Investigating rodents and environmental samples as possible sources of *Leptospira* infections in the Mnisi communal area, Bushbuckridge, South Africa

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Abstract

Leptospirosis is an important bacterial infection of both animals and humans, largely neglected worldwide. Leptospirosis occurs worldwide and is maintained in numerous animal hosts. The aim of this study was to investigate rodents as a potential source of pathogenic *Leptospira* spp. infection within the Mnisi community where a previous outbreak of human leptospirosis had occurred in 2018. The Mnisi community is a rural community, situated at the wildlife-livestock-human interface within the Bushbuckridge Local Municipality, Mpumalanga Province of South Africa. As part of the study, soil and water samples were also tested for the presence of pathogenic *Leptospira* spp. bacteria to investigate the potential of environmental sources negatively affecting human health.

Our research included three aspects: biobank samples collected from Athol, Gottenburg, Utha and Thlavakisa villages from September 2020 until March 2022; rats trapped from Welverdiend A in April 2022 and environmental samples collected from all five villages in April 2022. For trapping in Welverdiend A, eight traps were set per household, with a total of 25 households. These traps were set in the late afternoon, baited with peanut butter and oats, left overnight and checked in the early mornings, for a total of four trapping nights. The traps were placed in strategic areas around the household where they were safe from predators. After collection, the rats were euthanized and kidneys dissected for further processing. DNA was extracted making use of the Invitrogen PureLink Genomic mini kit, where-after a real-time PCR specific for pathogenic *Leptospira* was performed. Environmental samples included both soil (25 samples) and water (25 samples) collected from strategic areas throughout the five villages. DNA was extracted using the Zymo Quick-DNA Fecal/Soil Microbe MiniPrep Kit and a real-time PCR was performed. GPS coordinates and photographs were taken of all environmental sample collection sites.

A total of 158 rodent kidney samples was analyzed, comprising 124 biobank samples (from Athol, Utha, Gottenburg and Thlavakisa) and 34 fresh samples collected from Welverdiend A. None of the rodent kidney samples contained enough pathogenic *Leptospira* spp. bacteria for DNA to be detected. Twenty-five soil and 25 water samples were processed from all five villages (five of each from each village) and no pathogenic *Leptospira* spp. were detected.

This project was the first of its kind for the study of leptospirosis in the Mnisi community and forms part of the very limited information that is available on leptospirosis within South Africa. Although all samples returned a negative result, this study is still valuable as any information on the prevalence of *Leptospira* spp. and its potential reservoir hosts is important to guide further studies. The sample population was quite small due to limited funding and more in-depth research on pathognenic *Leptospira* spp. within the Mnisi Community, Bushbuckridge, would prove to be exceptionally valuable.

Various Lists

List of abbreviations

In order of appearance:

- SADC: Southern African Development Community
- NICD: National Institute for Communicable Diseases, South Africa
- ELISA: Enzyme-linked immunosorbent assay
- MAT: Microscopic agglutination test
- WHO: World Health Organization
- WOAH (formerly OIE): World Organization for Animal Health
- IHC: Immunohistochemistry
- PCR: Polymerase chain reaction
- DRC: Democratic Republic of the Congo
- SSW: Sabi Sand Game Reserve
- Great Limpopo TFCA: Great Limpopo Transfrontier Park & Conservation Area
- KNP: Kruger National Park

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Introduction

Background information

Leptospirosis is an important bacterial infection of both animals and humans, largely neglected worldwide. Leptospirosis is caused by pathogenic *Leptospira* spp., it occurs worldwide and is maintained in numerous animal hosts. For many years, rodents have been implicated as the primary source for human infection (Allan et al., 2015). Since then, studies have shown numerous domestic and wildlife maintaining or incidental hosts that may contribute to human infection.

Leptospira has historically been classified into pathogenic (*L. interrogans*) and non-pathogenic (*L. biflexa*) species (Farr, 1995). There are numerous classifications used for *Leptospira* spp. bacteria throughout the literature (Levett, 2001; Levett and Haake, 2010). In newer research *Leptospira* serovars as the basic subspecies taxon have been grouped as a function of their antigenic determinants (Caimi and Ruybal, 2020). The following species are primarily implicated in disease conditions of humans and animals: *L. interrogans, L. noguchii, L. borgpetersenii, L. santarosai, L. kirschneri, L. weilii* and *L. alexanderi* (Ahmed et al., 2006). This study was aimed at researching pathogenic *Leptospira* species.

There is a distinct lack of data concerning the true prevalence of leptospirosis in the Southern African Development Community (SADC) region, and also specifically in South Africa. This study aimed to fill that gap by providing data on the occurrence of *Leptospira* spp. infections in rodents from the Mnisi community, Bushbuckridge, South Africa. In a serological study on the prevalence of zoonotic disease in adults in Bushbuckridge, leptospirosis was diagnosed in 6.8% of patients presenting with febrile disease at the local clinic (Quan et al., 2014). In the same study 22% of cattle herders tested positive on serology for previous exposure to leptospirosis. Furthermore, there was an outbreak of leptospirosis in human patients in 2018 (three seropositive cases, apparently unlinked) in the same general area where this study was located (Anonymous, Communicable Diseases Communiqué, July 2018, Vol. 17(7)).

Cases of leptospirosis in humans within South Africa are sporadic, therefore this cluster of positive cases prompted an investigation as to the potential source/s of infection. A One Health approach was utilized for the investigation, with data collected from animals and the environment. The households with positive cases all showed opportunity for exposure to *Leptospira* spp. by animal contact such as rodents, livestock or dogs. All samples collected (household members (IgM), animals in close contact and water samples from the environment (PCR) tested negative for leptospires. These samples included only one rodent, five dogs, ten goats, four household members and fifteen water samples, and was insufficient to detect the source of infection in this outbreak. From this one can speculate that leptospirosis is a zoonotic disease of potential relative importance in the Bushbuckridge area although the source of exposure is yet undetermined.

This study aimed to fill some of the gaps in this information, by attempting to estimate the prevalence of pathogenic *Leptospira* spp. in rodents in the Mnisi area, South Africa. Some communities are known for poor sanitation and personal hygiene, with rodents gaining access to houses, feed stores and vegetable gardens, posing a great risk to human health. The collection of soil and water samples for *Leptospira* spp. analysis provided data on the risk associated with environmental contamination in these communities. This is an important factor to consider, as communities mostly make use of communal water and grow their own vegetables for household consumption. This study aimed to determine if there is a significant disease risk to humans associated with rodents and the environment. This can provide a guideline for further studies on leptospirosis to identify all major risk factors to human health and their possible control measures.

Research questions

- Are rodents a potential source of pathogenic *Leptospira* spp. infections for the Mnisi Community?
- Are soil and water potential environmental sources of pathogenic *Leptospira* spp.?

Aims and objectives

Aim

The aim of this study was to investigate rodents as a potential reservoir and source of pathogenic *Leptospira* spp. infection within the Mnisi community. Soil and water samples were also tested for the presence of pathogenic *Leptospira* spp. bacteria to investigate the potential of environmental sources posing a threat for human health.

Objectives

1. To determine the occurrence of pathogenic *Leptospira* spp. in rodents within the Mnisi Community, South Africa.
2. To visually depict the geographic distribution of infection within rodents in the study area.
3. To test soil and water samples from the same areas as indicators for environmental contamination with pathogenic *Leptospira* spp.

Literature review

Introduction

Leptospirosis is a zoonotic bacterial disease, not only causing economic losses in livestock farming, but also morbidity and mortality in humans. Leptospirosis has a world-wide distribution but remains under-diagnosed in Africa due to lack of resources and presence of other febrile illnesses such as malaria. It is estimated that >1 million cases occur annually worldwide (reported only). Of these, approximately 59 000 are fatal (Centers for Disease Control and Prevention) (reported only). With international travel becoming easier and more popular, the prevalence of tropical diseases has increased in returning travelers (Jensenius et al., 2013). Some tropical diseases can be fatal within weeks of symptoms presenting. This emphasizes the need for accurate information regarding disease, and proper diagnostic tests to diagnose and treat these cases in a timely fashion.

Leptospirosis is an infection caused by aerobic, Gram-negative bacterial spirochetes of the genus *Leptospira*. They are slow-growing, fastidious and move in a corkscrew-like fashion. The helical coiling is in a clockwise direction with two flagella per cell, enabling the organism to easily burrow into tissue (Goldstein and Charon, 1988). Leptospires survive optimally at 28 – 30°C environmental temperatures, being able to survive in soil, contaminated water, and animal- or human hosts (Breed et al., 1957). Leptospirosis therefore occurs more regularly in hot and humid areas of the world and is prevalent in tropical and sub-tropical regions (de Vries et al., 2014).

Leptospirosis has been regularly researched in the veterinary setting as it causes more severe economic losses in the farming industry (e.g. abortions, stillbirth, decreased milk production and death) (Myburgh and Otto, 1990) in comparison to human morbidity and mortality (de Vries et al., 2014). The infection is carried and maintained in the renal tubules (as a chronic renal infection), and sometimes genital tract, by numerous wild and domestic hosts. These carrier animals excrete leptospires intermittently or continuously in their urine, contaminating the environment. In most tropical areas, rodents are hypothesized to be the most important reservoir host leading to human and animal infection. Taking this into consideration, there is an increased risk in areas dominated by poor hygiene, urban slums, poor sewerage and regular floods, as this may expose humans and animals to rodent urine (Maze et al., 2018).
A literature study was performed on leptospirosis in the SADC (South African Development Communities) region, to provide a guideline on the amount of data currently available.

Human leptospirosis studies

Human infection was reported in 24 studies, spanning over seven countries in the SADC region. The country with the most abundant data available on human leptospirosis, is Tanzania, with a total of 13 articles published describing human disease. South Africa only had four studies describing human leptospirosis, with the rest of the countries having two or fewer published articles. Fever of unknown origin was the most common criterion for selection of study individuals. Numerous studies were done as part of a larger study screening for zoonotic diseases such as brucellosis, leptospirosis and Q fever. This low number emphasizes the lack of research available on human leptospirosis.

Prevalence of human leptospirosis

The prevalence of leptospirosis ranged from 0.1% (a cross-sectional prospective study in Madagascar) (Guillebaud et al., 2018) to 60% (a prospective cohort study in the Seychelles) (Yersin et al., 1998). The average prevalence in South African studies is 11.9%, with asymptomatic people in frequent contact with animals (“dip-tankers”, i.e. cattle herders) showing a prevalence of 21.9% according to Simpson et al. (2018). Mozambique had an average prevalence of 10.1% measured across the two studies available (Collares-Pereira et al., 1997, Ribeiro et al., 2017). An 11.5% prevalence was observed by using an enzyme-linked immunosorbent assay (ELISA) in conjunction with microscopic agglutination test (MAT) in one study (Ribeiro et al., 2017). Namibia had a disease prevalence of 3% when screening for *Leptospira*, Q

fever and brucellosis in numerous patients with Onyalai and those in close proximity with these patients (Wessels et al., 1986). These studies were selected to include due to their physical proximity to the study area.

