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Prevalence and characterization of *Leptospira* spp. in slaughter animals at abattoirs in Gauteng, South Africa and the zoonotic risk posed to abattoir workers

By

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A thesis submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy Veterinary Science Veterinary Tropical Diseases

In the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

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October 2020

Dedication

The dedication of my thesis is:

To God the supreme, who holds my future and is my entire being, in whom I believe and totally depend upon in all circumstances for He never sleeps nor slumbers!

To my ever loving and blessed family, (My wife: Mrs. Elizabeth Kasham Banenat Dogonyaro; my children Zugwai-pet name as Baby Zee (Blessing); Kazah'yet (David); Ahbusan (Daniel) and Syaai-Ryiah (Nitzachon-Victory). My prayers for you is, that, God will surely continue to watch over your going out and coming in, as he goes before you with showers of blessings in all your endeavours in Jesus Mighty name amen.

Declaration

I, *Banenat* ***Dogonyaro*** *Bajehson* fully aware of what plagiarism means and the policy of the University of Pretoria regarding plagiarism.

I hereby declare that, this thesis is my original work and where references are cited from other sources such as internet, printed sources etc, are other people's work.
All the cited work has been acknowledged and referenced appropriately based on the regulations of the University of Pretoria.

I have not used any other person's or student work in this thesis for my PhD research work.

No part of this thesis or this full thesis has been submitted elsewhere to any institution or university for another degree.

Signed:  ……

Date:……**October, 2020**……

Banenat Bajehson **Dogonyaro**

Acknowledgments

I would like to express my profound gratitude to the following people who play vital roles, especially in my life, scientific career and specifically, during this research project.

First of all, to God Almighty, my creator-who was, who is and who is to come, the lion of the tribe of Judah, the Alpha and the Omega of all things, for in you the solid rock I stand!

I am indeed very grateful to my lovely wife, (Mrs. Elizabeth Banenat Dogonyaro Bajeh) and children Zugwai-pet name: Baby Zee (Blessing), Kazah'yet (David), Ahbusan (Daniel) and Syaai-Ryiah (Nitzachon-Victory), for their endless prayers, support, encouragement and for standing in the gap while I was so many miles away from home during these years of research project. God bless and keep you healthy to fulfil his purpose while on earth amen. In addition, I am very thankful to my elder brothers and Sisters, especially to (Mr. Dauda Dogonyaro Bajeh and Mrs. Alherry Dauda Dogonyaro Bajeh-Sister in-law), for their true love and sacrifices, support and prayers. Special thanks to my beloved aged parents (RTD. Rev. Dogonyaro, Vvayin Bajeh and Mrs. Kogi Dogonyaro, Vvayin Bajeh) for making me who I am today through Jesus Christ our Lord. God bless and keep you healthy in Jesus Mighty name amen!

I am very grateful to my main supervisor, Professor Abiodun Adewale Adesiyun, for his excellent guidance and kindness and to my co-supervisors: Professor Henriette van Heerden for your wonderful mentorship and kindness and to Dr. Andrew David Potts, for his diligent guidance and support.

Words cannot express my heartfelt gratitude to you all (Supervisors) for making this Ph.D. thesis a huge success through your excellent positive criticisms and quality time that resulted in the completion of this thesis.

My profound gratitude also goes to Professor Albert I. Ko and Dr. Elsio Wunder Jnr., of the Yale University School of Public Health, College of Medicine, New Haven, Connecticut, USA for the award of Postgraduate Research Fellow at Professor Ko's Laboratory, Department of Epidemiology and Microbial Diseases, at the Yale School of Public Health, College of Medicine, to complete some important components of my Research and their very kind, friendly and excellent academic environment while I was at the Yale University School of Public Health. This was made possible through funds from the National Institute of Health (NIH), grant number: R01 AI121207, USA.

I am very thankful to Professor Lucile Blumberg, Dr. Jenny Rossouw and staff of the Bacterial Special Pathogens Unit of the National Institute for Communicable Diseases (NICD), in Johannesburg, South Africa for facilitating the initial human sera testing aspect of this Research project in your Laboratory.

I am very thankful to Dr. Bernice Harris from the Faculty of Health Sciences, University of Pretoria, who diligently and kindly collected the blood samples from consented abattoirs workers throughout the sampling period of this Project.

I am indebted to the Vice Chancellor and the Management of the University of Pretoria for the awards: University of Pretoria Postgraduate Doctoral abroad programme scholarship grant to the Yale University School of Public Health and the Doctoral support bursary for this thesis. These awards played an important role to enable me successfully accomplished an interesting component of my PhD., Research project at Yale University School of Public Health, USA. and supported me during my PhD., Research, respectively.

My appreciation also goes to the Dean and Management of the Faculty of Veterinary Science; My HOD, Professor Matjila P. Tshepo, Professor Marinda, C. Oosthuisen and the management of the Department of Veterinary Tropical Diseases, for all their kind support during my research at the Onderstepoort Veterinary Faculty. I am very thankful to Professor Folorunso Oludayo Fasina for his kind concern during my PhD. Programme in South Africa. To my Departmental Postgraduate coordinator and Faculty Postgraduate administrator in persons of Rina Serfontain and Mrs. Leonie Johnson, I am very thankful for your diligent and efficient in your duties.

I am very grateful to the staff of the Bacteriology Department, Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) for their kind and cordial relationship during my research work in their Laboratories.

To my fellow postgraduates, I am very grateful for the good company during this period of study. ‘‘We live to part and live to meet again’’.

This Ph.D. research programme wouldn’t have been possible without funds from the National Institute for Health (NIH) grant Number R01 AI121207, USA and the Gauteng Department of Agriculture and Rural Development (GDARD), South Africa. I would like to thank the Faculty of

Veterinary Sciences, University of Pretoria, for the Doctoral Abroad programme scholarship and the Doctoral support bursary awarded to me.

I thank the National Veterinary Research Institute (NVRI) Vom, Nigeria and the Onderstepoort Veterinary Research (OVR) for their support. I also thank the abattoir owners for the access to their facilities and the personnel that participated in this research by given us their consent.

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List of Abbreviations

%:	percentage
µm:	Micro milliliter
µM:	Micromolar
°C:	Degrees Celsius
ACID:	Acid citrate dextrose
AEC:	Animal ethics committee
AIC:	Akaike Information Criterion
ARC-OVR:	Agricultural Research Council -Ondersterpoort Veterinary Research
BLAST:	Basic local alignment search tool
BLS 2:	Biohazard cabinet class two
BP:	base pair
BSA:	Bovine serum albumin
DAFF:	Department of Agriculture, forestry and fisheries
DALYs:	Disability Adjusted Life Years
DFM:	Dark-field microscope
DNA:	Deoxyribonucleic acid
dNTP:	deoxyribonucleotide triphosphate
EDTA:	Ethylene-diamine-tetra-acetic acid
ELISA:	Enzyme-Linked Immunosorbent Assay
EMJH:	Ellinghausen McCullough Johnson and Harris
GDARD:	Gauteng Department of Agriculture and Rural Development
GEq/mL:	Genomic equivalents per gram
GIS:	Geographical information system
GPS:	Global positioning system
GTR+1:	General Time Reversible model
ID:	Identity number
IgG:	Immunoglobulin G (Gamma)
IgM:	Immunoglobulin M (Mu)
KB:	kilobase
Km^2:	Kilometre square
LipL32:	Outer membrane lipoprotein
MAFFT:	Multiple Alignment using Fast Fourier Transform
MEGA:	Molecular evolutionary genetics analysis

mg:	Milligrams
min:	Minute
ml:	Milliliter
mm:	Millimetre
mM:	Millimolar
n:	number
NICD:	National Infectious and Communicable Diseases
OIE:	World Organization for Animal Health
OR:	Odd ratio
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
pH:	Power of hydrogen or potential for hydrogen
QNORM:	quantiles formation of the normal distribution
qPCR (RT-PCR):	quantitative or real-Time Polymerase Chain Reaction
R2:	standard curve correlation efficient
REC:	Research Ethics committee
SA:	South Africa
SV:	Serovar
TBE:	Tris-borate-EDTA buffer
UDG:	Uracil DNA glycosylase
USA:	United states of America
WHO:	World Health Organization

Thesis Summary

Prevalence and characterization of *Leptospira* spp. in slaughter animals at abattoirs in Gauteng, South Africa and the zoonotic risk posed to abattoir workers

Candidate: Banenat Bajehson **Dogonyaro**
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Department: Veterinary Tropical Diseases
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Summary:

Leptospirosis is an important global re-emerging, occupational, environmental and zoonotic disease. It is an under-estimated disease of public health and veterinary importance caused by the pathogenic spirochetes belonging to the genus *Leptospira*. Currently in South Africa, there is limited information on leptospirosis and veterinarians' beliefs that leptospirosis is not an important disease in the country. The primary aim of the investigation was to determine the prevalence of *Leptospira* spp. in slaughtered livestock and workers at abattoirs in Gauteng province, South Africa. To achieve this aim, retrospective analysis of laboratory data and cross-sectional serological, bacteriological and molecular studies were conducted on livestock and abattoir workers during the study period.

The objective of the retrospective analysis of 11-year (2007 – 2017) data was to determine the seropositivity and infecting serovars of *Leptospira* in the sera of livestock (suspected or clinical cases of leptospirosis), submitted to the Agricultural Research Council (ARC)-Ondersterpoort Veterinary Research (OVR), Bacteriology serology laboratory. The overall seropositivity for leptospirosis in livestock was, 20.5% (1,425/6,945), using an eight-serovar microscopic agglutination test (MAT) panel. The frequency of seropositivity was 22.0% (1,133/5,168), 16.2% (286/1,763) and 0.0% (0/14) for cattle, pigs and sheep respectively ($p<0.00\ 01$). Australis (sv. Bratislava) was the predominant serovar having been detected in 29.4% (333/1,133) and 32.0% (91/286) of seropositive cattle and pigs respectively. The year 2016 of the 11 years retrospective data, had seroprevalence overall of 22.0% (102/466), with 100% (2/2) and 21.6% (101/466) for pigs and cattle respectively. It is

important to note that, this was the same period (2016) we conducted the current cross-sectional study.

A cross-sectional study was conducted on pigs and cattle slaughtered at Gauteng abattoirs in South Africa: Eighty-five (n=85) sera from slaughtered pigs at 5 consented abattoirs were analysed by MAT. The overall seropositive was, 24.7% (21/85) using 26 antigens panel for pigs in South Africa for the first time; Predominant serogroup was serogroup Australis-Bratislava reported as the predominant in seropositive pigs, 90.5% (19/21), 22.4% (19/85). For the cattle, 199 serum samples were analysed from slaughtered cattle from 11 abattoirs that consent was granted. Seropositive from cattle sera, 27.6% (55/199) with serogroup Sejroe (Hardjo), 10.5% (21/199) as the predominant circulating in the Country. The study demonstrated, for the first time in South Africa, the occurrence of four serovars, namely, Hardjo bovis strain lely 607; Topaz, 3.5% (7/199); Hebdomadis, 2.5% (5/199) and Medanensis, 1.5% (3/199) in slaughtered cattle. The vaccine used to prevent cattle leptospirosis in South Africa does not contain three of the newly detected serovars (Topaz, Hebdomadis and Medensis), an indication that the seropositive cattle acquired infection through natural exposure. There were statistically significant differences ($P<0.05$) in the detection of the serogroups of *Leptospira.* Of the five variables analysed, only one variable (abattoir) had statistically significantly ($P<0.001$) differences in the seroprevalence of leptospirosis in cattle.

With the bacteriological culture of 305 kidneys using Ellinghausen McCaullough Johnson Harris (EMJH) media, the isolation rate for *Leptospira* spp. was 3.9% (12/305), with species-rate being 4.8% (9/186), 4.1% (3/74) and 0.0% (0/45) for cattle, pigs and sheep respectively ($P>0.05$). The use of quantitative polymerase chain reaction (qPCR) assays detected *Leptospira* DNA in 27.5% (84/305) of the livestock kidneys tested. Of the animals tested, 26.9% (50/186), 20.3% (15/74) and 42.2% (19/45) of cattle, pigs and sheep kidneys respectively ($P=0.03$) were positive for *Leptospira* DNA. It was significant that, although all sheep samples tested for leptospirosis by isolation and serology were negative for *Leptospira* spp., a high frequency (42.2%) was positive for *Leptospira* DNA. Sequencing of DNA from isolates of *Leptospira* spp. and kidney tissues from cattle identified 13 as *L. interrogans* and 2 as *L. borgpetersenii*), from pigs 4 were *L. interrogans* and from sheep kidney tissues, 2 were *L. interrogans* and 1 was *L. borgpetersenii*. The phylogenetic tree analyses revealed that all the isolates and the kidney tissue samples grouped together with the pathogenic *L. interrogans* serovar Icterohaemorrhagiae and *L. borgpetersenii* serovar Hardjo bovis strain lely 607 from the GenBank retrieved sequences. This study is also the first reported genetic analyses of the pathogenic *L. interrogans* and *L. borgpetersenii*, in slaughtered livestock in South Africa.

To determine the exposure experience of *Leptospira* spp. in abattoir workers sampled from six abattoirs, two serological tests (MAT and IgM ELISA) and one molecular method (qPCR) were used. The seroprevalence of *Leptospira* in 103 workers was 10.7% and 7.8% by IgM ELISA and MAT respectively, and the prevalence of *Leptospira* DNA in whole blood by qPCR was 16.5% (P>0.05). The overall prevalence (serology and PCR) of *Leptospira* spp. was 30.1% (31/103). The predominant serovar detected in seropositive workers was Djasiman (50.0%) and the abattoir-related risk factors identified were working in high throughput (HT) abattoirs and exposure to blood and/or water splashes during and after slaughter. Antibodies to Serogroups sejroe (Sv. *Wolffi*) and Pomona (Sv. *Djasiman*) were both found in animals and abattoir workers. Although, the main serovars in abattoir workers were different from those in animals.

It was concluded that the detection of new serovars *Leptospira* spp. in South Africa which are not currently in the leptospirosis vaccine used in livestock coupled with the fact that these serovars are not in the diagnostic eight-antigen MAT panel indicate a need to re-assess the status of livestock leptospirosis, as well as to revisit the existing policy and practices on leptospirosis in the country. The use of a diagnostic strategy which included both serological and molecular methods will increase the sensitivity of such an approach. The zoonotic risk of leptospirosis to abattoir workers identified in the study is for the first time in South Africa and it indicates the need to introduce measures to mitigate abattoir-associated risk exposure to leptospirosis in abattoir workers in the country.

Keywords: ***Leptospira;*** **Leptospirosis; Serogroups/Serovars; Diagnosis; Slaughter livestock; Abattoir workers; Gauteng Province, South Africa**

CHAPTER 1

General Introduction

1.1 Introduction

Leptospirosis, also known as Weil's disease and red water of calves, amongst many other names, is an important bacterial zoonosis of international significance (Cachay and Vinetz, 2005). The disease is considered a re-emerging zoonosis, a forgotten zoonosis and the most widespread zoonosis in the world (Levett, 2004; Meites et al., 2004; WHO, 2003). Leptospirosis is related to socioeconomic or climatic conditions that favour animal reservoir and human exposure, especially in developing countries (Pappas et al., 2008). The disease has also been reported in industrialized countries (Bharti et al., 2003).

Leptospirosis has emerged as an important urban health problem worldwide, yet the dynamics of the environmentally transmitted *Leptospira* pathogen has not been well characterized, (Casanova-Massana et al., 2017). Humans become infected by leptospires through contact with animal reservoirs, environmental surface water and soil that are contaminated with infected urine (Ko et al., 2009) Considering the fact that *Leptospira* spp. have been known to be isolated from waste and effluents of abattoirs and slaughterhouses elsewhere (Saito et al., 2012), where infected livestock and shedders of the pathogen are slaughtered. Therefore, one of the objectives of the study was to determine the frequency of detection of *Leptospira* spp. from effluents from abattoirs in South Africa.

In sub-Saharan Africa, leptospirosis has been reported to be endemic in West Africa where the disease has been documented in Nigeria, Cameroun; Central Africa in the Democratic Republic of Congo and Gabon; Eastern Africa in Kenya and Tanzania and in Southern Africa, mostly reported in South Africa and Zimbabwe (de Vries et al., 2014).

The disease can occur in both subclinical and clinical states in animals and humans (Katz et al., 2003; Levett, 2004). Leptospirosis in humans is a multi-system disease presenting in various clinical manifestations (Levett, 2004; Martínez-García et al., 2000). Annually, approximately 1.3 million cases and about 60,000 deaths due to leptospirosis occur worldwide in humans (Costa et al., 2015). Human infection occurs via direct contact with the urine of infected animals or indirectly through interaction with a urine-contaminated environment or tissues (kidneys and blood) from carcasses of

infected animals could serve as a source of infection to abattoir workers. *Leptospira* spp., can survive outside the host if conditions (e.g. a warm, wet climate) are favourable (Bharti et al., 2003; Levett, 2001).

Leptospirosis is caused by the pathogenic *Leptospira interrogans* and to date there are over 17 pathogenic *Leptospira* spp. worldwide (Vincent et al., 2019), with some serovars adapted to certain animal species (Bharti et al., 2003; Katz et al., 2003; Levett, 2004). Many factors have made the eradication of leptospirosis in animal and human populations virtually impossible. Some of these factors include its occurrence in subclinical states (with infected, apparently healthy animals and humans shedding the pathogen, (OIE, 2018; Fang et al., 2014) and the availability of commercial vaccines which prevent disease but not infection. This factor has major implications because apparently healthy infected, vaccinated animals, serve as sources of infection to others (Katz et al., 2003; Rentko et al., 1992). In addition, the vaccines do not always offer cross-protection against other serovars (OIE, 2018; Suepaul et al., 2010).

The diagnosis of leptospirosis is very difficult and tasking as it may take many weeks to complete the isolation process. The disease is usually diagnosed in the laboratory by culturing the microorganism from tissues, blood, or urine using a modified Ellinghausen McCullough Johnson and Harris (EMJH) semi-solid medium containing bovine serum albumin fraction (Ellis, 1986), by detecting antibodies in serum samples, (Lavinsky et al., 2012), or by demonstrating the presence of leptospires in tissues using antibodies labelled with fluorescent markers. Other methods may be available in some laboratories, for example, the polymerase chain reaction (PCR). The quantitative PCR (qPCR) is the most modern, fast and specific method which is now commonly used to quantify leptospires (Branger et al., 2005). However, this method is very expensive (Levett et al., 2004).

In South Africa, reported data on leptospirosis in humans and animals are scarce, especially in the livestock industry where only the Microscopic Agglutination Test (MAT), the gold standard (OIE, 2018), is used routinely. The seroprevalence of leptospirosis has been reported in livestock with the prevalence ranging from 12.5 % to 22.2 % (Gummow et al., 1999; Potts et al., 1995); in wildlife, 1.7% to 12.0% (Hunter et al., 1988; Myburgh et al., 1990) and the seroprevalence of various serovars reported to range between 0.05% to 22.3 % % (Potts et al., 1995). The last isolation of leptospirosis in South Africa was reported over two decades ago by Gummow et al. (1999). In addition, there are no reported data using the qPCR assay as recommended by the World Health Organization (WHO) as being the most specific and sensitive method for the diagnosis of *Leptospira* spp. (WHO, 2003).

The livestock industry in South Africa serves as a source of income and employment to both owners and personnel working both in small- and large-scale farming and in feedlots following the slaughter of these animals at the abattoirs. Apart from abattoirs being used as facilities for slaughtering animals, they can also be used as facilities for active and passive surveillance of zoonoses, like leptospirosis, in animals and humans. It is also known that workers at abattoirs are exposed to zoonotic agents from slaughtered infected livestock (Olubunmi et al., 2017).

According to the National Institute of Communicable Diseases (NICD), there have been reported sporadic cases of leptospirosis in humans. A case of leptospirosis was reported in a human male in 2015 (http//www.nicd.ac.za, 2015). In December 2016, two inmates at the Pollsmoor prison were reported to have died as a result of leptospirosis linked to rats (http//www.nicd.ac.za, 2016). However, the disease is not considered a problem in South Africa. Therefore, the use of abattoirs to determine the status of the disease in livestock will provide invaluable information on the seroprevalence of leptospirosis and the risk it poses to abattoir workers. Such information may also contribute to the development of strategies to prevent exposure to the pathogen and mitigate the potential impact on public health in South Africa.

1.2 Problem Statement

Leptospirosis is a re-emerging important zoonosis which is highly prevalent in developing countries and is responsible for morbidity and mortality in humans and livestock (WHO, 2010). Infection in livestock causes abortion and other clinical manifestations, all resulting in economic losses. Rodents are considered important reservoir of infection for livestock, pet animals and in some cases, humans through contaminated water and foods, (Kruger et al., 2020).

There is a general dearth of current information on leptospirosis in South Africa. Furthermore, there exists a belief amongst veterinarians that leptospirosis is not important disease in the country. A study conducted by Potts et al. (1995) reported that livestock leptospirosis, especially in pigs, was not a major problem in the country, and overall, the frequency of vaccination of animals was low. Therefore, it is imperative to either substantiate or disprove this prevailing belief or assumption by providing current, meaningful empirical data through an epidemiological investigation on the occurrence and risk of leptospirosis (infection or diseases) in slaughter livestock and workers in abattoirs in the Gauteng province. The provision of such data by the proposed study will determine whether leptospirosis is under-diagnosed and/or under-reported and, if so, provide guidance in developing appropriate methods to reduce the incidence and prevalence of the disease in the country. The study will also provide invaluable data on whether there is a need to re-assess the

current practice (type and number of serovars) and policy (voluntary or mandatory) on the vaccination of livestock against leptospirosis in the country.

Therefore, based on the afore-mentioned issues, a study was designed with the following hypotheses:

1.3 Hypotheses

a. The seroprevalence of antibodies to *Leptospira* spp. in slaughtered livestock varies significantly among animal species
b. The serovars of *Leptospira* spp. circulating in livestock are the same as those frequently detected in abattoirs workers.
c. The antibodies to *Leptospira* spp. detected in slaughtered livestock are primarily the same as those contained in the vaccines used in the country.
d. Leptospirosis in livestock, humans and abattoir effluents is grossly under-diagnosed and therefore, poses a problem in the livestock industry and human population in South Africa.

1.4 Primary research questions

- Based on data from laboratory diagnosis of leptospirosis in livestock in South Africa between 2007 and 2017, what are the frequencies of diagnoses of leptospirosis and the serovars involved?
- What is the prevalence of leptospirosis in livestock slaughtered and abattoir effluents at the abattoirs in Gauteng Province, South Africa?
- What are the prevalent serovars of *Leptospira* circulating in livestock and abattoir workers in Gauteng Province of South Africa?

1.5 Secondary research questions

- What are the risk factors associated with exposure to *Leptospira* spp. in slaughtered livestock and abattoir workers in Gauteng province, South Africa?
- What are the characteristics (genotype) of the isolates of *Leptospira* spp. recovered from slaughtered livestock in South Africa?

1.6 Aim

The overall aim of this research project is to determine the prevalence and characteristics of *Leptospira* spp. in slaughtered animals, abattoir effluent at Gauteng province abattoirs and the zoonotic risks posed to abattoir workers.

1.7 Specific objectives

- To review the diagnostic laboratory records for evidence of suspected or confirmed cases of livestock leptospirosis over a period of 11 years (2007-2017) in South Africa.
- To determine the seroprevalence of leptospirosis in slaughtered livestock using the MAT and in abattoir workers using both the MAT, Panbio IgM ELISA Kit and qPCR in the Gauteng province.
- To determine the prevalence of pathogenic *Leptospira* spp. in the abattoir effluents by quantitative real-time PCR (qPCR).
- To detect pathogenic *Leptospira* spp. by isolation and polymerase chain reaction (PCR) from the kidneys of slaughtered livestock.
- To detect pathogenic *Leptospira* spp. in whole blood of abattoirs workers using qPCR.
- To determine important risk factors for exposure to *Leptospira* spp. in livestock and abattoir workers.
- To characterize pathogenic *Leptospira* isolates from livestock as to their genotypes targeting the *SecY* partial genes region.
- To compare the South African *Leptospira* serovars with other from other parts of the world using their phylogenetic relationship.

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CHAPTER 2

Literature Review

2.1 Aetiology and biology of *Leptospira* spp.

Adolf Weil's in 1886, first described leptospirosis as a disease caused by the pathogenic *Leptospira* spp. and in Japan, the microorganism was isolated from coal miners in 1908 (de Vries et al., 2014; Inada et al., 1915). Leptospires possess both Gram-positive and Gram-negative bacterial properties (Haake, 2000) and they have low endotoxic activity (Shimizu et al., 1987). Leptospires are corkscrew-shaped bacteria with amplitude of 0.1-0.15 and 0.5 wavelength (Faine, 1999; Mohammed et al., 2011). The bacteria are different from other spirochetes as they possess distinct hooks at both ends with ruffled and beaded surfaces when observed under high magnification (Bharti et al., 2003). They are 0.1 μm in diameter and 6-20 μm in length (Faine, 1999).

Spirochetes are highly mobile, thin and very small, therefore testing for leptospires by staining is not the ideal method; it requires a dark-field microscope (DFM) for direct observation of the organism. The movements of leptospires under the DFM are translational and non-translational, (Ellis, et al., 1993). The species of leptospires cannot be differentiate morphologically and isolated field strains of pathogenic leptospires are shorter and more tightly coiled compared to the leptospires that have been maintained in culture (Ellis et al., 1983; Faine, 1999). They are obligate and aerobic zoonotic organisms that grow at an optimal temperature between 28 - 30°C, in media supplemented with B1 and B2 amino acids and long chain of fatty acids, with low concentration of agar between 0.1-0.2 % (Mohammed et al., 2011).

The growth of leptospires is slow, especially at primary isolation, and it is recommended that the cultures be incubated for up to 13 weeks before being considered as negative and discarded. A maximum growth density in semi-solid media is a discrete zone under the surface of the media which becomes increasingly turbid as the incubation progresses. The optimum oxygen tension is related to the growth of leptospires and it is referred to as the Dinger's ring zone or disk (Mohammed et al., 2011).

Leptospires can survive in alkaline swamps, streams, rivers, soil, mud, diluted milk and tissues of live or dead animals (Mohammed et al., 2011) and while in the soil, they can survive and remain viable

for some weeks to many months and for several weeks in cattle slurry. The survival of pathogenic leptospires depends on several factors such as the temperature, inhibitory compounds and pH. They can survive at a pH between 7.2 – 8.0. Leptospires have been found unable to withstand drought, heat, acid and base disinfectants (Mohammed et al., 2011; Slack et al., 2006).

They are parasitic and therefore need hosts to survive in the environment and are killed by temperatures above 50°C and dehydration. The genome of leptospires has two chromosomes, the large and the small chromosomes with 4, 332,241 bp to 4, 277, 185bp and 358, 943 bp to 350, 181 bp respectively (Faine, 2007).

2.2 Classification of *Leptospira* species

Leptospirosis is caused by pathogenic leptospires that are classified (Noguciii, 1918) as follow:
Kingdom: Bacteria
Phylum: Spirochaetae
Class: Spirochaetes
Order: Spirochaetales
Family: *Leptospiroceae*
Genus: *Leptospira*
Leptospirosis, is caused by infection with the pathogenic, helical shaped, motile leptospires (Mohammed et al., 2011). *Leptospira* species are basically classified by two major methods, the first method is phenotypically (Vinetz, 2001), while the second method is genetic-based as updated in 1999 (Brenner et al., 1999). The basic unit of *Leptospira* taxonomy is the serovar. Serovars consist of closely related isolates based on serological reactions to the organism's lipopolysaccharide. More than 250 pathogenic serovars and at least 50 non-pathogenic serovars have been identified (Mohammed et al., 2011).

Before 1989, all pathogenic isolates belonged to the species, *Leptospira interrogans* and all non-pathogenic leptospires (saprophytes) were placed under *Leptospira biflex* (Faine and Stallman, 1982). Serovars were also classified into groups, using serological methods, into 24 serogroups (Mohammed et al., 2011). Saprophytic *L. biflexa* interestingly were described prior to the isolation of the pathogenic *Leptospira* spp. (Faine and Stallman, 1982). The genus *Leptospira* has since been reclassified, using genetic techniques, into 21 species, with over 17 pathogenic *Leptospira* spp. worldwide (Vincent et al., 2019). Species that have been detected in clinical cases include *L.*

interrogans, *L. borgpetersenii, L. alexanderi, L. alstonii, L. kirschneri, L. noguchi, L. santarosai, L. weilii* and *L. wolffii* (Picardeau, 2013).

Molecular taxonomy and the advent of many scientific questions and objectives led to major changes in the classification of *Leptospira* based on Deoxyribonucleic acid (DNA)-Deoxyribonucleic acid (DNA) i.e. DNA-DNA relatedness, which resulted in the former *L. interrogans* single specie being divided into seven species (Yasuda et al., 1987). Adler and de la Peña Moctezuma, (2010) reported that subsequent novel isolations and analyses of DNA have added several additional species of both pathogenic and saprophytic *Leptospira.*

2.3 Leptospirosis in animals including humans

Leptospirosis is a zoonotic disease worldwide and has been reported in both developed and developing countries, where the transmission of the disease is linked to multiple factors in animal to human at the ecological interface (Petrakovsky et al., 2014). The occurrences of outbreaks in animal and human populations are attributed to many factors involving animal husbandry, human behaviour and climatic changes, (Munoz-zanzi et al., 2020). Leptospirosis causes great economic loses in the livestock industry and in humans, it is a serious public health problem. According to Costa et al. (2015), the disease incidence ranged from 0.10 to 975 annual cases per 100,000 population, with a mean case fatality ratio of 6.85% in humans. Globally, approximately 2.90 million Disability Adjusted Life Years (DALYs) are lost per annum (Uls 1.25-4.54 million) (Torgerson et al., 2015), from the approximately 1.03 million cases of human leptospirosis reported previously (Costa et al., 2015). It was found that males were predominantly affected with an estimated 2.33 million DALYs (Uls 0.98-3.69) or approximately 80% of the total burden (Torgerson et al., 2015).

2.3.1 Reservoir hosts

Leptospirosis has various hosts that roam in the environment and leptospires circulate within the human and animal populations. These hosts include the reservoir hosts: rodents (rats and mice), cattle, pigs, sheep, horses, dogs, cats, and wildlife animals species (Levett, 2001). Based on the mode of transmission and the epidemiology of the disease, there are a number of high risk groups in the population because of their higher exposure potential to the pathogen as a result of their occupation (veterinarians, abattoirs workers, sewer workers, dairy farmers, mine workers), practices, habits (Levett, 2004; Meites et al., 2004) and water sport activities. Humans are usually infected by contact with urine of an infected host, contaminated drinking water or soil, or infected

animal tissue (de Vries et al., 2014). Studies have shown that some specific host reservoirs harbour specific serovars of *Leptospira* spp. (Table 2.1).

Table 2.1: Reservoirs of different serovars present in domestic animals and wildlife ***(Bharti et al., 2003)***

***Leptospira* serovar**	**Usual host**
Icterohaemorrhagiae and Ballum	Rats
Ballum	Mice
Grippotyphosa and Hardjo	Dairy cattle
Pomona and Tarassovi	Pigs
Pomona and Hardjo	Sheep
Canicola	Dogs

2.3.2 Transmission

Leptospirosis is a globally important zoonotic disease and its transmission is attributed to various factors at the animal-human ecological interface (Petrakovsky et al., 2014). Most frequent route of transmission is from infected mammalian species that excrete pathogenic leptospires in their urine. These pathogens are kept in sylvatic and domestic environments through transmission between species of rodents and other mammal species (Ko et al., 2009). It is important to note that these reservoirs, especially rodents, act as asymptomatic carriers and therefore, able to shed leptospires which can infect livestock and wildlife. Humans get infected by leptospires through contact with animal reservoirs, environmental surface water and soil that are contaminated with infected urine (Ko et al., 2009). Leptospires enter the body through broken skin and mucous membranes. Once in the bloodstream they spread throughout the body, causing a broad range of clinical manifestations. The transmission cycle is illustrated in (**Figure 2.1).**

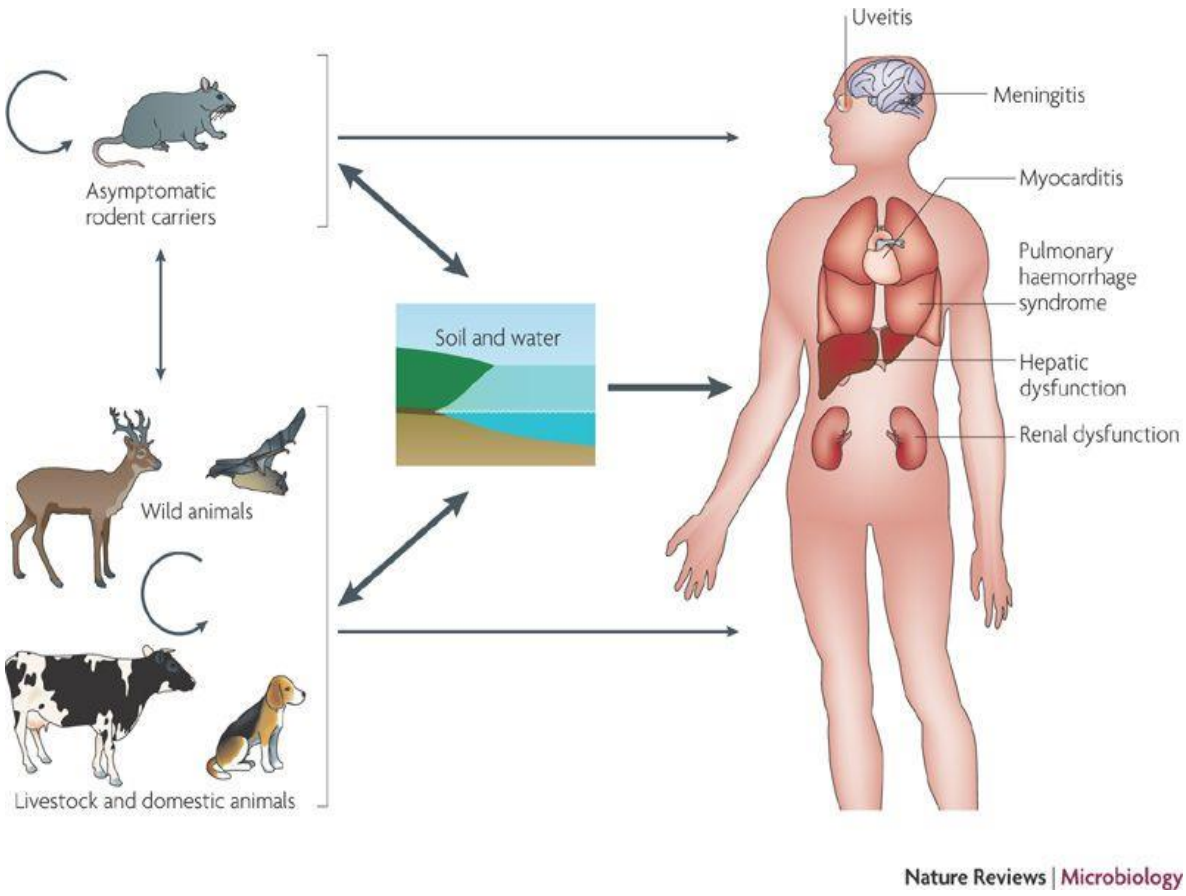

Figure 2.1: Transmission cycle of leptospirosis through the animal reservoir hosts especially rodent, through domestic animals and wildlife, as well as, environmental surface water, soil, to humans where the disease is established, (Ko, et al., 2009).

2.3.3 Pathogenesis

The mechanisms by which leptospires causes disease is not fully understood. Several putative virulence factors have been suggested, but with a few exceptions, their role in pathogenesis remains unclear. The clinical severity of the disease often appears to be out of proportion with the histopathological findings. Immune-mediated disease has been proposed as one factor that influences the severity of the symptoms (Levett, 2001).

There are no major differences in pathogenesis between animals and humans, although in animals, it can result in abortion especially in livestock (Sulliva, 1974). In susceptible hosts, systemic infection can lead to several severe multi-organ manifestations (Martínez-García et al., 2000). During the first phase of infection, symptoms include chills, fever, headache which may be severe and persistent, diarrhoea, or a rash (Mansour-ghanaei et al., 2005). Other symptoms include malaise, myalgia, conjunctivitis, retro-orbital pain and prostration. Symptoms of tender muscles

and lung involvement may appear quite abruptly after a period of incubation of close to 10 days (normally between 4 to 19 days), according to Mohammed and colleauges (2011).

2.3.4 Clinical signs and symptoms

Leptospirosis can easily lead to kidney and liver dysfunction and may be misdiagnosed, especially cases with symptoms that mimic other illnesses for instance, meningitis, mucous membrane, haemorrhage into skin, jaundice, myocarditis and hepatorenal failure.

In non-humans, the clinical signs observed include low milk production, abortion, stillbirth, infertility, death of animals, and a decrease in meat production (Martins et al., 2012; OIE, 2014). Other clinical signs are lethargy/depression, vomiting, fever, weight loss, polyuria/polydipsia, abdominal or lumbar pain, stiffness/arthralgia, renomegaly, diarrhoea, icterus, oculonasal discharge, petechiae, weakness and dyspnoea/cough (OIE, 2014).

In humans, leptospirosis may be confused with malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, melioidosis and others (Faine, 1982). However, there may be no symptoms at all. Human leptospirosis causes 'leptospiraemia' at the early stage and later causes severe multisystem manifestations in form of hepatic dysfunction, jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis, eye vitreous humour and meningoencephalitis (Ko et al., 2009). Humans serve as accidental hosts because they are not reservoirs and cannot spread a high number of leptospires for infection.

2.3.5 Prevention and control

Lesptospirosis control and prevention is a global challenge due to its complex transmission cycle and as a result, needs a multidisciplinary approach (‘‘One Health’’). Pathogenic serovars of *Leptospira* spp. cause significant clinical disease in both animals and humans.

Many commercial vaccines have been used to control human and animal leptospirosis in several countries with limited success, (Dellagostin et al., 2017). This was primarily due to the fact that some of the serovars used were not antigenic enough and because the prevalent serovars involved in infections in particular geographical locations were not included in the panel of serovars in the vaccine (Adesiyun et al., 2006).

Suepaul et al. (2010) in Trinidad and Tobago demonstrated that vaccines produced from locally prevalent serovars (Copenhageni and Mankarso) in the country were more effective in protecting vaccinated hamsters challenged with virulent strains of *Leptospira* than the commercially available vaccines. Therefore, it cannot be over-emphasized that there is a need to institute systems to monitor and ensure that the serovars responsible for clinical cases of leptospirosis be included in the vaccines used in respective countries or geographical locations.

In South Africa, despite the general belief amongst veterinary practitioners that leptospirosis is not a problem, vaccines for canine and some livestock are available in the market for voluntary use to vaccinate animals. The vaccines produced for use in pigs all contain four serovars (Canicola, Icterohaemorrhagiae, Grippotyphosa, and Pomona) of *Leptospira* while three of the four vaccines approved for use in cattle contain the same serovars as those used in pigs (Roach et al., 2010). All the seven vaccines approved for use in dogs contain only two serovars, Canicola and Icterohaemorrhagiae (Klaasen et al., 2003).

Animal vaccination is very important and treatment at an earlier stage is effective as they prevent the shedding of leptospires to healthy animals in a given population. Human vaccines are available in Japan, Russia, China, Vietnam and Cuba (Chen, 1985; Martínez et al., 2004). However, these existing vaccines have limitations such as short duration, lack of cross protection and side effects.

Control of rodents as reservoir host is very important to reduce the burden of leptospirosis. The lack of epidemiological evidence of rodent control in preventing leptospirosis need to be addressed as this will play an important role in the general control of leptospirosis in both animals and non-human populations.

In South Africa, there is no human vaccine available. Vaccines for animals are immunogenic and provide good protection, reduce infection, clinical signs and mortality (Peters et al., 2017). However, these human and animal vaccines have the limitation of offering protection to only specific serovars they contain; therefore they do not provide good cross-protect against serovars not contained in the vaccine (WHO, 2003). The use of antibiotics in the control of leptospirosis is applicable but may not be effective in all regions with the same regimens. In some cases, antibiotic used for leptospirosis mostly include doxycycline for chemoprophylaxis and in less acute cases, penicillin (Trot et al., 2017; WHO, 2003). The existing gaps in the available information on leptospirosis, the infecting *Leptospira*

spp. and the prevalent serovars implicated in humans and animals (livestock, dogs and rodents), make it imperative to conduct a comprehensive investigation of leptospirosis in the country.

