

Urban Small Mammal Pathogen Dynamics in Singapore - Prevalence,
Distribution, and Genetic Diversity

by

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Thesis submitted in partial fulfillment of
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ABSTRACT

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Abstract

The goals of this thesis are to identify pathogens present in the small mammal populations in urban environments of Singapore, to identify the distribution of these pathogens throughout the island, and to assess the genetic diversity and phylogenetic relationships of these pathogens. These three components of data collection are combined to assess the risk to human populations from zoonotic diseases. Small mammals were collected, throughout a total of ten trapping locations, over a period of three months. Rodents were euthanized according to guidelines specified by National Environmental Agency of Singapore. Organ and blood samples were harvested from collected rodents in order to extract DNA and RNA used in pathogen detection. PCR detection revealed several positive results for the presence of pathogens in families or genus belonging to *Hantavirus*, *Coronaviridae*, *Leptospira*, *Rickettsia*, *Trypanosoma*, *Borrelia*, and *Anaplasma*. These diseases are known to infect humans and may pose a threat to the public health of the Singaporean population.

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1. Introduction

Infectious diseases are the leading cause of death globally with emerging infectious diseases accounting for at least 12% of all human pathogens (Taylor, 2001; CDC, 2012). Furthermore, the incidence of emerging infectious disease (EID) 'events' has increased significantly over time (Jones, 2008). Emergence events can be characterized distinctly by either recognition of novel pathogens (in the cases of SARS and H5N1 avian influenza) and those that re-emerge in new locations and potentially as drug resistant or increasingly virulent forms (including West Nile, malaria, and tuberculosis).

These emergence events have been determined to occur globally in a non-random pattern with factors including ecological, environmental, and socio-economic (2008). Despite the high prevalence of novel and emerging infections, correlations between emergence and spatial and temporal variables are not fully understood (2008). Examining vector and reservoir species based on spatial and temporal variables as well as prevalence rates for diseases is a necessary element of delineating the roles of these factors. This examination will help assess the degree of risk such diseases present to human populations (Kang, 2007).

Zoonotic diseases have been previously identified in Singapore including the case of Nipah virus, which emerged in the spring of 1999 when over a hundred people in Malaysia and Singapore suffered from an encephalitic conditions and had a mortality rate of 40% in humans. Giant fruit bats were identified as the major reservoir for the

Nipah virus, however the virus manifested in pigs as a respiratory illness. All clinical cases were determined to have had some contact with pigs (CDC, 1999). Food-borne parasitic zoonoses have also been documented in 'rural slums' including *Clonorchis*, *Taenia* spp., hydatid disease, and *Toxoplasma* have also been identified in Singapore (Singh, 1991)

Infections derived from rodent borne pathogens are the cause of significant morbidity and mortality and remain an imminent threat to global health. For example, a rodent borne hantavirus was confirmed in 10 patients who visited Yosemite national Park with three of the confirmed cases ending in fatality. Infected visitors were residents of California, Pennsylvania, and West Virginia but had been exposed to the virus when lodging in tent cabins in the park (CDC, 2012). Specifically, the Asian house shrew (*Suncus murinus*) and members of the genus *Rattus* are natural reservoirs for hantaviruses, including etiological agents which cause failure of renal and pulmonary systems leading to severe hemorrhagic fever in humans (Kang, 2007). Additionally, these rodents harbor a range of other viruses, bacteria, and protozoa including rodent typhus and those in the genus of *Rickettsia*, *Borrelia*, *Anaplasma*, *Leptospira*, *Influenza*, *Trypanosoma* among others. However, under identification and misidentification are problematic in the areas of surveillance and risk assessment. It is substantially more difficult to allocate infectious disease prevention efforts in the most efficacious and

efficient means possible if the pool of infectious is under studied or neglected entirely.

Suncus murinus and *Rattus* species are ideal natural reservoir for zoonotic disease due to its wide geographic range, peridomestic affinities, social behavior, and high reproductive potential (Mills, 1999). Diseases associated with rodents are most commonly spread through inhalation of particles contaminated with excreta, although infections can also occur through bite wounds and direct contact from person to person (CDC, 2012).

The extent to which *Suncus murinus* and other small mammals are involved in the transmission of pathogens is not fully understood. The primary goal of this study is to elucidate pathogen prevalence and diversity in urban small mammal populations in Singapore. Singapore is an interesting location for studying rodent-borne infectious agents as pathogen species richness has been positively linked anthropization (Chaisiri, 2010). Identifying prevalence and diversity of zoonotic diseases and the corresponding reservoirs necessary is a necessary component of determining associated human risk (2010). This information is also compulsory for developing predictive models of ecological and environmental dynamics associated with pathogens that pose a threat to global public health (Singleton, 2003).

2. Literature Review

2.1 Rodents and Public Health

According to the World Health Organization, a zoonotic disease is defined as a disease or infection naturally transmitted from vertebrate animals to humans (Woolhouse, 2005). Small mammals have been natural reservoirs for zoonotic pathogens responsible for killing millions of people, including the plague and anthrax (Gubler 2012; CDC, 2012). Of the 1406 human pathogen species, around 175 are considered to be zoonotic, and 130 of those are labeled as emerging or reemerging (Woolhouse, 2005). Rodent and other small mammals are considered to be significant reservoirs for viruses, bacteria, protozoa, and fungi (2005).

The number of human diseases associated with animal reservoirs has risen in the last decades (Meerburg, 2009). As the human population expands into new environments and ecological niches, its exposure to new biodiversity and pathogens increases as well. In some instances, animals have fully adapted and integrated in to expanding human habitats and is the case with certain rodents. Rodents are among the most abundant and diverse groups of mammals in the world. Their opportunistic relationship with humans provides them with heightened contact, facilitating the inter-species transmission of a number of zoonotic infectious diseases. Mechanisms for disease transmission include both direct and indirect methods. Direct pathways of transmission include contact, e.g. biting) (Herbreteau 2012). Rodent-borne diseases are

usually transmitted indirectly; specifically hantaviruses and lassaviruses are shed in urine and as fomites (2009). Other indirect methods include transmission through arthropods such as between fleas and livestock (2009) Arthropod borne diseases are substantial to the Singaporean population including dengue, influenza, and rickettsias. Mosquitoes, ticks, and other blood feeding parasites have the capability of spreading numerous blood borne pathogens.