Prevalence of leptospirosis in animal hosts

South African data show an average prevalence of 35.4% of leptospirosis, with canines and bovines the most widely researched and also highest risk animals according to the data included in the literature review. Prevalence ranged from 2% (study on bovines in 1990, including 860 cows) (Myburgh and Otto, 1990) to 90.5% (study on prevalence of leptospirosis in pigs) (Hunter et al., 1987). Rodents were rarely included in studies performed in South Africa, also seemingly irrelevant as a potential host according to three case studies performed in 1999 (Gummow et al., 1999). Botswana only had two studies available; one researched the prevalence in numerous wild animals (17 of 69 African wildlife species tested positive for leptospirosis; 31.4% mammals, 27.8% avian and 6.3% reptiles) and rodents, showing a 23.4% prevalence of disease in rodents within that study (Jobbins and Alexander, 2015). The other study researched leptospirosis in bovines, with 24 out of 40 animals tested being positive (60% prevalence) (Herr and Winnen, 1983). Zimbabwe had an average prevalence of 21.3% across two studies done more than 30 years apart. Data was collected on bovines and canines with a 27% and 15.6% prevalence respectively (Feresu, 1987, Dhliwayo et al., 2012).

Diagnostic methods

Serological testing is the gold standard method of diagnosing leptospirosis. The microscopic agglutination test (MAT) is the diagnostic test recommended by the World Health Organization (WHO) as well as the World Organization for Animal Health (WOAH, formerly OIE) (Loureiro et al., 2013). This test is serogroup-specific, is inexpensive and widely available, but challenging due to the subjectivity of the diagnosis and the need to keep live *Leptospira* spp. cultures. One of the main limitations of MAT is a low sensitivity in the initial course of the clinical disease before the rise in specific immunoglobulins in blood. A test positive MAT can be difficult to interpret as vaccination, antibody cross-reactivity and lack of research about appropriate antibody cutoff titers complicate the diagnosis.

Serological testing is more appropriate when screening for infection at a herd level. Several tests have been developed to detect anti-*Leptospira* immunoglobulins that are more rapid and more commercially available than MAT. Enzyme-linked immunosorbent assays (ELISAs) are highly sensitive but less specific, and different ELISAs can distinguish between acute (IgM) and chronic (IgG) infection. The commercial kits are available only for specific animal species including dogs and cattle, but availability of tests to screen in other species is lacking, including pigs. These kits are also designed to detect IgM, which means that previous exposure in cases will not be detected and is thus not appropriate for surveillance cross-sectional studies in epidemiology of leptospirosis.

Immunofluorescence on tissue, blood or urine sediment can also be used to detect leptospires. The biggest limitation of these methods is the intermittent shedding of leptospires in the body, particularly in the chronic stages of disease. Formalin-fixed tissues can be submitted for immunohistochemistry (IHC), although the organisms may be scarce. This is not a commonly used method for surveillance studies.

Culture provides a definitive diagnosis of the leptospiral serovar. Samples submitted for culture are most commonly kidney tissue and urine. A special culture medium is necessary and diagnostic laboratories rarely use this method as the bacteria are extremely slow growing.

Molecular detection of leptospires has become increasingly more popular as methods become more streamlined, more rapid and more cost effective. Polymerase chain reaction (PCR) tests detect the pathogen's nucleic acid and may prove useful in the early stages of disease. Conventional PCR methods may also prove helpful in a clinical setting after antimicrobial therapy has been administered, as it detects both viable and non-viable organisms (Sykes et al., 2011).

PCR was found to detect a much higher prevalence of leptospirosis compared to MAT and culture (Mgode et al., 2005, Rahelinirina et al., 2010). Common genetic targets used for PCR testing includes *lipL32, secY, rrl* and *rrs* (Allan et al., 2015).

Real-time PCR provides a quantitative, rapid and sensitive test for the molecular detection and identification of pathogenic leptospires in clinical specimens. Real-time PCR assays using SYBR Green and melt-curve analysis have been developed to differentiate between *Leptospira* species (Levett et al., 2005, Merien et al., 2005, Moseley et al., 2020, Naidoo et al., 2020). Hydrolysis probe-based real-time PCR assays targeting the 16S ribosomal RNA (*rrs)* and *LipL32* genes have been shown to detect pathogenic *Leptospira* spp. (Slack et al., 2007; Gentilini et al., 2015), although the *rrs* real-time PCR assay also detects leptospires of intermediate pathogenicity. The rapid throughput of these methods allows for rapid decision making in a clinical setting, such as during outbreaks, as well as guiding clinicians in vaccine choices.

In summary, leptospirosis is an important, yet neglected zoonotic disease that has been identified in many animal species worldwide. This disease comprises a large range of symptoms and numerous clinical syndromes, once more complicating the diagnosis thereof. Febrile illness is a broad clinical syndrome, with much more prevalent diseases, such as malaria, gaining higher priority in previous research studies. There is limited data available on the prevalence and incidence of leptospirosis in sub-Saharan Africa, most likely due to lack of resources in these impoverished communities.

Leptospirosis was reported across numerous wildlife (Jobbins and Alexander, 2015) and domestic hosts, with some reports studying both human and animal infection. This would be helpful in linking possible animal infection with human disease but a vast amount of data is still lacking.

A study conducted in Tanzania in 2012, estimated the leptospirosis incidence to be 11-18 per 100 000 people annually (Maze et al., 2016), much lower than the previous sole incidence estimate of 75-102 per 100 000 people annually, as reported in 2007 (Biggs et al., 2013). The newer study discusses numerous theories regarding the decrease in annual incidence between 2007 and 2012, highlighting factors affecting disease transmission. One of these is severe climatic conditions. They hypothesize that the strong El Nino events of 2006-2007 may have transiently increased the annual incidence of disease as calculated in 2007. Supporting this hypothesis, numerous other studies also identified wet and humid conditions to be prominent risk factors in disease transmission. A study conducted in the Democratic Republic of the Congo (DRC) amidst a pneumonic plague outbreak among diamond miners during 2004, also reported leptospirosis in 53.7% of the individuals (Bertherat et al., 2014). This study also highlighted the poor conditions that these miners were working in. There were approximately 7000 people living in a small camp in the humid rain forest. Sanitation conditions were appalling and these miners were standing in pits dug in the ground, half-filled with stagnant water. Biscornet (2017) describes leptospirosis prevalence in humans in the Seychelles. A direct link with climatic conditions could be seen, with 11.6% and 5.4% prevalence recorded in the wet and dry season respectively (Biscornet et al., 2017). Poor sanitation conditions, humid climates and grazing of effluent-contaminated pastures, were also identified as risk factors in cases of leptospirosis in animal hosts. One study conducted in South Africa, described three case studies, highlighting the link between poor sanitation conditions and the high number of animals testing positive for leptospirosis (Gummow et al., 1999). This also demonstrated the spread of disease within multi-species farming units via water and effluent wastewater.

A study on disease prevalence in febrile patients and asymptomatic in-contact animal workers (“dip-tankers”), also showed that 21.9% of in-contact humans tested positive for leptospirosis (Simpson et al., 2018). This re-iterates the zoonotic potential of this disease.

Final Remarks

Within this review it has become apparent that leptospirosis is prevalent in both animals and humans within countries of the SADC region. This merely indicates that leptospirosis is present within the SADC region, with a high average prevalence even becoming apparent through the limited data available.

The study by Simpson et al. (2018) highlights the importance of numerous zoonotic diseases at the human-wildlife-livestock interface in the Mnisi community in Mpumalanga. In this study it was found that 6.8% of people with acute febrile illness (n=74) were IgM positive for *Leptospira*. The more shocking result was that 21.9% of people regularly in contact with animals (such as farmers, veterinary staff, and herdsmen) (n=64) tested IgM positive for *Leptospira* spp. The outbreak of three positive leptospirosis cases in Welverdiend A in 2018 as described by the NICD (Communicable Diseases Communiqué, July 2018, Vol. 17(7)) is also concerning, as this suggests that leptospirosis is present within the area and calls for further research to establish more accurate prevalence data.

Materials and methods

Ethics statement

This research was intended only for expansion of knowledge regarding a public health risk and is not intended for malicious or misuse of any kind. Information obtained from members of the public is confidential and will not be shared with any party other than for research purposes.

All ethical approval required for this research project was acquired prior to commencement of this project and can be found attached to this document (Appendix A to D). A Section 20 permit can be found in Appendix A. Research Ethics Committee and Animal Ethics Committee approval was acquired and all relevant amendments made (Appendix B and C). To travel with rodent and environmental samples (from Welverdiend A, Gottenburg, Athol, Utha and Thlavakisa to Hans Hoheisen Wildlife Research Station), as well as the extracted DNA (from Hans Hoheisen Wildlife Research Station to Department of Veterinary Tropical Diseases, University of Pretoria), travel permits were obtained from the Department of Land Reform and Rural Development (DALRRD) and signed by the state veterinarian (Appendix D).

Study area

This study was based within the Mnisi Community area, situated in the Mpumalanga Province of South Africa. It is situated within the Bushbuckridge Local Municipality with an estimated population of 40 000 – 50 000 people (Statistics South Africa, 2012). This area has a subtropical climate.

The study area is rural, consisting of approximately 29 500 ha of communal land. Approximately 75% of the area is bordered by conservation areas (private and provincial), including Manyeleti and Andover game reserves, and Sabi Sand Game Reserve (SSW). Manyeleti provincial game reserve and SSW form part of the Great Limpopo Transfrontier Park & Conservation Area (TFCA) as they have open access to the Kruger National Park (KNP) (Berrian et al., 2016). The location of this area is ideal for any research on zoonotic disease at the wildlife-livestock-human interface.

The targeted villages were Athol, Thlavakisa, Gottenburg, and Utha (included in a separate study funded by the National Institutes of Health (NIH)), as well as Welverdiend A, where the leptospirosis outbreak occurred in 2018 (see Figure 1).

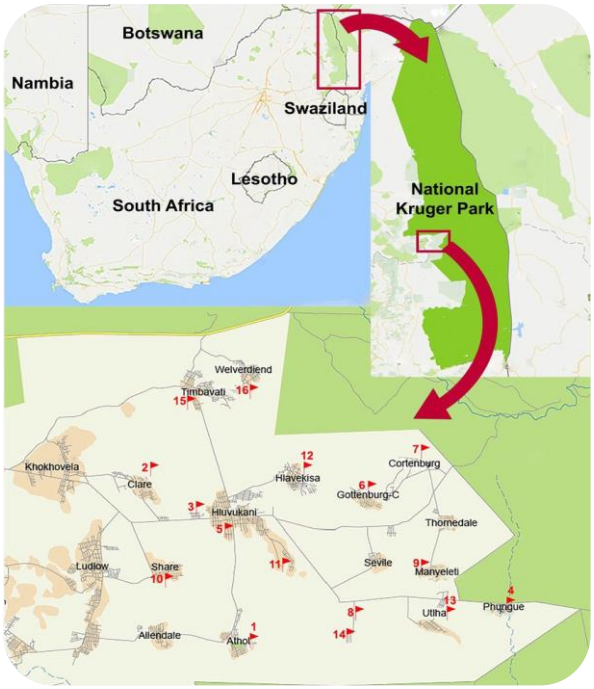

Figure 1: Map of the Mnisi study area, Bushbuckridge, Mpumalanga Province (Jongejan et al., 2020)

Sample size calculations

Sample size calculations for rodents

In this study, the prevalence of *Leptospira* species was investigated from rodents trapped in five different locations in the Mnisi Community, Mpumalanga Province, South Africa. The sample size required for the study was calculated using the formula by Thrusfield (2007):

$$n = [z_{\alpha/2}^2 * p_{exp} (1-p_{exp})]/d^2$$

Where n is the required sample size, z is the statistic for level of confidence, p_{exp} = estimated prevalence and d is the desired absolute precision for estimating prevalence. Prevalence of *Leptospira* species is not known in this area, and therefore a 50% figure is used to maximize the sample size. A sample size of 202 rodents was calculated considering a level of confidence of 95% (z = 1.96) and a precision of 6.9% (0.069).