The practice of active and passive surveillance using abattoirs should be a priority in the control of leptospirosis, as an occupational and environmental disease, using a multi-disciplinary approach of the ''One Health'' concept.

2.4 Epidemiology

2.4.1 Global Distribution of Leptospirosis

Leptospirosis has been reported to have a worldwide distribution in both developed, middle income and developing countries found in the following region: Europe, Asia, Australia/Oceana, North America, South America/Caribbean and Africa. Endemicity is mainly found in the Caribbean, South East Asia, Oceania and Central and South America (Pappas et al, 2008) and in Africa. The global burden of leptospirosis has been reported to be devastating, (Costa, et al., 2015; Torgerson, et al., 2015) **(Figure 2.2).**

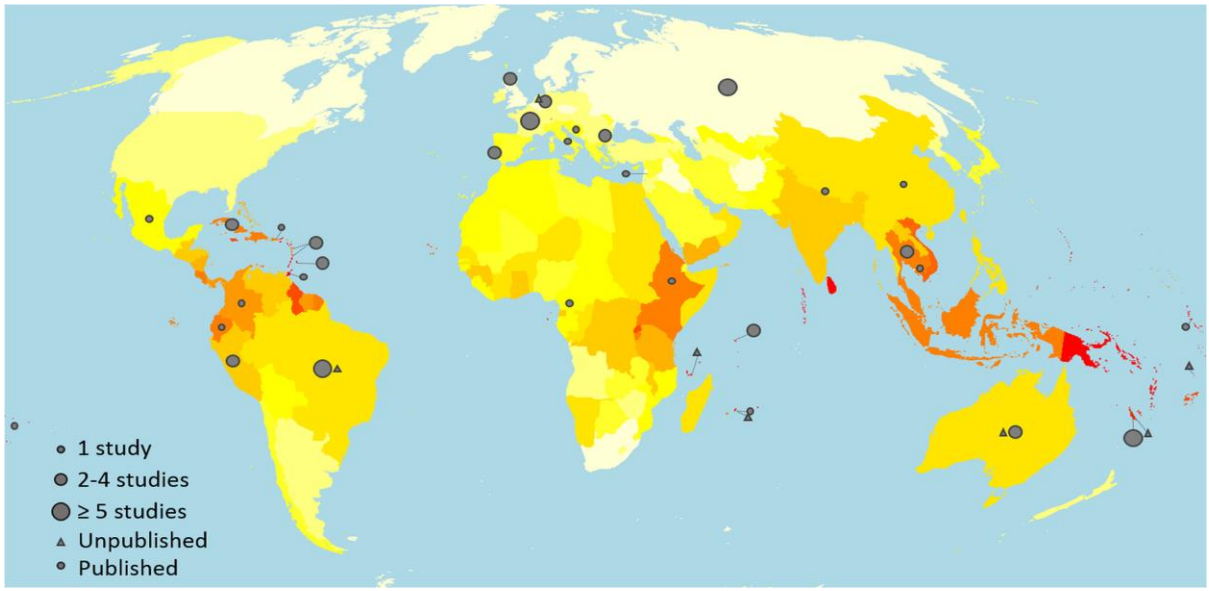

Figure 2.2: Estimated annual morbidity of leptospirosis by country or territory. Annual disease incidence is represented as an exponential colour gradient from white (0–3), yellow (7–10), orange (20–25) to red (over 100), in cases per 100,000 population. Circles and triangles indicate the countries of origin for published and grey literature quality-assured studies, respectively, (Costa et al., 2015). doi:10.1371/journal.pntd.0003898.g002.

Leptospirosis is a worldwide zoonosis that occurs in various epidemiological conditions especially in the tropics. In the tropical region, the incidence of human infection is higher than in temperate regions, but the occurrence of transmission exists in both developed and developing countries (Bharti et al., 2003). Incidence rates are not accurately estimated due to lack of knowledge of the disease, accessibility and sufficient rapid diagnostic application (Bharti et al., 2003).

The reported incidence rates of leptospirosis in human cases have been variable in several countries (Katz et al., 2001) but the disease is undoubtedly a major contributor to morbidity and mortality statistics internationally with economic and public health implications (Costa et al., 2015; Torgerson et al., 2015).

The geographic distributions of the pathogenic *Leptospira* species have been determined to be worldwide, in all continents. Leptospirosis is most common in warm, humid environments. Areas with a high disease incidence in humans include the Caribbean and Latin America, Oceania and parts of Asia. Leptospirosis also appears to be common in Africa, although surveillance is limited. Some *Leptospira* serovars are widely distributed, while others occur in limited areas. The predominant serovars in a host species can vary with the region. Also, of significance is the reported changing patterns of leptospirosis in several countries, emphasizing the need for active surveillance to effectively control the disease. Active surveillance will help in disease prevention and susceptibility to antimicrobial agents (Vinetz, 2001).

Furthermore, it has been established that leptospirosis is a more important zoonosis in developing than developed countries (Levett, 2004). Leptospirosis has been reported in developed countries where it is attributed to people living in slums (Ko et al., 1999; Vinetz & Diego, 1996). It is a recreational water activity disease, an occupational disease of abattoir workers and is associated with both crop and livestock farming activities. Also, in developing countries, the spread of the disease is facilitated by socioeconomic factors such as increased urbanization, infestation of rodents, poor sanitation and transmission through water contamination with leptospires from the urine of rodents or dogs (Vinetz, 2001; Ko et al., 1999).

Leptospirosis affects many wild and domestic animals and humans (El Jalii & Bahaman, 2004; Ko et al., 1999; Sulliva, 1974). These infections have resulted in global economic losses to both government and individuals stake holders.

2.4.2 Animal and Human leptospirosis in Europe

In Europe, a developed region, outbreaks of leptospirosis in humans and animals have been attributed to recreational activities such as swimming and to urbanization (Rodina et al.,2005). In the Republic of Greece, farm animals were tested for antibodies to *Leptospira* spp. with an overall seroprevalence of 11.8% (180/1527) detected as reported by Burriel & Woodward (2003). From their study, the seroprevalence according to animal species was as follows: cattle, 12.6% (35/277); pigs 17.8% (92/516); sheep, 5.7% (16/282); goats, 16.2% (32/198) and dogs 11.4% (29/254). Titres of 1:100 or higher were reported as positive using MAT, with serovars Bratislava, Copenhageni and Australis as the highest, followed by serovar Pomona, in cattle and goats.

In France, a 20-year seroprevalence study on animal leptospirosis was conducted where an overall seroprevalence of 22.3% (9,727/42,982) was reported (André-fontaine, 2016). The serogroups with the highest seroprevalence in cattle, using MAT at a cut-off titre of 1:100, were Sejroe, 34.0% (3,307/9727); Grippotyphosa, 29.9% (2,908/9,727) while those with low seroprevalences were Icterohaemorrhagiae, 17.3% (1,683/9,727) and Australis, 9.5% (942/9727). In pigs, an overall prevalence of 26.5% (11,265/42479) was found with serogroups Icterohaemorrhagiae, 50.3% (7, 872/15,651) and Australis, 42.6% (6,667/15,61) having the highest seroprevalence while Autumnalis, 18.1% (2,833/15, 651), Sejroe, 13.9% (2,175/15,651), Grippotyphosa, 11.0% (1,722/15,651) and Pomona, 5.9 % (923/15, 651) had lower seroprevalences. The disease was also reported in other animal species such as horses and dogs (André-fontaine, 2016).

In Germany, there was an increase in the prevalence of leptospirosis based on an epidemiological review of the patterns of the disease over a 40-year period which was attributed to an increase in the rat population and leptospirosis in dogs (Pappas et al 2008).

2.4.3 Animal and human leptospirosis in Asia

In Asia, there is a major problem of under-reporting of leptospirosis in the continent primarily because of the unavailability of data on the exact incidences of leptospirosis in many Asian countries (Pappas et al., 2008).

In India, there are no official data of leptospirosis from most of the provinces. Environmental factors, poor sanitary measures and over-crowded living areas are vital factors responsible for the high incidence rates of the disease. In countries near India, such as Bangladesh and Nepal (Larocque et

al., 2005), the disease was recognised as a differential diagnosis for dengue and febrile illnesses. Approximately 2,000 cases in India with case fatality rates ranging from 0.7 to 13.9% were reported, while in Thailand, up to 60 cases were documented with a fatality rate of 5% (Vinetz, 2001).

In Seychelles islands of India, serogroups Icterohaemorrhagiae and Hursbridge were reported to be the most prevalent (Pappas et al., 2008). It is evident that in countries (Pakistan, Afghanistan and Iraq) where there has been civil unrests such as war, data for leptospirosis is lacking (Pappas et al., 2008). There are limited leptospirosis data from Iran, although the disease is known to be present. A study reported that in 74 patients tested for a period of four months in (1999) in the northern region of the country, a high male seroprevalence of leptospirosis, 70.3% (52/74) using Immunofluorescence antibody (IFA) methods, was documented (Mansour-ghanaei et al., 2005).

Leptospirosis was reported in Thailand following the 21st outbreak after flood between 1996–2003, with a 90% cases reported in the Northeast region with fatality rate of 4.4%; these was associated with farmers between the age of 15 to 45 years old (Tangkanakul et al., 2005). In Vietnam, the seroprevalence of leptospirosis was reported to range from 12.8% (123/961) to 18.8% (263/1400) in people, including children with serogroup Bataviae being the most prevalent (Thai et al., 2006; Vanl et al., 1998). Malaysia has been reported to have seroprevalence of human leptospirosis of between 12.6% to 28% (El Jalii and Bahaman, 2004). In China, in 1999, over 500, 000 cases of leptospirosis were documented, and the case fatality rates ranged from 1 to 7.9% (Vinetz, 2001).

2.4.4 Animal and Human leptospirosis in Oceania

In Queensland, Australia, leptospirosis has been reported to be linked to livestock and farming of bananas and sugar cane industries with the peak in 1999 with 28 million incidence cases per year. This was associated with high rainfall and an increase in rodent population (Pappas et al., 2008). Also, in New Zealand, a serological survey was carried out between 2009 to 2010 on farm animals. The reported seroprevalence serovars were, 43.6 % for serovar Hardjo and 14.1% for serovar Pomona out of 3, 339 sheep while in 1,886 beef cattle, 45.6% for Hardjo and 19.6% for Pomona and 26.3% for serovars Hardjo, 8.8% for Pomona from 1, 870 deer (Dreyfus et al., 2018).

Benschop and colleagues (2009) during a sero-survey study of leptospirosis among workers in slaughterhouses reported a prevalence of 13.1% (19/145) in males and 4.1% (4/97) in females. In that study, 13.9% of the samples were positive for antibodies to *Leptospira* interrogans serovar

Pomona and *Leptospira borgpetersenii* serovar Hardjo, had odds ratios of 3.51 for exposure to leptospirosis in males more likely to be exposed to leptospirosis compared to females. In the same study, it was found that the median age for positive workers was 54 years (47-59 years) and for negative workers was 48 years (35-56 years). It was also reported that there had been epidemiological changing patterns of the disease, resulting from the appearance of a new serovar of *Leptospira borgpetersenii,* serovar Ballum which was associated with water activities (Thornley et al., 2002). In an update of human leptospirosis in New Zealand between 2010 and 2015, for the 442 cases investigated, the incidence rate of leptospirosis was reported to be 1.9% (2010); 1.6% (2011); 2.5% (2012); 1.3% (2013); 1.2% (2014) and 1.4% (2015) per 100,000 people, respectively (El-Tras et al., 2018). In the same update, it was reported that less than 10 serovars of *Leptospira* spp. were circulating in New Zealand out of the 230 leptospiral serovars species that have been identified worldwide (Levett, 2001). El-Tras et al. (2018), also they, reported that there were 110 human cases of *Leptospira borgpetersenii* serovar Hardjo, 85 cases of *Leptospira borgpetersenii* serovar Ballum, 30 cases of *Leptospira borgpetersenii*sv Tarassovi, 69 cases of *Leptospira interrogans* serovar Pomona, 8 cases of *Leptospira* interrogans serovar Canicola, 12 cases of *Leptospira interrogans* serovar Copenhageni, 2 cases of *Leptospira interrogans* serogroup Australis and finally 2 cases of *Leptospira kirschneri* serogroup Grippotyphosa were detected in patients between 2010 and 2015. There was one case of co-infection with Hardjo and Pomona.

2.4.5 Animal and Human leptospirosis in North America

In Mexico, a small increase was noticed in the annual cases in recent years from the Mexican Epidemiological data information available at http:/www.dgepi.salud.gob.mx/infoepi/index.htm accessed June 2007. In Mexico, a seroprevalence survey was conducted (Leon et al., 2008) in dairy cattle of the Tuluca Valley State using MAT. There was an overall seroprevalence of 10.3% (43/416) with serovar Hardjo (2.4%) as the most prevalent, followed by Canicola (1.6%). Titres detected in seropositive cattle were up to 1:1600.

Human leptospirosis has been reported in Yucatan, Mexico with a seroprevalence of 14.3% (57/400) using the MAT with no statistically significant differences ($P>0.05$) detected in the seroprevalence according to age group (Vado-solís et al., 2002). However, the difference in the seroprevalence by gender was statistically significant ($P<0.05$), with a seroprevalence of 11.6% (31/226) and 19.4% (26/134) in females and males, respectively.

In Hawaii, leptospirosis has been reported to have the highest annual incidence in the United State estimated at 1.29 million per 100, 000 population (Katz et al., 2002), with more cases in males.

2.4.6 Animal and Human leptospirosis in South America and the Caribbean

In Brazil, according to Martins et al. (2012), the seroprevalence of leptospirosis in small ruminants, namely, goats and sheep was 25.9% (145/560) and 47.4% (265/569) respectively. The predominant serovar was Hardjo which accounted for approximately 50% of all seropositive goats and sheep, followed by serovars Icterohaemorrhagiae, Grippotyphosa and Pomona. Additionally, Martin et al. (2013) conducted a study on a range of animal species animal and reported the following seroprevalences of leptospirosis and the predominant serovars detected comprised, rats (*Rattus norvegicus*), 36.2 % (17/47) with serovar Icterohaemorrhagiae detected; dogs, 73.3% (88/120) with serovar Icterohaemorrhagiae; cattle, 38.3% (335/875) with Sejroe; horses, 39.6% (275/695) with Icterohaemorrhagiae and Australis; goats, 14.9% (200/1,343) with Sejroe; sheep, (47.4% (146/308) with Sejroe; pigs 66.1% (232/351) with Icterohaemorrhagiae; wild mammals (except felines), 37.7% (29/77) with Icterohaemorrhagiae; wild felines, 13.3% (4/30) with Icterohaemorrhagiae and Pomona and finally golden lion tamarins, 15.1% (11/73) with Icterohaemorrhagiae.

Human leptospirosis has been increasing in Brazil due to urbanization. During an outbreak of leptospirosis in Salvador, there was an incidence of 12.5 cases per 100 000 with 80% (262/326) hospitalization of cases during the outbreak (Ko and colleagues, 1999). Furthermore, Vinetz (2001) reported that 28,360 cases had a case fatality rate of 0.8%. In a review of human leptospirosis carried out by Oliveira et al. (2017) in Minas Geraise state, Brazil, reported a seroprevalence of 50.1% (301/597) for leptospirosis antibodies in humans using the MAT, and of these results, 45.7% (273/597) had titres greater than 1:800, with 85.1% (508/597) in male patients while 39.4 % (235/597) of the patients were aged between 20 to 39 years. The serovars detected in seropositive patients were Icterohaemorrhagiae, Andamana, Patoc, Tarassovi, Copenhageni, Hardjo, and Australis. The serovars found with titres greater than 1:800 were to Icterohaemorrhagiae, Copenhageni, Amdamana, Tarassovi, Grippotyphosa and Canicola. In another study in Brazil (Daher et al., 2010), 79.1% (159/201) of positive cases were male with major clinical signs and symptoms being fever (96.5%), jaundice (94.5%), myalgia (92.5%), headache (74.6%), vomiting (71.6%), dehydration (63.5%), haemorrhagic cases (35.8%), active kidney injury (87%), platelet counts less than 100,000/mm^3 (74.3%), haematuria (42.9%) and death occurred in 31 cases (15.4%).

Leptospirosis has been documented in Argentina although data on the disease burden, the characteristics of leptospires and epidemiologic information on the disease are scarce (Vanasco et al., 2008). In their study on confirmed cases of leptospirosis in humans, the overall seroprevalence of leptospirosis was 22.4% (182/812) and antibodies were detected to *L. interrogans* serogroups Icterohaemorrhagiae, 31% (57/182) and Pomona, 15% (27/182), *L. borgpetersenii* serogroup Ballum, 14% (25/182), *L. interrogans* serogroup Canicola, 10% (18/182), *L. borgpetersenii* serogroup Sejroe 4% (8/182) and Tarassovi 2%. Two or more individuals out of 182 confirmed cases were infected with more than one serogroups (43/182) (Vanasco et al., 2008). Of the risk factors associated with leptospirosis in Argentina, 70% (74/182) were rural-based occupational risks, such as agricultural work (n = 42), dairy farming (n = 13), fishing (n = 9), abattoir work (n = 5), pig farming (n = 3), forestry (n = 2), agriculture (n = 1) and veterinary health care (n=1). Leptospires were confirmed from human clinical samples using PCR where 68.2% (58/85) were positive for the I6S rRNA, and serotyping revealed the predominant serovar to be Canicola (63%) while multi-locus sequence typing (MLST) identified the leptospires DNA as *L. interrogans* (Chiani et al., 2016).

In the Caribbean region, leptospirosis was reported to be endemic in Barbados, Jamaica, and Trinidad and Tobago with over 500 cases confirmed resulting to in a cumulative annual incidence of 100 per million population in 2000 according to the Caribbean Epidemiological Centre (CAREC) sourced at http://www.carec.org/annrep00/index.html (Pappas et al., 2008). Adesiyun and co-workers (2006) reported canine leptospirosis in Trinidad with a seroprevalence of 14.6% (61/419), with mixed infections detected in 5.5% (23/419); in suspected cases of clinical leptospirosis, 48.0 % (24/50) were confirmed seropositive using the MAT.

Also, in Trinidad and Tobago, an overall seroprevalence of 12.9% (154/1,192) was reported in livestock sampled where, 126 (21.5%) of 590 cattle tested were seropositive for leptospirosis. The serovars identified to be predominantly circulating in cattle at the time of study were Icterohaemorrhagiae (9.3%), Sejroe (4.1%), Ballum (4.1%) and Autumnalis (1.9%) (Suepaul et al., 2011). In sheep, the seroprevalence was 5.0% (11/222) with serogroups Autumnalis (2.7%) and Icterohaemorrhagiae (2.3%) detected. In goats, the seroprevalence was 3.3% (6/180) with the detection of serogroups Icterohaemorrhagiae (2.3%), Copenhageni (1.7%), Mankarso (1.1%) and Icterohaemorrhagiae (0.6%). In pigs sampled, the seroprevalence of leptospirosis was 5.0% (10/200) with the infecting serovars being Icterohaemorrhagiae (2.5%), Australis (2%) and Ballum (0.5%).

A comparison of the seroprevalence of leptospirosis in the livestock sampled by age and sex did not reveal a statistically significant differences (P>0.0) except for cattle where age had a statistically significant effect on the seroprevalence of leptospirosis. Furthermore, in Trinidad and Tobago, Suepaul et al. (2010) reported that serovar Copenhageni was the predominant serovar in dogs.

In a review of cases of human leptospirosis in the Caribbean, the seroprevalence reported in selected islands were Barbados (6.0%), Jamaica (47.0%), Suriname (19.0 %), and Trinidad and Tobago (19.0%) (Peters et al., 2017). Another study revealed that out of 3,455 human sera tested for leptospirosis, 13.1% (452) were seropositive for IgM antibodies to leptospirosis using ELISA, with significant differences among countries, gender and age groups (P<0.05) (Adesiyun and others, 2011). The frequency of detection of leptospirosis (23.1%) was significantly higher in the age groups 1-20 years and 31-40 years combined compared with other age groups. There was a significant difference in seropositivity between male patients (72.1%) and female patients (19.7%) (P< 0.05). In the same study, the MAT was used to test 100 ELISA positive sera and 98 (98%) were seropositive. The serogroups with high seropositivity rate were Copenhageni (70%), Icterohaemorrhagiae (67%), and Mankarso (29%).

2.4.7 Animal and Human leptospirosis in Africa

In Africa, a continent consisting of lower middle income communities (LMIC) and developing countries, there are limited data on leptospirosis in humans and animals (Mwachui et al., 2015; WHO, 2011). The distribution of leptospirosis varies across regions based on different climatic and environmental factors (de Vries et al., 2014), including highly populated settlements and lack of good sanitary practices.

In Nigeria, located in western Africa, the seroprevalence of leptospirosis by IgM ELISA kit in cattle was determined as 3.5% (5/142) with serovar Hardjo, and the infection rate in male was, 57.04% (81/142) and in female, 42.96 % (61/142) were reported (Ngbede *et al.,* 2012). In another study, *Leptospira* antibodies were reported in abattoir workers, 87.8% (231/263) using MAT and 81.0% (213/263) using IgM ELISA kit. The circulating serovars in abattoir workers in the country were Grippotyphosa (8.7%), Australis (6.9%), Hardjo (5.2%), Tarassovi (1.3%), Icterohaemorrhagiae (18.8%) (Abiayi et al., 2015).

In Cameroun, a model of classical statistical and multi-level prevalence study was carried out on leptospirosis in cattle which estimated that an unbiased seropositivity for leptospirosis at herd level was 95% and within herds was 35% (Scolamacchia et al., 2010).

In Burkina Faso, human leptospirosis was determined and confirmed among patients who presented with febrile jaundice at medical facilities. The combined prevalence using serological assays (IgM ELISA and MAT) and PCR methods was 3.5% (27/781), The seroprevalence of leptospirosis by MAT was 2.9% (23/781) with the detection of serovars Australis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona, and Sejroe. The seroprevalence using a commercial IgM ELISA kit was 5.8% (45/781) according to Zida et al. (2018). In addition, the study detected *LipL32* outer membrane gene for pathogenic leptospires in 0.5% (4/781) of patients.

In Eastern Africa, leptospirosis has been reported in Kenya, where the seroprevalence of human leptospirosis was found to be 13.4% (41/737) among abattoir workers using the commercial Panbio *Leptospira* IgM ELISA kit (Cook et al., 2017). The risk factors determined in their study included abattoir workers with wounds (OR 3.1; 95% CI 1.5 to 6.1), those who were smokers (OR 1.8; 95% CI 1.1 to 2.9); workers eating in between working hours (OR 2.1; 95% CI 1.2 to 3.6); workers cleaning offal (OR 5.1; 95% CI 1.8 to 15.0); and workers who used boreholes as a personal source of water (OR 2.3; 95% CI 1.1 to 4.7). A study was also conducted in Kenya on rodent kidneys using real time quantitative PCR, where 18.3% (41/224) of samples were positive for the presence of *L. interrogans* and *L. kircheneri* (Halliday et al., 2013).

In Tanzania, the overall seroprevalence of leptospirosis was reported for hospitalized febrile patients using the MAT, 33.3% (277/832) of the patient had evidence of leptospiral antibodies (Biggs et al., 2011). The predominant serovars detected in that study were Mini and Australis.

Data on leptospirosis in Uganda are not readily available for review. Milan and co-workers (2013) demonstrated a seroprevalence of 26.7% in dogs sampled around three National Parks in the country. In addition, a cross sectional study conducted by Alinaitwe et al., (2020) in cattle reported a seroprevalence using the MAT as, 27.8% (139/500). The most prevalent serovars reported in their study were serovars Tarassovi, (11.6%); Sejroe, (7.8%), and Australis, (5.2%).

In Ethiopia, a pilot study in Wanji reported a seroprevalence of 47.5 (28/59) for humans leptospirosis with a higher seroprevalence detected in males (30.5%) than females (16.9%) (Yimer et al., 2004).

In Southern Africa, a study in Zimbabwe conducted on volunteer subjects in farms where domestic rodents were trapped, reported a seroprevalence of 82.2% for leptospirosis in 182 farm workers and their family members sampled in Harare. A seroprevalence of 62.5% was detected in rodents on the same farms studied using the MAT (Dalu & Feresu, 1997). The predominant serovars circulating in the human and rodent infections were serovars Icterohaemorrhagiae, Pyrogenes and Grippotyphosa. The authors concluded that leptospirosis is a common occupational disease transmitted by rodents to human in the country.

Leptospirosis has been reported in other Southern African countries, such as in Angola where captured rodents were tested for leptospirosis. The species involved were as follows: *Rattus rattus*, 40.5% (15/37); *Rattus norvegicus*, 24.3% (9/37) and *Mus musculus* 35.2% (13/37). The use of DNA to detect leptospirosis in the study revealed that 21.6% (7/37) were positive for *Leptospira* DNA while bacteriological culture identified 10.8% (4/37) to be positive for *Leptospira* spp. which were confirmed by sequencing and analysis to be *L. interrogans* and *L. borgpetersenii* (Fortes-gabriel et al., 2016).

In Lusaka, Zambia, a seroprevalence of 6.6% (8/121) was reported for leptospirosis in pigs farms (Stafford et al., 1992). In Botswana, a prevalence of leptospirosis by PCR was reported to be 41.5 % (17/41) of renal carriage of *L. interrogan* in mongooses (Jobbins et al., 2014).

In South Africa, there is a dearth of information on human and animal leptospirosis, with few published reports (Taylor et al., 2008; Saif, 2012).

2.4.8 Diagnosis of Leptospirosis

For successful diagnosis of *Leptospira* species, it is important to know the type of samples, type of test, how and when to conduct the test for proper diagnostic results (Picardeau, 2013). Diagnosis of leptospirosis is generally very cumbersome and laborious, especially the conventional methods. However, clinical diagnosis of leptospirosis is inadequate for complete diagnosis of leptospirosis.

Several methods are used for the confirmatory diagnosis of leptospirosis. It is usually confirmed by MAT (MAT) (OIE, 2014). It is generally believed that leptospirosis is an under-reported disease in human and animal populations, as confirmed with the advent of more sensitive and specific diagnostic techniques, particularly the polymerase chain reaction (PCR) and other molecular methods (WHO, 2003).

The diagnostic methods of leptospirosis are therefore highlighted below:

2.4.8.1 Diagnosis of leptospirosis by clinical signs and symptoms

Clinical signs and symptoms of leptospirosis are inadequate to draw a conclusion of the disease in either animals or humans, due to the fact that in human cases, the disease is a febrile-illness similar to malaria, viral hepatitis, influenza, dengue fever and typhoid fever (Faine, 1982). Notable clinical signs and symptoms in animals include lethargy/depression, vomiting, fever, weight loss, polyuria/polydipsia, abdominal or lumbar pain, stiffness/arthralgia, renomegaly, diarrhoea, icterus, oculonasal discharge, petechiae, weakness and dyspnoea/cough (Adler & de la Peña Moctezuma, 2010). In humans the signs and symptoms consist of hepatic dysfunction, jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis, eye vitreous humour and meningoencephalitis (Ko et al., 2009). Therefore, there is need for differential diagnosis and application of other diagnostics methods such as the conventional bacteriological culture, MAT and PCR for confirmation (Picardeau, 2013).

2.4.8.2 Pathological findings

Pathogenic *Leptospira* spp. produce different clinical manifestations in the infected host ranging from subclinical infection to undifferentiated febrile illness, although the pathogenesis of leptospirosis is not yet fully understood (Bharti et al., 2003). In humans and animals, the major pathological changes are however the same. These pathological manifestations according to Bharti and colleagues (2003) include pulmonary lethal haemorrhage, conjunctival epithelium and renal failure. It has been reported that many factors such as the 4,768 predicted virulent genes, motility, and chemotoxin proteins (Ren et al., 2003; Lux et al., 2000; Faine, 1982) may contribute to acute and chronic infection process in humans and reservoir host (Bharti et al., 2003).

2.4.8.3 Bacteriological culture

Bacteriological culture of leptospires is a definitive method for the diagnosis of *Leptospira* spp. Leptospires can be isolated from human blood or urine during the first 7 days of an acute case of leptospirosis (Picardeau, 2013). From animals, leptospires can be isolated from the kidney, blood and urine of an infected animal within the first 7 days. This method involves the use of two common types of media (semi-solid and liquid), such as the Ellinghausen McCullough-Johnson Harris (EMJH) media as a conventional method (Ellinghausen, 1973). Some common antimicrobial agents such as neomycin sulphate, 5-flourouracil at 200 ug/ul, vancomycin, polymycin B and rifampicin are added to the media to prevent growth of contaminants. Some media contain 1% bovine serum to facilitate the growth of leptospires (Ellis et al., 1983; Ellinghausen, 1973). The culture methods have to be aseptically handled in a Biosafety Class II working cabinet and incubated at an optimum temperature of 28°C to 30°C for observation weekly for two to three months (Picardeau, 2013).

Contaminated cultures are subjected to filtration through 0.45 µm and/or 0.22 µm filters. Newly isolated leptospires always look shorter when observed under the dark field microscope (Ellis et al 1983). Some of the disadvantages of bacteriological culture method for leptospires are that it is prone to contamination, takes a long time, cumbersome, very tasking and needs the diagnostic capability of experienced technical personnel. Therefore, the isolation of leptospires is not easily successful in large numbers, for instance, in France, only 5 strains were cultured in a year (Picardeau, 2013). Generally, there is a limitation in acquiring data on leptospirosis based on isolation to determine the serovars circulating in animals and humans in a region, thus posing a major setback.

2.4.8.4 Serological methods

2.4.8.4.1 Microscopic Agglutination Test (MAT)

The Microscopic agglutination test (MAT), also known as the Martin and Petit test, was developed many decades ago, at the Pasteur Institute in France by Martin & Petit (1918). The MAT has been the gold standard for serological diagnosis of leptospirosis (WHO, 2003), with the aid of the dark field microscope. This test, as a conventional method, has its disadvantages which include false-positive and false-negative results, leading to reduced accuracy and results do not always correlate with the serogroup after identifying the strains isolated (Picardeau, 2013), the sensitivity of MAT is low in the early phase (first few days of the disease) and is cumbersome and time consuming to maintain the live antigens (Picardeau, 2013; WHO, 2003).

Maintaining of antigen strains is required when using the MAT and the MAT being serogroup-specific, cannot identify the leptospires detected to serovar level., requires paired samples after two weeks of the first sample collected, requires expertise to conduct, analyse and interpret the results (Picardeau, 2013; Smythe et al., 2009).

2.4.8.4.2 Enzyme-linked immunosorbent assay (ELISA) IgM

There are several IgM enzyme-linked immunosorbent assays (ELISAs) used for the diagnosis of leptospirosis globally. The principle of the ELISA is the detection of antibodies produced against *Leptospira* spp. using wells coated with genus-specific antigens (Picardeau, 2013). The PanBio ELISA kit, for example, is used as one of the commercially available IgM ELISAs. Its sensitivity has been reported to vary from region to region based on the populations studied and the exposure to different categories (pathogenic, intermediate or saprophytic) of leptospires (Panwala et al., 2011). In Thailand, the sensitivity and specificity of PanBio ELISA was determined to be 76% and 82% respectively (Desakorn et al., 2012), this sensitivity and specificity is low in the early (first few days) phase of the disease.

The IgM ELISAs have been confirmed to detect leptospiral antibodies earlier than MAT within the first 3 to 5 days of disease onset (Picardeau, 2013; Levett, 2001). The detection of IgM requires paired samples at an interval of two weeks after the first test and has the limitation of not detecting leptospiral antibodies to serogroup, unlike MAT. Therefore, there is always the need for serum samples that test positive for IgM antibodies to be subjected to confirmation by MAT, culture and/or PCR (Picardeau, 2013).

2.4.8.4.3 Diagnosis of leptospirosis using other serological techniques:

There are many serological tests used in the diagnosis of leptospirosis in addition to earlier techniques highlighted above (2.3.8.4.2 and 2.3.8.4.3). These different methods use the same principles of antibodies-antigen reaction.

Some of these serological techniques are Complement fixation test (CFT), Hemagglutination test, Macro-agglutination test, Latex bead agglutination test and Indirect immunofluorescence (Levett, 2001), other rapid diagnostic kits include Leptocheck-WB, LEPTO Dipstick and IgM dot ELISA dipstick test (DST). Although these various techniques are being used, it is known that they have low specificity and sensitivity in most cases (Picardeau, 2013).

2.4.8.5 Polymerase Chain Reaction (PCR)

Globally, it has been documented that cases of leptospirosis are under-diagnosed and therefore under-reported (WHO 2003). Modern methods for the diagnosis of leptospirosis have been developed based on the genes (*rrs, flab, omL1, SecY,* and many more of the house keeping genes) of interest for the targeted *Leptospira* species (pathogenic, intermediate and/or saprophytic or non-pathogenic). These methods include the conventional PCR that has been used for the diagnosis of leptospirosis for decades (Victoria et al., 2008; Branger et al., 2005; Kawabata et al., 2001; Merien et al., 1992). The efficient tool for the early diagnosis of leptospirosis in the first 10 days is the PCR, mostly when there is confusion of the disease expression clinically (Merien et al., 1995).

The most sensitive and specific diagnostic test currently used is the quantitative or real-time olymerase chain reaction (qPCR), (Espy et al., 2006; Slack et al., 2006; Waggoner, 2016). The qPCR is fast, reduces chances of contamination and is more accurate, especially when applied with the probe technology (Espy et al., 2006; Slack et al., 2006). Different types of real-time PCR are used in the diagnosis of leptospirosis including the SYBR green using the hybridization probes chemistry and the TaqMan assay using the hydrolysis probes chemistry (Levett et al., 2018; Schneider et al., 2018; Wunder et al., 2016; Stoddard et al.,2009; Espy et al., 2006; Slack et al., 2006; Merien et al., 2005; Palaniappan et al., 2005; Slack et al., 2005; Levett et al., 2005; Smythe et al., 2002). The qPCR targets important genes, such as the *ligA, rrs, gryB,* conserved hypothetical proteins and the *LipL32* gene region of the outer lipoprotein membrane that is contained in all pathogenic *Leptospira* spp. Although, a recent 16S PCR developed by two groups revealed that real-time RT-PCRs targeting the 16S rrs gene can improve detection of *Leptospira* spp. (Waggoner et al., 2016), in addition to the existing PCR methods for the diagnosis of leptospirosis.

The *LipL32* genes have been widely used in the screening of the pathogenic *Leptospira* spp. globally. PCR can reveal a positive case of pathogenic leptospires in a sample, provided the target is specific for the pathogenic *Leptospira* species. However, PCR does not permit direct identification to serovar level (Picardeau, 2013). It is therefore important to use PCR diagnostic methods, especially the TaqMan hydrolysis probe assays to target the pathogenic *Leptospira* spp., that cause serious veterinary and public health problem globally.

2.4.8.5.1 The role of *SecY* gene of the pathogenic Leptospira species

The *SecY* gene region is one of the house-keeping genes used in multi-locus typing and it is an important gene found in all the pathogenic *Leptospira* spp. The *SecY* gene encoding preprotein translocase for leptospires contains genes for ribosomal proteins that are located in the *S10-spc-alpha* locus (Victoria et al., 2008; Zuerner et al., 2000).

The *SecY* gene possesses two (alternating conserved and variable regions) which are major characteristics within its gene. This makes it appropriate to draw a logical conclusion on the primers that can produce PCR products with adequate sequence heterogeneity to allow the interpretation of phylogeny for *Leptospira* species (Victoria et al., 2008).

Furthermore, the *SecY* gene has been used for the identification of pathogenic leptospires and its detection has been shown to exhibit a very high taxonomic discriminatory level of differentiation according to Victoria and co-workers (2008). The primer set, *SecYII* (5'-GAA TTT CTC TTT TGA TCT TCG-3') and *SecYIV* (5'-GAG TTA GAG CTC AAA TCT AAG-3') that amplify *SecY* sequences from all the pathogenic *Leptospira* strains in conjunction with primer pair G1G2 is used (Zuerner al., 2000).

The PCR products analysed by sequencing can allow species identification and in some cases, the serovar (Cerqueira et al., 2010; Perez & Goarant, 2010)**,** where sequences generated from the genes *SecY, lipL41, rpoB and ligB*, were used to discriminate *Leptospira* isolates and a phylogenetic tree was constructed using the neighbour joining and Maximum likelihood methods (Cerqueira et al., 2010). These make the *SecY* partial gene region a unique and important gene for the genetic identification of pathogenic *Leptospira* spp.

2.4.9 The role of abattoirs

Abattoirs are facilities used for the slaughtering of animals, however, these abattoirs can be used for active and passive surveillance of zoonotic diseases, for example, leptospirosis, brucellosis, tuberculosis amongst many other zoonoses. Abattoirs can also serve as major sources of important information on animal diseases and an environment for the transmission of zoonotic diseases. Transmission may occur through the contamination of carcasses or meat, exposure of abattoir workers to fluids from slaughtered animals and therefore may constitute major public health and food safety problems (Fasanmi et al., 2017). Therefore, abattoirs play vital roles in the transmission of leptospirosis considering that leptospirosis is one of the most widespread zoonotic diseases,

which is of global medical and veterinary importance. It is also a re-emerging infectious disease (Mwachuii et al., 2015).

2.4.10 Leptospirosis as a ‘‘One Health’’ disease

A German physician and pathologist, Dr. Rudolf Virchow, (1821-1902), formally agreed to the connection between the health of humans and animals, gave rise to the concept of the ‘‘One Health’’ (Washington DC 2005). The term ‘‘One Health’’ describes a global strategy between various scientific disciplines at local, national and global levels in search of better health for humans, animals and the environment for mutual benefit (Gibbs et al., 2009; Klement et al., 2009). The concept has no boundaries as a multi-disciplinary approach to improve public health globally (Klement et al., 2009). In addition, the ‘‘One Health’’ needs Public Health awareness and regular capacity building of Researchers and Scientist on Public health professionals to better cope with zoonotic diseases such as leptospirosis amongst others.

Leptospirosis is not only a zoonotic pathogen but also an occupational and environmental disease that spans all animals and human niches. Leptospirosis is a disease that fits the concept of ‘‘One Health’’. It is a disease of global concern transmitted from animals to humans through the contaminated environment. For example, in the agricultural industry in developed countries, leptospirosis remains a major concern (Vinetz, 2001). The disease also causes a high livestock loss (Kingscote, 1985) and a public health problem to humans globally (Costa et al., 2015). Leptospirosis has been reported as a recreational disease in developed countries (Vinetz & Diego, 1996) yet a neglected disease. Furthermore, mechanisms of pathogenic *L. interrogans* for causing disease and transmission in cities sporadically without the presence of occupational and recreational exposure is not known Therefore, there is a need for a multi-disciplinary approach in the studies of this important zoonotic disease using the ‘‘One Health’’ approach for a better understanding, control and prevention of leptospirosis worldwide.

2.5 Leptospirosis in South Africa

2.5.1 Distribution of animal leptospirosis in South Africa:

A global disease known as leptospirosis is an important zoonotic disease of both animals and human in all the continents including Africa, where South Africa is located. There are limited data on leptospirosis in South Africa. Although, the disease has been reported in South African livestock using serological methods (MAT) (Potts et al., 2005), it has also been reported in mixed farming

(Gummow et al., 1999). The transmission of leptospirosis occurs from animal host reservoirs to humans through interaction in the environment.

2.5.2 Leptospirosis in cattle

Cattle serve as a good source of food and as well as income in South Africa. Therefore, the need to prevent and control zoonotic diseases such as leptospirosis is very vital. Clinical cases of leptospirosis have been reported in livestock including cattle (Gummow et al., 1999). Variable seroprevalences of leptospirosis due to several serovars ranging from 3% to 52.4% have been documented across the country. The frequently detected serovars were Tarassovi, Bratislava, Hardjo, Pomona, Canicola and Icterohaemorrhagiae (Myburgh et al., 1990; Hunter et al., 1988; Kaschula et al., 1978).

Reports on leptospirosis in South Africa were mostly on outbreaks and/or suspected cases. The seroprevalence of leptospirosis in cattle in Kwazulu Natal province was determined to be 19.4% (392/2021), with the predominant serovars being Pomona, followed by Tarassovi, Bratislava, Hardjo Canicola and Icterrohaemorrhagiae (Hesterberg et al., 2009). During an outbreak of leptospirosis in cattle, 52% (89/170) of the animals were seropositive and serovar Pomona was determined to be responsible (Gummow et al., 1999). Culture of samples obtained from clinical cases of leptospirosis during the outbreak yielded an isolation rate of 25% (3/25) with all isolates being serovar Pomona (Gummow et al., 1999).

