Seoul hantavirus as a cause of acute kidney injury in the United Kingdom

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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Memorandum

Apart from the help and advice acknowledged, this thesis represents the work of the author

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Abstract

Hantaviruses are a group of rodent-borne viruses. Seoul hantavirus is considered to be the only global hantavirus, although reported human cases outside of Asia are rare. Human infection occurs when breathing in aerosols of excreta from infected rodents. Hantaviruses have been listed as a major impacting factor leading to a rise in acute kidney injury (AKI) throughout the Western world.

In the UK, historically, there was evidence for human and animal exposure to hantaviruses demonstrated by the detection of specific antibodies and classic renal disease, however it is only during this study that existence of a UK hantavirus in wild rodent populations has been proven. Since 2012, several cases of acute AKI due to hantavirus infection in the UK have been confirmed. Two cases were from Yorkshire and had documented exposure to wild rats. Wild rodents were trapped from the farm belonging to one of the patient's and a strain of Seoul virus, named Humber virus, was isolated from rats.

Subsequent cases of AKI were in people with exposure to specially-bred pet fancy rats. Rats from one private breeding colony were tested and a second highly similar Seoul virus, named Cherwell virus, was described. Evidence for Cherwell virus was demonstrated in a human sample with genetic data of the virus recoverable from a serum sample. It was 100% identical to the pet rat strain, thus confirming SEOV as the causative agent of the patient's AKI. These findings have implications for public health as Seoul virus is capable of causing moderate-severe human disease. The overarching aim of the thesis was to confirm hantaviruses cause human infection in the UK and raise clinical awareness; this was achieved.

List of Abbreviations

Aa	Amino acid	FBS	Foetal bovine serum
Ab	Antibody	FDA	Federal Drug Association
ACDP	Advisory Committee on Dangerous Pathogens	FFA	Focus forming assay
A&E	Accident and Emergency	FFU	Focus forming units
Ag	Antigen	FITC	Fluorescein isothiocyanate
AKI	Acute kidney injury	FRNT	Focus reduction neutralisation test
BSA	Bovine serum albumin	Gn	N terminal glycoprotein precursor
BSL	Biosafety level	Gc	C terminal glycoprotein precursor
cDNA	Complementary DNA	HCPS	Hantavirus cardio pulmonary syndrome
$C0_2$	Carbon dioxide	HFRS	Haemorrhagic fever with renal syndrome
CPE	Cytopathic effect	HLA	Human leukocyte antigen
CRF	Chronic renal failure	ICTV	International committee on taxonomy of viruses
dH_20	Deionised water	ICU	Intensive care unit
DMEM	Dulbecco’s modified Eagle medium	IFA	Immunofluorescence assay
DMSO	Dimethylsulphoxide	IgA	Immunoglobulin A
DNA	Deoxyribonucleic acid	IgG	Immunoglobulin G
ECMO	Extracorporeal membrane oxygenation	IgM	Immunoglobulin M
EDTA	Ethylene-diamine-tetra acetic acid	Kb	Kilobase
EHF	Epidemic haemorrhagic fever	KHF	Korean haemorrhagic fever
ELISA	Enzyme-linked immunofluorescence assay	LFT	Liver function test
EM	Electron microscopy	MAb	Monoclonal antibody
FAM	6-carboxyfluorescein	MEM	Minimum essential media

MGB	Minor grove binder	PCR	Polymerase chain reaction
Ml	Millilitre	PRNT	Plaque reduction neutralisation test
MoD	Ministry of Defence	RNA	Ribonucleic acid
MSC	Microbiological safety cabinet	RT-PCR	Reverse transcriptase PCR
N	Nucleocapsid	Rpm	Revolutions per minute
NE	Nephropathia epidemica	S	Seconds
NHS	National Health Service	TBE	Trisborate EDTA
Nt	Nucleotide	µg	Microgram
OD	Optical density	µL	Microliter
ORF	Open reading frame	UV	Ultraviolet
PBS	Phosphate buffered saline		

ANDV	Andes virus	SAAV	Saaremaa virus
DOBV	Dobrava-Belgrade virus	SEOV	Seoul virus
HTNV	Hantaan virus	SNV	Sin Nombre virus
PHV	Prospect Hill virus	THAIV	Thailand virus
PUUV	Puumala virus	TPMV	Thottapalayam virus

Ap. agrarius	*Apodemus agrarius*	*Mu. domesticus*	*Mus domesticus*
Ap. flavicollis	*Apodemus flavicollis*	*My. glareolus*	*Myodes glareolus*
Ap. sylvaticus	*Apodemus sylvaticus*	*P. maniculatus*	*Peromyscus maniculatus*
Mi. arvalis	*Microtus arvalis*	*R. norvegicus*	*Rattus norvegicus*
Mi. pennsylvanicus	*Microtus pennsylvanicus*	*R. rattus*	*Rattus rattus*

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Adams, K., **Jameson, L**,. Meigh, R., and T. Brooks. 2014. "Hantavirus: an infectious cause of acute kidney injury in the UK." *BMJ Case Reports* 2014.

Newman, E.N.C., Johnstone, P., Bridge, H., Wright, D.W., **Jameson, L**., Bosworth, A., Hatch, R., Hayward-Karlsson, J., Osborne, J., Bailey, M.S., Green, A., Ross, D., Brooks, T., and R. Hewson. 2014. "Seroconversion for Infectious Pathogens among UK Military Personnel Deployed to Afghanistan, 2008–2011." *Emerging Infectious Disease Journal* 20(12).

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CHAPTER 1

Introduction

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are a globally distributed group of RNA viruses. The genus derived its name from the prototype strain Hantaan virus (HTNV), first detected near the Hantaan River in Korea. Several distinct species are known to circulate; those confirmed to cause human disease within Europe are: Dobrava-Belgrade (DOBV), Puumala (PUUV), Saaremaa (SAAV) and Seoul (SEOV) viruses. Andes (ANDV) and Sin Nombre (SNV) are the predominant hantavirus species responsible for serious human disease in South and North America, respectively.

Infectious virus particles are excreted in the urine, saliva and faeces of infected rodents.[1] Human infection most often occurs when breathing in aerosols of infectious rodent excreta. In general, two clinical syndromes are recognised in severe cases: haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas.[2] Historically known as a cause of 'field nephritis' in troops[3], hantaviruses are now recognised as a zoonosis of public health importance and have been listed as one of the impacting factors leading to a rise in acute kidney injury (AKI) throughout the Western world.[4]

1.1 Classification

Morphological analyses and molecular genomics led to the designation of hantaviruses within the family *Bunyaviridae*.[5] The *Bunyaviridae* family was formally established in 1975 and contains five genera of viruses: *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Topsovirus*, and *Hantavirus*.[6] Antigenic characterisation is used to separate the viruses within each genus. Arthropods, primarily mosquitoes, tick, and sand flies, are the predominant reservoirs for Bunyaviruses. Hantaviruses are the exceptions; these viruses are rodent-borne and are transmitted via aerosolised excreta.[7]

1.2 Historical overview

There are clinical reports in Chinese literature of maladies suggestive of hantavirus infection as early as 900 AD.[8] Similar reports exist from England and France dating back to the 14th and 17th century, respectively.[9] In more recent history, the disease was noted by Soviet researchers between 1913 and 1930 following sporadic outbreaks in Far Eastern USSR.[10] Though unclassified at the time, it was during the Korean War that hantaviruses first gained international attention.[11] United Nation troops suffered from symptoms including, fever, renal insufficiency, shock and haemorrhage. [12-14] The disease was initially termed Korean haemorrhagic fever (KHF); although a similar syndrome, termed epidemic haemorrhagic fever (EHF), had been recognised in China[15, 16] and countries from the eastern former Soviet Union.[10] Despite extensive investigation no aetiological agent was identified. It was not until 1976 that Lee *et al* (1978)[17] demonstrated sera from KHF patients reacted with an immunofluorescence assay composed of tissue sections of *Apodemus*

agrarius (striped-field mice), but the sera did not react with sections from other rodent species. The virus was successfully isolated from infected *A. agrarius* and KHF patient samples and named Hantaan virus after the river from which the rodents were collected.[17] Since then, all hantaviruses isolated have been named after the geographic locations in which they were discovered.

Concurrently, a similar though clinically milder disease was recognised in Scandinavia[18] and Russia[19] where it was known as nephropathia epidemica (NE)[16] Using the IFA method developed by Lee *et al.* (1978)[17], reactive antibodies to HTNV were demonstrated in sera from NE and EHF patients from Scandinavia, Finland, Russia, China, eastern and western Europe.[16, 20-24] Titres from samples obtained from Western Europe were lower than those from the rest of Asia, leading researchers to postulate that another hantavirus was responsible. Shortly afterwards a second hantavirus was isolated from *Myodes glareolus* (bank vole) captured in Puumala, Finland and therefore named Puumala virus.[23]

Following the early years of research on HTNV in Korea it became evident that urban cases of hantavirus disease were occurring in Korea, China and Japan. [25, 26] Sera tested positive for antibodies to hantavirus.[27] Antigen was demonstrated in the lungs of *Rattus norvegicus* (brown rat) and *Rattus rattus* (black rat) trapped around Seoul, the capital city of South Korea. At the time the virus was initially presumed to be HTNV, it was later attributed to Seoul virus (SEOV).[28]

More hantaviruses continued to be discovered with the first New World hantavirus isolated from *Microtus pennsylvanicus* (meadow vole) in Maryland, USA and named Prospect Hill virus (PHV).[29] Shortly after, two further hantaviruses were confirmed in Asia: Thailand virus (THAIV)[29] and Thottapalayam virus (TPMV).[31,32] A second European hantavirus was detected; Dobrava-Belgrade virus was isolated from *Apodemus flavicollis* (yellow-necked mouse) which was later shown to cause severe human disease in Eastern Europe.[33-35]

Electron microscopy of different hantavirus isolates at US laboratories revealed the morphology of a *Bunyavirus*.[36-37] CDC researchers went on to use Vero E6 cells for culture and neutralisation tests to confirm four distinct agents: HTNV, PHV, PUUV and SEOV.[22] *Hantavirus* was proposed and accepted as a new name for this genus.[38] In 1983, all viruses serologically related to HTNV, and demonstrated to cause similar clinical symptoms, were categorised under the generic term haemorrhagic fever with renal syndrome.[39]

A second human syndrome, hantavirus cardiopulmonary syndrome was documented in 1993 when Sin Nombre virus was discovered after 24 cases had been identified in the Four Corners region, USA. Antibodies to hantavirus were found and *Peromyscus maniculatus* (deer mouse) identified as the carrier of SNV.[40] This led to a new sub-group of hantaviruses characterised by severe disease with pulmonary and cardiac manifestations. Further investigation in the Americas led to the identification of Andes virus after it was noted that there was a high prevalence of hantavirus antibodies among native Americans in South America.[41,42] This

hantavirus is unique in that there is strong evidence for human to human transmission.[43]

The 2011 Ninth Report of the International Committee on Taxonomy of Viruses lists 24 *Hantavirus* species (Table 1.1) with a comparable number proposed as candidates for future inclusion.[44] It has been proposed that new *Hantavirus* species are only accepted on the list if the amino acid sequence differs by at least 10% for the S segment or 12% for the M segment from existing characterised species.[45]

Table 1.1 *Hantavirus* species accepted by ICTV listed according to their rodent host.

	Virus	**Abbreviation**	**Host species**	**Common name**
Family: *Cricetidae*	**Subfamily: *Arvicolinae* (voles and lemmings from Eurasia and N America)**			
	Puumala	PUUV	*Myodes glareolus*	bank vole
	Tula	TULV	*Microtus arvalis*	European common vole
	Topografov	TOPV	*Lemmus sibiricus*	lemming
	Khabarovsk	KHAV	*Microtus fortis*	Maximowicz vole
	Prospect Hill	PHV	*Microtus pennsylvanicus*	reed vole
	Isla Vista	ISLAV	*Microtus californicus*	meadow vole
	Subfamily: *Neotominae* (mice and rats of the New World)			
	Sin Nombre	SNV	*Peromyscus maniculatus*	deer mouse
	New York	NYV	*Peromyscus leucopus*	white-footed mouse
	El Moro canyon	ELMCV	*Reithrodontomys megalotis*	western harvest mouse
	Rio Segundo	RIOSV	*Reithrodontomys mexicanus*	Mexican harvest mouse
	Subfamily: *Sigmodontinae* (mice and rats of the New World)			
	Andes	ANDV	*Oligoryzomys longicaudatus*	long-tailed pygmy rice rat
	Bayou	BAYV	*Oryzomys palustris*	marsh rice rat
	Black Creek Canal	BCCV	*Sigmodon hispidus*	hispid cotton rat
	Cano Delgadito	CADV	*Sigmodon alstoni*	Alston’s cotton rat
	Laguna Negra	LANV	*Calomys laucha*	vesper mouse
	Muleshoe	MULV	*Sigmodon hispidus*	hispid cotton rat
	Rio Mamore	RIOMV	*Oligoryzomys microtis*	small-eared pygmy rice rat
Family: *Muridae*	**Subfamily: *Murinae* (mice and rats of the Old World)**			
	Hantaan	HTNV	*Apodemus agrarius coreae*	dark-striped field mouse
	Dobrava-Belgrade	DOBV	*Apodemus flavicollis*	yellow-necked field mouse
	Saaremaa	SAAV	*Apodemus agrarius agrarius*	striped field mouse
	Thailand	THAIV	*Bandicota indica*	great bandicoot rat
	Seoul	SEOV	*Rattus norvegicus, R. rattus*	brown rat, black rat
	Sangassou	SANGV	*Hylomyscus simus*	African wood mouse
Family: *Sorcidae*	**Subfamily: Corcidurinae and Soricinae (Insectivores)**			
	Thottapalayam	TPMV	*Suncus murinus*	Asian house shrew

1.3 Virus structure and replication

All *Bunyaviridae* have a negative-strand tripartite RNA genome composed of three segments (Figure 1.1). For hantaviruses this comprises the small segment (1530-2078 nt) encoding the nucleocapsid (N) protein[46], the medium segment (3534-3801 nt) encoding a polyprotein which upon cleavage produces the glycoproteins Gn and Gc that embed in the virus membrane, and the large segment (6529-6578 nt) which encodes the RNA dependent RNA polymerase.[47] The variable length of the three genome segments is mainly caused by the 3' noncoding region.

A putative non-structural protein NS has been described in hantaviruses associated with *Arvicolinae* (PHV, PUUV, TULV) and *Sigmodontinae* rodents (BCCV, ELMCV, SNV) but not for *Murinae* associated hantaviruses (DOBV, HTNV, SEOV).[48]

For all three segments the 3' terminal nucleotide sequence (AUCAUCAUCUG) are identical, conserved and complimentary to the 5' terminal nucleotide sequence.[46] This leads to the formation of a panhandle structure thought to play a role in viral transcription and replication.[49]

Electron microscopy studies demonstrate viruses belonging to the *Bunyaviridae* are spherical and generally have a diameter between 90 and 120 nm. Hantaviruses are however distinctive in the family due to their pleomorphic nature and have been demonstrated as tubular at different stages of replication ranging in size between 70 and 210 nm.[50, 51]

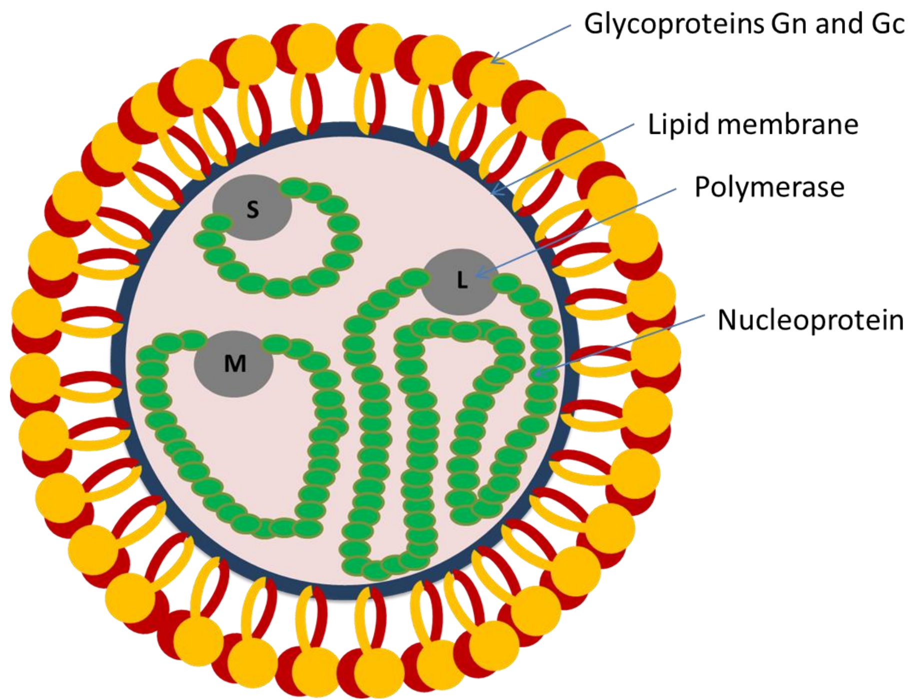

Figure 1.1 Graphic illustration of a hantavirus virion containing three vRNPs: small, medium and large

1.4 Life cycle

The hantavirus life cycle involves seven general processes: (1) attachment, (2) entry, (3) transcription, (4) translation, (5) replication, (6) assembly, and (7) progeny release. The glycoproteins facilitate attachment to and fusion with the host cell membrane, [52-54] thereafter cell entry occurs via receptor-mediated endocytosis.[55] Hantavirus replication takes place in macrophages and endothelial cells; particularly in the lungs and kidneys.[45] Intracellular replication occurs exclusively in the perinuclear region of the cytoplasm of infected cells. After assembly of the structural proteins and genomic RNA into virus particles, the virions mature by budding in the Golgi complex.[47, 56] The first newly formed virions can be detected within 24 hours post infection.[56]

1.5 Relationship between hantaviruses and their natural hosts

It took many years following the recognition of the resultant human disease to identify the reservoir of HTNV; a rodent, *A agrarius*. Until this isolation, all characterised viruses of the family *Bunyaviridae* were known to be arthropod-borne viruses. Each hantavirus is closely associated with a single (or a small number of closely related) Rodentia or Soricomorpha species.[57]

Phylogenetic analysis of all available sequences for hantavirus species demonstrates the close affinity between virus and carrier species with distinct clades formed by the viruses maintained by *Arvicolinae*, *Murinae* and *Sigmodontinae* species regardless of the geographic origin of the sample.

The exact mechanism is not well understood but it is accepted that hantaviruses maintain persistent infections of their host rodent species through horizontal transmission. Regulatory T-cells have been suggested to mediate SEOV persistence in *R. norvegicus*.[58] New-born animals are protected from infection by maternal antibodies transferred *in utero*.[59] Once infected, rodents shed virus in their excretions including saliva, urine and faeces despite the presence of specific antibodies.[60] Persistent infection in rodents is thought to occur with little or no pathogenic consequence but increasing evidence suggests that there are some detrimental effects. Weight gain and survival are reduced in SNV infected *P. maniculatus*.[62, 63] Winter survival is reduced in PUUV infected *My. glareolus*[64] and SEOV infected *R. norvegicus* demonstrate more aggressive behaviour than uninfected individuals.[65]

Hantaviruses are able to survive and remain infectious on materials for several weeks in ideal conditions of low temperatures, moist conditions and little UV exposure.[61] Other than inhalation of infectious particles, direct transfer by biting is thought be an important transmission route between rodents; this route is discussed further is section 1.16.4.

1.6 Distribution

Globally hantaviruses are an important cause of human illness with between 150, 000 and 200, 000 cases of HFRS and approximately 200 cases of HPCS resulting in hospital admission annually.[7] The majority of HFRS cases are reported from China, Korea, Russia and Scandinavia, followed by smaller numbers and sporadic

outbreaks in Japan, Central and Eastern Europe. The geographical distribution and pattern of human infections are driven by the natural rodent hosts for each hantavirus. The four hantaviruses responsible for the majority of clinical cases in Eurasia are DOBV, HTNV, PUUV, and SEOV.

1.6.1 Dobrava-Belgrade virus

DOBV causes a severe form of HFRS, however it is one of the most restricted hantaviruses in Europe with most cases reported from the Balkan region (Figure 1.2) where its carrier host *A. flavicollis* is abundant. Due to the habitat preferences of *A. flavicollis*, cases are almost exclusively in rural forested locations.

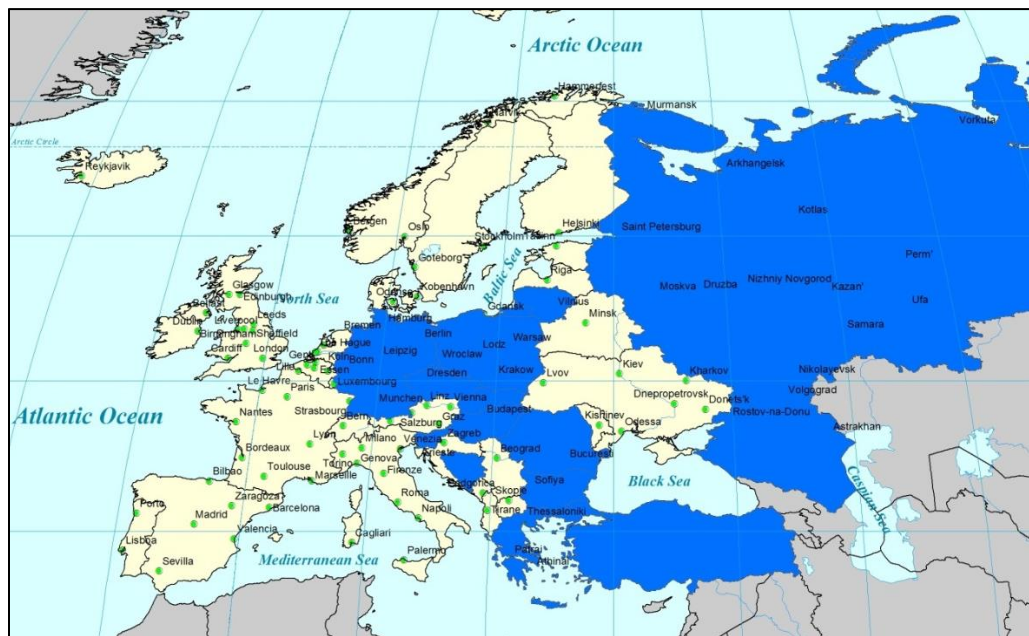

Figure 1.2 Current distribution of DOBV in Europe

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1.6.2 Hantaan virus

HTNV, the most severe agent of HFRS, is established in China, Korea and eastern Russia where the common field mouse *A. agrarius* is the carrier rodent. Adults working and living in rural areas have the highest risk for infection.

1.6.3 Puumala virus

PUUV, the aetiologic agent causing the clinically the mildest form of HFRS, remains the most prevalent hantavirus in Western and Central Europe (Figure 1.3) where its carrier host *My. glareolus* is common; again with most cases occurring in rural locations.

Figure 1.3 Current distribution of PUUV in Europe

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1.6.4 Seoul virus

SEOV is an exceptional hantavirus due to its potential for global distribution via its carrier host, the brown rat, *R. norvegicus*, which is ubiquitous on every continent with the exception of Antarctica. With the opportunistic behaviour of *R. norvegicus* human cases occur in both rural and urban settings. Further information on SEOV is presented in section 1.16.

1.7 Transmission

Viral transmission to humans typically occurs when materials contaminated with rodent excreta are disturbed, causing virus particles to aerosolise and be inhaled. Contact between broken mucosal membranes and virus contaminated materials or through a direct bite are also potential routes of transmission.[66]

Hantavirus infections in humans are reported throughout the year but often have seasonal peaks. In Scandinavia the majority of Puumala cases occur during winter when the rodent host, *My. glareolus* seeks shelter or food in human lodging. In contrast in Asia two peaks are evident - in spring and summer - related to planting and harvesting of crops which bring humans in close contact with rodents.[67]

Mites have been found to be PCR positive for HTNV in China, and laboratory experiments have shown that direct and transovarial transmission is possible. Further research is required to prove such mechanisms are possible in a natural setting.[68]

With the exception of ANDV[43] humans are dead-end hosts for hantaviruses. In 1996, the first possible inter-human transmission cases of ANDV were suspected[69] with mounting evidence supporting limited human-to-human transmission for ANDV in health care workers and close family members.[70]

1.8 Hantaviruses causing human disease

While hantavirus infections in rodents are largely asymptomatic they can result in severe disease in humans. Two partly overlapping clinical syndromes have been described: HFRS and HCPS.[71] Hantaviruses carried by *Sigmodontinae* rodents generally cause more severe disease in humans than those carried by *Murinae* and *Arvicolinae* rodents.

The incubation time is typically between 2 and 3 weeks but can be up to 8 weeks. Occupation is a dominant risk factor for hantavirus infection with animal workers, forestry workers, farmers and military personnel at greatest risk.[7, 72] Infection occurs more often in males than females with ratios of 2:1 to 3:1 reported. Cases occur in all age groups however the highest incidence is in 20-40 year olds.[73]

1.8.1 Haemorrhagic fever with renal syndrome

As the name suggests HFRS is a disease characterised by vascular haemorrhage and kidney dysfunction. Disease can manifest as mild, moderate or severe with case fatality rates up to 15% depending on the causative virus.[74] The initial symptoms have a rapid onset but are non-specific and include: intense headache, back and abdominal pain, fever, chills and nausea.[71] Severe disease may manifest with low

blood pressure, acute shock, vascular leakage and acute kidney injury. Recovery of renal function usually occurs after 2-3 weeks although may take months; long-term impairment of renal function can occur[75] as can hypertension.[76, 77] Severe disease is more often associated with infection with HTNV or DOBV; moderate disease characterised by lower mortality but increased likelihood of liver involvement is associated with SEOV infection.[78] PUUV infection results in the mildest form of HFRS and often leads to patients suffering from ocular manifestations.[74]

1.8.2 Hantavirus cardio-pulmonary syndrome

HCPS is characterised by acute onset of respiratory failure and cardiogenic shock. It bears resemblance to HFRS with vascular leakage, however the lungs are the primary target instead of the kidneys. In the early stages of disease non-specific symptoms such as fever, myalgia, malaise, headache and abdominal pain are indistinguishable from those of many other viral infections.[71] This febrile stage lasts for up to 2 weeks, but typically less than 1 week. Patients may then go on to the cardiopulmonary stage and develop acute shortness of breath, dizziness and hypoxia followed by pulmonary oedema, myocardial dysfunction, hypoperfusion and shock.[7] It is during this phase that the majority of HCPS patients die with mortality rates for HCPS higher than HFRS; rates are between 30 and 80% depending on the causative hantavirus. Patients who survive usually make a full though prolonged recovery with convalescence lasting several months during which time patients will experience fever and fatigue.

Table 1.2 Clinical Features of Clinically Relevant Hantaviruses[7, 73]

Hantavirus	ANDV	DOBV	PUUV	SEOV	HTNV	SNV
Severity	Severe	Severe	Mild	Moderate	Severe	Severe
Renal damage	±	+++	+	+	+++	±
Liver damage	±	+	No	++	+	±
Lung damage	+++	++	No	No	+	+++
Haemorrhage	±	++	±	+	+++	±
Mortality	35%	<10%	<1%	1-2%	5-15%	35%

± rarely reported; + some reports; ++ frequently reported, +++ often reported

1.9 Non-pathogenic hantaviruses

There are many hantaviruses that are yet to be linked to infection in healthy humans.[7] Included in this list are the growing number of novel hantavirus species detected in *Soricomorpha* (shrews) [79-87] and *Chiroptera* (bats). [88-90] Of the rodent-borne non-pathogenic hantaviruses in Europe, Tula virus (TULV) isolated from *Microtus arvalis* (European common vole) is the most likely candidate to be upgraded to a pathogenic species. While it has been associated serologically with human infection in the Czech Republic, Germany and Switzerland, it has not been unequivocally proven to cause human disease. [91-93]

1.10 Pathogenesis

Vascular dysfunction characterised by vasodilation and increased capillary permeability is thought to be the main factor in the pathogenesis of HFRS and HCPS. Both HFRS and HCPS are associated with rapid onset of thrombocytopenia and deranged vascular permeability and both can result in renal or pulmonary manifestations. Nevertheless, Old World hantaviruses predominantly result in renal dysfunction and the disease HFRS while New World hantaviruses are more frequently associated with cardiopulmonary failure - HCPS.

In recent years, the paradigm of separating HFRS and HCPS has been challenged by multiple authors.[94-100] Pulmonary symptoms including cough, pleural effusion and impaired pulmonary function have been reported for European cases of PUUV.[100-105] Acute kidney injury requiring dialysis has also been reported following SNV infection.[97] It has been proposed that hantavirus infections be referred to as hantavirus disease instead of being divided into HFRS and HCPS, however this is yet to be widely adopted.

It is widely accepted that human immunopathology, especially the cytotoxic T-lymphocyte response, rather than direct viral cytopathology, drives pathogenesis.[106-108] Host cells are not lysed by virions, as demonstrated by the lack of any visible CPE in endothelial cell culture.[109,110] The N protein is the immunodominant antigen inducing an early humoral immune response, but it does not stimulate a neutralising antibody response.[111,112] The formation of neutralising

antibodies and the protective immune response are instead induced by the glycoproteins.[113]

Hantavirus specific IgM antibodies are rapidly produced following infection then decline to an undetectable level within 4 months.[114] Specific IgG antibodies circulate shortly after infection and have been shown to persist for decades; providing life-long immunity as no re-infections have been reported.[112-114]

Patient genetic make-up is considered to be important in predicting severe outcomes. Patients with the HLA B8 DR3 haplotype demonstrate high levels of viral RNA whereas patients with the HLA B27 allele are more likely to develop a lower viraemia and thus develop a milder form of PUUV disease.[109] Integrins are cellular surface molecules commonly used by viruses as receptors for attachment and cell entry. They are expressed in many tissues including endothelial cells and platelets, which are thought to be the main targets during hantavirus infection. The usage of β3 versus β1 integrins for cell entry by hantaviruses seems to be an important pathogenicity determinant.[115] The pathogenic hantaviruses, ANDV, HTNV, PUUV, SEOV and SNV use β3 integrins, whereas non-pathogenic hantaviruses PHV and TULV use β1 integrins.

1.11 Treatment

Therapy generally consists of supportive care with no specific treatment for HFRS or HCPS available at present. Both syndromes benefit greatly from careful fluid management and control of blood pressure. Dialysis may be necessary for severe

cases of HFRS, with between one and two sessions of haemodialysis needed before sufficient recovery of renal function.[74] Mechanical ventilation is often required for HCPS patients with increasing evidence supporting the use of more aggressive support such as extracorporeal membrane oxygenation (ECMO) in severe cases.[116] Use of ECMO has been suggested to reduce long term complications.[99] Death with HCPS is more often caused by cardiac failure than pulmonary oedema.[117]

A lethal mouse model was developed for HTNV and utilised to show that ribavirin works by disrupting viral replication.[118] Several years later human trials were performed in China that demonstrated ribavirin is successful at reducing viral load and improving outcome if given early in the disease progress.[119] Ribavirin is itemised on the WHO Model List of Essential Medicines for HFRS treatment.[7]

Due to the annual case burden and global distribution, efforts are on-going to develop an effective hantavirus vaccine. To date there is no WHO or FDA approved vaccine; however, there are two rodent brain and cell culture inactivated vaccines that have been developed and utilised in China and Korea.[120, 121] Both are unlikely to gain widespread approval for use outside of Asia, with the most promising approach coming from a molecular approach using HNTV and PUUV plasmid DNA delivered by a gene gun.[122, 123]

1.12 Aetiological Diagnosis

1.12.1 Serology

Viraemia in humans suffering from HFRS, has a very short duration following onset of symptoms. Consequently, the diagnosis of hantavirus infection is typically made by the detection of hantavirus-specific antibodies. Serological techniques commonly used are immunofluorescence assays (IFA), enzyme linked immunosorbent assays (ELISA) and Western-blot analyses.[124] The confirmation of an acute or recent hantavirus infection relies on the detection of hantavirus specific, IgM or a rise of IgG serum antibodies in paired samples. Even during early days of infection IgM, and usually, IgG antibodies are present.[74] Due to the requirements for handling infectious ACDP hazard group 3 viruses and the lack of clinical isolates for the more novel hantaviruses, commercially available assays are almost exclusively based on recombinant hantavirus antigens. The N protein, either in native or truncated form, is often the antigen of choice for serological assays due to its ability to induce early and long term antibody responses.[125, 126]

Serological cross-reaction of antibodies between the different hantavirus species is a particular problem for diagnosis based solely on IFA and ELISA methods. Hantaviruses carried by the same rodent subfamily demonstrate the highest propensity to cross react: *Murinae* (DOBV, HTNV, SEOV), *Arvicolinae* (PHV, PUUV, TULV) and *Sigmodontinae* (ANDV, SNV).[126-128] In most cases, it is almost impossible to differentiate between two closely related species using simply IFA or ELISA techniques. Differentiation of the causative species is often achieved by endpoint titration using focus reduction neutralisation assays however this technique is

limited to identification of the most closely related virus in the assay. In this instance FRNT is used to titrate antibodies in human serum against a panel of hantavirus; the one which is able to be titrated to the greatest dilution is considered to be the most likely infecting hantavirus species. However if the antibody response is to a novel hantavirus or a virus species simply not represented on the FRNT panel then the results may be misleading. Only molecular sequencing techniques can unquestionably identify the causative virus species.

1.12.2 RT-PCR

Reverse-transcriptase polymerase chain reaction (RT-PCR) is a sensitive and specific method for detection of viral RNA and is possible for most hantavirus species; however, it is rarely used as the sole diagnostic indicator for hantavirus infection.[129] Viral titre in human bodily fluids is typically low,[130] thus a negative RT-PCR finding does not unequivocally provide a negative diagnosis.[131] Nonetheless, RT-PCR does have its use in parallel with serological methods and, should a molecular signal be detected it will allow for exact identification of the causative species which can only be achieved by analysis of viral RNA.[60,132] For human clinical samples nested RT-PCR is often necessary due to the low level of viraemia.[133] RT-PCR is most successful with HCPS cases and when using sera collected within the first ten days of infection.

1.12.3 Virus isolation

The gold standard for clinical confirmation is isolation of the causative agent. For hantaviruses virus isolation from human clinical samples is time consuming, requires containment level 3 facilities and is rarely successful. Several hantaviruses

have been propagated in Vero E6 cells however primary isolation is difficult due to viral growth presenting no cytopathic effect instead establishing a non-cytolytic infection.[134]

1.13 Control and Prevention

Rodent control and the minimisation of human exposure to rodents and their excreta are among the most effective ways of reducing human disease. Rodent-proofing of houses, food stores and careful removal of rodent waste are recommended control measures by the CDC.[7] When cleaning rooms or disposing of rodent nest materials it is advisable to wear gloves, a protective mask and use bleach –based (hypochlorite) disinfectant.