Sample size calculation for environmental samples

There is very little to no information available on environmental prevalence of *Leptospira* spp. in South Africa. This part of the study was a pilot study, of which the purpose was to provide information on the specific study area for further, more in-depth investigations.

The following formula was used to calculate the sampling size, as stated above (also described in Lemeshow et al. 1990):

$$n = \frac{z_{1-\alpha/2}^2 P(1-P)}{d^2}$$

Where n is the required sample size, z_(1-α/2) is the z-score for the desired level of confidence (95%), P, the expected prevalence, and d, the precision or margin of error (14%, 0.14). The prevalence is not known, and therefore we use 50% (0.5) to optimize the sample size.

$n=1.96^2 * 0.5(0.5)/0.142^2$

$n=49$

The margin of error is increased, with the power of analysis (1-β) 80%. This is acceptable as per published guidelines for preliminary soil sampling studies (EPA, 1989). This is on par with investigative studies on environmental sampling for leptospirosis in other parts of the world (Saito et al. 2013, Thibeaux et al. 2017).

Sampling methods

A cross-sectional study was conducted, using simple random sampling.

Rodent samples

There are ongoing projects that are collecting rodents in the Mnisi community. A National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIH) research project under Award Number R01AI136832 is investigating zoonotic tick-borne pathogens as a cause of acute febrile illness in the community, as well as the animal reservoirs of these pathogens. Rodents were also collected for another study on the epidemiology of toxoplasmosis in the Mnisi community (V0614-18). For the purpose of these studies, rodents have been collected from households, croplands and conservation areas for several years from four villages, namely Athol, Thlavakisa, Gottenburg and Utha. During rodent dissections, the kidney samples from the rodents were removed and stored in RNA-later. These samples were then stored in the Biobank at -80°C at Hans Hoheisen Wildlife Research Station (HHWRS). These kidney samples from rodents collected in households and croplands (n = 124) were utilized for further testing in this study on *Leptospira* spp. Rodent species collected from households so far for the NIH project include *Rattus* sp., *Mastomys* sp., *Gerbilliscus leucogastor* and *Lemniscomys rosalia*.

In addition, rats (*Rattus* sp.) from Welverdiend A village were collected from households, as this is the village where the outbreak of leptospirosis occurred in humans in 2018. A total of 34 rats were collected from Welverdiend A. Rodents were trapped overnight using Sherman and Tomahawk traps (Figure 2). Eight traps were placed in each household, with a total of 25 households. These traps were set in the late afternoon, baited with peanut butter and oats, left overnight and checked in the early mornings, for a total of four trapping nights. Verbal consent from each house owner was obtained before placing the traps. The traps were placed in strategic areas around the household where they were safe from predators.

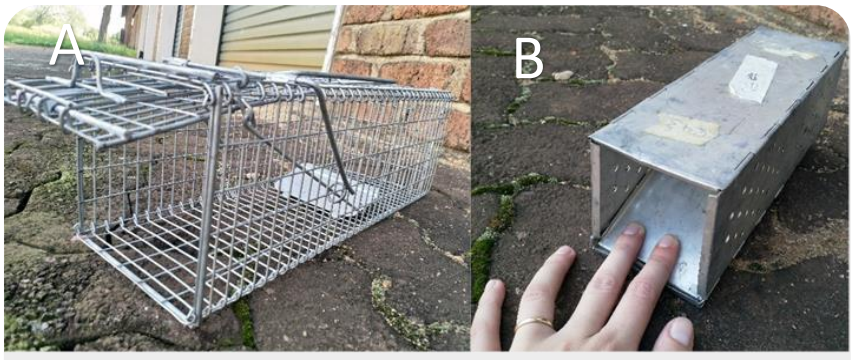

Figure 2: Traps used to capture rodents, A: Tomahawk trap, B: Sherman trap

After collection of the traps in the mornings, rodents of non-target species (*Mastomys* and *Aethomys*) were released on site. Traps containing rats were transported per vehicle to HHWRS (permit number 20220419ORK-VWP2, 2022-04-2019, as seen in Appendix D). Rats were euthanized by placing a cotton wool swab saturated with isoflurane in the traps, which were then sealed in a plastic bag. These plastic bags were properly labeled with the collection date, address of the house collected from and number of rats. All biosecurity measures were in place for working with rodents.

After euthanasia the carcasses were dissected in a biosafety flow cabinet (Figure 3), the kidney samples were collected (labelled left and right kidney) and stored in the -20°C freezer for DNA extraction. Data collected at dissection included weight of the rat, gender, scrotal/non-scrotal, perforated/non-perforated and any obvious abnormalities. This data was collected primarily for the potential use of these samples in the NIH project and were not utilized to draw any conclusions with regards to this study.

The key used for rodent samples:

Village-Rat-Number: Welverdiend-Rat-1		WR1
non-sc		Non-scrotal
sc		Scrotal
Perf		Perforated
Non-perf		Non-perforated
M		Male
F		Female

Figure 3: Biosafety flow cabinet used to dissect rodent carcasses

Environmental samples

To investigate environmental sources of *Leptospira* spp., soil and water samples were collected from muddy areas, shallow water bodies, dip tank areas, drainage lines and areas frequented by both humans and animals from the same villages where the rodent samples originated. Five samples of each soil and water were collected from each of the five villages. For both water and soil samples all people handling samples had to follow strict biosecurity measures such as wearing gloves and washing hands thoroughly after collection. All samples were collected in sterile containers and then bagged, with a label on the sample as well as the bag, for the protection of all personnel. These bagged samples were only handled by informed personnel, following biosecurity measures.

For the 25 soil samples, approximately 50 g topsoil was collected, ranging from 10 cm below to 1 meter above water level. The soil samples were collected from shaded areas and obtained by using a core drilling sterile spatula. Each soil sample was immediately placed into a 50 ml sterile Falcon tube and placed in an ice cooler, marked with the sample name, date, area collected and GPS coordinates, prior to transport to the laboratory.

For the 25 water samples, 50 ml water was collected in sterile screw top containers, from standing water. The samples were labeled as described above, placed in an ice cooler, and transported to the laboratory. When collecting

environmental samples, photos and the exact GPS coordinates were recorded to provide accurate tracing of positive samples.

The key used for environmental samples:

Village-Water-Sample number	
Athol-Water-1	AW1
Thlavakisa-Water-1	TW1
Gottenburg-Water-1	GW1
Utha-Water-1	UW1
Welverdiend-Water-1	WW1
Village-Soil-Sample number	
Athol-Soil-1	AS1
Thlavakisa-Soil-1	TS1
Gottenburg-Soil-1	GS1
Utha-Soil-1	US1
Weverdiend-Soil-1	WS1

Laboratory analysis

DNA extractions

Rodent kidney samples

DNA extractions were performed using the Invitrogen PureLink Genomic DNA Mini Kit (Invitrogen) as per manufacturer's instruction. Minced kidney tissue (25 mg) was placed into a sterile microcentrifuge tube; 180 µL PureLink® Genomic Digestion Buffer and 20 µL Proteinase K was added to the tissue and incubated on a heating block at 55°C for two hours. Next, 20 µL RNase A was added to the lysate, mixed by vortexing and incubated for two minutes at room temperature. PureLink Genomic Lysis/Binding buffer (200 µL) was then added and mixed before an additional 200 µL ethanol (100%) was added and vortexed for five seconds.

The lysate mixture (as prepared above) was loaded into a PureLink Spin Column and centrifuged (10,000 x g) for one minute (room temperature). The collection tube was discarded, and the spin column placed in a new collection tube. The column was then washed with 500 µL Wash Buffer 1 (prepared with ethanol as per instructions), centrifuged for one minute and placed into another collection tube. It was then washed with 500 µL Wash Buffer 2 (prepared with ethanol), centrifuged for three minutes and placed in a sterile microcentrifuge tube.

The final step was to elute the DNA by addition of 100 µL PureLink Genomic Elution Buffer, incubation at room temperature for one minute and then centrifugation at maximum speed for one minute. The purified DNA was then stored at -20°C prior to further processing.

Environmental samples

DNA extraction from both soil and water samples were performed using the ZR Soil Microbe DNA MicroPrep kit, as per manufacturer's instructions (Zymo Research, www.zymoresearch.com). According to the manufacturer, the kit is suitable for both soil and water samples, and has been used successfully by Naidoo et al. (2020) to detect *Leptospira* spp. in water samples in South Africa. Depending on the composition of the soil, up to 250 mg soil or 750 µL of sampled water was used per kit. The sample was added to the ZR BashingBead Lysis Tube with 750 µL buffer and vortexed, then centrifuged for one minute. This process homogenizes the sample by beads that are beating the soil. The supernatant (400 µL) was then transferred to a collection tube with a filter, and 1200 µL Genomic Lysis Buffer added

and mixed. Next, 800 µL of the mixture was transferred to a Zymo-Spin IICR Column in a collection tube and centrifuged, after which the flow-through was discarded. This step was repeated. The column was placed in a new collection tube, 200 µL DNA Pre-Wash Buffer added and the sample centrifuged. The g-DNA Wash Buffer (500 µL) was added, and the sample centrifuged. The flow-through was discarded, the column transferred to a microcentrifuge tube and 100 µL DNA Elution Buffer added. The sample was centrifuged to elute the DNA. The eluted DNA was then transferred to a prepared Zymo-Spin III-HRC Filter in a clean microcentrifuge tube and centrifuged again for three minutes. This process yielded 25 µL DNA eluted in 100 µL DNA elution buffer. The DNA was stored at -20°C until it was used.

Real-time PCR

A previously published quantitative real-time PCR (qPCR) assay that targets the subsurface lipoprotein 32 (*LipL32*) gene (Gentilini et al., 2015) was used in this study, with some modifications. Primers F_*LipL32* (5'-TCC CAG GGA CAA ACG AAA CCGT-3') and R_*LipL32* (5'-TGT TTC CAT CGG CTA AAC CGT-3') and probe *LipL32*_P (5'-[6FAM] ACG TAA AGC CAG GAC AAG CGC CG [BHQ1]-3') (Gentilini et al., 2015) were used to detect and quantify DNA of leptospires in rodent kidney, urine and water samples. The assay was optimized first by assessing performance with different primer (0.3, 0.4, 0.6, 0.9 µM) and probe (0.1, 0.2, 0.4 µM) concentrations, using five positive DNA samples (GD00258, GD00260, GG00185, GG00191, GG0196) extracted from rodent spleen, kindly provided by Dr. Jenny Rossouw, National Institute for Communicable Diseases (NICD) (Johannesburg, South Africa). Optimal conditions were those that resulted in highest fluorescence and lowest quantification cycles for the positive control samples. Each PCR reaction comprised 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa), 0.9 µM of each oligonucleotide primer, 0.2 µM of the FAM- and Black hole quencher 1 (BHQ-1)-labelled probe and 2.5 µl of DNA template in a total reaction volume of 20 µl. Thermal cycling was done in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Johannesburg, South Africa) under the following conditions: Uracil N-Glycosylase digest at 50°C for two minutes, followed by AmpliTaq Gold pre-activation at 95°C for 10 minutes and then 40 cycles of amplification at 95°C for 15 s and annealing at 57°C for one minute.