2.5.3 Leptospirosis in pigs

Data on leptospirosis in pigs are scarce and the little available information is based on serological studies. In a national survey of slaughtered pigs in abattoirs, Potts et al. (1995) reported a seroprevalence of 22% with the circulating serovars being Icterrohaemaorrhagiae, 12.6% (365/5041), Hardjo, 12.1% (609/5041) and Bratislava, 7.5% (378/5041).

In pigs that aborted, the seroprevalence of leptospirosis was 17% with the most prevalent serovars being Pomona, Harjo, Bratislava and Icterohaemorrhagiae (Gummow et al., 1999). The first isolation of *Leptospira* spp. in South Africa was from pigs (De Lange et al.,1987), followed 10 years later, with the isolation of the pathogen from cattle with serovar Pomona determined to be responsible for an outbreak of abortions on a farm (Gummow et al., 1999).

The implication is that for almost 20 years, no study has documented the isolation of *Leptospira* spp. in South Africa. Furthermore, none of the *Leptospira* spp. isolated in previous studies were characterized using molecular methods as an alternative to the gold standard MAT due to challenges of technical-know-how on the genetic analyses of *Leptospira* spp. in the country.

2.5.4 Leptospirosis in sheep

Sheep, as one of the most important livestock in South Africa, have the least amount of data available on leptospirosis. The sero-surveys conducted on leptospirosis in sheep sampled during an outbreak in two provinces (Mpumalanga and Gauteng) were 1.3% and 12.5%, respectively (Gummow et al., 1999). Currently, there is a wide knowledge gap on the status of leptospirosis in sheep in South Africa using isolation and molecular techniques.

2.5.5 Leptospirosis in rodents/wildlife

Rodents serve as major reservoirs and transmission hosts of leptospirosis through their urine when infected. A study conducted on trapped rodents in Durban, reported a seroprevalence of 10.8% (22/202) with the use of LeptoTekDri-Dot RTD and 12.6% (8/63) by conventional PCR (Taylor et al., 2008).

In a study of wildlife conducted at four national parks in the country, the seroprevalence of leptospirosis in rhinoceroses ranged from 1.2% to 8.8% and the predominant serovars detected were Grippotyphosa, Bratislava, Tarassovi, and Copenhageni (Fischer-tenhagen et al., 2000). Myburgh and colleagues (1990), detected antibodies to *Leptospira* spp., in 1.7% (7/406) of African buffalo with serovars Tarassovi and Hardjo being prevalent at the Kruger Ntional park in South Africa.

In 12 wild animal species sampled in Northern Natal, 12.0% (6/50) were seropositive for leptospirosis with serovars Tarassovi, Mini, Hardjo, Copenhageni and Pomona detected (De Lange et al., 1987). A national survey of Vervet monkeys revealed that 8.0% (4/50) were seropositive for leptospirosis (Kaschula et al., 1978).

2.5.6 Leptospirosis in dogs

Dogs are second to rodents as a leading reservoir and involvement in the transmission of *Leptospira* spp., through their urine. In a study conducted over six decades ago in the country, the seroprevalence of *Leptospira* spp. antibodies in dogs was reported to be 50% (3/6) (Malherbe & Kaschula 1953), with serovars Canicola and Sejroe detected using compliment fixation and agglutination-lysis tests. Several seroprevalence studies have been conducted on dogs in coastal areas and other provinces in the country with seroprevalence of leptospirosis ranging from 1.3% to 4.7% and the predominant serovars reported were Canicola, Pyogenes, Pomona and Tarassovi (Roach et al., 2010).

It is also known in many developing countries, including South Africa, particularly in rural communities, that dogs are not routinely vaccinated against leptospirosis. It is however not known whether this perception is the reality or that there is gross under-reporting of the disease because there is an abundance of rodents, the primary reservoir of *Leptospira,* in both urban and rural communities in the country.

2.5.7 Leptospirosis in horses

In horses, serological surveys for leptospirosis in three provinces, Gauteng, Kwazulu Natal and Western Cape, revealed seroprevalence of 48%, 37% and 32%, respectively and the two predominant serovars recorded were Bratislava (32%) and Djamani (15.4%), according to Simbizi and colleagues (2016). Furthermore, Gummow et al. (1999) reported a seroprevalence of 39% (5/13) for leptospirosis in horses tested using the MAT.

2.5.8 Human leptospirosis in South Africa

In South Africa, human leptospirosis data are scare (Taylor et al., 2008; Saif et al., 2012). In a rodent-related study (RatZooMan) conducted between 2003 and 2006, it was reported that 19.8% (43/217) of the humans sampled and tested from Durban, KwaZulu-Natal Province were seropositive for leptospirosis (Saif et al., 2012).

In most instances, only reported acute or suspected cases of leptospirosis were serologically diagnosed. In Durban, Taylor et al. (1987) reported a seroprevalence of 18.9% for apparently healthy humans using the MAT. It has also been reported that of the clinical samples from across the country sent to the Special Bacterial Pathogens Reference Unit and tested for IgM immunoglobulins to

detect acute leptospirosis, the seropositivity rate was 9% (16 of 176), 6.5% (14 of 215) and 12.5% (12 of 96), using an IgM ELISA kit. for samples collected during the period January to May in 2009, 2010 and 2011, respectively (Saif et al., 2012).

Recently, in South Africa, there have been reports of human leptospirosis cases in Gauteng province, Western Cape province and Mpumalanga province between 2015 and 2016, according to the National Infectious and Communicable Diseases (NICD) communique (http//www.nicd.ac.za). Considering these results, especially the RatZooMan study, it is possible that the prevalence of human leptospirosis might be higher than reported, due to the lack of active surveillance and knowledge of the disease. The under-diagnosis of leptospirosis may also be attributed, in part, to the predominant use of MAT and limited use of conventional bacteriological testing and PCR for the diagnosis of leptospirosis.

2.5.9 Distribution of leptospirosis in the environment in South Africa

Leptospirosis data from the environment are lacking, but risk factors in the informal settlements were assessed on environmental, socioeconomic diseases, and leptospirosis due to rodent was reported as 10.0% (Taylor et al., 2008). There is a need to investigate the South African environment, especially water bodies and soil in both urban and rural areas, considering that leptospirosis is an environmental disease.

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CHAPTER 3

Leptospirosis in livestock in South Africa: Review of laboratory data for the period 2007 to 2017

Manuscript under consideration: Microorganisms

3.1 Abstract

Leptospirosis is an important global zoonosis which causes losses in food production and income. The potential negative impact of leptospirosis on livestock production is also applicable to South Africa, due to inadequate information on leptospirosis in livestock in the country. This study reviewed the available laboratory diagnostic data on samples submitted to the Agriculture Research Council-Onderstepoort Veterinary Research Laboratory (ARC-OVR). The Microscopic Agglutination Test (MAT) with an eight-serogroup panel was used to test 6,945 livestock sera for leptospirosis. Data on the seropositivity for leptospirosis by year (2007-2017), province (9 provinces) and animal species (cattle, pigs, and sheep) were analysed. The highest number and frequency were 1, 549 (22.3%) and the lowest 212 (3.0%) was submitted in 2008 and 2012, respectively. The overall seropositivity for leptospirosis in livestock was 20.5% (1,425/6,945). The frequency of seropositivity in cattle and pigs varied significantly (P<0.001) by year of study, ranging from 6.5% (38/581) in 2009 as reference to 44.4% (233/502) in 2011; by Provinces, 71.% (123/1,744) in Gauteng as reference to 55.5% (297/535) in Mpumalanga province, while in pigs, the seropositivity frequency varied significantly by year from 8.5% (5/59) in 2010 as a reference to 34.3% (12/35) in 2012; by Province, 20.25 (77/753) in Gautnebd province as reference to 35.1% (20/57) and by animal species, 0.0% (0/14); 16.2% (286/1,763) for sheep, pigs and cattle respectively. It is however pertinent to mention that these comparisons are subjected to potential bias which may have originated from the heterogeneity of animals from which samples were obtained during different years and provinces. Among seropositive livestock, serogroup Australis was most predominant, accounting for 29.4% (333/1133) in cattle and 32.8% (94/286) in pigs. This is the first review of diagnostic data on leptospirosis in livestock in South Africa, thereby providing the predominant circulating serovars of *Leptospira* spp. It was evident that leptospirosis was widespread in cattle and pig populations tested. The predominance of antibodies to serogroup Australis, which is not included in the vaccines used in the country, has implications for prevention of leptospirosis in the country.

Key words: Serodiagnosis, Serogroup, Leptospirosis, Livestock, MAT and South Africa

3.2 Introduction

Leptospirosis is a zoonotic, re-emerging, and widespread infectious disease. The disease is an environmental problem with global public health and huge economic implications, especially in the developing countries. The disease distribution affects both domestic and wild animals, with a high incidence in tropical climates (Plank and Dean, 2000). Leptospirosis is caused by a pathogenic spirochete bacterium that belongs to the genus *Leptospira* (WHO, 2011), and its infection may result in abortion, diarrhoea, reproductive problems, low milk production or even death in cattle, pigs, sheep and goats, (World Organization for Animal Health (OIE) 2014; 2018). Rodents play an important role in the transmission of leptospirosis to both humans and animals. Infected animals display prolonged renal infection and shed the leptospires continuously in their urine, through which they contaminate the environment. In the process, the environmental contamination by the pathogen leads to the exposure and infection of human and other animals (Levett, 2001).

The diagnosis of leptospirosis and epidemiological studies rely on the use of serological methods, primarily the Microscopic Agglutination Test (MAT), as a an indirect technique and the officially recognised 'gold standard' to determine the serogroups of the infecting *Leptospira* (Levett, 2001., World Organization for Animal Health (OIE) 2014., 2018., WHO, 2003). The MAT results are regarded to be predictive of the serogroups of *Leptospira* species (spp.) circulating in countries or regions from where the samples originated. However, the MAT results could be inaccurate due to occurrence of cross-reactions among serovars that belong to the same serogroups (Petrakovsky et al., 2014). Moreover, the MAT is known to have several disadvantages which include low sensitivity in detecting early phase of the disease, its labour-intensive nature, the complicated procedure and a necessity to maintain *Leptospira* strains for preparing the live antigens (Budihal and Perwez, 2014; Picardeau, 2013). However, the MAT has always been used to provide adequate information on the epidemiological status of reservoirs as sources of infecting leptospires (Smythe et al., 2009). Other methods which include the polymerase chain reaction (PCR) technique and culture have been used for the diagnosis of leptospirosis (Budihal & Perwez, 2014; Ellinghausen, 1965).

It has been demonstrated that different animal species (cattle, pigs and goats), with and without observable clinical signs have been exposed and infected with *Leptospira* spp., using serological, bacteriological and polymerase chain reaction (PCR) diagnostic methods (Kurilung et al., 2017). Leptospirosis clinical signs are obviously observed in adult cattle and sheep during milking, with a sudden onset of agalactia (World Organization for Animal Health (OIE), 2018). In other species, the signs of disease were found like those of human infection as demonstrated by experimental

infections (Nally et al., 2004; Pereira et al., 2005). Natural reservoirs and carriers of *Leptospira* spp. have been identified to be domestic and wild animals which can also serve as accidental and maintenance hosts (Levett, 2001). Studies have demonstrated the prevalence of leptospirosis in various countries. In Malaysia for example, Bahaman et al, (1987) reported an overall seroprevalence of 25.5% (863/3377) in domestic animals, with variations amongst animal species: cattle, 40.5% (58/1378); buffalo, 31% (133/429); goats, 4.4% (29/657); sheep, 6.8% (3/44) and pigs, 16% (138/869). The study documented the presence of various serovars in cattle, including Icterohaemorrhagiae (1.0%), Pomona (3.4%), Tarassovi (7.2%), Canicola (1.1%) and Australis (0.9 %). In sheep, only serovar Canicola (6.8%) was found but in pigs, the seropositive serovars detected were Icterrohaemorrhagiae (0.1%), Pomona (3.3%), Tarassovi (1.4%), Grippotyphosa (0.6%), Canicola (6.2%), and Australis (0.9%) (Bahaman et al, 1987). Elsewhere, a preliminary survey for leptospirosis in livestock in northwest Morocco, using MAT, revealed a seroprevalence of 15.0% (19/126) with the predominance of the serogroups Ballum, Sejroe and Australis in cattle, and serogroups Ballum, Australis and Sejroe in sheep, 18.0% (5/28) (Benkirane et al., 2014).

It has been established that vaccination of animals against leptospirosis is used in the prevention of leptospirosis (World Organization for Animal Health (OIE), 2018). It has also been well documented that the predominant serovars of *Leptospira* spp. circulating within a geographical region must be included in the vaccines used in the same region to be effective (World Organization for Animal Health (OIE), 2018). However, the production of antibodies to vaccine serovars may interfere with surveillance of leptospirosis because it will be difficult to differentiate immunological response to vaccine serovars from that of natural exposure to the pathogen (World Organization for Animal Health (OIE), 2018). Hence, for the purpose of effective diagnosis of leptospirosis, vaccination status of tested animals must be known.

In South Africa (SA), using the MAT, the serological evidence of cattle leptospirosis was first demonstrated in the Western Cape Province in 1967, with a prevalence of 2.5% (Van der Merwe, 1967), while another study in the Eastern Cape Province (Gummow et al., 1999) revealed a seroprevalence of 52%, with the predominating serovar being Pomona. Recently, in a cross-sectional study on slaughtered cattle in some selected Gauteng abattoirs, Dogonyaro et al., (2020), reported leptospirosis seroprevalence of 27.6%, serogroups Sejroe (sv. Hardjo) and Mini (sv. Szwajizak) were predominant, 38.2% and 14.5%, respectively. A serological survey with slaughter pigs for *Leptospira* spp., from different provinces in South Africa, Potts et al., (1995) reported a seroprevalence of 22.0% with the following serovars: Icterrohaemorrhagiae (12.6%), Hardjo (12.1%) and Bratislava (7.5%). In

the same study, considering an outbreak situation for leptospirosis in pigs, the seroprevalence of the disease was 17% in aborted pigs and the predominant serovars were Pomona, Harjo, Bratislava and Icterohaemorrhagiae (Gummow et al., 1999).

In sheep from SA, the first available data on the seroprevalence of leptospirosis was 6.4% in 1967, in which some serovars among Australis, Autumnalis, Bovis, Canicola, Grippotyphosa, Hyos, Icterohaemorrhagiae, Pyrogenes and Saxkoebing were detected (Van der Merwe, 1967). In another study, Gummow et al., (1999) reported the seroprevalence of leptospirosis in sheep during outbreaks using the MAT and found a seroprevalence of 1.3 %. Similarly, in a survey of sheep in KwaZulu-Natal Province, Hesterberg et al., (2009) found sheep with a seroprevalence of 19.4% for leptospirosis with serovars Pomona (22%) and Tarassovi (18%) being the most prevalent.

To date, no comprehensive data have been analysed on leptospirosis in livestock in SA. There is also the need for a continuous country wide surveillance of leptospirosis in livestock for good information on the distribution of serogroups or serovars of *Leptospira* spp. circulating in South Africa. This study was conducted to comprehensively review and evaluate retrospective data on leptospirosis in the livestock industry available at the Bacteriology section where the leptospirosis data were retrieved at the Agricultural Research Council - Onderstepoort Veterinary Research (ARC-OVR).

3.2.1 Specific objectives:

The specific objectives of the current study are:

i) To collate and retrospectively analyse available data from samples that originated from both apparently healthy livestock (for export) and clinical cases (suspects and confirmed) in SA in order to determine the seropositivity for infections by *Leptospira* spp. in livestock.

2) To establish associations among seropositive categories (infecting serogroups and titres), considering the period of testing (2007-2017), the types of livestock (cattle, pigs and sheep), and the province of origin of the samples and

3) To identify existing data and knowledge gaps in information on leptospirosis in SA in order to make recommendations on interventions for improved diagnosis and control of leptospirosis in livestock in SA.

3.3 Materials and methods

3.3.1 Study area

The Eastern Cape Province has the highest concentration of cattle, sheep and goats in SA. KwaZulu-Natal Province is second in beef cattle production, while the Northern Cape Province is second in the production of sheep. Proportionally, the small-scale and communal sectors comprise 41% of the beef cattle, 12% of the sheep and 67% of the goats in the country (Meissner et al., 2013). For this study also, the current available data (2014/2015) on animal population in SA were inferred to, i.e., cattle (13.7 million), sheep (11 million) and pigs (1.5 million) (DAFF, 2016).

3.3.2 Government policy on livestock leptospirosis in South Africa

Leptospirosis is known to be endemic in livestock in South Africa (Botes and Garifallou, 1967), and reports of human cases exist. Since leptospirosis is not a reportable disease in SA, vaccination of animals against the disease is voluntary. However, the livestock destined for export market may be vaccinated based on the vaccination requirements of the importers. The vaccines used in SA for the prevention of leptospirosis in animals, including pet animals, contain five serovars, namely Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae and Pomona, and these may be administered by government or private veterinarians.

Clinical or suspect cases of leptospirosis in livestock are sampled by veterinarians who submit the blood samples to the serology section in Bacteriology Department of the ARC-OVR, Gauteng Province, SA, for leptospirosis diagnosis. In the country, laboratory diagnosis of leptospirosis is primarily based on the MAT, using eight-serogroup panel. Since the ARC-OVR is the only laboratory with repositories of information on leptospirosis in SA, data on leptospirosis in this laboratory are therefore accepted to be representative of the country.

3.3.3 Sources of data and criteria for inclusion and exclusion

Although the samples were processed and tested at the ARC-OVR laboratories based in Gauteng Province, they originated from livestock farms of private and state veterinary clinics located in all the nine provinces of SA (**Figure 3.1**). These samples originated from both apparently healthy livestock (for export) and clinical cases (suspects and confirmed).

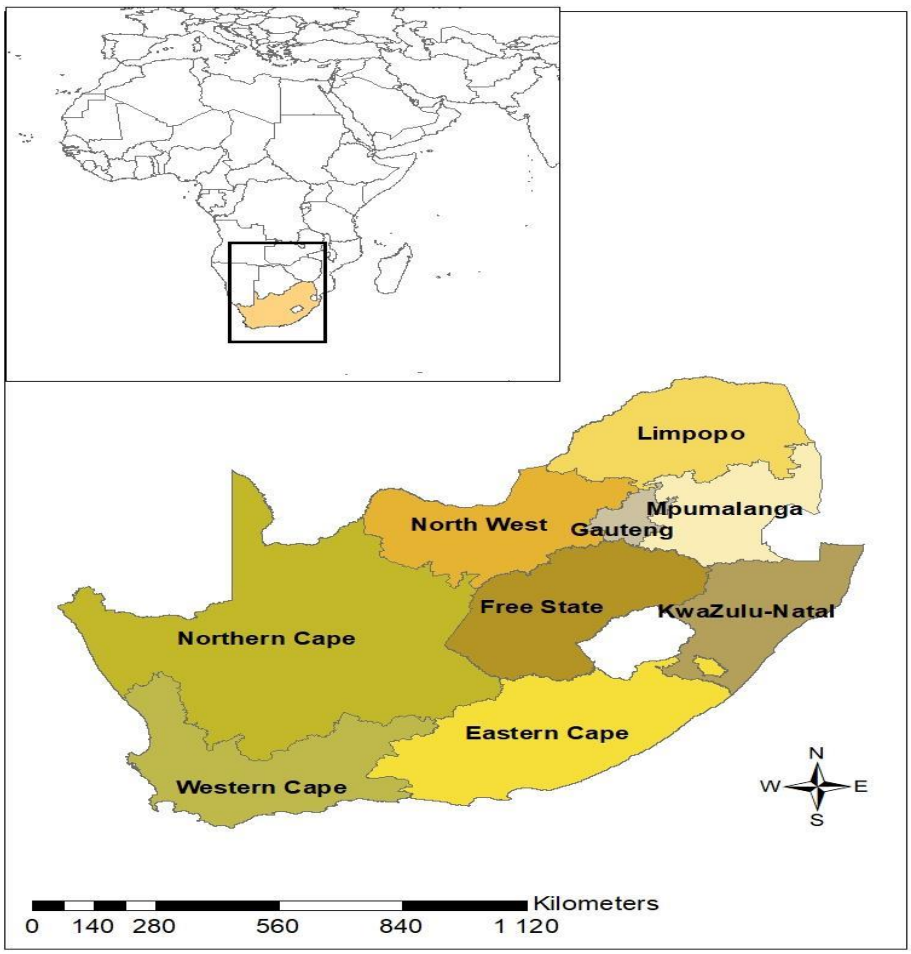

Figure 3.1: Map of South Africa showing the nine provinces where a total of 6,945 serum samples originated for the determination of prevalence of *Leptospira* species over a period of 11 years

The utilised data covered an 11-year period (2007-2017) and involved three animal species (cattle, pigs and sheep). All data in the records of the ARC-OVR on the diagnosis of leptospirosis were reviewed. The inclusion criteria for livestock data were (a) suspected or confirmed leptospirosis samples submitted during the period 2007 to 2017, (b) province of origin of the samples, (c) animal species of origin of the samples, (d) outcome data (positive or negative) available at ARC-OVR laboratory regarding the MAT results (including the infecting serogroups and their titres). For this study, any sample that lacked one or more of the criteria ('a – d') stated above was excluded from further analysis. Serologically positive samples obtained from cattle, pigs and sheep from all nine provinces and period of 11 years at the ARC-OVR were used in the study for the analyses.

3.3.4 Data collection

Data from the ARC-OVR were entered and collated using the Microsoft Excel® 2010. The data were filtered and classified by years, provinces, animal species, positivity for antibodies to a panel of eight

antigens of *Leptospira* spp.: {Canicola (Sv. Canicola); Pomona (Sv. Pomona); Sejroe (Sv. Hardjo prajitno); Grippotyphosa (Sv. Grippotyphosa); Icterrohaemorrhagiae (Sv. Icterrohaemorrhagiae); Tarassovi (Sv. Tarassovi); Australis (Sv. Bratislava) and Szwajizak (Sv. Szwajizak)} by MAT, serogroups, and titres of reactants. All data were retrieved from the sample forms and electronic data base which comprised the risk factors (year, province, and animal species) and MAT results (antibodies and titres to *Leptospira* spp.).

3.3.5 Ethical Approvals

Prior to the commencement of the study, approvals were obtained from the following bodies and committees: Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, University of Pretoria, South Africa (AEC: ***v084-16***), **Figure 3.2,** Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) (AEC: ***12-16***), **Figure 3.3** and Section 20 according to Act 35 of 1984 by the Director of Animal Health at the Department of Agriculture, Forestry and Fisheries (DAFF), (Number: ***FY2015/2016***), South Africa, **Figure 3.4**.

Figure 3.2: University of Pretoria Animal ethics committee approval certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Prevalence and characterization of Leptospirosis in slaughter animals at Gauteng Province abattoirs: Food safety implications for meat consumers and zoonotic risks posed to abattoir workers**
PROJECT NUMBER	**V084-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**BB Dogonyaro**

STUDENT NUMBER (where applicable)	**UP_28443782**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	***Hamster***	
NUMBER OF ANIMALS	**16**	
Approval period to use animals for research/testing purposes		**August 2016 –August 2017**
SUPERVISOR	**Prof. FO Fasina**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 29 August 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Figure 3.3: Agricultural Research Council-Onderstepoort Research Institute (ARC-OVR), Ethical committee approval certificate

APPROVED

ARC • LNR

Onderstepoort Veterinary Institute

AEC 12.16

Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

TRAIL PERIOD
STARTING DATE: 1 October 2016 ENDING DATE: 31December 2017

PROJECT NUMBER:				
PROJECT TITLE:	Prevalence and characterization of selected pathogens in slaughter animals in Gauteng Province abattoirs: Food safety implications for meat consumers and zoonotic hazards posed to abattoir workers			
PROJECT LEADER:	Dr Andrew Potts.			
DIVISION:	FFVH			
CATEGORY:	A			
SPECIES OF ANIMAL:	N/A			
NUMBER OF ANIMALS:	N/A			

RECOMMENDATIONS BY ANIMAL ETHICS COMMITTEE

Date of AEC meeting for consideration: 17 August 2016	Action Taken: APPROVED APPROVED	SIGNATURE: AEC-Chairperson Dr P. Mutowembwa

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

Figure 3.4: Department of Agriculture, Forestry and Fisheries (DAFF)-Section 20, Ethical committee approval certificate

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Dr Henriette van Heerden
Department of Veterinary Tropical Diseases
University of Pretoria
Tel: 012 529 8265
E-mail: henriette.vanheerden@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Dr van Heerden,

Your application sent with the email on 15 July 2016 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, "*Prevalence and characterisation of selected pathogens in slaughter animals at Gauteng Province abattoirs – Food safety implications for meat consumers and zoonotic risks posed to abattoir workers*" with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82) and the Meat Safety Act 2000 (Act No. 40 of 2000);

– 1 –

3. This section 20 approval covers the relevant sub-sections of the main research proposal as declared by the researcher, and does not extend beyond the scope of the specified protocol;
4. Samples must be packaged and transported in accordance the National Road Traffic Act, 1996 (Act No. 93 of 1996);
5. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the State Veterinarian of the area.
6. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study;
7. Only a registered waste disposal company may be used for the removal of waste generated by or during the study;
8. Records must be kept for five years for audit purposes.
9. No part of this study may commence until valid ethical approval has been obtained in writing from the relevant authority;
10. A dispensation for the storage of serum, bacterial isolates and extracted DNA is attached.

Title of research/study: *"Prevalence and characterisation of selected pathogens in slaughter animals at Gauteng Province abattoirs – Food safety implications for meat consumers and zoonotic risks posed to abattoir workers"*
Researcher: Dr Henriette van Heerden
Institution: Department of Veterinary Tropical Diseases, University of Pretoria
Our ref Number: 12/11/1/1/6
Your ref: FY 2015/2016

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2016 -09- 09

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Dr Henriette van Heerden
Department of Veterinary Tropical Diseases
University of Pretoria
Tel: 012 529 8265
E-mail: henriette.vanheerden@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "PREVALENCE AND CHARACTERISATION OF SELECTED PATHOGENS IN SLAUGHTER ANIMALS AT GAUTENG PROVINCE ABATTOIRS – FOOD SAFETY IMPLICATIONS FOR MEAT CONSUMERS AND ZOONOTIC RISKS POSED TO ABATTOIR WORKERS"

A dispensation is hereby granted on Point 10 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Serum samples collected from the respective slaughter animals at the specified abattoirs may be stored in the relevant ARC-OVI and UP serum banks;

ii) Bacterial isolates and extracted DNA obtained from the collected samples may be stored in the relevant ARC-OVI and UP serum banks;

iii) Stored samples may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016 -09- 09

3.3.6 Statistical analyses of data

3.3.6.1 Descriptive and univariate analysis:

Continuous data were assessed for normality by bar charts and descriptive statistics, while categorical data were presented as frequencies or percentages. The serological status (antibodies to *Leptospira* spp.) in cattle and pigs, as determined using MAT, was considered as a binary outcome (positive or negative). Proportions of positive animals were obtained, and the 95% confidence intervals were estimated using the quantiles formation of the normal distribution (qnorm) with the MASS package in R (Venables & Ripley, 2002). From the seropositive samples, the frequency of detection of each of the eight *Leptospira* serogroups, which are currently included in the MAT panel, was determined, and the frequencies were analysed for association with each of the variables: year, province and animal species using the chi-square or Fisher's Exact tests. The frequencies of the corresponding titres (1:100 to 1:3200) were also determiend and analysed for association with the serogroups.

3.3.6.2 Multivariable analysis:

The two explanatory variables, year (11 years, 2007 through 2017) and province (nine provinces), were analysed for association with *Leptospira* spp. seropositivity in multivariable logistic regressions, employing Generalised Linear Models, separately for cattle and pigs. The data were analysed using R Console version 3.2.1 (R Core Team, 2017) at 5% level of significance.

3.4 Results

Overall, a total of 6,945 samples were included in the study. For the study period (2007-2017), a total of 6,945 data points, which correspond to serum samples of individual animals, comprising 5,168, 1,763 and 14 from cattle, pigs, and sheep respectively, and which had been analysed using MAT, were retrieved. Seven samples were excluded in the analyses due to missing data.

3.4.1 Frequency distribution of samples tested by year (2007-2017), Province and animal type

The distribution of samples that had been tested at the ARC-OVR laboratory differed significantly ($P<0.05$) across the 11 years of sampling, the nine provinces and the three animals. By sampling period, the highest number of samples, 1,549 (22.3%), was submitted in 2008, followed by 940 (13.5%) in 2007, and the lowest number received was 212 (3.1%), in 2012 (**Table 3.1**).

Table 3.1: Frequency distribution of the sera of individual animals submitted that were used during a retrospective study for leptospirosis for 11 years (between 2007 and 2017) in South Africa

Year	No. of samples (%)*
2007	940 (13.5)
2008	1,549 (22.3)
2009	717 (10.3%)
2010	368 (5.3%)
2011	546 (7.9%),
2012	212 (3.1%),
2013	709 (10.2%)
2014	323 (4.7%)
2015	642 (9.2%)
2016	468 (6.7%)
2017	471 (6.8%)
Province	
Gauteng	2,508 (36.1)
KwaZulu-Natal	1,322 (19.0)
Northern Cape	4 (0.1%)
Western Cape	1,116 (16.1%)
Mpumalanga	588 (8.5%)
North West	517 (7.4%)
Orange Free State	464 (6.7%)
Cape Town	220 (3.2%)
Limpopo	206 (3.0%)
Animal species	
Cattle	5,168 (74.4%)
Pigs	1,763 (25.4%)
Sheep	14 (0.2%)

*The data was obtained from Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR), Pretoria, South Africa, and was from all the nine provinces of South Africa, and from three animal species.

By province, the highest frequency of samples submitted to the ARC-OVR laboratory was from Gauteng Province, 36.1% (2,508/6,945), followed by KwaZulu-Natal, 19.0% (1,322/6,945), and the lowest frequency recorded was from the Northern Cape Province, 0.1 % (4/6,945) (**Table 3.1**). The highest frequency samples submitted by animal type was from cattle, 74.4% (5,168/6,945), followed by pigs, 25.4% (1,763/6,945), and the lowest was from sheep, 0.2% (14/6,945) (**Table 3.1**).

3.4.2 Frequency of seropositivity for Leptospira in livestock (cattle and pigs) by year, province and animals species

Table 3.2: Proportions of cattle that tested positive for antibodies to *Leptospira* species and results of a multivariable logistic regression on seropositivity for *Leptospira* by year and provinces

Variable	No. of samples tested[a]	No. (%) seropositive[b]	95% CI of seropositivity	Odds ratio	*P* - value
Year					
2007	381	40 (10.5)[b]	7.4, 13.6	2, 3	<0.001[c]
2008	978	94 (9.6)	7.8, 11.5	1.2	0.31
2009[d] **Ref.**	581	38 (6.5)	4.5, 8.6		
2010	309	35 (11.3)	7.8, 14.9	1.0	0.87
2011	502	233 (46.4)	42.1, 50.8	3.3	<0.001[c]
2012	177	23 (13.0)	8.0, 17.9	1.0	0.94
2013	655	251 (38.3)	34.7, 42.2	6.2[e]	<0.001[c]
2014	282	112 (39.7)	34.7, 42.2	3.6[e]	<0.001[c]
2015	497	160 (33.2)	28.1, 36.3	3.7[e]	<0.001[c]
2016	467	103 (22.1)	18.3, 25.8	1.8	0.006[c]
2017	339	44 (13.0)	9.4, 16.6	0.8	0.52
Total	**5168**	**1133 (21.9)**			
Province					
ECP	180	45 (25.0)[b]	18.7, 31.3	3.1	<0.001[c]
GAUP[d] Ref	1744	123 (7.1)	5.9, 8.3		
KZN	1148	314 (27.3)	24.9, 30.1	4.4[e]	<0.001[c]
LIMP	149	48 (32.2)	24.7, 39.7	4.4[e]	<0.001[c]
MPHP	535	297 (55.5)	51.3, 59.7	11.5[e]	<0.001[c]
NCP	1	1 (100.0)	1, 1	2. 4	0.94
NWEP	333	83 (24.9)	20.3, 29.6	2.2	<0.001[c]
OFSP	318	79 (24.8)	20.1, 29.6	3.2	<0.001[c]
WCP	762	143 (18.8)	15.9, 21.5	2.0	<0.001
Total	**5168[a]**	**1133 (21.9)**			

[a] A total of 5,168 data units representing results of individual cattle samples, which were collected for a period of 11 years (2006-2017) from nine provinces in South Africa, were retrieved from the Agricultural Research Council -Onderstepoort Veterinary Institute (ARC-OVR)

[b] Seropositivity determined by the microscopic agglutination test (MAT)

[c] Statistically significant difference ($p<0.05$) between the level of a variable and the reference level for the same variable.

[d] Reference levels (year 2009; province GAUP).

[e] Highest Odds ratios obtained for the year 2013, 2014 and 2015; and provinces, KZN, LIMP and MPHP. GAUP: Gauteng, ECP: Eastern Cape, KZN: KwaZulu-Natal, LIMP: Limpopo, MPHP: Mpumalanga, NCP: Northern Cape, NWEP: North West, OFSP: Orange Free State, and WCP: West Cape.

Table 3.3: Proportions of pigs that tested positive for antibodies to *Leptospira* species and results of a multivariable logistic regression on seropositivity for *Leptospira* spp. by year and provinces

Variable	No. of samples tested[a]	No. (%) seropositive[b]	95% CI of seropositivity	Odds ratio	*P* - value
Year					
2007	555	91. (16.4)[b]	13.3,19.5	3.4	0.01[c]
2008	567	70 (12.3)	9.6, 15.1	1.8	0.27
2009	130	12 (9.2)	4.3, 14.2	2.9	0.07
2010[d] Ref	59	5 (8.5)	1.4, 15.6		
2011	44	8 (18.2)	6.8, 29.6	6.5[e]	0.004[c]
2012	35	12 (34.3)	18.6, 50.0	9.8[e]	<0.001[c]
2013	54	14 (25.9)	14.2, 37.6	4.3	0.019[c]
2014	41	5 (12.4)	2.2, 22.2	3.9	0.05[c]
2015	145	48 (33.1)	25.4, 40.8	4.2	0.01[c]
2016	1	0 (0.0)	0, 0	inf	0.97
2017	132	21 (15.9)	9.7, 22.1	1.2	0.71
Total	**1763**	**286 (16.2)**			
Province					
ECP	40	9 (22.5%)	9.6, 35.4	1.9	0.15
GAUP[d] Ref	753	77 (10.2)	8.1, 12.4		
KZN	176	47 (26.7)	20.2, 33.3	5.7[e]	<0.001[c]
LIMP	57	20 (35.1)	22.7, 47.5	4.4[e]	<0.001[c]
MPHP	53	9 (17.0)	6.9, 27.1	2.4	0.03[c]
NCP	3	1 (33.3)	-20.0, 86.7	4.[6]	0.25
NWEP	183	35 (19.1)	13.4, 24.8	2.9	<0.001[c]
OFSP	146	32 (21.9)	15.2, 28.6	3.1	<0.001[c]
WCP	352	56 (15.9)	12.1, 33.2	2.3	0.002[c]
Total	**1763[a]**	**286 (16.2)**			

[a] A total of 1,763 data units representing results of individual pig samples, which were collected for a period of 11 years (2006-2017) from nine provinces in South Africa, were retrieved from the Agricultural Research Council -Onderstepoort Veterinary Institute (ARC-OVR)

[b] Seropositivity determined by the microscopic agglutination test (MAT)

[c] Statistically significant difference ($p<0.05$) between a level of variable and the reference level for the same variable.

[d] Reference levels: year 2010; province GAUP).

[e] Highest Odds ratios were obtained for the years 2011 and 2012; and provinces KZN and LIMP.

GAUP: Gauteng, ECP: Eastern Cape, KZN: KwaZulu-Natal, LIMP: Limpopo, MPHP: Mpumalanga, NCP: Northern Cape, NWEP: North West, OFSP: Orange Free State, and WCP: West Cape.

For the cattle, all the two potential predictors (year and Province) were significantly associated ($p<0.001$) with seropositivity in the univariate analysis. Overall, the frequency of detecting cattle seropositive for *Leptospira* spp. for the 11-year period from the nine provinces was 21.9%

(1,133/5,168). In the pigs, all the two potential predictors (year and Province) were significantly associated (p<0.001) with seropositivity in the univariate analysis. Overall, the frequency of detecting pigs seropositive for *Leptospira* spp. for the 11-year period from the nine provinces was 16.2% (286/1,768), from the Maximum/highest MAT titre at 1:100 cut off point used for each sample and in the case of multiple cross-reacted samples, the sample with the highest titre was considered seropositive against others as a current practice in the ARC-OVR diagnostic laboratory for diagnostic purposes. No sheep out of the 14 sampled were seropositive for leptospirosis, therefore was not analysed.

The cattle proportion of seropositivity for *Leptospira* spp. was statistically significant among the years ($p < 0.001$). There was a spike in the frequency of leptospirosis in 2011, 46.3%, (233/502) as compared with the reference years (2009), whose occurrence was 6.5% (38/581) less than and the years beyond 2011 (2012 to 2017), whose occurrences were less than 40% (**Table 3.2)**. Between 2013 and 2017, there was a steady decline in frequency from 37.5% (266/709) in 2013 to 16.5% (78/471) in 2017. The lowest frequency of leptospirosis from the databank of 11 year was in 2009, 6.5% (38/581), (**Table 3.2).**

The pigs' proportion of seropositivity for *Leptospira* spp. was statistically significant among the years ($p < 0.001$). There was a spike in the frequency of leptospirosis in 2012, 34.3%, (12/35) as compared with the reference years (2010), whose occurrence was 8.5% (5/59) and the years, 2015 and 2013, whose occurrences were less than 34% (**Table 3.3)**. Between 2015 and 2013, there was a steady decline in frequency from 33.1% (48/145) in 2015 and 25.9% (14/54) respectively. The lowest frequency of leptospirosis from the databank of 11 year was in 2010, 8.5% (5/59), (**Table 3.3).**

In cattle, the proportion of seropositivity for *Leptospira* spp. was statistically significant among the province ($p < 0.001$). The highest frequency was detected in the Mpumalanga Province, 55.5% (297/535), compared with the reference Province Gauteng, 7.1% (123/1744), followed by the Limpopo Province, 32.2% (48/149); Kwazulu Natal Province, 27.3% (314/1148); Eastern Cape Province, 25.0% (45/180) as compared to the reference Province Gauteng. All the Provinces were statistically significant except Northern Cape Province, statistically insignificant, due to small number of samples submitted (**Table 3.2**). The frequencies were lower in the Northwest Province and Orange Free State Province, 24.9.2% (83/333) and 24.8% (79/318) respectively. The lowest frequency reported was in Gauteng Province 7.1% (123/1744), as the reference Province (**Table 3.2**).

In the pigs, the proportion of seropositivity for *Leptospira* spp. was statistically significant among the province ($p < 0.001$). The highest frequency was detected in the Limpopo Province, 35.1% (20/57), compared with the reference Province Gauteng, 10.2% (77/753), followed by the KwaZulu Natal Province, 26.7% (47/176) and Orange Free State Province, 21.9% (32/146) as compared to the reference Province Gauteng. Although, Northern Cape Province had 33.3% (1/3) but not statistically significant due to small samples submitted to the ARC-OVR. The lowest frequency reported was in Gauteng Province as the reference, 10.2% (77/53), (**Table 3.3**).

3.4.3 Risk factor analysis

In the cattle all the two variables, (year and province) were included in an initial logistic regression model, and the final multivariable model included 'year' and 'province'. The years 2013, (odd ratio [OR]=6.2, p<0.001), 2014, (odd ratio [OR]=3.6, p<0.001), and 2015, (odd ratio [OR]=3.7, p<0.001), showed significantly higher odds seropositivity for *Leptospira* species than 2009 (reference year for comparison), while the smallest odds were recorded in 2017 (OR=0.8, p=0.52) and Western Cape Province, (OR=2.0, p=0.001) **(Table 3.2).**

The two variables of pigs, (year and province) were included in an initial logistic regression model, and the final multivariable model included 'year' and 'province'. The years 2011, (odd ratio [OR]=6.5, p<0.001) and 2012, (odd ratio [OR]=9.8, p<0.001), showed significantly higher odds seropositivity for *Leptospira* species than 2010 (reference year for comparison, while the smallest odds were recorded in 2017 (OR=1.2, p=0.71) and Eastern Cape Province, (OR=1.9, p=0.15) (**Table 3.3**).