The loss of biodiversity may also be associated with the increasing incidence of emerging diseases as is the case in hemorrhagic fever caused by hantaviruses and arenavirus (Gubler 2001). One study found that outbreaks of rodent-borne hemorrhagic fevers occurred most frequently in anthropogenically disturbed habitats with low animal diversity (1999). Mills postulates that this is a result of divergent life history strategies. The classical ecologist dichotomization of mammalian adaptation patterns includes specialist species – those that are “highly adapted to a narrowly defined habitat and require one or a few specific food resources...have a longer life span, slower development to sexual maturity, and lower fecundity” (Mills, pg. 146) The alternative is “generalist” or “opportunistic” species – those that have “high adaptability to a wide range of habitats and can subsist on a variety of food sources...short lived, highly fecund, and have rapid development” (Mills, pg. 146). Some of the features such as short life span, high fecundity, and rapid development may overlap both generalist as well as specialist small mammals. However focal distinction is the diet limitation of specialist

animals, which limits or incapacitates them from thriving in human dominated environments and thereby limiting the passage of pathogens. Features of opportunistic species, specifically the ability to thrive in urban and agricultural environments, allow house shrews and certain member of the *Rattus* genus to coevolve with hemorrhagic fever viruses.

2.2 Vertebrate Reservoirs

2.2.1 Order Soricomorpha

Suncus murinus is a robust and adaptable species with established populations throughout eastern Africa, the Middle East, and Southeast Asia (Kang 2011). Both vertical and horizontal transmission of disease occurs in Asian shrew populations. Vertical transmission of hantavirus rarely occurs (Kang, 2010). An important route of horizontal transmission of hantavirus occurs between adult males during aggressive encounters (2010). Territorial behavior and aggression is affected by house shrew population density, and therefore larger populations may have higher incidences of disease spread through horizontal methods such as in hantavirus (Kang 2007). *Suncus murinus* is thrives throughout forested and urban areas throughout Singapore

2.2.2 Order Rodentia

Rats are a considerable threat to human health as they are reservoirs for several zoonotic diseases including the plague, rat typhus, lungworms, leptospirosis, arenaviruses, and hantaviruses (Singleton, 2009). The epidemiology of zoonotic diseases is not fully understood. Within Southeast Asia over 25 strains of hantaviruses and arenaviruses have been identified since 1995 (Mills, 1999). Little is known about specific species that are major reservoirs for bacteria, viruses, spirochetes, and helminthes that persists in urban environments. These pathogens are considered to be an increasing global public health threat due to increased globalization, specifically between rural and urban areas and habitat anthropization (Mills, 1999; Chaisiri, 2010). Predominant species in Singapore include *Rattus tanezumi*, *Rattus novergicus*, *Rattus annandalei*, *Hylopetes spadiceus* (red-cheeked flying squirrel), and *Callosciurinae callosciurus* (plains squirrel).

2.3 Pathogens of Interest

2.3.1 Hantaviruses

Hantaviruses have likely existed for approximately 30 million years and have at least 33 genotypes with an equivalent number of rodent reservoir species (Mills, 1999). Hantaviruses are enveloped negative sense RNA viruses in the Bunyaviridae family. Whereas infections do not appear to cause disease in the natural hosts, hantaviruses have an incubation time of two to four weeks before symptoms of infection occur. The

two most severe manifestations of hantavirus infections are hemorrhagic fever with renal syndrome (HFRS) during which increased vascular permeability and decrease in blood pressure occur as a result of endothelial dysfunction leading to tachycardia and hypoxemia (Kang, 2011). This syndrome is characterized by five phases including febrile, hypotensive, oliguric, diuretic, and convalescent phase during which the kidneys begin to fail and proteinuria occurs. Recovery occurs during the convalescent phase in cases that are not fatal. Additionally, hantaviruses are responsible for hantavirus pulmonary syndrome (HPS) with febrile illness and difficulty breathing in the later stages of the disease. This syndrome leads to tachycardia as well as difficulty breathing, coughing, and shortness of breath (2011).

Hantaviruses collectively cause up to 200,000 human clinical cases annually, though this is likely greatly underestimated (Kang 2007). Hantaviruses are a diverse group of viruses with six different strains found in rodent populations in Southeast Asia. More specifically, Thottapalayam (TPMV) virus has been found in *Suncus murinus* in Southeast Asia and Nepal (2007). Hantaviruses are transmitted by excretion in urine and feces from infected rodents, which present a risk to human health commonly through inhalation of excreta. Excretion of virus is likely to occur for months and possibly for the life of the rodent regardless of the presence of high titers of antibodies present in the sera.

2.3.2 Coronaviruses

Coronaviridae is a family of enveloped positive-sense RNA viruses. These viruses primarily infect the upper respiratory and gastrointestinal tract of mammals and birds and are thought to be the cause of common viral colds in human adults (Groot, 2011). Coronaviruses are often highly contagious and spread through aerosolized particles. Coronaviruses became highly publicized during the recent outbreak of Severe Acute Respiratory Syndrome (SARS) known to be caused by SARS-CoV. In rodents, Sialodacryoadenitis (SDA) virus is known to be a highly contagious virus that weakens the immune system and is characterized by cervical swelling, inflammation of submaxillary and parotid salivary glands, and ocular lesions (Yoo, 2000). However, SDA has a low mortality rate in rats and not all infected rats show symptoms (2000). Little is known about Coronavirus diversity in small mammals.

2.3.3 Trypanosoma

Trypanosoma is a multispecies genus of unicellular protozoa with using flagella for locomotion and are shaped like corkscrews (Barrett, 2003). As obligate parasites, they occupy the intestine and blood stream in mammalian hosts and are transmitted mainly by blood-feeding invertebrates. Human diseases caused by *Trypanosoma* include sleeping sickness, caused by *Trypanosoma brucei*, and Chagas disease which is caused by *Trypanosoma cruzi* (2003). *Trypanosoma evansi* is known to cause one form of the surra disease in animals, but rarely causes disease in humans (Powar, 2006).

2.3.4 Rickettsia

Rickettsia is a genus of bacteria that are gram-negative obligate intracellular parasites that are transmitted by various species of ticks, fleas, and lice. Rickettsia diseases are classified into three groups including spotted fever, typhus, and scrub typhus. New or reemerging rickettsioses have been documented, specifically tick borne lymphadenopathy and dermacentor borne necrosis eschar-lymphadenopathy (Walker, 2007).

Scrub typhus is an gram negative intracellular parasite that previously belonged to the genus *Rickettsia*, but is in *Orientia*. This pathogen is transmitted by species of trombiculid mites (Tseng, 2008). Scrub typhus is endemic in Southeast Asia and is a serious threat to human health as the disease is often fatal if left untreated

2.3.5 Borrelia

Borrelia is a genus of coiled helical spirochetes spread by ticks and lice. The bacteria invade the bloodstream of hosts after entering through mucus membranes. There are 36 known species, 12 of which cause Lyme disease or borreliosis (Steere, 2003). Common pathological signs of infection in humans include fever, headache, and fatigue. Infection may ultimately affect the brain, nerves, eyes, joints and heart if left untreated or inadequately treated (2003).

2.3.6 Anaplasma

Anaplasma is a genus of rickettsiales bacteria that reside in host red blood cells.

Anaplasma spp. may lead to Anaplasmosis in ruminants, in which the initial infection is tick borne and may lead to anemia from destruction of red blood cells (Mitchell, 1996)..

