


Figure 7.1. Fancy rat (*R. norvegicus*)

This study aimed to determine the proportion of pet rats from the breeder's colony that were infected with hantaviral RNA and provide evidence to assist in understanding the human health risk.

7.3 Methods

Samples

The owner's consent was obtained to euthanase the 21 pet rats (*R. norvegicus*) from their breeding colony in Oxfordshire and remove them for further testing at the Animal Health and Veterinary Laboratories Agency (AHVLA) and The University of Liverpool (UoL). Lung and kidney material were removed from all animals and the carcases were stored at -80 °C. Heart, liver, salivary gland and spleen were also removed from one individual 3784.

RNA extraction

50-100 mg of liver tissue was homogenised in 1 ml TRIzol® Reagent (Invitrogen, Life Technologies, Paisley, UK) with QIAGEN Stainless steel beads (5 mm) using a QIAGEN TissueLyser (Qiagen, UK). RNA was extracted from the homogenate as described in the Invitrogen TRIzol® Reagent instructions for animal tissues

(Invitrogen, Life Technologies, Paisley, UK). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) and stored at -80 °C until reverse transcription.

cDNA synthesis

RNA was reverse transcribed following a standard first strand cDNA synthesis protocol (Invitrogen, Life Technologies, Paisley, UK). Briefly, approximately 1 µl of RNA was reverse transcribed in a 20 µl reaction containing 50 ng/µl random hexamers, 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH), 5X First strand buffer, 0.1 M DTT, 40 U RNaseOUT and 200 U SuperScript™ III Reverse Transcriptase (Invitrogen, Life Technologies, Paisley, UK). Cycling parameters were 65 °C for 5 mins, 50 °C for 50 mins and 85 °C for 5 mins. The cDNA was stored at -20 °C.

Hantavirus PCR

Screening of the rodent lung samples for hantaviral RNA was performed using a nested pan-hantavirus PCR directed against partial polymerase (L) gene sequences (Klempa *et al.*, 2006). Briefly, 2 µl cDNA was synthesised in a 14.5 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pmol (each) of HAN-L-F1 and HAN-L-R1 primers (Eurofins MWG Operon, Germany) (Klempa *et al.*, 2006). Cycling parameters were 95 °C for 15 mins, 40 three step cycles (30 s at 95 °C, 45 s at 53 °C, 30 s at 72 °C) and at 72 °C for 6 mins.

Primers HAN-L-F2 and HAN-L-R2 were used in the second round PCR (Eurofins MWG Operon, Germany) (Klempa *et al.*, 2006). Thermal cycling conditions are as described above. Five µL of PCR product were analysed by 1.5 % agarose gel electrophoresis (120 V for 70 mins). Hantavirus positive samples gave a band at approximately 452 bp after the first round and 390 bp after the second round. Samples were repeated at two locations, UoL and AHVLA.

cyt b PCR

Morphological species determination of small mammals was confirmed by molecular identification using degenerate cyt *b* primers (Schlegel *et al.*, 2011).

Briefly, 1 µl cDNA was PCR-amplified in a 14.5 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 U Taq polymerase (ABgene) and 10 pM of the CytB Uni fw primer and CytB Uni rev primer (Eurofins MWG Operon, Germany) (Schlegel *et al.*, 2011). Cycling parameters were 94 °C for 3 mins, 40 three step cycles (30 s at 94 °C, 30 s at 47 °C, 1 min at 72 °C) and 72 °C for 10 mins. PCR product was analysed by 1.3 % agarose gel electrophoresis (120 V for 60 mins). Successful amplification of the cyt *b* gene gave a band at approximately 946 bp.

PCR product purification and DNA Sequencing

Primers and unincorporated nucleotides were removed from PCR products using an ExoSAP digest. Five µl PCR product was added to 2 µl mix containing 10X RX buffer, 0.2 U Shrimp Alkaline phosphatase (USB, UK) and 1 U Exonuclease I (New England BioLabs, UK). Cycling parameters were 37 °C for 45 mins, 80 °C for 15 mins. The cycle sequencing reaction was set up using the BigDye® Terminator v3.1 Cycle Sequencing kit. One µl of ExoSAP product was added to a 9 µl reaction mix containing 5X sequencing buffer, 0.75 µl BigDye 3.1 (Applied Biosystems, Life Technologies, Paisley, UK) and 1.6 pM of either LV forward, LV reverse, CytB Uni fw or CytB Uni rev primer. Cycling parameters were 25 three step cycles (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 mins). The sequencing product was then precipitated using 3 M sodium acetate prior to resuspension in HiDi™ formamide (Applied Biosystems, Life Technologies, Paisley, UK), and then run on an ABI3130xl.

Phylogenetic analysis

Multiple amino acid sequence alignments were generated in MEGA5 (Tamura et al., 2011). Sequence identities were compared using Geneious 5.6.5 (Biomatters: www.geneious.com, date accessed: 1/8/12). Maximum likelihood phylogenetic trees were produced in MEGA5 (Tamura et al., 2011) with bootstrap replications of 10,000 (Felsenstein, 1985). Optimum substitution models were estimated in MEGA5 (Tamura *et al.*, 2011).

7.4 Results

Screen

Screening of pet rat lung and kidney samples for the presence of Seoul hantavirus RNA showed an overall prevalence of 81 % (17/21) (Table 7.1). Of the pet rats 14/21 were found to be infected in the lung, 15/21 in the kidney and 12/21 in both (Table 7.1). Discrepancies between results are most likely due to low viral loads.

Of the single individual 3784 from which multiple organs were sampled, SEOV RNA was detected in the heart, kidney, lung, salivary gland and spleen but not in the liver.

Phylogenetic analysis

Partial hantavirus L segment was retrieved from the RT-PCR of ten of the 17 infected individuals (366 bp). All were 100 % identical and on blasting had greatest identity to Seoul strain Humber (97 %) which was recently isolated from UK wild rats (Jameson *et al.*, 2013a). Phylogenetic analysis of the SEOV-positive pet rat shows it clustering with the Humber strain with moderate bootstrap support of 58 (Figure 7.2).

Partial sequences of the cyt *b* gene (833 bp) were recovered from 18 of the 21 pet rat samples, 16 infected samples and two non-infected samples. Seven variable sites were located within this partial sequence. Pairwise comparisons among all sequences (including out groups) ranged from 0 to 12 %. All pet rat cyt *b* sequences in this study had an average genetic distance of 0.5 % (ranging between 0.1 to 0.8 %) and compared to wild UK rats an average genetic distance of 0.8 % (ranging between 0.4 to 1.3 %). Compared to brown rats worldwide (excluding individuals from mountainous areas of China, Lin *et al.*, 2012) the pet rat sequences had an average distance of 0.7 % (range between 0 to 2.0 %). The average genetic distance to the outgroup *R. tanezumi* and *R. rattus* was 11.1 % (ranging from 10.7 to 12 %). All partials assembled randomly amongst *R. norvegicus* sequences (Figure 7.3).

Table 7.1. Hantavirus RT-PCR results for the 21 pet rats (*R. norvegicus*) screened at two separate locations (UoL and AHVLA).