Data analysis

All collected data was entered into Excel. Rodent tables were set up for the Welverdiend A samples, with the village name, rodent species collected, sex, weight and positive or negative *Leptospira* spp. qPCR result. A separate table was set up for biobank samples, with collection date, collection area, sample ID and result. A separate table was also used for the environmental samples, which included the area of collection (village, GPS coordinate), whether it was a water or soil sample, a description of the area and the *Leptospira* spp. qPCR result.

Environmental samples from the surrounding villages were depicted on a map to allow for visual representation of the data. The terrain was included to provide more information regarding natural landmarks such as rivers or dams. The photographs of collection sites were correctly correlated to each GPS coordinate set in order to utilize in the event that samples returned a positive result. A few examples of these areas were included as demonstration.

All samples returned a negative result, more accurately defined as no pathogenic leptospiral DNA detected (or below the detection limit of the assay), therefore no further analysis on prevalence was conducted.

Results

Rodent kidney samples

Table 1 shows the CT value of positive control sample GD00258. A positive control sample was included with each batch of samples analyzed to ensure accurate testing. A similar result layout was obtained for all the samples analyzed, with a complete table of all results shown in Appendix E.

Table 1: CT value for positive control sample from the NICD

Sample ID	Target Name	Task	Reporter	Quencher	Cт
GD00258.10	leptol32	STANDARD	FAM	NFQ-MGB	35.93266

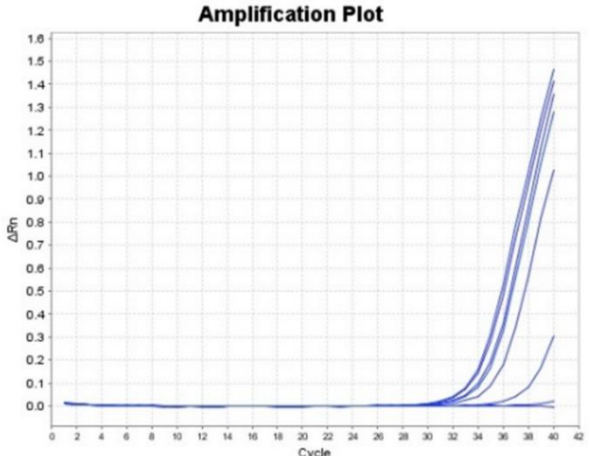

Figure 4: Amplification plot of positive control sample

The amplification plot obtained during optimization of the qPCR using the positive control samples is shown in Figure 4. This plot indicates that amplification only begins quite late in the amplification cycles, not presenting as the usual sigmoid-shape due to low concentration of *Leptospira* spp. DNA within the positive control DNA samples. These positive control samples from the NICD are *L. borgpetersenii* (previously identified using real-time PCR and a melt-curve analysis) from rodent spleen (Moseley et al., 2020).

Rodent kidneys included in this study consisted of freshly collected specimens from Welverdiend A (n=34) and biobanked samples (n=124). The samples from Welverdiend A were collected in April 2022, while the biobank samples included rodent kidneys from four villages, collected over various periods in the past two years. Details on the number of samples collected and their collection sites are shown in Tables 2 and 3, and give an indication of the distribution of our sample population. See Appendix E for details of all rodent samples collected.

Table 2: Summary of number of samples set out per village over multiple collection dates

	Athol	Utha	Gottenburg	Thlavakisa	Welverdiend A
October 2020	18	33	3	11	0
September 2021	17	3	7	15	0
March 2022	4	11	0	0	0
April 2022	0	0	0	0	34
Total	**39**	**47**	**10**	**26**	**34**
Percentage of total samples	**24.7%**	**29.7%**	**6.3%**	**16.5%**	**21.6%**

Table 3: Summary of rodent kidney samples analyzed and their corresponding qPCR result

Type of sample	Number of samples	Fresh/Biobank	Date collected	Pathogenic *Leptospira* bacteria detected (Yes/No)
Rodent kidney	67	Biobank	October 2020	No*
Rodent kidney	42	Biobank	September 2021	No
Rodent kidney	15	Biobank	March 2022	No
Rodent kidney	34	Fresh	April 2022	No
Total	**158**			

* No DNA detected or below the detection limit of the assay.

Figure 5 visually represents the proportion of samples from each of the villages included in this study. Athol, Utha and Welverdiend A are all very well represented, followed by Thlavakisa. Gottenburg had a relatively low representation in our sample population as fewer rodents were available from the biobank due to protests in the village during the collection dates.

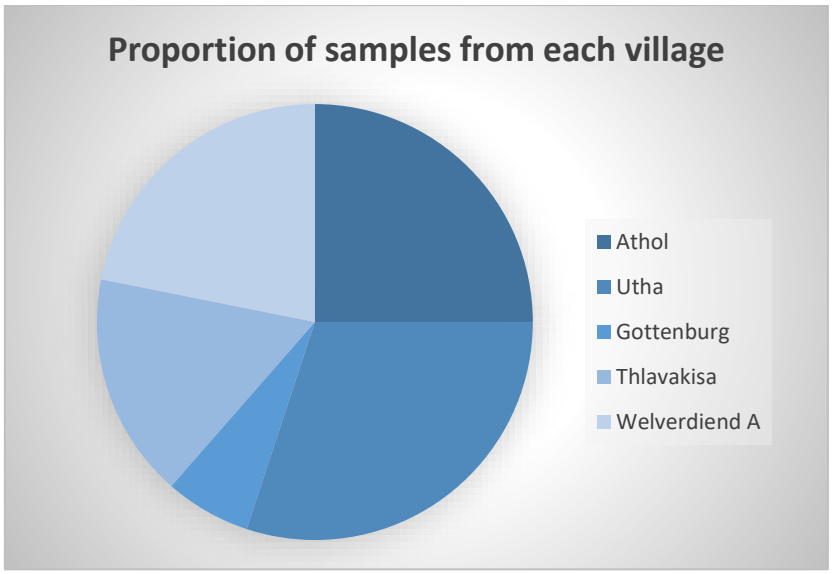

Figure 5: Proportion of rodent samples for each of the villages

Trapping within Welverdiend A in April 2022 proved to be successful in comparison to previous villages targeted by earlier projects. Over the four trapping nights, with eight traps per household and 25 households, 34 rats were captured for inclusion in this study. It can be noted that 13 rodents of other non-target species (*Mastomys* and *Aeuthomys*) were also captured and released on site. The number of rats captured per household proved quite interesting, as certain houses had a much higher trapping rate. See Table 4 below for a summary of rodents collected from the households of Welverdiend A.

Table 4: Rodents collected from Welverdiend A in April 2022

House number	Number of traps baited		Number of rats collected	Number of *Mastomys/ Aeuthomys* released	Notes	Pathogenic *Leptospira* bacteria detected (Yes/No*)
	Sherman	Tomahawk				
91 Thete	6	2	8		"kitchen" area most popular	No
92 Thete	6	2	0			N/A
67	6	2	5			No
76 (36?)	6	2	2			No
77	6	2	0			N/A
87	7	1	0			N/A
68	7	1	0	3		N/A
84 D.T. Thete	6	2	1	1	Livestock in the yard	No
27	6	2	1			No
59 (69?)	7	1	3			No
99	7	1	2			No
124 Masuku. E	7	1	0		Large pile of corn stored openly	N/A
123 Mnisi	7	1	0	4	Very clean house	N/A
66	7	1	1	1	Lots of free food available in the area, with fruit trees	No
21	7	1	1	2		No
24	7	1	2			No
43 Mashego shop	6	2	0	1	Populated area	N/A
52	6	2	5			No
45	6	2	1			No
44	6	2	2			No
41	6	2	0			N/A
6a	6	2	0			N/A
6b	6	2	0			N/A
100	6	2	0	1		N/A
42 Shilubani	6	2	0			N/A
Total collected			**34**	**13**		

* No DNA detected or below the detection limit of the assay.

Certain households had large amounts of forage available in the area, such as those with livestock on their premises, therefore lower trapping success was noted due to less interest in the baited traps. Other houses and yards were very successful for trapping as they had no alternative food sources available for the rodents other than the traps and possible kitchen areas where the members of the family cook and eat. Some examples are illustrated in Figure 6.

Environmental samples

Water and soil samples were collected from all five villages; Athol, Utha, Gottenburg, Thlavakisa and Welverdiend A. Samples were collected based on their surroundings and use within the community. Samples were collected throughout each of the villages and were not restricted to one area alone. A representative sample population was collected for both water and soil samples. A description of each of the samples was acquired, along with the GPS coordinates and a photo. Table 5 and 6 below indicate the coordinates and collection sites for both water and soil samples.

Figure 6: Examples of various households with different trapping success rates. A: Good trapping success (tidy kitchen area with limited food easily accessible to rodents), B: poor trapping success (corn freely available), C: poor trapping success (food freely available), D: poor trapping success (fruit tree with seed on the ground, next to the cropland).