The parasite resides inside of the blood cells making detection by the host immune system difficult. In humans, human granulocytic Anaplasmosis is caused by *Anaplasma phagocytophilum* (1996)

3. Methods

3.1 Trapping and Sample Collection

Fieldwork protocols for this project were approved by the Agri-Food & Veterinary Authority of Singapore (AVA) and the IACUC at the National University of Singapore including an agreement to euthanize all pest species caught. Additionally, authorization was given to euthanize the first 100 captured *Suncus murinus*. Before each trapping session, verbal permission was obtained from private property owners to conduct a trapping over a three day period. Eleven trapping sessions were conducted.

Supplies were organized and prepared at Duke-NUS facilities prior to departure for field sites and included the following: cleaning and labeling Sherman and Tomahawk traps (designed for live animal captures); preparing bait including fish, chicken, and pork; organizing locks and cables to secure the traps at the field site; preparing needles, syringes, respirators, gloves, anesthetic, glass slides, cotton swabs, biohazard bags, and eppendorf tubes for animal processing and collection of uncoagulated blood.

On the first day of each trapping session, traps were set and baited at 4 p.m. Traps were placed out of the field of direct sight including areas such as: roadside bushes, water drainage gutters, next to potential rodent dwellings identified by burrowing marks, areas of convergence and bottlenecks, next to fencing and walls, and next to dumpsters. Traps were secured with metal cables and locks whenever possible.

Traps were checked each morning at 7:00 a.m. in order to avoid exposing animals to direct sunlight and overwhelming heat. Long sleeves, long pants, rubber gloves, and N-95 respirator were used whenever handling animals. Upon encountering a trap that had been activated (i.e. shut door) the operator would hold the trap at arms length and open the door only enough to confirm the absence or presence of a rodent. In the event that the trap door was still open and the trap empty, the operator would close the door until resetting the trap at 4:00 p.m. to prevent daytime animal captures.

All traps containing rodents were removed and taken to an area secluded from human activity. The animals were euthanized by isoflurane exposure of 15 minutes. After 15 minutes, death was confirmed by lack of breathing and heartbeat. Up to 1 mL of blood was collected by cardiac puncture using a 27-gauge needle and syringe. Blood from this puncture was used to create a blood smear to later visually identify parasites in the blood.

Animals were collected from a period beginning on 15JUN12 to September. Rodent trapping took place over a non-consecutive eight week time period and over a diversity of urban environments including: food courts, neighborhoods, in the proximity of medical centers, along main road and walkways, and in communal living environment including HDB (Housing Development Board of Singapore) housing units. Under the recommendation of the small mammal specialist on staff at Duke-NUS, the primary baits used included a mixture of fried meats and noodles, rather than the

traditional oats and peanut butter commonly used to trap rodent in forest environments. A total of 11 trapping expeditions took place in 10 different settings. Local pest control companies also aided the collection of rodents by depositing occasional daily catches at Duke-NUS facilities according to a standard operating protocol (SOP) (see appendix a).

Following in-field processing, rodents were double bagged with biohazard bags and stored at Duke-NUS facilities at -80°C until necropsies could be performed. Necropsies were conducted inside a biosafety cabinet approved for use with BSL-2 pathogens. Two sets of latex or nitrile gloves were worn during necropsies. One incision was made from the rectum to the top of the thoracic cavity as to maintain the integrity of the hide. Some specimens were later donated to Raffles Museum of Biodiversity Research.

Two samples each of lung, kidney, spleen, large intestine, small intestine and colon were collected and placed in an empty 1.5 mL eppendorf tube or cryotube containing a viral transport media (WHO protocol) and then stored at -80°C.

3.2 PCR Detection

Prior to polymerase chain reactions (PCRs), harvested tissues were homogenized in PBS and DNA and RNA were extracted from blood and tissue samples using a Qiagen DNA Blood Mini Kit and a Qiagen QIAamp Viral RNA mini kit,

respectively. For RNA samples, RNA underwent reverse transcription using SuperScript™ II Reverse Transcriptase from Invitrogen to create double stranded complimentary DNA prior to PCR using. All assays were conducted according to the manufacturers protocols provided with the kits.

GoTaq® Long PCR Master Mix and polymerase was used in combination with pathogen DNA and RNA primers identified in publications to amplify pathogen nucleic acid. For hantaviruses, the PCR was targeted toward the partial large (L) segment of the genome.

Leptospirosis		
5' GCGGGCGCGTCTTAAACATG	3'	Forward
5' TTCCCCCATTTGAGCAAGATT	3'	Reverse
Borrelia		
5' TTATGAAAAAATATTTATTGGGAAT	3'	Forward
5' CTTTAAGCTCAAGCTTGTCTACTGT	3'	Reverse
Trypanosoma		
5' CGTCCCTGCCATTTGTACACAC	3'	Forward
5' GGAAGCCAAGTCATCCATCG	3'	Reverse
Bartonella		
5' GGGGACCAGCTCATGGTGG	3'	Forward
5' AAAGCAAAAAGAACAGTAAACA	3'	Reverse
Hantavirus		
5' TCATGNARRTTRAACATRCTYTTCCAC	3'	Forward
5' TYTTTGARTTTGCHCAYCAYTCWGATG	3'	Reverse
Anaplasma		
5' GGTACCYACAGAAGAAGTCC	3'	Forward
5' TAGACATCATCGTTTACAGC	3'	Reverse
Rickettsia		
5' GGGGGCCTGCTCACGGCG	3'	Forward
5' ATTGCAAAAAGTACAGTGAAC	3'	Reverse
Coronavirus		
5' GGTGGGACTATCCTAAGTGTGA	3'	Forward
5' CCATCATCAGATAGAATCATCAT	3'	Reverse
Coxiella burnetti		
5' CAACTGTGTGGAATTGATGA	3'	Forward
5' TTTACATGACGCAATAGCGC	3'	Reverse

Figure 1: A list of primers sets and sequences

4. Results

4.1 Trapping

Table 1.