		Hantavirus RT-PCR Result				
		UoL		AHVLA		
Sample	**Gender**	**Lung**	**Kidney**	**Lung**	**Kidney**	**Result**
3776	Female	Neg.	Neg.	Neg.	Neg.	**Neg.**
3777	Female	Pos.	Pos.	Pos.	Pos.	**Pos.**
3778	Female	Pos.	Neg.	Neg	Neg.	**Pos.**
3779	Female	Neg.	Pos.	Neg.	Pos.	**Pos.**
3780	Female	Pos.	Pos.	Neg.	Pos.	**Pos.**
3781	Female	Pos.	Pos.	Pos.	Pos.	**Pos.**
3782	Female	Pos.	Pos.	Pos.	Pos.	**Pos.**
3783	Female	Neg.	Pos.	Neg.	Pos.	**Pos.**
3784	Male	Pos.	Pos.	Pos.	Pos.	**Pos.**
3785	Female	Pos.	Pos.	Pos.	Pos.	**Pos.**
3786	Female	Neg.	Neg.	Neg.	Neg.	**Neg.**
3787	Male	Pos.	Pos.	Pos.	Pos.	**Pos.**
3788	Female	Neg.	Neg.	Neg.	Neg.	**Neg.**
3789	Female	Pos.	Pos.	Pos.	Pos.	**Pos.**
3790	Male	Pos.	Pos.	Pos.	Pos.	**Pos.**
3791	Male	Neg.	Pos.	Neg.	Pos.	**Pos.**
3792	Female	Pos.	Pos.	Pos.	Pos.	**Pos.**
3793	Male	Pos.	Pos.	Pos.	Pos.	**Pos.**
3794	Female	Neg.	Neg.	Neg.	Neg.	**Neg.**
3795	Male	Pos.	Pos.	Pos.	Pos.	**Pos.**
3796	Female	Pos.	Neg.	Pos.	Neg.	**Pos.**

Pos. = Positive, Neg. = Negative

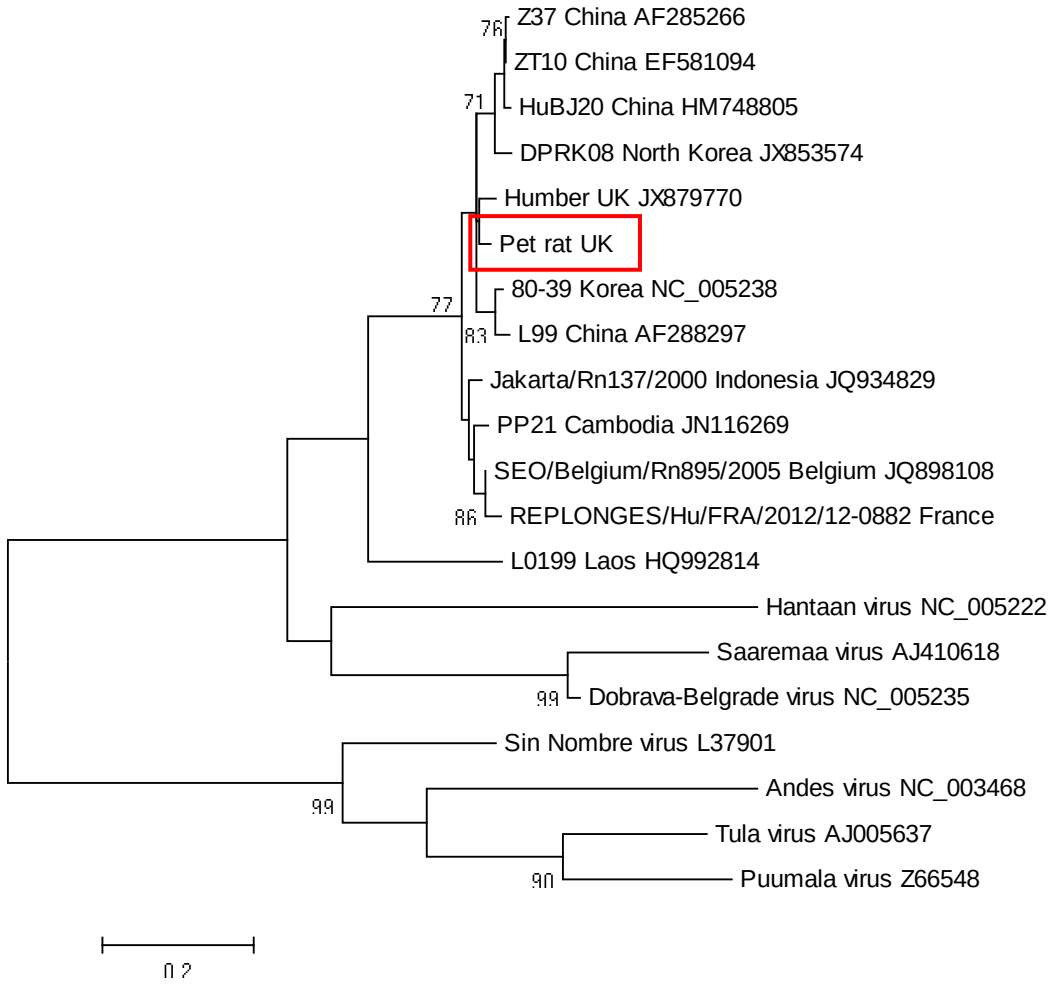


Figure 7.2. Maximum likelihood phylogenetic tree using model T92+Gamma (Tamura, 1992) for SEOV partial L segment sequences n = 20 in the MEGA5 (Tamura et al., 2011) package of software with bootstrap of 10,000 (Felsenstein, 1985). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates amino acid substitutions per site. Only bootstrap support of >70 % are shown. Positions with less than 95 % site coverage were eliminated. There were a total of 318 positions in the final dataset. The phylogenetic position of the UK pet rat is shown in relation to representative Seoul virus strains. Genbank accession numbers are shown next to taxa names.

Figure 7.3. Maximum likelihood phylogenetic tree using model HKY+Gamma (Hasegawa *et al.*, 1985) for partial cyt *b* segment sequences n = 77 in the MEGA5 (Tamura *et al.*, 2011) package of software with bootstrap (continued overleaf).

Figure 7.3 continued. of 10,000 (Felsenstein, 1985). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates amino acid substitutions per site. Only bootstrap support of >70 % are shown. Positions with less than 95 % site coverage were eliminated. Positions with less than 95 % site coverage were eliminated. There were a total of 833 positions in the final dataset. The phylogenetic position of the UK pet rats are shown in relation to representative *R. norvegicus* sequences. Genbank accession numbers are shown next to taxa names.

7.5 Discussion

Domesticated rats are not generally considered a public health problem. However we have found that a large proportion (81 %) of rats from a breeder's colony were infected with Seoul virus. Coupled with the recent human cases associated with the pet rats, hantaviruses may pose a greater public health risk than previously believed.

SEOV RNA was detected in 5/6 organs tested for the one individual 3784, including the salivary glands which have previously been shown to be a source of direct transmission between rats during aggressive encounters (Glass *et al.*, 1988). The liver did not yield viral RNA, although the individual may have had low viral loads in this sample. The liver was checked for inhibitors that could have affected the PCR. However detection of the cyt *b* gene confirmed this not to be the case.

Further analysis of partial L sequence retrieved from the RT-PCR shows it to be 97 % identical to the Humber strain, situated alongside it in the partial L segment tree (Figure 7.2). No L sequence was available for comparison from the recently described Cherwell strain that was isolated from the pet rat in North Wales (Jameson *et al.*, 2013b). The fact that this strain is so close to the wild UK strain raises questions as to the source of this virus; has it always been within the domestic rat communities or has recent contact been made with an infected UK rat? Whilst there have been no previously detected hantaviruses in pet rats, other UK studies have provided unconfirmed evidence for the circulation of SEOV in wild brown rats since at least the early 1980's (Walker *et al.*, 1984).