In the laboratory setting hantaviruses have been shown to be susceptible to inactivation via various methods including exposure to: acetone (100%), ethanol (70%), glutaraldehyde (2%), methanol (100%), paraformaldehyde (1%), sodium hypochlorite (1%) and UV irradiation.[135]

1.14 Hantaviruses in the United Kingdom

Laboratory confirmation of autochthonous and travel-related human hantavirus infection is provided by the Rare and Imported Pathogens Laboratory (RIPL), Public Health England (PHE), Porton Down. Due to the high levels of cross-reactivity between hantavirus species, and the lack of viral detection in any published United Kingdom (UK) study, it has previously been impossible to confirm and identify the presence of a single hantavirus species in the UK. Cases are likely to be under-

reported due to mild cases presenting with non-specific symptoms and a lack of awareness of hantavirus infection as a potential diagnosis in the absence of travel.

1.14.1 Potential carrier species

Each hantavirus species is considered to have a specific rodent species acting as its natural reservoir, several of which are present in the UK including: *R. rattus* (rare, mainly in cities i.e. London, Liverpool) and *R. norvegicus* (ubiquitous)-SEOV; *A. flavicollis* (mainly southern England & Wales) - DOBV; *My. glareolus* (ubiquitous)-PUUV; and *Mi. arvalis* (Orkney)- TULV. There is also mounting evidence that rodents are not the only source of infection for humans with both non-rodent wild animals (deer, hare, shrew) and domestic animals (cat, dog, cattle) implicated.[136]

1.14. 2 Serological evidence in animals

Several studies have revealed evidence for circulation of hantaviruses in wild animals in the UK: one sero-survey of wild, feral and domestic cats from across England and Wales tested between 1983 and 1989 demonstrated antibodies reactive to HTNV (76-118).[137] Overall 15 out of 157 (9.6%) domestic and feral cats were reported positive. From a further 198 chronically ill domestic cats, 30 (15.2%) had antibody to HTNV. Given their habit to kill and eat wild rodents, cats can be considered good sentinels for circulation of rodent-borne pathogens.

1.14. 2 Serological evidence in rodents

Studies examining rodent exposure continue to provide evidence for circulating of hantavirus in the UK. Antibodies against a panel of hantaviruses (HTNV, PUUV and

SEOV) were demonstrated in 4/100(4%) *R. norvegicus* and 1/102 (0.98%) mice (species not stated) during an epidemiological investigation in rural Somerset, England in 1992.[138,139] Furthermore, from 127 serum samples collected from *R. norvegicus* trapped on farms in Oxfordshire, North Wales and Hampshire, 5 (4%) had detectable hantavirus-specific antibodies.[140] Four of the samples reacted most strongly to SEOV (IR461) and one to HTNV (76-118). In 1996 similar results were demonstrated from Northern Ireland with antibodies against SEOV (R22) and HTNV (76-118) found among *R. norvegicus* (11/51 -21.6%) and *A. sylvaticus* (1/31 - 3.2%).[141]

1.14.3 Serological evidence in humans

Since the 1980s there has been growing evidence for hantavirus as a cause of human disease in the UK. Sero-studies between 1985 and 1989 in England and Scotland suggest exposure in risk groups (residents in rural areas or with professions involving contact with rodents) ranging from 4.3% in sewage and water workers to 21.5% in farmers.[142] At the time the sero-reactivity was considered to be PUUV-specific.

In 1992 an in-depth regional study in Somerset, England, demonstrated IgG antibodies specific to hantavirus in 29 patients who were suffering from a flu-like illness between January and November.[138] Symptoms described included: influenza-like illness (67%), pyrexia (87%), headache (76%), abnormal liver function (62%) with severe cases also developing a sore throat, swelling of the face, neck, hands and lower limbs and a macular erythematous rash. A panel of three hantaviruses

were used for IFA screening: HTNV, PUUV and SEOV, however it is not clear from the reported results to which virus, if any, the predominant sero-reactivity was directed.

The most recent sero-survey, published in 1999, discussed an investigation of 606 farmers, farm workers and their families in Herefordshire and Lancashire, England and their exposure to a variety of zoonotic pathogens including hantaviruses.[143] The authors reported a sero-prevalence of 4.7% during the first year of the study and 4.8% in the second year.[144] Again, a panel of hantaviruses was used for testing with no specific reactivity reported. In terms of pinpointing an exact hantavirus species causing human disease in the UK in the absence of molecular data, McKenna *et al* (1994)[145] provided the most conclusive serological evidence. Their sero-study of 627 Northern Irish patients presenting with symptoms suggestive of HFRS and 100 healthy controls found 16 patient samples (2.2%) reacted almost exclusively with SEOV (R22).

1.14.4 Acute human cases

Other than the symptomatic cases detected during the Somerset outbreak and during the Northern Ireland sero-study there have been eight other published cases of acute hantavirus infection most likely acquired in the UK. Two were reported from Glasgow, Scotland, one in 1983 [146, 147] and the other 1988, [148] five were reported during 1991, two from Sheffield, England[148] and three from Somerset, England[138,139,150,151] and one was reported from Nottingham, England during 1994.[152] Table 1.3 summarises the most common symptoms and laboratory

markers reported from these cases. Abdominal pain, raised creatinine, lowered platelets and a rash appear to be useful diagnostic indicators of HFRS disease in UK patients. Interestingly some case histories specifically mention recent contact with rats. Several of the cases were severe in nature, with renal replacement required, internal haemorrhage and hepatosplenomegaly occurring. All of the patients were otherwise healthy and had no recent travel history. 5/8 were male and 6/8 were aged 21 years old or under at the time of diagnosis.

Table 1.3 Common symptoms reported from published case histories of UK HFRS patients.

Symptoms/markers reported	No. of patients	Percentage of patients (%)
Abdominal pain	6/8	75
Proteinuria/elevated creatinine	6/8	75
Rash	5/8	62.5
Thrombocytopenia	4/8	50
Fever	4/8	50
Arthropathy	4/8	50
Headache	3/8	37.5
Vomiting	3/8	37.5
Hepatosplenomegaly	2/8	25
Acute kidney injury	2/8	25

1.14.5 Travel related cases

There have been occasional imported cases of hantavirus infection into the UK. The first known case occurred in a British business man returning from Malaysia in 1987.[153] He suffered from typical symptoms including: fever, nausea, vomiting, thrombocytopenia and markers of renal impairment (elevated creatinine and proteinuria). While the sero-reactivity results were not disclosed, it is most likely to have been a SEOV or HTNV infection based on the geographical origin of infection.

Several infections have been reported in British military personnel deployed to Bosnia in 1995[154] and Slovenia in 2002.[155] Symptoms and outcome were not shared for the three soldiers hospitalised in Slovenia but thought most likely to be due to DOBV infection. The case occurring in Bosnia required repatriation to the UK due to the patient's serious condition. Typical symptoms of HFRS ensued; fever, abdominal pain, renal impairment, nausea and vomiting but less commonly severe pulmonary distress occurred. The patient, a 19 year old male, developed pulmonary oedema and required intubation and ventilatory support. A panel of hantavirus strains were used in the IFA for diagnosis; SEOV titres were reported as high as 1:3200 but the titres for other strains were not disclosed. Given our current knowledge and the severe course of disease for this patient it is more likely that he suffered from a DOBV infection.

The most recent case prior to the commencement of this study occurred in a tourist during 2010, a 35 year old male, who had visited family in Estonia 3 weeks prior to his illness.[156] His clinical course was milder than earlier travel-related cases however

typical symptoms ensued including headache, abdominal pain, fever and mild proteinuria, thrombocytopenia and raised creatinine. He also described blurred vision a symptom more typical of PUUV infection. This was further supported by epidemiological questioning which highlighted that he had visited a rural, forested area and had spent time in a barn used for food storage - a typical winter shelter area for the PUUV host *My. glareolus*. Sero-reactivity results confirmed this by demonstrating PUUV specific IgG antibodies at a titre of 1:4096.

1.14.6 Laboratory related cases

Hantaviruses are capable of infecting laboratory workers directly when they are exposed through incorrect handling of the virus or when infected tissues are processed. To reduce potential exposure, all manipulation of potentially hantavirus infected material is undertaken under UK containment level 3 conditions. Since all laboratory animal collections were sourced from wild colonies there is the possibility that laboratory workers may have been exposed to hantaviruses when handling potentially infected rodents or during manipulation of cell cultures set up from rodent tissues. One notable occurrence of this was reported in in 1979 when laboratory rats were identified as the source of HFRS cases in Japanese workers.[157] Once published, Belgian researchers tested samples from three workers who handled laboratory rats and were hospitalised with interstitial nephritis in 1978.[158] They confirmed antibodies reactive to HTNV with IFA and PRNT and concluded that their illness was likely to be due to hantavirus infection. Follow up of other workers at the Belgian institution found 48/391 (12.3%) workers who handled the rats had reactive antibodies. The outbreak was not limited to Japan and Belgium; in 1983

retrospective testing of UK workers at a Cancer Research Institute found 3/4 workers (the fourth was unavailable for testing) who handled rats were sero-positive using IFA.[159] The three staff members were admitted to hospital in early 1977, suffering from symptoms including: fever, abdominal pain, back pain, proteinuria, respiratory complications and renal insufficiency. Two of the patients required haemodialysis. The fourth untested staff member, although not admitted to hospital, was ill at home for 1 week. Six additional staff members who worked at the institute between 1975 and 1979 were retrospectively tested and four demonstrated antibodies. All exposures were linked to the Louvain (Lou C) inbred strain of rats developed from a strain of Wistar origin.[160] The original colony had been imported from Japan via Belgium in 1975.[161] All IFA slides used to confirm infections were HTNV cultured in E6 cells and therefore the virus was assumed to be a hantavirus strain. It was not until the UK isolate (IR461) was sequenced in 2003 that it was reclassified as a strain of SEOV.[162] One of a number of isolates (IR461) was one of a group of UK hantavirus that appeared to be genetically distinct from other SEOV strains. Following destruction of associated laboratory rat colonies and cell lines no further human cases were detected.

1.15 Rationale

Prior to 2009 PUUV was considered to be the most likely hantavirus species responsible for HFRS cases in the UK due to the ubiquitous nature of its reservoir host and similar climatic conditions in the UK to PUUV endemic countries in Europe.[163] It was not until 2009 that SEOV was again highlighted as the possible agent responsible for an HFRS in England. A patient suffering from AKI who reported a recent rat infestation in their residence was found to have reactive antibodies against hantaviruses. The purpose of this study was to investigate SEOV as the causative agent of human infection in England.

1.16 Seoul hantavirus

1.16.1 Reservoir host – R. norvegicus

SEOV is a unique hantavirus in that it is considered to have the potential for global distribution due to its reservoir host, *R. norvegicus* (Berkenhout, 1769) - the common brown rat or Norway rat - being ubiquitous on all continents with the exception of Antarctica. In the UK *R. norvegicus* is distinguished from other rodents by its larger size (body length 150- 270mm and weight 200-600g), brown-grey fur, pointed muzzle and long naked tail.

In the wild they have an average lifespan of between 1 and 2 years; in captivity they may live as long as 4 years.[164] Their preferred habitat includes agricultural land and human settlements, where they live in large, male dominated colonies with a typical territory of 2000 m^2.[165] *R. norvegicus* are mostly nocturnal and known to be neophobic which can lead to difficulty when attempting to trap or poison them.

They are excellent swimmers and often take up residence near waterways and take advantage of flooded areas. Breeding occurs throughout the year but increases during warmer months. Females are capable of producing seven litters per year of which there are approximately 8 pups per litter. Males reach sexual maturity at 3 months old and females at 4 months. Originally this rat species was native to forests in what is now northern China and Mongolia. They arrived in Europe between the 16th Century and 18th Century.[166, 167] They are considered a pest species and a prolific public health problem, with one British study linking them to 13 zoonotic agents including hantaviruses.[140]

Figure 1.4 *Rattus norvegicus*.

Image credit http://spec-evo.wikia.com/wiki/File:Brown-rat.jpg

1.16.2 Human infection as a result of SEOV infection

Worldwide, approximately 150,000 cases of HFRS are thought to occur each year; 90% are reported from Asia[168] of which 25% are a result of SEOV infection.[169] With the global distribution of the reservoir host it is likely SEOV infections are under-reported outside of Asia. Serological evidence for SEOV infection in humans has been demonstrated in Bosnia,[170] Brazil,[171] France,[172] Japan,[173] Northern Ireland,[145] Russia[174] and USA. Patients suffering from HFRS induced by SEOV have a shorter clinical course with milder renal dysfunction but more severe abdominal symptoms and hepatic dysfunction than those with HTNV infection.[175] Symptoms reported from suspected European cases include: fever, myalgia, vomiting and diarrhoea, followed by acute renal failure and thrombocytopenia.[145, 170, 172]

1.16.3 Evidence for SEOV outside of Asia

Evidence for SEOV infection in rats has been demonstrated from Argentina,[176] Belgium,[177,178] Brazil,[171] Cambodia,[179] Egypt,[180] France,[167] Indonesia,[181] Japan,[25] North Korea,[182] Portugal,[183] Singapore,[184] South Korea,[185] Northern Ireland,[141] USA,[186] and Vietnam.[187] Antibody prevalence in wild rats ranges from 10% in Indonesia[188] to 31.6% in Buenos Aires[176] and 50% in Baltimore.[189] In Europe, sero-prevalence rates lie in-between those reported for Asia and America with 27% of rats antibody positive in Belgium[177] and 21.6% of rats in Northern Ireland.[141]

1.16.4 Transmission dynamics of SEOV

The main transmission route for hantaviruses is inhalation of infectious particles; however, laboratory experiments have indicated SEOV to be more effectively

transmitted via intramuscular injections.[190] Furthermore a correlation between presence of wounds and SEOV infection in wild rats has been observed.[191,192] These findings suggest that biting is the main route of transmission for SEOV. As *R. norvegicus* is a long established animal species for scientific research several studies have been undertaken to better understand SEOV. Experimental infection investigating viral shedding revealed presence of virus in saliva, urine, faeces and blood/organs at 10, 20, 30 and 40 days post infection.[193] Both sexes were equally susceptible to infection but male rats shed virus for longer, via more routes and also had higher levels of viral RNA in their blood.[193,194] Sero-positivity has been shown to increase with rat body mass[186] and age. Arikawa *et al*. (1986)[195] reported that a higher proportion of Norway rats over six months of age were sero-positive (64%) compared with younger animals (9%) in Japan. Similar findings were found in rats positive for SEOV in Argentina.[176]

1.17 Aims

Mounting evidence suggests that a hantavirus may be one of the infective causes of AKI in the UK. In the last four decades several acute cases in individuals without recent foreign travel have been detected and sero-prevalence studies in risk groups indicate continued exposure. Serological assays have been unable to determine the species of hantavirus responsible with atypical symptoms suggestive of more than one virus species or a novel virus circulating in the UK. Before commencement of this study no molecular evidence to confirm the causative species in wild rodents had been demonstrated, yet following the most recent case in 2009, SEOV was identified as the most likely agent.

The overarching aim of this study was to investigate hantaviruses as a cause of clinical disease in England. The specific objectives were to:

- identify and characterise a hantavirus circulating in rodents in the UK;
- generate a cell culture isolate of a UK hantavirus;
- confirm the hantavirus as a causative agent of human infection;
- investigate the extent of human exposure in key risk groups to the virus;
- develop a serological diagnostic technique for identifying hantavirus infection outside of containment.

CHAPTER 2

Materials and Methods

Unless otherwise stated, all plastic consumables were sourced from Fischer Scientific (Loughborough, UK); tissue culture media, supplements, chemicals and antibodies were from Sigma-Aldrich (Gillingham, UK). All incubations occurred in a humidified 5% $C0_2$ atmosphere and samples were stored at -80 °C. Manipulations with suspected or confirmed hantaviral infectious material were performed in a class III microbiological safety cabinet (MSC III) under ACDP containment level 3 (CL3) conditions at Public Health England (PHE), Porton Down in accordance with ACDP guidance on CL3 working practices. All molecular steps employed the use of nuclease free consumables.

2.1 Rodent sampling

2.1.1 Wild rodents

Following confirmation of an acute human case of hantavirus with no travel history in January 2012, wild rodents were trapped and sampled from the patient's residence in Yorkshire and the Humber, north-east England, UK during late February, early March 2012. A combination of live trapping (Longworth traps, Penlon LTD, Abingdon) and snap trapping (Rentokil snap traps) was employed. Traps were baited with sweet corn and peanut butter and checked twice per day to minimize stress on the animals. Live-trapped animal were humanely killed via cervical dislocation. Due to the high probability of exposure to infectious animals

and their excrement, personal protective equipment was utilised including: Tyvek suit, plastic boots, latex gloves, eye goggles and FFP3 mask. Disposables and waste were treated with 3% Virkon and transported as infectious clinical material. Animal carcases were packaged and transported according to HSE guidance for category B agents under the regulations for the Transport of Infectious Substances UN3373.

2.1.2 Pet rodents

After identification of domestic rats (*Rattus norvegicus*) as the likely source of a patient's hantavirus infection in January 2013, sampling was undertaken in February 2013, at the site of the breeding colony where the rats originated from in Oxfordshire, south England, UK. Blood sampling of the lateral tail vein or via cardiac puncture immediately following euthanisation was undertaken by a veterinarian. Urine samples when available were collected by use of pastette. With consent of the owner, rats confirmed to be RT-PCR positive for SEOV RNA were euthanized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) by a veterinarian.

2.1.3 Processing of rodent samples

Carcasses and samples were flash-frozen on solid carbon dioxide ($C0_2$) before being transported back to the laboratory under the same condition as described in section 2.1.1 for storage at -80 °C and necropsy. At dissection the following major organs were aliquoted individually: brain, bladder, heart, kidney, liver, lung, lymph node, pancreas, salivary gland and spleen. A sample of each tissue in 500 µL sterile PBS was homogenised at 5000 rpm for 30 s (x2) using a MagNA Lyser instrument

and ceramic beads (Roche, West Sussex, UK). Tubes containing homogenate were centrifuged at 900 x *g* for 1 min and supernatant collected. Serum was separated from the blood using standard centrifugation (2300 x *g* for 15 min).

2.2 RNA extraction

2.2.1 RNA extraction from animal samples

For purification of RNA from tissue, blood, serum and urine samples the samples were passed through QiaShredder columns (Qiagen, Manchester, UK) before using the Qiagen RNeasy Mini Kit in accordance with manufacturer's instructions with the exception that infectious samples were inactivated in Qiagen AVL buffer instead of RLT buffer. In brief 140 µL of sample was added to 560 µL AVL and incubated for a minimum of 10 min within the MSC III. Then 70% ethanol was added to the lysate and mixed before transferring the entire volume to the column and centrifuged at 9500 x *g* for 1 min to bind RNA to the column filter. The column was washed with proprietary buffers RW1 and RPE with centrifugation repeated as previously between steps and an additional centrifugation after to remove buffer residue. RNA was eluted with 50 µL nuclease free water after a 1 min incubation and final centrifugation.

2.2.2 RNA extraction from cell culture

For isolation of total RNA from cells and culture supernatant the QIAmp Viral RNA Kit (Qiagen, Manchester, UK) was used in accordance with manufacturer's instructions. In brief, 140 µL of sample was added to 560 µL AVL and incubated for a minimum of 10 min within the MSC III. Pure ethanol was added to the lysate and

mixed before transferring entire liquid volume to column and centrifuged as previously described to bind RNA to the filter. The column was washed with proprietary buffers AW1 and AW2 with centrifugation between steps and an additional centrifugation step afterwards to remove buffer residue. RNA was eluted using 50 µL proprietary AVE buffer after a 1 min incubation and final centrifugation.

2.3 Cells and viruses

2.3.1 Cells

Vero C1008 clone E6 (African green monkey epithelial kidney cell line: ECACC catalogue no. 85020205) cells were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 2 mM glutamine. Cells were sub-cultured after microscopic examination to ensure they were suitably confluent (80-90%) and free from bacterial or viral contamination. Cultures were routinely maintained in 75 cm^2 and 175 cm^2 filtered flasks. For routine passaging the growth medium was discarded and monolayer washed with PBS before a minimal volume of trypsin (0.25%) was added. The flasks were incubated for 5 min at 37 °C or until the cells visibly detached from the flask. Cells were mixed with new growth medium to produce an even suspension before being added to new culture flasks with a final split of between 1:3 and 1:10. Cultures were discarded after a maximum passage number of 30.

2.3.2 Viruses

All hantavirus strains (DOBV H119/99 0506241v, HTNV 76/118 0006112v, PUUV 851 0504101v, SEOV R22 0107222v, SNV HN107 9908151v and TULV 0504102v) were

kindly provided by the National Collection of Pathogenic Viruses (NCPV), PHE, Porton Down. Stock virus was diluted 1:10 in serum-free DMEM and adsorbed to Vero E6 cells by incubation at 37 °C for 90 min with the flask rocked every 15 min to ensure even coating. Viral inoculum was removed and minimal fresh culture media added. Cultures were incubated at 35.5 °C for ten days. Virus was harvested by subjecting the flask to two freeze-thaw cycles at -80 °C to disrupt cells. Flask contents were then centrifuged at 900 x *g* for 10 min to pellet cellular debris. Supernatant was aliquoted and stored at -80 °C.

2.4 Virus isolation

SEOV real-time RT-PCR positive lung samples were used for virus isolation attempts. Homogenised tissue supernatant was diluted 1:10 with serum free DMEM, passed through a 0.45 µM filter and 2 mL added to sub-confluent (60%) cultures in 25 cm^2 flasks. Cells inoculated with 2 mL DMEM only acted as a negative control. Virus was left to adsorb for 90 min at 37 °C before inoculum was removed, minimal fresh culture media added and the culture incubated at 35.5 °C for between 12 and 14 days. To minimise culture contamination from samples an antibiotic and antimycotic mix (2.5 µg/mL amphotericin B, 100 µg/mL kanamycin, 100 µg/mL penicillin streptomycin and 50 µg/mL gentamicin) was added to flasks at day 0, 5 and 9. After 12-14 days, samples of culture supernatant and cells were added to AVL buffer for RNA purification and real-time RT-PCR analysis. Cells were detached with trypsin and 2 mL of cells added to fresh sub-confluent (60%) cultures in 25 cm^2 flasks. The addition of antibiotics and antimycotics was ceased after 3 passages. Remaining cells were used to prepare slides for immunofluorescence assay (IFA)

analysis, the method for which is described later in section 2.10.3. Isolations were considered unsuccessful if after 8 passages there were no positive RT-PCR results from extracted material (see section 2.5.1) or positive IFA signals (see section 2.10.3)

2.5 Polymerase Chain Reaction (PCR)

2.5.1 One-step real-time RT-PCR

Real-time RT-PCR was performed in a BioRad Mini Opticon cycler using SuperScript III Platinum One-step qRT-PCR kit (Life Technologies, Paisley, UK) and a modified version of a previously published real-time RT-PCR assay for the dual detection of HTNV and SEOV.[128] The final master mix (20 µL) comprised 10 µL of 2X Reaction Mix, 0.9 µL of PCR-grade water, 1 µL of both a forward (5'-CATGGCWTCHAAGACWGTGGG-3' at 18 µM) and reverse primer (5'-TTKCCCCAGGCAACCAT-3'at 9 µM), 0.3 µL of a single Minor Groove Binder (MGB) -probe with a degenerate single base change (5' FAM-TCAATGGGRATACAACT-3' at 25 µM) in place of the two non-degenerate published MGB-probes, 0.8 µL of SuperScript III RT/Platinum *Taq* Mix, 1 µL of $MgSO_4$ and 5 µL of template. The cycling conditions used were 50 °C for 10 min, 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 40 s, with a final extension step at 40 °C for 30 s. The modification from a dual probe to a single probe arose during this study to reduce cost and reagents for each RT-PCR run. Nuclease free water was used for the negative control and viral RNA extracted from SEOV R22 culture used as positive control. Runs were repeated if either control did not perform as expected.

2.5.2 RT-PCR

RT-PCR amplification was performed using SuperScript III One-Step RT-PCR System with Platinum *Taq* (Life Technologies, Paisley, UK). The final master mix (50 µL) comprised 25 µL of 2X Reaction Mix, 14 µL of PCR-grade water, 2 µL of both a forward and reverse primer (at 10µM working concentration), 2 µL of SuperScript III RT/Platinum *Taq* Mix, and 5µL of template. Cycling conditions were: reverse transcription step was 50 °C for 15 min then initial denaturation was at 94 °C for 2 min followed by 45 cycles of; denaturation for 15 s at 94 °C, annealing for 50 s at 2 °C below the lower Tm, and extension at 68 °C for 1 min per kb. A final extension step at 68 °C was 5 min for products less than 1kbp, or 7 min for longer products. Two published assays are described in Table 2.1: one targeting the S segment[33] and a dual nested assay targeting the L segment.[132] The S segment assay was the preferred method for rodent tissues due to the larger fragment length amplified for sequencing. This assay was unsuccessful at detecting specific RNA in any of the human samples tested due to the weak signal. For all human samples instead the nested assay was the method of choice.

Table 2.1 List of RT-PCR primers used for pan hantavirus screening

Target	Use	Name	Primer sequence	Tm °C	Ref
S	Diagnostic	HAN F	GGC CAG ACA GCA GAT TGG	61	34
	pan-hanta	HAN R	AGC TCA GGA TCC ATG TCA TC	67	
L	Diagnostic	HAN-L-F1	ATG TAY GTB AGT GCW GAT GC	55	133
	1st pan-hanta	HAN-L-R1	AAC CAD TCW GTY CCR TCA TC	58	
L	Diagnostic	HAN-L-F2	TGC WGA TGC HAC IAA RTG GTC	62	133
	nested pan-hanta	HAN-L-R2	GCR TCR TCW GAR TGR TGD GCA A	64	

2.5.3 Electrophoresis of PCR products

Amplified DNA products were visualised by agarose gel electrophoresis in Tris – borate – EDTA (TBE) (100 mM Tris, 90 mM boric acid, 1 mM EDTA) with ethidium bromide (0.5 μg/mL) and then visualised in 302 nm wavelength UV light using G:BOX XT4 (Syngene, Cambridge, UK). Samples were loaded in 10x BlueJuice loading buffer (65% sucrose, 10 mM Tris-HCl, 10 mM EDTA, 0.3% bromophenol blue). Electrophoresis was carried out at 90 V for 45 min. Products were assessed against a quantitative 100 bp or 10 kb DNA ladder (New England BioLabs). Bands of interest were excised with a scalpel and purified using QIAquick Gel Extraction Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. In brief, after excision bands were frozen at – 20 °C for a minimum of 60 min then thawed at room temperature. 3 X band volume of proprietary Buffer QC was added to each sample tube and incubated on a heat block at 50 °C until the agarose dissolved. Sample was added to QIAquick spin column and centrifuged at 9500 x *g* for 1 min. Flow through discarded and 500 μL of Buffer QC added to column, centrifuged as previously and discarded. A second wash step with 750 μL proprietary buffer PE was carried out before centrifugation of the sample column in a clean collection tube to ensure all flow through was discarded. The sample was eluted from the column with 50 μL nuclease free water.

2.6 Sequencing of PCR products

Sequence analysis was performed on viral RNA sourced directly from lung tissue or human sera using the same primers used to produce the amplicons and same cycling conditions as previously described in section 2.5.2 and Table 2.1 Nucleotide

labelling was carried out using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Paisley, UK), unincorporated dye terminator was removed using DyeEx 2.0 Spin Kit (Qiagen, Manchester, UK), and sequencing of products was carried out on a 3130xl sequencer (Life Technologies, Paisley UK) according to manufacturer's instructions. FASTA nucleotide sequences were used as queries in BLASTN searches against the nucleotide collection database using the BLAST tool at http://blast.ncbi.nlm.nih.gov/Blast.cgi.[196] Following multiple nucleotide sequence alignments of available SEOV sequences in GenBank, primers were designed for each segment spanning overlapping intervals of ca. 500 bp as shown in Table 2.2.

2.6.1 Sequence comparison and phylogenetic analysis

The MEGA5 programme suite was used to perform alignments using ClustalW and phylogenetic analysis.[197] Phylogenetic trees were constructed using the neighbour-joining method, with bootstrap values obtained with 2,000 replicates from complete sequences available on GenBank (http://www.ncbi.nlm.nih.gov/genbank/ last accessed November 2014).

Table 2.2 List of PCR primers designed and used for Seoul virus sequencing

Target	Position	Primer sequence (5' – 3')	Tm °C
S	1 +	TAGTAGTAGRCTCCCTAAARAGCTA	53
S	267 -	GATGTTCTTCCCTGCTGCAATC	56
S	374 -	GTCAAGGCTATTCAGGTCCAGTG	57
S	493 -	AGTCTGCCTGCCTCTTGTTGTCA	60
S	507 +	GAGGATCAGATTCAAGGATGACAG	52
S	596 -	GCGTTTGGCATTGACACATACAG	68
S	977 +	CTGATAGGTGTCCACCAACAT	55
S	1073 -	GCCATAATTGTGTTCCTCATATCCTG	55
S	1201 +	ATGGTTGCCTGGGGAAAGG	68
S	1530 +	GCACAATCACTGCCATGTATAATC	54
S	1534 +	CTGCCATGTATAATCACGGG	62
S	1588 -	CCACCCTGTTCCCCATAYG	64
S	1616 +	GGATGTAGATTTCAATTGATCGAG	51
S	1813 -	TAGTAGTAGTATGCTCCTWAA	51
M	1 +	TAGTAGTAGACWCCGCAAGAAC	58
M	222 -	CTCTCTGGCACTAGCTGTTCTGC	59
M	295 -	CTTTCGCCATATGACCTTTGT	54
M	420 -	CTGATCTCCTGTTTCTATATG	47
M	474 +	GATTGTYCCWATWCATGCHTG	58
M	572 -	GTRGTRCARTAWGTYCTYTCRTAR	58
M	1105 +	GCACTYCCYTTRATYTGGAGGG	64
M	1166 -	GGRTGRAYTGCYTCATAGTAWCC	58
M	1537 +	CCTGCHTGYACATTRGCTRT	59
M	1685 -	CARATYTCACAHACCAKGARCC	58
M	2158 -	CWATRTGYARWGAKACACTYTGTTGRTC	59
M	2047 +	ATGCATACRGAYCTKGARTTWG	58
M	2596 +	ATGGAAGGAGGYGGWATWATC	58
M	2672 -	CCTRACATCACCAGGGTCTCC	64
M	3097 +	GAAAGTGDACACTNTCACGRGG	61
M	3232 -	CYARATGTGGDGCACTDGCYTG	62
M	3274 +	ATGATGATGGTGCACCTGAATG	55
M	3406 +	CCTGTTGAGCATTTTGTGTCCTG	56
M	3656 -	TAGTAGTAKRCTCCGCARRATGTC	57

Target	Position	Primer sequence (5' – 3')	Tm °C
L	1 +	TAGTAGTAGACTCCGGAAGAGAC	57
L	232 -	TCCCAGCAAACAGCAATA	53
L	441 -	GAGATATCTCAGACCTCGCTC	53
L	510 +	GTAGTTGCAGTCCGGACAGA	62
L	668 -	CATTGCTTCTAGTGCAGCTC	60
L	1038 +	GCACATGATATGGCACACAGA	64
L	1167 +	ATGATGCGACCTGAGTCAAAG	64
L	1281 -	CTGATTGTACAATATGACTACTGATG	58
L	1381 +	GCTAAGTCAACATGAACCTCGAG	63
L	1956 +	CACTACTTGCAACAGCTACATGG	63
L	2131 -	GATGTCACAGCAGGTATCAGATA	60
L	2745 +	ATGTCAGGCCAAGTACAAGAGG	64
L	2887 -	CTAGCTTGTGCCTTCTGATGTC	62
L	3144 +	GGATACTTATGAACCTCATGTCAGAG	62
L	3387 +	GGGCATCATGGAGAGGTA	61
L	3428 -	GTTACCCTGCAACCAATTG	60
L	3487 +	CTGCATGAGCATATTCTTTTGC	63
L	3678 -	GCAGCTGCAAGATCATCG	63
L	3812 +	CCTTCTTGCTCACTATCTGACCTAC	63
L	3943 -	CTAAGTGAGACTGCAAGTTGTGAG	61
L	4007 +	CTGGTGAGACATTTCAGCAT	60
L	4164 -	GTAATCATATGTAGGATGCTGTTC	58
L	4516 +	GGGTAACATTTAGAGAGGTACTTGC	61
L	4584 -	CTTGTAACCAATAACAGCTGG	58
L	4686 -	GCTTTGTTGGTATCACATCACAG	63
L	5195 +	GTTYCATGCATTATGGTTTGAAG	61
L	5280 -	CTGGATCAATAGGAGGCACAC	63
L	5780 +	GCACAGTATGATGAAGATAGYCCA	61
L	5850 -	GCGAACATCACGGATAGTATG	61
L	6263 +	GGATGAGAGTGCTTATACAGGTG	61
L	6318 +	GGGTGAGTCTGTTGTGGATCA	57
L	6422 -	GATGTTAACTGGGTTGTATACCTTTTC	62
L	6552 -	TAGTAGTAGACTCCGCAAAATG	57

2.7 Virus ultracentrifugation

Purified high-titre virus stocks were generated by culturing virus for 10 days in 175 cm^2 cell culture flasks. Virus was crudely harvested by subjecting the flask to two freeze-thaw cycles at -80 °C to disrupt cells. Flask contents were then centrifuged at 900 x *g* for 10 min to pellet cellular debris. Supernatant was ultracentrifuged through a 20% sucrose cushion for 4 hours at 90, 000 x *g*. Virus pellets were resuspended in ice-cold TNE buffer (0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris) by repeated vortexing.

2.8 Inactivation of hantaviruses by UV irradiation

Virus stock (0.5 mL) was placed in a plastic petri dish (Figure 2.1) and using a Bio-Link BLX crosslinker (5 x 8-watt tubes), exposed to UV irradiation for 5 min at 312 nm, equivalent to 1.4 J/cm^2 as detailed by Kraus *et al*, (2005)[135]. Inactivation was confirmed after 7 days of culture in Vero E6 cells where no detectable virus growth was demonstrated through in-house IFA as described in section 2.10.3.