Table 5: Soil samples with their collection sites for April 2022

ID	Village	GPS coordinates	Pathogenic *Leptospira* bacteria detected (Yes/No)	Short description
AS1	Athol	24° 42' 28.56'' S 31° 20' 39.79'' E	No	Puddle next to a kraal
AS2	Athol	24° 42' 29.45'' S 31° 20' 38.77'' E	No	Kraal
AS3	Athol	24° 42' 41.36'' S 31° 20' 22.34'' E	No	River used by community (nr. 1)
AS4	Athol	24° 42' 38.80'' S 31° 20' 50.80'' E	No	River used by community (nr. 2). Strong urine smell
AS5	Athol	24° 42' 50.28'' S 31° 21' 05.63'' E	No	Soil from the communal diptank crush
TS1	Thlavakisa	24° 37' 39.59'' S 31° 21' 59.94'' E	No	Drainage line
TS2	Thlavakisa	24° 37' 35.81'' S 31° 22' 22.34'' E	No	Soil close to community tap
TS3	Thlavakisa	24° 37' 30.37'' S 31° 22' 31.70'' E	No	T-junction, building new drainage next to road
TS4	Thlavakisa	24° 36' 53.90'' S 31° 22' 13.24'' E	No	Dam in a cow grazing camp
TS5	Thlavakisa	24° 37' 23.76'' S 31° 22' 27.30'' E	No	Private cropland
GS1	Gottenburg	24° 38' 03.61'' S 31° 23' 37.29'' E	No	Soil from the drainage line
GS2	Gottenburg	24° 38' 20.23'' S 31° 24' 35.72'' E	No	Personal kraal
GS3	Gottenburg	24° 38' 17.90'' S 31° 24' 54.57'' E	No	Soil collected from communal diptank
GS4	Gottenburg	24° 38' 37.13'' S 31° 24' 54.13'' E	No	Soil from personal cropland
GS5	Gottenburg	24° 38' 35.79'' S 31° 25' 00.00'' E	No	Soil from the drainage line
US1	Utha	24° 41' 25.25'' S 31° 27' 12.07'' E	No	Communal dam
US2	Utha	24° 41' 25.18'' S 31° 26' 48.22'' E	No	Soil from drainage of pit latrine at a bar
US3	Utha	24° 41' 48.58'' S 31° 26' 47.05'' E	No	Drainage line
US4	Utha	24° 41' 58.37'' S 31° 26' 43.43'' E	No	Soil from communal diptank crushpen
US5	Utha	24° 42' 04.51'' S 31° 26' 40.96'' E	No	Soil in drainage area of pig farm
WS1	Welverdiend	24° 34' 46.50'' S 31° 19' 29.50'' E	No	Communal water tank
WS2	Welverdiend	24° 34' 45.17'' S 31° 19' 25.06'' E	No	Soil next to Thete's kraal
WS3	Welverdiend	24° 35' 01.59'' S 31° 19' 29.28'' E	No	Soil next to communal diptank
WS4	Welverdiend	24° 34' 47.94'' S 31° 19' 29.59'' E	No	Communal dam
WS5	Welverdiend	24° 35' 13.98'' S 31° 20' 04.67'' E	No	Next to cropland in drainage line

Table 6: Water samples with their collection sites for April 2022

ID	Village	GPS coordinates	Pathogenic *Leptospira* bacteria detected (Yes/No)	Short description
AW1	Athol	24° 42' 28.56'' S 31° 20' 39.79'' E	No	Puddle next to a kraal
AW2	Athol	24° 42' 41.36'' S 31° 20' 22.34'' E	No	River used by community (nr. 1)
AW3	Athol	24° 42' 38.80'' S 31° 20' 50.80'' E	No	River used by community (nr. 2). Strong urine smell.
AW4	Athol	24° 42' 50.28'' S 31° 21' 05.63'' E	No	Puddle collected close to communal diptank
AW5	Athol	24° 43' 10.80'' S 31° 21' 05.35'' E	No	Small stream with standing water, used as communal water source
TW1	Thlavakisa	24° 37' 35.81'' S 31° 22' 22.34'' E	No	Standing water around community tap
TW2	Thlavakisa	24° 37' 30.37'' S 31° 22' 31.70'' E	No	T-junction, building new drainage next to the road
TW3	Thlavakisa	24° 36' 53.90'' S 31° 22' 13.24'' E	No	Dam in a cow grazing camp
TW4	Thlavakisa	24° 37' 23.76'' S 31° 22' 27.30'' E	No	Private cropland
TW5	Thlavakisa	24° 38' 03.61'' S 31° 23' 37.29'' E	No	Water from communal dam
GW1	Gottenburg	24° 38' 03.61'' S 31° 23' 37.29'' E	No	Water from drainage line
GW2	Gottenburg	24° 38' 08.66'' S 31° 23' 50.86'' E	No	Water collected around communal tap
GW3	Gottenburg	24° 38' 17.90'' S 31° 24' 54.57'' E	No	Water collected from communal diptank
GW4	Gottenburg	24° 38' 37.13'' S 31° 24' 54.13'' E	No	Water from municipal tap (borehole water)
GW5	Gottenburg	24° 38' 35.79'' S 31° 25' 00.00'' E	No	Water from drainage line
UW1	Utha	24° 41' 20.47'' S 31° 26' 43.43'' E	No	Puddle in the road
UW2	Utha	24° 41' 23.22'' S 31° 27' 02.49'' E	No	Drainage line
UW3	Utha	24° 41' 25.25'' S 31° 27' 12.07'' E	No	Communal dam
UW4	Utha	24° 41' 48.58'' S 31° 26' 47.05'' E	No	Drainage line
UW5	Utha	24° 41' 58.37'' S 31° 26' 43.43'' E	No	Standing water close to communal diptank crush
WW1	Welverdiend	24° 34' 46.50'' S 31° 19' 29.50'' E	No	Communal water tank
WW2	Welverdiend	24° 34' 45.17'' S 31° 19' 25.06'' E	No	Water next to Thete's kraal
WW3	Welverdiend	24° 35' 01.59'' S 31° 19' 29.28'' E	No	Standing water next to communal diptank
WW4	Welverdiend	24° 34' 47.94'' S 31° 19' 29.59'' E	No	Communal dam
WW5	Welverdiend	24° 35' 13.98'' S 31° 20' 04.67'' E	No	Next to cropland in drainage line

Each of the coordinates were plotted on aerial photographs, using the public platform Google Maps, to show distribution of these samples (Figures 7 and 8). The terrain is a valuable inclusion as it shows the location of bigger water bodies, housing areas and road infrastructure.

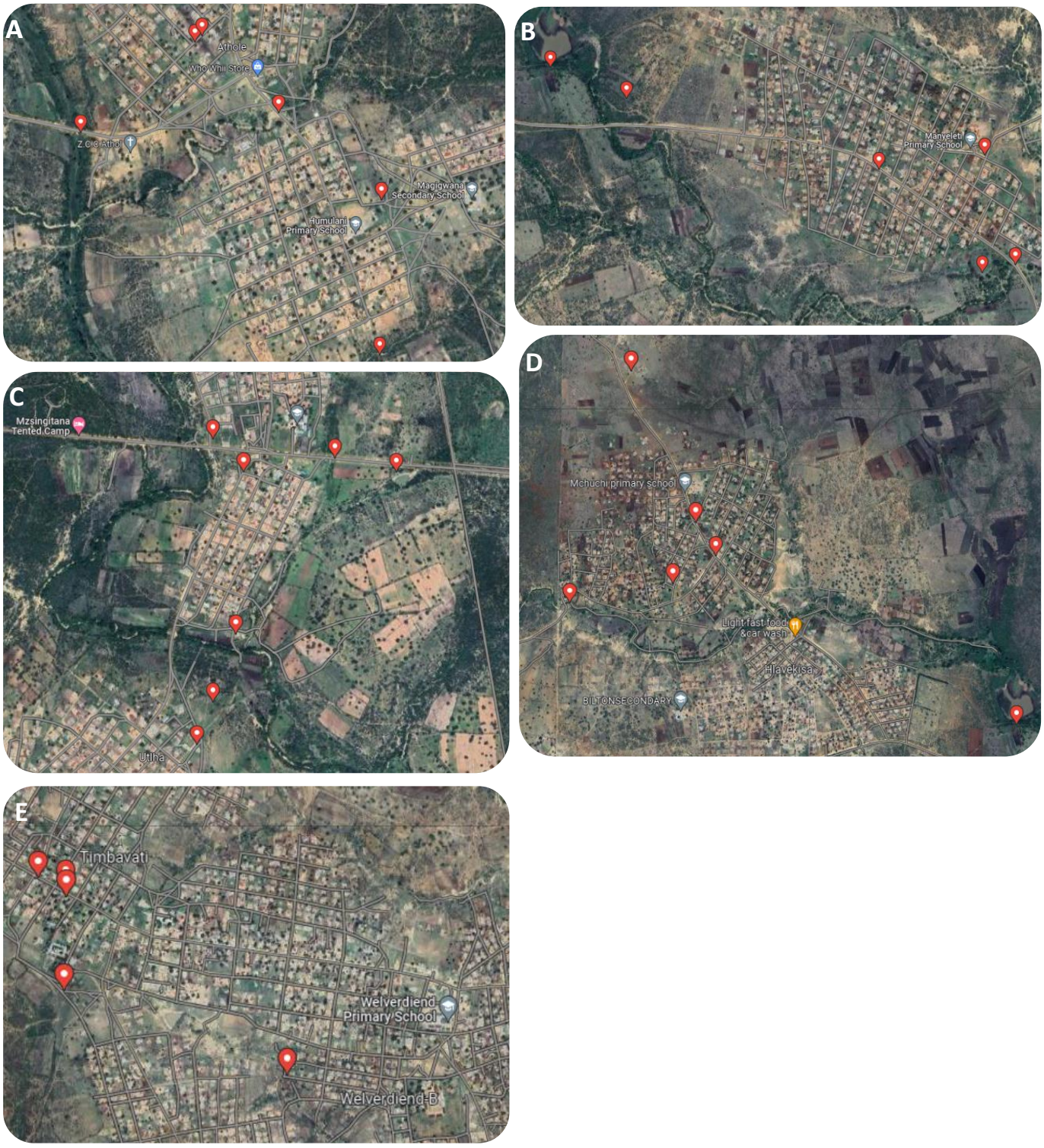

Figure 7: Environmental sample collection coordinates. A: Athol, B: Gottenburg, C: Utha, D: Thlavakisa, E: Welverdiend A. The red markers indicate the GPS location of each sample collected.

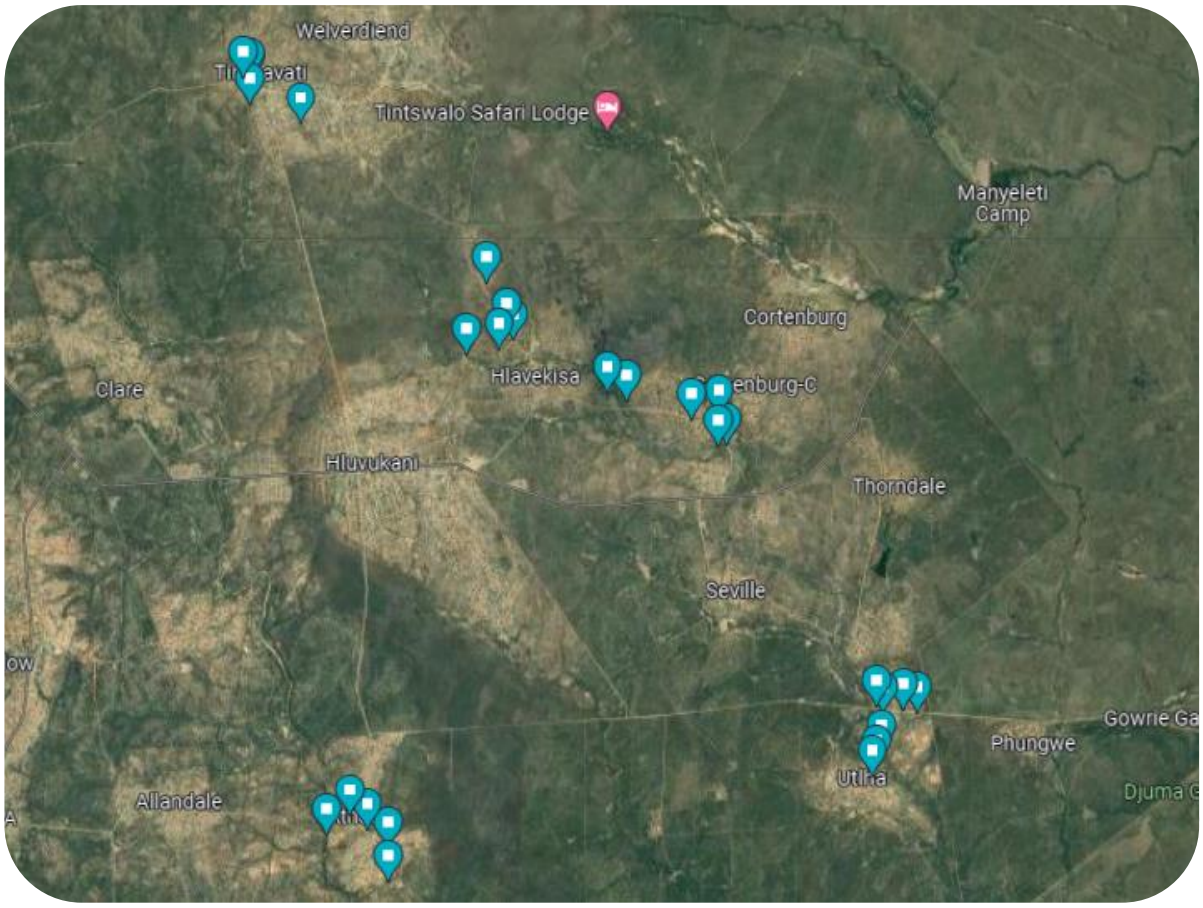

Figure 8: All coordinates where water- and/or soil samples were collected. Blue markers indicate GPS location of each sample collected.