The cyt *b* gene can not only be used for species identification but can also provide information on the relative genetic differences between a species. Based on cyt *b* analysis, the average genetic distances between pet rats and wild UK rats was 0.8 %, greater than that seen between pet rats and wild brown rats worldwide (0.7 %). The range however was larger when compared to brown rats worldwide. Also phylogenetic analysis of pet rats does not show any specific deviation from wild brown rats suggesting they are not that different (Figure 7.3).

Fancy rat hobbyists include a large network of individuals throughout the UK that meet up regularly for competitions and other social occasions. Such gatherings could give rise to possible transmission events; for example the transfer of virus contaminated urine and faeces between cages. There have been guidelines published by AHVLA and HPA on the safety precautions against hantaviruses to minimise the health risks to owners of pet rats (HPA, 2013).

We present here confirmation of the presence of Seoul virus RNA in a high proportion of rats from a breeding colony in Oxfordshire, UK. Sequence analysis of the partial L segment confirms it to be most similar to the recently isolated UK wild SEOV strain (Humber) and questions the origin and distribution of UK SEOV. We also demonstrate the widespread distribution of SEOV viral RNA in the majority of samples taken from a single individual including the salivary glands indicating another potential route for transmission. Further screening is required to determine the prevalence of SEOV in other breeding colonies around the UK. Until then safety precautions recently published should be followed to minimise the health risk to pet rat owners as well as transmission between pet rats.

Chapter 8. General Discussion

Zoonoses constitute the majority of infectious organisms that are known to be pathogenic to humans (Taylor *et al.*, 2001). They are not only a public health concern but also represent a considerable economic burden. More than 20 % of zoonotic pathogens are reported to have rodents as hosts (Cleaveland *et al.*, 2001). The order *Rodentia* represent an abundant and diversified order of mammals (Meerburg *et al.*, 2009), that can be found inhabiting most habitats, have the potential to get in close proximity to humans, and represent a link between wildlife communities and humans. The primary aim of this thesis was the targeted surveillance for hantavirus and Ljungan virus in rodent species from a range of habitats in the North West UK. Data from this study has also been applied to draw conclusions on rodent-borne zoonoses in the UK in general.

In 2011, Defra reported five zoonoses as currently circulating in the UK that are associated with rodents, bovine tuberculosis, cryptosporidiosis, HFRS, leptospirosis and Lyme disease (Defra, 2012). Whilst this figure may seem a fraction of the potential rodent-borne zoonoses that are known around the world (Meerburg *et al.*, 2009), it is by no means conclusive as more zoonotic pathogens are constantly emerging (Daszak, 2000) and as such can only be corroborated through further surveillance. Surveillance is important not only to gain insights into what is out there but also to assess the likely risks there might be to humans. Evidence of hantavirus circulation had been reported since 1983 (McCaughey & Hart, 2000; Walker *et al.*, 1984) but confirmation of a responsible agent did not follow. Hantaviral species can be difficult to distinguish due to detectable viraemia generally only being present in low levels in the acute phase of infection (Plyusnin *et al.*, 1997a) and also because hantaviruses exhibit cross-reactivity between closely related species. Whilst it is important to know the hantaviral agent responsible, there is currently no species specific treatment available and management tends to be on a case by case basis. It was only in 2012 following targeted hantavirus surveillance, that SEOV was first detected in wild brown rats in the UK (Jameson *et al.*, 2013a) and a novel hantavirus, Tatenale virus (TATV) was detected in a rural field vole in Cheshire (Chapter 5, this study). This highlights the benefits of fully investigating potential zoonotic episodes to appropriately evaluate the public health risk.

The main limiting factor in surveillance programmes, including this study, is one of resources, particularly financial. Whilst a comprehensive trapping and sampling programme for urban and rural rodents throughout the UK would provide better confidence in prevalence data, the financial costs of such a scheme are prohibitive. As the limited resources available were likely to impact on the outputs of the project, Defra provided us with a budget to hold a workshop in Liverpool. We invited international hantavirus experts from across Europe and the USA to advise us of the optimal field and laboratory approaches. The strategy of sampling and pan-hantavirus RT-PCR screening employed in this project were agreed at this workshop. The primary focus for surveillance was urban rodents, which resulted in the rural species e.g. bank voles and field voles, being under represented. Larger sample sizes would be necessary in the species tested in this project to have more confidence in the absence of either virus or the low prevalence and thus risk posed to humans. It is estimated that we would require sample sizes of approximately 60 or 300 to be convinced of detecting virus prevalence of 5 % or 1 %, respectively, at the 95 % confidence level. Also, hantaviruses have recently been detected in non-rodent species, such as bats and shrews (Klempa *et al.*, 2007; Weiss *et al.*, 2012), that were not sampled in this project but may in fact be carriers for other hantaviruses circulating in the UK and thus should be investigated. With regards to LV surveillance we found no evidence of LV in rodents collected for this study even though virus has previously been reported elsewhere in the UK (Salisbury *et al.*, 2013). Furthermore due to sensitivity and specificity validation of our hemi-nested RT-PCR using positive material provided by collaborators, we are confident that our assay would have detected LV if it were present in our samples.

Domesticated rats have generally been separate from their wild counterparts since the 19th century and have been largely regarded as no particular health concern for humans. Although the transmission of zoonotic pathogens between pet rodents and humans is rare, there have been several reported cases where it has occurred (Chomel, 1992), including lymphocytic choriomeningitis virus (Amman *et al.*, 2007), cowpox virus (Ninove *et al.*, 2009), hantavirus (Jameson *et al.*, 2013a), *Spirillum minus* (Shvartsblat et al., 2004) and *Leptospira* (Baer *et al.*, 2010). However, we have shown that the 'clean' image of pet rats should possibly be re-thought as a large proportion (81 %) of fancy rats in a breeding colony in

Oxfordshire were infected with Seoul virus (Chapter 7). Investigation of more breeding colonies would be required to determine the extent of prevalence geographically or if it happens to be a localised event. An anonymous surveillance of pet rats is planned by the Animal Health and Veterinary Laboratories Agency (AHVLA) and Public Health England (PHE).

Hantaviruses and Ljungan virus are but two zoonoses carried by rodents. It has been previously shown that rodents can be reservoirs for an array of pathogens as illustrated in brown rats (*R. norvegicus*) around the world and here in the UK (Adjemian *et al.*, 2008; Easterbrook *et al.*, 2007; Glazebrook *et al.*, 1978; Webster & Macdonald, 1995). As an output from this project through collaborations, our hantavirus infected field vole was also found to be infected with *Leptospira* (Chris Ball, pers. comms.), and *Capillaria hepatica* found in several urban brown rats (Appendix 1, McGarry, Manuscript in Prep), both serious zoonoses (Bhattacharya *et al.*, 1999; Ellis, 1999). Rodent samples from our urban surveillance, have been provided to Nottingham University (as part of the FP7 WildTech project) for the purpose of microarray validation to detect and identify infectious agents in rodent populations. This is an example of the sharing of rodent samples from the North West UK through collaborations to get a better understanding of the zoonotic pathogens actually carried by these otherwise healthy looking rodents (Appendix 1). Without other such exchanges from different areas of the UK we are at best likely to obtain only a patchy overview of rodent-borne zoonoses.