Figure 2.1 UV irradiation of hantaviruses

2.9 Fluorescent focus assay

To determine virus titres of stocks a fluorescent focus assay was used; in brief, confluent Vero E6 cells grown in 48-well plates were inoculated with 100 µL virus per well in 10-fold dilutions (in PBS 2% FBS) from neat to 1:10,000. After virus adsorption for 90 min at 37 °C the cells were overlaid (1 mL overlay per well) with a 1:1 mixture of 1.2% Avicel in demineralised water and 2X MEM supplemented with 4% FBS. Plates were then incubated at 35.5 °C for 7 days. Virus was inactivated and cells fixed with 1 mL of 8% formaldehyde/PBS per well at 4 °C for a minimum of 60 min. Wells were washed with PBS and residual liquid removed by inverting plates on tissue. Plates were permeabilised (0.5 mL 0.5% Triton-X100 and 20 mM glycine/PBS) for 30 min at room temperature to block residual aldehyde groups, and then washed with PBS. Staining was performed as described for in-house IFA section 2.10.3. For evaluation of the titres, the fluorescent focus units (FFU) of each well were counted and the titres calculated per mL.

2.10 Immunofluorescence assays

2.10.1 Commercial kits for human samples

Sera were screened for presence of hantavirus IgG using a commercially available anti-hantavirus indirect immunofluorescence test mosaic 1 from Euroimmun (Luebeck, Germany) in accordance with the manufacturer's recommendations. Each sample was compared to a known positive (Euroimmun CI 278h-0101-1G) and a negative control (human sera). The mosaic contains 6 biochips which allows for detection of antibodies against the most clinically relevant pathogenic hantavirus species (DOBV, HTNV, PUUV, SAAV, SEOV and SNV). Samples showing repeatable

characteristic cytoplasmic fluorescence at a dilution of 1:100 were considered positive. Where possible samples were titrated further up to 1:40,000. Where available a corresponding saliva sample for each positive serum sample was screened using the same assay and conditions. Slides were viewed at a total 200x magnification (10x eye piece and 20x lens) under blue-light using the manufacture's recommended EUROStar III Plus microscope. Other commercial serological assays including an ELISA (Hantavirus IgG Dx Select; Focus Diagnostics, USA) and a strip immunoassay (RecomLine HantaPlus IgG; Mikrogen, Germany) were briefly evaluated in accordance with manufacturer's instructions using control sera and deemed unsuitable due to and poor sensitivity and reproducibility.

2.10.2 Commercial kit modified for rat sera

Due to the high probability of rat sera containing infectious virus, blood samples were centrifuged inside of an MSC III in a CL3 laboratory and sera heat-inactivated at 60 ^{o}C for 60 min. Serum samples were then tested using the Euroimmun IFA as previously described in section 2.10.1 with the exception of the secondary antibody being anti-rat IgG (whole molecule)-FITC produced in rabbit (F1763).

2.10.3 In-house immunofluorescence slides

Sub-confluent (80%), 75 cm^2 Vero E6 culture flasks were washed with PBS and a minimal volume of trypsin (0.25%) added to disrupt the cell monolayer. Cells were pelleted in DMEM (no FBS) by centrifugation (900 x *g* for 10 min) and resuspended in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The supernatant was discarded. Then 10 µL of cells were spotted onto glass slides and

cells allowed to settle and air dry before fixing with ice-cold 100% acetone for a minimum of 10 min. Negative and infected cells were stained with mouse monoclonal anti-Seoul N-protein antibody (R31- Progen, Heidelberg, Germany) at 1:2 dilution, a secondary FITC-conjugated anti-mouse antibody (F8771) at 1:64 and counterstained with 0.1% Evans blue. Cultures were viewed at a total 200x magnification using EUROStar III Plus microscope. Infected cells were characterised by scattered, granular, punctate fluorescence when compared to negative control Vero E6 cells.

2.11 In-house ELISA

UV inactivated viruses: DOBV, HTNV, PUUV, SEOV, SNV and TULV (as described section 2.8) were used as the antigen for the ELISA. Protein estimation for each virus sample was performed using the NanoDrop 2000c (Thermo Scientific) Sufficient 96-well plates (Nunc MaxiSorp) were coated for 16 hours at 4 °C with 100 µL of inactivated virus diluted ten-fold 1:10 - 1:10,000 in carbonate-bicarbonate buffer (pH 9.6). After coating wells were washed once with PBS 0.05 % Tween20 and blocked for 60 min at 37 °C with BLOTTO Blocking Buffer (Thermo Scientific, Loughborough, UK). All subsequent wash steps were performed four times with 300 µL PBS 0.05 % Tween20 per well and a final wash of only PBS. 100 µL of human sera confirmed positive for anti-SEOV or anti-PUUV IgG antibodies were used as primary antibody at 1:100 and 1:1000 dilution (diluted in BLOTTO buffer) and incubated for 60 min at 37 °C. Human sera confirmed negative for anti-hantavirus antibodies was used a negative control. Wells were washed and 100 µL 1:20,000 anti-human IgG (whole molecule) - peroxidase antibody produced in goat (A8667) used as a

secondary antibody. The plate was incubated for 60 min at 37 °C after which wells were washed and 100 µL ABTS (Invitrogen, Paisley, UK) added and the plate covered in foil and incubated for 30 min at room temperature, followed by addition of 100 µL ABTS Peroxidase Stop Solution (Kirkegaard and Perry Laboratories, USA). Optical density (O.D.) of each well in the plate was measured at a wavelength of 405 nm using an automated ELISA plate reader (Multiskan EX, Thermo Scientific).

2.12 Sero-prevalence studies

2.12.1 Farmers – prospective sero-study

Subjects included in the study were adult volunteers (≥18 years old on day of sampling) who verbally confirmed they fitted the eligibility criteria which was: (1) must reside in North Yorkshire & Humber, (2) their main occupation is working on a farm or their main residence is within a farm, and (3) who had consented to blood donation for the purpose of anonymous screening for the presence of antibodies against hantavirus. Volunteers were provided with study information and a consent form before sample collection. A questionnaire (Appendix I) was completed by face to face interview.

Sample size calculation was based on the most recent and comparable survey where 4.7% of farmers were sero-positive.[143] A minimum sample size of 73 was calculated to be sufficient to estimate the proportion sero-positive, assuming the true prevalence is 5%, with 95% confidence level and 5% precision. As sampling was planned to occur at farmer meetings and markets, the sample size was recalculated to account for clustering assuming an intra-cluster correlation coefficient of 0.1 as

$n1 = n(1 + p(m - 1))$, where n is the estimated sample size assuming simple random sampling, n1 is the new estimate of the required sample size, p is the intra-cluster correlation coefficient and m is the number of clusters (here assumed to be 5). Hence, the revised sample size was approximately 100.

The study was approved by NHS National Research Ethics Service reference 05/Q2008/7.

2.12.2 Military – retrospective sero-study

Volunteers were recruited from either British Army regiments or Royal Marines units prior to deploying to Helmand, Afghanistan. For each deployment, a research nurse visited the unit and presented study information to the troops. Volunteers were required to give their informed consent and to have a single 'pre-deployment' blood sample taken. Upon return from their six month tour of duty, volunteers were visited and asked to give a second 'post-deployment' blood sample. This study had Ethical Approval from both NHS National Research Ethics Service (reference 05/Q2008/7) and MOD Research Ethics Committee.

2.12.3 Renal patients – retrospective sero-study

Samples included in the survey were adults (≥18 years old) admitted to Portsmouth Renal Unit during 2013 who had been clinically diagnosed with either acute kidney injury (AKI) or chronic renal failure (CRF) of undetermined aetiology. Patients were randomly selected and archived samples (stored at -20 °C) aliquoted anonymously

before shipping to PHE, Porton Down for testing. A date and diagnosis of AKI or CRF was provided with each sample.

Sample size calculation was based on the most comparable survey where 2.1% of renal patients in Northern Ireland were sero-positive.[144] A minimum sample size of 207 was calculated to be sufficient to estimate the proportion sero-positive, assuming the true prevalence is 2.1%, with 95% confidence level and 2% precision.

2.13 Human sera and saliva sampling

Blood was collected by venepuncture (8 mL) and serum (2 - 4 mL) separated from the blood using standard centrifugation.[198] Saliva was collected using the Salivette system (Sarstedt Ltd., Leicester, UK) with saliva separated as described by Lamey and Nolan (1994).[199] Serum and saliva samples were stored frozen at −80 °C until tested.

2.14 Data Processing and Statistical Analysis

Statistical analyses were performed with the software program Minitab version 16 (Minitab Inc., State College, PA, USA) and the R language for statistical computing (http://www.R-project.org). Fisher's exact test was used to compare proportions when sample size was low and expected values ≤5. Significance was set at $p < 0.05$; 95% confidence intervals for proportions were calculated using the Wilson Method.[200]

CHAPTER 3

Characterisation of a UK hantavirus isolate and confirmation of a human pathogen

3.1 Introduction

In humans, the timeframe to detect viral nucleic acid in blood can be relatively short for certain infections including those caused by hantaviruses and detection by this route has rarely been successful as a diagnostic tool. In contrast carrier rodents are believed to be persistently infected and testing of their tissues offer the greatest opportunity for molecular characterisation of the infecting hantavirus species and strain. Taking this into consideration the study aims were to investigate rodents linked to recent human cases of hantavirus infection on the UK to determine and characterise the likely causative agent of infection.

In addition to the acute human cases and serological evidence discussed in Chapter 1, immediately prior to the commencement of this study, two acute human cases were diagnosed, one in Yorkshire and one in London with no recent travel history (pers. comm. Dr. Tim Brooks, PHE Porton). One of the cases showed strongest reactivity with SNV and the other with SEOV. Upon epidemiological questioning the latter case from Yorkshire disclosed recent exposure to wild rats at his residential dwelling.

3.2 Hantavirus in Yorkshire – exposure to wild rats

3.2.1 Detection of acute human infection

In December 2011, a 59 year old man presented to his local Accident and Emergency Department with a history of fever, chills and a cough. The patient was a resident on a small livestock farm in North Yorkshire and Humber where he occasionally assisted with farm work, however his main occupation was office-based. The patient disclosed regular exposure to rats on his home premises, where the rat population had increased in recent months. Two weeks prior to his visit to A&E, he had been prescribed flucloxacillin by his General Practitioner (GP) to treat a cut on his wrist which he acquired from falling on a metal pig ark at the farm.

Following admission to the hospital's Infectious Disease ward with suspected leptospirosis he was treated with intravenous benzylpenicillin and oral ciprofloxacin. Blood tests revealed deranged liver enzymes, lymphopenia, and thrombocytopenia and indicated AKI with creatinine peaking on day four, following admission, at 378 µmol/L (normal range 50-110 µmol/L). Physical examination demonstrated increasing lower abdominal pain. Tests for *Legionella, Leptospira* and hepatitis virus A, B, C and E were negative. Two serum samples submitted to RIPL at PHE Porton confirmed acute hantavirus infection with rising IgG titres across the majority of hantavirus species on the IFA (Figure 3.1). The highest titres were seen for SEOV and HTNV (Table 3.1) at 1:40, 000 and 1:20, 000 respectively. This was considered to be the second documented case of hantavirus infection in Yorkshire linked to rats. A full recovery was made and after two months all of the patient's haematological and serum biochemical measurements had normalised.

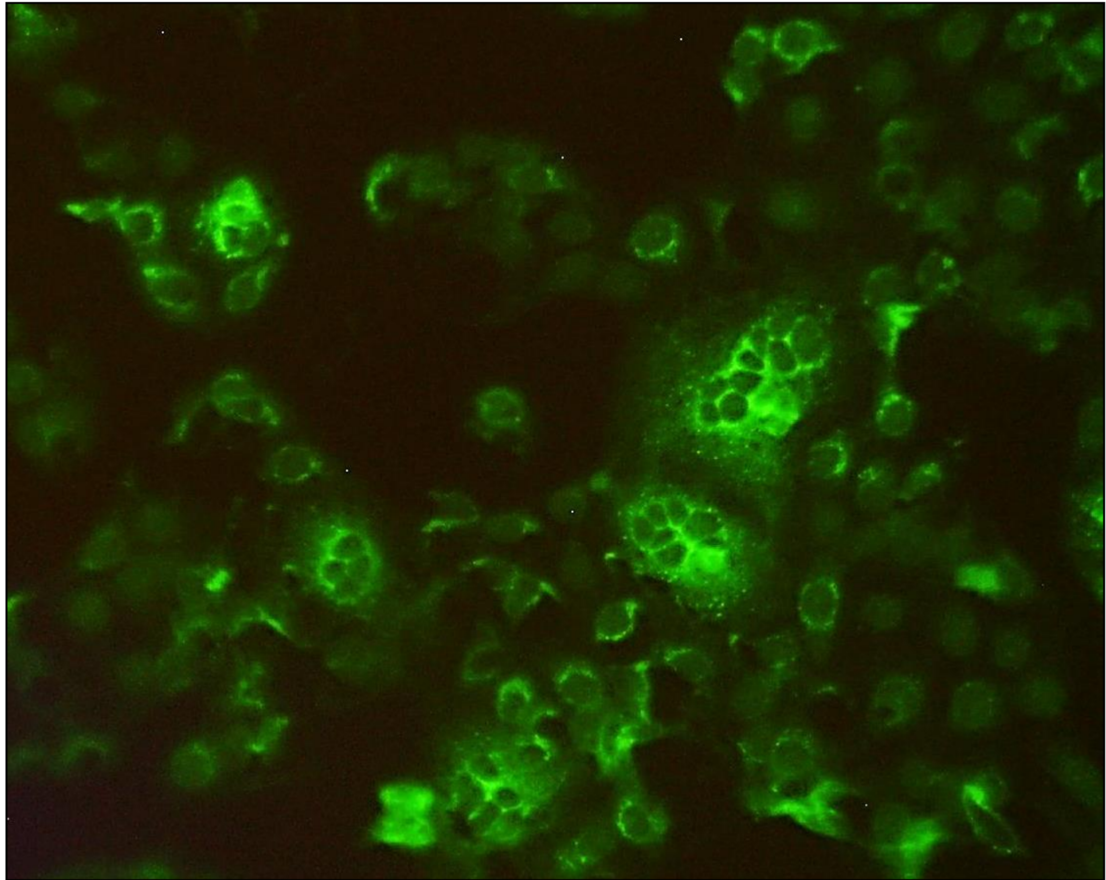


Figure 3.1 Typical fluorescence pattern seen with presence of anti-SEOV IgG antibodies using Euroimmun mosaic 1 slides viewed at total 200x magnification using EUROStar III plus microscope

Table 3.1 Results of patient's hantavirus serology

Hantavirus	**06/01/12**	**18/02/12**
DOBV	1:1000	1:3200
HTNV	1:10, 000	1:20, 000
PUUV	1:1000	1:1000
SAAV	1:1000	1:3200
SEOV	1:10, 000	1: 40, 000
SNV	1:100	1:1000

3.2.2 *Wild rat sampling*

In February 2012, after obtaining permission, trapping of small mammals was commenced on the patient's farm. Eleven rodents in total were dissected and their organs tested: five *Apodemus sylvaticus* (wood mouse), four *R. norvegicus* and two *My. glareolus*. Extracted RNA from each rodent lung was subjected to a genus-specific RT-PCR assay targeting the S segment. Two *R. norvegicus* (RN1 and RN4) samples produced amplicons of the expected size of ca. 850 bp (Figure 3.2). RT-PCR of extracted nucleic acid from organs of the positive rats, found detectable RNA in the heart, lung, lymph node and salivary gland tissue of both rats, and the brain, bladder, kidney, liver, and spleen of one rat. No RNA was detected in the pancreatic tissue of either rat (Figure 3.3). The cDNA from both amplicons was sequenced and showed the highest level of similarity (97%) to Seoul hantavirus IR461 (GenBank accession no. AF329388) in a standard nucleotide BLAST analysis (NCBI/Blast http://blast.ncbi.nlm.nih.gov/Blast.cgi last accessed November 2014). Complete sequencing of all three segments directly from RN1 and RN4 lung tissue confirmed a SEOV. Detailed phylogenetic analysis is discussed in section 3.4.

3.2.3 *Family follow up*

Other family members and one farm worker were invited to be screened for exposure to hantaviruses. Seven in total volunteered: 4 females and 3 males. No hantavirus reactive IgG antibodies were detected in any of the serum samples using the standard IFA.

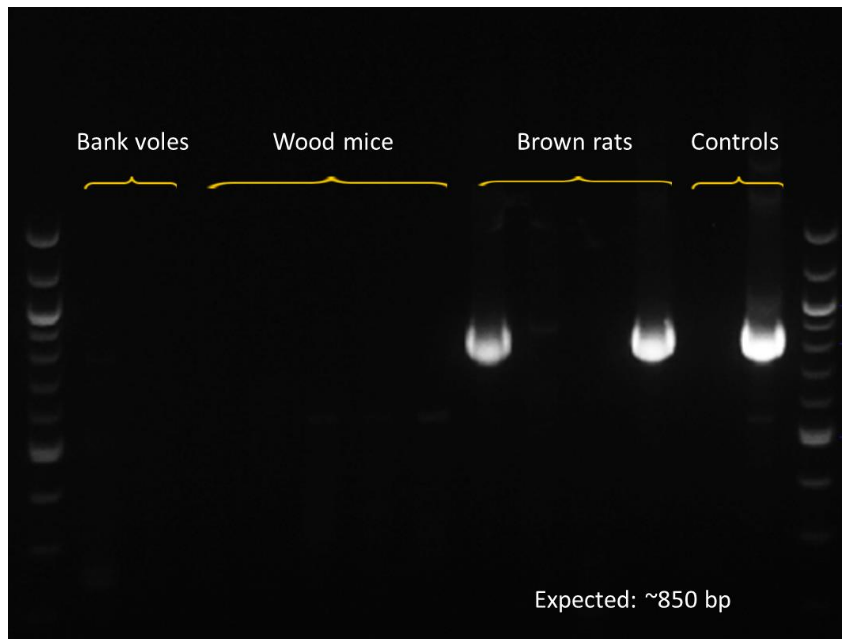


Figure 3.2 RT-PCR results of RNA extracted fromwild mammal lung tissue from Yorkshire farm

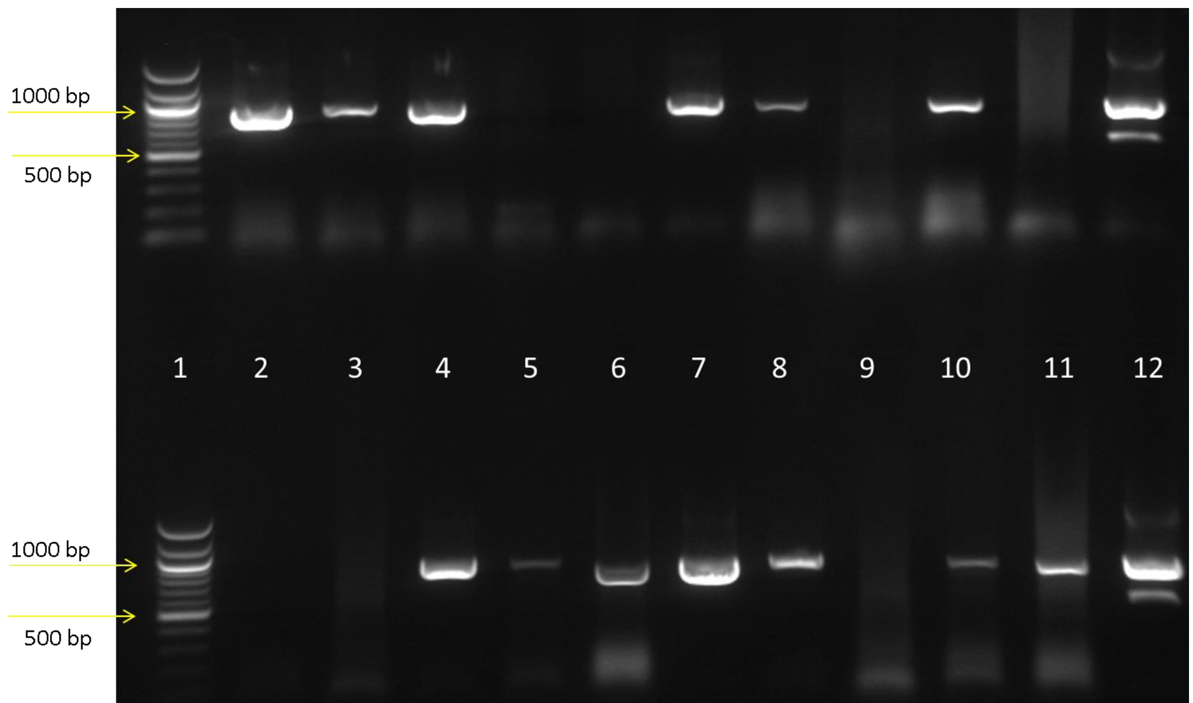


Figure 3.3 RT-PCR results of RNA extracted from RN1 (top) and RN4 (bottom) individual organs. (1) DNA ladder, (2) brain, (3) bladder, (4) heart, (5) kidney, (6) liver, (7) lung, (8) lymph node, (9) pancreas, (10) salivary glands, (11) Spleen, (12) R22 positive control

3.3 Investigation in Oxfordshire – exposure to pet rats

3.3.1 Detection of acute human infection

In November 2012, a 28 year old man was admitted to hospital in North Wales with a four day history of fever, chills, diaphoresis and vomiting. Laboratory investigation revealed thrombocytopenia, deranged liver enzymes, AKI and hypoxia. His condition worsened and he required aggressive fluid replacement, 38 days of ventilatory support and 21 days of renal replacement therapy within the intensive care unit. A stored serum sample taken one month before admission (for an unrelated health event), and a sample taken whilst in the ICU confirmed a sero-conversion to hantaviruses, specifically SEOV and HTNV (Table 3.2). After 52 days in hospital the patient made a full recovery and was discharged from hospital.

Table 3.2 Results of patient's hantavirus serology

Hantavirus	Oct 2013	Dec 2013
DOBV	Neg	1:1000
HTNV	Neg	1:10, 000
PUUV	Neg	1:100
SAAV	Neg	1:1000
SEOV	Neg	1: 10, 000
SNV	Neg	1:100

Epidemiological investigation identified pet agouti rats (*R. norvegicus*) kept by the patient's partner as a probable source of infection. As the patient was unwell soon after his admission, the pet rats were returned to the care of the original breeder in Oxfordshire, south England.

3.3.2 Pet rat sampling

The two rats linked to the index patient were housed in a separate building to that of the breeder's pet rat colony. A blood sample was taken by a veterinarian from the tail vein of the animals and a urine sample collected from one. All three samples were positive for hantavirus via real-time RT-PCR using a specific SEOV/HTNV dual MGB probe. The decision was made to euthanize the two rats with the permission of the owner and take blood samples from the breeder's larger rat colony from which the two rats originated.

Real-time RT-PCR and serology results, where available, are detailed in Table 3.3 In brief, 7 additional rats were found to be RT-PCR positive and 21/21 rats tested demonstrated IgG antibodies against SEOV and HTNV; 2 rats were unable to be tested. Dissection and RT-PCR analysis of individual organs from rat 001 demonstrated extensive infection (Figure 3.4): Bladder, heart, kidney, liver, lung and lymph nodes had detectable RNA. Viral RNA sourced directly from lung tissue was subjected to additional characterisation and sequencing of all three genomic segments completed. Detailed phylogenetic analysis is discussed in section 3.4.

Table 3.3 Molecular and serological results of pet rats tested from Oxfordshire

ID	Sex	RT-PCR (Ct)	Serology
001	M	32.19	Pos
002	M	31.25	Pos
003	M	Neg	Pos
004	M	Neg	Pos
005	M	Neg	Pos
006	M	30.58	Pos
007	M	Neg	Pos
008	M	Neg	Pos
009	F	35.14	Pos
010	F	Neg	Pos
011	F	33.09	Pos
012	F	Neg	Pos
013	F	34.36	Pos
014	F	Neg	Pos
015	F	38.57	Pos
016	F	38.25	Pos
017	F	Neg	Pos
018	F	Neg	Pos
019	F	Not tested	
020	F	Neg	Pos
021	F	Neg	Pos
022	F	Neg	Pos
023	F	Neg	Pos
024	F	36.56	Pos
025	M	Not tested	

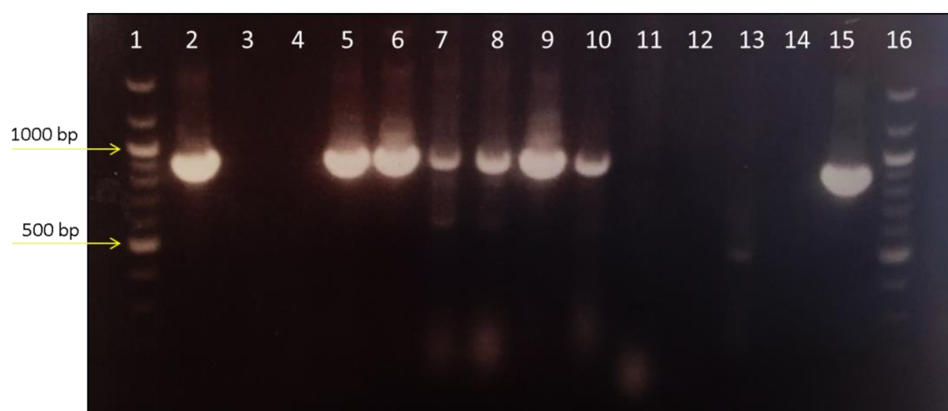


Figure 3.4 RT-PCR results of RNA extracted from pet rat 001 individual organs. (1) DNA ladder, (2) PUUV positive control, (3) negative control, (4) brain, (5) bladder, (6) heart, (7) kidney, (8) liver, (9) lung, (10) lymph node, (11) pancreas, (12) salivary glands, (13) spleen, (14) negative control, (15) DNA ladder and (16) PUUV positive control

3.3.3 *Family follow up*

Following routine epidemiological questioning of the patient it became apparent that the partner of the Oxfordshire breeder may have suffered from undiagnosed HFRS. In November 2011, one year prior to the previous case, he was hospitalised with fever, thrombocytopenia, splenomegaly and AKI. At the time he was diagnosed with a virus of unknown aetiology. An archived serum sample from his admission was compared to a sample taken in January 2013 and confirmed a hantavirus infection with anti-SEOV IgG titre of 1:1000 rising to 1:10,000. The breeder was also tested in January 2013 and found to have a low IgG titre of 1:100 against SEOV. The partner of the index case had no detectable antibodies against hantaviruses.

3.4 Genomic analysis of UK SEOV strains

Sequence data was obtained from RNA extracted directly from rat lung tissue. The exception being for isolate IR461; viral RNA was extracted from cell culture stocks banked in 1984. As is common practice for hantaviruses the strains were name after the location from which they were collected, Humber for the Yorkshire samples and Cherwell for the Oxfordshire sample. All sequences were submitted to GenBank and accession numbers as detailed in Table 3.4.

Table 3.4 GenBank accession numbers for UK SEOV strains. *previously available via GenBank[161]

	Humber RN1	**Humber RN4**	**Cherwell**	**IR461**
S	JX879769	KM948598	KC626089	AF329388*
M	JX879768	KM948597	KM948593	AF458104*
L	JX879770	KM948596	KM948594	KM948595

3.4.1 *Comparison of S segment sequences*

The complete S segments of RN1, RN4 (Yorkshire) and 001 (Oxford) were determined to be 1768 and 1772 nucleotides (nt) compared with IR461 in between with a length of 1769 nt. Each contains a single ORF of 1289 nt (nt 43 – 1332) encoding the N protein of 429 aa in length. As expected, the ORFs of all of the sequences were found to have a high degree of nucleotide and amino acid homology (range from 35 to 38 nt and 1 to 7 aa differences):

- 97.29% nt and 99.77% aa between Yorkshire and Oxford,
- 97.05% nt and 98.6% aa between Yorkshire and IR461,
- 97.21% nt and 98.37% aa between Oxford and IR461.

3.4.2 *Comparison of M segment sequences*

The M segments of all three samples and IR461 were found to be 3651 nt in length. All contain a single ORF of 3401 nt (nt 47 – 3448) encoding the GPC protein of 1133 aa. Although a high degree of homology still occurs a greater degree of diversity at the nucleotide level can be seen between the ORFs as compared to that between the S segments (range from 120 to 161 nt and 9 to 23 aa differences):

- 96.47% nt and 99.2% aa between Yorkshire and Oxford,
- 95.85% nt and 97.97% aa between Yorkshire and IR461,
- 95.27% nt and 98.06% aa between Oxford and IR461.

3.4.3 *Comparison of L segment sequences*

The L segments of the Yorkshire samples were found to be 6530 nt in length and the Oxfordshire sample 6531 nt. L segment data for IR461 was not available

therefore complete sequencing was undertaken in order to have this for comparison. It too was found to be 6530 nt in length. All contain a single ORF of 6455 nt (nt 37 – 6492) encoding the polymerase protein of 2151 aa. Sequence homology between the isolate ORFs was comparable to that found for the M segments (range from 246 to 278 nt and 19 to 22 aa differences):

- 96.19% nt and 99.12% aa between Yorkshire and Oxford,
- 95.99% nt and 98.98% aa between Yorkshire and IR461,
- 95.69% nt and 99.02% aa between Oxford and IR461.

3.5 Phylogenetic analysis

Complete nucleotide sequences for each segment were aligned using ClustalW and the molecular evolutionary genetics analysis (MEGA5) programme suite was used to construct neighbourhood joining trees. The Bootstrap method with 2000 replicates was chosen for the test of phylogeny with maximum composite likelihood selected for the substitution model with uniform rates. Trees were visually rooted with the most divergent reference strain TPMV.[201]

For all trees, the UK SEOV strains form a distinct cluster (Figures 3.5a, b, c). IR461 is suggested as being the ancestral strain for segments M and L. The Chinese strain Gou3 forms the basal clade of the S segment tree; complete sequence data of this strain is not available for M and L segment analyses. The lack of complete sequence data available for M and L segments limits further analysis.