3.4.4 Frequency distribution of antibodies to serogroups of Leptospira spp. in cattle and pigs by year

During the 11-year review period, eight serogroups were detected (**Figure 3.5** and **Figure 3.6**). The overall frequency of antibodies to one or more of the eight serogroups of *Leptospira* spp. in the livestock (cattle and pigs) under study and tested by the MAT was 2.9% (1133/5,168) and 16.2% (286/1768) respectively.

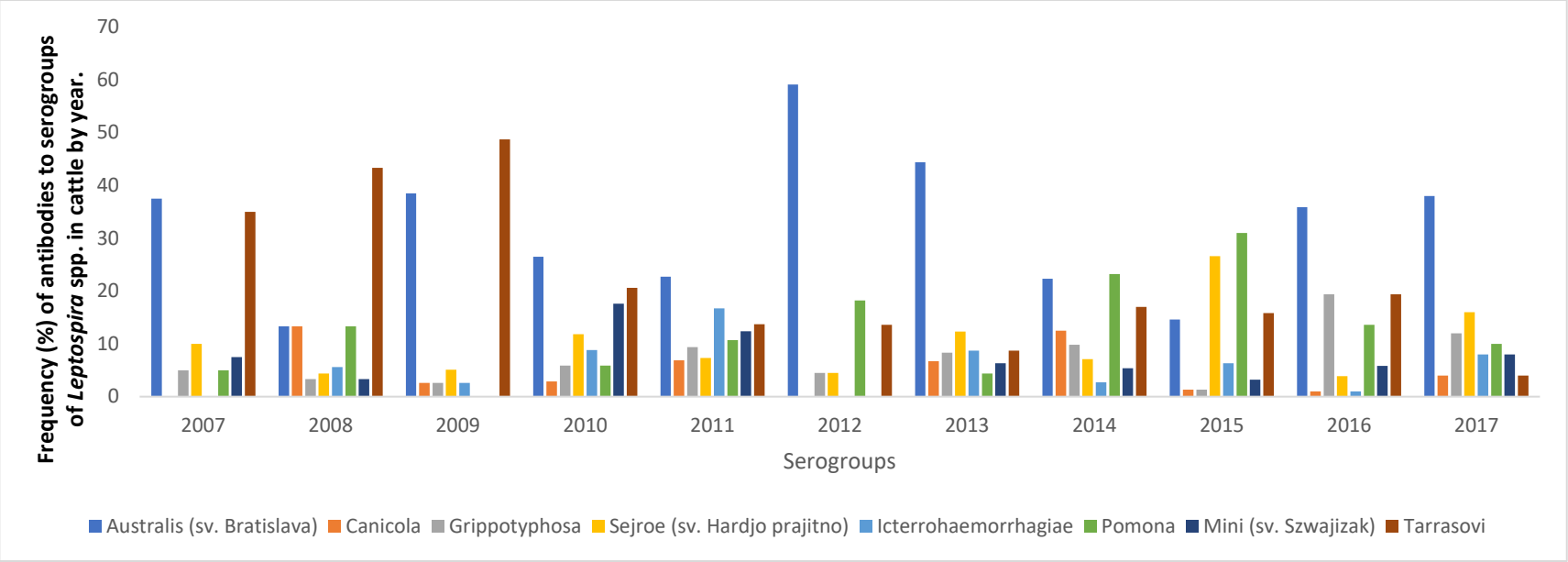

Figure 3.5: Frequency of detection of antibodies to serogroups of *Leptospira* in cattle by year. A retrospective study, in which data for 11 years (2007-2017) were obtained from a database on leptospirosis at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVR), where a total of 1, 133 sera samples from nine provinces of South Africa were analysed with MAT using eight *Leptospira* reference antigens

*p<0.001; there were significant differences when the proportions of the various serogroups in each year of study were compared (using the chi-square or Fisher's Exact tests at 5% significance level).

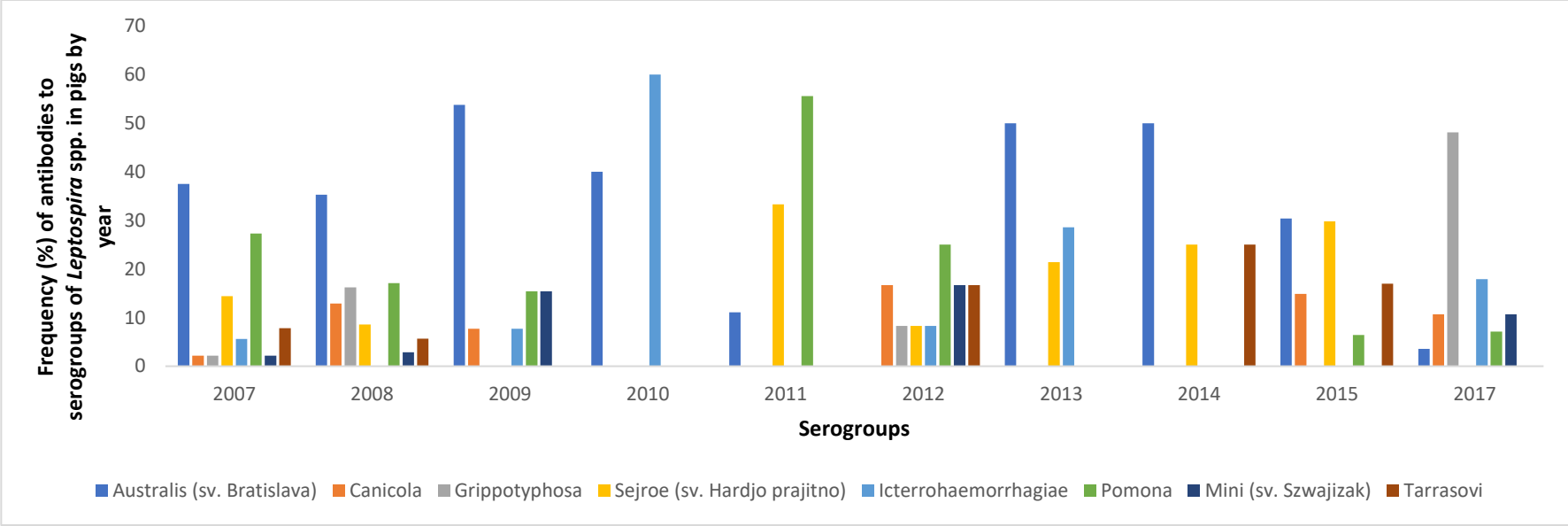

Figure 3.6: Frequency of detection of antibodies to serogroups of *Leptospira* in pigs by year. A retrospective study, in which data for 11 years (2007-2017) were obtained from a database on leptospirosis at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVR), where a total of 286 sera samples from nine provinces of South Africa were analysed with MAT using eight *Leptospira* reference antigens.

There was no positive sample from the submitted samples to Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVR), in the year 2016 therefore, no serogroup positive as well.

*$p<0.001$; there were significant differences when the proportions of the various serogroups in each year of study were compared (using the chi-square or Fisher's Exact tests at 5% significance level).

The annual frequencies of antibodies to the various serogroups in each of the 11 years under study (2007 to 2017) for cattle and pigs are shown in **Figure 3.5** and **Figure 3.6**. There was a significant association between the frequency of occurrence of the various serogroups with the years of study ($p<0.001$); also, the difference in the frequency of the various serogroups in each year was statistically significant ($p<0.001$) (**Figure 3.5** and **Figure 3.6**).

For the cattle, out of the total 1,133 seropositive samples, the 11-year total frequency, in descending order, for each serogroup was: 29.4% (333) for Australis (sv. Bratislava), 17.8% (202) for Tarassovi, 13.2% (150) for Pomona, 11.0% (125), for Sejroe (sv. Hardjo prajitno), 8.0% (91), for Grippotyphosa, 7.8% (88) for Icterohaemorrhagiae, 6.9% (78) for Szwajizak and 5.8% (68) for Canicola, (**Figure 3.5**). Australis (sv. Bratislava), was the most predominant serogroup detected in each of nine out of the 11 years studied, while the serogroups Tarassovi and Sejroe (sv. Hardjo) were the most frequently detected in 2008 and 2015 respectively. Serogroup Canicola had the lowest frequency of detection in two of the 11 years (2015, 1.3% and 2018), while serogroup Icterohaemorrhagiae was least detected in three of the 11 years (2016, 1.0%; 2009, 2.6% and 2014, 2.7%). Serogroup Grippotyphosa had the lowest frequency of detection in two of the 11 years (2009, 2.6% and 2015, 1.3%). Amongst the total 1,133 *Leptospira* spp. seropositive samples from cattle analysed for serogroups over the 11 years, the total number of all serogroups by year was as follows: 40 (3.5%, 2007), 50(7.9%, 2008), 39 (3.4%, 2009), 34(3.0%, 2010), 233 (20.5%, 2011), 22 (1.9%, 2012), 252 (22.2%, 2013), 112 (0.8%, 2014), 158 (13.9%, 2015), 103 (9.0%, 2016) and 50 (4.4%, 2017). The difference in the proportions of the total seropositive serogroups by year was statistically significant ($p<0.05$).

In the pigs, out of the total 286 seropositive samples, the 11-year total frequency, in descending order, for each serogroup was: 32.2% (91) for Australis (sv. Bratislava), 17.8% (51) for Pomona, 14.0% (41), for Sejroe (sv. Hardjo prajitno), 9.9% (29), for Grippotyphosa, 8.2% (24) for Canicola, 7.5% (22) for Tarassovi, 6.5% (19) for Icterohaemorrhagiae and 3.8% (11) for Szwajizak, (**Figure 3.6**). Australis (sv. Bratislava), was the most predominant serogroup detected from nine years of the 11 years studied, the remaining serogrougs across the years had between one to five years without a serogroup detected. Amongst the total 286 *Leptospira* spp. seropositive samples from pigs analysed for serogroups over the 11 years, the total number of all serogroups by year was as follows: 88 (30.7%, 2007), 68(23.7%, 2008), 13 (4.4%, 2009), 5(1.7%, 2010), 9(3.4%, 2011), 12(4.2%, 2012), 14(4.8%, 2013), 4(1.3%, 2014), 46 (16.0%, 2015) and 27(9.4%, 2017). The difference in the proportions of the total seropositive serogroups by year was statistically significant ($p<0.05$). It is

important to note that, there was no positive serogroup for the year 2016 from the pigs' samples submitted to the ARC-OVR Laboratory.

3.4.5 Frequency distribution of antibodies to serogroups of Leptospira spp. in cattle and pigs by Province

The frequency of detection of the various serogroups by cattle and pigs in each of the nine provinces of South Africa is shown in **Table 3.4** and **Table 3.5.** The association between the frequency of serogroups with province of origin was significant ($p<0.001$). In cattle, of the 1,133 samples that were seropositive for antibodies to *Leptospira* spp. serogroups, the highest frequency (considering the total of all serogroups) was detected in KwaZulu-Natal Province, 27.3% (310/1,133), followed by Mpumalanga Province, 26.3% (298/1,133) and the lowest frequency was in Northern Cape, 0.08% (1/1,133) (**Table 3.4**). For the pigs, out of the 286 samples that were seropositive for antibodies to *Leptospira* spp. serogroups, the highest frequency (considering the total of all serogroups) was detected in Gauteng Province, 26.2% (75/286), followed by Western Cape Province, 21.3% (61/286) and the lowest frequency was in Northern Cape, 0.3% (1/1,133) (**Table 3.5**).

Table 3.4: Frequency of serogroups of *Leptospira* as determined in a retrospective study in cattle by nine provinces for 11 years in South Africa

	Frequency (%)[a] of seropositivity for serogroups of *Leptospira* species by province									Total no. (%) in all provinces
Serogroup	ECP[b]	GAUP	KZN	LIMP	MPHP	NCP	NWEP	OFSP	WCP	
Total no. per province (%) out of the overall 1,133	45 (5.1)	124 (11.0)	310 (27.4)	48 (4.2)	298 (26.3)	1 (0.1)	80 (7.0)	79 (7.0)	148 (13.1)	1, 133
Australis (sv. Bratislava)	33.3	22.6	26.1	39.6	21.1	100	48.8	57.0	28.4	333 (29.4)
Icterohaemorrhagiae	11.1	8.9	4.5	0.0	13.8	0.0	13.9	2.5	2.7	88 (7.8)
Pomona	15.6	3.2	18.7	25.0	12.1	0.0	10.0	3.8	14.9	150 (13.2)
Tarassovi	13.3	19.0	19.0	27.1	14.8	0.0	0.0	8.9	28.4	202 (17.8)
Sejroe (sv. Hardjo)	11.1	4.0	18.1	2.1	8.7	0.0	0.0	10.1	16.2	125 (11.0)
Grippotyphosa	6.7	12.9	4.2	2.1	12.8	0.0	10.0	5.1	5.4	91 (8.0)
Canicola	2.2	10.5	4.5	0.0	7.4	0.0	10.0	5.1	2.7	66 (5.8)
Mini (sv. Szwajizak)	6.7	12.9	4.8	4.2	9.4	0.0	7.5	7.6	1.4	78 (6.9)

[a]Frequency in each cell for each serogroup was obtained by dividing the number of samples positive for a serogroup in a province by the total number of positive serogroups in a province.

[b]ECP=Eastern Cape, GAUP=Gauteng, KZN=Kwazulu-Natal, LIMP=Limpopo, MPHP=Mpumalanga, NCP=Northern Cape, NWEP=North West, OFSP=Orange Free State and WCP = Western Cape.

Table 3.5: Frequency of serogroups of *Leptospira* as determined in a retrospective study in pigs by nine provinces for 11 years in South Africa

	Frequency (%)[a] of seropositivity for serogroups of *Leptospira* species by province									**Total no. (%) in all provinces**
Serogroup	**ECP[b]**	**GAUP**	**KZN**	**LIMP**	**MPHP**	**NCP**	**NWEP**	**OFSP**	**WCP**	
Total no. per province (%) out of the overall 286	**9 (3.4)**	**75 (26.2)**	**46 (16.1)**	**19 (6.6)**	**9 (3.1)**	**1 (0.3)**	**34 (11.8)**	**32 (11.2)**	**61 (21.3)**	**286**
Australis (sv. Bratislava)	22.2	28.0	23.0	68.4	77.8	100	44.0	34.4	17.5	92 (32.0)
Icterohaemorrhagiae	33.3	3.9	0.0	0.0	0.0	0.0	8.6	3.1	14.3	19 (6.5)
Pomona	0.0	23.4	27.7	0.0	0.0	0.0	34.3	21.9	2.3	51 (17.8)
Tarassovi	11.1	10.4	17.0	5.3	0.0	0.0	2.9	0.0	4.8	21 (7.3)
Sejroe (sv. Hardjo)	11.1	7.8	14.9	26.3	22.2	0.0	8.6	15.6	19.0	40 (14.0)
Grippotyphosa	22.2	2.6	10.6	0.0	0.0	0.0	0.0	9.4	27.0	28 (9.8)
Canicola	0.0	15.6	6.4	0.0	0.0	0.0	0.0	9.4	9.5	24 (8.2)
Mini (sv. Szwajizak)	0.0	7.8	0.0	0.0	0.0	0.0	0.0	6.3	4.8	11 (3.8)

[a]Frequency in each cell for each serogroup was obtained by dividing the number of samples positive for a serogroup in a province by the total number of positive serogroups in a province.

[b]ECP=Eastern Cape, GAUP=Gauteng, KZN=Kwazulu-Natal, LIMP=Limpopo, MPHP=Mpumalanga, NCP=Northern Cape, NWEP=North West, OFSP=Orange Free State and WCP = Western Cape.

In six out of the nine provinces of the South Africa, all the eight serogroups currently used as *Leptospira* spp. standard antigens for MAT were detected and the remaining three provinces, two to seven serogroups were not detected in cattle, **Table 3.4**. Limpopo Province, serogroups Canicola and Icterohaemorrhagiae were not detected, and in Northern Cape Province, antibody reaction was detected against only serogroup Australis (sv. Bratislava). Australis (sv. Bratislava) was the most predominant serovar in each of the nine provinces, with a frequency ranging from 57.0% (79/1133) of the total serogroups in Orange Free State Province to 48.8% (80/1155) of the North-western Province (**Table 3.4**). Tarassovi was the second most predominant serogroup in Western Cape, (28.4%, 42/145), Limpopo (27.1%, 13/48), Gauteng, 25.0% (31/124)**.** Generally, considering all provinces, serogroups Canicola and Szwaiizak were the least detected, comprising 5.8% (66/1133) and 6.9% (78/1133) respectively of the total overall 1,133 seropositive samples (**Table 3.4**).

In two out of the nine provinces of South Africa, all the eight serogroups currently used as *Leptospira* spp. standard antigens for MAT were detected in pigs, while in the remaining 7 Provinces; two to seven serogroups were not detected (**Table 3.5**)**.** Only Gauteng and Western-cape Provinces have all the eight serogroups antigens of *Leptospira* spp., used for MAT in South Africa. The highest serogroups were detected in Gauteng and Western-cape Provinces, 25.2 (75/286) and 21.3% (61/286) respectively (**Table 3.5).** In Northern Cape Province, antibody reaction was detected against only serogroup Australis (sv. Bratislava). Australis (sv. Bratislava) was the most predominant serogroup, (0.3%.1/286), **(Table 3.5).** Pomona was the second most predominant serogroup in North-Western cape, (34.5%, 12/34), followed by KwaZulu Natal, (27.7%, 13/49) (**Table 3.5**). The lowest was detected, serogroup Szwajizak, (6.3%, 2/32), (**Table 3.5**).

3.4.6 Frequency of detection of antibodies to serogroups of Leptospira spp. by provinces in cattle and pigs

Cattle: Of the overall 1, 419 livestock samples determined to be seropositive for antibodies to the serogroups of *Leptospira* spp., 1133 (79.8%) were from cattle and 286 (20.1%) were from pigs. There was a significant association between the proportions of the various seropositive serogroups and the category of animal species ($p<0.001$). All the eight serogroups in the panel used for the MAT assay were detected in the seropositive cattle and pigs (**Figure 3.7**)**.**

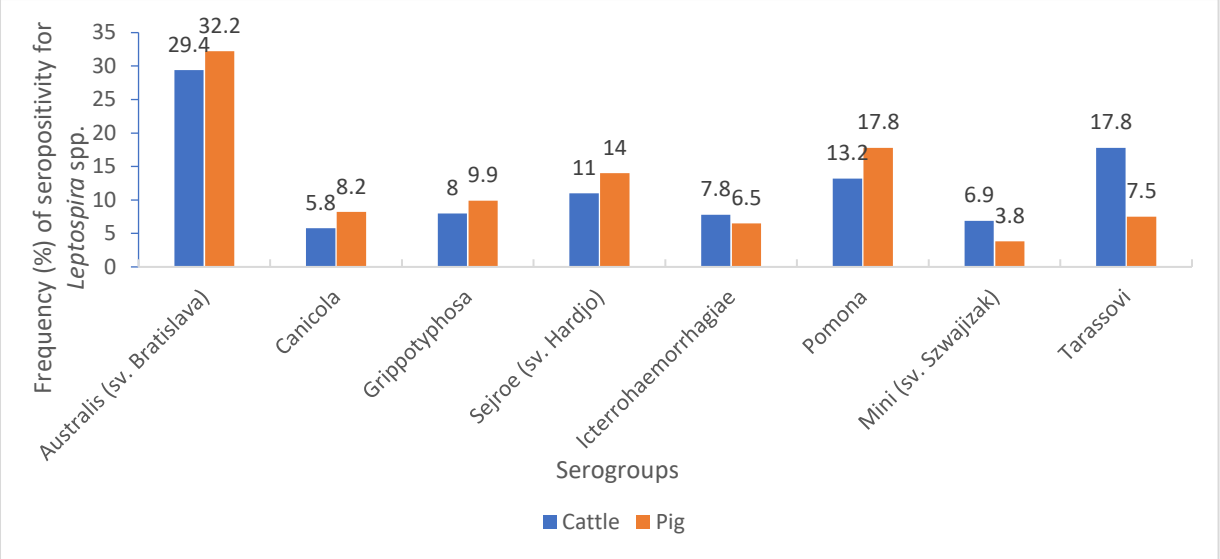

Figure 3.7: Frequency of detection of antibodies to serogroups of *Leptospira* species in cattle and pigs in nine provinces of South Africa. Results obtained from retrospective data of *Leptospira* seropositive samples (overall total 1,419) from the OVRI. Antibody detection had been done using the microscopic agglutination test (MAT) over a period of 11 years (2007 to 2017).

The highest frequency of antibodies in cattle was detected to serogroup Australis (sv. Bratislava) (29.4%, 333/1133), followed by Tarassovi (17.8%, 202/1133) and Pomona (13.2%, 150/1133), while the least detected antibodies to serogroups in cattle were Canicola (5.8%, 66/1133) and Szwajizak (6.9%, 78/1133) (**Figure 3.7**).

Pigs: The most predominant serogroups detected in pigs were Australis (sv. Bratislava) (32.2%, 94/292), Pomona (18.1%, 52/286) and Sejroe (sv. Hardjo), (14.3%, 41/286), while serogroup Szwajizak was least detected (3.8%, 11/286) (**Figure 3.7**). Of the eight serogroups tested, higher seropositivities were detected in pigs than cattle to five serogroups, namely Australis (sv. Bratislava), Canicola, Grippotyphosa, Sejroe (sv. Hardjo) and Pomona (**Figure 3.7**).

3.4.7 Frequency distribution of titres of antibodies to serogroups/serovars of Leptospira spp. by cattle and pigs

The frequency distribution of various titres for each serogroup for the review period was determined for both cattle and pigs. There were statistically significantly differences between proportions of the various titres for each serogroup, and between proportions of each titre across the various serogroups (p<0.001), in both cattle and pigs (**Table 3.6**).

In cattle serum samples, the predominant titres for all the eight serogroups were 1:100, 46.2% (523/1133), 1:400, 32.7% (3700/1133) and 1:200, 16.9% (191/1133) **(Table 3.6)**. Overall, a total 419 (36.9%) of the 1133 seropositive cattle samples had relatively high titres of ≥1:400 (**Table 3.6)**. For the three serogroups with a highest frequency of detection, namely Australis (sv. Bratislava), Tarassovi and Pomona, the frequencies of samples that showed titres ≥1:400 were 35.7% (119/333), 29.2% (59/202) and 54% (81/150), respectively, (**Table 3.6**).

Table 3.6: Frequency (%) distribution of antibody titres to each serogroup of *Leptospira* spp. in cattle and pigs

Animal species	No. (%) of samples positive for various titres of antibodies down each serogroup										
Cattle	**Titre**	**Australis (sv. Bratislava[a])**	**Canicola**	**Grippotyphosa**	**Sejroe (sv. Hardjo)**	**Icterrohaemorrhagiae***	**Pomona**	**Mini (sv. Szwajiza)**	**Tarassovi**	**Total**	***P*- value**
	100[b]	152 (45.6)	33 (50.0)	44 (48.4)	59 (47.2)	46 (52.3)	43 (28.7)	36 (46.2)	110 (54.5)	523 (46.2)	<0.001
	200[b]	62 (18.6)	14 (21.2)	14 (15.4)	15 (12.0)	14 (15.9)	26 (17.3)	13 (16.7)	33 (16.3)	191 (16.9)	<0.001
	400[b]	104 (31.2)	17 (25.8)	31 (34.1)	47 (37.6)	25 (28.4)	71 (47.3)	27 (34.6)	48 (23.8)	370 (32.7)	<0.001
	800	12 (3.6)	2 (3.0)	2 (2.2)	4 (3.2)	2 (2.3)	9 (6.0)	2 (2.6)	9 (4.5)	42 (3.7)	<0.001
	1600[c]	3 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)	0 (0.0)	0 (0.0)	2 (1.0)	6 (0.5)	<0.001
	3200[d]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	1 (0.1)	<0.001
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
	Sub-total	**333**	**66**	**91**	**125**	**88**	**150**	**78**	**202**	**1133**	
Pigs	100[b]	43 (45.7)	14 (58.3)	14 (48.3)	15 (36.6)	12 (63.2)	18 (34.6)	7 (63.6)	6 (27.3)	129 (44.2)	<0.001
	200[b]	24 (25.5)	3 (12.5)	6 (20.7)	12 (29.3)	4 (21.1)	15 (28.8)	1 (9.1)	4 (18.2)	69 (23.6)	<0.001
	400[b]	17 (18.1)	6 (25.0)	7 (24.1)	9 (22.0)	2 (10.5)	14 (26.9)	3 (27.3)	12 (54.5)	70 (24.0)	<0.001
	800	8 (8.5)	1 (4.2)	2 (6.9)	4 (9.8)	0 (0.0)	4 (7.7)	0 (0.0)	0 (0.0)	19 (6.5)	<0.001
	1600[c]	2 (2.1)	0 (0.0)	0 (0.0)	1 (2.4)	1 (5.3)	1 (1.9)	0 (0.0)	0 (0.0)	5 (1.7)	<0.001
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
	Sub-total	**91**	**24**	**27**	**41**	**19**	**51**	**11**	**22**	**286**	

Analysis for the titres of the serogroups was made from retrospective data of 1,419 (cattle, n=1133; pigs, n=286) seropositive samples for *Leptospira* species, as determined using microscopic agglutination test (MAT). Retrospective data from OVI-ARC, Pretoria, South Africa

[a] Serogroups with highest titres

[b] Predominant titres

[c] Intermediate titres

[d] Highest titres

* Icterrohaemorrhagiae

For pig sera, the most frequently detected antibody titre for all the eight serogroups was 1:100, 44.2% (129/292), followed by 1:400, 24.0% (70/292) and 1:200, 23.6% (69/292) **(Table 3.6).** Among the three most frequently detected serogroups in pigs, namely Australis (sv. Bratislava), Pomona and Sejroe (sv. Hardjo), 2.1% (2/94), 1.9% (1/52) and 2.4% (1/41) respectively of the samples showed the highest titre of 1:1,600, and the difference between the three proportions was not statistically significant (P=0.09). All the predominant titres for the cattle and pigs comprised all the 8 eight antigens used for MAT at ARC-OVR laboratory.

3.4.8 Comparison of the seropositivity and titres of vaccine and non-vaccine serovars

In South Africa, the vaccine used to prevent leptospirosis in livestock is a killed adjuvant, concentrated cultured vaccine combined with other pathogens such as *Trichomonas foetus* and *Campylobacter foetus*, in the same vial of vaccines. The vaccine contains serovars Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae and Pomona. These five vaccine serovars are included in the antigen panel used in the MAT, in addition to the following serovars, Bratislava, Tarassovi and Szwajizak (non-vaccine serovars).

For the vaccine serovars used for MAT typing, the frequency distribution among the total of 1425 seropositive samples was: Canicola (6.3%, 90/1419), Grippotyphosa (8.4%, 120/1425), Hardjo (11.6%, 166/1419), Icterohaemorrhagiae (7.5%, 107/1419) and Pomona (14.2%, 202/1418) (**Table 3.4**), while for the three non-vaccine serovars, namely Bratislava, Tarassovi and Szwajizak, their frequencies were 30% (427/1425), 15.7% (224/1425) and 6.2% (89/1425) respectively. Overall, there was no statistically significantly difference (p=1.0) between the frequencies of vaccine serovars (48.1%, 685/1425) and on-vaccine serovars (51.9%, 740/1425). Similarly, the frequency of the three non-vaccine serovars with titres of 400 and above (33.6%% (249/740) was statistically insignificant (p=0.06) to the corresponding frequency of vaccine serovars (38.5%, 264/685) used throughout South Africa and covered in the current study.

3.5 Discussion

It is significant to note that the sera tested and analysed in the current review of the laboratory-generated data originated from livestock suspected of leptospirosis, associated with outbreaks or destined for exportation. Due to the purposive nature, the seropositivity for *Leptospira* spp. in this study may therefore be higher than the expected country-level prevalence for apparently healthy livestock, which should be the true national seroprevalence for leptospirosis in animals. However,

the patterns of seropositivity detected for leptospirosis in this review provide information about the infecting serogroups of *Leptospira* spp. in both apparently healthy livestock (for export) and clinical cases (suspects and confirmed) sampled from the nine Provinces of SA. These outcomes have both clinical and economic implications for the livestock industry in South Africa.

Overall, a seropositivity rate of 20.5% (1,425/6,945) was obtained, with statistically significant higher rate in cattle, approximately, 25.0% (1,293/5,168) compared with pigs, 16.2% (286/1,763) and sheep, 0.0% (0/14) ($p = 0.001$). There is an over-representation of cattle in the test samples (74.4%, 5,168/6,945) compared with pigs (25.4%, 1,763/6,945) and sheep (0.2%, 14/6,945) ($p<0.05$). The disparity in the number of samples submitted for testing reflected the relative importance of cattle compared with other stock and the slaughter rates in South Africa in 2014/2015 (DAFF, 2016). All the risk factors/predictors (year, Province and animal species) assessed in the current study were statistically significantly in association with seropositivity for leptospirosis. Although, there is a widely varied seropositivity for antibodies to *Leptospira* spp. among the years considered, the underlying reason(s) for this observation was not immediately available. This was also partially because the data on outbreaks, suspect cases and apparently healthy animals tested for exportation were not immediately available.

It should be noted that the variations in seropositive rates among Provinces agreed with previous published studies which indicated that the differences in the environmental sanitation, occurrence of animal reservoirs amongst other factors are essential for the transmission of leptospires and affect the exposure potential for animals and seroprevalence to leptospirosis (Bharti et al., 2003; Gummow et al., 1999; Ko, Goarant and Picardeau, 2009; Suepaul et al., 2010; Vinetz, 2001). Other factors that may influence seropositivity for *Leptospira* spp. include degree of urbanization, poor settings, agricultural activities, hygiene, sample type and handling, antibodies presence or absence during the period of sampling, the number of serogroups in the panel of *Leptospira* antigens used as well as the diagnostic titre for the diagnosis and the technical ability of the personnel responsible for performing the MAT, among other factors (Adler and de la Moctezuma, 2010; Ko et al., 2009; Picardeau, 2013; Vinezt, 2001).

In this study, the animal species was risk factor and cattle have higher seropositivity (25.0%) than pigs (16.2%) (**Table 3.2** and **Table 3.3**). Similar findings were reported in Trinidad by Suepaul et al., (2011) who found a seroprevalence of leptospirosis of 21.5%, 5.0% and 5.0% in cattle, pigs, and sheep respectively. These findings may be associated with the different systems of livestock

management (Gummow et al., 1999). Other studies have documented the effect of animal species on the occurrence of leptospirosis (Kingscote, 1985; Potts et al., 1995; Suwancharoen et al., 2013; Suepaul et al., 2010). Factors such as differences in husbandry and management practices, vaccination practices, among others may be responsible, in part, for the animal species effect (Adesiyun et al., 2006; Gummow et al., 1999).

The overall seropositivity (20.5%) of antibodies to *Leptospira* spp. detected in this study is comparable to the 25.6% (863/3,377) reported for livestock in Malaysia by Bahama et al., (1987) but much higher than the 11.5% detected in 1635 livestock tested in Thailand by Suwancharoen et al., (2013).

In our study, seropositive cattle have the predominating *Leptospira* spp. being serogroup Australis (sv. Bratislava). Other studies from South Africa have reported a lower seroprevalence of 19.4% (392/2,021), with serogroups Tarassovi, Australis (sv. Bratislava), Sejroe (sv. Hardjo prajitno), Canicola and Icterrohaemorrhagiae in KwaZulu Natal Province (Hersterberg et al., 2009) or higher seroprevalence of 27.6% (55/199) with predominant serogroups, Sejroe (sv. Hardjo) and Mini (sv. Szwajizak) (Dogonyaro et al., 2020), and 52% (89/170), with predominance of serogroup Pomona (Gummow et al., 1999). The changes may reflect the changing patterns of serovars of *Leptospira* in the country as documented in other countries (Vinetz, 2001). This finding is slightly higher than 21.5% seropositivity in 590 apparently healthy cattle sampled in the Caribbean. In outbreaks of leptospirosis in cattle in Nicaragua, the seroprevalence of leptospirosis ranged from 15.4% to 26.4% and in Padua in Brazil, 41.5% (39/94) were documented to be seropositive (Mughini-Gras et al., 2014). For pigs, a seropositivity of 16.2% (286/1,763) was determined. This is similar to the 16% reported for pigs tested in Malaysia (Bahaman et al., 1987) and higher than the 5.0% detected in 200 pigs tested in Trinidad by Suepaul et al., (2011). Varying seropositivity for leptospirosis have been reported in apparently healthy pigs and in clinical cases (Bahaman et al., 1987). A considerably higher seroprevalence, 40.5% (558/1,378) of leptospirosis was reported in apparently healthy cattle in Malaysia ((Bahaman et al., 1987). A study in North West Morocco reported a seroprevalence of 15% (19/126) from the cattle tested (Benkirane et al., 2014). A lower seroprevalence of 9.9% was also detected in cattle tested in the provinces of Thailand by Suwancharoen et al., (2013). All these variations in seropositivity of leptospirosis in pigs reported, may be due to different factors such as system of management, sanitation, vaccinations, laboratory technical-know-how on the diagnosis and the environment (Adesiyun et al., 2006; Adler & de la Moctezuma, 2010; Gummow et al., 1999; Ko et al., 2009; Picardeau, 2013; Vinezt, 2001).

It was of interest that none of the sheep tested over the 11-year period was seropositive for leptospirosis. Although, the total number of sheep tested was small comparatively (n = 14/6,945) and this may not reflect the actual frequency of leptospirosis in the sheep population in the country. There is also the possibility that the use of only eight antigens in the MAT panel may have failed to detect antibodies to serovars infecting sheep but not tested for. In a study on sheep sampled in Malaysia, Bahaman et al., (1987) reported that 6.8% of the sheep tested were seropositive for leptospirosis while in Morocco, 18.0% of the sheep were seropositive for leptospirosis (Benkirane et al., 2014).

The MAT used to detect leptospirosis in this study has advantages and disadvantages (Picardeau, 2013) and the standardised cut-off diagnostic titre used was 1:100 which has been documented (Suepaul et al., 2011; World Organization for Animal Health (OIE), 2014; 2018). For diagnostic MAT, titres used for clinical cases varied geographically depending on the endemicity of leptospirosis in the animal population; a titre of over 400 has been used elsewhere (Faine, 1982). In this review, we evaluated for seropositivity at different titres and although the titre of 100 was the most prevalent in cattle (46.2%) and pigs (44.2%); the titres detected in seropositive cattle and pigs were relatively high with titres of 400 and higher found in 37.0% (419/1,133) and 32.2% (94/292) respectively. These findings suggest the clinical involvement of the infecting serogroups.

Although at least a 20-serogroup panel of antigens has been recommended for use to screen for leptospirosis to avoid under-reporting of the seroprevalence of leptospirosis (World Organization for Animal Health (OIE), 2014; 2018; WHO, 2003), only eight antigens in the MAT panel was used to detect antibodies to *Leptospira* spp. in this study. The cost of purchasing and maintaining > 20 serogroups (antigens) in the international panel may not be affordable for many laboratories in Sub-Saharan Africa thereby resulting in the use of fewer but carefully selected serogroups based on predominant or circulating serogroups (Levett et al., 2013). The ARC-OVR use eight serogroups as a policy based on the assumption that these were the predominant serogroups circulating in livestock population in South Africa and the perception that it is a cost-effective strategy.

Although the vaccination history of the livestock tested for leptospirosis in this study is unknown, the majority of the animals tested were suspect or clinical cases of leptospirosis, and it is unlikely that they were adequately vaccinated since vaccination is known to prevent clinical disease in animals (World Organization for Animal Health (OIE), 2014; 2018). Furthermore, the predominant serogroups detected in the 1,425 seropositive livestock in our current study included Australis (sv.

Bratislava), (30.0%) and Tarassovi (15.7%), which are not in the any of the vaccines used in South Africa. This is an indication that the seropositive livestock were naturally exposed to field strains of *Leptospira* spp. in the country. Furthermore, it cannot be over-emphasized that in comparing the serological data on leptospirosis reported in several studies it is important to consider the practice and types of serovars/antigens in vaccines used in the different countries and regions. This is because if serovars contained in the vaccines are also included in the panel used to perform the MAT, the occurrence of cross-reaction with the antibodies produced in response to vaccination may occur (World Organization for Animal Health (OIE), 2014; 2018) and can influence serological surveillance for leptospirosis.

The four vaccine serovars contained in the vaccine sold in South Africa, were in the panel of eight serogroups used to perform the MAT. Three of the serogroups: Australis (sv. Bratislava), Tarassovi and Szwajizak) used in screening for leptospirosis by MAT in the current study were not in the vaccines. Therefore, the seropositivity detected was most likely due to natural exposure to field strains of the serogroups. It is also important to consider the titres of the serogroups in the vaccines in the interpretation of the serological findings in the current study. To emphasize the role played in the positivity of antibodies to three non-vaccine serogroups, they contributed a non-statistically significantly (P = 1.0) higher frequency, 53.3% (760/1,425) than the five vaccine serogroups, 48.1% (685/1,425). Additionally, the frequency of the three non-vaccine serogroups with titres of 400 and above, 29.5% (421/1,425) was not statistically significantly ($p<0.06$) higher than the 17.0% (242/1,425) for the five vaccine serovars. Both findings are both indicators that the livestock in the current study were naturally exposed to the three non-vaccine serogroups: Australis (sv. Bratislava), Tarassovi and Szwajizak) and may have contributed significantly to the seropositivity and possibly, the clinical leptospirosis in the animals sampled, although, not statistically significant.

It is significant to have detected serogroup Australis (sv. Bratislava) at an overwhelming frequency considering the three risk factors (year, Province and animal species) investigated were significantly associated with the seropositivity for leptospirosis. Serogroup Australis (sv. Bratislava) was detected at significantly higher frequency in 9 (81.8%) of the 11 years of the review and in all nine provinces. Furthermore, Australis (sv. Bratislava) was the only serogroup detected in Northern Cape Province and in both cattle (29.4%) and pigs (32.2%), although the number of samples submitted from Northern Cape is insignificantly small (n = 4) to make statistical meaning in this analysis. The data suggest that this serogroup is important in causing infection and clinical cases in cattle and pigs in South Africa. More importantly, serogroup Australis (sv. Bratislava) is presently not included in the

vaccines used in livestock in South Africa, thus supporting the evidence for natural exposure. Therefore, it is imperative to consider inclusion of serogroup Australis (sv. Bratislava) in the vaccines in use in livestock in South Africa.

In published studies, different predominant serogroups have been detected in cattle and pigs, with some results in agreement or at variance with the findings in the current study. For example, the predominance of serogroups Icterohaemorrhagiae, Sejroe, Ballum and Autumnalis were documented in the Caribbean (Suepaul et al., 2011). Icterrohaemorrhagiae, Pomona, Tarassovi, Grippotyphosa, Canicola, Pomona, and Australis in Malaysia (Bahama et al., 1987); Sejroe (sv. Hardjo) in the Southern part of Uganda (Atherstone et al., 2014), Ballum, Australis and Sejroe, in North West Morocco (Benkirane et al., 2014) and Ranarum, Sejroe, Mini and Australis (sv. Bratislava) in Thailand (Suwancharoen et al., 2013).

Currently, in South Africa, there are no published reports on retrospective analyses of a large laboratory data on leptospirosis, therefore a comparison of the current study with existing data in the country was not possible. However, it is evident that analysis of the 11-year data on livestock leptospirosis regarding the seroprevalence of leptospirosis and some risk factors that affected its occurrence in the country has been very informative.

3.5.1 Limitations of the study

Several limitations were identified during the collation and analysis of the data. These include the following: (i) biodata (age, sex, breed, etc.) on the livestock from which the serum samples that were tested originated were either not easily accessible which limited our ability to assess the possible effects of more risk factors on seropositivity for leptospirosis; (ii) non-availability of information on the reasons the samples were submitted to ARC-OVR namely for diagnosis of leptospirosis, for monitoring of negative herds, suspicion of infections based on clinical signs, investigating potential reasons for low reproductivity of herds, export requirement, etc. (iii) lack of information on the vaccination history of the animals tested, therefore not possible to determine if the seropositivity and titres of the antibodies to the serogroups detected by the MAT resulted from vaccination due to cross-reactivity with vaccine serogroups or natural exposure to the pathogen, (iv) the use of only eight serogroups in the MAT diagnostic panel may have resulted in under-diagnosis of leptospirosis in the livestock teste and (v) currently, no active and passive surveillance of leptospirosis in the country is undertaken to lead to a better understanding of the distribution of the disease. Finally,

there were some observable inconsistencies in data over the eleven years period since it is not a controlled study.