Many zoonoses originate from wildlife species (Cleaveland *et al.*, 2001; Kruse *et al.*, 2004) so it might be speculated that urban rodent populations may pose a lower risk to humans despite a higher transmission risk due to the close proximity. For the most part evidence presented in this thesis would support this as we found no evidence of HV or LV in urban rodents in Liverpool, and the novel hantavirus was detected in a rural rodent (Chapter 5). However the Lyon study (Chapter 6) contrasts this as 14 SEOV infected urban and four rural brown rats were found. This may be more attributable to the fact that SEOV, carried by members of the *Rattus sp*., distribution is known to be mediated by human travel e.g. boats, and as such are more likely to be associated with urban areas. Other zoonotic pathogens such as *Bartonella*,

Coxiella and *Rickettsia* have also been shown to be maintainable in urban areas (Comer *et al.*, 2001).

Whilst this thesis has focused primarily on rodents as sources of zoonoses and a cause for concern, it must not be forgotten that the picture is mirrored in that of other animal orders, which in some cases are reported to have the potential to harbour greater proportions of zoonotic pathogens than rodents e.g. ungulates and carnivores (Cleaveland *et al.*, 2001). Non-rodent species that predate on rodents can also be useful in potentially acting as sentinels for disease systems. There have been several examples of where the surveillance of sentinel species such as cats, dogs, foxes and owls have been shown to predict the risk of hantaviruses to the human population (Dobly *et al.*, 2012; Escutenaire *et al.*, 2000; Heyman *et al.*, 2013). Companion animal (cats and dogs) sera samples were available through the pet travel scheme for this study, however resources did not allow for them to be serologically tested. As such it should be ensured that surveillances cover all animal orders to get a more complete assessment of zoonoses in the UK.

Next generation sequencing (NGS) is a relatively novel technique that has many applications in research especially virology. Its usefulness mainly comes as a result of the vast amount of information it can produce in a small amount of time, and with currently financial constraints being the only main drawback which will inevitably cease as technology improves and becomes more popular (Radford *et al.*, 2012). The benefits of NGS application is most apparent when dealing with epidemics (Radford *et al.*, 2012). These specifically require a faster response in order to ascertain treatment, control and preventative measures against the causative agent. Whilst the genome sequences obtained in Chapter 4 were not received as a consequence of an epidemic but instead were for the academic purpose of characterising two further LV genomes, it provides a good example of the value of NGS. A limitation found in the analysis of these full LV genomes was however the lack of availability of any sequence information from spatially and/or temporally separate isolates despite the virus being reported in different locations and years. In the case of LV (Chapter 4), it may have supported or contested the finding of LV exhibiting surprisingly slow evolution for a picornavirus. Examples where NGS have already been valuable include the molecular epidemiology of foot-and-mouth disease outbreaks in the UK

(Knowles & Samuel, 2003), rapid identification of a novel arenavirus in South Africa within 72 hours of receiving samples (Briese *et al.*, 2009), a yellow fever virus outbreak in Uganda (McMullan *et al.*, 2012), Schmallenberg virus in Europe (Hoffmann *et al.*, 2012), characterisation of an *E. coli* outbreak within 62 hours (Mellmann *et al.*, 2011) and of the 2009 influenza A pandemic (Kuroda *et al.*, 2010). Of the list of notifiable zoonoses in the UK (www.defra.gov.uk/animal-diseases/notifiable, date accessed 1/2/13), avian influenza, bovine spongiform encephalopathy, bovine tuberculosis and European bat lyssavirus have been reported since 2008. The risk from newly emerging zoonotic outbreaks in the UK might seem insignificant but this may be due to the resources that are spent to control and prevent outbreaks (Corry & Hinton, 1997; de la Rua-Domenech, 2006; Westrell *et al.*, 2009). Realistically, we should focus our attention on those zoonoses that are circulating within the UK as they have the potential to impact on our health and the economy. With this NGS technology, we are now fully equipped to act upon the first signs of an outbreak as well as pre-empting future outbreaks and emerging pathogens.

Whilst obtaining full genomes are often the goal for many pathogens, sequence marker information from the host can be just as useful for understanding the dynamics of the virus and its host. In Chapters 5, 6 & 7, the mitochondrial cytochrome *b* (cyt *b*) gene, a gene used for the purpose of species identification (Bradley & Baker, 2001) was used to infer if non-indigenous brown rats had been introduced into the UK and that may provide an explanation to the presence of SEOV. In this study we found divergences of 0.3 % between SEOV infected and non-infected brown rats from around Lyon, 0.6 % between Lyon rats and UK brown rats (Chapter 5) and 0.8 % between UK and pet rats (Chapter 6). These low divergences were very similar regardless of their country of origin, infection status or domestication, thus making it difficult to distinguish within species differences. A limitation was that only partial sequences were obtained, although this did correspond to approximately 73 % of the entire cyt *b* gene, but also differences may not be associated with that particular region of the mitochondria. Whilst useful for species identification, instead perhaps other potential markers, nuclear or mitochondrial could be used instead to compare and improve the resolution within a species. Although two reports have shown the cyt *b* gene from *R. norvegicus* and *R.*

rattus to be sufficient in determining genetic diversity on a global scale (Aplin *et al.*, 2011; Lin *et al.*, 2012), it was inadequate to detect differences within species in this study to highlight the potential introduction of non-indigenous individuals to the UK. A literature search turned up no other markers commonly used for phylogenetic analysis of rodents.

Whether it be whole genome (Chapter 4) or partial sequences (Chapters 5, 6 & 7) it seems clear from this study that all should be obliged to publish their sequence data for research to progress.

Conclusions

This study aimed to primarily assess the presence and prevalence of two zoonotic viruses carried by rodents, in the North West UK, so as to provide assistance in assessing the relative human risk. In addition, this study 1) characterised two new LV isolates using next generation sequencing techniques, and 2) in collaboration with the FP7 WildTech project determined the prevalence of hantaviral RNA in urban brown rats from Lyon, France and 3) in collaboration with AHVLA and HPA determined the prevalence of hantaviral RNA in a breeding colony of pet rats. The results revealed no evidence of Ljungan virus circulating in North West UK rodents (Chapter 2). The characterisation of two new Swedish LV isolates exhibited no significant variations from the current five genomes available although only small amounts of positive selection was found, a characteristic unusual for RNA viruses (Chapter 3). Hantavirus surveillance resulted in the detection of a novel virus, Tatenale virus, in a field vole (Chapter 4). This study also revealed that a proportion of brown rats from Lyon (14.1 %) and pet rats (81 %) from a breeding colony were infected with SEOV hantaviral RNA (Chapter 5 and Chapter 6). Moreover, I demonstrated that the application of next generation sequencing techniques can be used to obtain full RNA viral genome sequences for genomic characterisation (Chapter 3 and Chapter 5) and molecular methods for the successful surveillance of RNA viruses in wild and domestic rodents (Chapter 2 and Chapter 4).

In summary, these findings provide new evidence for the circulation of hantaviruses and Ljungan virus in UK and domestic rodents, and as such has also highlighted the importance for the continued investigation of zoonoses in the UK.