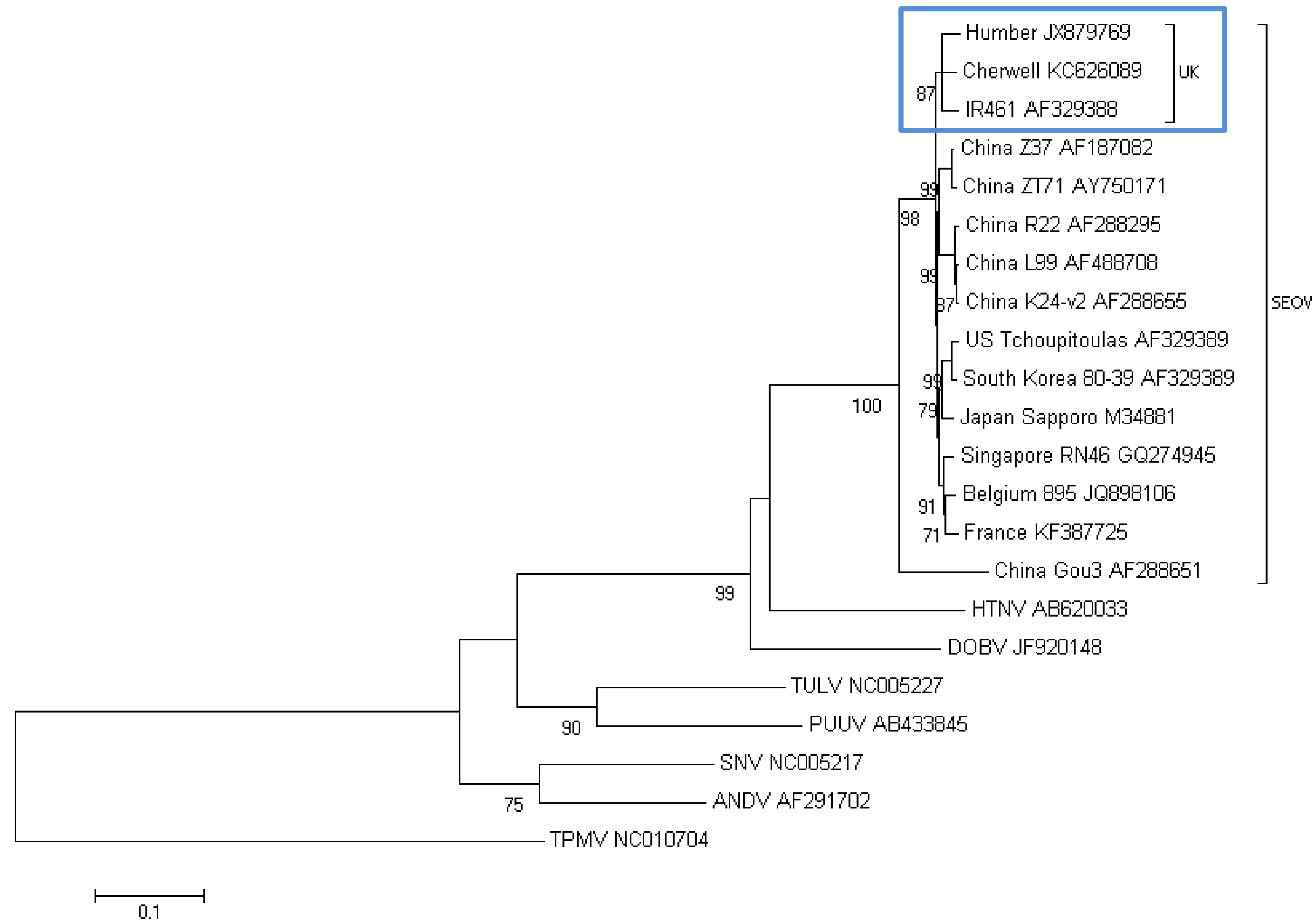


Figure 3.5a. Phylogenetic analysis of complete S segment sequences

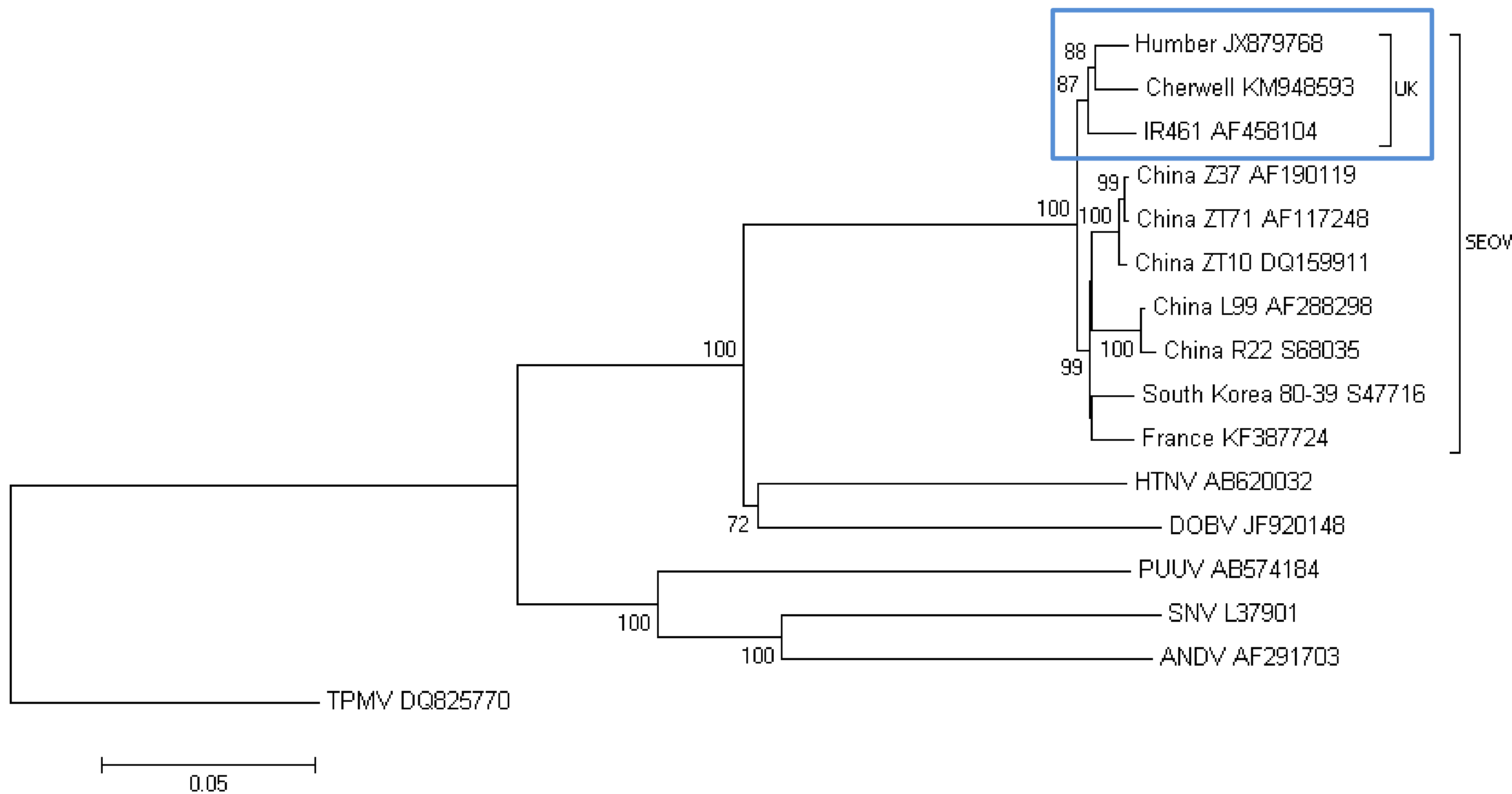


Figure 3.5b Phylogenetic analysis of complete M segment sequences

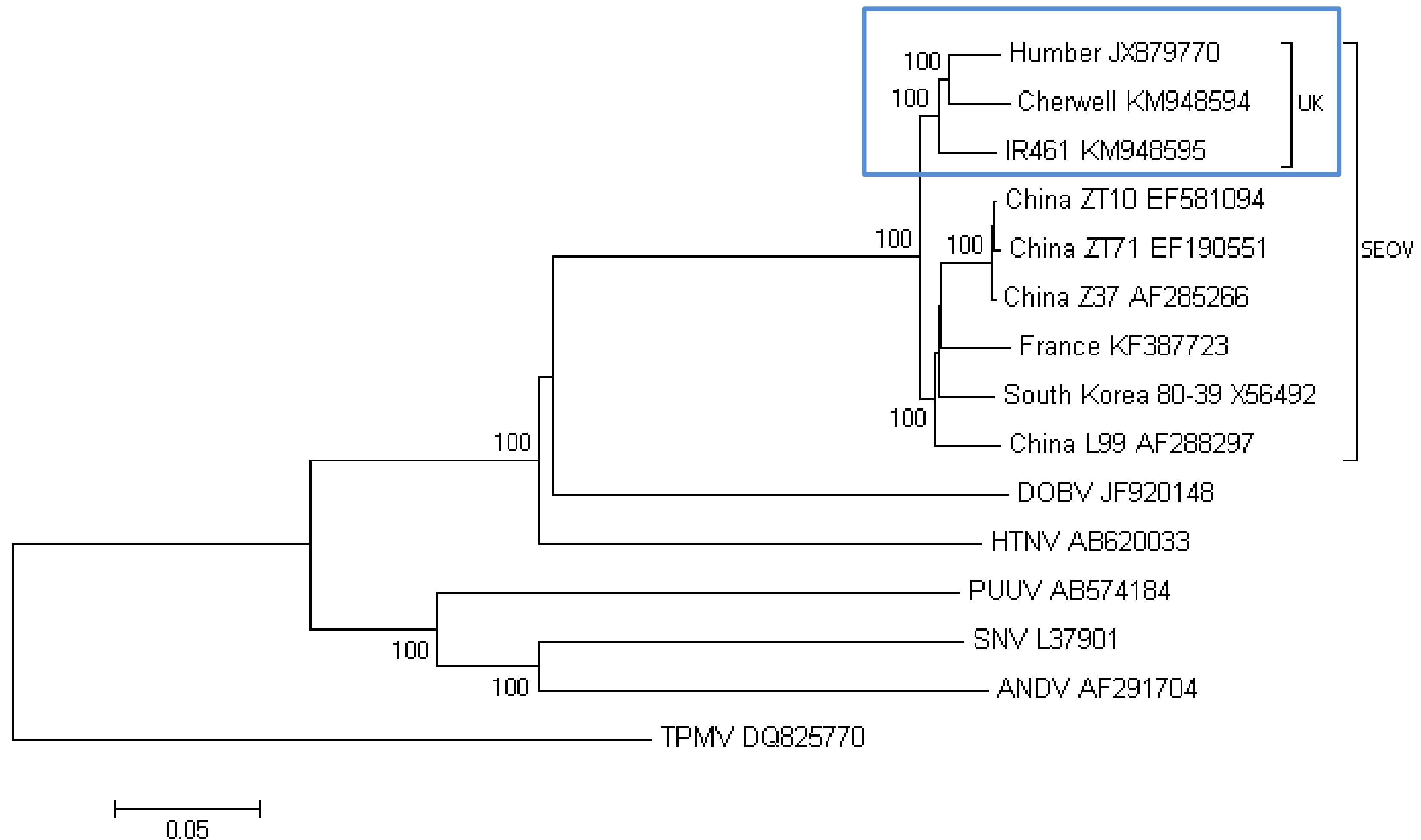


Figure 3.5c. Phylogenetic analysis of complete L segment sequences

3.6 Molecular detection of hantaviral RNA in a human sample

In August 2013, an 11 year old female presented to A&E in Gloucester with a headache, malaise, fever and anorexia. Laboratory investigation showed haematuria, abnormal LFTs and thrombocytopenia. After learning that pet rats were kept in the property where the child was resident, hantavirus serology was requested. A serum sample taken four days after onset tested positive for hantavirus antibodies: 1:3200 against SEOV and HTNV and 1:320 against DOBV and SAAV. Crucially the serum sample was also positive by real-time RT-PCR for SEOV and HTNV virus, using an MGB dual probe (CT 37). Further rounds of RT-PCR and nested PCR of the L segment confirmed SEOV with 100% homology to the Cherwell SEOV strain previously described from pet rats.

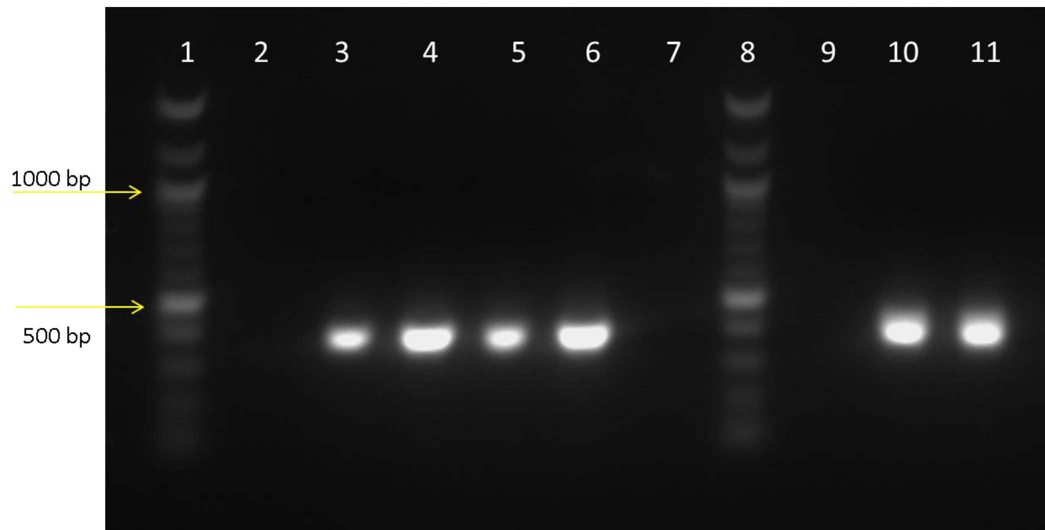


Figure 3.6 Nested RT-PCR results of RNA extracted from patient sample. (1) DNA ladder, (2) negative control, (3-6) replicate patient samples, (7) empty lane, (8) DNA ladder, (9) empty lane, (10-11) replicate SEOV positive control

3.7 Discussion

Prior to this study hantaviruses were strongly suspected of causing human disease in the UK but yet to be confirmed with only serological data available prior to the research described herein. The data presented in this chapter firmly demonstrate the existence of a pathogenic SEOV hantavirus in the UK wild and pet rat population. Three acute human cases resulting in serious illness were recorded and characterised, and follow-up investigation led to the discovery of two new strains of SEOV; Humber and Cherwell. A fourth acute case was diagnosed retrospectively. Importantly none of the patients had recently travelled outside of the UK.

Noteworthy are the typical symptoms and laboratory markers reported from these four cases: all suffered from fever, deranged liver enzymes and thrombocytopenia; 3/4 also developed AKI. Whilst these markers are likely to be skewed towards detection of severe cases they could be used as a preliminary case definition to identify patients for hantavirus testing. Early diagnosis is important for improving patient outcome; in countries where clinical awareness is high, the need for dialysis following hantavirus-induced AKI has fallen to less than 5%.[202] Correct and prompt diagnosis has also been shown to reduce inappropriate antibiotic treatment in Europe.[203]

The vast majority of hantavirus sequences are from rodent material, with those obtained from human cases extremely rare. This is largely due to patient samples being taken too late in the course of infection, but also due to low levels of viral RNA in blood in humans.[204] In this study for the first time, hantavirus genetic

material has been directly detected in a specimen from an acutely ill human patient in the UK with classic HFRS manifestations and confirmed exposure to carrier rodents. Since the material isolated from the patient showed 100% homology to the Cherwell strain characterised from pet rats we can be confident that this strain of SEOV is pathogenic to humans.

Additionally, whilst SEOV was detected in wild rats, an interesting finding was the role of pet rats in hantavirus transmission. Pet rodents are a known risk for transmission of zoonotic pathogens [205,206] but this is the first time they have been reported as carriers of a hantavirus. Biologically domestic rats are the same as their wild counterparts therefore it is not unexpected they have the capability to transmit SEOV. From a public health perspective however this is an important finding as they are likely to present a greater risk for transmission to their owners due to the increased contact time and exposure to potentially infectious excrement in confined spaces. Even with strict hygiene measures implemented, the risk is unlikely to be eliminated from infected pet animals due to the aerosolisable nature of the virus. This is corroborated by the fact that 7/7 contacts with the Yorkshire case related to wild rats tested sero-negative whereas 2/3 contacts with the Oxford case linked to pet rats were sero-positive, one of whom also was clinically ill with typical HFRS disease.

Remarkably most SEOV variants identified thus far are genetically homogeneous and closely related to each other with up to 95% nucleotide sequence identity.[201] Analysis of other hantavirus species usually provides clear evidence for origin with

geographical clustering as seen for PUUV.[207-209] Typically SEOV does not follow such a pattern with clusters made up of strains isolated from Korea, Japan and USA. The exception here appears to be the UK strains. Phylogenetic analysis showed that the UK SEOV strains form a distinct cluster consisting of the three strains from the laboratory (IR461), wild (Humber) and pet (Cherwell) rats. The finding that all three form such a distinct cluster reduces the likelihood of their having been separately introduced into the UK.

Comparison of the nucleotide sequences demonstrates little difference between the strains. Humber and Cherwell appear to be more closely related and most likely to have evolved from IR461 or a common ancestor yet to be characterised. It should be emphasised that, since 1995, no work had been undertaken at PHE Porton with SEOV strain IR461. Sequencing of the Yorkshire strain was completed before IR461 was retrieved from the archive freezer. None of the sequences matched any positive control virus strains handled in the laboratory therefore we can be confident no contamination occurred.

For hantaviruses the L segment is typically the most conserved, followed by the M segment and then the S segment.[210] Interestingly the homology between Humber and Cherwell follow the opposite pattern with greater nucleotide and amino acid changes seen in the S and M and then the L segment. Sequencing of both rats from Humber (RN1 and RN4) was undertaken to investigate any differences between individual rats. Minimal divergence was found between the two with only 6

nucleotide and no amino acid changes found across all three segments (1 S, 1 M and 4 for L- segment).

As this study was focused on detection of hantaviruses in humans and rodents and not specific dynamics within hosts quantitative, real-time RT-PCR was not employed. None-the-less, comparisons can be drawn; when detected, viral RNA appears to be highest within lung, heart, bladder and brain tissue. Specific RNA was not consistently detected in all rat tissues. Virus shedding is known to fluctuate due to viral replication cycles within rodent hosts.[60] Indeed, when investigating the rat colony from Oxfordshire all rats tested were found to have a high titre of hantavirus reactive antibodies; however, only nine (including two rats from the premises of the index case) had detectable viral RNA on the day of sampling. For future testing of rats, heart and lung tissue appears to be the best sample types for consistently detecting high levels of hantavirus RNA.

CHAPTER 4

Sero-prevalence of hantavirus in potential high risk populations

4.1 Introduction

Numerous sero-epidemiological studies have shown occupations that include human-rodent contact are associated with a higher risk of hantavirus infection.[1, 7, 74, 205,211-213] In comparison to control groups a significantly higher number of sero-conversions have been demonstrated amongst forestry workers, hunters, farmers and military personnel.

During the winter months when *My. glareolus* seek shelter in buildings, people have an increased risk of exposure and infection with PUUV as they come into contact with materials contaminated with rodent excreta in confined spaces.[212] Severe disease is more frequently detected in persons between the ages 20-50 years old with clinical cases in children rare.[1] Hantavirus disease has been reported in both sexes; however, there is a significantly higher prevalence in males. This skew in prevalence for both gender and age is thought to be mainly a result of increased occupational exposure. Unsurprisingly, given the common sequelae associated with HFRS, higher sero-conversion rates have been shown in both chronic renal failure patients and those suffering from AKI compared to the general population.[214, 215]

With SEOV a confirmed cause of HFRS in the UK, further investigations were warranted to determine the extent of human exposure to this virus. The main focus was in the county of Yorkshire due to the detection of two recent severe acute cases there. A sero-epidemiology survey was designed specifically for a risk group in this region; farmers.

In respect of the above findings, a serological survey of renal patients in the Yorkshire region would also have been an ideal study to undertake within the duration of this PhD. However, due to time constraints for ethical approval and a similar national survey of renal units being planned following publication of the virological findings, this was not possible. Instead an archive of serum samples was made available from acute and chronic renal patients who had been admitted to Portsmouth hospital. This group of patents provided a reasonable comparison to be made between Hull with Portsmouth both being cities having an active port. It also provided a snapshot of potential hantavirus seroprevalence in a risk group from another UK location and add to knowledge on whether SEOV was restricted to one geographical location within the UK.

The final risk group identified, and for which samples were readily available, was active British military personnel. The main purpose for inclusion of this group was to build a pool of seropositive samples for assay validation at a later stage.

Populations tested were:

1. Farmers and rural residents in North Yorkshire and Humber
2. British military personnel
3. Patients with confirmed AKI or CRF in Portsmouth

Screening for hantavirus-specific antibodies was undertaken using a commercially available immunofluorescence assay that enabled the simultaneous detection of DOB, HTN, PUU, SAA SEO, and SN virus nucleocapsid IgG in human sera. The IFA technique has been used extensively for the sero-diagnosis of hantavirus infections, screening of animals and selected human populations, and is still the most widely used assay. Besides the detection of specific IgG antibodies in serum, IFA can also be used to provide an indication for the hantavirus subtype involved by comparing serum antibody titres against the respective hantavirus antigens.

In addition, for one of the study populations (Yorkshire farmers) saliva was investigated as a suitable sample medium for future sero-prevalence studies. The use of saliva is an increasing area of research for diagnostic sampling as it is easy to collect, non-invasive to the patient and readily available, with healthy adults producing between 500-1500 mL of saliva per day.[216] Whilst the predominant immunoglobulin isotype in saliva is IgA, IgG is known to be biologically active within the oral cavity; it is mainly derived from gingival cervicular fluid and mucosal transudate.[216,217] A correlation between saliva IgG and serum IgG has been demonstrated for many viruses including; human immunodeficiency virus (HIV), hepatitis A virus (HAV), hepatitis C virus, Epstein Barr virus (EBV), cytomegalovirus

(CMV) and rubella virus.[217] No published survey has researched the use of saliva collection for detection of IgG levels specific to hantaviruses.

4.2 Sero-prevalence in Yorkshire farmers and rural dwellers

4.2.1 Study objectives

- To estimate the sero-prevalence of hantavirus in adults aged 18 years or above, who live or work on a farm in North Yorkshire and Humber.
- Determine whether sero-prevalence varies with age, gender, farm type or risk activity.
- Investigate whether hantavirus specific IgG is detectable in saliva samples and comparable to levels of IgG detected in serum.

4.2.2 Study population

North Yorkshire and the Humber is located in the region of Yorkshire, North East England 53°57′30″N, 1°4′49″W (York). The distribution of farm types varies across the county: livestock is predominant in the north-west, cereal and general cropping in the east/south-east, with the north, north-east and central areas generally more fragmented with a mixture of farm types. It has a population of approximately 1, 700,000 within which there is generally an even gender ratio, with the exception of those aged 80+ years where the female: male ratio is ~1.8:1. North Yorkshire has a high proportion (24%) of its population over the age of 65 years. The study area incorporated the following locations: Craven, East Riding of Yorkshire, Hambleton, Harrogate, Hull, North East Lincolnshire, North Lincolnshire, North Yorkshire, Richmondshire, Ryedale, Scarborough, Selby and York (Figure 4.1).

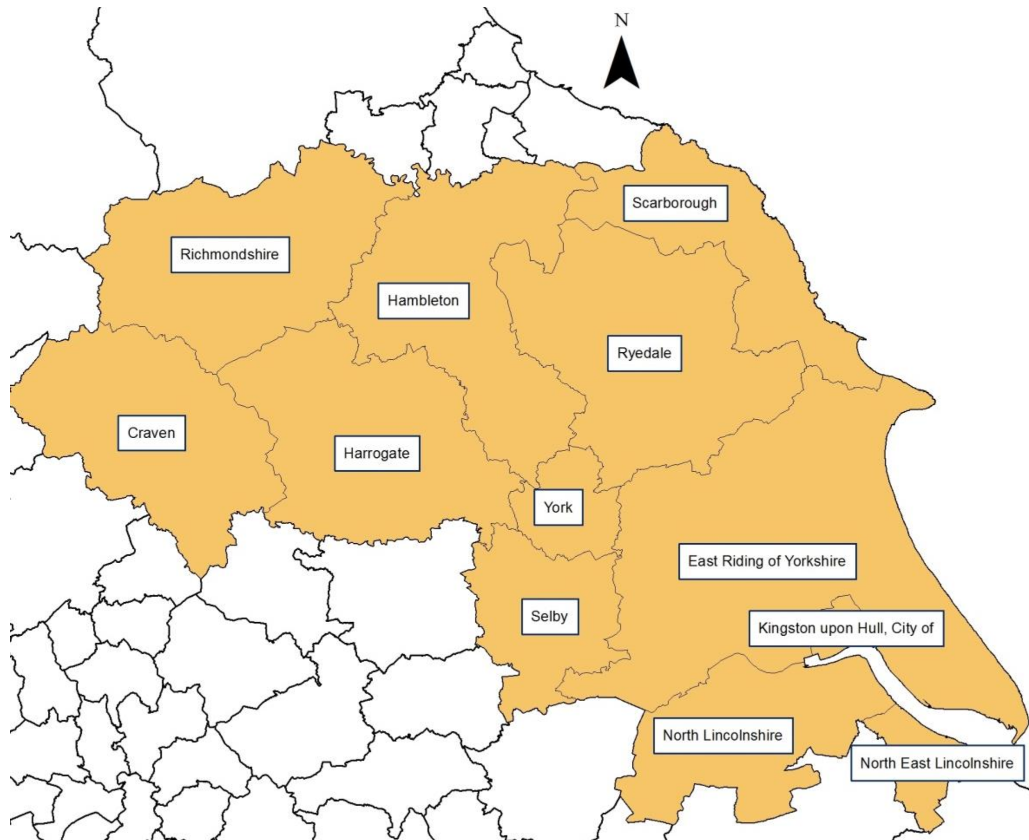


Figure 4.1 Target areas of North Yorkshire and Humber for inclusion

4.2.3 Serological results

128 volunteers were recruited, with nine excluded from analysis because of ineligibility. Of the 119 eligible volunteers sampled and tested, nine (7.6%; 95% CI 4.0 - 13.8%) had hantavirus specific IgG antibodies detectable at 1:100 dilution. 7/9 positive sera were considered specific to HTNV and/or SEOV and 2/9 positive sera were specific to SNV and/or PUUV.

112/119 disclosed some form of travel outside of the UK in their lifetime; crucially the two volunteers whose serum showed cross-reactivity with SNV/PUUV had not travelled outside of Europe. While PUUV has been extensively reported as a

pathogenic hantavirus in Europe SNV has not, however PUUV and SNV are known to serologically cross-react using IFA techniques. The majority of volunteers (98/119) disclosed their main or full-time occupation to be farming; the remaining volunteers (21/119) merely reside on a farm.

4.2.4 Saliva sampling

Collection of a corresponding saliva sample was achieved from 117/119 eligible volunteers. No other alterations were made to the assay other than sample type. From individuals demonstrating a positive serum sample 8/8 of their saliva samples were also found to have characteristic positive fluorescence. A subset of saliva samples (13) for which their corresponding serum sample was negative were also tested to ensure the 8/8 positive saliva samples were indeed true positives and not a reaction with standard saliva. None of these 13 saliva sample demonstrated characteristic fluorescence. One saliva sample was unable to be collected from a volunteer with positive serum and therefore was not available for testing. A slight decrease in fluorescence in comparison to serum samples was noted for all samples. In addition specificity for individual hantavirus species was reduced with 8/8 showing reactivity for HTNV, SEOV and SNV.

4.2.5 Epidemiological analyses

Table 4.1 details analysis of individual risk variables investigated from the compiled questionnaire results. No significant association could be drawn between any measured demographic or farm specific variable and sero-status. Analysis was limited due to the low number of positive samples and those taken from women, in

conjunction with the study population incorporating a convenience rather than random sampling; hence results may be both imprecise and subject to bias. Therefore care should be drawn when interpreting the results.

All sero-positive individuals reported rodents on their property with 9/9 reporting rats and 6/9 mice. Differences in rodent contact may have identified a statistical significance protective variable ($p > 0.001$, OR 0.006) that being reporting the presence of rodent during the day. Only 2/9 positive individuals noticed rodents during the day compared to 9/9 seeing rodents at night. Further investigation of which rodent species are seen highlighted that reporting mice on the farm was also associated with decreased risk ($p= 0.02$, OR 0.1, 95% CI 0.02 to 0.96). In the absence of an extremely large population, rats are rarely seen during the day whereas mice are less constrained by such population dynamics. Rats predate on mice therefore mice are naturally averse to their presence.[218] Where you find mice you are less likely to find rats. This in turn may decrease the potential for exposure of humans to SEOV. This is further supported by volunteers who reported seeing mice on their property being less likely to be sero-positive.

Category	No. positive (%)	No. Negative	No. tested	Odds ratio	Confidence interval	P- value*
Age (years)						
< 30	3 (14.3)	18	21	2.5	0.4 to 13.2	0.2
≥ 30	6 (6.1)	92	98			
Median	45	51				
Gender						
Male	7 (7.0)	93	100			
Female	2 (10.5)	17	19	1.6	0.1 to 9.2	0.6
Farmer						
Yes	8 (8.2)	90	98	1.8	0.2 to 82.7	>0.99
No	1 (4.8)	20	21			
Farm type						
Animal	4 (11.4)	31	35			0.7
Arable	1 (7.1)	13	14	1.7	0.1 to 88.8	
Mixed	4 (5.7)	66	70	1.9	0.3 to 11	
Farm classification						
Cereal	5 (6.3)	75	80	0.7	0.1 to 3.9	0.7
Cropping	3 (7.5)	37	40	1.1	0.2 to 5.5	>0.99
Horticulture	0	4	4	NA		
Pig	1 (5.9)	16	17	0.8	0.02 to 6.7	>0.99
Poultry	3 (15.0)	17	20	3.0	0.4 to 15.6	0.1
Dairy	1 (5.6)	17	18	0.8	0.02 to 6.2	>0.99
Livestock	7 (8.1)	79	86	1.8	0.3 to 18.2	0.7
Other	0	3	3	NA		
Farm size						
< 500	6 (7.2)	77	83			
≥ 500	3 (9.4)	29	32	2.0	0.5 to 11.5	0.4
Median	330	296				
Materials						
Silage	9 (9.8)	83	92	NA	0.8 to Inf	0.06
Bedding	9 (8.6)	96	105	NA	0.4 to Inf	0.4
Feed	9 (9.1)	90	99	NA	0.6 to Inf	0.2
Hay	8 (9.3)	78	86	4.2	0.5 to 190.1	0.3
Timber	7 (6.7)	97	104	0.8	0.1 to 8.4	0.7
Coal	6 (9.8)	55	61	2.3	0.5 to 15.0	0.3
Rodents seen						
Mice	**6 (5.1)**	**112**	**118**	**0.1**	**0.02 to 0.96**	**0.02**
Rats	9 (7.6)	109	118	NA	0.2 to Inf	>0.99
During day	**2 (1.7)**	**117**	**119**	**0.006**	**0.0004 to 0.05**	**<0.001**
At night	9 (7.6)	109	118	NA	0.2 to Inf	>0.99
Rodent control						
Professional	4 (14.3)	24	28	2.8	0.5 to 14.2	0.2
Self	5 (5.6)	85	90			

Table 4.1 Analysis of factors associated with sero-positivity to hantaviruses
* Calculated using Fischer's exact test

Postcode information was collected for 115/119 eligible participants allowing geographical visualisation of sero-positive individuals; 2/4 volunteers who did not provide this information were sero-positive, with both samples identifying exposure to PUUV/SNV-like hantavirus. 6/34 districts included in the survey had at least one positive sample (Figure 4.2). One of the 6 regions was where an acute case was detected, however five did not have any previous information on hantavirus occurrence.

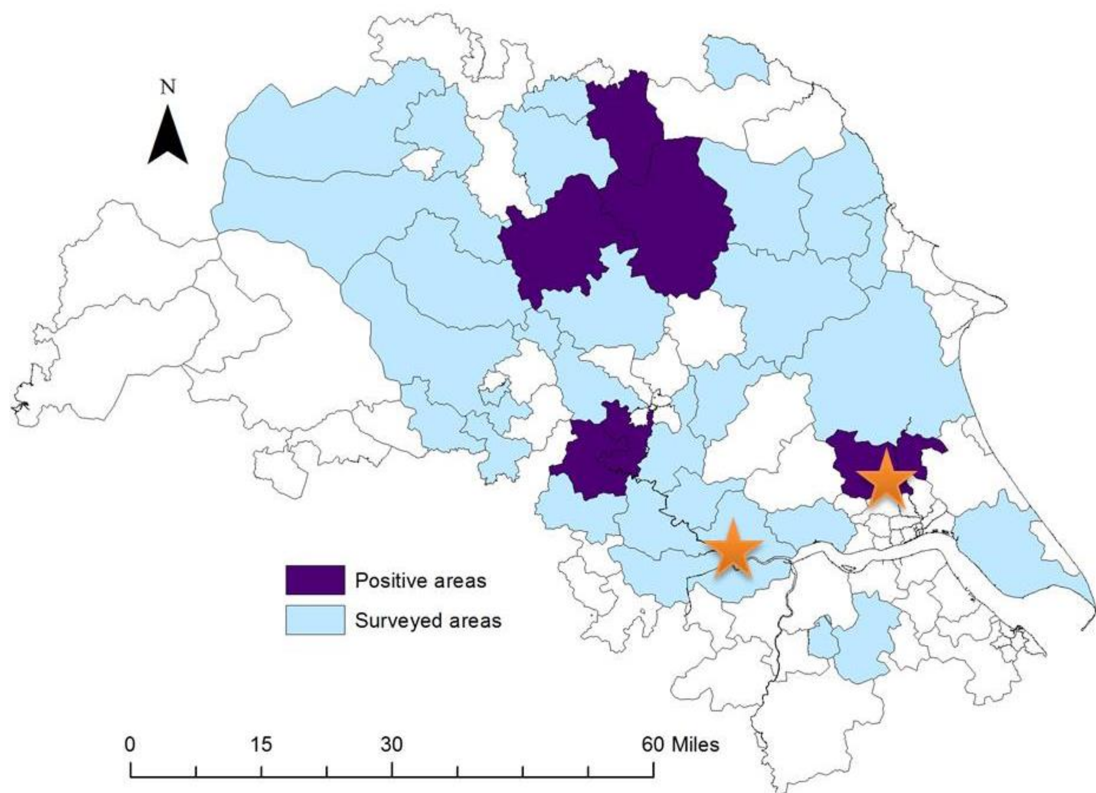


Figure 4.2 Surveyed areas and areas with a minimum of one positive serum sample recorded. Stars indicate location of previous acute cases.

4.3 Sero-prevalence in British military personnel

4.3.1 Study objectives

To determine sero-prevalence of hantavirus in British military personnel deployed to Afghanistan.

4.3.2 Study population

Access was granted to samples collected for general arbovirus surveillance. Volunteers were recruited between 2008 and 2011 from either British Army regiments or Royal Marines units prior to deployment to Helmand, Afghanistan. A serum sample was taken before and after deployment to determine if sero-conversion occurred during recent tour.

4.3.3 Serological results

Over the period of the study March 2008 to October 2011, 453 volunteers gave paired samples, (i.e. both pre-deployment and post-deployment samples) which were tested for presence of hantavirus antibodies. The sampled population consisted of seven Army units deployed across all years and one Royal Marine unit deployed in 2010. Overall 11/453 (2.43%) individuals demonstrated reactive antibodies specific to hantaviruses at a dilution of 1:1000. Of those 11, five had detectable antibodies in their first sample; therefore, six (1.33%) can be considered to have sero-converted during their tour. Separating the two classifications of military personnel it is interesting to find Royal Marines appear to have a much greater risk of being sero-positive for hantavirus 8/128 (6.25%) compared to 3/325 (0.92%) Army recruits. No positive samples were detected from tours occurring in

the years 2008 and 2009 during which 196 samples were collected compared to 257 taken during 2010 and 2011. Nine out of the 11 positive samples demonstrated a serological cross-reactivity to DOB, HTN and SEO. The remaining two samples were indicative of PUU infection.

Table 4.2 Prevalence of sero-conversion against hantavirus in military personnel deploying to Afghanistan. Data shown as raw numbers against sample size with percentage prevalence in parentheses. Total prevalence is broken down to show those that sero-converted on tour (i.e. negative before deployment, positive on return) and those who already had detectable antibody prior to deploying.

Regiment	Year	N =	N = +ve before tour	N = +ve during tour	Total +ve
Army	2008	39	0	0	0
Army	2009	157	0	0	0
Army	2010	63	1 (1.59%)	0	1
Royal Marines	2010	128	4 (3.1%)	4 (3.1%)	8
Army	2011	66	0	2 (3.0%)	2
Total		**453**	**5 (1.1%)**	**6 (1.3%)**	**11**

4.4 Sero-prevalence in renal patients

4.4.1 Study objectives

To determine sero-prevalence of hantavirus in a subset of renal patients.

4.4.2 Study population

Patients admitted to Queen Alexandra hospital, Portsmouth, UK with AKI or CRF between July and December 2013. Specimens of serum archived after being taken for blood-borne virus screening were randomly selected and anonymised by NHS staff. They were tested retrospectively at Porton in 2014. Portsmouth is situated on the southern coast of Hampshire, South England 50°81′67″N, 1°08′33″W. Figures taken from the 2011 Census shows the city has a population of approximately 207, 000 people with an even gender ratio and a predominance of adults between 24-49 years old (35%).

4.4.3 Serological results

From 243 samples tested, 17 (7.0%) demonstrated IgG antibodies reactive against hantavirus. Of the 17, 3/41 (7.3%) were from AKI classified patients and 14/202 (6.9%) from CRF patients. Virtually all, 16/17, were found to show the strongest cross reaction to HTN and SEO with only one sample from an AKI patient reactive across the entire panel of virus species. Samples were able to be titrated to higher dilutions; six at 1:10,000 and 11 at 1:1000.

4.5 Discussion

Three distinctive populations were tested with the common ground of having an increased risk for hantavirus specific antibodies compared to the general population: farmers, military personnel and patients with renal disease. The sero-prevalence rates for each were found to be 7.6%, 2.43% and 7.0% respectively. A previous comparable study looking at a subset of the farming population in England 15 years prior found sero-prevalence for hantaviruses to be 4.7%.[143] Although the hantavirus sero-positive frequency of this current study appears to be higher at 7.6%, the previous estimate lies within the 95% confidence interval.

It is interesting that prevalence rates for a separate risk group (patients with renal disease) from a region of the country not previously associated with HFRS is nearly identical to that for farmers but much higher than a comparable study from Northern Ireland which found rates of 2.1% (15/727).[145] Due to the lack of conclusive data on human infection in the UK, the propensity for subclinical infection and the non-specific and diverse symptoms it is likely that clinicians are in most cases unaware that hantaviruses are a potential cause of human disease and therefore cases are undetected or misdiagnosed as generalised AKI. When an infectious cause is considered leptospirosis is the prime candidate. Hantavirus disease is rarely considered part of the differential diagnostic investigations requested.