Trap site	# of traps	# of nights	# of rodents caught
Blair Road	13	1	1
Chinatown	15	2	2
Pasir Ris Rood Court	20	2	2
Everton Road Alley	20	2	1
Little India (Buffalo Road Alley)	20	2	3
Old Airport Road Food Courts	20	2	3
Blair Road (second trapping)	20	2	3
Long House Food Court	20	2	2
Geylang (Lor 15-17)	20	2	1
Duke-NUS	20	2	8
Outram Bridge	20	2	3
Pest Control Companies (Various locations)	n/a	n/a	4
		Total	33

trapping sites

$$\frac{\Sigma(\# \text{ of small mammals caught})}{\Sigma((\# \text{ of traps}) * (\# \text{ of nights per session}))} = \text{Trapping Success Rate (TSR)}$$

Figure 2: Trapping success rate calculation

The trapping success rate for cumulative expeditions listed above was 12.21%, which was within the published ranges of between 5-15% (Weihong, 1999). This equates to approximately one animal caught per every 9.2 hours of trapping when using 20 traps. The following image shows the geographic distribution of trappings performed in this fieldwork throughout the island of Singapore:

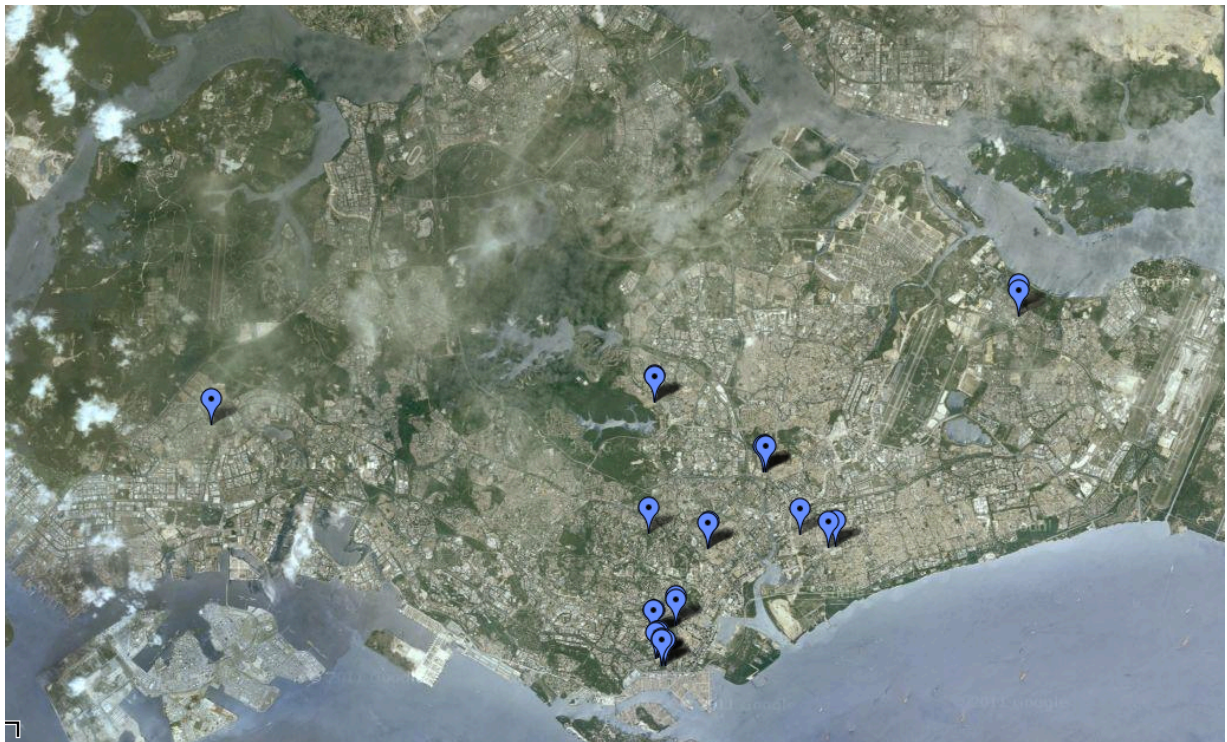


Figure 3. Rodent trapping locations on the island of Singapore

Species	# Collected									
		Leptospirosis	Barotonaella	Borrelia	Anaplasma	Coxiella burnettii	Trypanosoma	Babesia	Hantavirus	Coronavirus
<i>Suncus murinus</i>	18	2	1	5	8	0	1	0	2	3
<i>Rattus tanezumi</i>	6	1	2	1	4	0	0	0	1	0
<i>Rattus novergicus</i>	5	1	0	1	2	0	0	0	0	0
<i>Mus custaneus</i>	4	0	1	0	1	0	0	0	0	0
Total	33									

Table 2. Rodents collected and respective pathogen preliminary positives

Primers used from previously published research were used for PCR pathogen detection (Masiga, 1992; Regnery, 1991; Me rien 1992, Parola, 2000; Nocton 1994; Vijgen 2008). Preliminary positives were designated based on band presence, size and pattern. Sequencing data was used to confirm or reject preliminary assessments.

4.2 PCR

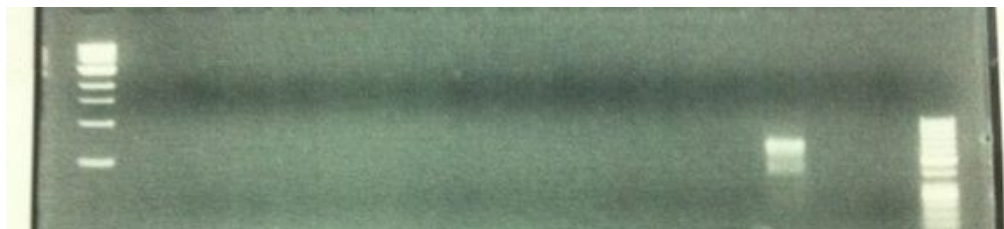


Figure 4. Preliminary Positive band detection of *Trypanosoma* in *S. murinus*



Figure 5. Preliminary positive band detection of *Rickettsia* in *S. murinus*

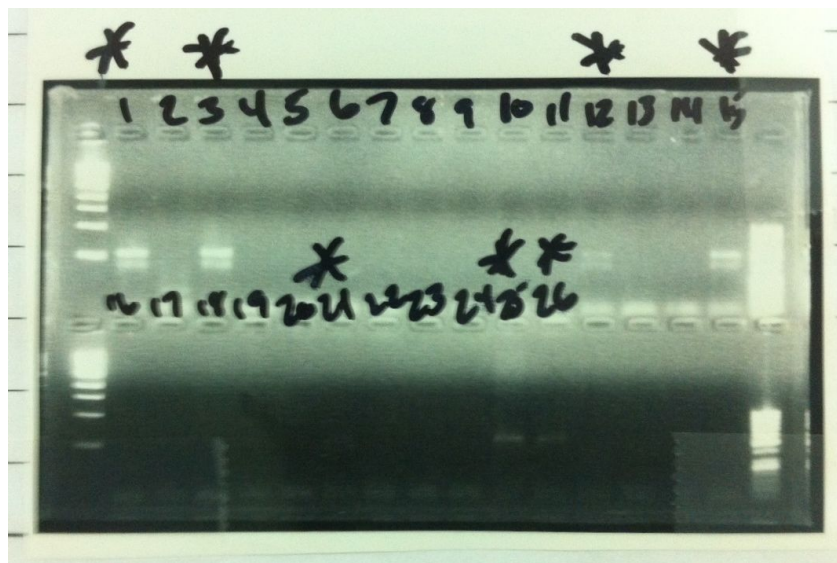


Figure 6. Preliminary positive band detection of *Borrelia* in *S. murinus* and *R. novergicus*