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Appendix 1

Veterinary Laboratory Services

Necropsy Report

pcwww.liv.ac.uk/vetpathology

UNIVERSITY OF LIVERPOOL

FACULTY of VETERINARY SCIENCE

Species: wild rat	Species: *Rattus norvegicus*	Pathology Lab No: 09L-4539
LabNo (SoB): K44	Age: adult	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk Professor Michael Begon / Kieran Pounder School of Biological Science 208 Bio Sciences Building		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination:
Liver: focal firm white areas

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: mild neutrophilic conjunctivitis
Heart: not examined
Lungs: not examined
Spleen: not examined
Liver: moderate bile duct coccidiosis

B) Kidneys: no histological abnormality is recognised
Urinary bladder: not examined
Testes: not examined

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

U Hetzel, Dr.med.vet., Dr.rer.nat., DiplBiol, FTA Pathologie, MRCVS
16/12/09

UH emailed 16/12/09

Veterinary Laboratory Services

Necropsy Report

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Species: wild rat	Species: *Rattus norvegicus*	Pathology Lab No: 09L-4588
LabNo (SoB): K45	Age: adult Body condition: / ++	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormalities were detected.
Left thorax with focal pleural adhesions
Colonic nematodes

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: focal pleural adhesions, moderate BALT activation
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: multiple intraepithelial and luminal nematodes
Ovary: no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: mild diffuse infiltration by heterophils, lymphocytes, plasma cells
Large intestine: mild diffuse infiltration by heterophils, lymphocytes, plasma cells, multiple nematodes
Pancreas: no histological abnormality is recognised

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UH emailed 16/12/

Veterinary Laboratory Services

Necropsy Report

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K 46 Pathology Lab No: 09L-4589 Parasitology Lab No:
Age: adult	Body condition: ++	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: focal granuloma, focally mild heterophilic, lympho-plasmacytic interstitial pneumonia
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: mild heterophilic and lympho-plasmacytic pyelitis
Urinary bladder: no histological abnormality is recognised
Ovary / Testes: no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: mild heterophilic and lympho-plasmacytic duodenitis
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised, several luminal nematodes
Pancreas: no histological abnormality is recognised

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UH emailed 17/12/09

Veterinary Laboratory Services

Necropsy Report

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Species: Wild rat	Species: *Rattus norvegicus*	SoB Lab No: Pathology Lab No: Parasitology Lab No:	K47 09L-4621
Age: Subadult	Body condition: Moderate	Sex: F	
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: 15/12/09 Pathologist(s): UH Typed: sg	

GROSS FINDINGS

External / internal examination: No abnormality was detected.

Lung: Pulmonary abscess.

Colon: Small numbers of intraluminal nematodes.

HISTOPATHOLOGY

A) Brain: No histological abnormality is recognised.
Eye: No histological abnormality is recognised.
Heart: No histological abnormality is recognised.
Lungs: No histological abnormality is recognised.
Spleen: Mild hyperaemia, no further histological abnormality is recognised.
Liver: Focal mild lymphoplasmacytic pericholangitis, mild hyperaemia.

B) Kidneys: Focally mild perivascular lymphoplasmacytic infiltration.
Urinary bladder: Multifocal intraepithelial ? trematode / nematode infestation.
Ovary: No histological abnormality is recognised
Uterus: No histological abnormality is recognised
Adrenal: Not provided.

C) Stomach: Pars non-glandularis, moderate numbers of intra-epithelial nematodes (? Capillaria).
Small intestine: No histological abnormality is recognised.
Large intestine: No histological abnormality is recognised.
Mesenteric lymph nodes: No histological abnormality is recognised.
Pancreas: No histological abnormality is recognised.

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8 January 2010

Veterinary Laboratory Services

Necropsy Report

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Species: Wild rat	Species: *Rattus norvegicus*	SoB LabNo: K48 Pathology Lab No: 09L-4622 Parasitology Lab No:
Age: Adult	Body condition: Moderate	Sex: F
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: 15/12/09 Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: No histological abnormality is recognised.
Eye: No histological abnormality is recognised.
Heart: No histological abnormality is recognised.
Lungs: Moderate BALT hyperplasia, moderate peribronchial lymphoplasmacytic infiltration.
Spleen: No histological abnormality is recognised.
Liver: Not provided.

B) Kidneys: No histological abnormality is recognised.
Urinary bladder: No histological abnormality is recognised.
Ovary: Not provided.
Uterus: No histological abnormality is recognised.
Adrenal: No histological abnormality is recognised.

C) Stomach: Pars non-glandularis, focal evidence of intraepithelial ? Capillaria eggs.
Small intestine: No histological abnormality is recognised.
Large intestine: Colon focal intraluminal nematodes.
Mesenteric lymph nodes: No histological abnormality is recognised.
Pancreas: No histological abnormality is recognised.

COMMENT

Neutrophilic peri-bronchiolitis infestation, nematode infestation of stomach and colon.

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8January 2010

Veterinary Laboratory Services

Necropsy Report

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UNIVERSITY OF LIVERPOOL

FACULTY of VETERINARY SCIENCE

Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K 49 Pathology Lab No: 10L- 18 Parasitology Lab No:
Age: adult	Body condition: +++	Sex: m /
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: focally mild subpleural lympho-plasmacytic infiltration, focal granuloma (ZN:
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: + nematodes (John McGarry: *Trichomosoides crassicauda:* Oviparous worms which are situated in the bladder wall release the larvated eggs in urine. These penetrate and hatch in the stomach and are carried around the body (esp lungs) but only fully develop in the bladder
Testes: + spermiogenesis, no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

U Hetzel, Dr.med.vet., Dr.rer.nat., DiplBiol, FTA Pathologie, MRCVS

Veterinary Laboratory Services

Necropsy Report

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UNIVERSITY OF LIVERPOOL

FACULTY of VETERINARY SCIENCE

Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K 50 Pathology Lab No: 10L- 19 Parasitology Lab No:
Age: subadult	Body condition: ++	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: multiple pulmonary abscesses.

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: abscess (purulent pneumonia (ZN for mycobacteria: negative)
Spleen: no histological abnormality is recognised
Liver: focal perivascular neutrophilic infiltration zone 1 (acute purulent hepatitis, focal, mild)

B) Kidneys: no histological abnormality is recognised
Urinary bladder: no histological abnormality is recognised
Ovary: focal corpora lutes, no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Necropsy Report

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K 51 Pathology Lab No: 10L-132 Parasitology Lab No:
Age: / adult	Body condition: / +++260g	Sex: m /
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: Lungs: moderately diffusely firm, pale red in colour (? atelectasis)
U-bladder: ++ nematodes c.f. *Trichomosoides crassicauda*
Colon descendens: + nematodes

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: moderate BALT activation, moderate diffuse interstitial fibrosis (? Interstitial pneumonia)
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: ++ *Trichomosoides crassicauda*
Testes: + spermiogenesis, no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: ++ nematodes, no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Veterinary Laboratory Services

Necropsy Report

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K52 Pathology Lab No: 10L-131 Parasitology Lab No:
Age: / adult	Body condition: + / ++ / +++260g	Sex: m /
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination:
Lung: Multiple poorly demarcate white pulmonary processes
Stomach: moderate thickening of wall pars nonglandularis

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: focally mild lympho-plasmacytic and neutrophilic conjunctivitis
Heart: no histological abnormality is recognised
Lungs: multiple pulmonary granulomas ./ granulomatous pneumonia (ZN: negative)
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: ++ *Trichomosoides crassicauda*
Ovary / Testes: no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: mild hyperkeratosis, + nematodes
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: Wild rat	Species: *Rattus norvegicus*	SoB LabNo: K55 Pathology Lab No: 10L-643 Parasitology Lab No:
Age: juv	Body condition: / +++ 93.3 g	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: Liver with multiple yellow areas.

HISTOPATHOLOGY

A) Brain: No histological abnormality is recognised.
Eye: No histological abnormality is recognised.
Heart: No histological abnormality is recognised.
Lungs: Mild catharrhal-purulent broncho-pneumonia, mild BALT hyperplasia.
Spleen: Mild follicular hyperplasia.
Liver: Multifocal pyogranulomatous hepatitis with large numbers of intralesional nematode eggs c.f. *Capillaria hepatica* and peripheral fibrosis.