With the Yorkshire region recording some of the highest rates of admission for AKI in the UK[219] and with recent hantavirus cases resulting in AKI this disease warrants

further exploration in renal patients with an unknown aetiology. A 2009 review by the National Confidential Enquiry into Patient outcome and Death found evidence of one or more complications in patients who died of AKI in 2007. From 564 patients included in the study 130 exhibited respiratory failure and 35 haemorrhage. Retrospective analysis also showed the above complications were missed in 12 and 3 patients respectively.

As expected, in both the farming and renal groups sero-reactivity was heavily skewed towards SEOV and HTNV. Both study areas encompass cities (Hull and Portsmouth) with large ports. Two samples belonging to farmers showed reactivity to PUUV and SNV. This was not unforeseen; others have reported similar findings with three samples reacting to SNV in a study of Swedish farmers.[220] It does however raise the prospect of another circulating hantavirus in the UK, most likely PUUV for which the environmental factors have already been demonstrated to be suitable and the reservoir host is widespread.[163] However, in contrast to the samples from the farming population the samples from renal patients were able to be titrated to higher dilutions; six at 1:10,000 and 11 at 1:1000. This is most likely due to the exposures being more recent, if not very recent, and the likely cause of their admission; therefore antibody titres would be expected to be higher than for a population which may have mounted a response decades prior to testing.

Hantavirus disease is not a new hazard to military populations. Several acute cases have previously been reported within the British military.[154, 155] While sero-prevalence rates are lower than the other two risk groups it is higher than reported

from military populations in other countries. Similar studies have been undertaken in Switzerland where they found rates between 0.2 and 1.9%.[221] It was interesting to look for sero-conversion while troops were on tour and indeed six did convert during the sampling period. Five samples were also positive pre and post deployment. As it was unlikely to be the first tour for most individuals it is impossible to determine if their antibody response was due to exposure in the UK or another tour. However, apart from two samples which were almost exclusively reactive for PUUV and positive pre and post deployment, the other nine showed reactivity for DOBV, HTNV and SEOV. These highly cross-reactive samples will be useful for future serological assay validation.

Saliva looks to be promising for determining a simple positive or negative sample but appears to be less suitable for identifying individual species. Nonetheless, collection of saliva is a convenient and less-invasive sampling method for sero-surveillance surveys with proven effectiveness for other viruses, and its potential use for hantavirus sero-prevalence and screening studies is worth further investigation.

Overall these small sero-surveys, in particular those investigating the farming community and renal patients, indicate hantavirus is prevalent in risk groups in the UK and may be responsible for inducing AKI and CRF in patients in Portsmouth.

CHAPTER 5

Virus isolation and use of inactivated virus in serology assays

5.1 Introduction

5.1.1 Diagnostic methods for hantaviruses

Diagnosis of hantavirus infection in humans relies on serological methods, with detection of specific IgM antibodies, or a four-fold rise in specific IgG antibodies from paired samples, confirming an acute infection.[222] No detection of IgM or comparable levels of IgG in paired samples taken greater than two weeks apart indicates past infection, with specific antibodies considered to be life-long.

Several commercial assays are available for hantavirus serological testing but they are expensive, and mostly based on IF methods that use fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies (MAbs) or an ELISA targeting one specific hantavirus species.

Detection of viral antigens using IFAs has been employed since the 1970s and if performed correctly provides reasonable specificity and sensitivity. However, such assays are demanding, require expertise for interpretation of test results, and rarely fit easily into high-throughput laboratory workflows. ELISA's were also developed in the 1970s and have the advantage of being comparatively cheap, highly sensitive due to the enzymatic detection properties, and high throughput.

For both types of assay, live, and inactivated (irradiated) whole virus can be used but production of these requires ACDP level 3 facilities and irradiation requires specialist and costly equipment. Instead of whole virus, the majority of commercial assays have relied on recombinant hantavirus nucleoprotein expressed by baculoviruses, *Escherichia coli*, vaccinia virus or yeast systems. These have the benefit of mass production of antigen outside of high containment facilities.

5.1.2 Current diagnostic methods employed at PHE Porton for hantaviruses

Public Health England, Porton, is the Reference Laboratory for hantavirus testing in the UK, a commercially available IFA detecting specific IgG is the main tool for clinical diagnosis. Virus isolation and focus reduction neutralisation testing (FRNT) are techniques not routinely undertaken in recent times at Porton and such protocols needed developing for further characterisation of positive samples.

5.1.3 Diagnostic challenges with hantaviruses

Viral isolation has traditionally been the 'gold standard' for the diagnosis of human infection but as it requires a high level of expertise, is labour intensive and slow, it is progressively being replaced by rapid molecular methods. Isolation of hantaviruses, in particular, is rarely successful but it is necessary to investigate antigenic change, confirm virus viability, perform animal studies and assess antiviral susceptibility. Efforts are enhanced by using fresh tissue from reservoir host animals as noted in Chapter 4.

It should be emphasised that given the length of time required for isolation of hantaviruses the use of this technique is of limited benefit to immediate patient management. Virus isolation is of value to longer term clinical research and to contribute to molecular epidemiological and evolutionary knowledge on circulating hantavirus species. As discussed in Chapter 1 different hantavirus strains are responsible for a variety of clinical outcomes therefore it is useful to identify the causative species of hantavirus infections to improve clinical responses to patient care. Rapid molecular techniques, such as RT-PCR, are able to provide a prompt, reliable and specific differential laboratory diagnosis but the effectiveness of the technique is constrained by the short term viraemia produced by hantavirus infection.

In the absence of virus isolation or molecular detection, a better indication of the causative hantavirus species for human infection can be gained through FRNT. Comparison of levels of neutralising antibody against a panel of hantaviruses identifies the species the antibodies are most reactive against. The main disadvantage of this technique is it may lead to the inaccurate classification of a novel hantavirus. It is also a burdensome and slow technique again requiring ACDP level 3 facilities and is therefore rarely used for diagnostic purposes. Furthermore, the best results are obtained with convalescent serum taken a minimum of 3 weeks after onset of illness which again is of little benefit to acute patient management. The ideal situation would be for a high throughput serological assay to be developed which could confidently differentiate between different hantavirus species.

5.1.4 Aims of the Chapter

The aims were to generate a cell culture isolate of a UK SEOV strain and to use this virus for the development of antigen for future serological assays.

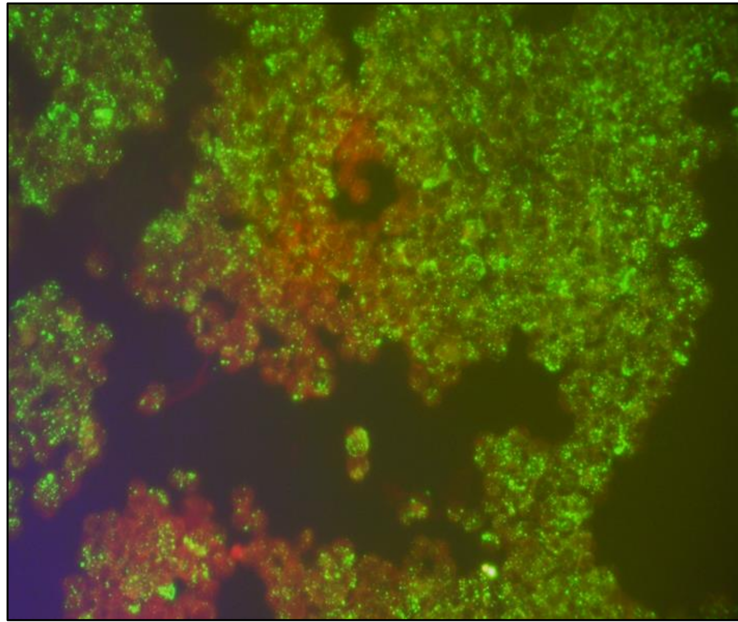
5.2 Isolation of Seoul virus strain Humber

Lung tissue of wild rat number 1 from Yorkshire (RN1) was homogenised in sterile PBS and inoculated on confluent Vero E6 cells. In the beginning cultures grown at 37 °C were passaged by splitting 1:5 every 5-7 days when cells looked over-confluent. This protocol was amended to cultures being grown at 35.5 °C, then splitting every 10-14 days between 2 and 5 mL of inoculated cells were added to 60% confluent fresh cells. Real-time RT-PCR and IFA was performed for each split until at least 8 passages had been performed.

Cultures were discarded after this time if no positive signals were detected. Several attempts were made including: homogenisation of different tissue types in PBS, 1% BSA solution and DMEM; inoculations with a range of tissue dilutions from neat to 1:1000; and, a range of inoculation times from 60 min to overnight.

At no time was viral growth successful using RN1 samples despite strong molecular signals from neat tissue. Attempts were then focused on tissue samples from wild rat RN4. From the first passage infected Vero E6 cells showed positive IF staining to a commercial monoclonal antibody (Progen, Germany) against the nucleocapsid protein of SEOV R22 strain after ten days in culture. After a further three passages

all cells demonstrated positive staining (Figure 5.1), the cells were harvested and the virus was considered isolated.



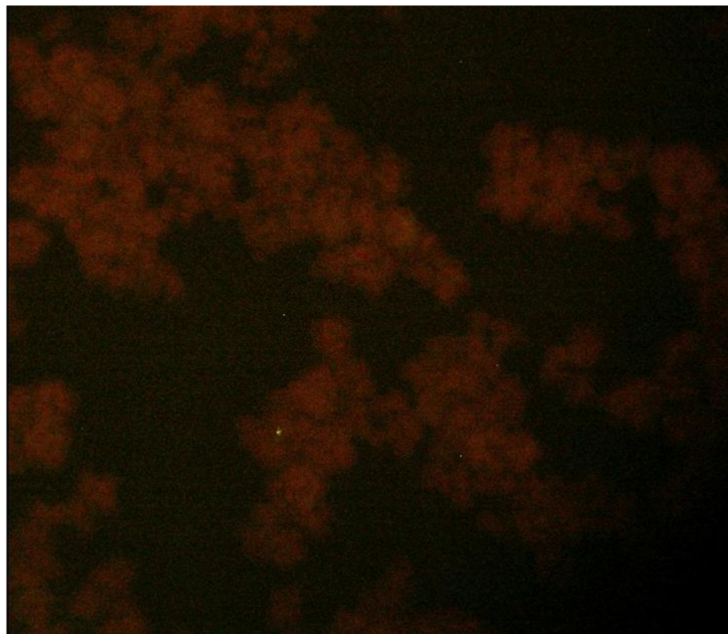


Figure 5.1 Vero E6 cells infected with Humber virus (top picture) showing positive FITC staining and negative control Vero E6 (bottom picture). Viewed at total 200x magnification using EUROStar III plus microscope

The isolate was designated Seoul hantavirus, strain Humber. Negative Vero E6 cells showed no specific staining.

From reviewing the literature of successful isolations for hantaviruses and knowledge gained from these isolation attempts the protocol detailed in Chapter 2 section 2.4 is the method of choice for future attempts.

5.3 Unsuccessful isolations

The isolation protocol detailed in Chapter 2 was utilised for rats 001 and 002 from Oxfordshire. Homogenised brain, heart, lung and liver tissue were attempted with no success.

After serological testing and one aliquot taken for molecular testing there was no sample available from the only RT-PCR positive patient sample. Further serum and urine samples taken had no detectable hantavirus RNA and therefore culture was not attempted.

5.4 Quantification of Humber virus

The plaque assay technique[223] was attempted to quantify virus in isolation material and for future use with positive serum in PRNT. Despite following the protocol exactly, plaques were never successfully visualised. Different concentrations of DMSO, various overlay substrates (Agarose, CMC and Avicel), and a range of inoculation times and infection times were tried. Attempts were also unsuccessful for HTNV virus. In particular, due to the length of time hantaviruses require to

grow, cell monolayers were negatively affected and it was impossible to differentiate between plaques caused by virus and normal cell death due to over-confluence. As hantaviruses do not produce cytopathic effects in Vero E6 cells, it is unsurprising the plaque assay technique is not efficacious. In current times the focus forming assay (FFA) is the method of choice. The FFA is a variant of the plaque assay beneficial for quantifying viruses that do not lyse infected cells. Results of the FFA are expressed as focus forming units per millilitre, or FFU/mL. Using the same FITC-MAb as for IFA, the FFA demonstrated 1.1×10^6 FFU per 1 mL for Humber virus.

5.5 UV inactivation of Humber virus

Stocks of Humber virus at 1.1×10^6 FFU per 1 mL were successfully inactivated (Figure 5.2a and b) using the method described in the literature[135] and in Chapter 2. Even at neat concentration, no positive staining for viable virus following inoculation with inactivated samples was visible. Native Humber virus was used as positive control. Once inactivation was proven to be effective stocks were removed from the CL3 laboratory and handled on the bench under CL2 conditions.

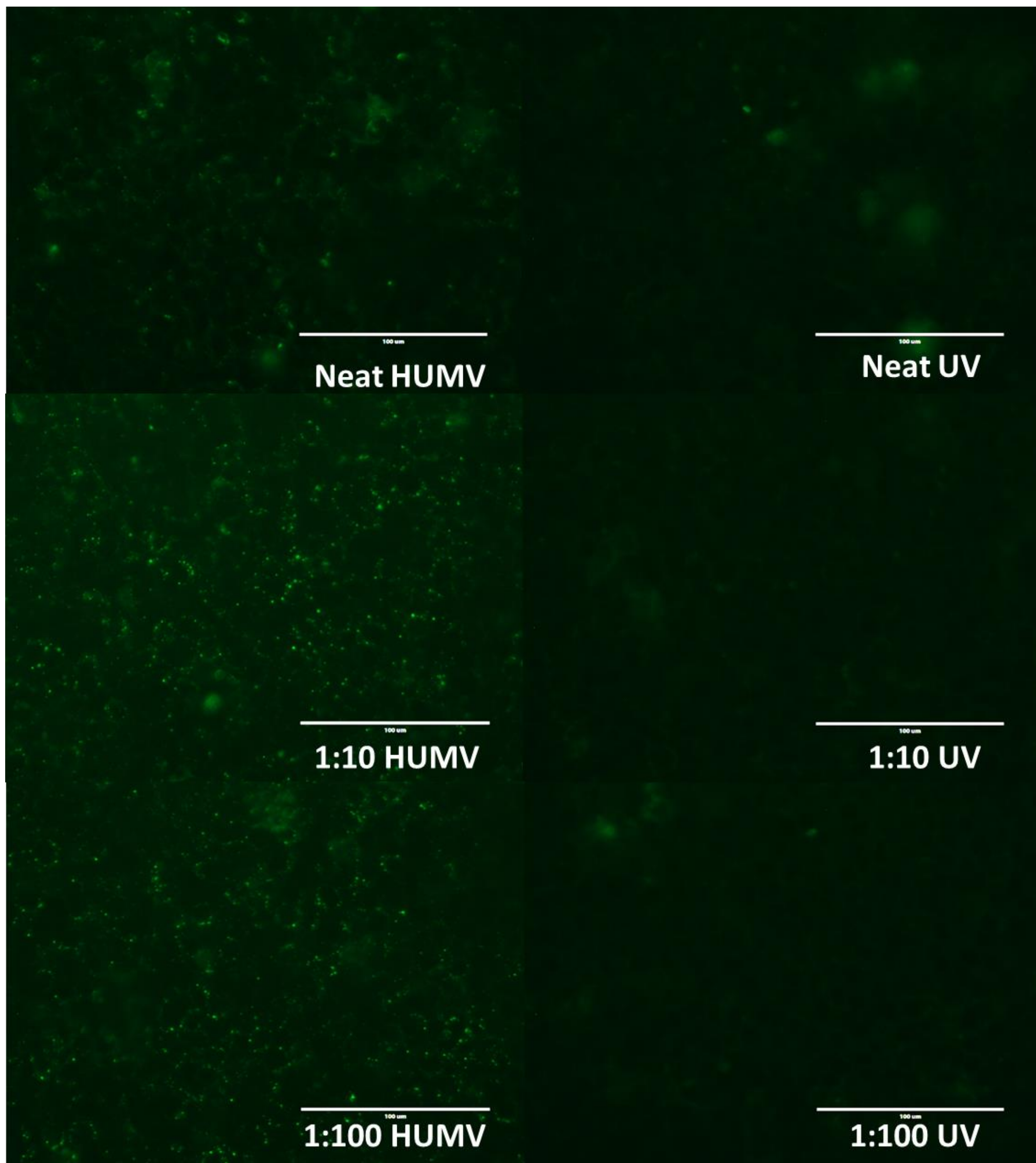


Figure 5.2a. In-house IFA showing positive staining for native HUMV virus and no staining for UV inactivated virus at neat, 1:10 and 1:100 dilutions. Viewed at total 200x magnification.

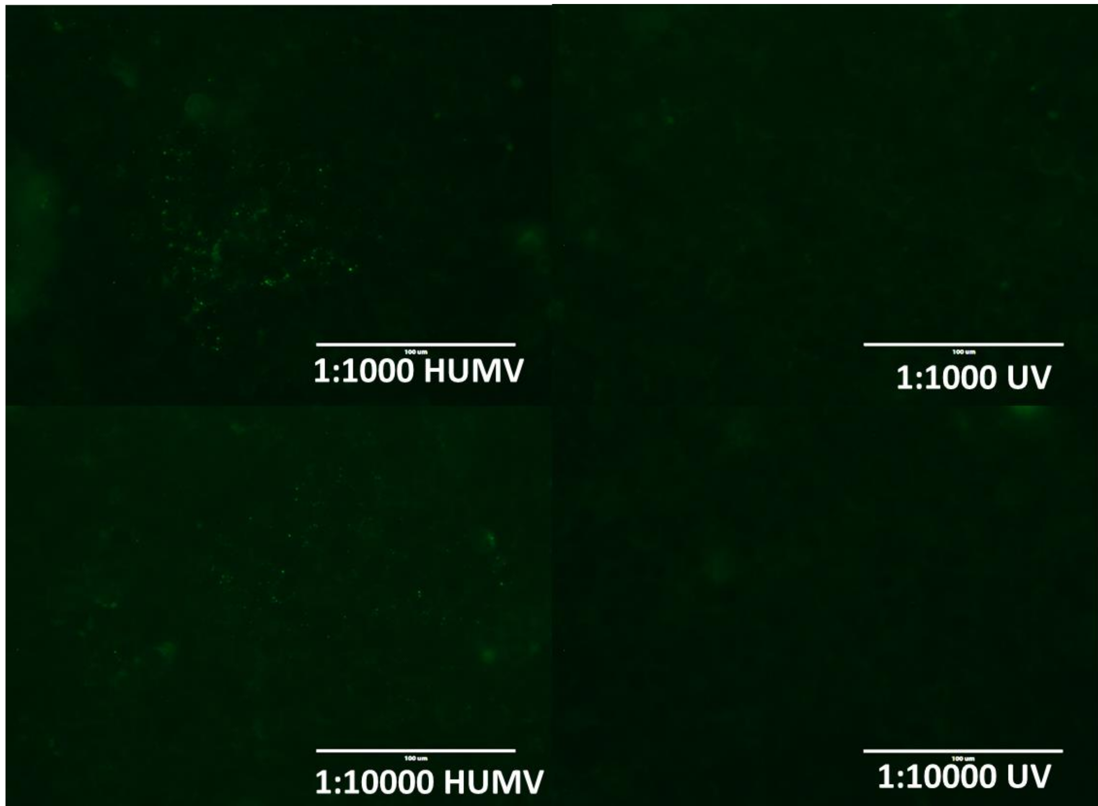


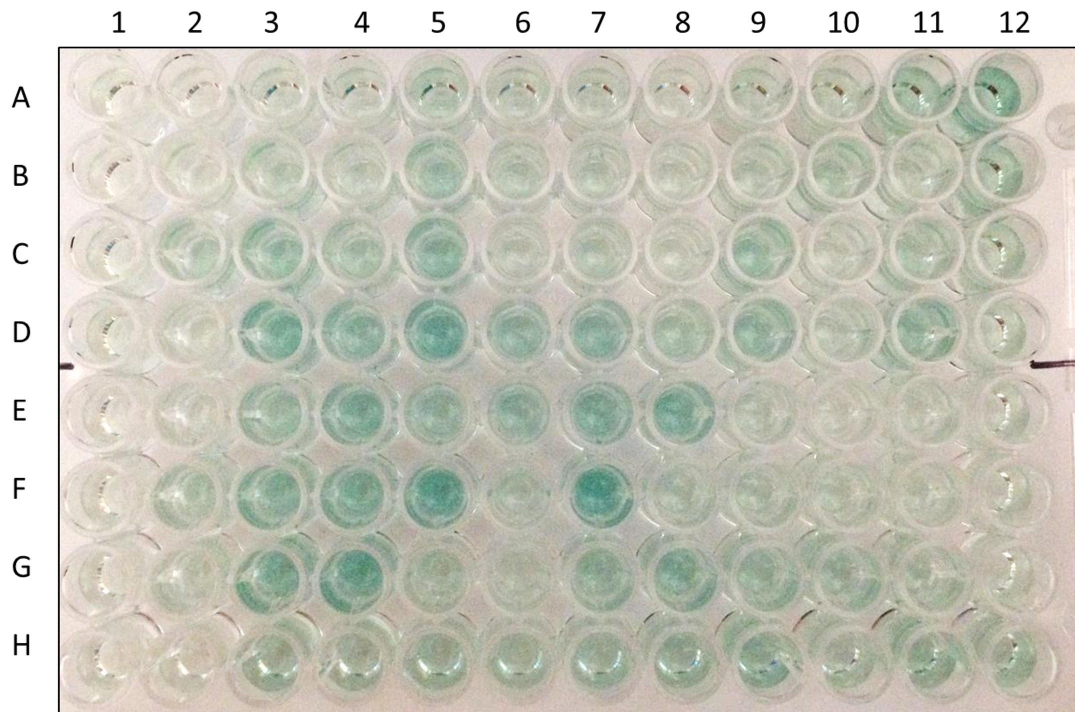
Figure 5.2b. In-house IFA showing positive staining for native HUMV virus and no staining for UV inactivated virus at dilutions 1:1000 and 10,000. Viewed at total 200x magnification.

5.6 Inactivated whole virus as antigen in an ELISA

The results of the UV inactivate virus as antigen using the method described in Chapter 2 are demonstrated in Figure 5.3. Two serum samples confirmed positive for hantavirus specific antibodies, SEOV and PUUV, via IFA were tested at 1:100 and 1:1000 dilution. UV-inactivated hantaviruses, including the Humber strain isolated during this study, were coated to the plate in a dilutions 1:10, 1:100, 1:1000 and 1:10,000. In addition a negative serum sample and an equivocal sample were tested at the same dilutions.

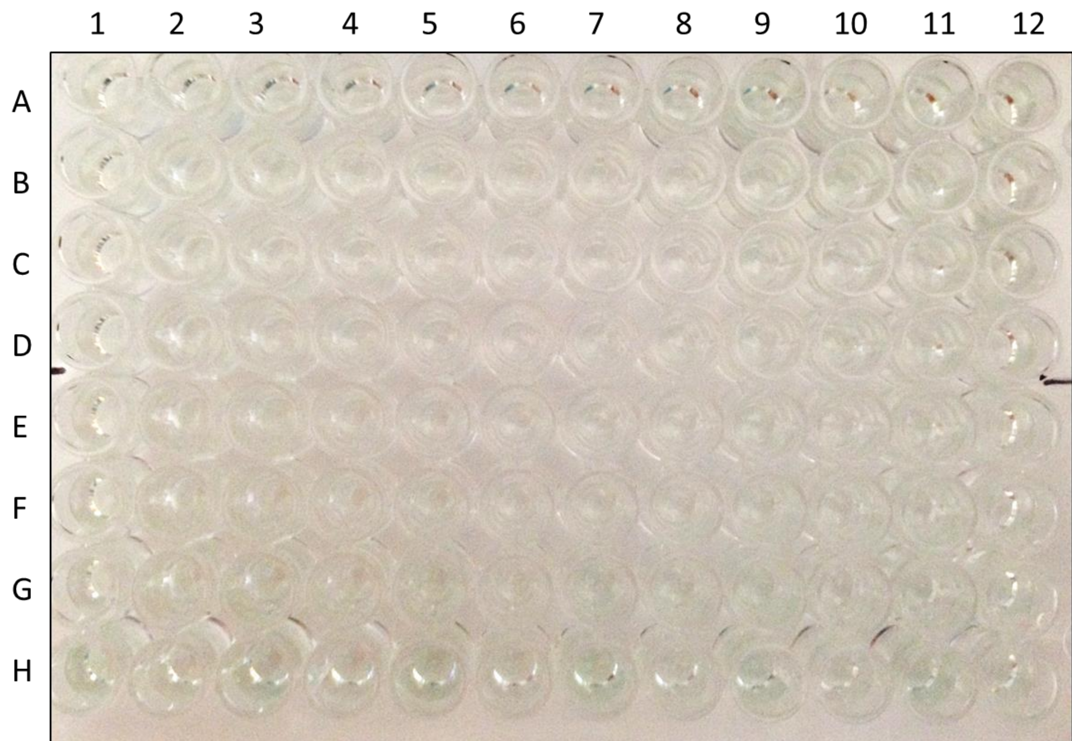
ELISA development was initially hampered by the lack of whole virus sticking to the plate during coating. A variety of buffer types and coating incubation times and temperatures were trialled. Overnight coating at 4 °C in carbonate-bicarbonate buffer (pH 9.6) was found to be the most successful.

OD values for the final method tested during this study are shown in Figures 5.3a and b. Both visual and OD values demonstrate reactivity for positive serum samples in comparison to little reactivity for the negative and equivocal serum. More detailed analysis and refinement was not possible at this time, as time constraints prevented further work-up. However these preliminary results indicate UV-inactivated hantavirus is a potential antigen for use in an ELISA format.



	SEOV		HTNV		DOBV		PUUV		SNV		TULV	
	1 100	**2** 1000	**3** 100	**4** 1000	**5** 100	**6** 1000	**7** 100	**8** 1000	**9** 100	**10** 1000	**11** 100	**12** 1000
A 10	0.137	0.025	0.182	0.182	0.281	0.115	0.182	0.076	0.140	0.181	0.328	0.500
B 100	0.015	0.047	0.171	0.171	0.388	0.232	0.171	0.152	0.187	0.271	0.158	0.306
C 1000	0.064	0.278	0.216	0.216	0.469	0.419	0.216	0.116	0.370	0.126	0.273	0.223
D 10k	0.096	0.095	0.417	0.417	0.592	0.566	0.417	0.276	0.383	0.151	0.425	0.165
E 10	0.039	0.096	0.375	0.537	0.369	0.364	0.473	0.501	0.117	0.080	0.140	0.136
F 100	0.062	0.247	0.520	0.510	0.659	0.190	0.691	0.219	0.191	0.137	0.160	0.126
G 1000	0.075	0.159	0.559	0.637	0.251	0.190	0.366	0.435	0.344	0.317	0.219	0.086
H 10k	0.047	0.041	0.291	0.204	0.262	0.268	0.345	0.297	0.349	0.173	0.323	0.282

Figure 5.3a. Adjusted (minus negative values in Figure 5.3b rows A to D) OD values of ELISA plate incubated with patient serum reactive for SEOV (rows A to D of plate) and patient serum reactive for PUUV (rows E to H plate). X axis shows hantavirus species coated on plate and patient sera dilution. Y axis shows the dilution of the virus coated onto the plate.



	SEOV		HTNV		DOBV		PUUV		SNV		TULV	
	1 100	**2** 100	**3** 100	**4** 100	**5** 100	**6** 100	**7** 100	**8** 100	**9** 100	**10** 100	**11** 100	**12** 100
A 10	0.108	0.111	0.123	0.100	0.096	0.116	0.104	0.123	0.157	0.113	0.111	0.111
B 100	0.122	0.145	0.143	0.105	0.114	0.128	0.134	0.098	0.125	0.132	0.112	0.105
C 100	0.105	0.122	0.113	0.106	0.111	0.103	0.116	0.107	0.113	0.102	0.100	0.107
D 10k	0.104	0.122	0.111	0.148	0.119	0.103	0.114	0.101	0.124	0.125	0.104	0.118
E 10	0.117	0.130	0.164	0.126	0.124	0.114	0.108	0.118	0.125	0.091	0.111	0.092
F 100	0.113	0.127	0.136	0.106	0.131	0.105	0.113	0.135	0.182	0.093	0.114	0.093
G 100	0.134	0.114	0.151	0.118	0.165	0.132	0.157	0.116	0.221	0.126	0.137	0.100
H 10k	0.173	0.112	0.172	0.120	0.175	0.106	0.172	0.129	0.182	0.104	0.164	0.109

Figure 5.3b. OD values of ELISA plate incubated with negative patient serum (rows A to D of plate) and an equivocal human sample with weak IFA signal but considered negative (rows E to H plate). X axis shows hantavirus species coated on plate and sera dilution. Y axis shows the dilution of the virus coated onto the plate.

5.7 Ultracentrifugation of Humber virus

Various methods were trialled in order to purify and concentrate HUMV as use for future antigen rather than using crude cell lysate stocks. Maximum centrifugation speed and time was balanced against protecting the virus to maintain infectivity. The method described in Chapter 2 was deemed to be the most successful and in-house IFA confirmed purified virus was still capable of infecting E6 cells.

5.8 Discussion

In addition to the molecular evidence for a SEOV, presented in Chapter 3, herein the chapter reports the successful isolation of the SEOV strain Humber from wild *R. norvegicus* trapped in Yorkshire; thereby proving the presence of an infectious SEOV in the UK. This isolate is certified mycoplasma free and available to accredited laboratories via the National Collection of Pathogenic Viruses (catalogue no. 1211221v). The reason why virus could only be cultured from RN4 tissue and not RN1 is unknown as both yielded comparably strong RT-PCR signals. It is conceivable that RN1 was trapped earlier in the night than RN4 and therefore had more time for tissue and virus degradation before being collected and frozen the following morning.

Despite numerous attempts with SEOV strain Cherwell, no isolation was made. This is most likely a direct adverse effect of the Pentobarbitone used to humanely euthanize the pet animals as tissue samples yielded strong RT-PCR signals and samples were transported and handled using identical methods to those from Yorkshire. Pentobarbitone is an alkylating agent which in high concentrations will

interfere with viral replication and protein synthesis. It is unfortunate there was not enough sample available for an isolation attempt from the RT-PCR positive patient, however given the few amino acid differences between Humber and Cherwell it was concluded that the two are antigenically identical thus would not impact on future assay development.

To quantify virus in aliquoted samples for future FRNT testing an FFA protocol was adopted. Focus forming assays have the drawback of incurring a higher cost due to the conjugated antibody required than the plaque assay but the substantial advantage of a shorter time for results. Hantaviruses are slow-growing and if the assay relies on cell lysis it will be between 10 and 14 days before results are available. For this study infected cells for the FFA were incubated for 7 days but preliminary data indicates this can be reduced and work will continue to determine the optimum time this can be achieved. Seven days is still a long period of time for acute diagnosis of infection and is unlikely to be routinely requested by clinicians. Instead it is useful to quantify material for research purposes and for use in FRNT for a better indication of causative hantavirus species if this is required once a diagnosis is made. It will also aid epidemiological tracing and identification of the rodent source of infection.

It is encouraging that the published protocol for inactivating hantaviruses using UV irradiation was reproducible. For safety reasons and to comply with internal risk assessments, proof of inactivation was required. Even neat virus stocks were inactivated as demonstrated no evidence of positive staining following culture. As

apparent for native virus (Figure 5.2a shows decreased staining for neat virus compared to virus diluted 1:10) if hantaviruses are added to cultured cells at too high a concentration defective particles are produced and virus proliferation decreased. A dilution series of both native and inactivated virus was performed to ensure inactivation was successful and lack of staining was not as a result of viral self-inhibition.

The viral antigens for the IgG ELISA were, respectively, a whole-virus preparation of UV inactivated DOBV, HTNV, PUUV, SEO, SNV and TULV from cell culture. The cell lysate was chosen over purified virus as this method shows less background than purified antigen[224] however ultracentrifuged antigen may be trialled in the future to compare if this improves sensitivity and adherence of the antigen to the micro wells. ELISAs using native whole virus have also been shown to have higher sensitivity than those employing partial or recombinant antigen although conversely may show an increase in cross-reaction across the hantavirus species.[226-227] The reactive pattern of adjusted OD values for both SEOV positive and PUUV positive serum is similar. Interestingly, OD values increase in parallel with virus dilution with the highest values often recorded for virus dilution at 1:1000 and 1:10, 000. Perhaps similar to results seen for FFA, too high a concentration of virus produces viral interference. When bunyaviruses reach a certain concentration they produce defective viral particles rather than replicating whole virus.[47]

Protein values were retrospectively obtained for each hantavirus to attempt to understand the difference in cross-reactivity of the ELISA plate and explain why the

serum reactive for SEOV via IFA reacted so weakly with the HUMV coated wells. Total protein concentration was found to be similar so did not explain the weak reaction for HUMV however they did indicate protein levels were too high (between 3.8 and 5.0 mg/mL). While this is total protein and not necessarily pure viral protein it is far higher than that which is ideal for use in ELISA plates and the concentration would be greatly diluted for future assay work-up. Too high a concentration of antigen can negatively affect coating leading to oversaturation of the plate which results in inhibition of antibody binding.

It may be possible to reduce such cross-reactivity and interference with full validation and ELISA work-up, including dilution series of optimum antigen, antibody and virus concentration. Once the chosen antigen has been fully characterised further refinements to the ELISA may include progression to a capture ELISA. This has the advantage of allowing quantification of antibody in the serum sample.

Any future development should be mindful that the ELISA is intended to provide laboratory differential diagnostic capability, and that cross-reactivity between different virus antigens may, in fact, be a useful property. The initial ELISA test would ideally pick up all strains of hantavirus and simply provide a sensitive and specific positive or negative result. ELISAs are rarely suitable for sero-typing when such cross-reactivity between virus species exists. Characterisation of the exact hantavirus species can then be undertaken on the vastly reduced number of positive samples rather than on all suspected samples.

The purpose of this crude ELISA and use of UV inactivated virus as antigen was to provide a proof of principle. This limited data confirms the antigen is reactive to positive serum containing hantavirus specific antibodies compared with negative hantavirus control serum. If time had permitted it would have been interesting to mix the UV inactivated virus strains to determine their combined reactivity against different hantavirus serum antibody profiles. UV works as an inactivation method by damaging viral nucleic acids resulting in cross linkage of pyrimidine bases; prior to this test it was not known if the cross-linkage would interfere with sero-reactivity.