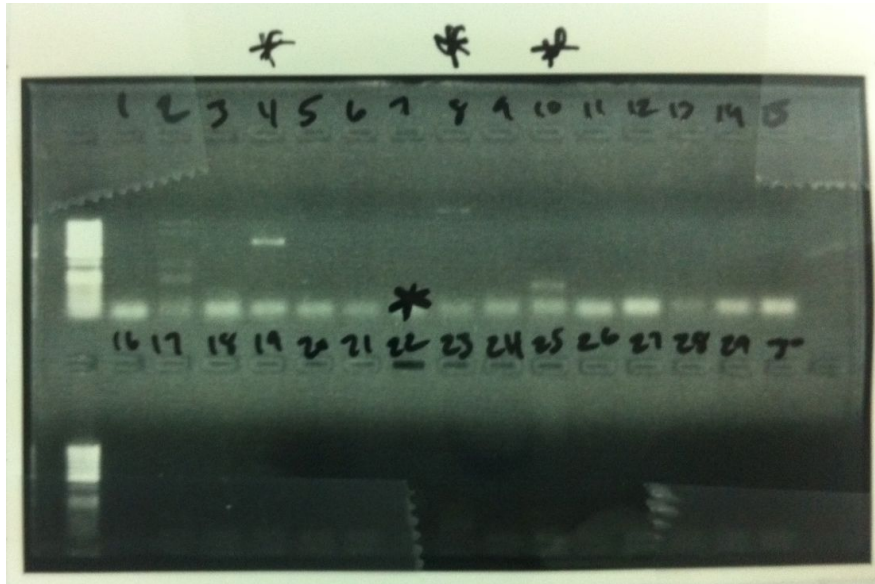


Figure 7. Preliminary positive band detection of *Leptospira* in *S. murinus* and *R. novergicus*

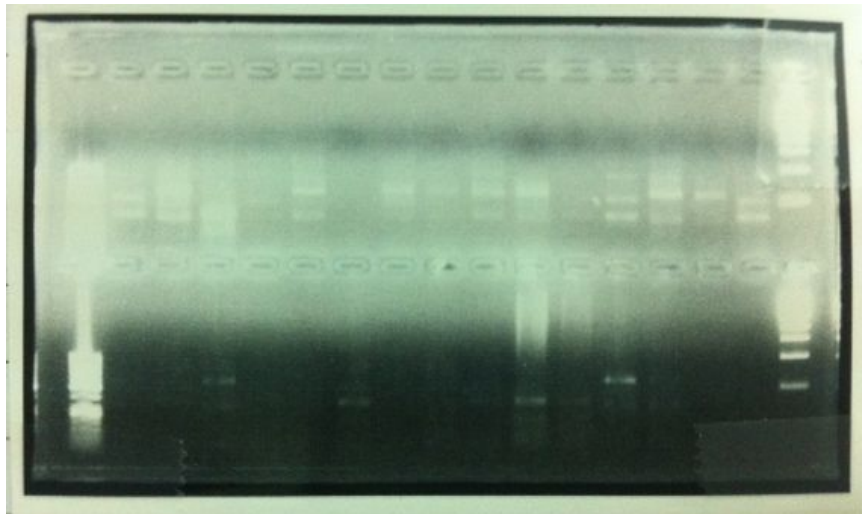


Figure 8. Preliminary positive band detection of *Anaplasma* in *S. murinus*, *R. novergicus*, *R. tanzumi*, and *M. castaneus*.

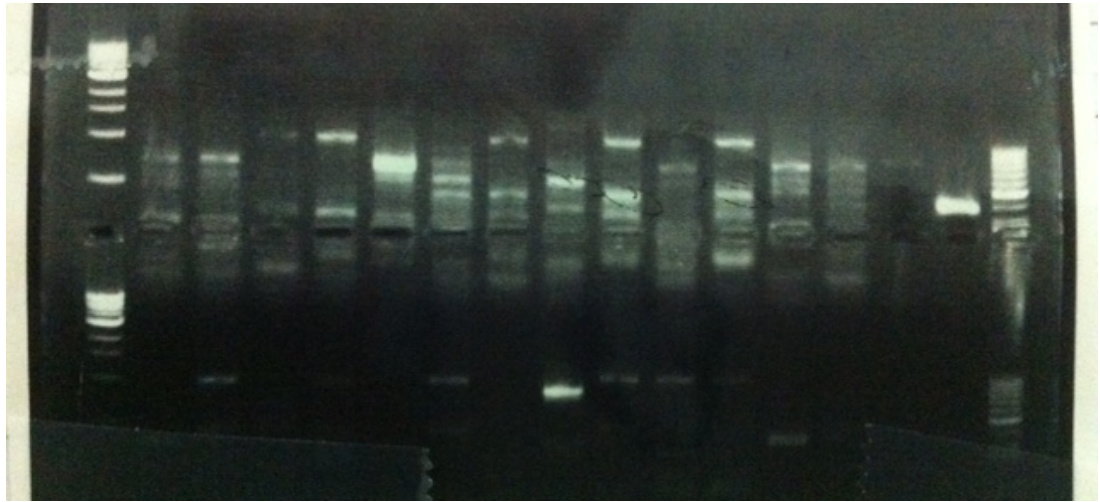


Figure 9. Preliminary positive detection of *Bartonella* in *Rattus norvegicus*

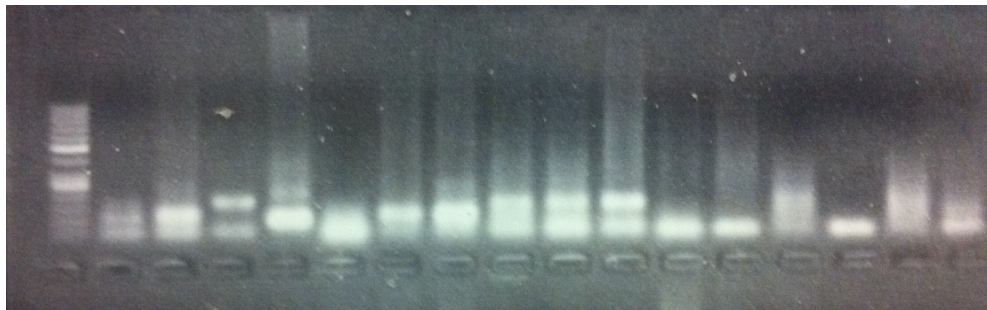


Figure 10. Preliminary positive band detection of *Coronaviridae* in *S. murinus*