B) Kidneys: No histological abnormality is recognised.
Urinary bladder: No histological abnormality is recognised.
Ovary: No histological abnormality is recognised.
Uterus: No histological abnormality is recognised.
Adrenal: No histological abnormality is recognised.

C) Stomach: No histological abnormality is recognised.
Small intestine: No histological abnormality is recognised.
Large intestine: small numbers of luminal nematodes, no histological abnormality is recognised.
Mesenteric lymph nodes: No histological abnormality is recognised.
Pancreas: No histological abnormality is recognised.

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Species: Wild rat	Species: *Rattus norvegicus*	SoB LabNo: K57 Pathology Lab No: 10L-644 Parasitology Lab No:
Age: adult	Body condition+++ 310 g	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: Apart from a mildly dilated duodenum, no abnormality was detected.

HISTOPATHOLOGY

A) Brain: No histological abnormality is recognised.
Eye: No histological abnormality is recognised.
Heart: No histological abnormality is recognised
Lungs: Mild catarrhal purulent broncho-pneumonia, mild BALT hyperplasia, ? mild diffuse interstitial fibrosis.
Spleen: No histological abnormality is recognised.
Liver: Focally mild periportal pyogranulomatous hepatitis.

B) Kidneys: No histological abnormality is recognised.
Urinary bladder: No histological abnormality is recognised.
Ovary: No histological abnormality is recognised.
Uterus: No histological abnormality is recognised.
Adrenal: No histological abnormality is recognised.

C) Stomach: Mild T. crassicauda nematode infection.
Small intestine: mild lympho-plasmacytic and neutrophilic infiltration L. mucosa.
Large intestine: No histological abnormality is recognised.
Mesenteric lymph nodes: No histological abnormality is recognised.
Pancreas: No histological abnormality is recognised.

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Species: Wild rat	Species: *Rattus norvegicus*	SoB LabNo: K58 Pathology Lab No: 10L-645 Parasitology Lab No:
Age: juv	Body condition: +++ 56.56 g	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: No histological abnormality is recognised.
Eye: No histological abnormality is recognised.
Heart: Multifocal mild ventricular interstitial lympho-plasmacytic myocarditis with degeneration of single cardiomyocytes.
Lungs: Mild diffuse interstitial fibrosis,
Spleen: Mild follicular hyperplasia, mild extramedullary hyperplasia.
Liver: focally mild lympho-plasmacellular pericholangitis.

B) Kidneys: No histological abnormality is recognised.
Urinary bladder: No histological abnormality is recognised.
Testes: No spermiogenesis, no histological abnormality is recognised.
Adrenal: Not examined.

C) Stomach: No histological abnormality is recognised.
Small intestine: No histological abnormality is recognised.
Large intestine: No histological abnormality is recognised.
Mesenteric lymph nodes: Mild sinus histiocytosis, no histological abnormality is recognised.
Pancreas: No histological abnormality is recognised.

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Species: Wild rat	Species: *Rattus norvegicus*	SoB LabNo: K59 Pathology Lab No: 10L-646 Parasitology Lab No:
Age: adult	Body condition: +++ 283.20 g	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: No histological abnormality is recognised.
Eye: No histological abnormality is recognised.
Heart: Focally mild left ventricular lympho-plasmacytic myocarditis.
Lungs: Focally moderate BALT hyperplasia, mild catarrhal purulent brochiolitis, mild diffuse interstitial fibrosis.
Spleen: Mild extramedullary haematopoiesis.
Liver: Multifocal granulomatous hepatitis with large numbers of intralesional nematode eggs c.f. *Capillaria hepatica* and peripheral fibrosis.

B) Kidneys: No histological abnormality is recognised.
Urinary bladder: No histological abnormality is recognised.
Testes: Moderate spermiogenesis, no histological abnormality is recognised.
Adrenal: No histological abnormality is recognised.

C) Stomach: Mild T. crassicauda infection.
Small intestine: No histological abnormality is recognised.
Large intestine: No histological abnormality is recognised.
Mesenteric lymph nodes: Not examined.
Pancreas: No histological abnormality is recognised.

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K60 Pathology Lab No: 10L-2848 Parasitology Lab No:
Age: / adult	Body condition: / ++ / 290g	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: focally mild lympho-plasmacytic interstitial infiltration, focally mild interstitial fibrosis
Lungs: mild BALT hyperplasia, mild mediahypertrophy of smaller arteries
Spleen: no histological abnormality is recognised
Liver: multifocal mild to moderate periportal lympho-plasmacytic hepatitis

B) Kidneys: multifocal mild lympho-plasmacytic interstitial nephritis
Urinary bladder: moderate epithelial hypertrophy, focally multinucleated transitional epithelial cells, ++ *Trichomosoides crassicauda*

Ovary: no histological abnormality is recognised
Uterus: moderate diffuse submucosal neutrophilic and lympho-plasmacytic endometritis
Adrenal: multifocal mild to moderate cortical acute haemorrhages, multifocal moderate cortical lympho-plasmacytic infiltrates

C) Stomach: mild intraepithelial *Mastophorus muris* infection, mild submucosal neutrophilic and lympho-plasmacytic infiltration
Small intestine: mild diffuse submucosal neutrophilic and lymphoplasmacytic enteritis
Large intestine: one section of mucosallly attached nematode
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K61 Pathology Lab No: 10L-2849 Parasitology Lab No:
Age: / adult	Body condition: / ++ / 325g	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: Spleen: mild follicular hyperplasia
Colon: ? mild nematode infection

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: mild diffuse interstitial fibrosis
Spleen: moderate follicular hyperplasia
Liver: focal single cell necroses with mild peripheral neutrophilic and lymphoplasmacytic
infiltration (focally mild purulent hepatitis)

B) Kidneys: multifocal mild lympho-plasmacytic interstitial nephritis
Urinary bladder: moderate polypous transitional cell hyperplasia, mild diffuse submucosal neutrophilic and lympho-plasmacytic cystitis

Testicle: spermiogenesis present, no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: mild submucosal neutrophilic and lympho-plasmacytic gastritis
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K 62 Pathology Lab No: 10L-2850 Parasitology Lab No:
Age adult	Body condition: ++ 277.28g	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination: Lungs: diffusely poor retraction,? pleuritis

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: mild BALT hyperplasia, mild interstitial fibrosis, mild pneumocyte type 2 hyperplasia,
focally moderate mesothelial hypertrophy
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: no histological abnormality is recognised
Ovaries: no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: mild intraepithelial *Mastophorus muris* infection
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K68 Pathology Lab No: 10L-2852 Parasitology Lab No:
Age: juv	Body condition: + 75.33g	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH Typed: sg

GROSS FINDINGS

External / internal examination: Liver: diffuse pale cream discolouration

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: mild lympho-plasmacytic and neutrophilic conjunctivitis
Heart: no histological abnormality is recognised
Lungs: no histological abnormality is recognised
Spleen: no histological abnormality is recognised
Liver: mild diffuse lympho-plasmacytic pericholangitis

B) Kidneys: no histological abnormality is recognised
Urinary bladder: no histological abnormality is recognised
Testes: no spermiogenesis, no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K72 Pathology Lab No: 10L-2853 Parasitology Lab No:
Age: adult	Body condition: ++ 264.398g	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination: Liver: white areas
Spleen: mild follicular hyperplasia

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: mild interstitial fibrosis and pneumocyte type 2 hyperplasia, mild BALT hyperplasia, focal multinucleated macrophages, focal moderate subintimal arterial calcification
Spleen: mild follicular hyperplasia
Liver: focally mild purulent-necrotising hepatitis, mild diffuse sinusoidal leucocytostasis
Kidneys: no histological abnormality is recognised
Urinary bladder: focally mild epithelial hypertrophy with intraepithelial nematode (?*Trichomosoides crassicauda*) eggs / protozoa
Ovaries: no histological abnormality is recognised
Uterus: mild neutrophilic endometritis
Adrenal: no histological abnormality is recognised

B) Stomach: mild intraepithelial *Mastophorus muris* infection
Small intestine: mild lympho-plasmacytic and neutrophilic infiltration of lamina propria
Large intestine: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: K96 Pathology Lab No: 10L-2854 Parasitology Lab No:
Age: subadult	Body condition: ++ 134.33g	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Professor Michael Begon / Kieran Pounder School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination: No abnormality was detected.