If a rodent sample returned a positive result, these samples could potentially be linked to the environment and used as a prediction for further risk factors. For example, if water from a communal water source had tested positive, further deductions regarding risk can be made. Photographs of collection sites for environmental samples were important, as these are ever-changing areas due to varying weather conditions. Some photographs of the collection sites are shown in Figure 9.

Figure 9: Examples of soil and water collection sites. A: WW4 and WS4, B: UW5 and US4, C: GW1 and GS1, D: GW5 and GS5, E: AW3 and AS4, F: GW3

Discussion

The aim of this study was to determine whether rodents are a potential source of pathogenic *Leptospira* spp. infections for the Mnisi Community. As previously mentioned, the Mnisi Community is at the wildlife-livestock-human interface and previous leptospirosis cases in humans were reported by the NICD in 2018. As an additional aspect, environmental samples (specifically water and soil samples) were collected to gain a better understanding of pathogenic *Leptospira* spp. and their role in the environment. Research on leptospirosis remains lacking in Southern Africa, with limited data available in South Africa and none for the Mnisi Community.

Rodents have primarily been researched in the past, alongside other species, to gain more knowledge regarding leptospirosis in various hosts. Rodents were rarely included in studies performed in South Africa, also seemingly doubtful as a potential host according to three case studies performed in 1999 (Gummow et al., 1999). Another article described an interesting phenomenon, where no rodents tested positive in a wide-spread cross-sectional study performed in the Moshi District of Tanzania (Allan et al., 2018). This can be directly compared to studies conducting research on leptospirosis in livestock, which have also had variable results; one study conducting research on the prevalence of leptospirosis in pigs had a 90.5% prevalence using leptospiral isolation (Hunter et al., 1987), another study on leptospirosis in bovines (including 860 cows) only had 2% testing positive via MAT (Myburgh and Otto, 1990). For this reason, the dominant reservoir in pathogenic leptospirosis is still mostly unknown and more research in this area is warranted. Any information obtained on leptospirosis is helpful in increasing public awareness and knowledge on this topic.

In this research study, a representative sample was obtained, including rodents from multiple households within five villages within the Mnisi Community Area. These rodents were trapped from within and around houses where they had close contact with livestock, dogs and humans. These rodents were from areas where possible urine contamination of food and/or water could have occurred. The environmental samples collected in this study were from high-risk areas such as communal water sources, vegetable gardens, drainage lines, dip tanks and kraals where livestock are regularly present, and humans could potentially be infected. The rodent samples were collected over a period of two years and various seasons. The environmental samples were collected after a period of heavy rainfall, with large amounts of standing water present in the communities. The Welverdiend A rodent samples were collected in autumn, when rodents are more common in households due to the limited food availability in the surrounding croplands. Rodent kidneys were dissected within a few hours of collection, DNA extracted and stored at -20°C before PCR analysis was performed. Environmental samples were collected, stored on ice until return to the lab, where DNA was extracted and stored at -20°C for further analysis. A wide range of rats was collected, with specimens of both sexes and various weights, thus providing a completely random sample population with no risk of bias regarding weight, sex or age.

All rodent kidney samples and environmental samples had no pathogenic *Leptospira* spp. bacteria in high enough concentration for the qPCR to detect leptospiral DNA. All samples were analyzed alongside positive control DNA samples from the NICD, collected from rodent spleen. The results suggest that there are no pathogenic *Leptospira* species present, which our test is designed to detect, within all the samples collected for this study. This includes the rodent and environmental samples collected from Welverdiend A. In a parallel study, conducted by another researcher, using a subset of the samples included in this study, a SYBR Green qPCR assay was utilized which makes use of the same primers but includes SYBR Green instead of the Taqman probe for *Leptospira* spp. detection (Clara Maurizzi, MSc student, Department of Veterinary Tropical Diseases, UP, personal communication). These samples are returning positive results, with many melting curves fairly similar to those of the positive control samples from the NICD. This research is still ongoing, but one can speculate that the positive results seen on the SYBR Green qPCR assay could be non-pathogenic *Leptospira* spp. since all these samples were negative on the Taqman qPCR assay. The next step for these SYBR Green qPCR positive samples would be to perform sequencing to determine the origin of those positive samples. This finding was quite interesting as one would expect pathogenic *Leptospira* spp. in the Mnisi area, especially in Welverdiend A following the outbreak of leptospirosis in humans in 2018 (Anonymous, Communicable Diseases Communiqué, July 2018, Vol. 17(7)); however the presence of non-pathogenic leptospires would be interesting to pursue further in determining which serovars are present and whether they are more, less, or equally prevalent in the rodent kidney samples as the water or soil samples.

Some limitations of this study included a relatively small sample size and restricted availability of funding. The original objective of this study was to determine the prevalence of *Leptospira* spp. within the Mnisi Community, and the sample size was calculated accordingly as demonstrated on page 14. The sample size calculated at that point was 202 samples to determine the prevalence of *Leptospira* spp. if the assumed prevalence of disease is set to 50% to maximize the number of samples required. Due to limited trapping success within Welverdiend A, only 34 rats were collected, therefore bringing the total rodent kidney samples analyzed to 158. Since the samples collected in this study all tested negative, one would be inclined to say that no *Leptospira* spp. is present within the study population. This, however, might be an untrue statement as the sample size was not large enough to certify freedom of disease. In order to prove freedom of disease in a very large population (such as the rodent population within the Mnisi Community), one would need 332 samples, calculated by making use of an online calculator (https://epitools.ausvet.com.au/freedomss) where the design prevalence, unit sensitivity and required population sensitivity needed to be entered. These values were set to 0.01 (1%) (to have one sample out of the population test positive), 0.9 and 0.95 respectively. The population size is large and impossible to determine; therefore, the binomial method needs to be utilized for this sample size calculation. Another point to note is that this sample size calculation assumes 100% test specificity, which is unknown in this study. Therefore, within the parameters of this study one would not be able to draw the conclusion that this study certifies freedom of disease.

The restricted funding that was available for this study limited the number of samples that could be tested. A more focused approach, with Welverdiend A the only village included, would be more valuable in order to draw more specific conclusions. However, to trap a larger number of rodents from a single village one would need a few months of trapping, and this was not possible for this study.

A valuable extension to this project would be to test livestock alongside rodents and environmental samples. This should include bovines, porcines and canines due to the proximity with humans within these communities. One study conducted in South Africa, described three case studies, highlighting the link between poor sanitation conditions and the high number of animals testing positive for leptospirosis (Gummow et al., 1999). This also demonstrated the spread of disease within multi-species farming units via water and effluent wastewater.

This study area, the Mnisi community, is well suited to ongoing research on leptospirosis, as the area has a warm climate, beneficial for bacterial proliferation. This community is rural, with the population still largely reliant on the communal water sources and cultivation of their own vegetables.

The apparent absence of pathogenic *Leptospira* spp. on PCR in this study may be disheartening; however, due to the overall lack of available research on leptospirosis in South Africa, any information gathered is valuable. This study encountered many hurdles along the way to completion, which emphasized the difficulty of pioneering research on a disease condition such as leptospirosis due to the overall lack of resources, lack of knowledge regarding the perfect testing methods and unavailability of the tests themselves. There is still a long way to go to learn about and understand leptospirosis within South Africa.

Concluding Remarks

This study on pathogenic *Leptospira* in the Mnisi Communal area, Bushbuckridge, served as a building block for ongoing research with regards to zoonotic infections, increasing scientific and public awareness of this neglected disease. As heavily emphasized in the literature review included in this project, leptospirosis remains underdiagnosed and very limited information is known regarding its potential reservoirs, hosts and severity of disease. It is of the utmost importance that research in this area is continued to better understand this important disease and increase the knowledge within the general public. This is especially important within rural communities, where the population is still heavily reliant on the resources of the land – communal water sources, rivers for bathing, self-cultivating vegetables and livestock farming. Leptospirosis has been known to cause production loss due to high morbidity in livestock worldwide. This has not been thoroughly addressed in the literature, creating a misconception about the importance of this disease in the livelihood of people in southern Africa. With continued research on leptospirosis, its reservoirs, hosts and leptospiral serovars, one might be able to better control the prevalence of this neglected zoonotic disease.

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Appendix A: Section 20 permit

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Mama Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/1A (2139 MG) (JD)

Dr Ilana van Wyk
Hans Hoheisen Wildlife Research Station, Faculty of Veterinary Science, University of Pretoria
Email: liana.vanwyk@up.ac.za

Dear Dr van Wyk,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Title of research project / study: "An investigation into the presence of Leptospirosis infection in rodents within the Mnisi community, Bushbuckridge, South Africa"

Your application requesting an amendment of the Section 20 permit issued by the Director of Animal Health on 2022-02-24 for the study mentioned above refers. I am pleased to inform you that the amendment is hereby granted with the following conditions:

Conditions:

1. As part of the study, the collection and testing of water samples, in addition to soil samples, may also be included;
2. As part of the study, rats sampled from Welverdiend A where the outbreak occurred, in addition to the rodent samples from the RO1 project in the HHWRS Biobank, may also be included;
3. DNA extracts (after the extraction has been conducted at HHWRS), may be sent to Onderstepoort DVTD Laboratory for rt-PCR;
4. This amendment does not relieve the researcher of any of the other conditions as contained in the Section 20 permit issued on 2022-02-24 for this study;

5. Written permission from the Director of Animal Health must be obtained prior to any deviation from the conditions approved for this study under the Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za

Kind regards,

pp.