3.5.2 Conclusions

It is concluded that since the data from the study determined the seropositivity for leptospirosis in mostly clinical and suspect cases, the livestock data evaluated cannot be representative of the entire livestock population in South Africa but a subset of animals presented (clinical case, suspect cases and animal meant for export). The outcome of this study from the data has clinical and economic significance for the livestock industry.

Although MAT, the gold standard, was used for the diagnosis of all the serum samples tested, the low sensitivity reported for MAT coupled with the use of only eight antigens in the panel of serogroups which is considerably lower than the recommended use of over 20 in the international panel, may have resulted in the under-diagnosis and under-reporting of leptospirosis in the livestock tested. All the 14 samples of sheep tested and were used in this study were seronegative for leptospirosis which may reflect the true seropositivity for leptospirosis in this animal species or the effect of the small sample size tested.

3.5.3 Recommendations

It is recommended that future livestock samples submitted to the ARC-OVR laboratory should be accompanied by relevant biodata of animals (age, sex, breed, reasons for submitting samples for diagnosis of leptospirosis and vaccination history) to enable more informed risk analysis and better control and prevention strategies, (ii) Efforts should be made to enlighten the livestock farmers in the country on the negative impact leptospirosis could have on animal production and the potential economic losses. This approach should encourage the livestock farmers across the country to screen their animals for leptospirosis by submitting samples to the ARC-OVR laboratory, (iii) A panel containing more antigens (serogroups), preferably 24 as recommended by the WHO, should be used to screen animals for leptospirosis or to validate a smaller panel (lower number of serogroups but with appropriated selection to coverage of the local Leptospira spp. circulating strains for use in SA. This approach should result in the MAT providing more accurate data on the status of leptospirosis and the circulating serogroups in livestock in the country; (iv) There should be active and passive surveillance of leptospirosis within the country for better understanding of the distribution of the diseases in South Africa, (v) Finally, there is a need for additional diagnostic techniques such as

bacteriological isolation of the pathogen, molecular diagnostic methods including both conventional and quantitative real-time PCR. Such an approach will lead to a better control and prevention strategy to boost agriculture and to curtail possible human exposure to leptospirosis.

3.5.4 Connecting statement to the next chapter

Based on the limitation and the recommendations of the retrospective analysis of laboratory data highlighted above in Sections 3.5.1 and 3.5.3, a cross-sectional study was conducted on slaughtered pigs in Gauteng province abattoirs, to determine the seroprevalence of leptospirosis and the potential risk posed to humans in the country.

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CHAPTER 4

Occurrence of antibodies to *Leptospira* spp. in slaughtered pigs at abattoirs in Gauteng Province, South Africa

Manuscript in preparation

4.1 Abstract:

Leptospirosis is an important global re-emerging, occupational and zoonotic disease. It is an under-estimated disease of veterinary importance caused by the pathogenic *Leptospira*. The disease causes reproductive problems and economic losses in the pig industry. However, little current information on leptospirosis in South Africa is available. A cross-sectional study was conducted in five Gauteng province abattoirs in South Africa to determine the seroprevalence, infecting serogroups and risk factors for leptospirosis in slaughtered pigs. At visits to selected abattoirs, blood samples were collected from 85 pigs alongside with demographic data on slaughtered pigs. The microscopic agglutination test was performed on sera using a 26-serovar (antigen) panel at a cut-off titre of 1:100. Variables were analysed for association with seropositivity for leptospirosis. The seroprevalence of leptospirosis was 24.7% (21/85), and antibodies were detected to only 2 (7.7%), Bratislava and Pomona, of the 26 serovars. This is the first reported study to use 26 antigens panel of Leptospira for testing pigs in South Africa. Serovar Bratislava was predominantly detected in seropositive pigs, 90.5% (19/21). Two variables, (abattoir and age), were significantly ($p<0.05$) associated with seroprevalence of leptospirosis in slaughtered pigs. Bratislava is the overwhelming *Leptospira* serovar circulating in pigs in South Africa and should be a vaccine candidate for pigs in the country and may be zoonotic to abattoir workers.

Keywords: Occurrence; Leptospirosis; Microscopic Agglutination Test (MAT); Pigs; Abattoirs; South Africa

4.2 Introduction

Leptospirosis is a significant emerging disease which affects human and animals, including livestock, causing significant morbidities and mortalities with impactful economic losses (Bharti et al., 2003; Costa et al., 2015). It is an occupational disease of abattoir workers and is associated with both crop and livestock farming activities, as well as being associated with recreational water activity. In developing countries, the spread of the disease is facilitated by socioeconomic factors such as

increased urbanization, rodent infestation, and poor sanitation. It has been established that leptospirosis is a more important zoonosis in the developing than developed countries (Vinetz, 2001). However, it has also been reported in developed countries where it is attributed to people living in slums (Ko et al., 1999; Vinetz et al., 1996). The transmission of the disease is through water contamination with leptospires from the urine of rodents or dogs (Ko et al., 1999; Vinetz, 2001).

In animals, the clinical signs observed include low milk production, abortion, stillbirth, infertility, death of animals, and a decrease in meat production (Martins et al., 2012; OIE, 2014). Other clinical signs are lethargy/depression, vomiting, fever, weight loss, polyuria/polydipsia, abdominal or lumbar pain, stiffness/arthralgia, renomegaly, diarrhoea, icterus, oculonasal discharge, petechiae, weakness and dyspnoea/cough (OIE, 2014). In severe leptospirosis, the mortality rate may be as high as 15% and antibiotic treatment can only be effective at early stage of leptospirosis diagnosis (Ko et al., 1999). It has been reported that leptospirosis is responsible for serious reproductive failure in the pig population globally (Cléia et al., 2002).

The seroprevalences of leptospirosis in pigs has been reported in many regions of the world. In Mexico, 25% seroprevalence was reported with serovars Bratislava, (51.0%) and Panama, (10.0%) as the predominant in seropositive animals (Vado-solis et al., 2002), and 66.1% seroprevalence with a predominance of serovar Icterohaemorrhagiae, (Martins et al., 2013). However, low seroprevalences have been reported in pigs from farms in Trinidad, 5.0% with serogroups Icterohaemorrhagiae (2.5%), Australis (2%) and Ballum (0.5%) (Suepaul et al., 2011). Seroprevalences of leptospirosis as an occupational disease have been reported from abattoirs (Cook et al., 2019). These abattoirs are facilities used routinely to slaughter animals, however, these abattoirs can be also used for active and passive surveillance of zoonotic diseases, such as leptospirosis. Abattoirs can also serve as major sources of important animal disease information or an environment for zoonotic disease transmission. This may be through the exposure of abattoir workers to body fluids (blood, and urine) of slaughtered infected livestock (Fasanmi et al., 2017). In slaughtered pigs, seroprevalences of leptospirosis in Brazil were 66.7%, serogroups Icterrohaemorrhagiae having the highest frequency of 79.1% in seropositive pigs (Cléia et al., 2002). In St. Kitts 64.8%; the result revealed serogroup Mankarso (Shiokawa et al., 2019) and in Kenya 32.9%; serovar Lora with the highest frequency (21.4%) (Ngugi et al., 2019). Seroprevalences of leptospirosis in abattoir workers have been confirmed in Western Kenya (Cook et al., 2019), New Zealand (Benschop et al., 2009; Dreyfus et al., 2014), Argentina (Chiani et al., 2016), and Nigeria (Abiayi et al., 2015). Most of these

seroprevalences were carried out using the MAT as a ''gold standard'' for the diagnosis of leptospirosis (OIE, 2018).

The microscopic agglutination test (MAT), also known as the Martin and Petit test, has been the 'gold standard' for serological diagnosis of leptospirosis (Faine, 2004; OIE, 2014, WHO, 2003). The advantages of this conventional test are: testing both individuals and herds, increased sensitivity in acute stage of infection and antibodies of other bacteria do not cross-react (OIE, 2018) but its limitations include issues of specificity and sensitivity (false-positive and false-negative results) may be in chronic stage, low accuracy, and poor-serogroup correlation after identification of the isolated strains (Picardeau, 2013). It is also cumbersome and time consuming (Picardeau, 2013; WHO, 2003).

In South Africa, De Lange et al. (1987), first reported the isolation of *Leptospira* serovar Pomona from porcine foetuses as well as, from the renal lymph nodes of slaughtered pigs showing chronic nephritis and detected this serovar in 17% (9/52) of pigs. Hunter et al., (1987), isolated serovar Pomona from 'white spot' lesions in kidneys in slaughtered pigs. Potts et al., (1995) reported a seroprevalence of 22.2% in slaughtered pigs from abattoirs in South Africa with the predominant serovars being Icterohaemorrhagiae (12.6%), Hardjo (12.1%) and Bratislava (7.5%). Gummow et al., (1999) identified the predominant serovar Pomona using serological and bacteriological methods on aborted cases studied, but identified other serovars (Harjo, Bratislava and Icterohaemorrhagiae). This was the last documentation of leptospirosis in South Africa in pigs. As a policy in South Africa, vaccination of animals, including pigs against leptospirosis is voluntary. However, in situations when an importer demands that pigs should have a history of vaccination against leptospirosis pre-shipment from South Africa, the pigs are vaccinated, and a certificate is issued by the attending veterinarian. Vaccines available in the country contain five serovars, namely, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae and Pomona. Testing for leptospirosis is currently performed at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) Bacterial Serology Laboratory, where the MAT is performed using an eight-serovar panel (Bratislava, Canicola, Icterohaemorrhagiae, Tarassovi, Pomona, Szwajijak, Hardjo prajitno and Grippotyphosa) to diagnose leptospirosis in the country. Bacteriological and molecular methods are not used in the diagnoses of leptospirosis in animals.

Currently, there are considerable knowledge gaps of leptospirosis in the country primarily due to the lack of any organized passive or active surveillance for leptospirosis in animals and humans, which is further exacerbated by the possibility of under-diagnosis and under-reporting of leptospirosis. This is

because, as the only diagnostic laboratory test uses an eight-serovar MAT panel and there is limited availability of qualified technical personnel to diagnose leptospirosis in the country. Therefore, the objectives of this study are stated bellow.

4.2.1 Specific objectives:

i). To use 26 panel of *Leptospira* spp. antigens to determine the seropositivity for infections and/or exposure by *Leptospira* spp. in pigs slaughtered at the abattoirs in Gauteng province, SA.

ii). To determine the seroprevalence of infecting serogroups and titres for leptospirosis in slaughtered pigs in Gauteng province, SA.

iii). To explore the risk factors for leptospirosis in slaughtered pigs in Gauteng province, SA.

4.3 Materials and methods

4.3.1 Study area

The study was conducted in Gauteng province, one of the nine provinces in South Africa. The province has the highest number of abattoirs in the country, comprising high throughput (HT), low throughput (LT) and rural abattoirs. In our study, we sampled pigs from five abattoirs distributed across the province **(Figure 4.1).** These pigs were from farms and feedlots outside Gauteng province but from within the country.

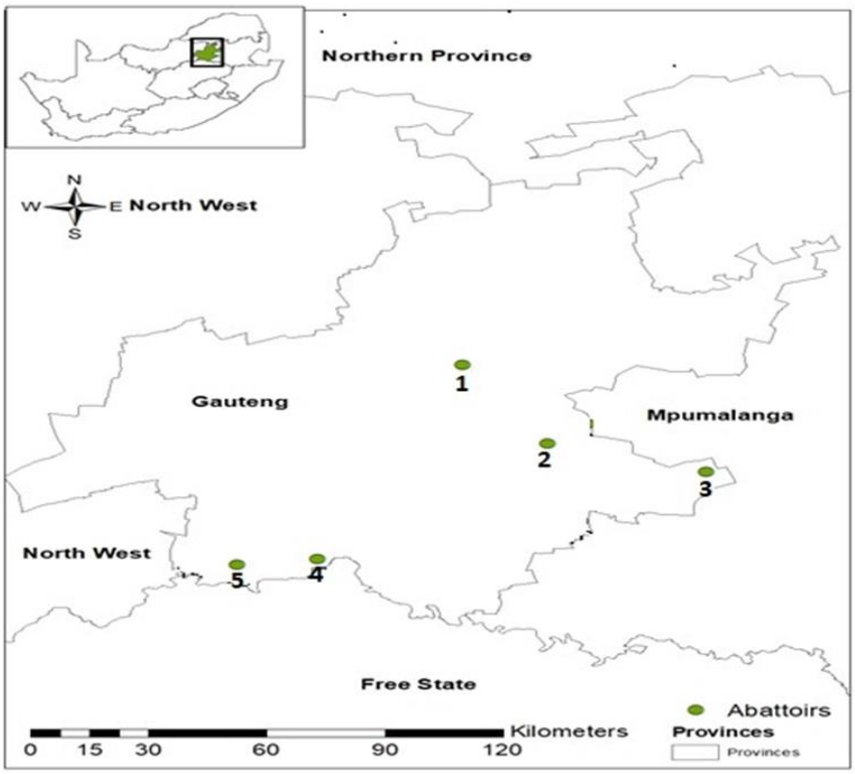

Figure 4.1: Map showing the locations of the 5 abattoirs in Gauteng Province, South Africa from where slaughtered pigs were sampled

The pig population in the country was reported to be approximately 1.5 million in 2014/2015 (DAFF, 2016). Geographic information system (GIS) data were collected using the nuvi® GPS navigator (Garmin, 2689 LMT., U.S.A.) during each visit to the abattoirs. The readings were entered into the Arc GIS program version 13.0 and the data used to plot **Figure 4.1**.

4.3.2 Sample size determination

To estimate the minimum sample size for the current study at a 95% confidence interval, the formula of Thrusfield, (2007) was used as follows: $n = [1.96^2 \ P_{exp} \ (1-P_{exp})]/d^2$, where n = required sample size, P_{exp} = estimated prevalence of leptospirosis and d = desired absolute precision. For the study, P_{exp} was 22.2% (Potts et al., 1995) and d was 9%. The estimated minimum sample size for the study was 82 and a total of 85 samples were sampled for the study.

4.3.3 Study design and abattoir selection

To conduct this cross-sectional study, a list of (n=28) functional abattoirs was obtained from the Veterinary Public Health section of Gauteng Department of Agriculture and Rural Development

(GDARD). We randomly selected 14 abattoirs and only five abattoirs slaughtered pigs solely or in combination with other animal species (cattle and sheep); efforts were made to use more pigs abattoirs but these were the only five abattoirs (Hight throughput (n=3); Low throughput (n=2); that their managers or owners consented to the collection of samples from their abattoirs which resulted to the small sample size. The locations of the five abattoirs are indicated in **Figure4. 1.** Samples from these abattoirs were collected between September 2016 and April 2017.

4.3.4 Demographic data

Demographic data were obtained from the abattoir managers at the five consented abattoirs. These included the abattoir-level information, the location of abattoirs (1-5), throughput (HT = slaughtering more than 20 pigs per/day and LT = slaughtering 20 or less than pigs per/day) and animal species slaughtered per abattoir (multi-species = slaughtering any two or more of cattle, pigs and sheep; and mono-species = slaughtering only pigs). The pig variables obtained were age (young = less than 1 year and adults = 1 year and above) and sex (male and female). All the pigs slaughtered belonged to the Large White breed or its crosses only.

4.3.5 Collection and processing of samples

At slaughter, whole blood samples were collected aseptically using sterile 50 mL screw capped sterile plastic cups. Approximately 10 mL of the whole blood was aliquoted into 10 mL sterile yellow screw-capped vacutainer tubes, without anticoagulant, from where sera were harvested after centrifugation at 8000 rpm for 5 minutes in an Eppendorf centrifuge 5810R (Germany®). Sera were stored in properly identified cryovials at -20°C for further testing.

4.3.6 Detection of antibodies to Leptospira spp. using the microscopic agglutination test (MAT)

The microscopic agglutination test (MAT) was performed following standard protocols (OIE, 2018, WHO, 2003) at the ARC-OVR using Sorenson's medium with 8 reference antigens of *Leptospira* spp., and at the Department of Epidemiology and Microbial Diseases, Yale University School of Public Health, New Haven, Connecticut, USA using (a 26 serovar panel (**Table 4.1**).

Table 4.1: The 26 Leptospira reference antigens used for Microscope Agglutination Test (MAT) in the study

Serial No.	Serogroup	Serovar	Strains
1	Djasiman	Djasiman	Djasiman
2	Mini	Szwajizak[b]	Szwajizak
3	Hebdomadis	Hebdomadis	Hebdomadis
4	Tarassovi	Topaz	94-79970/3
5	Ballum	Arborea	Arborea
6	Javanica	Javanica	Veldrat B-46
7	Medanensis	Medanensis	Hond HC
8	Sejroe	Hardjo	Lely 607
9	Panama	Panama	CZ 214 K
10	Icterohaemorrhagiae	Icterohaemorrhagiae[a,b]	RGA
11	Sejroe	Hardjo[a,b]	Hardjo prajitno
12	Tarassovi	Tarassovi[b]	Perepelitsin
13	Bataviae	Bataviae	Swart
14	Pomona	Pomona[a,b]	Pomona
15	Celledoni	Celledoni	Celledoni
16	Canicola	Canicola[a,b]	Hond Ultrecht IV
17	Cynopteri	Cynopteri	3522C
18	Grippotyphosa	Grippotyphosa[a,b]	MoskvaV
19	Ballum	Ballum	Mus 127
20	Shermani	Shermani	1342 K KIT
21	Australis	Bratislava[b]	Jez Bratislava
22	Pyrogenes	Robinsoni	Robinsoni
23	Hebdomadis	Kremastos	Kremastos
24	Autumnalis	Bulgarica	Nikolaevo
25	Pyrogenes	Zanoni	Zanoni
26	Australis	Australis	Balico

[a]Serovars in the vaccines used in South Africa

[b]Eight serovars used in the MAT panel at ARC-OVR

Briefly described, the sera were diluted at 1:50 with Sorensen's media for preliminary screening for antibodies against *Leptospira* spp. using live culture antigens (approximately 2×10^8 leptospires per mL) of *Leptospira* serovars (OIE, 2018). Prior to the use of the 26 panel of antigens in the MAT, the strains were sub-cultured, weekly, in 10 mL of Ellinghausen, McCullough, Johnson and Harris medium (EMJH) (Becton-Dickinson Biosciences, Sparks, Maryland, USA) in sterile transparent screw-cap tubes and incubated at 29°C (Ellis, 1986; OIE, 2018). These cultures were checked for bacterial growth density of 1 - 2×10^8 *Leptospira* per mL and used for weekly testing after 5-7 days of post inoculation. The serogroups (antigens-) (**Table 4.1**) used in the study were obtained from the Royal Tropical Institute, Amsterdam, Netherlands and at the Department of Epidemiology and Microbial Diseases, Yale University School of Public Health, New Haven, Connecticut, USA.

All samples that were seropositive at the screening dilution of 1:50 were thereafter subjected to a two-fold dilution titration (1:100 to 1:3,200) to determine the final titre. The end point observed under the dark field microscope (Carl Zeiss microscope, Imager 2®, USA.) was the dilution of serum samples that showed 50% agglutination, leaving 50% free leptospires compared with the control culture diluted at 1:2 with phosphate buffered saline (PBS). The tested sera had the same dilution ratio with the control test. Any sample that was positive at a titer ≥1:100 to any of the serovars by one or both laboratories were classified as positive for leptospirosis. The results were described as the presumptive infective serogroup based on the serovar with the highest titer for each animal. In case there was multiple serovars belonging to multiple serogroups that had the highest titer, the animal was considered positive with unknown presumptive serogroup.

4.3.7 Statistical analyses

4.3.7.1 Descriptive and Univariate analysis

Univariate analysis was conducted to determine the association between the serological status of pigs with the identified potential risk factors. The serological status was considered as a binary outcome (positive or negative), and the predictor variables for pigs were abattoir location (no. 1 to 5), type of abattoir (multi-species, mono-species), throughput of abattoir (low throughput (LT), high throughput (HT), sex (male, female), and age (adult, young). Each predictor variable was tested for significant associations with the serological status using the chi-square test or Fisher exact test.

4.3.7.2 Ethical Approvals

Prior to the commencement of the study, approvals were obtained from the following bodies and committees: Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, University of Pretoria, South Africa (AEC: ***v084-16***), **Figure 3.2,** Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) (AEC: ***12-16***), **Figure 3.3** and Section 20 according to Act 35 of 1984 by the Director of Animal Health at the Department of Agriculture, Forestry and Fisheries (DAFF), (Number: ***FY2015/2016***), South Africa, **Figure 3.4,** as shown in **Chapter 3, *section 3.3.5 (Ethical Aprrovals).***

4.4 Results

4.4.1 Descriptive and univariate association analysis

Overall, the seroprevalence of leptospirosis in pigs was 24.7% (21/85, 95% Cl 15.5-33.9). The descriptive statistics and univariate associations between individual animal-level risk factors and seropositivity for *Leptospira* spp. antibodies amongst pigs from the five abattoirs (nos. 1, 2, 3, 4 and 5) are shown in **Table 4.2.**

Table 4.2: Descriptive statistics and univariate associations between potential animal-level risk factors and infection with *Leptospira* species as determined by Microscopic Agglutination Test (MAT) in pigs from five abattoirs in Gauteng Province, South Africa

Variable	Category	No. positive/No. tested (%)	*p*-value
Abattoir location	1	7/10 (70.0)	0.001
	2	0/15 (0.0)	
	3	1/10 (10.0)	
	4	7/20 (35.0)	
	5	6/30 (20.0)	
Abattoir type	Mono[a]	6/30 (20.0)	0.60
	Mult[b]	15/55 (27.3)	
Throughput	HT	7/40 (17.5)	0.21
	LT	14/45 (31.1)	
Age	Adult	8/53 (15.1)	0.01
	Young	13/32 (40.6)	
Sex	Male	16/58 (27.6)	0.43
	Female	5/27 (18.5)	

[a]Mono: Mono species

[b]Multi: Multi-species

4.4.2 Risks factors analysis

Of the five variables investigated (abattoir, throughput, abattoir type, age, sex), only two (abattoir, age) showed statistical significance ($p<0.05$) with serological status of pigs in the univariate analysis, as shown in **Table 4.2.** Out of the five abattoirs, four had at least one pig positive for anti-*Leptospira* spp. antibodies. The highest exposure to *Leptospira* spp. was observed in abattoir no. 1, with 7 positive out of the 10 pigs sampled, followed by abattoir no. 4 (7 positive out of 20 pigs sampled), while no pig was positive in abattoir no. 2 (n=15 pigs sampled). A bigger proportion of *Leptospira* spp. seropositivity was observed in young pigs (40.6%, 13/32) than in adult pigs (15.1%, 8/53).

4.4.3 Serogroups/serovars and titres of Leptospira spp.

Of the 26 *Leptospira* spp. antigens contained in the MAT panel, antibodies were detected to only 2 antigens, Bratislava and Pomona, and the positive result for the two antigens was observed in 21 pigs out of the overall 85 pigs sampled (**Table 4.3**).

Table 4.3: Titres of *Leptospira* antibodies to serogroups/serovars determined from sera of pigs slaughtered at abattoirs in Gauteng province, South Africa.

	Percentage of positive samples (no. positive and total of positive samples for serogroups[a]			
Titre[b]	**Bratislava**	**Pomona**	**Total**	**p-value**
100	47.4 (9/19)	0.0 (0/2)	42.9 (9/21)	p=0.001
200	36.8 (7/19)	50.0 (1/2)	38.1 (8/21)	P=0.01
400	5.3 (1/19)	0.0 (0/2)	4.8 (1/21)	P=0.001
800	10.5 (2/19)	0.0 (0/2)	9.5 (2/21)	P=0.001
1600	0.0 (0/19)	50.0 (1/2)	4.8 (1/21)	P=0.001
Total no. of pigs	19	2	21	

[a] Of a total of 26 serovars included in the MAT assay, 2 (8%) antibodies were detected in pig sera
[b] The highest titre was observed for the serovar Pomona,1,600 comprising 5% of the positive samples (1/21)

The proportions of pigs seropositive for two serogroups were 22.4% (19/85) for Bratislava and 2.4% (2/85) for Pomona **(Table 4.3).** Of the 21 sera positive for antibodies of serovars to *Leptospira* spp., serogroup Bratislava was detected in 90.5% (19/21), and Pomona in 9.5% (2/21).

The titres of antibodies to the *Leptospira* serovars, Bratislava and Pomona, in the pigs are shown in **Table 4.3,** ranging from 100 to 1,600, and these showed a statistically significant difference (P<0.05) when the proportions of pigs positive for each titre between the two serovars were compared. Overall, the most frequently detected seropositivity was found in titres 100 (42.8%, 9/21) and 200 (38.1%, 8/21). The highest seropositivity for Bratislava serovar was at titre 100 (47.4%, 9/19), followed by titre 200 (36.8%, 7/19), while equal frequency 50.0%, (1/2) of seropositivity in pigs was recorded for titres 200 and 1,600 as the highest titre for serogroup Pomona (**Table4. 3**).

4.5 Discussion

The seroprevalence of leptospirosis in pigs sampled from the five abattoirs in Gauteng province was 24.7% (21/85). This is comparable to an earlier report by (Potts et al., 1995) who confirmed 22.2% seroprevalence for leptospirosis in slaughter pigs in South Africa 25 years ago, although (De Lange et al., 1987) had earlier reported a slightly lower seroprevalence of 17%. Despite the 25 years period apart, and the use of eight and 26-serovar panel in 1995 and 2017 respectively, the results were comparable. These findings suggest that the use of a panel of serovars which contain the predominant serovars circulating in the pig population in the province may be adequate. It has been recommended by previous workers that serovars included in the MAT panel should represent the prevalent serovars in animal reservoirs such as rodents or livestock present in different geographical locations or countries, (Cruz-Romero et al., 2018; Ngugi et al., 2019; Shiokawa et al., 2019).

Our data also suggest that there has not been a significant change in the occurrence of leptospirosis in slaughter pigs in Gauteng province over the last 25 years. However, using a larger sample size will be required to accurately determine the current status of infections by *Leptospira* spp. in pigs in the country. The 24.7% seroprevalence of leptospirosis in slaughtered pigs may be representative of the rate of infections of pigs at different pig farms in Gauteng province. Leptospirosis is known to cause clinical manifestations such as lethargy, depressions, fever, weight loss and stiffness as well as reproductive problems including infertility, stillbirths, abortion and death (Ko et al., 1999; Martins et al., 2012; OIE, 2014) which all have negative economic consequences for the pig industry in the country. Compared to other developing countries where the seroprevalence of leptospirosis in pigs at abattoirs or pig farms was determined by MAT using a diagnostic titre of 1:100, the findings have varied considerably as reflected in Trinidad, 5.6% (Suepaul et al., 2011) and Thailand, 11.3% (Chadsuthi et al., 2017) which are lower than found in our study, but higher seroprevalences have been documented in Kenya, 32.9% (Ngugi et al., 2019), Columbia, 55.8% (Calderón et al., 2014), Mexico, 61.0% (Cruz-Romero et al., 2018) and St. Kitts, 64.8% (Shiokawa et al., 2019). It is important to consider factors that may aggravate or hamper the seroprevalence of leptospirosis which include management systems (intensive, semi-intensive and extensive), sanitary conditions and rodent control on farms, and the MAT (number and type of serovars in circulation) (OIE, 2014; Picardeau, 2013; Shiokawa et al., 2019; Smythe et al., 2009).

It was of diagnostic relevance that of the 26 serovars present in the MAT panel, antibodies were detected to only two (Bratislava and Pomona). In this current study, it is important to note that, there were multiple reactions of Leptospira antibodies serogroups in one or more samples, in the

case of multiple reactions, we considered the highest titre of multiple serovars/serogroups that reacted as positive, therefore at the end we had only serogroups Bratislava and Pomona as the most prevalent.

The preliminary inference that can be drawn was that the remaining 24 serovars may not be circulating in the pig populations around Gauteng. This has cost and labor implications regarding the purchase and maintenance of serovars irrelevant to infection of pigs in Gauteng province by serovars of *Leptospira* spp. It will be necessary to investigate circulating serovars in South Africa so as to prioritize those with diagnostic importance to South Africa and to preferably include such in the test panel. Differences in the detection of antibodies to serovars in the MAT panel have been documented which have led to the recommendations that in addition to using serovars common in a geographical area, the sensitivity of the test is enhanced if the local isolates of the serovars are included in the panel (OIE, 2014; Picardeau, 2013; Smythe et al., 2009).

Bratislava is unquestionably the predominant serogroup circulating in the pig population in Gauteng province and possibly across the country. This is because the serovar had a seroprevalence of 22.4% (19/24) and it accounted for 90.5% of all MAT positive results. This serovar could therefore be an important aetiological agent of porcine leptospirosis with potential economic implications for the livestock industry in the country. In the three earlier reports on leptospirosis in pigs in South Africa, the predominant serovars in apparently healthy slaughtered pigs were Pomona (De Lange et al., 1987) and Icterohaemorrhagiae and Hardjo (Pott et al., 1995) while in the three case studies conducted on pigs diagnosed of clinical leptospirosis, Pomona was the predominant serovar detected both serologically and bacteriologically (Gummow et al., 1999).

Our findings therefore suggest that although the overall seroprevalence of leptospirosis did not change significantly over a 25-year period (1995 versus 2020), the change in predominantly infecting serovars was evident, a finding that may need further validation. Changes in the patterns of infecting serovars of *Leptospira* serovars have been documented in livestock elsewhere (Chadsuthi et al., 2017; Hartskeerl et al., 2011; Thornley et al., 2002). Varying predominance of serovars of *Leptospira* have been reported in pigs. The predominance of serovar Bratislava in pigs in this study agreed with reports from other studies, for example, in Mexico (Vado-solís et al., 2002), Germany (Strutzberg-Minder and Kreienbrock 2011), and St. Kitts (Shiokawa et al., 2019). Serovar Bratislava has been isolated from pigs and associated with clinical porcine leptospirosis by others (Arent et al., 2016; Boqvist et al., 2003; Naito et al., 2007). However, different serovars were prevalent in other

studies, for instance, Icterohaemorrhagiae in Trinidad (Suepaul et al., 2011), Sejro in Poland (Wasiński and Pejsak 2010), Pomona in Mexico (Cruz-Romero et al., 2018), Tarassovi in Brazil1 (Fernandes et al., 2020), Lora in Kenya (Ngugi et al., 2019) and Shermani and Ranarum in Thailand (Chadsuthi et al., 2017).

It was no surprise that serovar Pomona was detected in 2.4% of the pigs sampled, albeit at a fairly low seroprevalence, since the same serovar has been predominant in apparently heathy, slaughtered pigs and clinical cases in previous studies in South Africa Africa (De Lange et al., 1987; Gummow et al. 1999). This serovar, Pomona, is recognized as an important agent for reproductive problems (Gummow et al., 1999; Strutzberg-Minder and Kreiebrock 2011).

Although a majority, 85.7% (18/21) of the titres detected to the two serogroups were low (400 and lower), significant titres (800 and above) for disease were detected in 3 (14.3%) which may have been associated with clinical leptospirosis undetected at the abattoir (Bertelloni et al., 2018; Gummow et al., 1999). It cannot be ignored that one of the samples positive for antibodies to serogroup Pomona had a titre of 1:1600 which could reflect active infection or recovery from recent clinical infection.

The potential interference of vaccination with surveillance cannot be ignored, although it has been reported to be minimal (Balakrishnan and Roy, 2014; Júnior et al., 2007; Martin et al., 2018). It is pertinent to mention the fact that the predominant serovar Bratislava detected in our study is not part of the serovars included in the vaccines used in pigs and other livestock in South Africa. It can therefore be inferred that the antibodies detected to the serovar were elicited as a result of natural exposure, this may be of clinical and diagnostic importance. The fact that serovar Bratislava is highly prevalent and circulating in the pig population in Gauteng province and because it is not contained in any of the vaccines currently used in the country are strong indications that it should be a strong candidate for inclusion in future vaccines for use in pigs in the country. It has been demonstrated that the titres of antibodies to *Leptospira* spp. induced following vaccination of livestock is for a short or variable duration (Balakrishnan and Roy, 2014; Martin et al., 2018) and in most adult livestock the titres detected are likely due to natural exposure. Unfortunately, vaccination of animals against leptospirosis is voluntary in the country and there was no history of vaccination in the slaughtered pigs in our study.

The seroprevalence of 24.7% (21/85, 95% Cl 15.5-33.9), was indicative of risk of exposure to *Leptospira* spp. The location of abattoirs, farms of origin of pigs sent for slaughter, topography of the farm and the management systems, have been documented to significantly affect infection of pigs by leptospirosis (Cruz-Romero et al., 2018; Gummow et al., 1999; Ngugi et al., 2019; Shiokawa et al., 2019). Furthermore, the age of pigs also influences exposure to *Leptospira* spp., although this finding is at variance with the observation of Suepaul and colleagues. (2011) who did not detect a significant association between age of pigs and leptospirosis. Ngugi and co-workers, (2019) however reported that older pigs had an odds ratio of 1.9, indicative that they were 1.9 times more likely to be exposed to *Leptospira* spp.

4.5.1 Limitations, Conclusions and Recommendations

A limitation of the study is our inability to obtain the history of vaccination against *Leptospira* spp. and evidence of prior clinical leptospirosis, and the low sample size which was dictated by the number of pigs slaughtered and available for sampling during the study period. Serogroup/serovar Australis (sv. Bratislava) remains the most prevalent circulating serogroup of *Leptospira* spp. in the pig population studied in Gauteng province, South Africa but while inference can be drawn on the predictors and risk factors, none was significant in this study. The occurrence of swine leptospirosis in slaughtered pigs in abattoirs in Gauteng province poses zoonotic risk to abattoir workers and is of economic significance to pig producers in the country. A more comprehensive and representative study is recommended for South Africa. This may influence the decision of vaccine content and inclusive serovars in future vaccine production for South Africa.

4.5.2 Connecting statement to the next chapter

Based on the limitation and the recommendations of the cross-sectional study in pigs as mentioned in section 4.6, a cross-sectional study was conducted on slaughtered cattle in Gauteng province abattoirs, to determine the seroprevalence of leptospirosis and the potential risk posed to humans in the country.

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CHAPTER 5

Seroepidemiology of *Leptospira* infection in slaughtered cattle in Gauteng province, South Africa

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Tropical Animal Health and Production,* https://doi.org/10.1007/s11250-020-02417-0*) online.

5.1 Abstract

Leptospirosis is an important economical disease of livestock globally, especially in Asia, the Caribbean and the African continent. Its presence has been reported in a wide range of livestock. However, information on leptospirosis in South Africa is scanty. We conducted a cross-sectional study in 11 randomly selected abattoirs to determine the seroprevalence and risk factors for leptospirosis in slaughtered cattle in Gauteng province, South Africa. During abattoir visits to selected abattoirs, blood samples were collected from 199 cattle and demographic data obtained on the slaughtered animals. The microscopic agglutination test (MAT) was performed on all sera using a 26-serotype panel using cut off titre ≥ 1:100. Animal- and abattoir-level risk factors were investigated for their association with seropositivity for leptospirosis. The seroprevalence of leptospirosis in the cattle sampled was 27.6 % (55/199). The predominant serogroups detected in seropositive cattle were *Sejroe (sv.* Hardjo*)* (38.2%) and Mini sv. Szwajizak) (14.5%) but low to Canicola (sv. Canicola) (1.8%) and Pomona (sv. Pomona) (1.8%). The differences were statistically significant (P<0.05). Of the five variables investigated, only one (abattoirs) had statistically significantly (P<0.001) differences in the seroprevalence of leptospirosis among abattoirs. The study

documented for the first time in South Africa, the occurrence of serogroups Sejroe (Hardjo bovis strain lely 607), Tarassovi, Hebdomadis and Medanensis in slaughtered cattle. It was concluded that six of the nine serovars (representing seven serogroups) of *Leptospira* spp. circulating in cattle population in South Africa are not vaccine serogroups. The clinical, diagnostic and public health importance of the findings cannot be ignored.

Key words: Seroepidemiology**,** Leptospirosis**,** Cattle, MAT**,** Abattoirs and South Africa.

5.2 Introduction

Leptospirosis is an important zoonotic disease of public and animal health importance worldwide and is caused by pathogenic spirochete of the genus *Leptospira* (Haake, 2000). Leptospirosis is an environmentally transmitted disease and a susceptible host is infected when in contact with water or soil contaminated with urine of a reservoir animal. However, infection can also occur after direct exposure to tissues and fluids of infected animals (Faine et al., 1999; Dhewantara et al., 2019). There is a wide range of animals from livestock, companion animals and wildlife that have been identified as carriers or reservoirs for pathogenic *Leptospira* spp. and can shed the bacteria in their urine without symptoms (Bharti et al., 2003; Adler and Moctezuma 2010).

Leptospirosis is a life-threatening disease for humans. Recently it has been reported that there are over 1 million cases of leptospirosis around the globe and the mortality is up to 60,000 death per year (Costa et al., 2015; Torgerson et al., 2015). Levett, (2001) reported the existence of over 300 serovars of *Leptospira* spp. categorized into 25 serogroups. It has also been reported that 17 pathogenic *Leptospira* spp. circulate worldwide with 21 intermediates that cause non-severe clinical manifestation (Vincent et al., 2019). Mortality rates of leptospirosis in animals and humans have reported 40% aborted cases and above in cattle (Spickler and Leedom, 2013) and over 60,000 death per year in humans (Costa et al., 2015).

Leptospirosis is mostly under-diagnosed given its non-specific flu-like symptoms at early stages of the disease and the lack of good diagnostic methods (Levett, 2001). The microscopic agglutination test (MAT) is considered the “gold standard” for serological diagnosis of leptospirosis, especially in epidemiological studies (World Organization for Animal Health (OIE) 2014; 2018). The advantages and disadvantages of the MAT as a diagnostic test are well documented in the literature (Brandáo et al., 1998; Levett, 2001; Smythe et al., 2009; World Organization for Animal Health (OIE) 2014).

Although the major risk of the disease is upon inhabitants of developing countries living in conditions of poverty and/or lack of basic sanitation leptospirosis, being a zoonosis, has been transmitted to abattoir workers (Almasri et al. 2019; Cook et al. 2017; Dreyfus et al. 2015). Abattoirs used for the slaughter of livestock in any country are vital to conduct active and passive surveillance for diseases, particularly zoonoses such as leptospirosis (Ngugi et al. 2019).

In South Africa, the serological evidence of cattle leptospirosis was first reported in the Western Cape province by van der Merwe, (1967) using the MAT with a seroprevalence of 2.5%, while Gummow et al. (1999) reported a seroprevalence of 52% with a predominance of serovar Pomona in the Eastern Cape province. Hesterberg et al., (2009) documented a seroprevalence of 19.4% for leptospirosis in cattle in rural communities in KwaZulu-Natal province and found serovar Pomona to be most frequently detected. To date in South Africa, the antigens that have been used in previous studies are Pomona, Tarassovi, Bratislava, Canicola, Hardjo, Icterohaemorrhagiae, Szwajizak, Grippotyphosa, Bulgarica, Hyos, Robinsoni, and Saxkoebing (Gummow et al., 1999; Hersterberg et al., 2009; Van der Merwe, 1967).

In the country, current data are unavailable in cattle, which is created primarily due to the lack of active surveillance, the use of only eight serovars in the panel of antigens for the serodiagnosis of leptospirosis, limited technical-know-how on the serological testing and culture of leptospires.

5.2.1 Specific objectives

Therefore, the objectives of the study were to use the international panel of 26 serovars with MAT to determine the seroprevalence of leptospirosis in cattle slaughtered at abattoirs in Gauteng province, to compare the seropositivity for leptospirosis using both 8- and 26-serovar serovar panels, to determine the types and titers of serogroups of *Leptospira* spp. circulating in livestock and finally, to investigate the risk factors associated with infection by *Leptospira* spp. in cattle at the abattoirs.