CHAPTER 6

Discussion and Conclusion

Previous research between the 1970s and 1990s had provided strong serological evidence in human patients for at least one hantavirus species circulating in wild rodents and one hantavirus associated exclusively with laboratory rats. This PhD aimed to build on these findings and through use of a combination of molecular and serological techniques characterise and confirm a hantavirus responsible for current human renal disease in the UK.

6.1 Hantavirus as a confirmed human pathogen in the UK

6.1.1 Seoul hantavirus

The investigation of rodents linked to acute human cases of HFRS during this study led to the isolation and characterisation of one SEOV strain (Humber) from wild *R. norvegicus* and molecular characterisation of a second closely related SEOV strain (Cherwell) from pet *R. norvegicus*. Partial sequence data, obtained from a human serum sample, matched with 100% homology to the L segment of the Cherwell strain firmly establishing this SEOV is pathogenic to humans. Within the timeframe of this PhD a pathogenic hantavirus circulating in wild and pet rats has been confirmed in the UK.

Seoul virus is a known cause of renal disease in Asia but data on its role in causing renal disease in Europe are scarce. Following the publication of the UK findings

presented in this thesis the first molecularly confirmed human case of SEOV infection was reported from France.[228] In addition multiple SEOV positive wild *R. norvegicus* were detected in the Lyon region of France.[229] Further afield a highly similar SEOV has been recently reported from wild *R. norvegicus* in New York.[230] The findings from New York reported by Firth *et al.* (2014)[230] are of particular interest as not only is the Baxter strain closely related to HUMV but the authors estimated it was introduced to the region 3-16 years prior. It seems a logical step to perform similar analysis of the three UK strains: HUMV, CHEV and IR461. While it is tempting to speculate IR461 is the ancestral strain from the interpretation of the phylogenetic trees and the fact that IR461 was proven to be introduced via imported rats from Belgium via Japan (simultaneously resulting in human infection in both countries); molecular clock analysis would provide more substantial evidence to confirm this.

6.1.2 Reservoir hosts

While it was not surprising to identify wild rats as carrier hosts, the involvement of pet rats was unexpected and appears to be the first report of pet animals responsible for transmission of a hantavirus to humans. A separate sero-study that followed the initial discovery of the involvement of pet rats in human disease was undertaken as part of a public health investigation led by Public Health England. Between October 2013 and June 2014 people with likely exposure to wild and pet rats were sampled and tested for hantavirus antibodies. The key finding of this study was 32.9% (26/79) of the sera from pet rat owners were found to have hantavirus antibodies when tested using the commercial IF assay described in

Chapter 2. The strongest reactivity were observed against SEOV antigen. This implies SEOV is circulating within pet rat populations in the UK and could present a significant health risk to owners, breeders, veterinarians and retail suppliers who handle these rodents.

It was not possible to access medical records for those rat owners who were seropositive. The next step therefore would be to understand what proportion of the 33% were ever clinically ill or is there a subset of people who seroconvert but have subclinical infection. If the majority of pet rat owners develop AKI then the situation requires more attention than if only a marginal number of owners develop severe disease. There is a delicate balance required for future investigations as these animals are treasured pets and/or a source of income. An appropriate testing strategy needs to be devised to identify positive animals in both the trade and domestic setting.

Following the publication of the UK pet rat findings, researchers conducted a subsequent investigation in Sweden and demonstrated SEOV positive rats in a colony which included a rat imported from the UK. [230] This demonstrates such findings are again not limited to the UK and the virus can be imported and exported via the pet rat trade.

6.2 Circulation of other hantavirus species

The results presented in this thesis alongside the subsequent findings of SEOV in France, Sweden and the USA continues to provide evidence for SEOV as a pathogen

outside of its origin in Asia. As shown two decades prior, it is important to include Seoul antigen in the panels for hantavirus screening in Europe.[145] The majority of sero-conversions in the Northern Ireland study would have been wrongly classified as negative had the SEOV R22 antigen not been utilised. This is however not to suggest SEOV is the only hantavirus of importance in the UK.

There is strong evidence for an additional hantavirus species circulating in the UK. Two of the farmers investigated in Chapter 4 demonstrated reactive antibodies to SNV and PUUV, not SEOV. In addition, three sero positive sera from the public health investigation mentioned in section 6.1.2 were reactive to other hantaviruses: two reactive against DOBV antigen and one reactive against PUUV. This is in contrast to pet rat owners which only demonstrated seropositivity for SEOV and HTNV.

Research by others in the UK may have in part alluded to this second hantavirus species. Pounder *et al.* (2013)[233] provided preliminary findings of a hantavirus detected in a *Microtus agrestis* (field vole) in Northern England. Limited molecular data was recovered but enough to indicate a hantavirus and subsequent serology was cross reactive for PUUV. As the species of vole tested was not *My. glareolus* it is unlikely to be PUUV but antigenically a closely related hantavirus species.[23]

6.3 Clinical definition and raised awareness

6.3.1 Case definition

Compiling the clinical findings of the most recent UK cases discussed in Chapter 3 and those reported previously in the literature, SEOV hantavirus infection should be suspected and tested for when the following symptoms and laboratory markers are present in conjunction with relevant epidemiological exposure to wild or pet rats:

- Abdominal pain
- Fever
- Chills
- Headache
- Vomiting
- Elevated creatinine
- Thrombocytopenia

6.3.2 Raised awareness

Throughout this study findings have been disseminated via peer-reviewed publication, press releases and through oral presentations at national and international conferences. Awareness of hantavirus as a cause of AKI has been raised among the UK medical community. This is evident by the rise in requests for hantavirus testing through RIPL, PHE Porton.

Table 6.1 Number of hantavirus IFA tests performed at PHE Porton.

Year	No. of IFA tests
2010	28
2011	41
2012	271
2013	256
2014	259

6.4 Future work

The findings presented in this thesis have highlighted several areas for future research; in particular further investigation is necessary to clarify the samples with reactivity to hantavirus species other than SEOV (sero-results detailed in Appendix II). The FFU technique discussed in Chapter 5 could easily be expanded to other hantavirus species and be used for the titration of clinical samples via FRNT by the hantavirus reference laboratory at PHE, Porton. This would aid epidemiological and outbreak investigations when molecular data is not available.

Completion of the UV inactivated virus ELISA which would pick up sero-conversion to all clinically relevant hantavirus species would provide a useful first-line screening assay for reference laboratories. The current commercially available assays are prohibitively expensive, only target one or a limited number of antigenically related hantavirus species and are too labour intensive for large scale screening studies or busy reference laboratories.

Considering the prevalence of hantavirus infection in pet rats and the propensity of the fancy rat community in showing and sharing animals potentially chronically infected, it is impracticable to think SEOV will be eradicated in the UK. Within such a community, control and prevention strategies are challenging. They range from the provision of awareness advice to vaccine development. The introduction of any vaccine in the UK could be directed to a number of risk groups such as pet rat owners, farmers, military personnel and veterinarians. As previously mentioned in Chapter 1 there are vaccines currently on the Asian market [120, 121] but these are unlikely to ever gain approval for use in Europe as they are not manufactured to EU regulatory standards. The DNA based vaccine currently at clinical trial stage in USA is based on PUUV and ANDV and therefore will not guarantee protection against SEOV.[122, 123] A recently developed recombinant candidate vaccine using an attenuated poxvirus vector, Modified Vaccinia virus Ankara, has been shown to be highly effective for another *Bunyaviridae* - Crimean-Congo haemorrhagic fever virus.[232] This use of this methodology for production of a hantavirus vaccine has potential.

6.5 Conclusion

Knowledge on hantavirus as a cause of AKI in the UK requires further characterisation. The findings presented in this thesis provide conclusive evidence for the presence of SEOV in the UK and a starting point for future investigations in the UK into human disease and enzootic cycles in rodent hosts.

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Appendices

Appendix I Questionnaire for sero-survey

Page 1 of 4

Unique Identifier:

A HANTAVIRUS RESEARCH STUDY INVOLVING THE FARMING COMMUNITY IN THE NORTH YORKSHIRE AND THE HUMBER REGION, 2013

For completion on all recruited cases in North Yorkshire and the Humber, 2013

Inclusion Criteria	Exclusion Criteria
• at least 18 years of age • resident in North Yorkshire and the Humber • main occupation is working on a farm and/or their main residence is within a farm in North Yorkshire and the Humber	• < 18 years of age • live outside North Yorkshire and the Humber • do not live or work on a farm in North Yorkshire and the Humber • do not provide consent • have previously provided a sample for this study

Does the volunteer meet the above criteria? ☐ Yes ☐ No

Has the volunteer read and understood the information sheet? ☐ Yes ☐ No

Consent given and form signed? ☐ Yes ☐ No

Blood specimen collected? ☐ Yes ☐ No

Saliva specimen collected? ☐ Yes ☐ No

Name of nurse collecting specimens:	

Additional Notes:

Final Version

HANTAVIRUS INVESTIGATION IN NORTH YORKSHIRE AND THE HUMBER

Date Form Completed: Location:	Completed by:

SECTION 1 PERSONAL DETAILS				
Age in years:				
Gender: ☐Male ☐Female		Residential postcode:		
Main occupation:				
1.0. Have you ever travelled outside of the UK?				☐Yes ☐No
If yes please tick all that apply	☐Africa	☐ Asia	☐Europe	☐South America
	☐Antarctica	☐ Australia/Oceania	☐ North America	
1.1. Have you travelled outside the UK in the last 3 months?				☐Yes ☐No
If yes please tick all that apply	☐Africa	☐ Asia	☐Europe	☐South America
	☐Antarctica	☐ Australia/Oceania	☐ North America	

SECTION 2 EXPOSURE TO FARMS		
2.0. Do you live on a farm?		☐Yes ☐No
2.1. Do you work on a farm?		☐Yes ☐No
If yes to both	2.2. Do you work and live on the same farm?	☐Yes ☐No
IF NO TO Q2.2 PLEASE CONTINUE NEXT SECTIONS FOR PRIMARY FARM AND REPEAT SECTION 3-7 FOR ADDITIONAL FARM ON SEPARATE SHEET		

SECTION 3 PRIMARY FARM DETAILS	
3.0. Please tick what general type of farm it is:	☐Animal ☐Arable ☐Mixed ☐Don't know
3.1. Please tick what specific type of farm it is: (tick all that apply)	☐Cereals ☐General Cropping ☐Horticulture ☐Specialist Pigs ☐Specialist Poultry ☐Dairy ☐Grazing Livestock ☐Mixed ☐Other....................................
3.2. Farm size (approximate acreage): .... ☐Don't know	
3.3. Main connection to farm: ☐Principal farmer ☐Relation of principal farmer ☐ Regular hired worker ☐Seasonal/Part-time hired worker ☐Other (please specify)	

SECTION 4 EXPOSURE ON PRIMARY FARM

4.0. Do you have contact with the following materials?
(If yes, please tick method for each material. Tick 1 if handled by bare hands, 2 if handled with gloved hands, 3 if by hand tool and 4 if by machinery)

	Silage				Animal bedding				Animal feed				Bales of hay				Wood/ timber				Coal			
Never																								
If yes, is it	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Most days																								
Every Week																								
Every month																								
Rarely																								

SECTION 5 WATER SOURCES ON PRIMARY FARM

5.0. What are the sources of water on the farm?

Mains water	☐ Yes	☐ No	**☐Don't know**
Well water	☐ Yes	☐ No	**☐Don't know**
Spring water	☐ Yes	☐ No	**☐Don't know**
River	☐ Yes	☐ No	**☐Don't know**
Other	☐ Yes	☐ No	

Please specify...

SECTION 6 EXPOSURE TO RODENTS ON PRIMARY FARM				
6.0. Have you ever seen rodents (alive or dead) or evidence of rodents on the farm			☐Yes	☐No
If yes please continue Q6, If no continue to Q7	Have you seen live rodents (tick all that apply)	☐day	☐ night	☐ never
	Have you seen evidence of damage caused by rodents		☐Yes	☐No
	Have you seen rodent excrement		☐Yes	☐No
	What type of rodents have you seen:			
	Mice		☐Yes	☐No
	Rats		☐Yes	☐No
	Don't know		☐Yes	☐No

SECTION 7 RODENT CONTROL ON PRIMARY FARM				
7.0. Is rodent control used on this farm?		☐ **Don't know**	☐Yes	☐No
If yes, please tick all that apply:	A professional pest control company		☐Yes	☐No
	self administered poisoned bait,		☐Yes	☐No
	self administered traps,		☐Yes	☐No
	Other please specify			
7.1. Do you ever handle rodents			☐Yes	☐No
If yes	Live animals		☐Yes	☐No
	Dead animals,		☐Yes	☐No
	Do you wear gloves when handling rodents: ☐ Never ☐ Occasionally ☐ Most times ☐ Always			

This is the end of the questionnaire for participants from one farm.

Please thank the participant for completing this questionnaire and inform them the information they have provided may be stored on computer and will be used for public health purposes. All information will be handled according to strict National Health Service confidentiality guidelines.

Please continue questionnaire for sections 3-7 for additional farms

Appendix II Serology results

ID	Sample	Max Dilution	SAAV	DOBV	SNV	SEOV	PUUV	HTNV
27	Farmer sera	100			POS		POS	
40	Farmer sera	100				POS		POS
48	Farmer sera	100				POS		
50	Farmer sera	100					POS	
53	Farmer sera	100				POS		POS
63	Farmer sera	100				POS		POS
81	Farmer sera	100				POS		POS
87	Farmer sera	100				POS		POS
129	Farmer sera	100				POS		POS
27	Farmer saliva	100			POS	POS	POS	POS
40	Farmer saliva	100	POS		POS	POS	POS	POS
48	Farmer saliva	100			POS	POS	POS	POS
50	Farmer saliva	100			POS	POS		POS
53	Farmer saliva	100		POS	POS	POS		POS
63	Farmer saliva	100		POS	POS	POS		POS
81	Farmer saliva	100			POS	POS	POS	POS
87	Farmer saliva	100		Not	available	to	test	
129	Farmer saliva	100	POS	POS	POS	POS	POS	POS
QAH003	AKI	10,000				POS		POS
QAH020	CRF	1000						POS
QAH022	CRF	10,000				POS		POS
QAH031	CRF	1000				POS		POS
QAH041	CRF	1000				POS	POS	POS
QAH070	AKI	1000	POS	POS	POS	POS	POS	POS
QAH074	AKI	1000						POS
QAH078	CRF	1000						POS
QAH082	CRF	1000				POS		
QAH098	CRF	10,000				POS	POS	POS
QAH130	CRF	10,000	POS	POS		POS		POS
QAH161	CRF	1000				POS		POS
QAH167	CRF	1000				POS		POS
QAH171	CRF	10,000				POS		POS
QAH173	CRF	1000						POS
QAH200	CRF	1000				POS		POS
QAH233	CRF	10,000				POS	POS	POS
7460	MoD sera	1000				POS		
7589	MoD sera	1000				POS		
7592	MoD sera	1000				POS		POS
7616	MoD sera	1000		POS		POS		
7666	MoD sera	1000				POS		POS
7667	MoD sera	1000		POS		POS		POS
7693	MoD sera	1000		POS		POS		
7694	MoD sera	1000			POS		POS	
7696	MoD sera	1000					POS	
7767	MoD sera	1000						
7791	MoD sera	100				POS		POS

Appendix III Publications

The continued emergence of hantaviruses: isolation of a Seoul virus implicated in human disease, United Kingdom, October 2012

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Citation style for this article:
Jameson LJ, Logue CH, Atkinson B, Baker N, Galbraith SE, Carroll MW, Brooks T, Hewson R. The continued emergence of hantaviruses: isolation of a Seoul virus implicated in human disease, United Kingdom, October 2012. Euro Surveill. 2013;18(1):pii=20344. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20344

Article submitted on 04 December 2012 / published on 03 January 2013

Following a suspected case of hantavirus in a patient suffering from acute kidney injury, rodents from the patient's property in Yorkshire and the Humber, United Kingdom (UK) were screened for hantaviruses. Hantavirus RNA was detected via RT-PCR in two *Rattus norvegicus*. Complete sequencing and phylogenetic analysis established the virus as a Seoul hantavirus, which we have provisionally designated as strain Humber. This is the first hantavirus isolated from wild rodents in the UK and confirms the presence of a pathogenic Seoul virus in Europe.

In January 2012, as part of routine diagnostic services, the Rare and Imported Pathogens Laboratory (RIPL) at the Health Protection Agency (HPA) Porton, detected a suspected case of hantavirus infection in a patient diagnosed with acute kidney injury (AKI) from Yorkshire and the Humber, United Kingdom (UK). Positive serology by way of indirect immunofluorescence (Euroimmun, Germany) showed evidence of hantavirus antibodies specific to Hantaan virus (HTNV) and Seoul virus (SEOV) with rising IgG titres >1:10,000. The patient disclosed regular exposure to rodents at their home and noted that the rat population had increased in recent months. With permission from the patient, trapping of rodents in the vicinity of the family residence and farm was undertaken in February/March 2012, with the aim of confirming the presence of an aetiological agent in these rodents that might be responsible for the patient's AKI.

Rodent sampling

Permission was obtained from the patient and family to trap rodents at their residence. Between 28 February and 1 March 2012, rodents were trapped using a Longworth trap (Penlon Ltd., Oxford) or snap traps and, where necessary, humanely killed via cervical dislocation before being flash frozen on dry ice.

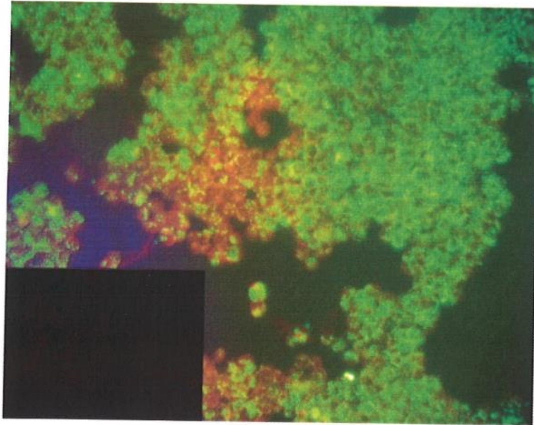
Eleven rodents were analysed for the presence of hantavirus RNA: five *Apodemus sylvaticus* (wood mouse), four *Rattus norvegicus* (Norway rat) and two *Myodes glareolus* (bank vole). Following tissue harvest by necropsy, lung tissue was homogenised and total RNA was extracted using QIAamp viral RNA mini kit (Qiagen). RNA from each rodent lung was subjected to a genus-specific RT-PCR assay using hantavirus primers targeting the S segment [1]. Two *R. norvegicus* (RN1 and RN4) samples produced amplicons of the expected size of ca. 850 bp, with SEOV R22 used as the positive control. cDNA from both amplicons was sequenced and showed the highest level of similarity (97%) to Seoul hantavirus IR461 (GenBank accession no. AF329388) in a BLAST analysis. Further evidence the virus was Seoul-like was obtained through virus culture of homogenised lung from RN4. Infected Vero E6 cells (ECACC Vero C1008, clone E6) showed positive staining to a commercial monoclonal antibody (Progen, Germany) against the nucleocapsid protein of SEOV R22 strain after ten days in culture. After a further three passages the majority of cells demonstrated positive staining (Figure 1), the cells were harvested and virus isolated as previously described [2]. The isolate was designated Seoul hantavirus, strain Humber. Attempts to culture virus from RN1 were unsuccessful.

Molecular analysis

Hantaviruses are enveloped viruses with a tripartite, negative-strand RNA genome encoding the small (S), medium (M) and large (L) segments. Following multiple nucleotide sequence alignments of available SEOV sequences in GenBank, primers were designed for each segment spanning overlapping intervals of ca. 500 bp. Between two and seven independent runs were completed for each amplicon with 100% sequence conformity. Complete sequences for all three segments (RN1) were generated using standard Sanger sequencing on

Figure 1

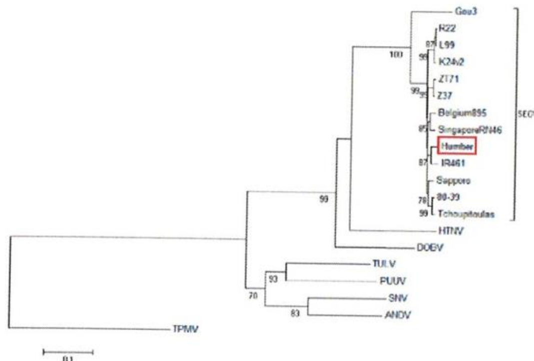
Vero E6 cells infected with Seoul hantavirus, strain Humber showing positive immunofluorescence, United Kingdom, October 2012



Negative and infected cells were stained with mouse monoclonal anti-Seoul N-protein antibody (Progen, Heidelberg, Germany) at 1:2 dilution, a secondary FITC-conjugated anti-mouse antibody at 1:64 (Sigma-Aldrich) and counterstained with 0.1% Evans blue. Viewed at 20x magnification under ultraviolet light; scattered, granular, punctate fluorescence (green) when compared to negative control Vero E6 cells only (inset, red) signified a positive reaction.

Figure 2

Phylogenetic analysis of the complete S segment of Seoul hantavirus, strain Humber, United Kingdom, October 2012



Horizontal distances represent the number of nucleotide differences. Bootstrap confidence limits exceeding 70% are shown for each branch node. Accession numbers for sequences extracted from GenBank: SEOV Gou3 AF288651, IR461 AF329388, K24v2 AF288655, R22 AF288295, L99 AF488708, Sapporo M34881, 80-39 NC005236, SingaporeRN46 GQ27495, ZT71 AY75171, Z37 AF187082, DOBV JF920150, HTNV AB620031, TULV Z49915, PUUV AB433845, SNV L2578, ANDV AF291702 and AY526097.

a 3130xl sequencer (Life Technologies); they comprised 1,768 nt, 3,651 nt and 6,530 nt, each encoding one open reading frame. These sequences were deposited in the GenBank database under accession numbers JX879768-70 designated as Seoul virus, strain Humber. Sequence data from RN4 were compared with deposited sequence data from RN1, this demonstrated 1 nt difference each in the S and M segments at positions 1,264 and 2,458 respectively.

The MEGA5 programme suite [3] was used to perform alignments using ClustalW and phylogenetic analysis. Phylogenetic trees were constructed from complete SEOV sequences using the neighbour-joining method, with bootstrap values obtained with 2,000 replicates. Subsequent genetic analysis of the S and M segments (RN1) confirmed their similarity to SEOV IR461 (Figures 2 and 3), with 63 (3.6%) and 149 (4.1%) nucleotide substitutions, respectively. The high homology and grouping of SEOV Humber with SEOV IR461 is surprising as IR461 is a distinctive strain [4] linked only to infections in laboratory workers in the UK and Belgium [5,6]. IR461 has not been detected in wild rodents.

Sequence data are not currently available for the L segment of SEOV IR461, however enough L segment sequences are available from isolates in the SEOV group to show that the L segment of the Humber strain (RN1) also clusters within the SEOV phylogenetic group of the hantavirus family (Figure 4).

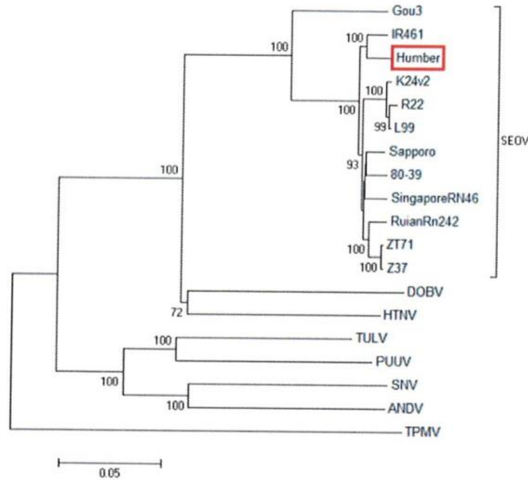
Discussion

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are a group of rodent-borne viruses with a wide global distribution. Human infection most often occurs when breathing in dried aerosolised excreta from infected rodents. Two clinical syndromes are associated with severe disease: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) [7]. In the UK there has been growing evidence for human and animal exposure to hantaviruses demonstrated by the detection of specific antibodies and classic HFRS disease.

As hantaviruses are wholly associated with their rodent and insectivore hosts, their distribution is limited to that of their respective host species. SEOV is an exceptional hantavirus in that it has a global distribution owing to the dispersal of its carrier host, rats, through global trade. Outside of Asia, SEOV has been confirmed by molecular methods in rats in Africa [8], the Americas [9] and Europe [10-12]. There are several ports located on the Humber estuary including: the UK's largest port by metric tons, Grimsby and Immingham, Kingston upon Hull, and the UK's most inland port, Goole. It is possible that the importation of infected rats has led to the establishment of SEOV in local populations of *R. norvegicus* in the Yorkshire and Humber region. Few acute human cases caused by infection with SEOV have been confirmed outside of Asia [13] and none have simultaneously reported identification

FIGURE 3

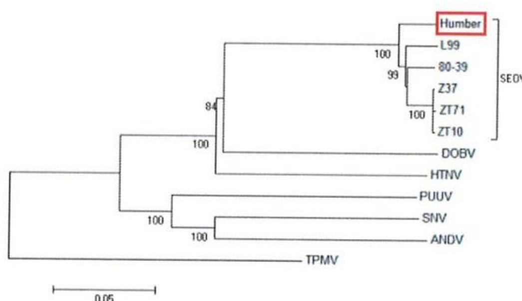
Phylogenetic analysis of the complete M segment of Seoul hantavirus, strain Humber, United Kingdom, October 2012



Horizontal distances represent the number of nucleotide differences. Bootstrap confidence limits exceeding 70% are shown for each branch node. Accession numbers for sequences extracted from GenBank: SEOV Gou3 AF145977, IR461 AF458104, K24v2 AF288654, R22 S68035, L99 AF288298, Sapporo M34882, 80-39 S47716, SingaporeRN46 GQ274943, RuianRn242 GU592928, ZT71 EF117248, Z37 AF190119, DOBV JF920149, HTNV AB620032, TULV NC005228, PUUV AB433852, SNV NC005215, ANDV AF291703 and TPMV NC010708.

FIGURE 4

Phylogenetic analysis of the complete L segment of Seoul hantavirus, strain Humber, United Kingdom, October 2012



Horizontal distances represent the number of nucleotide differences. Bootstrap confidence limits exceeding 70% are shown for each branch node. Accession numbers for sequences extracted from GenBank: SEOV L99 AF288297, 80-39 X56492, Z37 AF285266, ZT71 EF190551, ZT10 EF581094, DOBV JF920148, HTNV AB620033, PUUV AB574184, SNV L37901, ANDV AF291704 and TPMV DQ825770.

of the causative virus from rodents. Taking into consideration the patient's AKI, typical of SEOV, the high titre of antibodies detected specifically to the SEOV/HTNV group, the isolation of the virus from *R. norvegicus* from the patient's property, and its similarity to other pathogenic SEOV strains, it is highly likely the Humber strain is pathogenic to humans.

Due to the high levels of cross-reactivity between hantavirus species and the lack of viral detection in any published UK study, it has previously been impossible to confirm and identify the presence of a hantavirus in the UK. This represents the first isolation of a UK hantavirus from wild rodents and further confirmation of SEOV as a human pathogen outside of Asia. Given that *R. norvegicus* are ubiquitous in the UK, research is ongoing to determine the extent of human exposure to this virus in the region. Furthermore, with serological evidence specifically for SEOV previously reported in rats in Northern Ireland [14], it is unlikely this virus is restricted to north-east England.

Conclusion

Here we report the first detection and characterisation of a UK strain of Seoul hantavirus from wild rats. We feel it is important to raise awareness of hantavirus as a potential cause of renal disease in the UK, as Seoul hantaviruses are capable of causing moderate HFRS and we have strong indications this virus is linked to human disease. Our findings confirm the existence of a hantavirus in the UK and will allow further studies to evaluate its prevalence as a human pathogen.

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Pet rats as a source of hantavirus in England and Wales, 2013

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We report the detection of a strain of Seoul hantavirus (SEOV) in pet rats in England and Wales. The discovery followed an investigation of a case of haemorrhagic fever with renal syndrome in Wales. Hantavirus RNA was detected via real-time reverse transcription-polymerase chain reaction (RT-PCR) and classic RT-PCR in pet rats belonging to the patient. Sequencing and phylogenetic analysis confirmed the virus to be a SEOV that is similar, but not identical, to a previously reported United Kingdom strain from wild rats.

In January 2013, a male patient in north Wales suffering from acute kidney injury and clinically presenting with haemorrhagic fever and renal syndrome, tested seropositive (IgG 1:10,000) for Seoul (SEOV) and Hantaan (HTNV) hantavirus using indirect immunofluorescence (Euroimmun, Germany). A previous blood sample from October 2012, taken for unrelated purposes, was retrospectively obtained and tested. This sample demonstrated no antibody to hantavirus thus confirming serconversion as defined by Heyman et al., 2007 [1]. Epidemiological assessment identified the patient's two pet agouti rats (*Rattus norvegicus*) as a possible source of the hantavirus.

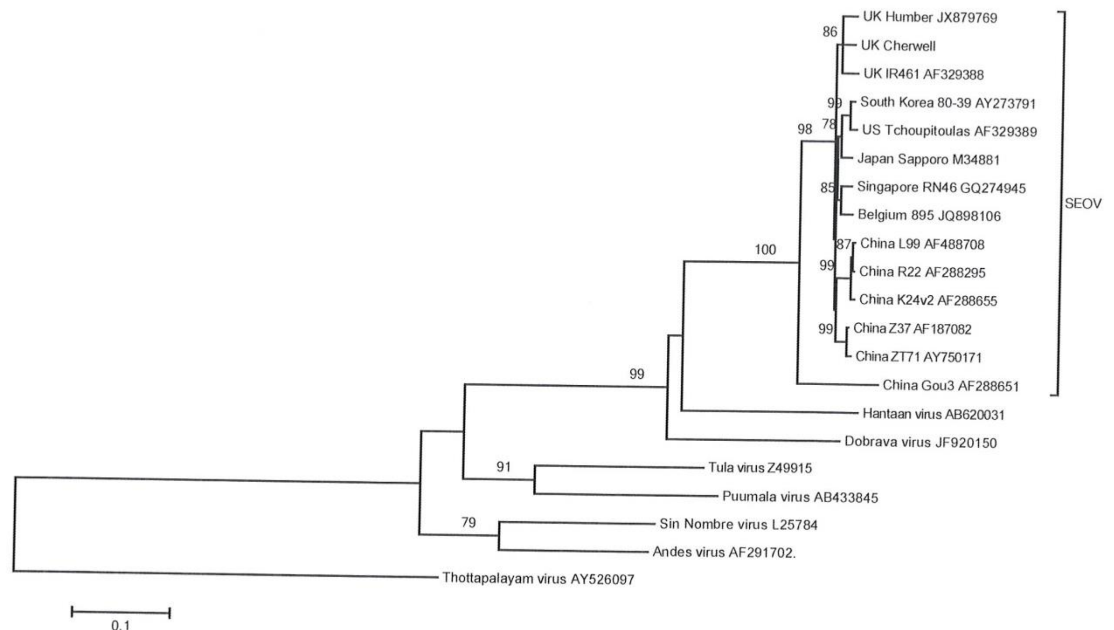
Virus investigation

Due to the patient's serious clinical condition the pet rats were in the care of the original breeder in Oxfordshire, south England. They were housed in a separate building to that of the breeder's pet rat colony. Blood samples from both rats, and urine from one of the two rats, were obtained and processed for RNA extraction using RNeasy kit (Qiagen). The extract was tested using a modified version of a previously published real-time reverse transcription-polymerase chain reaction (RT-PCR) assay for the dual detection of HTNV and SEOV [2]. The modifications adopted were: (i) use of a single Minor Groove Binder (MGB) -probe with a degenerate single base change (5' FAM-TCAATGGGRATACAACT-3') in place of the two non-degenerate published MGB-probes and (ii) use of the SuperScript III Platinum One-step qRT-PCR kit (Invitrogen) in accordance with the manufacturer's specifications. In-house validation confirmed that these changes had no detrimental impact on assay sensitivity and reduced the cost of the test.

The urine and blood samples tested positive and results were rapidly fed back to the incident control team, which included representatives from Health Protection Agency (HPA, Porton and Colindale), Thames Valley Health Protection Unit (HPU), Public Health Wales (PHW), Environmental Health (EH) and Animal Health and Veterinary Laboratories Agency (AHVLA). Due to uncertainties regarding the prevalence of this virus within the pet rat population in the United Kingdom (UK), the nature of its transmission, and the potential seriousness of human disease in this particular instance, the owner's consent was obtained to euthanase the two pet rats and remove them for further testing at the HPA Porton. One of the rats was processed as previously described [3] and viral RNA sourced directly from lung tissue was subjected to additional characterisation of the virus through standard Sanger sequencing on a 3130xl sequencer (Life Technologies). Sequencing of the S segment was achieved, confirming the virus was indeed a strain of SEOV similar, but not identical, to previously isolated UK SEOV strains: Humber (wild rats) [3] and IR461 (laboratory rats) [4]. We have provisionally designated this strain 'Cherwell'. Alignments of sequences from the virus strains were

FIGURE

Phylogenetic analysis of S segment sequence of the Seoul hantavirus Cherwell strain derived from a pet rat, England, February 2013



SEOV: Seoul hantavirus; UK: United Kingdom; US: United States.

Horizontal distances represent the number of nucleotide differences. Bootstrap confidence limits exceeding 70% are shown for each branch node. The phylogenetic tree was based on S segment sequences of previously published SEOV strains and other major hantavirus species as well as the sequence derived from the pet rat (*Rattus norvegicus*). The available geographical origin of the viral sequences, the viruses' names and respective GenBank accession numbers figure on the tree.

conducted using ClustalW and the molecular evolutionary genetics analysis (MEGA5) programme suite [5] was used to perform phylogenetic analysis. Comparisons between Cherwell and Humber S segments highlighted a total of 47 nucleotide differences, 36 within the open reading frame (ORF) resulting in one amino acid difference (methionine to isoleucine) at position 247. Phylogenetic analysis using the neighbour-joining method in MEGA5, with bootstrap values (2,000 replicates), placed the Cherwell S segment within the same group as the corresponding segments of Humber and IR461 (Figure). The sequence (1,769 nucleotides) was released to GenBank under accession number KC626089.

Following confirmation of hantavirus infection in these two rats, blood samples were obtained with the owner's permission, from 21 of the breeding colony rats and processed in the same way. Guidance was provided to the breeder to minimise the potential risk of infection while caring for the remaining rats, should they be positive. On the day of sampling, 7/21 rats had detectable RNA specific to HTNV/SEOV in blood. Preliminary sequence data indicates the same Cherwell strain in these rats.