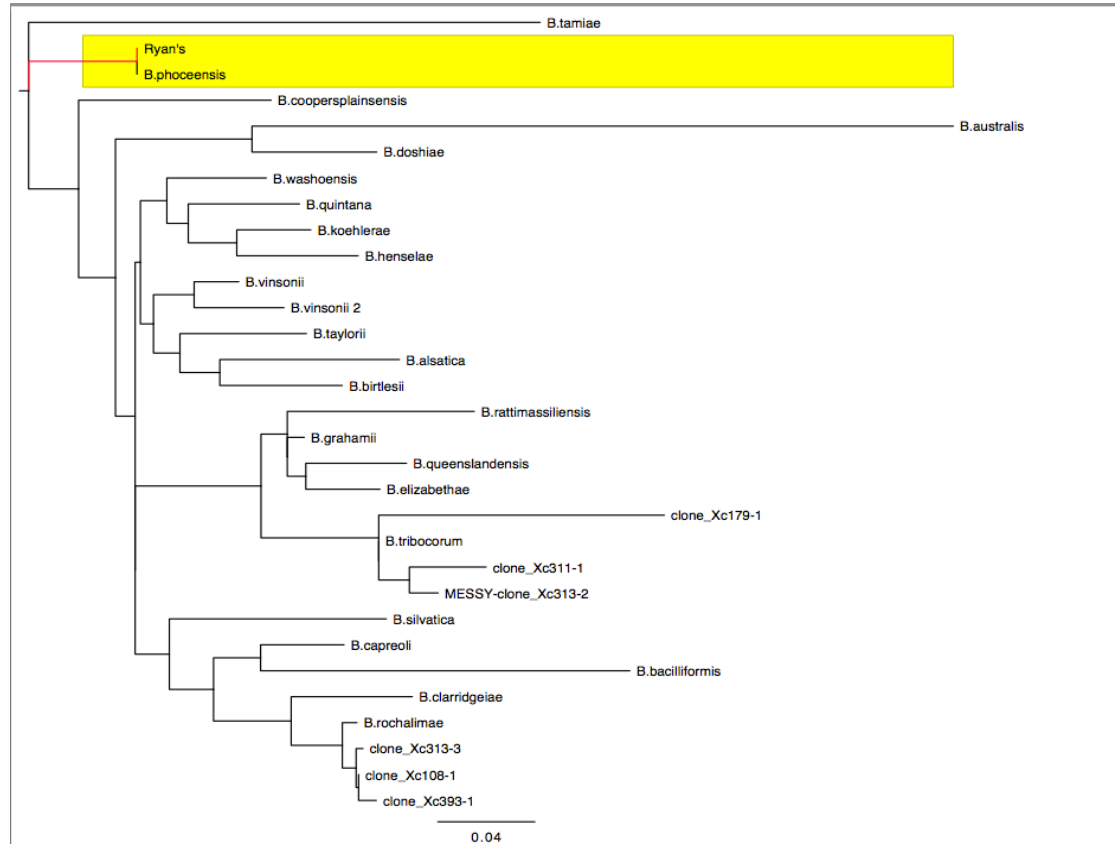


Figure 11. Phylogenetic identification of Bartonella phoceensis

DNA extracted from blood samples were used in detection assays for *Leptospira*, *Bartonella*, *Borrelia*, *Anaplasma*, *Coxiella burnetti*, *Trypanosoma*, and *Babesia*. RNA extracted from pooled samples of lung, kidney and spleen tissues was used in the detection of hantavirus. RNA extracted from pooled large and small intestinal tissues were used to detect coronavirus. Extractions were performed with either the Qiagen QIAamp DNA Blood Mini Kit or the QIAamp Viral RNA kit.

4.3 Sample Size Calculation

In order to compare the prevalence rates of *Bartonella phoceensis*, the following calculation demonstrates the sample size needed in order to assess whether or not prevalence rate differences are statistically significant in a particular cluster of animals relative to urban populations:

The Equation:

$$\text{Sample Size} = n/[1-(n/\text{population})]$$

$$n = Z^2 Z [p(1-p)/(D^2 D)]$$

p = the expected prevalence value of Bartonella in urban Singapore

D = the maximum difference between the sample prevalence rate and the urban population rate.

Z = the area under a normal curve with a confidence level of 95%

The Calculation:

$$n = 1.96^2 \cdot 1.96 [0.0667(1-0.0667)/(0.0333^2 \cdot 0.0333)]$$
$$n = 215.13$$

$$SS = 215.13 \cdot [1+(215.13/1000)]$$
$$\underline{SS = 177}$$

5. Discussion

The initial goal of this thesis was to capture in the range of 100-200 rodents to have a strong representation of the urban rodent populations. Given the time line of this project, this goal was an over estimate. Previous studies calculated trapping success rates (# of animals caught per trap per trapping night) ranging from 5-15%. Our trapping success rate was an average of 8.1% per night. However, when trapping locations are subjectively dichotomized into high human population density and low human population density settings, the TSR between the two settings is appreciable. In high human population density settings the TSR was approximately 5.6%, whereas in the low human population density settings the TSR was approximately 13.8%.

Initially this discrepancy appeared counterintuitive given the natural coevolutionary history of humans and rodents as well as their dependency on human trash as a source of food. This however, may in part be explained by the presence and rapid expansion of the pest control industry in Singapore within recent years, totaling over 220 companies on the island. As a precursor to this study, I also assisted in a small mammal surveillance study in the forested areas in Singapore. While pathogen sampling was also a part of this study, the ecological component of the study demonstrated that urbanization appears to be decreasing the biodiversity of small mammal populations in Singapore. Anecdotal evidence that humans actively control the rodent populations through trap setting is present through conversations I've had with locals on the

nuances of pest control (i.e. what type of traps and bait yield the highest success). It is possible that these proactive measures are effective in reducing rodent populations in areas of high human density or that rodents, through negative reinforcement, have learned to avoid traps while more rural rodent populations have not learned such behavior. While I contacted numerous pest control companies in order to obtain supplementary samples for analysis, many pest control companies did not conduct live trapping and utilized poison traps for small mammal control.

Additional challenges to this study included the sociological interactions and specifically the language barrier. In obtaining verbal permission to conduct trapping I found that many private business and landowners either did not speak English or were not fully proficient. This limited the location sites to where I could effectively communicate and agree upon a trapping schedule with the appropriate private owners.

The contrast between these two scenarios is significant. In the former, in which rodent populations are more strictly controlled in proximal areas of high human density, the risk of rodent borne diseases is intuitively lower. If, however, the latter is true and rodents have simply adapted novel behaviors to help avoid human trapping, the threat of rodent borne pathogens remains constant, while efforts to minimize contact with human becomes increasingly difficult.