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date:
2022 -03- 30

SUBJECT: Amendment of Section 20 approval in terms of Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984)

Appendix B: Certificate of Animal Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

25 February 2022

Approval Certificate
New Application

AEC Reference No.:	REC134-21
Title:	An investigation into zoonotic Leptospira infections in rodents within the Mnisi communal area, Bushbuckridge, South Africa
Researcher:	Dr I Van Wyk
Student's Supervisor:	Dr NE Collins

Dear Dr I Van Wyk,

The **New Application** as supported by documents received between 2021-10-25 and 2022-01-31 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-01-31.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Rats	79
Samples	**Number**
Kidney samples (Samples from live animals)	79
Kidney samples (Stored- Historic/Retrospective) V023-19	123

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-02-25.
3. Please remember to use your protocol number (REC134-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8414
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix C: Certificate of Research Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 2 June 2022

AMENDMENT LETTER OF APPROVAL

Ethics Reference No	**REC134-21 Line 1**
Protocol Title	**An investigation into zoonotic Leptospira infections in rodents within the Mnisi communal area, Bushbuckridge, South Africa**
Principal Investigator	**Dr I Van Wyk**
Supervisors	**Dr NE Collins**

Dear Dr I Van Wyk,

We are pleased to inform you that the **Amendment** conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC134-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

NOTES: Conditionally approved pending addressing the following point – Please give reasons for requesting additional animals/samples?.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

Appendix D: Travel with samples consent letter

Permit in	Permit out
	/

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: 20220419CKK – VUUR2

DEPT. LANDBOU

LITIKO LETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	ILANDA VAN WIJK	ID No.:	7705260034084
Address: / Adres: / Likheli:	HANS HOHEISEN WILDLIFE RESEARCH STATION		
to move with: / om te beweeg met: / uvunyelwa yekuhambisa:	NINETY SAMPLES	RATS + ENVIRONMENTAL	LIVE / PRODUCTS
identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	WELVERDIEND A 40 X RATS PLUS WATER + SOIL. OTHER VILLAGES WATER + SOIL SAMPLES		
from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibini/endzaweni:	WELVERDIEND A, GOTTENBURG, ATHOL, UTHLA + THLAVEKISA	in the district of / in die distrik / esigodzini sase	BOHLABELA
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzaleni/endzaweni:	HANS HOHEISEN WRS	in the district of / in die distrik / esigodzini sase	BOHLABELA

On the following conditions:
1. This permit: 19–30 APRIL
(a) is valid for ~~10 days~~ from date of issue and for one consignment only;
(b) must accompany the animals / products mentioned above;
(c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
(d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. Other conditions:

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een beweging;
(b) moet die diere / produkte hierbo vermeld vergesel;
(c) moet vir inspeksie getoon word op aanvraag van enige grond- of dieresienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir wild / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsynsriglyne vervoer word.
5. Ander voorwaardes:

Ngaphansi kwemibandzela lechazwe ngaphansi:
1. Lemvumo:
(a) [illegible] 10 [illegible]
(b) [illegible]
(c) [illegible]
(d) [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]

6. Retention for / Quarantine for / Direct slaughter within: days after arrival at destination

Seal no/s	/	/	Place / Plek / Indzawo:	HANS HOHEISEN
Vehicle reg. no/s	DY 77 VH GP	/	Date / Datum / Lusuku:	2022 04 19

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

DR HKM RAMPF
PRINT NAME
Issuing Veterinary Official

SIGNATURE
State Veterinarian / Staatsveearts / Dokotela wetilwane

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Loneleemvumo longalandzeli lemvumo kumbe mephi umbandzela wayo uphule umtsetfo.

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF AGRICULTURE
OFFICIAL VETERINARIAN
2022 -04- 19
DR H.K.M. RAMPF
REG. NO D18/15940
MPUMALANGA PROVINCE

Permit in	Permit out
	/

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: 20220615CRK-UWP3

DEPT. LANDBOU

LITIKO LETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngekugunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	ISHMAEL KGOHLOANE	ID No.:	8008115376084
Address: / Adres: / Likheli:	UNIVERSITY OF PRETORIA		
to move with: / om te beweeg met: / evunyelwa yekuhambisa:	500 FLUCURED / TONGUE THREE	RODENT, SOIL + WATER	LIVE / PRODUCT
identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	WATER: DNA EXTRACTIONS X25 SOIL: DNA EXTRACTIONS X25 RODENT KIDNEY DNA EXTRACTIONS : X		
from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibhini/endzaweni:	HANS HOHEISEN	STOCK CARD NUMBER	in the district of / in die distrik / esigodzini sase: BOHLABELA
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzelen/endzaweni:	DUTB-PARACLINICAL FAO: C. BYARUHANGA		in the district of / in die distrik / esigodzini sase: TSHWANE

On the following conditions:
1. This permit:
(a) is valid for 10 days from date of issue and for one consignment only
(b) must accompany the animals / products mentioned above;
(c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
(d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. Other conditions:

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een besending;
(b) moet die diere / produkte hierbo vermeld vergesel;
(c) moet vir inspeksie getoon word op aanvraag van enige grond-of dieraeienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir wild / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsynsriglyne vervoer word.
5. Ander voorwaardes:

Ngaphansi kwalemibandzela lelandzelako ngaphansi:
1. Lemvume:
(a) [illegible]
(b) [illegible]
(c) [illegible]
(d) [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]

6. Retention for / Quarantine for / Direct slaughter within: days after arrival at destination

Seal no/s	/	/	Place / Plek / Indzawo:	HANS HOHEISEN
Vehicle reg. no/s	FBB309L	/	Date / Datum / Lusuku:	2022-06-15

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

DR. HELM JOHAN...F
Issuing Veterinary Official:

SIGNATURE
State Veterinarian / Staatsveearts / [illegible]

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzeli lemvumo lesobe ngukhi umbandzela wayo uphule umtsetfo.

DISTRIBUTION:

VETERINARY OFFICIAL AT DESTINATION

MPUMALANGA DEPT. OF AGRICULTURE
BOHLABELA DISTRICT
VETERINARY SERVICES:
BUSHBUCKRIDGE EAST/ORPEN
2022 -06- 15
D18/1590
NAME: DR HELM JOHAN... SIGN:
P/BAG X1321, THULAMAHASHE 1365
TEL NO. 013 773 1150 FAX NO. 013 773 0336

Appendix E: Samples analyzed with corresponding PCR results

All samples analyzed with their corresponding collection date, collection area, sample type and PCR results:

Date analyzed	Sample ID	Sample type	Pathogenic *Leptospira* DNA detected	Village	Date collected
Sep 15 2022	1	Rodent Kidney	No	Welverdiend A	Apr-22
	9	Rodent Kidney	No	Welverdiend A	Apr-22
	17	Rodent Kidney	No	Welverdiend A	Apr-22
	25	Rodent Kidney	No	Welverdiend A	Apr-22
	33	Rodent Kidney	No	Welverdiend A	Apr-22
	13**	Rodent Kidney	No	Athol	Sep-21
	25**	Rodent Kidney	No	Athol	Sep-21
	61**	Rodent Kidney	No	Thlavakisa	Sep-21
	71**	Rodent Kidney	No	Thlavakisa	Sep-21
	83**	Rodent Kidney	No	Gottenburg	Sep-21
	34-	Rodent Kidney	No	Athol	Mar-22
	67-	Rodent Kidney	No	Utha	Mar-22
	2	Rodent Kidney	No	Welverdiend A	Apr-22
	10	Rodent Kidney	No	Welverdiend A	Apr-22
	18	Rodent Kidney	No	Welverdiend A	Apr-22
	26	Rodent Kidney	No	Welverdiend A	Apr-22
	34	Rodent Kidney	No	Welverdiend A	Apr-22
	14**	Rodent Kidney	No	Athol	Sep-21
	26**	Rodent Kidney	No	Athol	Sep-21
	62**	Rodent Kidney	No	Thlavakisa	Sep-21
	72**	Rodent Kidney	No	Thlavakisa	Sep-21
	84**	Rodent Kidney	No	Gottenburg	Sep-21
	42-	Rodent Kidney	No	Utha	Mar-22
	63-	Rodent Kidney	No	Utha	Mar-22
	3	Rodent Kidney	No	Welverdiend A	Apr-22
	11	Rodent Kidney	No	Welverdiend A	Apr-22
	19	Rodent Kidney	No	Welverdiend A	Apr-22
	27	Rodent Kidney	No	Welverdiend A	Apr-22
	1**	Rodent Kidney	No	Uncertain	Sep-21
	15**	Rodent Kidney	No	Athol	Sep-21
	30**	Rodent Kidney	No	Athol	Sep-21
	63**	Rodent Kidney	No	Thlavakisa	Sep-21
	73**	Rodent Kidney	No	Thlavakisa	Sep-21
	85**	Rodent Kidney	No	Gottenburg	Sep-21
	43-	Rodent Kidney	No	Utha	Mar-22
	69-	Rodent Kidney	No	Utha	Mar-22
	4	Rodent Kidney	No	Welverdiend A	Apr-22
	12	Rodent Kidney	No	Welverdiend A	Apr-22
	20	Rodent Kidney	No	Welverdiend A	Apr-22
	28	Rodent Kidney	No	Welverdiend A	Apr-22

Date analyzed	Sample ID	Sample type	Pathogenic *Leptospira* DNA detected	Village	Date collected
	4**	Rodent Kidney	No	Athol	Sep-21
	16**	Rodent Kidney	No	Athol	Sep-21
	33**	Rodent Kidney	No	Athol	Sep-21
	65**	Rodent Kidney	No	Thlavakisa	Sep-21
	74**	Rodent Kidney	No	Thlavakisa	Sep-21
	86**	Rodent Kidney	No	Thlavakisa	Sep-21
	44-	Rodent Kidney	No	Utha	Mar-22
	24*	Rodent Kidney	No	Uncertain	Uncertain
	5	Rodent Kidney	No	Welverdiend A	Apr-22
	13	Rodent Kidney	No	Welverdiend A	Apr-22
	21	Rodent Kidney	No	Welverdiend A	Apr-22
	29	Rodent Kidney	No	Welverdiend A	Apr-22
	6**	Rodent Kidney	No	Athol	Sep-21
	21**	Rodent Kidney	No	Utha	Sep-21
	34**	Rodent Kidney	No	Athol	Sep-21
	66**	Rodent Kidney	No	Thlavakisa	Sep-21
	75**	Rodent Kidney	No	Uncertain	Sep-21
	11-	Rodent Kidney	No	Utha	Mar-22
	45-	Rodent Kidney	No	Utha	Mar-22
	6	Rodent Kidney	No	Welverdiend A	Apr-22
	14	Rodent Kidney	No	Welverdiend A	Apr-22
	22	Rodent Kidney	No	Welverdiend A	Apr-22
	30	Rodent Kidney	No	Welverdiend A	Apr-22
	7**	Rodent Kidney	No	Athol	Sep-21
	22**	Rodent Kidney	No	Athol	Sep-21
	36**	Rodent Kidney	No	Utha	Sep-21
	69**	Rodent Kidney	No	Gottenburg	Sep-21
	77**	Rodent Kidney	No	Thlavakisa	Sep-21
	12-	Rodent Kidney	No	Utha	Mar-22
	46-	Rodent Kidney	No	Utha	Mar-22
	7	Rodent Kidney	No	Welverdiend A	Apr-22
	15	Rodent Kidney	No	Welverdiend A	Apr-22
	23	Rodent Kidney	No	Welverdiend A	Apr-22
	31	Rodent Kidney	No	Welverdiend A	Apr-22
	11**	Rodent Kidney	No	Athol	Sep-21
	23**	Rodent Kidney	No	Athol	Sep-21
	55**	Rodent Kidney	No	Thlavakisa	Sep-21
	60**	Rodent Kidney	No	Thlavakisa	Sep-21
	78**	Rodent Kidney	No	Thlavakisa	Sep-21
	17-	Rodent Kidney	No	Athol	Mar-22
	47-	Rodent Kidney	No	Utha	Mar-22
	8	Rodent Kidney	No	Welverdiend A	Apr-22
	16	Rodent Kidney	No	Welverdiend A	Apr-22
	24	Rodent Kidney	No	Welverdiend A	Apr-22
	32	Rodent Kidney	No	Welverdiend A	Apr-22