Investigation of human contacts with rats

A blood sample was obtained from the patient's partner in Wales; this was negative for IgG antibodies. Blood samples were also obtained from the breeder and her spouse in England. The breeder, who had most contact with the rats in the breeding colony, had a low positive titre to HTNV and SEOV (IgG 1: 100). The breeder's spouse tested strongly positive with an IgG titre of 1: 10,000 to HTNV and SEOV, strongly suggesting hantavirus infection. Retrospective investigation of his medical records showed that he had been admitted to hospital in late 2011 with an undiagnosed viral illness resulting in acute renal impairment and thrombocytopenia. It is now considered highly likely that this was due to hantavirus infection; an archived blood sample from this admission was retrospectively tested

and demonstrated an IgG titre of 1: 1,000 to HTNV and SEOV. Detailed clinical findings of these cases will be published shortly [6]. In summary, of four people exposed to this particular population of rats, two had been clinically ill with renal impairment and were strongly seropositive, one had a low level of antibody with no clinical illness, and one was seronegative.

Control measures

Transmission of hantaviruses to humans most often occurs through breathing in aerosols of excreta from infected rodents [7]. Large quantities of infectious virus are intermittently excreted in the urine, saliva and faeces of infected rodents. The unique finding in this investigation of a strain of SEOV in pet rats, rather than wild rats, posed a challenge for infection control and involved a multi-disciplinary panel including medical/scientific experts from HPA, HPU, PHW, EH, and veterinarians from AHVLA. As part of this investigation the pet rats were euthanised, with owner's consent, in order to further scientific understanding of hantavirus infection in pet rats. Recommendations for management of any future incidents would be made on a case for case basis. Further studies are planned to gather evidence on the prevalence of this virus in the pet rat community which will inform future risk assessment and the provision of appropriate public health guidance. Interim guidance on minimising the infection to the pet rat community has been prepared [8] and will continue to be updated as the investigation progresses.

Discussion and conclusions

In January 2013, we reported the isolation of a UK strain of SEOV (Humber) from wild rats in north-east England [3]. We now report a second SEOV strain from the UK which is similar, but genetically distinct from the Humber isolate and laboratory-associated IR461 isolate. Further research will continue to investigate the relationship of these three strains. The pet rats identified in this investigation are part of a wider pet rat community which partakes in national and international shows and fosters international sharing of rats. Currently, the prevalence of SEOV in the UK pet rat community is unknown, but if SEOV infection was widespread, there would be implications for the wider (non-UK) pet rat community. The HPA is continuing to investigate this newly recognised source of hantavirus infection in collaboration with the AHVLA. Should overall findings indicate that further health protection advice is necessary, the HPA will work with the relevant partners to provide this.

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Conflicts of interest

None declared.

Authors' contributions

Lisa Jameson, Barry Atkinson and Roger Hewson were responsible for the virological analysis and the interpretation of laboratory results. Surabhi Taori, Jane Osborne and Tim Brooks coordinated the national public health response and were responsible for clinical diagnosis. Peter Levick, Charlotte Featherstone and Guda van der Burgt performed the blood sampling. Noel McCarthy and Judy Hart coordinated the local public health response. Amanda Walsh contributed to the national public health response and prepared public health guidance for dissemination. Lisa Jameson wrote the draft manuscript and all authors revised and approved the final version.

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Case Report

UK hantavirus, renal failure, and pet rats

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In November, 2012, a 28-year-old man, presented with a 4-day history of fever, shivers, sweating, and vomiting. He had type-2 diabetes, which was being treated with sitagliptin and metformin. On admission he had evidence of a systemic inflammatory response (temperature 39·3°C, pulse 160 bpm, respiratory rate 30 per min, white cell count $15·0\times10^9$ per L, with 12·3 neutrophils and 0·2 myelocytes), abnormalities of blood clotting (INR 1·6, PTT 57 s, fibrinogen 0·99 g/L (normal range 1·5–4·5); platelets 19×10^9 per L), multi-organ failure (creatinine 167 μmol/L, raised alanine aminotransferase 511 U/L and bilirubin 87 μmol/L), progressive hypoxia, hyperglycaemia glucose 20·6 mmol/L), and lactic acidosis (PH 7·29, lactate 7·5 mmol/L). He was diagnosed with overwhelming sepsis and transferred to the intensive care unit. Initial treatment was with piperacillin-tazobactam, insulin, oxygen, and aggressive fluid replacement, including platelet infusions, fresh frozen plasma, and cryoprecipitate. Ventilatory support was required 15 h after admission, at which time he was anuric. Renal replacement therapy was needed for 21 days and ventilatory support for 38 days, partly because of pseudomonas superinfection of the chest that was diagnosed on day 17.

Tests for legionella and leptospira and initial blood cultures were negative. Serum taken 30 days after admission had a high IgG titre to Seoul hantavirus (1:10 000 by IFA, Euroimmun, Medizinische Labordiagnostika AG), although serum from 1 month before admission (sent for hepatitis screening because of a mild transaminasaemia) was negative. Hantavirus RNA was not detected in either sample. We learnt that he kept two pet agouti rats (*Rattus norvegicus*) that he had acquired from a larger pack bred in England. Seoul hantavirus RNA was detected by RT-PCR[1] in blood taken from these two rats and from seven of the larger group. In November, 2011, one of the English owners had been hospitalised with fever, renal impairment, splenomegaly, and thrombocytopenia that was secondary to an unidentified viral illness. Retrospective Seoul hantavirus serological tests showed an increase in IgG titre (1:1000 to 1:10 000) from November, 2011, to January, 2013, suggesting a hantaviral cause for this episode. The second owner had a low IgG titre (1:100, January, 2013), suggesting past hantavirus infection but not recalling any notable illness. Low-level cross-reactivity to other hantaviruses was frequently seen but was disregarded since Seoul hantavirus RNA.[1] was identified from the rats. Our patient left hospital on day 52 with fully recovered renal function.

Hantavirus infection presents as hantavirus pulmonary syndrome in the Americas and haemorrhagic fever with renal syndrome in Eurasia.[2] Seoul hantaviruses can show a spectrum ranging from benign undifferentiated febrile illness without renal failure to diffuse haemorrhage, intractable shock, and multiorgan failure. When confronted with such an illness, a differential diagnosis of hantavirus infection should be considered, especially if history suggests contact with rats, even in the absence of recent foreign travel. Viraemia is very short and viral RNA is detectable only in early disease. Diagnosis is therefore usually done by serological testing. Supportive therapy is the mainstay of care for patients with haemorrhagic fever with renal syndrome.[3] In 2012, a hantavirus infection acquired in the UK was traced to wild rats and challenged the notion that hantavirus infection is not indigenous to the UK.[4] The two cases reported here are both associated with pet rats and provide strong evidence of transmission to the community that owns pet rats. Since there is a regular interaction between different rat colonies by way of cross-breeding, pet-sitting, and rat exhibitions, this virus may be more widespread than recognised. Hantaviruses are transmitted by direct contact with rodents or their excrement,[2] and prevention is targeted towards reducing this exposure. Specific guidance on preventive measures is available from the Health Protection Agency[5] and should be made available to rat owners.

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Figure: Photograph of pet agouti rat

Contributors

AC, PD looked after the patient, SKT, LJJ, PJD wrote the report. SKT, LJJ, NDM, JH, JCO, MS, TJGB did the investigations and analysis. Written consent to publication was obtained.

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Article

Prevalence of Antibodies against Hantaviruses in Serum and Saliva of Adults Living or Working on Farms in Yorkshire, United Kingdom

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Abstract: Hantaviruses are an established cause of haemorrhagic fever with renal syndrome (HFRS) in Europe. Following a confirmed case of HFRS in the UK, in an individual residing on a farm in North Yorkshire and the Humber, a tidal estuary on the east coast of Northern England, and the subsequent isolation of a Seoul hantavirus from rats trapped on the patient's farm, it was considered appropriate to further investigate the public health risk of this virus in the region. Of a total 119 individuals tested, nine (7.6%) were seropositive for hantavirus antibodies. Seven of the seropositive samples showed a stronger reaction to Seoul and Hantaan compared to other clinically relevant hantaviruses. Observation of rodents during the day, in particular mice, was associated with a reduced risk of seropositivity. In addition to one region known to be at risk following an acute case,

five further potential risk areas have been identified. This study supports recently published evidence that hantaviruses are likely to be of public health interest in the region.

Keywords: farmers; hantavirus; serology; saliva; serosurveillance; haemorrhagic fever with renal syndrome; Seoul virus

1. Introduction

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are a globally distributed group of emerging rodent- and insectivore-borne RNA viruses named after the prototype strain, Hantaan virus (HTNV) discovered in Asia. Several distinct species are known to circulate; those confirmed to cause human disease within Europe are: Dobrava (DOBV), Puumala (PUUV), Tula (TULV), Saaremaa (SAAV) and Seoul (SEOV) viruses [1]. Andes (ANDV) and Sin Nombre (SNV) are the predominant hantavirus species responsible for serious human disease in South and North America, respectively [2].

Each hantavirus has a specific rodent or insectivore species acting as its natural reservoir which intermittently excretes infectious virus in urine, saliva and faeces [3,4]. Seoul is a unique member of the hantavirus family in that it is considered to have a potential for global distribution due to its reservoir host, the brown rat (*Rattus norvegicus*) being ubiquitous on all continents with the exception of Antarctica. Viral transmission to humans typically occurs when materials contaminated with rat excreta are disturbed causing virus particles to aerosolise and be inhaled. Contact between broken mucosal membranes and virus contaminated materials or through a direct bite are also potential routes of transmission [1].

Known risk populations for contracting haemorrhagic fever with renal syndrome (HFRS) are rural workers, military personnel, underground workers and pest controllers; as their occupation brings them in close contact with rodents [2,5]. Severe disease is more frequently detected in persons between the ages 20–50 years old. The disease has been reported in both sexes; however, possibly linked to occupational risk there is a significantly higher prevalence in males [1,3].

The most common and convenient method for determining exposure to hantaviruses is detection of specific antibodies [6]. In the United Kingdom (UK) several studies have used this method for determining seroprevalence of hantavirus immunoglobin G (IgG) in apparently healthy persons. One assessment of serostatus in Northern Ireland found 1.2% (4/320) of farmers had antibodies against hantavirus [7]. The most recent serosurvey of 606 farmers, farm workers and their families in Herefordshire and Lancashire, England, demonstrated a seroprevalence of 4.7% in the first year and 4.8% in the second year of the study [8]. Both studies have provided evidence for hantavirus exposure in the UK; however, it was not until 2012 that a causative hantavirus species was confirmed. Rodent trapping on the farm of an acutely ill patient led to the identification and isolation of the first UK strain of hantavirus from wild rats (*Rattus norvegicus*); a SEOV designated Humber [9]. The patient was a resident on a small livestock farm in North Yorkshire and Humber. The patient disclosed regular exposure to rats with a noticeable increase in rat numbers in the months preceding their illness. Two of four rats trapped and tested from the farm were positive for SEOV RNA.

With a confirmed autochthonous case of HFRS and virus isolation from the local rat population, further investigations were undertaken to determine the extent of human exposure to this virus in the region. The primary objective of the present study was to determine the seroprevalence of hantavirus antibodies in an occupationally exposed group in a known risk area of the UK.

A further objective was to identify a more convenient method of sampling for future studies. The use of saliva is an increasing area of research for diagnostic sampling as it is easy to collect, non invasive to the patient and readily available with healthy adults producing between 500–1,500 mL of saliva per day [10]. Whilst the predominant immunoglobulin isotype in saliva is immunoglobin A (IgA), IgG is also active within the oral cavity where it is mainly derived from gingival cervicular fluid and mucosal transduate [10,11]. A correlation between saliva IgG and serum IgG has been demonstrated for several viruses including human immunodeficiency virus, hepatitis A virus, hepatitis C virus, Epstein Barr virus, cytomegalovirus and rubella virus [10]. To the best of our knowledge, no published survey has researched the use of saliva collection for detection of IgG levels specific to hantaviruses.

2. Results

128 volunteers were recruited but nine were excluded from analysis because of ineligibility. Nine (7.6%; 95% CI 4.0 to 13.8%) of the 119 eligible volunteers had detectable levels of IgG serum antibodies against hantaviruses. As demonstrated in Table 1, 7/9 positive sera reacted strongly to HTNV and/or SEOV and 2/9 positive sera reacted strongly to SNV and/or PUUV. 112/119 disclosed some form of travel outside of the UK in their lifetime; crucially the two volunteers whose serum cross-reacted with SNV had not travelled outside of Europe. From the 119 eligible volunteers, 117 also supplied a saliva sample. From the serum positive individuals 8/8 corresponding saliva samples showed characteristic positive fluorescence compared to negative saliva samples (13/13) tested. One saliva sample was not collected from a volunteer with positive serum and therefore was unable to be tested.

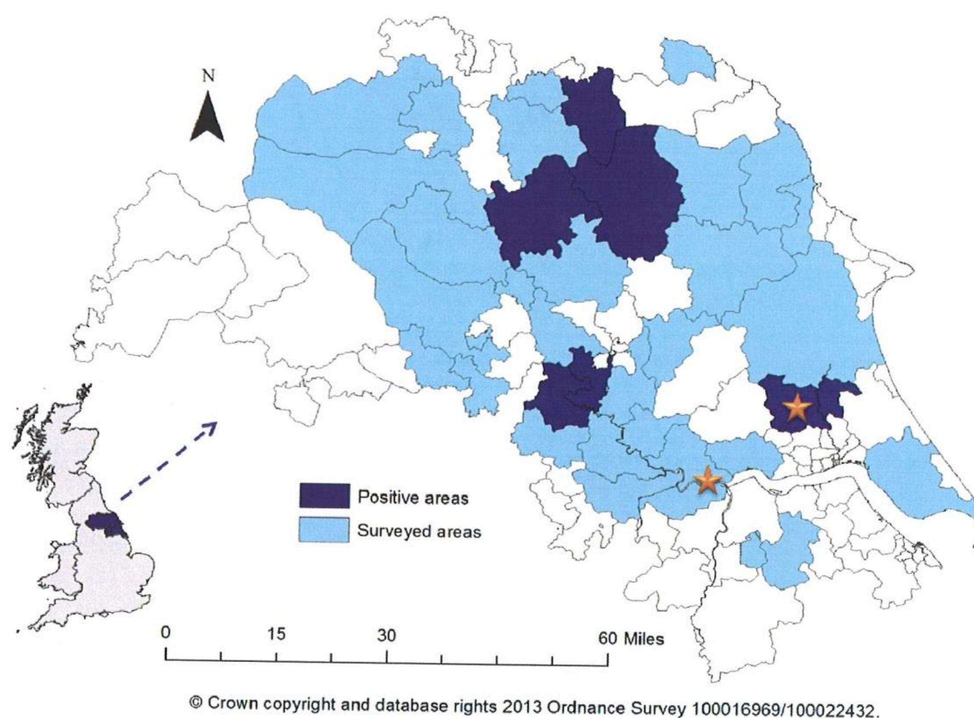
Table 1. Pattern of reactivity for immunofluorescence assay at 1:100 dilution in sera and (saliva).

Sample	DOBV	HTNV	PUUV	SAAV	SNV	SEOV	Result
1	− (−)	− (+)	++ (+)	− (−)	++ (+)	+ (++)	PUUV/SNV
2	− (−)	++ (+)	− (+)	+ (+)	− (+)	++ (+)	HTNV/SEOV
3	− (−)	+ (+)	+ (+)	− (−)	+ (+)	++ (+)	SEOV
4	− (−)	− (+)	+++ (−)	− (−)	− (+)	+ (+)	PUUV
5	− (+)	+++ (+)	− (−)	− (−)	− (+)	++ (+)	HTNV/SEOV
6	− (+)	++ (+)	− (−)	− (−)	− (+)	+ (+)	HTNV/SEOV
7	− (−)	+++ (++)	− (+)	− (−)	− (+)	++ (++)	HTNV/SEOV
8	−	++	−	−	−	+	HTNV/SEOV
9	− (+)	++ (++)	− (+)	− (+)	− (+)	+ (++)	HTNV/SEOV

Reactivity score: − negative, + weak, ++ moderate, +++ strong.

The majority of volunteers (98/119) disclosed their main or full-time occupation to be farming; the remaining volunteers (21/119) reside on a farm. Postcode information was collected for 115/119 eligible participants allowing geographical mapping of seropositive individuals; 2/4 volunteers who did not provide this information were seropositive, with both samples identifying exposure to PUUV/SNV-like hantavirus. 6/34 districts included in the survey had at least one positive sample (Figure 1). One of the six regions was previously known to be a risk area due to one of the acute cases occurring there, however five did not have any previous information on hantavirus prevalence.

Figure 1. Map of Yorkshire and the Humber showing surveyed areas and areas with a minimum of one positive serum sample recorded. Stars indicate location of previous acute cases. Insert shows location of Yorkshire within the United Kingdom.



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As detailed in Table 2, analyses from the questionnaire results showed little association between any measured demographic or farm specific variables and serostatus. In contrast, differences in rodent contact identified potential areas for future investigation. All seropositive individuals confirmed they had rodents on their property with 9/9 reporting rats and 6/9 mice. Seeing rodents during the day appears to give a significant reduction in risk ($p > 0.001$, OR 0.006) with only 2/9 positive individuals noticing rodents during the day compared to 9/9 seeing rodents at night. Further investigation of which rodent species are seen highlighted that reporting mice on the farm was also associated with decreased risk ($p = 0.02$, OR 0.1, 95% CI 0.02 to 0.96).

Table 2. Analysis of factors associated with seropositivity to hantaviruses.

Category	No. positive (%)	No. Negative	No. tested	Odds ratio	CI	p-value*
Age (years)						
<30	3 (14.3)	18	21	2.5	0.4 to 13.2	0.2
≥30	6 (6.1)	92	98			
Median	45	51				
Gender						
Male	7 (7.0)	93	100			
Female	2 (10.5)	17	19	1.6	0.1 to 9.2	0.6
Farmer						
Yes	8 (8.2)	90	98	1.8	0.2 to 82.7	>0.99
No	1 (4.8)	20	21			
Farm type						
Animal	4 (11.4)	31	35			0.7
Arable	1 (7.1)	13	14	1.7	0.1 to 88.8	
Mixed	4 (5.7)	66	70	1.9	0.3 to 11	
Farm classification						
Cereal	5 (6.3)	75	80	0.7	0.1 to 3.9	0.7
Cropping	3 (7.5)	37	40	1.1	0.2 to 5.5	>0.99
Horticulture	0	4	4	NA		
Pig	1 (5.9)	16	17	0.8	0.02 to 6.7	>0.99
Poultry	3 (15.0)	17	20	3.0	0.4 to 15.6	0.1
Dairy	1 (5.6)	17	18	0.8	0.02 to 6.2	>0.99
Livestock	7 (8.1)	79	86	1.8	0.3 to 18.2	0.7
Other	0	3	3	NA		
Farm size						
<500	6 (7.2)	77	83			
≥500	3 (9.4)	29	32	2.0	0.5 to 11.5	0.4
Median	330	296				
Materials						
Silage	9 (9.8)	83	92	NA	0.8 to Inf	0.06
Bedding	9 (8.6)	96	105	NA	0.4 to Inf	0.4
Feed	9 (9.1)	90	99	NA	0.6 to Inf	0.2
Hay	8 (9.3)	78	86	4.2	0.5 to 190.1	0.3
Timber	7 (6.7)	97	104	0.8	0.1 to 8.4	0.7
Coal	6 (9.8)	55	61	2.3	0.5 to 15.0	0.3
Rodents seen						
Mice	**6 (5.1)**	**112**	**118**	**0.1**	**0.02** to **0.96**	**0.02**
Rats	9 (7.6)	109	118	NA	0.2 to Inf	>0.99
During day	**2 (1.7)**	**117**	**119**	**0.006**	**0.0004** to **0.05**	**<0.001**
At night	9 (7.6)	109	118	NA	0.2 to Inf	>0.99
Rodent control						
Professional	4 (14.3)	24	28	2.8	0.5 to 14.2	0.2
Self	5 (5.6)	85	90			

* Calculated using Fischer's exact test.

3. Discussion

In the UK, there is a lack of contemporary data available on the prevalence of hantavirus antibodies in at-risk populations. Only isolated cases have been reported with the majority of seroprevalence studies undertaken in the 1980s and 1990s. Uncertainty surrounding the presence of a hantavirus in the UK has been resolved following a recent cases of acute hantavirus infection in North Yorkshire and the Humber region which led to the characterisation and isolation of a UK variant of SEOV from wild *R. norvegicus*. As brown rats are the most likely known source for hantavirus infections in the region, farmers and those who reside on a farm were chosen for this study as they have an increased risk for contact with rats and their excrement. While it was expected contact between the study population and rats would be higher than the general population it was surprising to find 92.4% of questionnaire respondents regularly seeing rats on their residence. From a public health perspective this is of concern given that SEOV is not the only pathogen of concern transmissible from rats as previously reviewed by Webster [12].

The most comparable study, over 15 years ago, looking at exposure to a variety of zoonotic organisms in English farmers found seroprevalence to hantaviruses to be 4.7% [8]. Although our current study found the frequency to be slightly higher at 7.6%, the previous estimate lies within our 95% confidence interval. Nevertheless, the current study may be expected to identify a higher prevalence as the study area included a known risk area whereas the previous study's locations had no link to symptomatic hantavirus infection.

We found no significant association between contact with different agricultural materials and hantavirus seroprevalence. Neither did we find any significant association between hantavirus infections and various demographic factors such as age or gender. This may be a direct result of the low number of positives, low uptake of women and older volunteers and the study population encompassing a convenience rather than random sampling strategy. Due to the low number of total positive volunteers the analysis of individual factors such as gender and farm type was limited and therefore may inaccurately imply there is no correlation. In retrospect it would have been valuable to have sampled more volunteers under the age of 30. This may have provided some indication as to whether SEOV was introduced recently to the region or has been circulating for some time.

Saliva samples from 8/9 (1 sample not collected) volunteers with positive serum samples demonstrated reactive IgG antibodies against hantaviruses. No other alterations were made to the assay other than sample type. Whilst saliva appears to be promising for determining a simple positive sample it appears to be less suitable for identifying serotype. Most samples showed a decrease in recorded fluorescence in comparison to serum samples and were less specific with at least mild reactivity recorded for SNV, SEOV and HTNV for all eight samples tested. A further weakness of the use of saliva was discovered following repeated testing of the samples up to 11 months after collection; samples which originally demonstrated reactivity no longer did so. It is most likely this is due to sample degradation as saliva contains many micro-organisms and proteases which may affect sample stability. Repeated freeze thaws and a lack of addition of additives to reduce degradation such as, sodium azide or protease inhibitors may have contributed to this. Nonetheless collection of saliva is a convenient and less-invasive sampling method for serosurveillance surveys with proven effectiveness for other viruses, and its potential use for hantavirus seroprevalence studies is worth further investigation.

It was expected that the majority of serum samples reactive to SEOV would show considerable cross-reactivity with HTNV, a known issue using this method. The finding of antibodies to hantaviruses other than SEOV in two individuals was not unexpected. An acute human case in 2010 with symptoms typical of HFRS produced a similar result with serological assays indicating cross-reaction with a PUUV/SNV-like hantavirus [13]. In addition, Ahlm *et al.* [14] reported similar findings with three samples reacting to SNV in a study of Swedish farmers. This raises the prospect of another circulating hantavirus in the UK, most likely PUUV as SNV has not been detected outside of the Americas, for which the environmental factors have already been demonstrated to be suitable and the reservoir host (*Myodes glareolus*) is ubiquitous [15]. One further possibility is a novel UK hantavirus. Recently, Pounder *et al.* [16] described detection of a vole-associated hantavirus in north-west England. Blood from the field vole (*Microtus agrestis*) demonstrated cross-reactivity with PUUV using an indirect fluorescent antibody test but molecular techniques suggested it to be distinct from other classified hantavirus species. Neutralisation is not a technique routinely used at PHE Porton for examination of hantavirus infection. However, determination of the specific hantavirus in individual samples that are reactive to would be of interest particularly for the PUUV/SNV serum samples, and this will be considered in future.

One variable of statistical significance, highlighted by this serosurvey was the presence of rodents during the day; surprisingly this appeared to be protective. An explanation for this may reside with the questionnaire design. Volunteers were asked if rodents were observed during the day, this was not further explored as to whether it was rats or mice seen during the day. In the absence of an extremely large population, rats are rarely seen during the day whereas mice are less constrained by such population dynamics. Mice are naturally averse to the presence of rats, therefore their presence may indicate a lower likelihood of rats and in turn a decreased potential exposure to SEOV. This is further supported by volunteers who reported seeing mice on their property being less likely to be seropositive.

Future investigations should look to include analysis of risk areas for hantavirus and flood zones which was out-with the scope of this preliminary study. This is particularly prudent given that North Yorkshire and the Humber is a high risk area for floods within the UK. Flooding is a recognised trigger for outbreaks of rat-borne diseases, in particular leptospirosis but also hantavirus, due to changes in rat behaviour leading to increased contact with humans [17].

4. Materials and Methodology

4.1. Study Location

North Yorkshire and the Humber is located in the region of Yorkshire, North East England 53°57′30″N 1°4′49″W (York). The distribution of farm types varies across the county: livestock is predominant in the north-west, cereal and general cropping in the east/south-east, with the north, north-east and central areas generally more fragmented with a mixture of farm types.

It has a population of approximately 1, 700,000 within which there is generally an even gender ratio, with the exception of 80+ where females: male ratio is ~1.8:1. North Yorkshire has a high proportion (24%) of its population over the age of 65. The study area incorporated the following

locations: Craven, East Riding of Yorkshire, Hambleton, Harrogate, Hull, North East Lincolnshire, North Lincolnshire, North Yorkshire, Richmondshire, Ryedale, Scarborough, Selby and York.

4.2. Study Subjects

Subjects included in the study were adult volunteers (≥18 years old on day of sampling) who verbally confirmed they live or work on a farm within the study area and who consented to blood donation for the purpose of anonymous screening for the presence of antibodies against hantavirus. This group was selected because of their presumed increased risk of exposure to potential reservoirs and as representative of the population to which the acute case belonged.

Recruitment was undertaken between February and April 2013. A convenience sample of volunteers was obtained through local press releases, newsletters and recruitment drives at local meetings and markets. Volunteers were provided with study information and a consent form before sample collection. A questionnaire was designed and piloted following informed discussions with members of the farming community. The questionnaire was completed by face to face interview and included sections on occupation, working conditions, travel history and contact with rodents.

Blood was collected by venepuncture and serum separated from the blood using standard methods of density gradient centrifugation [18]. Saliva was collected using the Salivette system (Sarstedt Ltd., Leicester, UK) with saliva separated as described by Lamey and Nolan [19]. Serum and saliva samples were stored frozen at −80 °C until tested. This study was approved by NHS National Research Ethics Service reference 05/Q2008/7.

4.3. Data Processing and Statistical Analysis

Sample size calculation was based on the most recent and comparable survey where 4.7% of farmers were seropositive [8]. A minimum sample size of 73 was calculated to be sufficient to estimate the proportion seropositive, assuming the true prevalence is 5%, with 95% confidence level and 5% precision. As sampling was planned to occur at farmer meetings and markets, the sample size was recalculated to account for clustering assuming an intra-cluster correlation coefficient of 0.1 as $n1 = n(1 + p(m - 1))$, where n is the estimated sample size assuming simple random sampling, n1 is the new estimate of the required sample size, p is the intra-cluster correlation coefficient and m is the number of clusters (here assumed to be 5; [20]). Hence, the revised sample size was approximately 100.

Statistical analyses were performed with the software program Minitab version 16 (Minitab Inc., State College, PA, USA) and the R language for statistical computing [21]. Fisher's exact test was used to compare proportions. This approach did not account for the clustering within the data (due to the selection process) and hence may result in increased risk Type I errors. However, the goal of this analysis was hypothesis generation rather than hypothesis testing and hence this limitation was accepted. The results of theses comparisons must be interpreted in light of this, and the limited statistical power of the study to detect differences in seropositivity between exposure groups. Significance was set at $p < 0.05$; 95% confidence intervals for proportions were calculated using the Wilson Method [22].

4.4. Serological Test

All sera were screened for presence of hantavirus IgG using anti-hantavirus indirect immunofluorescence test mosaic 1 (Euroimmun, Luebeck, Germany) in accordance with the manufacturer's recommendations. Each sample was compared to a known positive (Euroimmun CI 278h-0101-1G) and a negative control (human sera). The chosen assay screens for antibodies against the most clinically relevant pathogenic hantavirus species (DOBV, HTNV, PUUV, SAAV, SEOV and SNV). Samples showing repeatable characteristic cytoplasmic fluorescence at a dilution of 1:100 were considered positive. For each positive serum sample the corresponding saliva sample was screened using the same test and conditions as that for the sera. Thirteen random saliva samples were screened as negative controls.

5. Conclusions

While it is expected that farmers will have higher seroprevalence rates than the general population, this study is useful in furthering the understanding of hantaviruses, and most likely SEOV, in North Yorkshire and Humber and will aid future studies with a view to reducing risk. Seoul virus has previously been considered to be mainly an urban hantavirus almost exclusively reported in Asia. However, our results support the assumption of widespread rural circulation of SEOV in the region. In addition to the recent acute clinical case, five further areas of the county demonstrate seropositivity and regular contact with a common carrier host; therefore hantavirus should be included in the differential diagnosis of patients with suspected leptospirosis in the North Yorkshire and Humber region.

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Author Contributions

L.J.J., A.N., and E.N.C.N. assisted with recruitment of volunteers, collection of samples and data collection. L.J.J. performed the experiments. L.J.J., L.C., and R.M.C. performed data analysis. N.J.B. and L.J.J. designed saliva sampling methodology. A.N., R.H., and M.W.C. were responsible for overseeing the study and initiation of collaborations. All authors contributed to study design, final interpretation of results and assisted in writing the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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CASE REPORT

Hantavirus: an infectious cause of acute kidney injury in the UK

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SUMMARY

We present a case of an undifferentiated febrile illness in a 59-year-old man from East Yorkshire. He was initially treated for leptospirosis due to the fact that he had farm exposure and the findings of acute kidney injury (AKI), thrombocytopenia and a raised alanine transferase (ALT) on his initial blood results. Serology tests later proved him to have had another rodent-borne illness: hantavirus. An investigation by Public Health England (formerly known as Health Protection Agency) (PHE) went on to prove the presence of the same serotype of hantavirus in rats caught on the patient's property. After an initial deterioration, the patient made a relatively uneventful recovery and all his blood tests returned to normal levels.

BACKGROUND

Hantavirus is a recognised cause of acute kidney injury (AKI) and has been listed as 1 of the 15 major factors leading to a rise in absolute incidence of AKI throughout the Western world.[1] Although common in Europe, there have been relatively a few recent case reports of hantavirus in the UK other than those associated with exposure abroad.[2] In the absence of a travel history the diagnosis is rarely if ever thought of or tested for by general practitioners (GPs) or hospital physicians. It is our opinion that hantavirus is underdiagnosed in the UK and should be considered in the differential diagnosis whenever leptospirosis is considered. Our case report in conjunction with the isolation of the virus from rats caught on the patient's property strongly supports the presence of a pathogenic UK hantavirus. It is our hope that this case report will increase awareness of hantavirus as a potential diagnosis in the UK. Experience from other endemic countries shows that early diagnosis of hantavirus provides a twofold benefit: (1) reducing the cost of unnecessary treatment and (2) improving the clinical outcome for the patient. Where clinical awareness is high the need for dialysis as a treatment for hantavirus-induced AKI has fallen to less than 5%. Complications such as hyperkalaemia and severe uraemia can often be avoided with careful fluid balance.[3] In Europe, patients with early and correct diagnosis were significantly less likely to be hospitalised and given inappropriate antibiotic treatment compared with those with a delayed diagnosis.[4]

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CASE PRESENTATION

In December 2011, a 59-year-old man presented to Hull Royal Infirmary with a 2-day history of fever, rigours, anorexia and a dry cough. He lived on a small hold farm in East Yorkshire. Approximately 2 weeks prior to admission, he had been treated with oral flucloxacillin by his GP for mild cellulitis following a cut to his wrist on some metal in a pig field. The wound had initially gone unnoticed by the patient and therefore there was some exposure of the wound to the dirt and untreated water in the pig field. Along with pigs, there were horses and cows on the farm and with hindsight the patient and his wife noted that there had been an increase in the numbers of rats seen around the farm in the weeks prior to his illness. He had a medical history of psoriasis and pulmonary sarcoidosis, which was quiescent and he was on no regular medication. He had no travel outside the UK for at least 3 years. Apart from a temperature of 38.1°C, examination on admission was normal. The wound on his wrist had completely healed with no residual signs of any cellulitis. The patient was transferred to the infectious diseases ward in Castle Hill hospital where further tests were performed.

INVESTIGATIONS

Initial blood results demonstrated AKI, deranged liver enzymes, lymphopenia and thrombocytopenia (table 1). His chest X-ray (CXR) was clear. Three sets of blood cultures were negative, as was a throat swab for respiratory viruses and urinary *Legionella* antigen.

During his admission hepatitis A, B, C and E and *Leptospira* serology were checked and were all negative. Owing to the clinical picture and the farming history, blood was sent to Rare and Imported Pathogens, Public Health England (PHE), Porton Down for hantavirus serology. Serum was confirmed strongly positive particularly to Seoul and Hantaan IgM (strip-immunoassay) and IgG (immunofluorescence assay, IFA) as detailed in table 2. Serology was repeated 12 days later and pointed towards this being a Seoul serotype (1:40 000) by IFA. No detectable hantavirus RNA was found in either blood or urine samples. However, further circumstantial evidence that this patient's infection was caused by a Seoul hantavirus was provided when a subsequent PHE investigation isolated a new UK strain of Seoul hantavirus from rats on the patient's property.[5]

DIFFERENTIAL DIAGNOSIS

In cases of undifferentiated fever such as this there is generally a wide differential diagnosis including bacterial and viral infections, autoimmune processes and malignancy. Given this patient's previous

Table 1 Selected blood results

	25/12/11	26/12/11	28/12/11	29/12/11	31/12/11	01/01/12	04/01/12	06/01/12	13/01/12	27/02/12
Haemoglobin, g/dL	16.1	15.4	15.5	14.6	14.9	14.2	13.1	12.5		14.0
White cell count×10^9/L	2.6	4.5	7.1	5.5	5.8	6.6	9.4	5.7		6.0
Lymphocytes×10^9/L	0.52	0.79	1.98							
Platelets×10^9	49	58	70	179	210	262	271	256		225
Prothrombin time, s		11.2			10.4	10.9	12.7	11.6		
APTT, s	32.2	30.2			22.4	23.8	26.9	26.4		
Sodium, mmol/L	131	131	129	129	136	139	136	141	142	140
Potassium, mmol/L	4.0	3.8	3.9	3.5	3.9	4.0	3.5	4.0	5.0	4.5
Bicarbonate, mmol/L	24	26	20	20	22	23	23	27	25	23
Urea, mmol/L	8.3	8.1	15.0	20.1	19.2	15.5	6.5	5.5	5.2	7.3
Creatinine, μmol/L	132	144	299	378	333	277	148	134	131	96
Bilirubin, μmol/L	20	20	41	57	38	26	22	19	13	13
Alkaline phosphatase, iu/L	81	76	106	163	323	385	263	229	189	58
Alanine aminotransferase, iu/L	305	221	245	248	143	120	64	57	47	18
Albumin, g/L	32	30	26	26	22	21	22	23	29	39
CRP, mg/L	135	97	113	94	27	22	189	71	8.4	1.2

APTT, activated partial thromboplastin time; CRP, C reactive protein.

history of sarcoidosis a flare-up of this also needs to be considered. However, his farm exposure and in particular the history of his wrist injury in conjunction with the AKI, thrombocytopenia and deranged liver enzymes meant that the main differential diagnosis was leptospirosis.