With the total of 33 rodents available to sample, we detected preliminary positive results for pathogens relevant to public health including most notably leptospirosis,

hantavirus, coronavirus, and rickettsia. Further analysis through nucleic acid sequencing to confirm the positive presence and species of these diseases and others will help clarify the disease threat indicated in these preliminary results present to the human populations in Singapore. As many of the listed pathogens are present in rodent excreta, exposure to these substances is a potential threat to human health. Further research is needed on the patterns of human exposure to rodent excreta, and the pathogens they contains, to more clearly elucidate the threat of rodent borne diseases on humans and to provide a better understanding of how infectious diseases emerge. One limitation of this study was the lack of ectoparasites for Analysis. In the design of the project, I planned on collecting ectoparasites in the lab immediately prior to necropsy. However, many of the rodents, specifically shrews, were wet, slimy, and dirty which made searching for ectoparasites nearly impossible. I suspect the reason for this observation is that the grease and ingredients from the bait covered the fur of the rodents. Rarely was the bait fully consumed by the trapped animal and was often spread around the trap at the time that anesthetic was applied to the rodent.

Despite preliminary positive PCR detection of numerous pathogens, sequencing data adequate for analysis was only obtained for sample, which was a region of the citrate synthase genome of Bartonella. Phylogenetic analysis of this segment was used to determine the species strain was *B. phoceensis*. This species has previously been isolated from European Rattus Novergicus, however has not been hugely studied for its

physiological manifestations and its threat to the human population. Based on common banding patterns, it appears that there are two positive bands for *B. phoceensis*. Therefore the average prevalence rate of 0.0667 was used in the calculation for sample sizes necessary for comparison of prevalence rates of *B. phoceensis* to in urban areas to other clusters such as forest areas. Although some strains of Bartonella have been detected in Singapore, including *B. henselae* that causes cat scratch fever, there is limited literature on current prevalence rates of Bartonella in both human and animal populations.

The sequence data files (.ab1) for many of the samples showed an electropherogram lacking clearly defined peaks or unevenly spaced peaks. One possible source of error is that the template concentration was lower than the threshold. Many of the bands visualized with ethidium bromide and ultra-violet light appeared faint and therefore the entire amount the PCR product was sent for sampling, yet the amount sent may still have been lower than the optimal 125 nanograms. Another problem was the use of degenerate primers. When running a BLAST analysis of the sequence samples we received, we found that the amplified sequence matched to genomic DNA for host species of *Mus castaneus*, and *Rattus norvegicus*. The use of nested primers designed for the detection of specific species of pathogens rather than family specific primers may help negate the problem of undesirable amplification. In addition, PCR cloning could be

used for more accurate and specific identification.

6. Appendix A



Title: **Overnight Drop Off and Storage of Rodents**

Prepared by: Ryan C Garrett and Ian H Mendenhall
Version 1.0 Number: 1

Approved by: Viji Vijayan and Mahi RN

Effective date:

1.0 OBJECTIVE

The objective of this standard operating procedure (SOP) is to detail methods to be employed in the drop off of rodents at Duke-NUS storage facilities during non-operational hours.

2.0 SCOPE

These procedures are applicable for all supplemental workers depositing rodents for nightly storage at Duke-NUS storage facilities during non-operational hours. Third party collaborators have stipulated that in order to contribute rodents to our research, deposits must be made between the hours of 19:00 and 00:00.

3.0 RESPONSIBILITIES

3.1 Principal Investigator

These individuals are responsible for ensuring that this SOP is disseminated and adhered to by all research personnel involved in the deposition, collection, and sampling of the rodents of Singapore. It is the responsibility of Principal Investigator to make all relevant personnel aware of these procedures and safety hazards, arrange applicable training, have appropriate vaccinations, are included on relevant permits, and oversee the work for all research staff and students.

3.2 Staff/Students/Collaborators/Research Personnel

All field research personnel working must be aware of the potential human and environmental safety hazards; must be knowledgeable of this SOP and its contents (and other applicable NUS-wide and nation-wide guidelines, SOPs, and regulations); must obtain necessary training and work under supervision until proficient in the practices and techniques required to handle such material safely. All field personnel are responsible for reporting safety incidents and potential human and environmental safety hazards.

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4.0 DEFINITION

Lean-to Cage: A cage-like structure in which the small rodent cages will be placed during drop offs, located at 2, Jalan Bukit Merah.

Rodent Handlers: Rodent trappers that are not employed by Duke-NUS

Urban Tomahawk Traps: A live capture trap used to collect small mammals

5.0 PROCEDURES

5.1. Drop Off Correspondence

Prior to the drop off of any specimen, the rodent handler will contact a member of the Duke-NUS EID-small mammals group (Appendix A) to confirm that the lean-to cage is available for drop off. The rodent handler must receive affirmation prior to drop off.

5.2. Third Party Access to Facilities

Upon entering the Duke-NUS storage facility grounds the rodent handler will convene with security personnel to ensure that their presence is known and that they are on a list of acceptable handlers (appendix A). Said list will be agreed upon and disseminated to relevant security personal as decided prior to the entry of handlers.

5.3. Deposit of Rodents

Rodent handlers will deposit animals in the lean to cage with a latched door located the behind the Duke-NUS storage shed. A secure wire cage covered with a tarp and located at the back of the shed on 2 Jalan Bukit Merah will be used to temporarily house the rodents until Duke-NUS staffs are available for processing. This will have a lock that only the Laboratory of Virus Evolution and the third party rodent trapping agency will have the key to. This cage will prevent any animals that escape from their cages, thus establishing two levels of containment.

5.4 Recording specimen Information and Euthanasia

The rodent handler will record information including date and location for each specimen on a clipboard provided by EID staff. The rodent handler will assign each specimen a unique number, which will be associated with information recorded on a data collection sheet. A laminated card with the identifying number will be placed on top of the corresponding cage. Individual cages will be removed from the storage cage and placed into a bag containing cotton balls soaked in isoflurane to euthanize the rodent. The animal will then be double bagged in biohazard bags, placed into a cooler, and brought to a -80C freezer in the Emerging Infectious Disease laboratory. Isoflurane and the cotton balls should be brought back to laboratory to be disposed appropriately.