Date analyzed	Sample ID	Sample type	Pathogenic *Leptospira* DNA detected	Village	Date collected
	12**	Rodent Kidney	No	Athol	Sep-21
	24**	Rodent Kidney	No	Athol	Sep-21
	58**	Rodent Kidney	No	Gottenburg	Sep-21
	70**	Rodent Kidney	No	Gottenburg	Sep-21
	79**	Rodent Kidney	No	Gottenburg	Sep-21
	33-	Rodent Kidney	No	Athol	Mar-22
Sep 16 2022	16-	Rodent Kidney	No	Athol	Mar-22
	37*	Rodent Kidney	No	Uncertain	Uncertain
	145+	Rodent Kidney	No	Athol	Oct-22
	229*	Rodent Kidney	No	Utha	Oct-22
	247*	Rodent Kidney	No	Utha	Oct-22
	268*	Rodent Kidney	No	Utha	Oct-22
	299*	Rodent Kidney	No	Thlavakisa	Oct-22
	311*	Rodent Kidney	No	Manyeleti	Oct-22
	365*	Rodent Kidney	No	Utha	Oct-22
	406*	Rodent Kidney	No	Gottenburg	Oct-22
	8-GW3	Water	No	Gottenburg	Apr-22
	6-GW1	Water	No	Gottenburg	Apr-22
	15-TW5	Water	No	Thlavakisa	Apr-22
	42*	Rodent Kidney	No	Athol	Oct-22
	155+	Rodent Kidney	No	Athol	Oct-22
	233	Rodent Kidney	No	Utha	Oct-22
	248*	Rodent Kidney	No	Utha	Oct-22
	280	Rodent Kidney	No	Athol	Oct-22
	300*	Rodent Kidney	No	Thlavakisa	Oct-22
	313*	Rodent Kidney	No	Thlavakisa	Oct-22
	366*	Rodent Kidney	No	Utha	Oct-22
	408*	Rodent Kidney	No	Thlavakisa	Oct-22
	9-GW4	Water	No	Gottenburg	Apr-22
	7-GW2	Water	No	Gottenburg	Apr-22
	24 TW4	Water	No	Thlavakisa	Apr-22
	92*	Rodent Kidney	No	Athol	Oct-22
	159+	Rodent Kidney	No	Athol	Oct-22
	235	Rodent Kidney	No	Utha	Oct-22
	251	Rodent Kidney	No	Utha	Oct-22
	281	Rodent Kidney	No	Athol	Oct-22
	301*	Rodent Kidney	No	Manyeleti	Oct-22
	333*	Rodent Kidney	No	Utha	Oct-22
	392*	Rodent Kidney	No	Utha	Oct-22
	AW1	Water	No	Athol	Apr-22
	10-GW5	Water	No	Gottenburg	Apr-22
	UW3	Water	No	Utha	Apr-22
uw2?	NG3	Water	No	Utha	Apr-22
	98*	Rodent Kidney	No	Gottenburg	Oct-22
	173+	Rodent Kidney	No	Utha	Oct-22

Date analyzed	Sample ID	Sample type	Pathogenic *Leptospira* DNA detected	Village	Date collected
	238	Rodent Kidney	No	Athol	Oct-22
	252	Rodent Kidney	No	Utha	Oct-22
	282*	Rodent Kidney	No	Athol	Oct-22
	302*	Rodent Kidney	No	Utha	Oct-22
	334*	Rodent Kidney	No	Utha	Oct-22
	393*	Rodent Kidney	No	Utha	Oct-22
	AW2	Water	No	Athol	Apr-22
	1-WW1	Water	No	Welverdiend A	Apr-22
	UW4	Water	No	Utha	Apr-22
	AS1	Soil	No	Athol	Apr-22
	130	Rodent Kidney	No	Utha	Oct-22
	174+	Rodent Kidney	No	Utha	Oct-22
	243	Rodent Kidney	No	Utha	Oct-22
	254	Rodent Kidney	No	Athol	Oct-22
	283*	Rodent Kidney	No	Athol	Oct-22
	303*	Rodent Kidney	No	Utha	Oct-22
	335*	Rodent Kidney	No	Utha	Oct-22
	395*	Rodent Kidney	No	Utha	Oct-22
	AW3	Water	No	Athol	Apr-22
	3-WW3	Water	No	Welverdiend A	Apr-22
	UW5	Water	No	Utha	Apr-22
	AS3	Soil	No	Athol	Apr-22
	131*	Rodent Kidney	No	Utha	Oct-22
	175+	Rodent Kidney	No	Utha	Oct-22
	244	Rodent Kidney	No	Utha	Oct-22
	256	Rodent Kidney	No	Utha	Oct-22
	284*	Rodent Kidney	No	Athol	Oct-22
	304*	Rodent Kidney	No	Utha	Oct-22
	337*	Rodent Kidney	No	Utha	Oct-22
	401*	Rodent Kidney	No	Thlavakisa	Oct-22
	AW4	Water	No	Athol	Apr-22
	2-WW2	Water	No	Welverdiend A	Apr-22
	11-TW1	Water	No	Thlavakisa	Apr-22
	AS2	Soil	No	Athol	Apr-22
	135*	Rodent Kidney	No	Athol	Oct-22
	222	Rodent Kidney	No	Thlavakisa	Oct-22
	245	Rodent Kidney	No	Utha	Oct-22
	257*	Rodent Kidney	No	Athol	Oct-22
	296	Rodent Kidney	No	Thlavakisa	Oct-22
	306*	Rodent Kidney	No	Utha	Oct-22
	346*	Rodent Kidney	No	Athol	Oct-22
	404*	Rodent Kidney	No	Thlavakisa	Oct-22
	AW5	Water	No	Athol	Apr-22
	4-WW4	Water	No	Welverdiend A	Apr-22
	12-TW2	Water	No	Thlavakisa	Apr-22

Date analyzed	Sample ID	Sample type	Pathogenic *Leptospira* DNA detected	Village	Date collected
	138*	Rodent Kidney	No	Utha	Oct-22
	227*	Rodent Kidney	No	Utha	Oct-22
	246	Rodent Kidney	No	Athol	Oct-22
	263	Rodent Kidney	No	Athol	Oct-22
	298*	Rodent Kidney	No	Thlavakisa	Oct-22
	310*	Rodent Kidney	No	Uncertain	Oct-22
	355*	Rodent Kidney	No	Utha	Oct-22
	405*	Rodent Kidney	No	Gottenburg	Oct-22
	UW1	Water	No	Utha	Apr-22
	5-WW5	Water	No	Welverdiend A	Apr-22
	13-TW3	Water	No	Thlavakisa	Apr-22
Sep 17 2022	TS1	Soil	No	Thlavakisa	Apr-22
	TS3	Soil	No	Thlavakisa	Apr-22
	TS4	Soil	No	Thlavakisa	Apr-22
	TS5	Soil	No	Thlavakisa	Apr-22
	TS2	Soil	No	Thlavakisa	Apr-22
	G5 5	Soil	No	Gottenburg	Apr-22
	G5 4	Soil	No	Gottenburg	Apr-22
	G5 3	Soil	No	Gottenburg	Apr-22
	G5 2	Soil	No	Gottenburg	Apr-22
	G5 1	Soil	No	Gottenburg	Apr-22
	5-WS5	Soil	No	Welverdiend A	Apr-22
	4-WS4	Soil	No	Welverdiend A	Apr-22
	3-WS3	Soil	No	Welverdiend A	Apr-22
	2-WS2	Soil	No	Welverdiend A	Apr-22
	1-WS1	Soil	No	Welverdiend A	Apr-22
	GS5	Soil	No	Gottenburg	Apr-22
	GS4	Soil	No	Gottenburg	Apr-22
	GS3	Soil	No	Gottenburg	Apr-22
	GS2	Soil	No	Gottenburg	Apr-22
	GS1	Soil	No	Gottenburg	Apr-22
	AS5	Soil	No	Athol	Apr-22
	AS4	Soil	No	Athol	Apr-22

Rat collection data from Welverdiend A in April 2022: (20/04/2022 - 23/04/2022)

ID	Date collected	House number	Weight (gram)	Sex (M/F)	Pathogenic *Leptospira* bacteria detected (Yes/No)	Notes
WR1	20/04/2022	91 Thete		M	No	
WR2	20/04/2022	91 Thete		F	No	
WR3	20/04/2022	92 Thete		M	No	
WR4	20/04/2022	67		F	No	
WR5	20/04/2022	84 D.T. Thete		F?	No	
WR6	20/04/2022	99		F	No	
WR7	20/04/2022	21		M	No	
WR8	20/04/2022	24		M	No	
WR9	20/04/2022	52		F	No	
WR10	20/04/2022	52		F	No	
WR11	20/04/2022	45		F	No	Mildly anorexic
WR12	20/04/2022	44		F	No	
WR13	20/04/2022	44		F	No	
WR14	21/04/2022	91 Thete	110	M (Sc)	No	
WR15	21/04/2022	91 Thete	55	F	No	
WR16	21/04/2022	76 (36?)	58	M (non-SC)	No	
WR17	21/04/2022	27	140	M (Sc)	No	
WR18	21/04/2022	59 (69?)	50	F (perf)	No	
WR19	21/04/2022	66	23	F (non-perf)	No	
WR20	21/04/2022	52	120	F (perf)	No	
WR21	22/04/2022	91 Thete	46	M (non-SC)	No	
WR22	22/04/2022	76 (36?)	55	M (non-SC)	No	Big testes
WR23	22/04/2022	24	33	F (non-perf)?	No	
WR24	22/04/2022	52	73	M (Sc)	No	
WR25	23/04/2022	91 Thete	115	F (perf)	No	
WR26	23/04/2022	92 Thete	64	M (Sc)	No	
WR27	23/04/2022	67	34	F (non-perf)?	No	
WR28	23/04/2022	67	36	F (non-perf)?	No	
WR29	23/04/2022	67	31	M (non-SC)	No	
WR30	23/04/2022	67	33	M (non-SC)	No	
WR31	23/04/2022	59 (69?)	145	M (Sc)	No	
WR32	23/04/2022	59 (69?)	90	F (perf)	No	
WR33	23/04/2022	99	71	F (perf)	No	*Mastomys?*
WR34	23/04/2022	52	21.5	M (non-SC)	No	