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: no histological abnormality is recognised
Lungs: moderate interstitial fibrosis and pneumocyte type 2 hyperplasia, mild BALT hyperplasia, moderate arterial mediahypertrophy
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: focally mild epithelial hypertrophy
Testes: mild spermiogenesis, no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: C79 Pathology Lab No: 11L-1061 Parasitology Lab No:
Age: juv / subadult / adult	Body condition: + / ++ / +++	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Michael Begon / Kieran Pounder / Giovanni Pellegrini School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination:
Lung: multifocal pale, 0.2cm firm nodules (?parasitic granuloma)
Eye: unilateral microphthalmos
Spleen: mild splenomegaly, follicular hyperplasia

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: secondary microphthalmos with mild diffuse lympho-plasmacytic (neutrophilic) panophthalmitis
Heart: focally mild fibrosing lympho-plasmacytic myocarditis
Lungs: multifocal to coalescing pulmonary granuloma, moderate interstitial fibrosis, mild arterial mediahypertrophy
Spleen: mild follicular hyprtrophy
Liver: focally mild to moderate periportal lympho-plasmacytic hepatitis, mild hepatocellular lipidosis

B) Kidneys: mild to focally moderate interstitial lympho-plasmacytic nephritis
Urinary bladder: mild diffuse neutrophilic (purulent) cystitis
Ovary: no histological abnormality is recognised
Uterus: involution, mild diffuse lympho-plasmacytic and neutrophilic endometritis
Adrenal: no histological abnormality is recognised

C) Stomach: multifocal intraepithelial Capillaria like , partly embryonated eggs and fertile nematodes
Small intestine: no histological abnormality is recognised
Large intestine: one nematode section, no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: C80 Pathology Lab No: 11L-1062 Parasitology Lab No:
Age: juv / subadult / adult	Body condition: + / ++ / +++	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Michael Begon / Kieran Pounder / Giovanni Pellegrini School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination: Left thoracic wall mass approx 1cm pertruding from body wall, adherent to lungs (no histology sample), mild splenomegaly with follicular hyperplasia

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: focally mild interstitial lympho-plasmacytic and neutrophilic myocarditis.
Lungs: moderate BALT hyperplasia, mild interstitial fibrosis, mild arterial mediahypertrophy
Spleen: mild follicular hyperplasia
Liver: multifocal mild periportal lympho-plasmacytic hepatitis

B) Kidneys: multifocal mild periglomerular / interstitial lympho-plasmacytic nephritis
Urinary bladder: focally mild epithelial hyperplasia, focal multinucleated cells
Testes: no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: not investigated

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: C81 Pathology Lab No: 11L-1063 Parasitology Lab No:
Age: juv / subadult / adult	Body condition: + / ++ / +++	Sex: m
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Michael Begon / Kieran Pounder / Giovanni Pellegrini School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination: mild splenomegaly with follicular hyperplasia

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: multifocal mild interstitial lympho-plasmacytic myocarditis
Lungs: mild interstitial fibrosis, mild BALT hyperplasia
Spleen: mild follicular hyperplasia
Liver: focally mild periportal lympho-plasmacytic hepatitis

B) Kidneys: no histological abnormality is recognised
Urinary bladder: no histological abnormality is recognised
Testes: no histological abnormality is recognised
Bulbourethral glands: moderate acute purulent adenitis
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

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Species: wild rat	Species: *Rattus norvegicus*	SoB LabNo: C82 Pathology Lab No: 11L-1064 Parasitology Lab No:
Age: juv / subadult / adult	Body condition: + / ++ / +++	Sex: f
E-mail: mbegon@liverpool.ac.uk; kpounder@liv.ac.uk; McGarry, John Michael Begon / Kieran Pounder / Giovanni Pellegrini School of Biological Science / 208 BioSciences Building John McGarry, Veterinary Parasitology		Received: Pathologist(s): UH

GROSS FINDINGS

External / internal examination: Tail, approx. 2cm distally from anus, hard swelling (? fracture + callus)

HISTOPATHOLOGY

A) Brain: no histological abnormality is recognised
Eye: no histological abnormality is recognised
Heart: multifocally mild interstitial lympho-plasmacytic (neutrophilic) myocarditis
Lungs: very mild interstitial fibrosis
Spleen: no histological abnormality is recognised
Liver: no histological abnormality is recognised

B) Kidneys: no histological abnormality is recognised
Urinary bladder: no histological abnormality is recognised
Ovary / Testes: no histological abnormality is recognised
Uterus: no histological abnormality is recognised
Adrenal: no histological abnormality is recognised

C) Stomach: no histological abnormality is recognised
Small intestine: no histological abnormality is recognised
Large intestine: no histological abnormality is recognised
Mesenteric lymph nodes: no histological abnormality is recognised
Pancreas: no histological abnormality is recognised

D) Tail: moderate fibrosing and lympho-plasmacytic panniculitis, myositis and periostitis with evidence of bone remodelling (callus, old fracture)

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Appendix 2

children who received no vaccination (22%, 6/27) than among those with unknown vaccination history (10%, 15/157). Of 5 vaccinated children, 1 had JE; however, verification of this child's vaccination was not possible. Among 71 children who had no evidence of JE but for whom serum samples were available for testing, 5 had antibodies against mumps virus, 8 against echoviruses, and 5 against coxsackieviruses. Viral cultures of CSF from all 189 children were negative.

Our finding of 10.4 JE cases per 100,000 children ≤15 years of age in Dehong Prefecture is higher than the estimated incidence of 5.4 cases per 100,000 population among children ≤14 years of age in JE-endemic countries (*2*). Nevertheless, the true JE population incidence for Dehong Prefecture might be underestimated if some children received no medical care or were admitted to other hospitals. Adults were not studied; however, ≈90% of JE cases in China are reported among children <15 years of age (*5,6*). Unfortunately, accurate age-adjusted JE vaccination coverage data for Dehong Prefecture are not available. Although vaccination programs have markedly lowered JE incidence in China in recent years (*5,6*), the finding of continuing high JE incidence in Dehong Prefecture warrants further attention.

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Novel Hantavirus in Field Vole, United Kingdom

To the Editor: Hantaviruses (family *Bunyaviridae*) are transmitted to humans by inhalation of aerosolized virus in contaminated urine and feces, mainly from rodents of the families Cricetidae and Muridae. Although infections in rodents are asymptomatic, infections in humans can lead to hemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome (*1*).

In Europe, 5 rodent-borne hantaviruses have been detected: Dobrava-Belgrade, Saaremaa, Seoul, Puumala, and Tula (*1*,*2*). The most common and widespread hantavirus in Europe is Puumala virus, which is associated with the mildest form of hemorrhagic fever with renal syndrome (*1*).