5.3 Materials and methods

5.3.1 Policy on prevention and surveillance for leptospirosis in South Africa

It has been documented that leptospirosis is endemic in animal and human populations in South Africa (Botes and Garifallou, 1967). Leptospirosis is not a reportable disease in the country and vaccination and testing for the disease is voluntary. The commercially available vaccines to prevent the disease contain five serovars namely, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae and

Pomona. The diagnosis of leptospirosis in the country is centralized at the leptospirosis reference laboratories based at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) in Pretoria, South Africa. The MAT is the standard test used to confirm the diagnosis of leptospirosis using an 8-serovar panel consisting the five vaccine serovars mentioned above plus serovars Bratislava, Tarassovi and Szwajizak. Sera for testing for leptospirosis are normally submitted by individual livestock owners, private and government veterinarians.

5.3.2 Study area

The cross-sectional study was conducted in Gauteng province of South Africa. Gauteng province is the smallest province in South Africa and has the highest number of abattoirs in the country, consisting of both high throughput (HT) and low throughput (LT) abattoirs which slaughter animals originating from all the nine provinces of the country.

5.3.3 Sample size determination

To estimate the sample size for the current study, with a 95% confidence interval, the following formula (Thrusfield, 2007) was used: $n = [1.96^2 P_{exp} (1-P_{exp})]/d^2$, where n = required sample size, P_{exp} = estimated prevalence of leptospirosis and d = desired absolute precision. This is because there is a dearth of current data on livestock leptospirosis in the country. For the study, P_{exp} was estimated at 50% and d was 7.0%. The estimated minimum sample size for the study was therefore 196 animals.

5.3.4 Selection of abattoirs

The list of red meat abattoirs including their names, throughput, location and operational status (active or non-active) was obtained from Gauteng Department of Agriculture and Rural Development (GDARD). From the list, 11 abattoirs were randomly selected from a total 35 abattoirs for the study from where samples were collected between September 2016 and April 2017 **(Figure 5.1**).

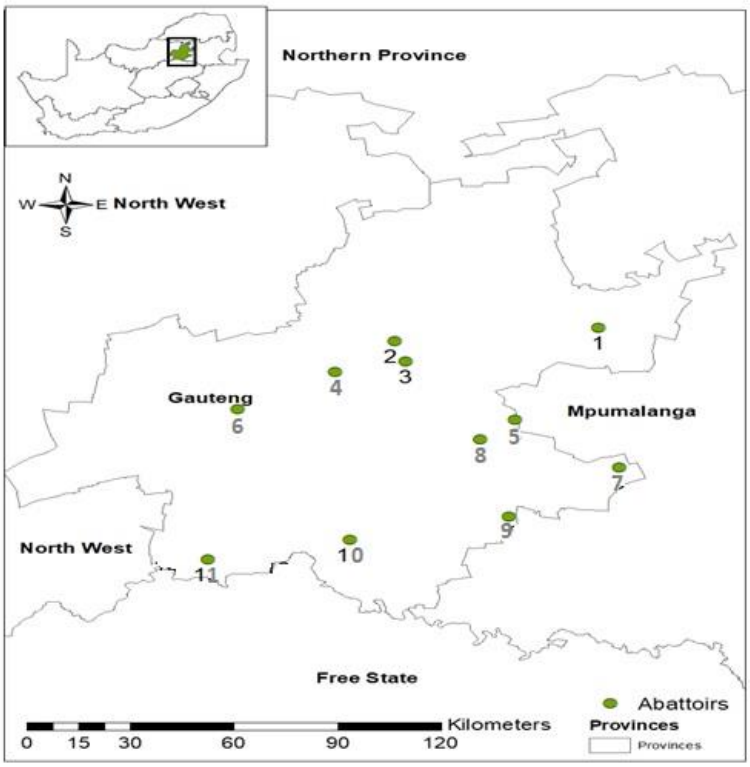

Figure 5.1: Map showing the locations of the 11 abattoirs in Gauteng Province from where slaughter Cattle were sampled

The selected abattoirs were visited once during the study period and cattle being slaughtered on the day of the visits were sampled. During each abattoir visit for sampling, the geographic information system (GIS) data (geo coordinates) were collected using the nuvi® GPS navigator, (Garmin, 2689 LMT., U.S.A.). The readings were entered into the Arc GIS program, version 13.0 and the data used to produce the map.

5.3.5 Collection and processing of samples

At slaughter, whole blood was collected aseptically from the selected animals into 10 mL yellow capped tubes, containing serum separator, and the tubes were identified by the ID number on the tag of each animal. Overall, a total of 199 blood samples were collected from slaughtered cattle. Sera harvested from the clotted blood through centrifugation were stored at -20°C for further analysis.

5.3.6 Collection of demographic data

The cattle arriving at the abattoirs for slaughter originated from farms throughout South Africa based on the information obtained from the abattoir managers. The abattoir-related information

included the location in Gauteng province, throughput (HT and LT) and number of animal species slaughtered (multi-species and mono-species). The animal-related information collected comprised the age (young and adult), sex (male and female) and breed. Information was unavailable on the leptospirosis vaccination status of each animal and the herd history of occurrence of leptospirosis, thereby making trace back investigation of animals to the farm origin impossible in the current study. It is pertinent to mention that the MAT is unable to differentiate between titres of vaccinated versus infected animals (OIE, 2014).

5.3.7 Detection of antibodies to *Leptospira* spp. using the microscopic agglutination test (MAT)

The microscopic agglutination test (MAT) was initially performed at the Leptospirosis Reference Laboratory at the ARC-OVR laboratory using as antigen the eight serovars as described on the standard protocols for MAT in South Africa. The same samples were also tested at the Yale University School of Public Health, Department of Epidemiology and Microbial Diseases, New Haven, Connecticut, USA using a 26 serovar panel **(Table 5.1**).

Table 5.1: The 26 Reference antigens of *Leptospira* spp. used for MAT in this study

S/no.	Serovar	Serogroup
1	Djasiman	Djasiman
2	▶Szwajizak	Mini
3	Hebdomadis	Hebdomadis
4	Topaz	Tarassovi
5	Arborea	Ballum
6	Javanica	Javanica
7	Medanensis	Medanensis
8	Hardjo-Lely 607	Sejroe
9	Panama	Panama
10	* ▶Icterohaemorrhagiae	Icterohaemorrhagiae
11	* ▶Hardjo-Prajitno	Sejroe
12	▶Tarassovi	Tarassovi
13	Bataviae	Bataviae
14	* ▶Pomona	Pomona
15	Celledoni	Celledoni
16	* ▶Canicola	Canicola
17	Cynopteri	Cynopteri
18	* ▶Grippotyphosa	Grippotyphosa
19	Ballum	Ballum
20	Shermani	Shermani
21	▶Bratislava	Australis
22	Robinsoni	Pyrogenes
23	Kremastos	Hebdomadis
24	Bulgarica	Autumnalis
25	Zanoni	Pyrogenes
26	Australis	Australis

*Serovars contained in the vaccine sold in South Africa for livestock.

▶Serovar used for routine diagnosis in the ARC-OVR Central Laboratory, South Africa

To perform the MAT at the ARC-OVR, the sera were diluted at 1:50 (OIE,2018), using Sorensen's media for the first screening for antibodies against *Leptospira* spp. using live culture antigens (approximately 2×10^8 leptospires per mL) of eight reference antigens of *Leptospira* serovars. To standardize the antigens prior to use in the MAT, the strains were sub-cultured in 10 mL of Ellinghausen, McCullough, Johnson and Harris medium (EMJH) (OIE, 2018) in sterile transparent screw-cap tubes and incubated at 29°C and were checked weekly for the bacterial growth density of $1 - 2 \times 10^8$ *Leptospira* per mL after 5-7 days of inoculation. The serovars (antigens) used in this phase of the study were Bratislava, Canicola, Icterohaemorrhagiae, Tarassovi, Pomona, Swazajak, Hardjoprajitno and Grippotyphosa and were obtained from the Royal Tropical Institute, Amsterdam, Netherlands. All samples that were seropositive at the screening dilution of 1:50 were thereafter

subjected to a two-fold dilution titration (1:100 to 1:3,200) to determine the final titer. The end point observed under the Dark Field Microscope (Leitz Wetzlar®, Model number 963225, Germany) was the dilution of serum samples that showed 50% agglutination, leaving 50% free leptospires compared with the control culture diluted at 1:2 with phosphate buffered saline (PBS). At the Yale University the MAT was also used to determine the antibodies to *Leptospira* spp. in the serum samples, but we used an extended panel of 26 representative serovars of *Leptospira* spp. as antigen **(Table 5.1).** The MAT was performed as previously described (OIE, 2018).

Any sample that was positive at a titer ≥1:100 to any of the serovars by one or both laboratories was classified as positive for leptospirosis (OIE 2014, 2018). The results were described as the presumptive infective serogroup based on the serovar with the highest titer for each animal. In case there was multiple serovars belonging to multiple serogroups that had the highest titer, the animal was considered positive with unknown presumptive serogroup.

5.3.8 Statistical analyses

Univariate analysis of associations was conducted considering the serological status of the cattle as a binary outcome (positive or negative). The predictor variables for cattle were abattoirs (n=11), throughput of abattoir (LT, HT), sex (male, female), age (adult, young) and breed (n=5). Each predictor variable was tested for significant associations with the serological status using the chi-square test or Fisher exact test of association. The proportions of positive animals for various levels of the variables were also calculated.

Statistical analysis was carried out using R Console version 3.2.1 (R Core Team 2017) at 5% level of significance. Microsoft Excel software was used to plot bar charts of frequency of seropositivity of the variables generated from the univariate analyses.

5.3.9 Ethical Approval

Animal ethical clearances were approved and received from the Department of Agriculture Forestry and Fisheries (DAFF) through the Section 20 approval, (Number: ***FY2015/2016***), **Figure 3.4,** the University of Pretoria Animal Ethics Committee (AEC: ***v084-16***) of the Faculty of Veterinary Science, **Figure 3.2** and from the ARC-Onderstepoort Veterinary Research (ARC-OVR), (ARC-OVR) (AEC: ***12-16***), **Figure 3.3,** for this research, as shown in **Chapter 3, *section 3.3.5 (Ethical Aprrovals).***

5.4 Results

5.4.1 Seropositivity of sera of cattle using 8- and 26-serotypes panels for MAT

Overall, a total of 27.6 % (55/199) of the cattle were seropositive for leptospirosis using the 26-antigen MAT panel. Of a total of 199 cattle tested, only 19 (9.5%) were seropositive using the 8-serovar MAT panel while the 26-serovar panel classified 55 (27.6%) as seropositive. The difference was statistically significant (P=0.01). All the 19 cattle determined to be seropositive for leptospirosis by the 8-antigen panel were also classified as seropositive using the 26-antigen which included the same antigens in both panels, i.e. 100% agreement. Therefore, the use of 8-serovar panel alone resulted in 18.1% (36/155) of the samples being classified as false-negative results.

5.4.2 Analysis for leptospirosis seroprevalence in cattle

The data analyzed were based on the results obtained from the 26-serovar panel MAT. **Table 5.2** shows the descriptive statistics and univariate associations between variables and seropositivity for antibodies to *Leptospira* spp. in cattle at abattoirs.

Table 5.2: Descriptive statistics and univariate associations between potential animal-level risk factors and infection with *Leptospira* species as determined by MAT in cattle abattoirs in Gauteng Province in South Africa

Variable	Category	No. positive/Total tested (%)	*P*-value
Abattoir	1	6/8 (75)	<0.001
	2	1/25 (4.0)	
	3	9/20 (45.0)	
	4	1/4 (25.0)	
	5	2/30 (6.7)	
	6	20/30 (66.7)	
	7	9/30 (30.0)	
	8	3/10 (30.0)	
	9	1/7 (14.3)	
	10	0/10 (0.0)	
	11	3/25 (12.0)	
Throughput	HT	34/115 (29.6)	0.52
	LT	21/84 (25.0)	
Breed	Nguni	12/31 (38.7)	0.29
	Brahman	4/10 (40.0)	
	Holstein	1/6 (16.7)	
	Bonsmara	37/141 (26.2)	
	Jersey	1/11 (9.1)	
Sex	Male	35/118 (29.7)	0.52
	Female	20/81 (24.7)	
Age	Adult	53/183 (29.0)	0.24
	Young	2/16 (12.5)	

The abattoir-level seroprevalence of leptospirosis was 90.9% (10/11). For the five variables investigated for cattle-level seroprevalence, statistically significantly difference was detected in only one, the abattoirs. The cattle-level seroprevalence ranged from 0.0% (0/10) in Abattoir 10 to 75.0% (6/8) in Abattoir 1 and the differences were statistically significant (P<0.001). The differences in the seroprevalence of leptospirosis were not statistically different by the throughput (HT versus LT) of abattoirs, breed, sex and age of cattle.

5.4.3 Frequency distribution of antibodies to serogroups of *Leptospira* spp. in cattle

The seroprevalences of leptospirosis in cattle by serogroups was as follows: Sejroe (sv. Hardjo), 10.1% (20/199), Mini (sv. Szwajizak), 4.0% (8/199), Tarassovi (sv. Tarassovi), 3.5% (7/199) and (sv. Topaz), 3.5% (7/199) but low to Pomona (sv. Pomona), 0.5% (1/199) and Canicola (sv. Canicola), 0.5% (1/199) **(Figure 5.2).**

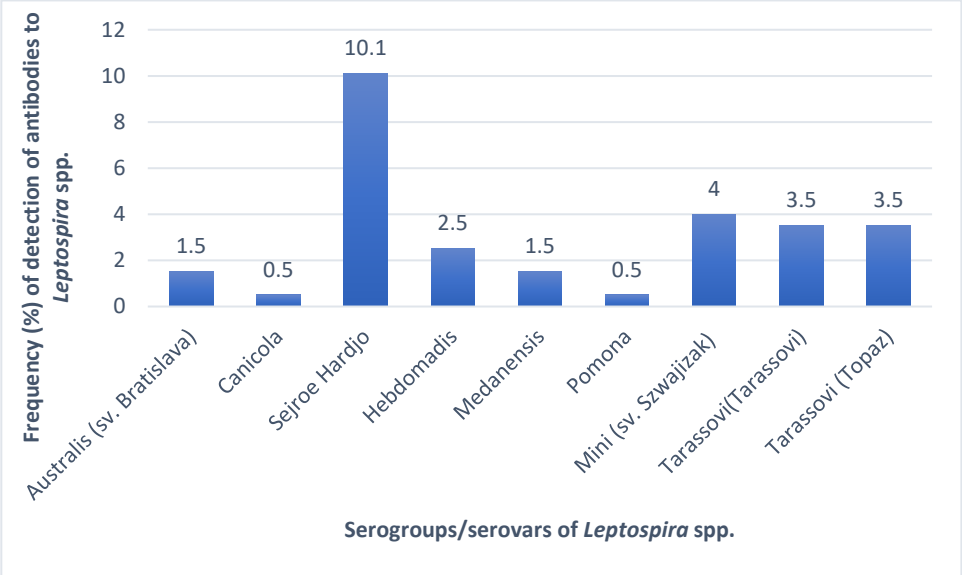

Figure 5.2: Frequency distribution of serogroups/Serovars of *Leptospira* spp. detected in Cattle

5.4.4 Seropositivity to vaccine antigens (serovars) of *Leptospira* spp.

The 26 serogroups tested by MAT included the five serovars (Grippotyphosa, Icterohaemorrhagiae, Canicola, Hardjo and Pomona) in the commercial vaccines used to prevent leptospirosis in the country. Antibodies were detected to only three (Canicola, Hardjo and Pomona) of the five serovars **(Table 5.1).** The frequency of detection of antibodies to the three vaccine serovars was 40.0% (22/55) compared to 60.0% (33/55) found for the six non-vaccine serovars. The difference was statistically significant (P=0.01).

5.4.5 Distribution of titers of antibodies to serogroups of *Leptospira* in cattle

For the seven serogroups detected, the antibody titers (ranged from 100 to 3200 in seropositive cattle and the frequencies were statistically significantly different (P<0.05) **(Table 5.3).**

Table 5.3: Titres of antibodies to serogroups (serovars) of *Leptospira* spp. in cattle

Animals type	Titre	Serogroups (Serovar)									Total / p-value
		Australis (sv.Bratislava)	**Canicola (sv.Canicola)**	**Sejroe ([a]sv.Hardjo)**	**Hebdomadis (sv.Kremastos)**	**Medanensis (sv.Medanensis)**	**Pomona (sv.Pomon)**	**Mini (sv.Szwajizak)**	**Tarassovi ([a]sv.Tarassovi)**	**Tarassovi (sv.Topaz)**	
Cattle	100	100 (3/3)	100 (1/1)	40.0 (8/20)	80.0 (4/5)	33.3 (1/3)	100 (1/1)	37.5 (3/8)	14.3 (1/7)	71.4 (5/7)	49.1(27/55) (p<0.05)
	200	0.0 (0/3)	0.0 (0/1)	35.0 (7/20)	0.0 (0/5)	33.3 (1/3)	0.0 (0/1)	37.5 (3/8)	28.6 (2/7)	28.6 (2/7)	27.3(15/55) (*p*<0.05)
	400	0.0 (0/3)	0.0 (0/1)	0.0 (0/20)	20.0 (1/5)	0.0 (0/3)	0.0 (0/1)	0.0 (0/8)	14.3 (1/7)	0.0 (0/7)	3.6 (2/55) (*p*<0.05)
	800	0.0 (0/3)	0.0 (0/1)	20.0 (4/20)	0.0 (0/5)	33.3 (1/3)	0.0 (0/1)	0.0 (0/8)	14.3 (1/7)	0.0 (0/7)	10.9 (6/55) (*p*<0.05)
	1600	0.0 (0/3)	0.0 (0/1)	0.0 (0/20)	0.0 (0/5)	0.0 (0/3)	0.0 (0/1)	25.0 (2/8)	14.3 (1/7)	0.0 (0/7)	5.5 (3/55) (*p*<0.05)
	3200	0.0 (0/3)	0.0 (0/1)	5.0 (1/20)	0.0 (0/5)	0.0 (0/3)	0.0 (0/1)	0.0 (0/8)	14.3 (1/7)	0.0 (0/7)	3.6 (2/55) (*p*<0.05)
	P-value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	
Total No.		**3**	**1**	**20**	**5**	**3**	**1**	**8**	**7**	**7**	**55**

[a] Highest titre in cattle, (3,200), 3.6 % (2/55) with, 14.3% (1/7) for Tarassovi (sv.Tarassovi).

The predominant titers (100 and 200) in the 55 seropositive cattle were detected at a frequency of 49.1% (27/55) and 27.3% (15/55) respectively (P=0.02). The serogroups with the highest titers (3200) was detected at a frequency of 3.6 % (2/55) comprising 1.8% (1/55) for Tarassovi (sv. Tarassovi) and 1.8% (1/55) for Sejroe (sv. Hardjo).

5.5 Discussion

In the current cross-sectional study conducted in abattoirs throughout Gauteng province, the seroprevalence for leptospirosis in cattle was 27.6%. Comparable seroprevalences of 21.5% and 22.3% have been reported by others (Suepaul et al., 2011; André-fontaine, 2016), while considerably lower seroprevalences of leptospirosis (3.5% to 10%) have been reported in abattoir studies by others (Leon et al., 2008; Ngbedi et al., 2012). Higher seroprevalences of 40.0% leptospirosis in slaughter cattle at abattoirs have been documented in Egypt (Horton et al., 2014) and in St. Kitts, 79.8% (Shiokawa et al., 2019), using MAT. It is however pertinent to mention that in comparing seroprevalence data obtained from livestock, factors such as the type (serogroups and serovars), spectrum (number) of serovars and the diagnostic titers used in the MAT, inability of the MAT to different between antibody titers generated in vaccinated and naturally infected animals (OIE, 2014), technical proficiency of the personnel preforming the tests, the vaccination history of the animals tested, amongst others, should be taken into consideration (Picardeau, 2013; Smythe et al., 2009; World Organization for Animal Health (OIE) 2014). The findings in this study have a national significance because the cattle slaughtered in abattoirs located in Gauteng province originated from several provinces across South Africa. Plausibly, the data may be representative of the seroprevalence of leptospirosis (27.6%) in cattle in the country, with resultant negative economic impact on livestock production and zoonotic risk to abattoir workers.

In our study, the seroprevalence of leptospirosis differed significantly across the 11 abattoirs in Gauteng province. The situation in South Africa may be explained, in part, by the differences in the seroprevalence of leptospirosis in cattle from different types of farms (feedlot and communal) in the Gauteng province and the country at large. It also depends on factors such as farm exposure to reservoirs of leptospirosis, particularly rodents, environmental contamination, management systems and level of sanitation (Vinetz, 2001).

The infecting serogroups of *Leptospira* spp. in livestock have both epidemiological and diagnostic significance. This is because if the number and type of serotypes included in the panel used for the

MAT are inadequate, the findings may lead to under-reporting of leptospirosis in the country. In this study, the use of 8-serotypes panel for the MAT on the 199 samples revealed a statistically significantly lower seroprevalence of 9.5% compared to the 27.6% detected with the 26-serotypes panel. The diagnostic implication is that the use of an 8-serotypes MAT panel only would have resulted wrongly classifying 18.18.5% of the samples as negative for leptospirosis. In addition to increasing the number of serotypes in the MAT panel, it has been suggested that the sensitivity of the MAT may be increased by the use of serotypes of *Leptospira* spp. isolated from the geographical area, for example the country, where the sera were being tested (Pinto et al. 2015) and the use of lower cut-off titers for classifying MAT results, for example, ≥1:40 or ≥1:48 (Dreyfus et al., 2018; Ngugi et al., 2019), instead of the recommended titre of 1:100 (World Organization for Animal Health (OIE) 2018).

Overall, with the 26-serovar panel, antibodies to *Leptospira* spp. were detected to 9 (34.6%) and the predominant serogroup in the 55 seropositive cattle was Sejroe (sv. Hardjo), with a seropositivity of 36.4% (20/55) and an overall seroprevalence of 10.1% (20/199). Data on the serological surveys for cattle leptospirosis in the country are limited, with the first report originating from the Western Cape (Van der Merwe, 1967), using the MAT where a seroprevalence of 2.5% (108/4,305) was detected. The serogroups observed in that study were Australis, Autumnalis, Bovis, Canicola, Grippotyphosa, Hyos, Icterohaemorrhagiae, Pyogenes and Saxkoebing. These findings date back to the mid-1960's which might indicate a change in the distribution of the circulating serovars currently in the country. It has been documented that sejroe (sv. Hardjo) was the most frequently detected serovar in slaughter cattle and cattle sampled from farms by others in Mexico (Vado-solís et al., 2002), Southern Uganda (Atherstone et al., 2014) and Tanzania (Schooman and Swai, 2013). The widespread predominance of antibodies to serogroup sejroe (sv. Hardjo) is based on reports that cattle are a reservoir for the serovar and that the serovar causes leptospirosis in cattle (Balamurugan et al., 2018; Bharti et al., 2003). However, other serogroups of *Leptospira* spp. have been documented to be predominant in other countries as indicated by Shiokawa et al., (2019) who reported that the highest seroprevalence was observed to serogroup Mankarso in cattle slaughtered in abattoirs in St. Kitts. Suepaul et al., (2011) also reported the predominance of serogroup Icterohaemorrhagiae in cattle sampled from farms in Trinidad, and serogroup Shermani and Ranarum in cattle in Thailand (Chadsuthi et al., 2017).

Of potential clinical relevance is the fact that the titers of antibodies detected in the seropositive cattle were also high to the predominant Sejroe (sv. Hardjo), with 34% of the samples seropositive

for the serogroups having titers of 800 and over. These titers are considered significant for current or acute disease (Adesiyun et al., 2006; Gommow et al., 1999). Similarly, high titers of antibodies were detected to other serogroups tested in our study. The limitation of this cross-sectional study can however not be ignored since although all the slaughter animals were apparently healthy, the recovery of animals from recent exposure to *Leptospira* spp. which could have led to increased titers, could not be ascertained in this study. Furthermore, the limitation of the MAT used in the current study which is unable to differentiate between antibody titers produced following vaccination and natural exposure (OIE, 2014), should however be considered in discussing the importance of the titers of antibodies to Sejroe (sv. Harjo) detected in our study.

The other serogroups to which antibodies were detected in cattle in the current study, Australis, Canicola, Hebdomadis, Medanensis, Pomona, Mini and Tarassovi, have also been documented in cattle by others (Adesiyun et al., 2006; Dreyfus et al., 2018; Gummow et al., 1999; Schoonman and Swai, 2010; Suepaul et al., 2011;; Vallée et al., 2018). The seroprevalence of antibodies to serogroups of *Leptospira* spp., within and across countries and regions, may be affected by the policy on vaccination and the types of serovars of *Leptospira* in the vaccines, the serotypes used in the MAT panel, infecting serovars in animal reservoirs and environmental contamination. Unfortunately, in our study the vaccination history of the animals sampled was unavailable and vaccination for livestock against leptospirosis is voluntary in the country. It is important to note that of the five vaccine serovars which are also included in the panel of 26 serotypes used for MAT, antibodies were detected to only three (Canicola, Hardjo and Pomona) in our cross-sectional study. Additionally, antibodies were detected to 6 non-vaccine serovars (Topaz, Hebdomadis, Medanensis, Bratislava, Szwajizak, Tarassovi). It is noteworthy that for the 55 cattle seropositive for leptospirosis (titers of 100 or higher), 33 (60.0%) had antibodies to the six non-vaccine serovars at a significantly higher frequency compared to the 22 (40.0%) which exhibited antibodies to the three vaccine serovars. It is therefore indicative that the seropositivity detected in our study was primarily be due the natural exposure of the cattle to *Leptospira* spp. Additionally, considering the absence of history of voluntary vaccination of cattle against leptospirosis, it cannot be assumed that the cattle positive for antibodies against the three vaccine serovars were due to vaccine exposure rather than natural exposure to the pathogen. Although the potential interference of vaccination with surveillance has been reported, there are documentations that it is minimal (Balakrishnan and Ro, 2014; Júnior et al., 2007; Martin et al., 2018). It is also important to consider the fact that MAT does not differentiate between the titers produced by vaccinated and naturally infected animals (OIE 2014).

Of the variables and risk studied, the throughput of abattoirs, sex and breed of animals did not have a significant effect on the seroprevalence of leptospirosis in the cattle studied. Ngbede et al., (2012) reported a similar finding regarding the sex and breed of cattle slaughtered at an abattoir in Nigeria where no significant association with the seropositivity for leptospirosis in the slaughtered animals was detected. Similarly, Suepaul et al. (2010) reported that sex of cattle was not significantly associated with the occurrence of leptospirosis in cattle in Trinidad.

The age of cattle tested in our study was not significantly associated with seropositivity for leptospirosis, a finding at variance with the report of Ngbede et al. (2012) who reported that the age of slaughtered cattle was statistically significantly (P=0.0313) associated with the seropositivity for leptospirosis as follows, <2 yrs. (0.0%), 2-5 yrs. (1.82%) and >5 yrs. (12.5%). The authors attributed the differences to increased exposure to the pathogen over time. The difference between both studies could be due, in part, to the fact that in South Africa most of the cattle slaughtered originated from feedlots where animals are slaughtered at approximately 1-2 years of age, while most of those slaughtered in Nigeria are primarily from extensively and semi-intensively managed farms and are considerably older, >2 year old cattle constituted 94.4% of the 142 cattle tested. Suepaul et al. (2011) in a farm-based study in Trinidad had also reported that age of cattle had a significant effect on seropositivity for leptospirosis.

5.5.1 Conclusions and recommendations

It is concluded that serogroups Sejroe, Mini and Tarassovi are circulating in cattle in Gauteng province and therefore may be of clinical importance**.** The finding of a high frequency of detection of serogroups of *Leptospira* that are neither in the vaccines nor in the MAT antigen panel used in the country may have both clinical and diagnostic implications. The potential public health significance of serological evidence of leptospirosis in slaughtered cattle to abattoir workers as well as the economic impact on livestock farmers through animal morbidity and mortality cannot be ignored.

It is recommended that the spectrum and types of serotypes in the panel used to diagnose livestock leptospirosis with the MAT in the country be increased from the current 8-antigen panel to reduce the under-reporting of leptospirosis in the country. Secondly, the vaccines used to prevent leptospirosis in South Africa should be re-considered to, in addition, contain the predominant serovars (particularly Bratislava, Topaz, Tarassovi and Szwajizak) detected to be currently circulating

in livestock in the country. Finally, it will be prudent to conduct a national abattoir-based study on leptospirosis in the country.

5.5.2 Connecting statement to the next chapter:

Following the knowledge gap which included the use of only MAT for the diagnosis of leptospirosis in review of the diagnostic laboratory data at the ARC-OVR (Chapter 3), the cross-sectional study (Chapters 4 and 5) and in agreement with the recommendation by the WHO for the use of different diagnostic methods (isolation and molecular detection/characterization) in addition to the MAT, it is imperative to conduct Isolation and molecular study to determine the prevalence and characteristics of *Leptospira* spp. from kidney tissues and abattoir effluents in Gauteng province, South Africa.

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CHAPTER 6

Frequency of isolation, molecular detection and characterization of *Leptospira* spp. from kidneys of slaughtered livestock in abattoirs in Gauteng Province

Manuscript in preparation

6.1 Abstract

The confirmatory diagnosis of leptospirosis plays an important role in understanding the distribution of the serovars causing the disease and its effect on both humans and animals as a zoonotic, environmental and a neglected tropical disease. There is a dearth of information on leptospirosis in South Africa. Therefore, the study was conducted to determine the prevalence of *Leptospira* spp. in the kidneys of slaughter livestock (cattle, pigs and sheep) in Gauteng province using bacteriological and molecular methods. The isolates of *Leptospira* spp. were also characterized using the polymerase chain reaction (PCR). A cross-sectional study was conducted in 14 abattoirs from where 305 kidney samples were collected and inoculated into semi-solid Ellinghausen McCaullough Johnson Harris (EMJH) medium for the isolation of *Leptospira* spp. using standard methods. The qPCR assay was used to detect *Leptospira* DNA in kidney samples collected using PCR that targeted the pathogenic outer membrane lipoprotein (*LipL32*) gene region. Furthermore, the pathogenic *SecY* gene regions of qPCR *LipL32* positive kidney samples or Leptospira isolates from kidney samples were amplified, sequenced and phylogenetically analysed. The overall frequency of isolation of *Leptospira* spp. from livestock was 3.9% (12/305) and the animal species specific rate was 4.8% (9/186), 4.1% (3/74) and 0.0% (0/45) from cattle, pigs and sheep respectively (P>0.05). With the use of *LipL*32 qPCR, the overall frequency of detection *Leptospira* DNA was 27.5% (84/305) and the animal species specific rate was 26.9% (50/186), 20.3% (15/74) and 42.2% (19/45) for cattle, pigs and sheep respectively (P=0.03). Of the 22 sequences generated from the *SecY* gene region, 6 were from *Leptospira* spp. isolates comprising 5 from cattle (4 *L. interrogans* and 1 as *L. borgpetersenii*) and 1 *L. interrogans* from pigs and 16 sequences from kidney tissue with 10 from cattle (9 *L. interrogans* and 1 *L. borgpetersenii*), 3 from pigs identified as *L. interrogans* and 3 from sheep (2 *L. interrogans* and 1 *L. borgpetersenii*). The phylogenetic tree of the sequenced *L. interrogans* and *L. borgpetersenii SecY* gene sequences from this study grouped with the pathogenic *L. interrogans* serovar Icterohaemorrhagiae and *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 GenBank sequences, respectively. Of diagnostic relevance is the fact that *L. borgpetersenii* serovar Hardjo

bovis strain Lely 607 is not part of the 8 serovar panel used in the microscopic agglutination test (MAT) for leptospirosis diagnostic by the veterinary laboratory in South Africa. The pathogenic *L. interrogans* and *L. borgpetersenii* are circulating in the livestock population of apparently healthy cattle, sheep and pigs slaughtered at abatoirs in Gauteng province, South Africa. This study is the first molecular characterization of *Leptospira* spp. from livestock in South Africa in combination with bacteriological method. Results in this study demonstrated that, the diagnostic use of molecular method will eliminate or reduce the under-reporting of leptospirosis in livestock, particularly in sheep, in South Africa.

Key words: Isolation, Molecular characterization, *Leptospira* spp., Livestock, Abattoirs and South Africa.

6.2 Introduction

Pathogenic *Leptospira* spp. are the cause of leptospirosis in humans and animals worldwide. The disease is transmitted through exposure to the urine of an infected animal host or reservoir host containing the pathogenic leptospires. Leptospirosis has emerged as an important urban health problem worldwide, yet the dynamics of the environmentally transmitted pathogenic *Leptospira* has not been well characterized, (Casanova-Massana et al., 2017). It can also be contracted from the environment through contact with animal reservoirs, environmental surface water and soil that are contaminated with infected urine (Ko et al., 2009). A systemic infection due to the pathogen can affect vital organs of the animal (Martínez-García et al., 2000). This disease could cause major economic loss, especially to the livestock industry and a threat to human livelihood, as these livestock serve as a source of income and food (OIE, 2018; WHO, 2003).

In South Africa, a report in 2014/2015 indicated a population of 13.7 million cattle of which 24,476 were slaughtered consisting of both adult and calves (DAFF, 2016). The pig population consisted of 1,5 million pigs of which 2,926 were slaughtered and a sheep population of 11 million with 5,141 slaughtered sheep (DAFF, 2016). Given the importance of livestock to the economy of the country, there is a need to understand the potential negative effect of leptospirosis on the livestock industry.

Leptospires (diameter of 0.1 μm, length of 6-20 μm) are distinct from other spirochaetes due to the presence of hooks which are distinct at both ends when observed under the microscope with high magnification (Bharti et al., 2003). Leptospires grow at an optimal temperature between 28-30°C in EMJH containing agar (0.1 to 0.2%) they also survive in the environment under favourable

conditions, according to Mohammed and co-workers (2011). Leptospires are very slow growing organisms with a maximum growth density in semi-solid media where the optimum oxygen tension related to growth of the organism is known as the Dinger's ring zone (Mohammed et al., 2011).

Leptospirosis is caused by the infection with the pathogenic *Leptospira* spp. (Mohammed et al., 2011). All pathogenic isolates belong to *Leptospira interrogans* and all non-pathogenic organisms (saprophytes) were placed under *Leptospira biflex* (Faine and Stallman, 1982). The genus *Leptospira* has been re-classified using genetic methods into 21 species with *L. interrogans*, *L. borgpetersenii. L. alexanderi, L. alstonii, L. kirschneri, L. noguchi, L. santarosai, L. weilii* and L. wolffii. detected in clinical cases (Picardeau, 2013).

The transmission of leptospirosis is attributed to many environmental factors (Petrakovsky et al., 2014). This is through the excretion of leptospires in the urine of infected reservoir animals where the pathogens are in close contact with domestic animals and rodents (Ko et al., 2009). The pathogenesis of leptospirosis is not yet fully understood but it has been reported that the pathogenic *Leptospira* spp. can result in different clinical manifestations in the infected host, ranging from subclinical infection to undifferentiated febrile illness (Bharti et al., 2003; Vinetz, 2001). The clinical signs of leptospirosis in animals include low milk production, abortion, stillbirth, infertility, decrease in meat production and death of animals (Martins et al., 2012; OIE, 2018).

In humans, the clinical signs and symptoms comprise lethargy/depression, vomiting, fever, weight loss, polyuria/polydipsia, abdominal or lumbar pain, stiffness/arthralgia, renomegaly, diarrhoea, icterus, oculonasal discharge, petechiae, weakness and dyspnoea/cough (Faine, 1982). Clinical signs and symptoms are insufficient to confirm leptospirosis in animals and humans (Ko et al., 2009; Picardeau, 2013). Therefore, definitive diagnosis of the disease involves the use of specific and recommended diagnostic tools such as bacteriological, serological (MAT) and molecular methods, which are considered mandatory to detect the causative agent, pathogenic *Leptospira* spp. (WHO, 2003, 2011).

The type of samples processed for the detection of *Leptospira* spp. is important (Picardeau, 2013). Some of these diagnostic methods, such as bacteriological culture, are cumbersome, time-consuming, easily contaminated and require skilled personnel. More importantly, the isolation rate is frequently low and not sensitive (Picardeau, 2013). These limitations pose a major problem to obtaining data on leptospires circulating in animals, humans and the environment in different

regions. However, the advantage of the isolation method is that it is a definitive technique for the confirmation of infecting serovars from individual animals or humans (OIE, 2014; 2018).

The use of molecular diagnostic methods for leptospirosis is highly recommended (WHO, 2003) to reduce the problem of under-diagnosis of the disease. The methods include the qPCR detection of the pathogenic *Leptospira* spp. *LipL32* partial gene region for screening (Wunder et al., 2016) and the *SecY* partial gene region with its alternating conserved and variable regions that makes it appropriate for heterogeneity interpretation of *Leptospira* spp. phylogeny (Victoria et al., 2008). In addition, the amplified *SecY* partial gene region using the G1G2 internal primers (Zuerner et al., 2000) fowllowed by sequence analysis has allowed identification of some serotypes or serovars (Cerqueira et al., 2010; Perez and Goarant, 2010), as well for the identification of pathogenic leptospires (Victoria et al., 2008). The advantages of the qPCR compared to the conventional methods are that it is fast, reduces chances of contamination, specific and sensitive, especially with the use of the hydrolysis probes, has a high throughput (Smythe et al., 2009; Wunder et al., 2016). The qPCR assay has been found to detect as low as 10^2 and 10^3 bacteria/ml of pure culture, whole-blood, plasma, and serum samples targeting the *LipL32* and *SecY* gene regions (Stoddard et al., 2009). In three independent experiments, they found a slightly higher sensitivity of the qPCR in plasma than in whole blood and serum. However, the disadvantages include the fact that it is expensive, needs good skills and cannot identify leptospires to serovars level (Picardeau, 2013).

Bacteriological isolation, serological assays and PCR have been used singly or in combination for the diagnosis of leptospirosis in animals and humans to increase the sensitivity and specificity of the diagnostic strategy (Picardeau, 2013; WHO, 2003). Da Silva and co-workers (2012) reported an isolation rate of 38.2% (13/34) from a Brazilian sheep slaughterhouse, 46.2% (6/13) from kidneys, and 53.9% (7/13) from the liver. In the Nan province of Thailand, bacteriological isolation and molecular methods were used to detect pathogenic *Leptospira* spp. from asymptomatic domestic animals, humans and water (Kurilung et al., 2017). In their study, the overall frequency of detection from cattle was 12.21 % (16/131), while the frequency of isolation of *Leptospira* spp. from urine samples from cattle was 0.76% (1/131), but the isolate was not characterized. With the use of rrs nested PCR and sequencing, targeted cattle and pigs urine samples, 1.5% (2/131) were determined to be *L. interrogans* and 8.39% (11/131) were *L. weilii.* For pigs, the overall prevalence was 7.89% (12/152), while culture yielded 3.94% (6/152) as positive, which were not characterized. Using rrs nested PCR for detection and sequencing, 1.9% (3/153) of cultures were identified as *L. interrogans* and 5.92% (9/152) were identified as *L. weilii.* For the environmental water samples, the overall

frequency of positive samples was 21.42 % (3/14). The cultures of the water samples were all negative 0.0 % (0/14). By subjecting the water samples to the rrs nested PCR and sequencing, 7.1% (1/14) of cultures were identified as *L. interrogans* and 14.3% (2/14) as *L. weilii* (Kurilung et al., 2017).

In another study on slaughtered livestock in New Zealand, the overall frequency of detection of *Leptospira* DNA from both kidneys and liver by qPCR was reported as 27.0% (145/545) (Fang et al., 2014). They detected the average *Leptospira* spp. DNA from kidneys of slaughtered cattle at a frequency of 21.0% (30/148). In Botswana, a prevalence was reported to be 41.5 % (17/41) for renal carriage of *L. interrogan* in mongooses using PCR (Jobbins et al., 2014). The isolation rate for leptospires from the kidneys of slaughtered pigs in Colombia was detected to be 0.8 % (3/383) (Romero-vivas et al., 2013), while in Brazil all the kidney samples were negative 0.0% (0/36) (Freitas et al., 2004). Amplification of the DNA was confirmed by sequencing in two kidney samples, 5.9% (2/43), and these sequences had 100 % homology with sequences of *L. interrogans* in GenBank.