TREATMENT

As leptospirosis was the working diagnosis he was given intravenous benzyl penicillin and oral ciprofloxacin. Initially, he remained unwell with a spiking temperature and his renal function deteriorated (see table 1). On day 4 his temperature settled and shortly afterwards his blood tests started to improve. The antibiotics were stopped and at his request he was discharged home for close outpatient follow-up. Shortly after discharge, he complained of increasing lower abdominal pain and his C reactive protein (CRP) rose sharply. A repeat CXR remained clear, urine culture was negative and a CT scan of his abdomen and pelvis was reported as being normal. His abdominal pain and CRP settled over the next few days without further intervention.

OUTCOME AND FOLLOW-UP

Over the next few weeks, he continued to make an uneventful recovery. By the end of February 2012, 2 months after the initial illness, all his blood tests had normalised. Interestingly as part of the PHE investigation following this case, all family members and farm workers had serology tests performed for hantavirus and all these subsequently came back negative.

Table 2 Results of patient's hantavirus serology

Serotype	06/01/12	18/01/12
Puumala virus	Positive 1:1000	1:1000
Dobrava virus	Positive 1:1000	1:3200
Sin Nombre virus	Positive 1:100	1:1000
Saaremaa virus		
Seoul virus	Positive 1:10 000	1:40 000
Hantaan virus	Positive 1:10 000	1:20 000

DISCUSSION

Hantaviruses are a group of over 40 rodent-borne viruses, which are globally widespread. Most hantaviruses can only be passed to humans through direct contact with rodents or material contaminated with rodent waste (urine and faeces).[6] The disease presents in different forms with the severity and target organs largely dependent on the causative serotype: hantavirus cardiopulmonary syndrome (HCPS) in the Americas caused predominantly by infection with serotypes Sin Nombre (SINV) and Andes (ANDV) and haemorrhagic fever with renal syndrome (HFRS) caused predominantly by infection with Hantaan (HTNV) and Seoul (SEOV) in Asia and Dobrava (DOBV) and Puumala (PUUV) in Europe. The different serotypes are carried by different rodents. Most of the serotypes are carried by various species of mice or voles. In contrast, SEOV is carried by the brown rat *Rattus norvegicus*, which is widespread throughout Europe including the UK. Although there is some serological evidence of SEOV infection in Europe there have been very few proven human cases outside of Asia. To the best of our knowledge, our case is the first reported case of SEOV infection from a wild rat population in the UK.

The clinical course of HFRS is classically divided in to five phases: febrile, hypotensive, oliguric, diuretic and convalescent.[6] However, some or all of these phases may not be evident as HFRS can present as a spectrum from subclinical to fatal. Generally infections caused by HTNV and DOBV are clinically more severe than those caused by SEOV. PUUV is considered to cause only mild or subclinical illness. A large Chinese study of the different clinical characteristics of HTNV and SEOV infections found a significantly higher mortality rate and a higher requirement for dialysis in the HTNV group.[7] Interestingly however, they found that patients with SEOV infection had a longer duration of fever and were more likely to have liver enzyme derangement, sometimes even in the absence of AKI. The lack of typical HFRS features in patients infected with SEOV also meant that they were more likely to be initially misdiagnosed. Other than in south-east Asia, where intravenous ribavirin has been demonstrated to be efficacious when given early, there is no specific treatment for HFRS.[8 9] Management is supportive and careful fluid management is crucial. Severe cases may require renal replacement therapy.

Although widespread in Europe, there have been only sporadic reports of indigenous hantavirus cases in Scotland and England.[10–17] Since 1980s there has been growing evidence for endemic hantavirus infection in the UK. Most recently there has been a case report of hantavirus infection in a patient in Wales.[18] Interestingly serology from this case also pointed towards it being SEOV. Subsequently, SEOV RNA was detected from the patients two pet rats and from several others from a larger breeding pack in England that the pet rats had been sourced from.[19] The evidence from our case and the Welsh case would seem to show that SEOV is present in both the wild and the pet rat populations in the UK and therefore there can be little doubt that it is also causing human infections. With recent reports of SEOV being detected in France[20] and pet rats in Sweden[21] our findings are of interest beyond the UK.

Despite this hantavirus is rare if ever thought of as a diagnosis or tested for by GPs or hospital physicians in the UK. This case demonstrates the need for all frontline physicians to be aware of hantavirus as a potential diagnosis in patients presenting with fever, AKI and thrombocytopenia, particularly if there is a history of potential rodent contact. It should also be considered in cases of febrile illness with liver enzyme derangement with or without AKI. Indeed, it should be thought of and tested for whenever leptospirosis is a differential diagnosis.

Learning points

- Hantaviruses are a group of over 40 rodent-borne viruses, which can be spread to humans by close contact with rodents or material contaminated with rodent waste.
- There appears to be a new Seoul serotype hantavirus, which is present in the UK wild rat population and has the potential to cause human infections.
- Seoul serotype hantavirus cause a clinical picture of haemorrhagic fever with renal syndrome.
- Hantavirus should be considered as part of the differential diagnosis in patients presenting with fever, acute kidney injury and thrombocytopenia, especially if there is a history of possible rodent contact.
- Management of hantavirus is supportive and careful fluid balance is crucial.

Contributors KA was responsible for this patient and she drafted the manuscript and approved the final draft. LJ drafted the manuscript and approved the final draft. RM was involved in this case and approved the final draft. TB was involved in the analysis of specimens from this case at Porton Down and approved the final draft.

Competing interests None.

Patient consent Obtained.

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Reply

Reply to Comment Clement *et al.*: (Prevalence of Antibodies against Hantaviruses in Serum and Saliva of Adults Living or Working on Farms in Yorkshire, United Kingdom)

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We acknowledge Clement and colleagues for their comments [1] on our paper [2]. We agree that many controversies are being discussed by the hantavirus community, particularly surrounding the interpretation of serological results and the designation of new species and strains. Within this setting, we are grateful for the opportunity to respond to the key factual and methodological points raised by Clements *et al.*

The decision of whether Saaremaa virus (SAAV) is a separate species of hantavirus is not within the scope of our study: we followed the guidelines of the International Committee on Taxonomy of Viruses (ICTV) which lists SAAV separately from Dobrava virus. Serological cross-reactions between certain hantavirus species are widely acknowledged and referred to in our discussion of results. Given the published molecular evidence of a Seoul hantavirus in the region [3,4] and unpublished confirmation

of virus amplified from a human sample with 100% homology to Cherwell [5], we are confident in assuming that the samples reactive for both Seoul and Hantaan represent Seoul infection.

We did not intend to suggest that insectivore-related hantaviruses are pathogenic to humans and our text does not state this. It does assume that transmission occurs (even if only between reservoir animals) through excreted virus, which is the known route for all studied hantaviruses. Transovarial transmission is not an effective route for maintenance of hantaviruses, so excretion seems the most likely manner for insectivore-borne hantaviruses to be sustained in nature.

We acknowledge that it might have been useful for readers less familiar with the history of hantavirus research in the UK if we had cited the work of McKenna *et al.* [6]. However, that study focused on symptomatic patients and ours used a subset of healthy volunteers, limiting the validity of direct comparison. We believe that the PHLS farmers cohort study [7] provides a more appropriate comparator group. Regarding the inclusion of IgM titres, it is unreasonable to compare high titre acute samples, as reported by McKenna *et al.* [6], Jameson *et al.* [3] and Taori *et al.* [8], to samples from persons who may have been exposed decades prior to sampling. Given that our volunteers were not sick, we are unable to see the benefit of testing for IgM in our anonymised study.

The principal aim of our study was to provide a preliminary investigation of the seroprevalence to hantaviruses among farm workers and dwellers in a region of Great Britain in which a recent case of HFRS had occurred. As part of this investigation, a limited questionnaire was answered by participants to explore some possible risk factors. In order to limit the burden to participants, the questionnaire was deliberately kept short and only a limited set of questions was included. The results only pertain to the study sample (*i.e.*, farmers and farm dwellers) and the potential impact of using a convenience sample, rather than a random sample, is noted in our discussion. Further studies to evaluate the role of a wider range of potential risk factors are now warranted.

Within Table 2, "NA" is used to denote "not available" (rather than not assessed). Several odds ratios were not available, as the calculations involved category values of zero; for example, all 9 seropositive participants reported seeing rats and none reported not seeing rats.

The commercially available Euroimmun IFA (Luebeck, Germany) is a useful and convenient diagnostic assay for acute samples and serosurveys as it covers a panel of hantavirus species. It is not designed to confirm the causative species. We agree with Clement *et al.* that only molecular methods are able to do this. Thus we were cautious in interpreting the results of the samples reacting to the other hantavirus species and at no point suggested they were likely to be result of a Sin Nombre (SNV) infection, instead we described them as SNV/Puumala-like hantaviruses. We and peer reviewers at the time understood that this subtle statement alluded to the inexactness of the IFA technique at this level. We focused attention on the results of SEOV, since this is where we had provided molecular evidence of its existence, as indicated above.

While acknowledging the concerns of Clements *et al.*, we stand by the key findings and design of our study, which has provided new data on the seroprevalence of hantavirus infection in a farming community in which the first molecularly confirmed case in the UK had recently been detected [2]. More detailed studies logically follow on from these preliminary findings and are now in progress.

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Seroconversion for Infectious Pathogens among UK Military Personnel Deployed to Afghanistan, 2008–2011

Edmund N.C. Newman, Penelope Johnstone, Hannah Bridge, Deborah Wright, Lisa Jameson, Andrew Bosworth, Rebecca Hatch, Jenny Hayward-Karlsson, Jane Osborne, Mark S. Bailey, Andrew Green, David Ross, Tim Brooks, and Roger Hewson

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe seroconversion of UK military personnel deployed to Afghanistan between 2008 and 2011 to various vector-borne and zoonotic diseases, based on a biosurveillance study
- Distinguish asymptomatic and symptomatic cases associated with seroconversion of UK military personnel deployed to Afghanistan between 2008 and 2011 against various vector-borne and zoonotic diseases
- Assess clinical and public health implications of the findings from this biosurveillance study on seroconversion of UK military personnel deployed to Afghanistan between 2008 and 2011 to various vector-borne and zoonotic diseases.

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Military personnel are at high risk of contracting vector-borne and zoonotic infections, particularly during overseas deployments, when they may be exposed to endemic or emerging infections not prevalent in their native countries. We conducted seroprevalence testing of 467 UK military personnel deployed to Helmand Province, Afghanistan, during 2008–2011 and found that up to 3.1% showed seroconversion for infection with *Rickettsia* spp., *Coxiella burnetii*, sandfly fever virus, or hantavirus; none showed seroconversion for infection with Crimean-Congo hemorrhagic fever virus. Most seroconversions occurred in personnel who did not report illness, except for those with hantavirus (70% symptomatic). These results indicate that many exposures to infectious pathogens, and potentially infections resulting from those exposures, may go unreported. Our findings reinforce the need for continued surveillance of military personnel and for education of health care providers to help recognize and prevent illnesses and transmission of pathogens during and after overseas deployments.

Military personnel represent a population that travel to and work in environments where they are exposed to endemic or emerging infections that are not prevalent in their native country. Groups of military personnel on international deployments thus form a useful naive sentinel group for infectious diseases; these groups may serve as indicators of the infectious disease agents to which the local populations are exposed and of the diseases that may be encountered by other visitors traveling to the area (e.g., tourists, as well as staff of nongovernmental organizations, aid organizations, and other government agencies). Study of infectious disease exposures and illnesses among military personnel may provide insight into the epidemiology of emerging and reemerging infections and highlight what pathogens could be imported back to the home nations of visitors to these areas.

The recognition that military personnel may act as disease sentinels is not a new concept. Infectious diseases have beleaguered international military operations for centuries, with historic campaigns facing substantial loss of life after soldiers succumbed to infections endemic to the areas in which they were operating at the time (*1*). Examples from British military history include many diseases that may be categorized as "undifferentiated febrile illnesses" (*2*). The causative agents for these fevers, often unidentified at the time they occurred, were predominantly infectious pathogens established in the geographic area in which troops were operating. For example, in the late 19th century, the zoonotic disease brucellosis and the vector-borne parasitic diseases leishmaniasis and malaria were common in British colonial troops deployed to the tropics and subtropics. In India, 24% of troops were admitted to the hospital for malaria in 1908 alone (*3*). During World War I, cases of leptospirosis (*4*) and trench nephritis (in retrospect thought to be caused by hantavirus, which resulted in >35,000 illnesses) (*5*) were also documented.

More recently, since World War II, UK military operations in Korea, Malaya, Borneo, Sierra Leone, Liberia, East Timor, and Haiti have resulted in illnesses among military personnel caused by vector-borne pathogens; these illnesses have included Japanese encephalitis, malaria, and dengue (*2,6*). This incidence of infectious disease is not limited to the tropics; campaigns in the Balkans and the Arabian Gulf during the past 25 years resulted in reports of cases of a range of vector-borne and zoonotic infections (*7–9*).

Although information about endemic infectious disease in Afghanistan is limited, previous historical reports indicate that several endemic vector-borne and zoonotic diseases, including rickettsial diseases, Crimean-Congo hemorrhagic fever (CCHF), and pappataci fever (now known as sandfly fever) (*10*). Given the country's geographic location, other pathogens endemic to the central Asian region could also be expected to be of concern to military personnel; these pathogens include Sindbis, chikungunya, and West Nile fever viruses (*11*). Although infections with many of these pathogens can be prevented by drugs (chemoprophylaxis) or vaccination, and the risk for exposure can often be reduced by physical measures, the ongoing UK military campaign in Afghanistan has still resulted in infectious diseases occurring in deployed personnel. Severe cases have required restrictions of duties or even hospitalization or evacuation to the United Kingdom, and high illness rates can affect the operational capability of the military force (*12*).

In 2011, Bailey et al. documented numerous cases of "undifferentiated febrile illness" in British military personnel serving in Helmand Province, Afghanistan, during the summer of 2008 (*13*). These cases, for which no organ focus could be determined on clinical and radiologic assessment and no positive results were obtained from microbiological investigations (e.g., blood cultures and malaria antigen tests), have been given the colloquial term "Helmand fever."

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For this study, we conducted surveillance among UK military personnel to determine the prevalence of several vector-borne or zoonotic infectious agents that were suspected to be the causative agents of some of these cases of undifferentiated fever: *Rickettsia* spp., *Coxiella burnetii*, sandfly fever virus, hantavirus, and CCHF virus (CCHFV). We conducted serosurveillance testing of 467 military service members who were deployed to Helmand Province during March 2008–October 2011. In this article, we describe the pathogens for which study participants showed seroconversion before and after a tour of duty and the incidence of those seroconversions within the military population.

Methods

Recruitment and Sampling

Study volunteers were recruited from either British Army regiments or Royal Marines units before deployment to Helmand Province, Afghanistan. For each deployment, a research nurse visited the unit and gave study information to the troops. Volunteers were required to give informed consent and to have 1 predeployment blood sample taken. After return from the 6-month tour of duty, volunteers were visited and asked to give a second postdeployment blood sample. At the postdeployment visit, volunteers were also asked to complete a short questionnaire detailing any "flu-like" illness or symptoms they experienced while deployed and any contact they had with livestock, wildlife, or insect vectors. The primary location of each company (i.e., group of ≈100 personnel in which the volunteer was posted) while deployed was also noted. Ethical approval for this survey was provided by the UK National Health Service National Research Ethics Service and Ministry of Defence Research Ethics Committee.

Blood samples were taken by venipuncture using BD SSTII Advance 10-mL vacutainers (Becton Dickinson, Oxford, UK). After a minimum 30-min incubation at room temperature, samples were centrifuged at 3,500 rpm for 15 min to separate the serum. This serum fraction was then separated and stored at -80°C for subsequent serologic testing.

During the study period, March 2008–October 2011, a total of 467 volunteers gave paired serum samples (i.e., predeployment and postdeployment samples). In all cases, samples were kept anonymous (by unique identification numbers), and questionnaire data/confidential information were kept in a secure database with restricted access.

Serologic Testing

Postdeployment samples were tested first for antibodies to all pathogens of interest. If results were positive, the corresponding predeployment sample was then tested to ascertain whether the volunteer seroconverted during the tour of duty for which they were recruited to the study or if they were already seropositive (i.e., exposed/seroconverted to that pathogen) before being recruited for the study. A volunteer whose sample was negative before deployment but positive after deployment was deemed to have seroconverted during that particular tour of duty. For each pathogen of interest, diagnostic tests were used in line with Public Health England's diagnostic laboratory procedures. All assays used have been validated locally for diagnostic use, are approved by UK regulatory authorities, and have been awarded Communauté Européenne marking by the European Union.

C. burnetii ELISA

Antibodies against *C. burnetii*, the causative agent of Q fever, were detected by using a commercial ELISA to detect phase 2 IgG and IgM human antibodies (Serion/Virion, Würzburg, Germany). ELISAs were conducted by using the DS2 Automated ELISA workstation (DYNEX Magellan Biosciences, Chantilly, VA, USA) according to the manufacturer's instructions. All serum samples were tested at an initial dilution of 1:100, as recommended by the manufacturer's product insert for diagnostic samples. If results were calculated as positive (above the automated threshold on DS2 software) for either IgG or IgM, the test was repeated in duplicate for confirmation. Because of the nonacute nature of the survey, all samples showing at least Phase 2 IgG positivity were deemed positive.

Hantavirus Immunofluorescence Assay (IFA)

Antibodies against the hantavirus group of pathogens were detected by using a commercial Hantavirus IIFT Mosaic 1 IgG IFA (Euroimmun, Lübeck, Germany). All serum samples were tested at an initial dilution of 1:100, as recommended by the manufacturer's product insert for diagnostic samples. If results were calculated as positive (for any species/subspecies of hantavirus), the test was repeated and confirmed by using the Recomline Bunyavirus IgG/IgM Line Assay (Mikrogen, Neuried, Germany). All IFA slides were set up by using the AP16 IF Plus automated slide processor (Euroimmun) and viewed by using a conventional fluorescent light microscope.

Rickettsia IFA

Antibodies against *Rickettsia* spp. were detected by using a commercial Rickettsia IgG and IgM IFA (Focus Diagnostics, Cypress, CA, USA). This assay is specific for rickettsial infection; that is, antibodies against *Coxiella* spp. do not cross-react in this test (Public Health England, unpub. data). All serum samples were tested at an initial dilution of 1:64, as recommended by the manufacturer's product insert

for diagnostic samples. If results were calculated as positive, the test were repeated and confirmed by titrating down a 2-fold dilution series to 1:512 (data not shown) to ensure that fluorescence diminished with antibody dilution (as recommended in the product insert). Because samples were not obtained during an acute illness phase, any sample that showed a consistent signal at 1:64 dilution was considered to be positive, even if this diminished by the 1:128 dilution. All IFA slides were prepared in accordance with the manufacturer's instructions and viewed by using a conventional fluorescent light microscope

Sandfly Fever Virus IFA

Antibodies against sandfly fever viruses (genus *Phlebovirus*) were detected in serum by using a commercial sandfly fever assay (IIFT Mosaic 1 IgG immunofluorescence assay; Euroimmun). All serum samples were tested at a dilution of 1:100, as recommended by the manufacturer's product insert for diagnostic samples. Given the nonacute nature of the samples, a positive result was defined as any sample that showed a consistent signal (for any of the 4 species/strains of sandfly fever virus) at 1:100 dilution. All IFA slides were set up by using the AP16 IF Plus automated slide processor (Euroimmun) and viewed by using a conventional fluorescent light microscope.

Crimean-Congo Hemorrhagic Fever ELISA

Antibodies against CCHFV nucleoprotein were detected by using an in-house CCHF recombinant ELISA (*14*). ELISAs were carried out by using the DS2 Automated ELISA workstation (DYNEX Magellan Biosciences). All serum samples were tested at an initial dilution of 1:100, and any tests that gave any signal above background were repeated in duplicate.

Results

The Table shows the results of the respective serology assays performed. For each pathogen (with the exception of CCHFV, for which no seroprevalence was reported), 2 categories of a seropositive volunteer were identified: those who showed detectable levels of antibody before and after deployment; and those who only showed positivity on return, with no detectable antibody in predeployment samples. During deployment, the troops showed the highest seroconversion rates for sandfly fever virus (3.1%) and rickettsiae (2.7%). However, seroconversions for hantavirus and *C. burnetii* (1.3% and 1.7%, respectively) also occurred.

The group of volunteers who were positive for each pathogen before deployment showed that there was a background level of seroprevalence for these pathogens within the UK military. The group of initially antibody-negative personnel seroconverted to the pathogens while on deployment, which suggests that military personnel are being exposed to such diseases while on operations in Afghanistan and that these diseases are being transmitted in the region.

Figure 1 shows the prevalence of volunteers who seroconverted during each deployment period. This comparison enabled us to plot prevalence as a function of season: winter deployments (October–March) compared with summer deployments (March–October). This analysis showed a higher prevalence of seroconversion in the Northern Hemisphere summer months, when the deployment area is hotter and drier than over the winter. Sandfly fever virus showed the greatest seasonal variation, with most cases in the summer months, and *C. burnetii* showed the least variations, findings that are consistent with these pathogens' known modes of transmission.

Many seroconversions for the studied pathogens appeared to be asymptomatic. All volunteers took part in a return questionnaire when the postdeployment sample was taken. This questionnaire was used to determine whether those who seroconverted after deployment experienced a "flu-like" illness during deployment. Figure 2 shows that, for all 4 pathogens acquired (*Rickettsia* spp., *C. burnetii*, sandfly fever virus, and hantavirus), a proportion of volunteers showed seroconversion but did not report feeling ill. As expected, sandfly fever virus and rickettsial infections showed the highest proportion of asymptomatic cases; just 6.5% and 7.4%, respectively, of those who seroconverted reporting feeling ill (i.e., 93.5% of sandfly fever virus exposures and 92.6% of *Rickettsia* spp. exposures were asymptomatic). The less common but often more clinically significant pathogens hantavirus and *C. burnetii* resulted in more reports of illness; 64.7% of *C. burnetii* seroconversions and 30.8% of hantavirus seroconversions were asymptomatic.

Table. Results of antibody testing for 5 infectious pathogens among UK service personnel before and after deployment to Helmand Province, Afghanistan, March 2008–October 2011*

Pathogen	No. persons tested	No. (%) with detectable antibody before deployment	No. (%) with seroconversion after deployment	Total no. (%) with positive antibody test
CCHFV	466	0	0	0
Sandfly fever virus	459	8 (1.7)	14 (3.1)	22 (4.8)
Rickettsia spp.	446	10 (2.2)	12 (2.7)	22 (4.9)
Hantavirus	453	5 (1.1)	6 (1.3)	11 (2.4)
Coxiella burnetii	467	7 (1.5)	8 (1.7)	15 (3.2)

*Assays were run sequentially on samples from all persons tested; some sample sizes were insufficient for testing for all agents. CCHFV, Crimean-Congo hemorrhagic fever virus.

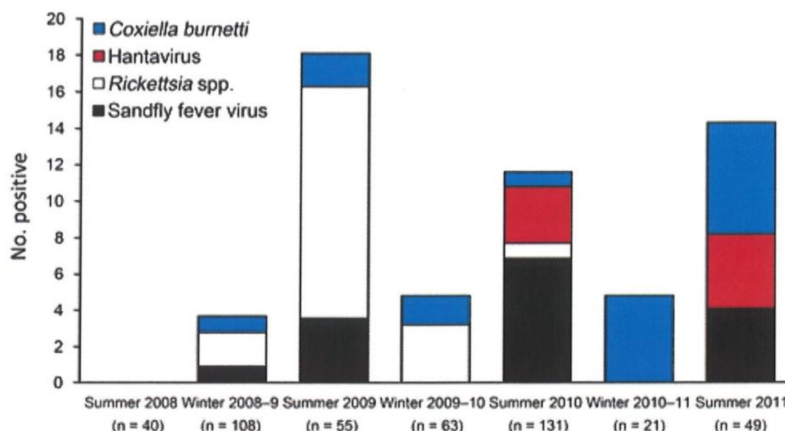

Figure 1. Results of antibody testing for 4 infectious pathogens, by tour of duty, among 467 UK service personnel deployed to Helmand Province, Afghanistan, March 2008–October 2011. n values indicate number of volunteers tested from each tour of duty. Assays were run sequentially on samples from all persons tested; some sample sizes were insufficient for testing for all agents.

Postdeployment questionnaires showed that nearly one fifth (90/467, 19.3%) of all volunteers reported experiencing "flu-like" symptoms while on operations in Helmand Province. A total of 56.7% of those who recorded ≥1 episodes of feeling unwell reported experiencing fever only, whereas 12.2% had no additional/specific symptoms; the remaining 31.2% reported either diarrhea and vomiting (15.6%) or "other" symptoms (Figure 3).

Discussion

UK military personnel consist of ≈237,190 persons (estimate of combined regular and volunteer reserve military personnel in UK, 2010 [UK Ministry of Defence, unpub. data]). Our results show that military personnel are being exposed to, at minimum, *Rickettsia* spp., *C. burnetii*, sandfly fever virus, and hantaviruses while on operations in Afghanistan, a region where these agents and resulting diseases are endemic. These results suggest that human or animal (for the zoonotic pathogens) reservoirs likely exist in the local populations of the Helmand region for these diseases.

Troops often patrol through vegetation and farm land where both reservoir and vector populations are high, which can easily result in exposure to pathogens and further supports the hypothesis that the nature of their work makes armed forces personnel a group with an increased incidence of exposure to such vector-borne or zoonotic pathogens. However, this group may also act as a good sentinel cohort for other populations of nonindigenous workers from governmental and nongovernmental organizations.

UK troops do sometimes stop over at British military bases in Cyprus during their return to the United Kingdom. However, these stopovers are typically very short in duration (2–3 days), and they do not occur in an environment where exposure is likely to occur, with the possible exception of sandfly bites. In addition, for each pathogen studied, a proportion of troops showed antibody positivity before deployment, suggesting that they may have been exposed to these pathogens on previous deployments to Afghanistan (before being recruited to the study) or on other exercises and operations around the world.

Overall, seroprevalence for these pathogens appears to be low, although this is hard to determine without a comprehensive study of nondeploying troops. These results may reflect good discipline or education of UK troops in the use of personal protection measures such as the use of DEET-containing insect repellent and mosquito nets, both of which are actively promoted by the UK military. A higher proportion of those deployed over the summer months showed seroconversion, corresponding with an increase in the numbers of biting vectors such as the *Phlebotomus* sandfly and ticks (rickettsial vectors). Further study of seasonality might show whether these

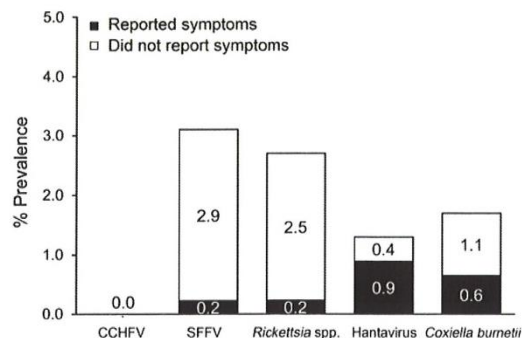

Figure 2. Percentages of UK service personnel who sero-converted to 1 of 5 infectious pathogens who reported feeling unwell or did not report illness during deployment to Helmand Province, Afghanistan, March 2008–October 2011. A total of 90 (19.3%) of 467 deployed service members reported feeling unwell during deployment. CCHFV, Crimean-Congo hemorrhagic fever virus; SFFV, sandfly fever virus.

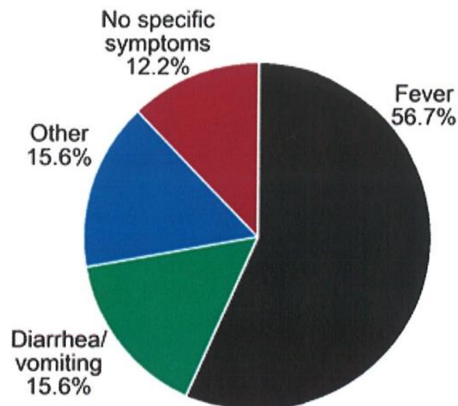

Figure 3. Distribution of signs and symptoms among 90 UK service personnel who seroconverted to 1 of 4 infectious pathogens (sandfly fever virus, hantavirus, *Coxiella burnetii*, *Rickettsia* spp.) and who reported feeling ill during deployment to Helmand Province, Afghanistan, March 2008–October 2011.

increases correlate with increased military activity (e.g., military patrolling and combat operations increase during the summer months), environmental/entomologic changes (e.g., decreases in rain and/or increases in temperature conditions favorable to agent, reservoir, or host density), or possible lapses in discipline regarding personal protective measures.

At first glance, summer seasonality may seem odd for an increase in hantavirus seroconversion, for which incidence would be more likely to correlate with closer proximity of humans with the rodent reservoir. However, UK summer deployments extend to late September and in some cases the first week or two of October; thus, these deployment continue into early autumn, when rodents are actively moving into buildings. In addition, the apparent lack of rickettsial infections after summer 2010 raises questions of whether this might correlate with a change in vector life-cycle, abundance of reservoir species, changes in insect bite prevention regimens, or changes to local livestock management regimens.

The increased incidence of rickettsial and sandfly fever virus infections that we found among deployed troops does not necessarily mean that these vector-borne pathogens are more prevalent in the environment and local population than the zoonotic pathogens *C. burnetii* or hantavirus. Rather, these results may mean that mode of transmission and incidence of encounters with the vectors and reservoirs differ. However, our results do indicate that these pathogens may pose the greatest risk to UK troops.

Although most of the diseases we detected are relatively self-limiting after the initial acute infection, our results suggest ways to improve control measures to reduce the rate of transmission. UK military personnel are already given medical advice and education before deployment, emphasizing the prevention of insect bites while deployed by use of arthropod repellent, insecticide-impregnated clothing, and mosquito nets. The observation that rickettsial infections and Q fever might account for a sizeable proportion of cases of undifferentiated febrile illness seen in military field hospitals has led to the empirical use of doxycycline in such cases because *Rickettsia* and *Coxiella* species are sensitive to this drug. This practice justifies further research on use of doxycycline for chemoprophylaxis in these instances.

More than half (56.7%) of volunteers who reported feeling unwell specifically reported a fever without other specific symptoms, which is consistent with the "undifferentiated febrile illness" that the pathogens we investigated can cause. The 15.6% of volunteers reporting illness who reported diarrhea and vomiting could have had gastrointestinal infections; localized outbreaks of norovirus and similar infections are common in military operating bases) (*15*). Of the many volunteers who experienced fever but did not show seroconversion for the 5 pathogens tested here, exposure to influenza virus or other respiratory infections should be considered. In 2011, Eick et al. reported that deployed US troops experienced 30.1% seroconversion for influenza and 6 other respiratory infections (*16*). However, uptake of influenza vaccination, which is offered to UK military personnel before winter deployments, has increased for each of the past 4 years (*2*). Data from the militaries of other countries, which might have different deployment patterns and protective measures, should be compared only with caution. In addition, although some volunteers who seroconverted for all 4 pathogens reported flu-like symptoms while deployed, we cannot ascertain whether these symptoms were a result of exposure to the pathogen of interest.

Some volunteers who seroconverted for the pathogens tested were asymptomatic for the duration of their deployments. Clinical disease probably did not develop in these patients, despite evidence of an exposure and an antibody response. Our finding that 65% of acute *C. burnetii* infections were asymptomatic is consistent with previous reports (*17*,*18*), but even in asymptomatic persons infected with this pathogen, long-term complications such as chronic Q fever and Q fever fatigue syndrome may develop. Overall, ≈5% of acute cases progress to chronic Q fever, and complications can include endocarditis requiring prolonged antimicrobial drug treatment and possibly heart valve surgery (*19*). In addition, Q fever fatigue syndrome may develop in ≈20% of those infected (*17*), which is generally incompatible with a military career and has substantial effects on patients' quality of life.

Royal et al. (*20*) recently reported *C. burnetii* seroprevalence in US troops deployed to the Al Asad region of Iraq in 2005; a known Q fever outbreak occurred in this region at this time. This smaller study (n = 136) reported a 7.2% prevalence of *C. burnetii* infection among troops located in that area at the time of the outbreak. The report further supports (in addition to original work by Bailey et al. [*13*]) the *C. burnetii* seroprevalence reported here. Although we did not see as high an incidence of seroconversion, we were not specifically looking in an area with a known Q fever outbreak, merely an area in which Q fever is believed to be endemic.

Although this study did not find seroconversion for the viral hemorrhagic fever agent CCHFV, military and public health reports demonstrated that the virus is circulating in the region (*21*). In 2009, a US Army soldier in the neighboring Kandahar Province died of CCHFV infection (*22*), and in 2012, a UK citizen returning from the northwest of the country also died (*23*,*24*).

In conclusion, this study highlights and confirms the potential for vector-borne and zoonotic diseases that are endemic in southern Afghanistan to emerge or reemerge to pose a substantial public health threats as the country rebuilds its public health infrastructure. A study of this type cannot give a specific indication of prevalence for these pathogens in the local population, but this surveillance can provide a valuable way of exploring emerging disease epidemiology, particularly of vector-borne and zoonotic infections, in areas with poor public health reporting and infrastructure. Our findings of seroconversion for 4 of these pathogens among deployed UK troops reinforce the need for continued surveillance and continued education of health care providers so that, should military operations or environmental factors change in such a way that these modest incidence numbers increase, costly outbreaks can be avoided. This study also highlights the need for rapid, field-capable, point-of-care diagnostics in regions or situations for which full laboratory diagnostic facilities are not practical or available.

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