In the United Kingdom, only a few cases of hantavirus infection in humans have been reported and confirmed serologically, but the causative virus species were not identified (*3*,*4*). Subsequent longitudinal studies reported considerable hantavirus seropositivity among healthy human cohorts, suggesting past exposure to hantaviruses or subclinical infection (*3*). Serologic surveys of rodents (rats

and mice) and cats also supported the presence of a hantavirus indigenous to the United Kingdom (*3*). To determine whether hantaviruses are circulating in wild rodents in the United Kingdom, we conducted molecular analyses on rodent tissues.

From September 2009 through November 2011, a total of 495 wild rodents consisting of 133 brown rats (*Rattus norvegicus*), 269 wood mice (*Apodemus sylvaticus*), 50 house mice (*Mus musculus*), 35 bank voles (*Myodes glareolus*), and 8 field voles (*Microtus agrestis*) were caught live across northwestern England (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/19/4/12-1057-Techapp1.pdf). Animals were euthanized in the field by use of isoflurane inhalation, according to UK Home Office Guidelines (http://webarchive.nationalarchives.gov.uk/+/http://www.homeoffice.gov.uk/docs/hc193.html). Within 2 hours, kidney, liver, and lung tissues were removed. When field conditions allowed, blood samples were collected; otherwise, heart tissue was collected. Samples, and carcasses that could not be processed within 2 hours, were stored at −80°C.

RNA was extracted by using TRIzol Reagent (Invitrogen, Life Technologies, Paisley, UK). To detect hantavirus RNA, we used a nested pan-hantavirus reverse transcription PCR selective for partial polymerase large segment (L) gene sequences (*5*). With the exception of 1 male field vole (B41) collected near Tattenhall, Cheshire (online Technical Appendix Figure), all lung samples were negative for hantavirus RNA. The positive amplicon was sequenced by using a BigDye Terminator 3.1v Cycle Sequencing Kit on an ABI3130xl genetic analyzer (Applied Biosystems/Life Technologies, Paisley, UK) (GenBank accession no. JX316008). Partial small segment (S) sequences were also recovered from lung RNA from vole B41 (GenBank accession no. JX316009) (online Technical Appendix Table). Established reverse transcription PCRs for the medium segment were unsuccessful.

Comparisons of nucleotide and amino acid sequence identities demonstrated, as expected, that the Arvicolinae-associated hantaviruses showed the highest similarity to the UK sequence at the nucleotide (65.7%–78.8% for S and 76.6%–77.5% for L) and the amino acid (66.4%–86.3% for S and 80%–88% for L) levels (online Technical Appendix Table).

Phylogenetic analyses of partial L (Figure, panel A) and partial S sequences (Figure, panel B) confirm the inclusion of the viral sequence from vole B41 as a distinct member of the Arvicolinae-associated hantaviruses. In the partial L tree (Figure, panel A), viral sequence B41 clustered with Prospect Hill and Tula viruses with good support, although in the partial S tree (Figure, panel B), B41 seems to be more closely related to the Asian

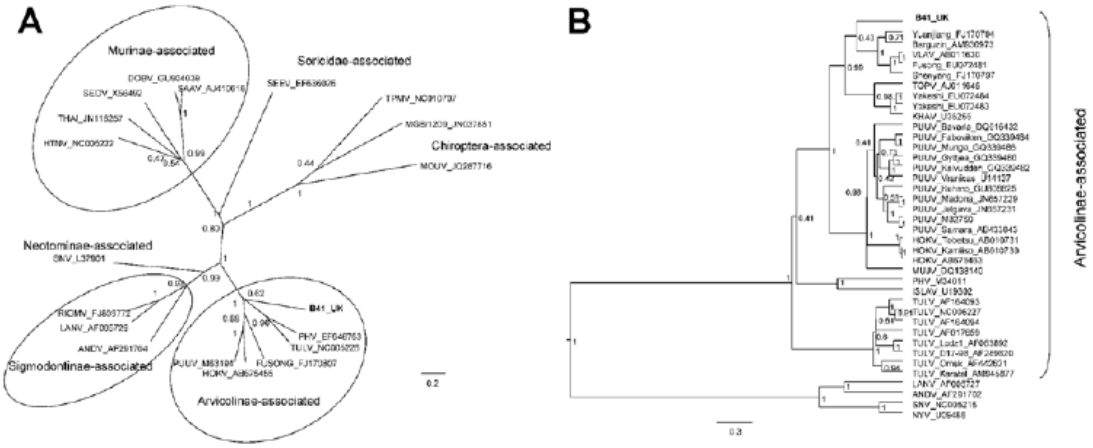

Figure. Bayesian phylogenetic trees constructed by using the models HKY+gamma for partial large segment sequences (n = 19) (A) and GTR+gamma for partial small segment sequences (n = 39) (B) within BEAST software (*6*) with Markov chain Monte Carlo chain lengths of 10 million and strict clock. Optimum substitution models were estimated by using MEGA5 (*7*). The trees are drawn to scale; branch lengths are measured in the number of substitutions per site. The numbers at each node are posterior probabilities. All effective sample size values exceeded 150 for partial L and 1,600 for partial S sequences. The phylogenetic position of virus isolated from field vole B41 (in **boldface**) is shown in relation to representative hantaviruses (A) and more closely related Arvicolinae-associated hantaviruses (B). GenBank accession numbers are shown next to taxonomic names. Scale bars indicate nucleotide substitutions per site. VLAV, Vladivostok virus; TOPV, Topografov virus; KHAV, Khabarovsk virus; PUUV, Puumala virus; HOKV, Hokkaido virus; MUJV, Muju virus; PHV, Prospect Hill virus; ISLAV, Isla Vista virus; TULV, Tula virus; LANV, Laguna Negra virus; ANDV, Andes virus; SNV, Sin Nombre virus; NYV, New York virus.

Microtus vole–associated hantaviruses, albeit with low posterior probability values. These differences in tree topologies probably reflect different compositions of the sequence datasets.

Blood collected from vole B41 was positive for hantavirus-specific antibodies (indirect fluorescent antibody test that used Puumala antigen) (*8*), suggesting cross-reactivity, as would be expected for Arvicolinae-associated hantaviruses. Hantavirus RNA was detected in the kidneys but not the liver of vole B41 and not in the lungs, liver, or kidneys of the 7 other field voles. Degenerate cytochrome B gene PCR and sequencing (*9*) were used to confirm the morphologic identification of the field voles (B41 CytB GenBank accession no. KC222031).

The nucleotide and amino acid sequence divergences between B41 and the most related hantaviruses correspond to that typically found between hantavirus species (*5*). The phylogenetic analyses further support B41 as a distinct hantavirus. Thus, we propose to name this novel virus Tatenale virus, reflecting the medieval name of its place of origin.

M. agrestis voles, among the most numerous mammals in mainland Britain, have not been shown to be primary carriers of a specific hantavirus, although recent studies suggest that they might be involved in the maintenance of Tula virus in Germany (*10*). Further surveillance is needed to confirm that *M. agrestis* voles are the reservoir hosts of Tatenale virus, provide an estimate of virus prevalence, and determine zoonotic risk. Current knowledge of other *Microtus* vole–borne hantaviruses suggests that although they might infect humans, their pathogenic potential is generally low (*1*). Future work will involve attempts to isolate Tatenale virus and generate its full-genome sequence.

Because hantavirus diseases have such broad clinical features, many cases among humans in the United Kingdom might be misdiagnosed. The confirmation of a novel hantavirus in indigenous wildlife in the United Kingdom might promote inclusion of hantavirus infection in the differential diagnosis for patients with acute renal failure, undiagnosed febrile illness, and exposure to rodents (*4*).

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