In a study on naturally infected animals in Brazil, Freitas et al., (2004), reported the isolation of leptospires from bovine urine at a frequency of 66.7% (2/3), the 36 liver samples from pigs, the isolation rate was, 5.6 % (2/36), while the remaining uterus body, kidney and the ovary tissues from swine all tested negative by isolation, (0/36). Barbante and co-workers, (2014) isolated leptospires from 25.0% (5/20) of the sheep kidney tissues tested in Brazil. In Zimbabwe, 10.4 % (50/480) of the kidney samples of cattle slaughtered at an abattoir near Harare were positive for *Leptospira* spp., by culture in EMJH. The isolates belonged to the following serogroups using MAT: 64.0% (32/50) belonged to the Sejroe serogroup; 14.0% (7/50) to Pyogenes; 8.0% (4/50) to Hebdomadis; 4.0% (2/50) to Tarassovi and 2.0% (1/50) to each of serogroups: Australis, Bataviae, Grippotyphosa, Icterohaemorrhagiae and Pomona (Feresu et al., 1992).

In South Africa, the last reported isolation of leptospires was documented in 1987 (Gummow, et al., 1999) where 25.0% (3/12) of bovine tissues cultured for *Leptospira* spp. were positive but the isolates were not serotyped. For over the past two decades, no published studies exist on livestock either on farms or at abattoirs which determined the prevalence of leptospirosis either by culture, serology or by PCR. Therefore, the aim of the chapter was to isolate and characterize *Leptospira* spp. from slaughtered livestock in abattoirs in Gauteng province by molecular methods.

6.2.1 Specific objectives

To achieve this aim, the following specific objectives were investigated:

i) To determine the frequency of isolation of Leptospira spp., from the kidneys of slaughtered cattle, pigs and sheep at abattoirs in Gauteng province, South Africa.

ii) To determine the frequency of detection of pathogenic *Leptospira* spp. from the kidneys of slaughtered livestock) using the *Leptospira* TaqMan Hydrolysis qPCR assay targeted the *LipL32* gene region.

iii) To detect pathogenic *SecY* partial gene region and quantify pathogenic *Leptospira* spp. in the kidneys of cattle, pigs, sheep, abattoir effluents and *Leptospira* spp., cultured or isolates from cattle and pigs using the *LipL32* gene region qPCR assay

iv) To sequence amplified *SecY* gene region and phylogenetically analyse the isolates of *Leptospira* from kidneys and in the *Leptospira* DNA from kidney tissues.

6.3 Materials and Methods

6.3.1 Country of study

6.3.1.1 South Africa

South Africa is a country located in the southern tip region of the African continent, with a population of approximately 57.78 million people as of 2018. It has three capital cities namely: Cape Town, Pretoria, and Bloemfontein**.**

6.3.1.2 Gauteng province

Gauteng Province, the study area, is in the Highveld and the smallest province in South Africa accounting for only 1.5% of the land area (18.178 Km^2) of the South African total area of 1,220.813 Km^2**.** These cities have agricultural and food companies. Gauteng province has the highest number of abattoirs in the country, comprising both High throughput (HT) and Low throughput (LT) abattoirs slaughtering animals from Gauteng province as well as from other provinces in the country. Therefore, the slaughtered animals sampled at the abattoirs in Gauteng province in the current study may be representative of the country since they originated from provinces across South Africa. The population of livestock per million in 2014/2015 in Gauteng province was reported to be 13.7, 11 and 1.5 for cattle, sheep and pigs respectively (DAFF, 2016). The three species of livestock were sampled in the current study.

6.3.1.3 Location of abattoirs included in the study

A list of functional red meat abattoirs (mono- and multi-species) in Gauteng province was provided by the Department of Veterinary Public Health (VPH) of Gauteng Department of Agriculture and Rural Development (GDARD). Overall, 14 abattoirs comprising 7 HT and 7 LT, were randomly selected from abattoirs whose owners approved the conduct of the study at their facilities. The distribution of abattoirs in Gauteng province from which livestock were sampled is shown in (**Figure 6.1).** The geographic information system (GIS) data were collected using the nuvi® GPS navigator (Garmin, 2689 LMT., U.S.A.). The readings were entered into the Arc GIS program version 13.0 and the data used to plot figures and produce maps.

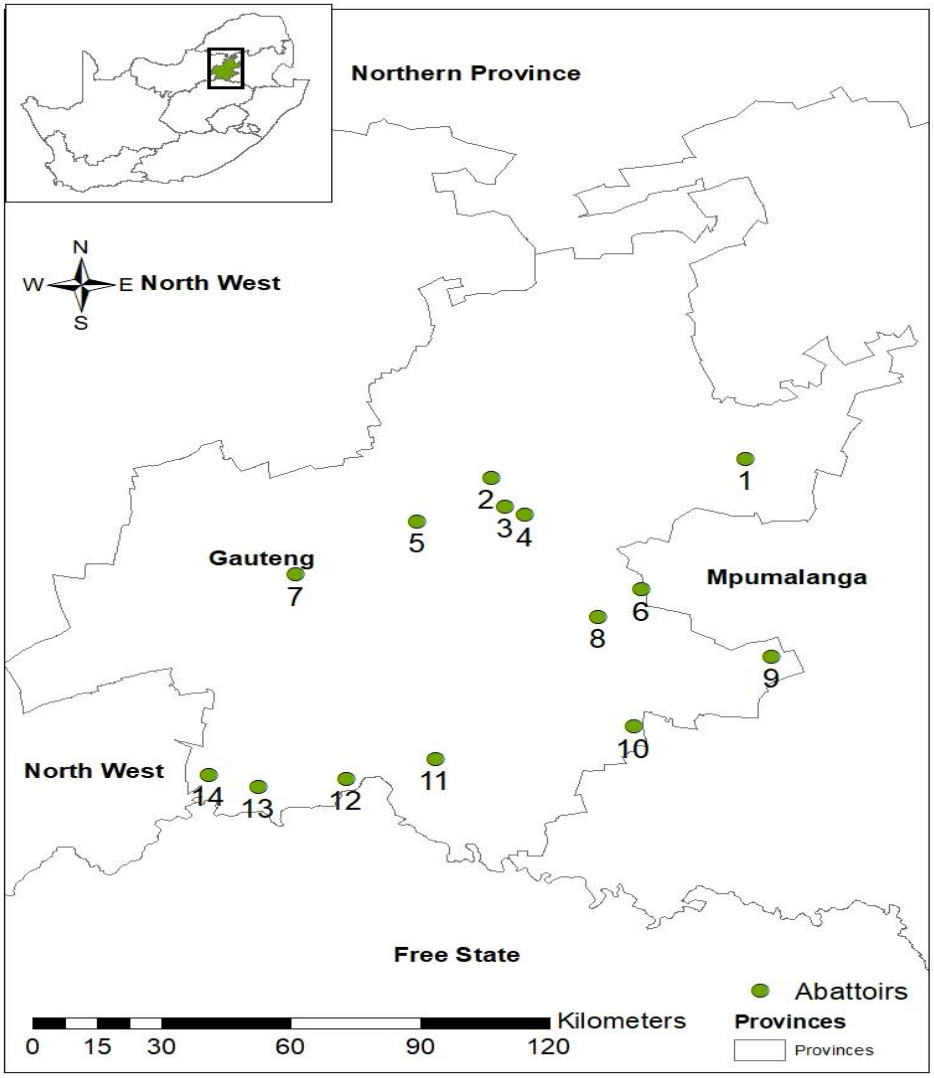

Figure 6.1: Small insert map shows the location of Gauteng province in South Africa and main map displaying the locations of the 14 abattoirs in Gauteng province from which samples were collected.

6.3.2 Type of study:

The study conducted was cross-sectional in design.

6.3.3 Type of sampling:

A convenience sampling approach was used in this study where 14 red meat abattoirs used for the slaughter of livestock were randomly selected from the Gauteng province in South Africa and sampled between September 2016 and April 2017. These abattoirs were the ones where the abattoirs owners or abattoir managers consented to facilitate the study.

6.3.4 Demographic data and risk factors for livestock sampled at the abattoirs

The demographic data obtained from the abattoirs included the type of abattoir (HT or LT) and location of abattoirs using the Global Positing System (GPS) within the Gauteng province. The animal-level risk factors obtained to investigate their potential effects on the frequency of detection of *Leptospira* spp. included the animal species (cattle, pigs and sheep), sex (male and female), age (adult and young), and breed.

6.3.5 Type and source of animals and number of samples collected:

6.3.5.1 Type of animals:

The animals sampled in this cross-sectional study were cattle, pigs and sheep.

6.3.5.2 Type and number of samples collected:

Kidneys of livestock were collected at slaughter. Overall, a total of 305 kidney samples were collected (one kidney per animal). The number of animals sampled comprised 186, 74, 45 and 14 from cattle, pigs, sheep, and abattoir effluents, respectively.

6.3.5.3 Methods of samples collection:

The kidney samples were aseptically removed from each selected carcass into individual sterile Ziploc bags which were individually identified. Abattoir effluents samples were also aseptically collected in three different location at random within the abattoir and were pulled together in a 50 mL plastic cup and labelled. All collected samples were transported on ice to the laboratory within 2 - 4 hr of collection.

6.3.6 Processed samples

Each kidney sample from the 305 animals was processed by both bacteriological culture (isolation of *Leptospira* spp.) assay and molecular methods (detection of *Leptospira* DNA) assay. Abattoir effluent samples (n=14) were centrifuged into pellets for DNA extraction.

6.3.7 Media used for isolation of *Leptospira* spp.

Ellinghausen McCaullough Johnson Harris (EMJH) semi-solid medium **(**Difco™ BD *Leptospira* Enrichment EMJH, USA) was prepared by the addition of 1% agar to the basal broth media and used for the culture of leptospires. EMJH liquid medium was used for the purification of leptospiral cultures for further typing and characterization.

6.3.8 Isolation of *Leptospira* spp. from kidney tissues

In a class II Biohazard cabinet (BSL 2), 50 mg of kidney tissue containing the cortex and medulla portion was collected. Each of the kidney tissues used in this study was aseptically cut using a sterile scalpel blade and sterile petri dish and then added to a sterile 5 mL syringe plunger containing 3 mL of liquid EMJH medium to macerate the tissues. From a 2 mL screw cap cryovial, approximately 2 mL of the macerated kidney content was transferred aseptically for homogenization using the Precellys® 24 lysis homogenizer at 4500 rpm for 2 minutes. Thereafter, 200 µL of the supernatant was aseptically inoculated into 5 mL of semi-solid EMJH medium (Johnson and Harris, 1967), containing 200 µg/mL 5-flourauracil in a labelled 10 mL sterile tube. The inoculated EMJH media tubes were incubated at 29°C and aspirate from the tube was observed weekly for a period of 3-6 months under a dark field microscope (Nikon Labophot® Japan; Model number:277602) for the presence or absence of leptospires. Samples without leptospiral growth by the end of the 6th month incubation period were classified as negative for *Leptospira* spp. The isolation of *Leptospira* spp. from the kidney samples was conducted at the Agricultural Research Centre-Onderstepoort Veterinary Research laboratory, Onderstepoort Gauteng province, South Africa.

6.3.9 Typing of isolates of *Leptospira* spp.:

At the ARC-OVR, where MAT is currently conducted using eight serovars for diagnostic serological purposes but isolation of the leptospires has not been carried out over two decades due to unavailability of antisera for serotyping of *Leptospira* isolates. Therefore, the isolates of *Leptospira* spp. recovered in this study were then sent to Professor Ko's Laboratory at Yale University, College of Medicine, School of Public Health where antisera for serotyping *Leptospira* isolates were

available. However, contamination of the cultures posed an insurmountable challenge and repeated efforts to purify them were unsuccessful. Since pure cultures of leptospires were required to successfully serotype the isolates with rabbit monoclonal antisera, this could not be done in this study. Therefore, molecular methods were used for the identification of the isolates of *Leptospira* spp., in this chapter.

6.3.10 Polymerase chain reaction (PCR)

6.3.10.1 DNA extraction

Fifty milligrams (50 mg) of kidney tissues were aseptically cut from each kidney (n=305) using a separate scalpel blades, forceps and a Petri-dish in a BLS 2 for the extraction of DNA. Abattoir effluents were processed as described by Riediger et al. (2016). Briefly, samples were homogenized by inversion and a 40-mL aliquot was centrifuged (15,000 × g for 20 min at 4°C) and the supernatant was discarded. The pelleted abattoir effluents were resuspended with 2 mL PBS for extraction. Briefly, DNA was extracted from each of the kidney samples and abattoir effluent using the ISOLATE II Genomic DNA (Bioline) extraction kit as described by the manufacturers, with minor modifications, specifically, the use of 50 mg of tissue and 2 h of incubation instead of 25 mg of tissue and 3 h of incubation of the sample with the addition of pre-lysis buffer and proteinase K. These extractions were carried out at the Department of Veterinary Tropical Diseases Laboratory, Faculty of Veterinary Science, University of Pretoria, South Africa.

6.3.10.2 Detection of Leptospira spp. by real-time PCR (qPCR) using the pathogenic *LipL32* gene region

Extracted DNA from *Leptospira* spp. isolates and the kidney tissues were tested using the qPCR TaqMan Hydrolysis assay with the Cador®*Leptospira* qPCR (Commercial kit) detecting the *LipL32* gene region in pathogenic *Leptospira* spp. on the Rotor Gene® Q (Whitehead Scientific, Germany®). The Cador®*Leptospira* qPCR (Commercial kit) and the assay used at the Yale University School of Public Health both targeted the *LipL32* gene region outer membrane of the pathogenic *Leptospira* spp. The confirmation of the presence of *Leptospira* DNA in the extracts of the isolate was initially done at the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Sciences, University of Pretoria, South Africa, following the manufacturer's instructions. Briefly, a total volume of 20 µl reaction was used containing 1× Pathogen Master Mix, 1× *Leptospira* Primer/Probes, 1× Internal Control Assay, 1× Internal Control DNA and RNase-free water. Both positive and negative controls used for the assay were obtained from the Cador®*Leptospira* PCR commercial kit for pathogenic *Leptospira* spp. (Whitehead Scienfic Campany, South Africa). The condition of the PCR reaction for

the assay was as follows: Activation at 95°C for 5 min in 1 cycle, 95°C and 60°C for annealing and extention for 15 sec and 30 sec respectively, for 40 cycles. Following the confirmation of the presence of *Leptospira* DNA at DVTD, University of Pretoria further investigations on the extracted *Leptospira* DNA were conducted by the graduate student at the Yale University, College of Medicine, School of Public Health, New Haven, Connecticut, USA.

6.3.10.3 Construction of standard curve of qPCR for quantification of *LipL32* gene region

A *LipL32* qPCR, not using a commercial kit, was conducted at the Yale University School of Public Health, USA using a standard stock positive control genomic DNA (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 isolated by, Nascimento et al., (2004) for a standard curve calibration of the genomic DNA, prior to performing the qPCR of the extracted DNA from the kidney samples of the slaughtered livestock. The standard curve was constructed using the serial dilution of the DNA stock starting at $Log10^1$ to $Log10^7$ genomic equivalents per gram (GEq/mL). The samples including the abattoir effluents extracted DNA, were tested in duplicates alongside each dilution of the standard curve. A non-template negative control was also tested with all samples. The genomic equivalents per mg of kidney DNA was used to express the results as earlier suggested (Lourdault et al., 2009).

After the standardization of the standard curve, the *Leptospira* DNA extract was subjected to a qPCR targeting the *LipL32* gene to screen for pathogenic *Leptospira* spp. (Wunder et al., 2016). The PCR reaction consisted of a 25 µL final volume containing 1 x Platinum Quantitative PCR Super mix Rox-UDG (Invitrogen®), 10 µM of each primer (*LipL*32-45F and *LipL32*-286R), 5 µM TaqMan probe (*LipL32*-189P) and 5µL of extracted DNA. The cycling conditions were as previously described, with a holding stage of 95°C for 10 min, 45 cycles of 95°C for 15 seconds and 60°C for 1 min using a TaqMan-based quantitative PCR assay in ABI 7500 system (Thermo Fisher Scientific, 171 Inc.Real-time PCR ABI 7500). The Ct-value ≤ 40 was regarded as positive while Ct-value ≥ 40 was regarded as negative. The Excel software was used to determine the standard curve correlation efficient (R^2). The results of the *LipL32* commercial kit qPCR describe in 5.4.10.2 and *LipL32* qPCR describe in section 5.4.10.3 by Wunder et al. (2016) will be reported as *LipL32* qPCR results since both qPCR methods has the same target and identify pathogenic *Leptospira* spp.

6.3.10.4 Detection and characterization of *Leptospira* spp. *SecY* gene region PCR and sequencing

6.3.10.4.1 Detection of Leptospira spp. isolated using the *SecY* gene region PCR and sequence analysis

Two of the 12 isolates of *Leptospira* spp. were contaminated and could not be used for *SecY* gene region PCR and sequencing. Thus 10 isolates of *Leptospira* spp. were subjected to the pathogenic *SecY* gene PCR and sequences to discriminate the pathogenic *Leptospira* spp. as described by Victoria et al. (2008).

6.3.10.4.2 Detection of Leptospira spp. in livestock kidneys using the *SecY* partial gene region by PCR:

The kidney samples positive on *LipL32* gene target qPCR were further subjected to pathogenic *SecY* PCR assay to discriminate the pathogenic *Leptospira* spp. as described by Victoria et al. (2008). The kidney DNA with concentrations over Log10gc/g 4.23 as quantified by the qPCR were further subjected to *SecY* gene region PCR followed by sequencing to discriminate the pathogenic *Leptospira* spp. as described by Victoria et al. (2008).

6.3.10.4.3 Performance of the pathogenic Leptospira spp. *SecY* partial gene region nested PCR assay

The pathogenic *SecY* partial gene region was amplified using the *SecY*II and *SecY*IV primer sets: *SecY* II (5'-GAATTTCTCTTTTGATCTTCG-3') and Sec IV (5'-GAATTTCTCTTTTGATCTTCG-3') for the first step PCR with a final volume of 25 µL containing 1 x buffer, 200 µM dNTP's, 400 µM primer pair *SecY*II and *SecY*IV each, 0.2 µg bovine serum albumin (BSA) (Ambion), 1.25U Taq polymerase, (Thermo Scientific) and 3 µL extracted DNA template (Victoria et al., 2008).

The nested PCR was performed using the G1G2 pair of primer sets for pathogenic *Leptospira* spp., with a total volume of 25 µL containing 1× buffer, 200µM dNTP's, 400 µM of each primer *SecYII* and *SecYIV*, 0.2 µg bovine serum albumin (BSA), (Ambion), 1.25 U Taq polymerase (Thermo Scientific) and 3µL of the first PCR amplicon (Victoria et al., 2008). The positive control used for the amplification was *Leptospira interrogans,* serovar Copenhageni strain Fiocruz L1-130 (Nascimento et al., 2004) and the ultra-pure water (Thermo Scientific) was used as the negative control.

Both the first *SecY* partial gene region and the second nested *SecY* G1G2 primer pairs were run in a PCR reaction condition in a My Cycler™ Thermal Cycler (BioRad) of 94°C for 5 min (1 cycle), 94°C for

30 sec, 55°C for 45 sec, for (35 cycles) and 72°C for 60 sec (1 cycle), (Victoria et al., 2008). The agarose gel electrophoresis was run using 3 µL of amplicons in 1.5% agarose gels in TBE buffer for 35 min at 110 V, using ethidium bromide (10 mg/µL). Images were captured using the Bio-Rad-Chemi-Doc-XRS.

6.3.10.4.4 Unidentified isolates of Leptospira spp.

Of the 12 isolates of *Leptospira* spp. recovered from kidney tissues four *Leptospira* spp. isolates observed under the Dark field microscope could not be detected using the *LipL32* gene region primers and probes of the qPCR assays used. Therefore, we then excluded them as unidentified, which will require future follow-up study to confirm them as either intermediate or non-pathogenic *Leptospira* spp.

6.3.10.4.5 Purification of amplicons

The Qiaquick PCR purification kit was used according to manufacturer's specifications to purify the generated 670 bp initial *SecY* PCR products and nested *SecY* PCR products (285 bp). The generated amplicons were then sent to Eurofins Genomic (Kentucky®), USA for Sanger sequencing.

6.3.10.4.6 Sequence analyses of *SecY* partial gene region of Leptospira isolates and kidney tissue samples and phylogeny

The resulting sequences obtained from PCR products obtained from *Leptospira* spp. isolates and kidney samples were edited using the CLC Genomic workbench version 7.5.1. Reference sequences were blasted using the basic local alignment search tool (BLAST) (http//www.ncbi.nih.gov). The sequenced *SecY Leptospira* and *Leptospira* reference sequences retrieved from GenBank were aligned using MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/) and trimmed using the BioEdit (http://www.mbio.ncsu.edu/BioEdit/page2.html). A phylogenetic tree was constructed using the maximum likelihood method in MEGA 7.0.2 with 1000 boostraps value.

6.3.10.4.7 Statistical analyses

i. Univariate analysis

Univariate analysis of associations was conducted using the serological status of the animal as a binary outcome (positive or negative). The predictor variables were abattoir (14 abattoirs), type of abattoir (multi-species and mono-species), throughput of abattoir (LT and HT), animal species (cattle, sheep and pigs), sex (male, female), and age (adult and young). Each predictor variable was

tested for significant associations with the serological status using the chi-square test of association. Prevalence ratio for each animal level potential risk factor was obtained, and 95% confidence intervals estimated using the quantiles formation of the normal distribution (qnorm) with MASS package in R (Venables and Ripley, 2002).

ii. Multivariable analysis

Significant variables ($p<0.05$) in the univariate analysis were assessed for collinearity by means of the chi-square statistic; variables were considered collinear if $p<0.05$. When a pair of variables was found to be collinear, only the more biologically plausible variable was kept for further analysis in the binary logistic regression. Analysis was carried out considering the serology result as determined by the MAT for individual animals as a binary outcome. Out of the three statistically significant variables (abattoir, breed, species) from the univariate analysis, the pairs: *breed and species, abattoir and breed*, and *abattoir and species* were found to be collinear, and therefore only *species* and abattoir were retained in the final model.

Given the likelihood that some animals slaughtered in the same abattoir may have originated from the same farm/herd/flock, leading to dependence, intra-cluster correlation within abattoirs was tested at the beginning of the regression process. To test if seropositivity for antibodies to *Leptospira* spp. by the MAT were clustered in abattoirs, a Log Ratio test between a model with the *'abattoir'* as *random effect* and a null model was performed. The p-value from the Log Ratio test was found to be less than 0.05, meaning that the results of *Leptospira* spp. are clustered inside the abattoir.

A mixed effect logistic regression model, therefore, was used in the multivariable analysis, with the species as the *'fixed effect'* and the abattoir as the *'random effect'*. Hosmer-Lemeshow χ^2 was used as a goodness of fit test. Statistical analysis was carried out using R Console version 3.2.1 (R Core Team, 2017) at 5% level of significance. For the cleaning of data and frequency determination of the predictor variables of the livestock slaughtered, Microsoft Excel 2010 was used, for descriptive statistics to plot the bar chart and to determine the frequency of all the variables used as mentioned in the risk factors analyses.

6.3.10.4.8 Ethical approvals

Animal ethical clearances were received from the Department of Agriculture Forestry and Fisheries (DAFF) through the section 20 approval (Number: **FY 2015/2016**) as shown in (**Figure 3.4**), by the

University of Pretoria the Animal Ethics Committee of the Faculty of Veterinary Science **(**Number: **v084-16**) **(Figure 3.2)** and from the ARC-OVR (AEC: **12-16)** as shown **(Figure 3.3),** as shown in **Chapter 3,** ***section 3.3.5 (Ethical Aprrovals).***

6.4 Results

6.4.1 Detection of leptospires from livestock kidneys by isolation

The overall frequency for isolation of leptospires from slaughtered livestock kidneys in 14 Gauteng abattoirs was 3.9% (12/305). The Dingers ring zone was observed 3 to 8 weeks post-inoculation in EMJH media inoculated with kidney samples with leptospiral growth **(Figure 6.2).**

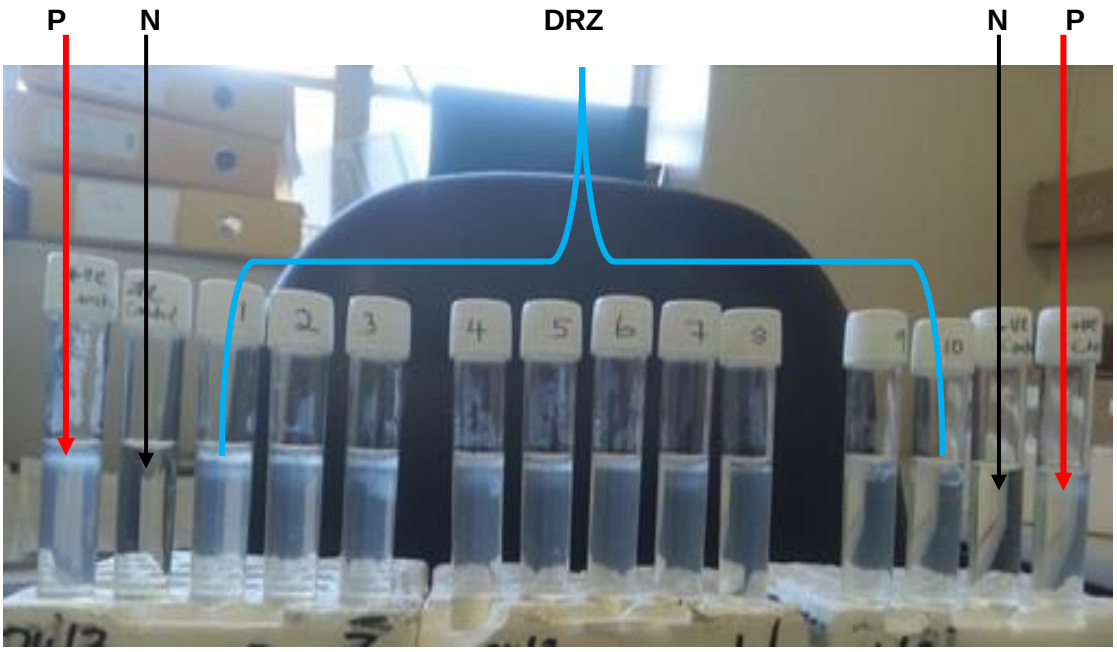

Figure 6.2: EMJH semi-solid medium inoculated with kidney tissues after 3 to 8 weeks incubated at 29°C in this study. The positive controls (P) using *Leptospira* serovar, Grippotyphosa are indicated by red arrows, negative controls (N) using phosphate buffer saline (PBS) are indicated by the black arrows and the Dinger's ring zone of leptospiral growth is indicated in inoculated sample by the blue bracket. This morphological observation was further confirmed by observation under the dark field microscope for the active movement of leptospires. **Photo: By Dogonyaro, B.B. (graduate student Researcher), on the 24th March 2017.**

6.4.2 Unidentified isolates of *Leptospira* spp.

Of the 12 isolates of *Leptospira* spp. recovered from kidney tissues and observed under DFM, four could not be detected using the *LipL32* gene region pathogenic primers and probes of the qPCR assays used. This might be an indication that they might be intermediate or non-pathogenic

Leptospira spp. isolates because the PCR assay was developed specifically to be sensitive and specific for only for the detection of the pathogenic *Leptospira* spp. The four isolates were excluded and classified as unidentified, thus requiring future investigation in another study.

6.4.3 Risk factors for isolation of *Leptospira* spp. from livestock kidneys in Gauteng province

6.4.3.1 Frequency of isolation of Leptospira spp. by abattoir, abattoir type and throughput in Gauteng province

Of the 14 abattoirs tested, only 7 (50.0%) had livestock that yielded isolates of *Leptospira* spp. The frequency of isolation of *Leptospira* spp. by type of abattoir was 91.7% (11/12) and 8.3 % (1/12) for multi- and mono- type abattoir, respectively. The difference was statistically significant (p=00001). For the throughput of abattoirs, the frequency of isolation of *Leptospira* spp. was 50.0% (6/12) and 50.0% (6/12) from HT and LT abattoirs respectively.

6.4.3.2 Frequency of isolation of Leptospira spp. by animal species

The frequency of isolation by animal species was 4.8% (9/186), 4.1% (3/74) and 0.0% (0/45) in cattle, pigs and sheep respectively as shown in **Figure 6.3** but the differences were not statistically significant (P>0.05). Of the 12 isolates of *Leptospira* spp., 9 (75.0%), 3 (25.0%) and 0 (0.0%) originated from cattle, pigs and sheep respectively.

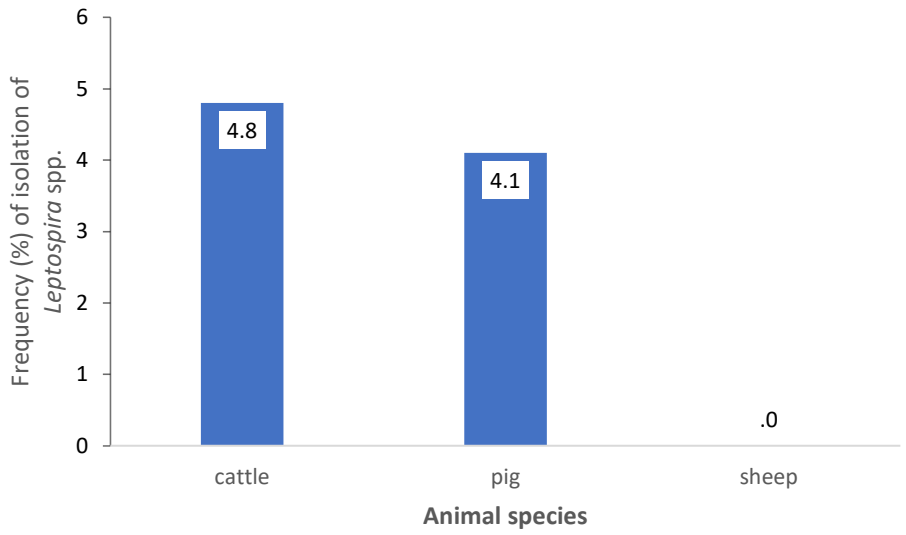

Figure 6.3: Frequency of isolation of *Leptospira* spp. from the livestock kidneys by animal type (cattle, pigs and sheep) slaughtered in Gauteng Province abattoirs in South Africa

6.4.3.3 Frequency of isolation of Leptospira spp. by age of animal

For cattle, the frequency of isolation of *Leptospira* spp. was 5.3% (9/170) and 0.0% (0/16) for adult and young animals respectively but the difference was not statistically significant (P>0.05). All the 9 isolates of *Leptospira* spp. originated from adult cattle. For pigs, the frequency of isolation of *Leptospira* spp. was 2.3% (1/43) and 6.5% (2/31) for adult and young animals respectively (P>0.05). Of the 3 isolates recovered from pigs, 33.3% (1/3) were from adult and 66.7% (2/3) from young pigs.

6.4.3.4 Frequency of isolation of Leptospira spp. by sex of animal

For cattle, the frequency of isolation of *Leptospira* spp. was 1.8% (2/110) and 9.2 % (7/76) for male and female cattle respectively (P=0.0209). Of the 9 isolates recovered from cattle, 22.2% (2/9) were from males and 77.8% (7/9) from females. For pigs, the frequency of isolation of *Leptospira* spp. was 2.0% (1/50) and 8.3% (2/24) for male and female pigs respectively P>0.05). Of the 3 isolates recovered from pigs, 33.3% (1/3) were from males and 66.7% 2/3) from females.

6.4.3.5 Frequency of isolation of Leptospira spp. by breed of animal

For cattle, the frequency of isolation of *Leptospira* spp. was 13.3% (4/30) and 3.9% (5/129) from Nguni and Bonsmara cattle respectively (p=0.28). Of the 9 isolates recovered from cattle, 55.6% (5/9) were of Nguni breed and 44.4% (4/9) were of Bonsmara breed. For pigs, the frequency of isolation of *Leptospira* spp. was 4.1% (3/74) but all the pigs slaughtered and sampled were of White large breed.

6.4.3.6 Distribution of Leptospira-positive livestock by isolation in Gauteng province:

The distribution of livestock positive for *Leptospira* spp. by isolation is shown in **Figure 6.4.** Most of the *Leptospira*-positive livestock (cattle and pigs) originated from abattoirs located in the central part of Gauteng province. Five, was the highest number of isolates of *Leptospira* spp. recovered from one abattoir, followed by two from another abattoir while one isolate each was recovered from five abattoirs. Data on the origin of the animals were unavailable.

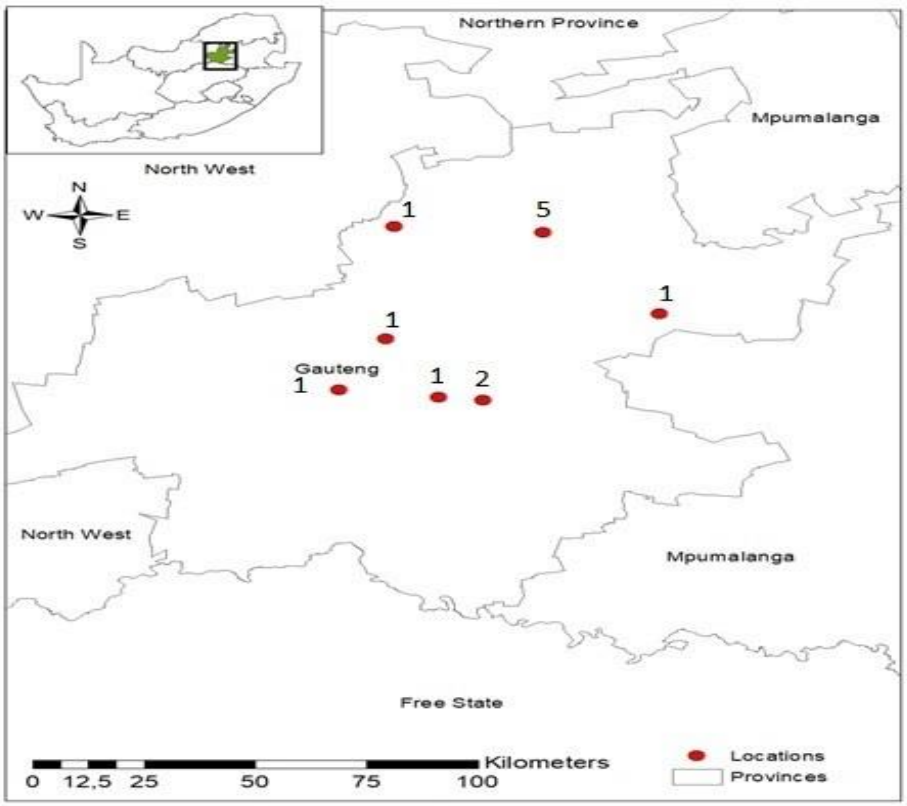

Figure 6.4: The distribution of livestock positive for *Leptospira* spp. by isolation in abattoirs at the Gauteng Province showing the number of *Leptospira* spp. recovered by abattoirs.

6.4.4 Detection of *Leptospira* spp. in kidneys of livestock and abattoir effluents by qPCR

6.4.4.1 Standardization of the qPCR methods:

The qPCR was standardized by serial dilution of the DNA stock starting at $Log10^{1}$ to $Log10^{6}$ GEq/mL targeting the *LipL32* gene region at Yale University School of Public Health, USA (**Figure 6.5.**). The detection criteria were that any sample with a Ct value < 40 was regarded as positive and any sample with a Ct value > 40 was classified as negative.

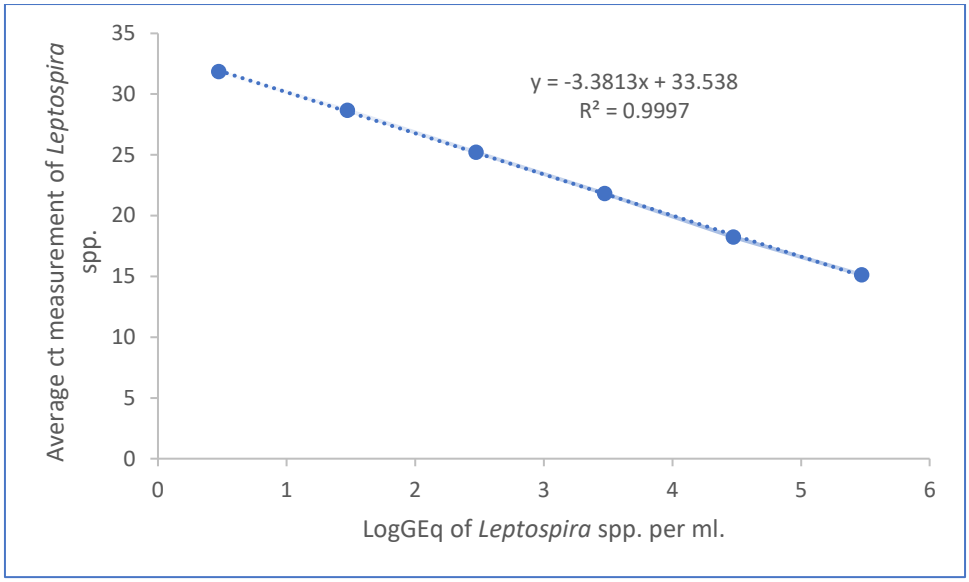

Figure 6.5: Standardized qPCR curve used for the quantification of the concentration of A standard stock positive control genomic DNA (*Leptospira interrogans,* serovar Copenhageni strain Fiocruz L1-130) in GEq/ml targeting the *LipL32* gene region of the pathogenic *Leptospira* spp.

6.4.4.2 Detection of pathogenic Leptospira spp. using the *LipL32* gene region qPCR

6.4.4.2.1 Frequency of hybridization of leptospires in kidney tissues of slaughtered livestock in South Africa:

The overall frequency of *LipL32* gene region present in pathogenic *Leptosipra* spp. detected with pPCR in kidney tissues of livestock (cattle, pigs and sheep) was found to be 27.5% (84/305) for the kidneys tissues samples analysed and all the 14 abattoir effluents samples were negative. These samples were tested in the Department of Veterinary Tropical Diseases Laboratory targeting the *LipL32* gene region of the pathogenic *Leptospira* spp. prior to Yale testing targeting the same *LipL32* gene region.

6.4.4.2.2 The *LipL32* gene region qPCR for pathogenic Leptospira spp. in cattle

The frequency of *LipL32* gene region present in pathogenic *Leptospira* spp. detected with pPCR in kidney tissues of cattle was found to be 6.9% (50/186) (**Figure 6.6).**

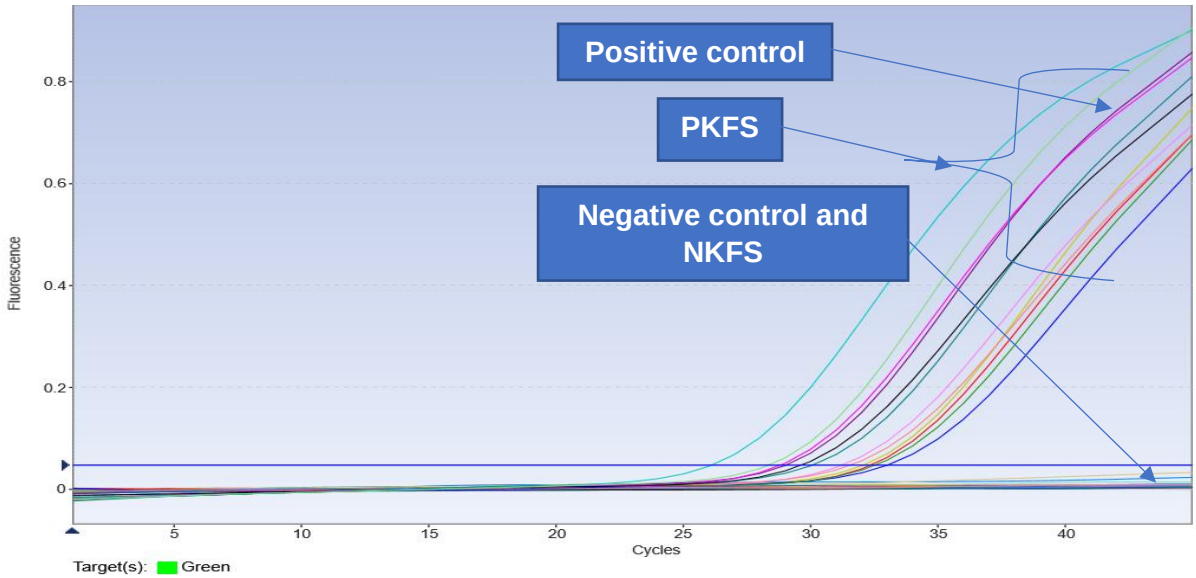

Figure 6.6: Fluorescence of hybridisation of pathogenic *Leptospira* spp. Lipoprotein L32 gene (*LipL32*) probe using qPCR from cattle kidney samples, positive control (*Leptospira interrogans,* serovar Icterohaemorrhagiae strain) and negative control (ultra-pure water).