7. References

- Barrett MP, Burchmore RJ, Stich A, et al. (November 2003). "The trypanosomiasis". *Lancet* 362 (9394): 1469–80
- Blasdell, K, J Cosson, Y Chaval, V Herbreteau, B Douangboupouha, S Jittapalapong, A Lundqvist, J Hugot, S Morand, and P Buchy. "Rodent-Borne Hantaviruses in Cambodia, Lao PDR, and Thailand." *Ecohealth* (2011):
- Chaisiri, K, W Chaeychomsri, J Siruntawinetti, F Bordes, and S Morand. "Human-dominated Habitats and Helminth Parasitism in Southeast Asian Murids." *Parasitology Research* 107.4 (2010): 931-937
- Centers for Disease Control and Prevention. (2012). Infectious Diseases. Retrieved from <http://www.cdc.gov/ncidod/about.htm>
- Centers for Disease Control and Prevention. (2012). Infectious Diseases. Retrieved from <http://www.cdc.gov/rodents/diseases/direct.html>
- Centers for Disease Control and Prevention. (2012). Update: Outbreak of Nipah Virus – Malaysia and Singapore 1999. Retrieved from <http://www.cdc.gov/mmwr/preview/mmwrhtml/00057012.htm>
- Groot RJ, Baker SC, Baric R, Enjuanes L, Gorbalenya AE, Holmes KV, Perlman S, Poon L, Rottier PJM, Talbot PJ, Woo PCY, Ziebuhr J, "Family Coronaviridae". In: Ninth Report of the International Committee on Taxonomy of Viruses. AMQ King, E Lefkowitz, MJ Adams, and EB Carstens (Eds), Elsevier, Oxford, (2011): 806-828.
- Gubler D, Reiter P, Ebi K, Yap W, Nasci R, Patz J. "Climate variability and change in the United States: potential impacts on vector- and rodent-borne diseases." *Environ Health Perspect.* 2001 May; 109(Suppl 2): 223–233.
- Herbreteau V, Bordes F, Jittapalapong S, Supputamongkol Y, Morand S. "Rodent-borne diseases in Thailand: targeting rodent carriers and risky habitats." *Infect Ecol Epidemiol.* 2012; 2
- Johansson, P, G Yap, H Low, C Siew, R Kek, L Ng, and G Bucht. "Molecular Characterization of Two Hantavirus Strains from Different Rattus Species in Singapore." *Virology Journal* 7.15 (2010)

- Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P. Global trends in emerging infectious diseases. *Nature*. 2008 Feb 21;451(7181):990-3.
- Kang, H J., M Y. Kosoy, S K. Shrestha, M P. Shrestha, J A. Pavlin, and R V. Gibbons. "Short Report: Genetic Diversity of Thottapalayam Virus, a Hantavirus Harbored by the Asian House Shrew (*Suncus Murinus*) in Nepal." *Am J Trop Med Hyg* 85.3 (2011): 540-545.
- Kang, H J., M Y. Kosoy, S K. Shrestha, M P. Shrestha, J A. Pavlin, and R V. Gibbons. "Thottapalayam Virus Is Genetically Distant to the Rodent-borne Hantaviruses Consistent with Its Isolation from the Asian House Shrew (*Suncus Murinus*)." *Virol J* 4.80 (2007)
- Kang, H, Satoru Arai, A Hope, J Cook, and R Yanagihara. "Novel Hantavirus in the Flat-Skulled Shrew (*Sorex Roboratus*)." *Vector-Borne and Zoonotic Diseases* 10.6 (2010): 593-597
- Masiga, D. K., A. J. Smyth, P. Hayes, T. J. Bromidge, and W. C. Gibson. "Sensitive detection of trypanosomes in tsetse flies by DNA amplification". *Int. J. Parasitol.* (1992): 22, 909– 918.
- Me'rien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. "Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples". *J Clin Microbiol.* (1992) 30: 2219–2224.
- Meerburg B, Singleton G, and Kijlstra A. "Rodent-borne Diseases and Their Risks for Public Health." *Crit Rev Microbiol* 35.3 (2009): 221-270
- Mills, J. "The Role of Rodents in Emerging Human Disease: Examples from the Hantaviruses and Arenaviruses." *Australian Centre for International Agricultural Research* (1999): 134-160
- Mills, J, G Singleton, L Hinds, H Leirs, and Z Zhang. "The Role of Rodents in Emerging Human Disease: Examples from the Hantaviruses and Arenaviruses." *Ecology Based Rodent Management* (1999): 134-160.
- Mitchell P, Reed K, Hofkes J "Immunoserologic Evidence of Coinfection with *Borrelia burgdorferi*, *Babesia microti*, and Human Granulocytic Ehrlichia Species in Residents of Wisconsin and Minnesota". *Journal Of Clinical Microbiology*, (1996): p. 724–727.

- Nocton J.J., F. Dressler, B.J. Rutledge, P.N. Rys, D.H. Persing and A.C. Steere. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *N. Engl. J. Med.* (1994): 330: 229–234.
- Parola, P, Roux, V, Camicas, JL, Baradji, I, et al. Detection of Ehrlichiae in African ticks by polymerase chain reaction. *Trans R Soc Trop Med Hyg* (2000) 94:707–708.
- Powar RM; Shegokar, VR; Joshi, PP; Dani, VS; Tankhiwale, NS; Truc, P; Jannin, J; Bhargava, A (1 January 2006). "A rare case of human trypanosomiasis caused by *Trypanosoma evansi*". *Indian J Med Microbiol* 24 (1): 72-74
- Regnery, M. "Genotypic identification of Rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes." *Journal of Bacteriology* (1991): 173. 1576-1589.
- Taylor, L. et al. (2001). Risk factors for human disease emergence *Philosophical Transactions of the Royal Society B*, 356(1411):983-9.
- Tseng BY, Yang HH, Liou JH, Chen LK, Hsu YH (February 2008). "Immunohistochemical study of scrub typhus: a report of two cases". *Kaohsiung J. Med. Sci.* 24 (2): 92–8.
- Singh M, Hian Y, Lay-Hoon C. "Current Stats of Food-Borne Parasitic Zoonoses in Singapore." *Southeast Asian J Trop Med Public Health.*
- Singleton, G, L Smythe, G Smith, D Spratt, K Aplin, and A Smith. "Rodent Diseases in Southeast Asia and Australia: Inventory of Recent Surveys." *Rats, Mice and People: Rodent Biology Management* 10.6 (2003): 25-29.
- Song, J, H Kang, S Gu, S Moon, S Bennet, K Song, L Baek, H Kim, M O'Guinn, S Chong, T Klein, and R Yanagihara. "Characterization of Imjin Virus, a Newly Isolated Hantavirus from the Ussuri White-Toothed Shrew (*Crocidura lasiura*)." *Journal of Virology* 83.12 (2009): 6184-6191.
- Steere AC; Sikand VK, Schoen RT, Nowakowski J (2003). "Asymptomatic infection with *Borrelia burgdorferi*". *Clin. Infect. Dis.* 37 (4): 528–532
- Vijgen. *Methods in Molecular Biology: SARS and other Coronaviruses - Laboratory manual.* (2008): 454 p1-10

Walker DH. "Rickettsiae and rickettsial infections: the current state of knowledge". Clin Infect Dis. Jul 15 2007;45 Suppl 1:S39-44.

Weihong J, Veitch C, Craig J. "An Evaluation of the Efficiency of Rodent Trapping Methods: the Effect of Trap Arrangement, Cover Type, and Bait New Zealand Journal of Ecology (1999) 23(1): 45-51

Woolhouse, M, and S Gowtage-Sequeira. "Risk Factors for Emerging Zoonoses." Centre for Infectious Diseases. United Kingdom: University Of Edinburgh, 2005.

Yoo D, Pei Y, Christie N, Cooper M. "Primary Structure of the Sialodacryoadenitis Virus Genome: Sequence of the Structural-Protein Region and Its Application for Differential Diagnosis: Journal of Clinical Diagnostics Lab Immunology (2000): 568-573.