Note: PKFS=Positive kidney field samples; NKFS=Negative Kidney field samples.

6.4.4.2.3 The *LipL32* gene region qPCR for pathogenic Leptospira spp. in pigs

The frequency of *LipL32* gene region present in pathogenic *Leptospira* spp. detected with pPCR in kidney tissues of pigs was found to be 20.3% (15/74) as shown in **(Figure 6.7).**

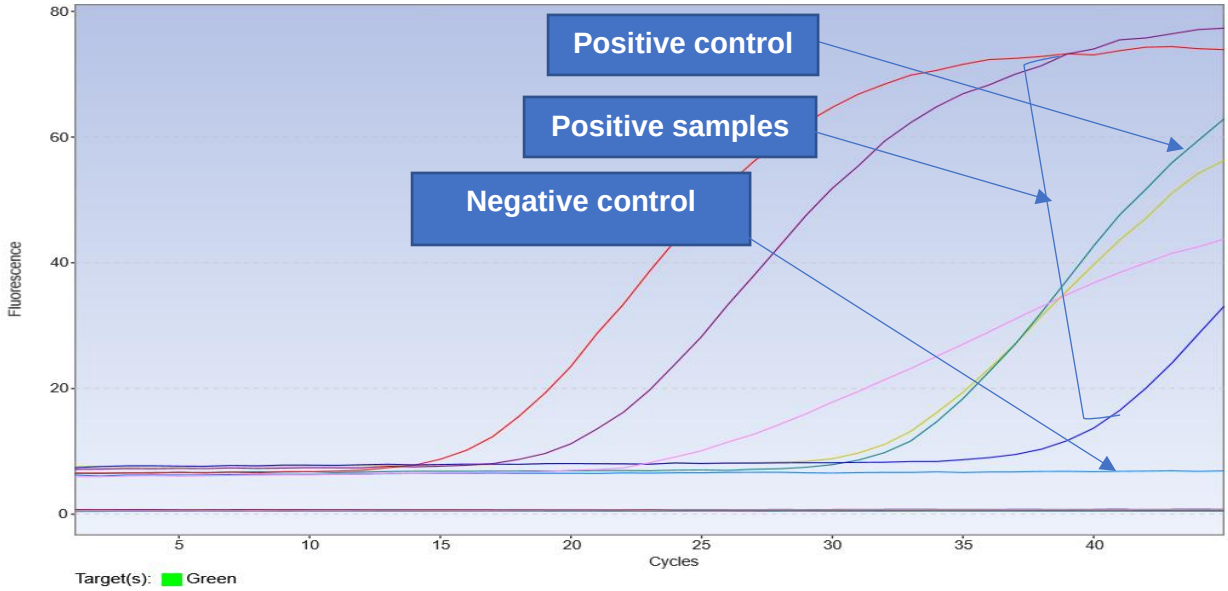

Figure 6.7: Fluorescence of hybridisation Lipoprotein L32 gene (*LipL32*) probe of pathogenic *Leptospira* spp. using qPCR from pig kidneys, positive control (*Leptospira interrogans,* serovar Icterohaemorrhagiae strain and the negative control (ultra-pure water).

6.4.4.2.4 The LipL32 gene region qPCR for pathogenic Leptospira spp. in sheep:

Of the three animal species tested, sheep had the highest frequency *LipL32* gene region present in pathogenic *Leptospira* spp. detected with pPCR in kidney tissues at 42.2% (19/45) as shown in **Figure 6.8.**

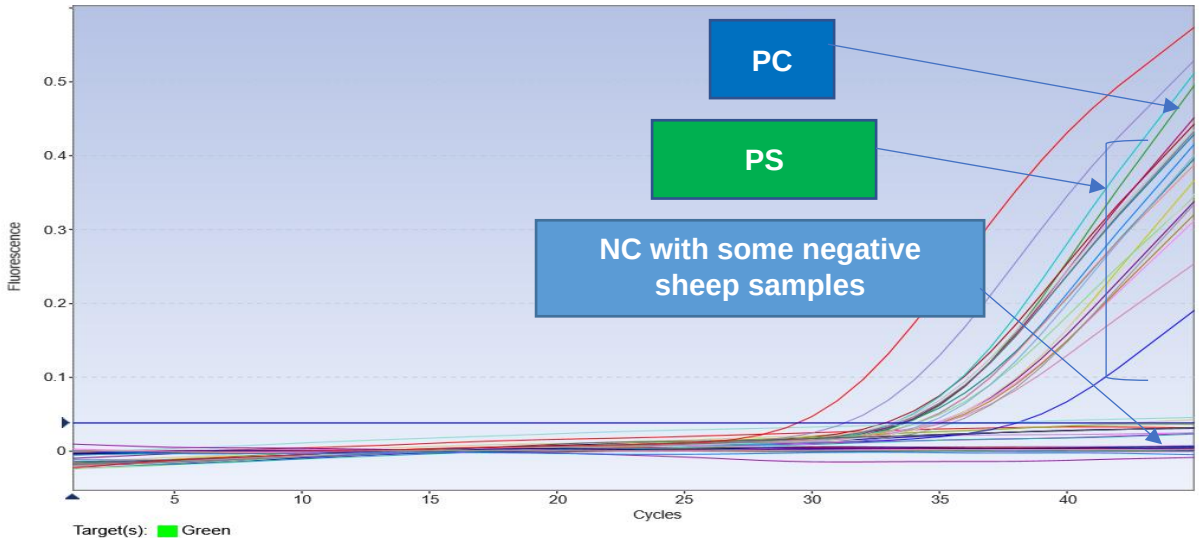

Figure 6.8: Fluorescence of hybridisation Lipoprotein L32 gene (*LipL32*) probe of pathogenic *Leptospira* spp. using qPCR from sheep kidneys, positive control *Leptospira interrogans,* serovar Icterohaemorrhagiae strain (PC) and the negative control (NC) with other NKFS.

Note: PC= *Leptospira interrogans,* serovar icterohaemorrhagiae strain, Positive control; PS=Positive samples and NC=Negative control with some negative sheep samples.

6.4.4.2.5 *Detection* of pathogenic/virulence *LipL32* gene region using qPCR in isolates of *Leptospira* spp. and kidney tissues from livestock:

The overall detection of the *LipL32* gene region using qPCR present in pathogenic *Leptospira* spp. was positive in 84 (27.5%) of 305 kidney samples tested. The positivity rate was 3.3% (10/305) for *Leptospira* isolates observed under the Dark field Microscope (only 10 of the 12 *Leptopsira* isolates were observed as pure and 2 were contaminated and could not be used in this assay). Of the 10 isolates of *Leptospira* spp. observed under the Darkfield microscope, 6 were identified as pathogenic *Leptospira* spp. by the *LipL32* gene region qPCR assay and the remaining 4 isolates were unidentified as described in *section 6.4.2* of this Chapter. Furthermore, from these positive genomic DNA quantified, the *SecY* gene region of *Leptospira* spp. was used to generate a total of 22 sequences, six originated from the isolates and 16 from the kidney tissues. **Figure 6.9 and 6.10** shows the amplification of the first and nested PCR of the *SecY* partial gene region using PCR.

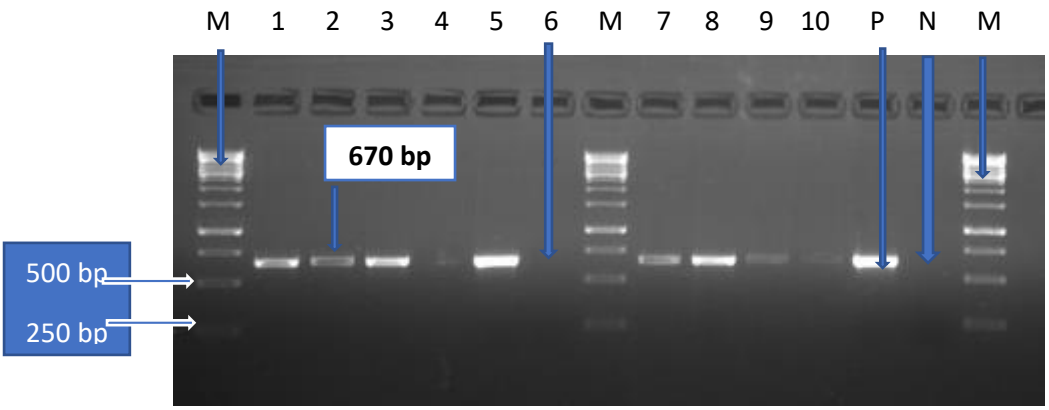

Figure 6.9: Agarose gel image of the first amplification of the 670 bp *SecY* partial gene region using PCR with primers (*SecY*II and *SecY*IV). The marker (M) is the O' Gene Ruler 1Kb DNA Ladder (Thermo Fischer). M=Marker; 1, 2, 3, 4, 5, 7, 8, 9 and 10 = Samples positive; 6= Sample negative; P=Positive control (*Leptospira interrogans,* serovar Copenhageni strain Fiocruz L1- 130) and N=Negative control (ultra-pure water).

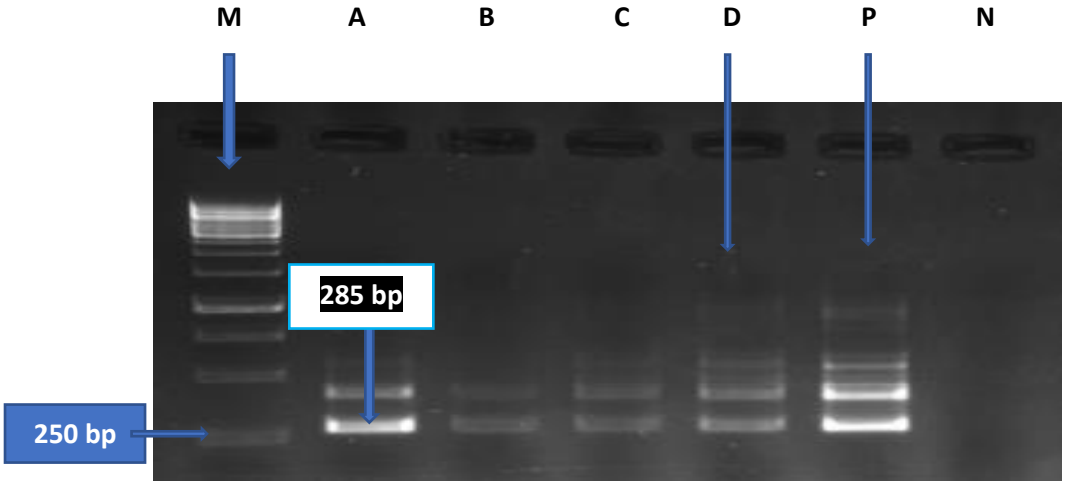

Figure 6.10: Agarose gel image of the nested amplification of the 285 bp *SecY* partial gene region using PCR with primers (G1G2). The O' Gene Ruler 1Kb DNA Ladder (Thermo Fischer) was used as marker (M). M=Marker; A to D=Samples positive for *SecY* gene region nested PCR; P=Positive control (*Leptospira interrogans,* serovar Copenhageni strain Fiocruz L1- 130) and N=Negative control (ultra-pure water).

6.4.4.3 Phylogeny of *SecY* sequences of Leptospira isolates and kidneys samples tissue

6.4.4.3.1 Sequences of *SecY* gene region:

Twenty-two sequences from the *SecY* gene region amplified and sequences from six (6) isolates and 16 from kidney tissues were identified. The *SecY* gene region sequences from the 6 isolates included 5 from cattle (4 *L. interrogans* and 1 *L. borgpetersenii*), 1 *L. interrogans* from pig. The *SecY* gene region sequences from the 16 kidney tissue samples included 10 from cattle of which 9 were *L. interrogans* and 1 was *L. borgpetersenii.* Three *SecY* gene region sequences from pig kidney samples were identified as *L. interrogans* and the 3 sequences from sheep were identified as 2 *L. interrogans* and 1 *L. borgpetersenii*

6.4.4.4 Phylogenetic tree analyses of the 22 sequences generated from *SecY* partial gene region of slaughtered livestock

6.4.4.4.1 Phylogenetic tree for isolates of Leptospira spp. and kidney tissues of cattle

The phylogenetic tree analysis of *SecY Leptospira* gene sequences from cattle consisting of *L. interrogans* and *L. borgpetersenii* clustered into two different clades (clade A and B) according to their serovars (**Figure 6.11).** The 4 *SecY L. interroga*ns sequences (4 from isolates indicated by red dots) and 9 from kidney samples (in bold without dots) with **Figure 6.11 clade A**) were identical to each other and to GenBank sequences of *L. interrogan*s serovar Icterrohaemorrhagiae A 20 (KU219597), *L. interrogan*s Lai 56601 and *L. interrogan*s serovar Copenhageni (**Figure 6.11 clade A**) and clustered with identical 9 sequences from kidney samples (in bold without dots) with **Figure 6.11 clade A**). The 2 *SecY L. borgpetersenii* sequences from cattle samples (1 isolate with red dot and 1 kidney tissue sample in bold without dot) were identical to each other and to Genbank *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 (EU365953), *L. borgpetersenii* serovar Hardjo 105A and *L. borgpetersenii* Tunis P 2 25 sequences (**Figure 6.11 clade B**).

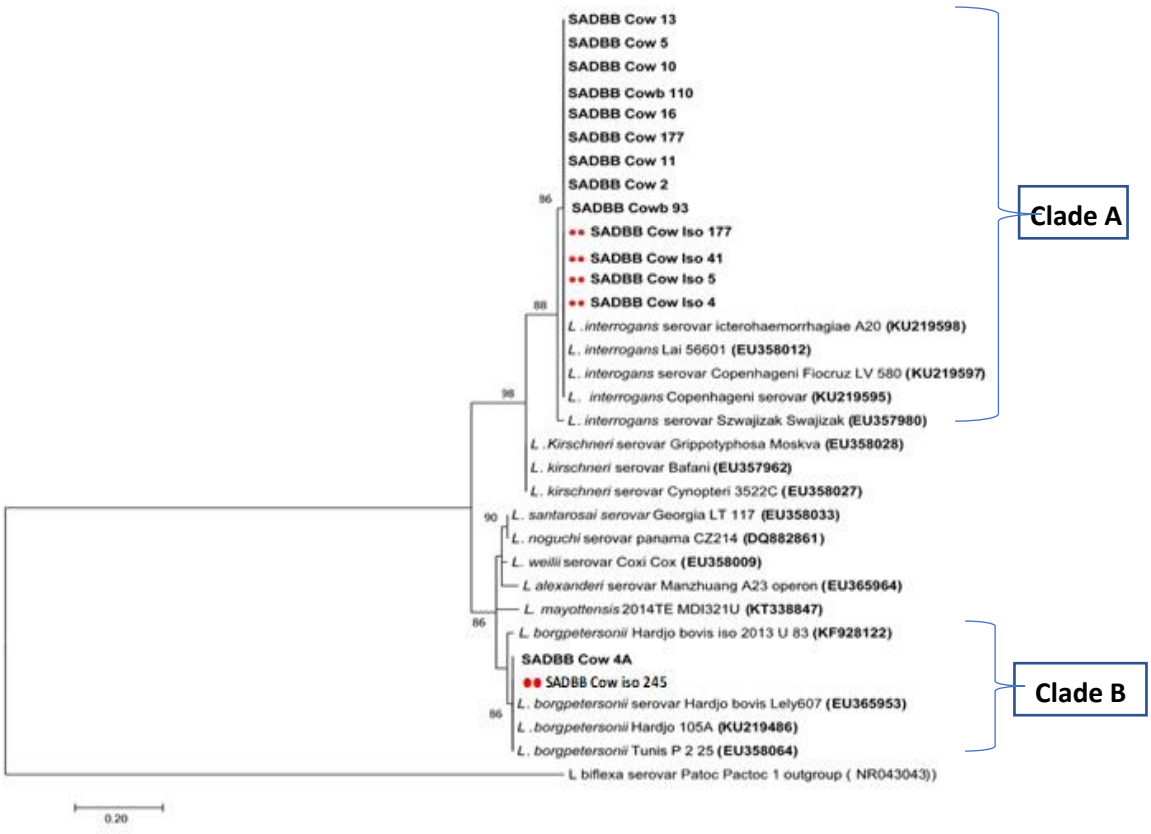

Figure 6.11: Phylogenetic tree of *SecY* partial gene region of pathogenic *Leptospira* spp. sequences using the maximum likelihood methods based on the General Time Reversible (GTR+1) model. *SecY* sequences were obtained from 15 cattle slaughtered at Gauteng abattoirs indicated in bold, which include sequences obtained from *Leptospira* cultures or isolates indicated by red dots as well as GenBank reference sequences of pathogenic *Leptospira* species with *L. bifexa* as outgroup. Bootstrap of 1000 replicates with values above 75 % were considered.

6.4.4.4.2 Phylogenetic tree for isolates of Leptospira spp. and kidney tissues of pigs:

The phylogenetic tree analysis of *SecY Leptospira* gene sequences from pigs identified as *L. interrogans* clustered into clade C (**Figure 6.12).** Sequences *SecY* SADBB_pig_62 and SADBB_pig 51 from pig kidney samples were identical with *L. interrogan*s serovar Icterrohaemorrhagiae A 20 (KU219597), *L. interrogan*s Lai 56601 and *L. interrogan*s serovar Copenhageni, while *SecY L. interrogan*s sequence from an isolate (SADBB_pig_iso 290 indicated by 2 red dots) and SADBB_pig_41 (from pig kidney sample) were identical to each other but differed slightly from the other *L. interrogans* sequences (**Figure 6.12 Clade C**).

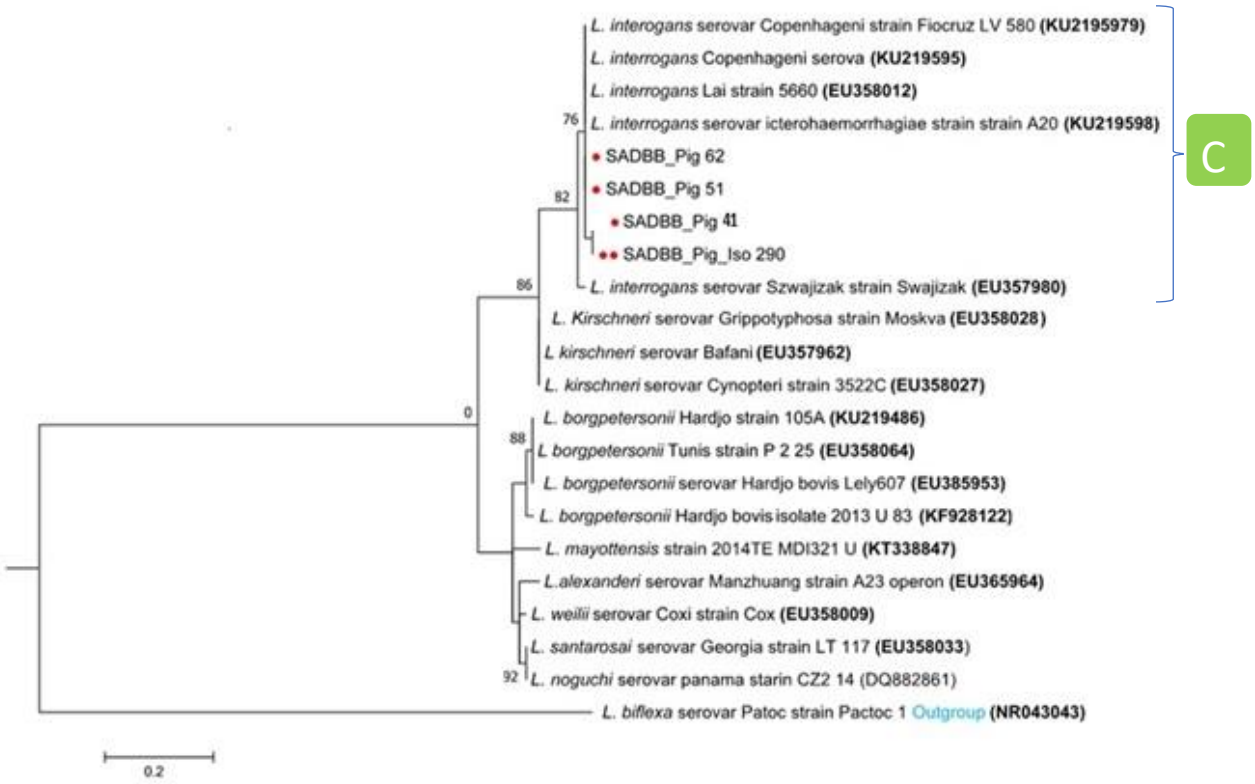

Figure 6.12: Phylogenetic tree of *SecY* partial gene region of pathogenic *Leptospira* spp. sequences using the maximum likelihood methods based on the General Time Reversible (GTR+1) model. *SecY* sequences were obtained from 4 pigs slaughtered at Gauteng abattoirs indicated in bold single dot (kidney tissues) which include sequences obtained from *Leptospira* cultures or isolate indicated by two red dots as well as GenBank reference sequences of pathogenic *Leptospira* species with *L. bifexa* as outgroup. Bootstrap of 1000 replicates with values above 75 % were considered.

6.4.4.4.3 Phylogenetic tree for isolates of Leptospira spp. and kidney tissues of sheep:

The phylogenetic tree analysis of *SecY Leptospira* gene sequences from sheep consisting of *L. interrogans* and *L. borgpetersenii* clustered into two clades (clade D and E) according to the different serovars (**Figure 6.13).** The *SecY L. interrogan*s SADBB sheep 1 sequence from kidney sample (in bold with 1 red dot, **Figure 6.13 clade D**) was identical to GenBank sequences of *L. interrogan*s serovar Icterohaemorrhagiae A 20 (KU219597), *L. interrogan*s Lai 56601 and *L. interrogan*s serovar Copenhageni (**Figure 6.13 clade D**) which clustered but differed slightly from *SecY L. interrogan*s SADBB sheep 2 sequence from kidney sample (in bold with 1 red dot, **Figure 6.13 clade D**). The *SecY L. borgpetersenii* SADBB sheep 3 sequence (in bold with 1 red dot) from sheep kidney sample was identical to Genbank *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 (EU365953), *L. borgpetersenii* serovar Hardjo 105A and *L. borgpetersenii* Tunis P 2 25 sequences (**Figure 6.13 clade E**).

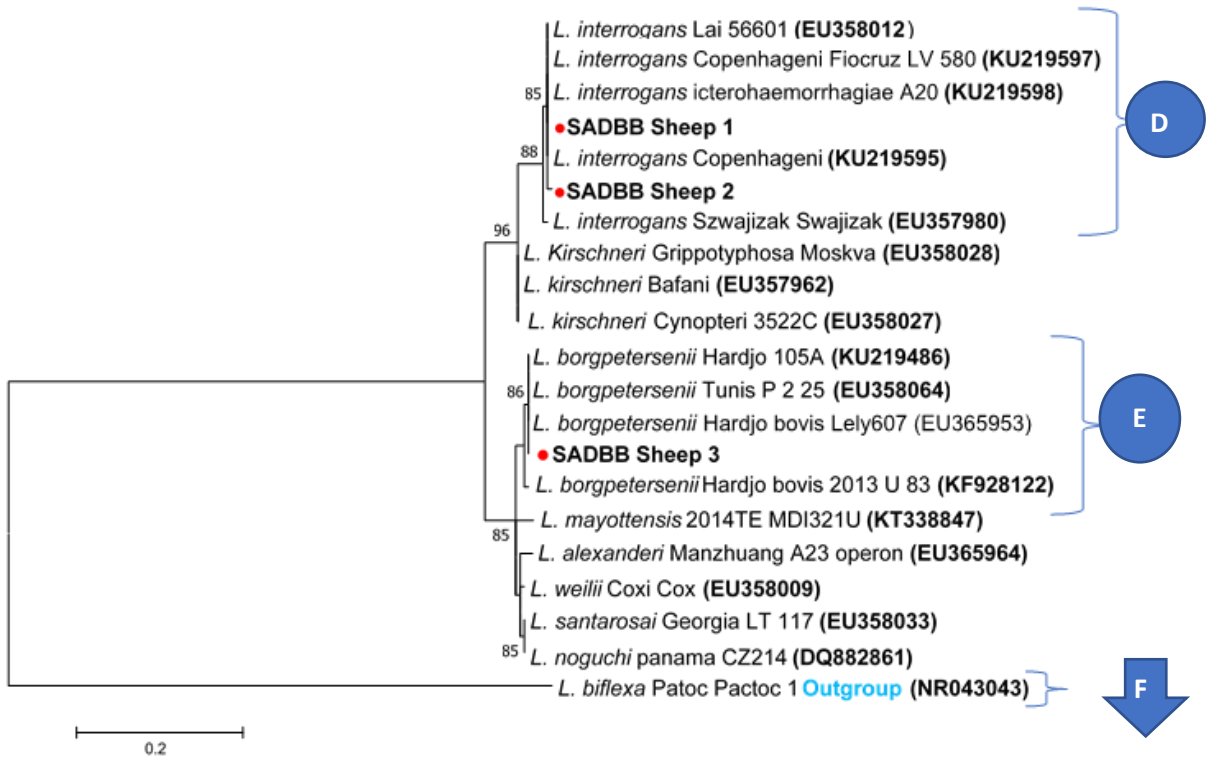

Figure 6.13: Phylogenetic tree of *SecY* partial gene region of pathogenic *Leptospira* spp. sequences using the maximum likelihood methods based on the General Time Reversible (GTR+1) model. *SecY* sequences were obtained from 3 slaughtered sheep at Gauteng abattoirs indicated with 1 red dot as well as GenBank reference sequences of pathogenic *Leptospira* species with *L. bifexa* as outgroup. Bootstraps of 1000 replicates with values above 75 % were considered.

6.4.4.4.4 Phylogenetic analysis for isolates of Leptospira spp. and kidney tissues from livestock (cattle, pigs and sheep):

The phylogenetic tree analysis of *Leptospira* spp. *SecY* partial gene region sequences from cattle, pigs and sheep clustered with Genbank partial *SecY* sequences of *L. interrogans* and *L. borgpetersenii* into two clades **(G and H), (Figure 6.14).** The *SecY* partial gene sequences from isolates recovered from cows' kidneys SADBB_cow_iso4, SADBB_cow_isof5, SADBB_cowb_isot and SADBB_cow_isof177 (**Figure 6.14,** G1 marked with red dots written in red ink boldly), pigs kidney tissues samples, SADBB_Pig_62 and SADBB_Pig_51, **(G1 in blue ink in Figure 6.14)** and sheep kidney sample, SADBB_sheep_26 **(G1 in green ink in Figure 6.14)** were identical to *L. interrogans* serovar icterohaemorrhagiae strain A20 (KU219598), *L. interrogans* Lai strain 56601 (EU358012), *L. interrogans* Copenhageni serovar (KU219595) and *L. interogans* serovar Copenhageni strain Fiocruz LV 580 (KU219597) **(Figure 6.14 G1).** These identical *L. interrogans* South African sequences (**G1**) were from pigs and sheep kidney tissues and isolates from cattle kidneys. Nine *SecY* partial sequences from cattle kidney tissue were identical **(G2 subclade in Figure 6.14)** and different slightly

from sequences in G1 subclade. The *SecY L. interroga*ns sequences from pig culture (SADBB_Pig_iso290, indicated by blue arrow and written in blue ink in bold), SADBB_pig_41 and SADBB_Sheep_30 {from pig kidney tissue (blue ink in bold) and sheep kidney tissue (green ink in bold) samples} were identical and clustered together subclade G3 and different slightly to G2 subclade, **(Figure 6.14).** The *SecY L. borgpetersenii* SADBB_Cow_4, SADBB_Cow_iso245 and SADBB_Sheep_329 sequences **(Figure 6.14 in clade H**) were identical with Genbank L. *borgpetersonii* serovar Hardjo bovis Lely607 **(EU365953),** *L. borgpetersonii* Hardjo strain 105A **(KU219486)** and *L. borgpetersenii* Tunis strain P 225 (EU 358064) sequences.

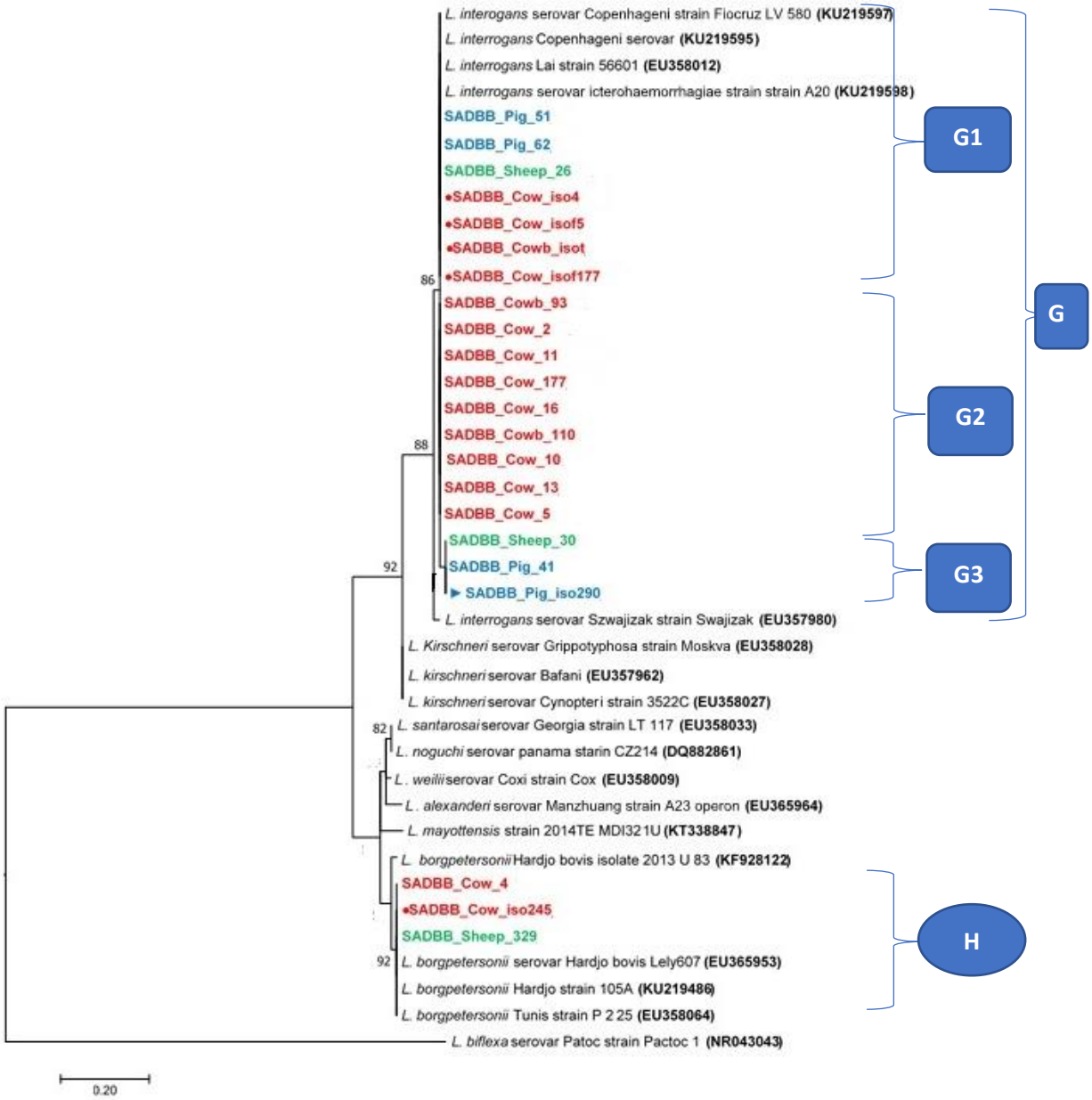

Figure 6.14: Phylogenetic tree of *SecY* partial gene region of pathogenic *Leptospira* spp. sequences using the maximum likelihood methods based on the general time reversible (GTR+1) model. *SecY* sequences were obtained from 22 livestock [cattle (red ink in bold), pigs (blue ink in bold) and sheep (green ink in bold)] slaughtered at Gauteng abattoirs. Cattle isolates sequences are indicated with a red dots and red ink in bold, while cattle kidney tissue samples sequences are indicated by red ink in bold without dot; pigs isolates are indicated by a blue arrow and written in blue ink in bold while the pigs' kidney tissue samples sequences are indicated with blue written in bold ink without the arrow and for the sheep kidney tissues sequences written in bold green ink. The GenBank reference sequences of pathogenic *Leptospira* species with *L. bifexa* as outgroup were used. Bootstrap of 1000 replicates with values above 75 % were considered.

6.5 Discussion

Accurate diagnosis of leptospirosis in livestock, such as cattle, pigs and sheep being important for the well-being of animals, the economy of the country, small stakeholders and healthy environment. It is also invaluable for the control and prevention of the disease spill-over to humans being a zoonotic disease, especially to veterinarians, abattoir workers and farmers. It is therefore important to have detected in this cross-sectional study that the overall frequency of isolation of *Leptospira* spp. in slaughtered livestock was 3.9% (12/305) and for detection of *Leptospira* DNA by PCR, was 27.5% (84/305). The overall frequency of isolation of *Leptospira* spp. (3.9%) from all livestock (cattle, pigs and sheep) in our study is considerably lower than the 38.2% (13/34) reported for slaughtered livestock in a Brazilian slaughterhouse (Da Silva et al 2012). The difference may reflect livestock management and sanitary practices among other factors (Gummow et al., 1999).

Considering that the cattle population in South Africa during the 2014/2015 period was 13.7 million of which 24,476 were slaughtered for consumption and for economic empowerment (DAFF, 2016), the isolation of *Leptospira* spp. from 4.8% (9/186) of the slaughtered cattle in Gauteng province is important. The slaughtered cattle in Gauteng province originated from various provinces in South Africa and not solely from Gauteng province. Furthermore, infected livestock could be shedders of the pathogen capable of contaminating the environment (farms and abattoirs), therefore exposing the farmers, veterinarians and abattoir workers to leptospirosis, thus posing a zoonotic risk (Fang et al., 2014). It has been documented that humans could become exposed to *Leptospira* spp. through the mucous membranes and skins (Ko et al., 2009).

The isolation rate (4.8%) of *Leptospira* spp. from the kidneys of slaughtered cattle in the current study is considerably higher than the 0.76% (1/131) reported in slaughtered cattle in Nan province, Thailand (Kurilung et al., 2017) but lower than the 10.4% isolation rate for *Leptospira* spp. reported for cattle slaughtered in abattoirs near Harare in Zimbabwe (Feresu et al., 1992). Differences in the isolation rates of *Leptospira* spp. in studies conducted in different countries and environments may be explained in part, by the fastidious growth requirements of leptospires during isolation, the media used, isolation method, the technical abilities of individuals involved and the risk of contamination during the long incubation period associated with the isolation process (OIE, 2018; Picardeau, 2013).

In South Africa, data on isolation of leptospires in cattle and from abattoirs are lacking. In an investigation of an outbreak of leptospirosis on livestock farms, Gummow and co-workers, (1999)

isolated the pathogen from 25% (3/12) of samples of cattle urine and aborted foetus. The higher frequency of isolation of leptospires from their study compared to our study might be due to the clinical cases during an outbreak investigated by Gummow et al., (1999) while we sampled apparently healthy slaughter cattle in abattoirs. The possible effects of environmental factors such as rodent population and urine contamination, as well as sanitary practices on farms, cannot be ignored (Petrakovsky et al., 2014).

In the current study, *LipL32* gene region qPCR detected *Leptospira* DNA in 26.9% (50/186) in the kidney tissues of slaughtered cattle which is like the reported detection of *Leptospira* DNA in 21.0% (30/148) of the cattle kidney samples tested in New Zealand, also by qPCR (Fang et al., 2014). Similarly, the overall frequency of detection of *Leptospira* DNA from the livestock (cattle, sheep and pigs) in our study, 27.5% (84/305), is almost the same frequency of 27.0% (145/545) in sheep and cattle in New Zealand. It may reflect a similarity of the exposure of livestock to leptospirosis in both South Africa and New Zealand.

Pigs also play a role in the economy of South Africa being a source of food and job opportunities and based on the statistics provided on the annual number of pigs reared in 2014/2015 being 1,523,000, the production was 233,000 tonnes with 4.7 million per capita kg/year. In this study, the frequency of isolation of *Leptospira* spp. from slaughtered pigs was 4.1% (3/74) which is comparable to the 3.94% (6/152) reported for pigs' urine sampled in Thailand (Kurilung et al., 2017). However, the isolation rate for leptospires; from slaughtered pig kidneys in Colombia, 0.8 % (3/383) (Romero-Vivas et al., 2013) and in Brazil, 0.0% (0/36) (de Freitas et al., 2004) are considerably lower than detected in the current study**.** The difference in the finding of these studies might be due to the difficulties associated with culture of leptospires, contamination, skills of personnel and rodent population in the different regions of studies (Ko et al., 2009; OIE, 2014; Picardeau, 2013).

In this study, *Leptospira* DNA was detected in 20.3% (15/74) of the kidneys of pigs tested using qPCR which is much higher than the 0.8% (3/383) reported for a similar study in pigs in Colombia using PCR (Romero-Vivas and co-workers, 2013). This may be due to management system and environmental factors, sensitivity and specificity of the PCR assay, the type of samples collected as well as the time of samples collection, how it was collected and stored alongside with the experience of the personnel in PCR diagnosis (Bharti et al., 2003; Gummow et al., 1999; Johnson et al., 2004; Picardeau, 2013; Vinetz, 2001).

The sheep population in South Africa is low compared to the cattle and pigs, therefore, the number of sheep as at 2014/2015 was 21,033. No prior isolation of *Leptospira* spp. from sheep had been documented in South Africa and it is of interest that the current study failed (0.0%) to isolate leptospires from the 45 kidney samples tested. This may reflect the low level of infection with *Leptospira* spp. in the sheep population in the country and possibly due to the failure of the isolation process to recover leptospires in the kidney tissues tested. A study in Brazil however reported a considerably higher frequency of isolation of *Leptospira* spp. from 46.2% (6/13) of sheep slaughtered in abattoirs (Da Silva et al., 2012). The difference between both studies may again be due to the exposure of sheep to leptospires as a result of management practices and exposure to rodents, which are known important reservoirs of *Leptospira* spp. (Gummow et al., 1999; Ko et al., 1999).

Of diagnostic relevance is the finding that the isolation rate of *Leptospira* spp. was significantly lower than the detection rate for *Leptospira* DNA in kidney tissues in cattle (4.8% versus 26.9%, $p<0.0001$), pigs (4.1% versus 20.3%, $p=0.0025$) and sheep (0.0% versus 42.2%, $p<0.000001$). Furthermore, the overall frequency of isolation of *Leptospira* spp. from kidney tissues in the livestock tested was 3.9% (12/305) which was statistically significantly ($p<0.00001$) lower than the rate of detection rate of 27.5% (84/305) of *Leptospira* DNA in kidney tissues. The implication is that the use of the isolation procedure alone to determine the prevalence of *Leptospira* spp. in livestock in South Africa, if done routinely, will grossly under-estimates the status of the disease. Of importance is the fact the all the sheep kidney tissue samples were negative for *Leptospira* spp. while the same kidney samples were positive for *Leptopira* DNA. These differences were due to the considerably higher sensitivity, specificity and accuracy of qPCR assay compared to any other diagnostic methods used for the diagnosis of leptospirosis (Bourhty et al., 2011; Picardeau, 2013; WHO, 2011). It is however important to mention that qPCR is unable to differentiate between the existence of live and dead leptospires in kidney tissues which limits its application for risk assessment for human and animal exposure to the pathogen and the contamination of the environment with viable leptospires (Picardeau, 2013; Wunder et al., 2016).

The risk of exposure of abattoir workers to *Leptospira* spp. is of zoonotic significance, considering the findings of the isolation studies which determined that 7 (50.0%) of the 14 abattoirs from where the livestock samples originated, slaughtered *Leptospira*-positive animals. Additionally, 27.5% (84/305) of the 14 abattoirs slaughtered livestock positive for *Leptospira* DNA. In our study, workers who worked at abattoirs that slaughtered cattle appeared to have a higher exposure potential to *Leptospira* spp., compared to abattoirs that slaughtered pig and sheep based on the frequency of isolation of the pathogen from cattle kidneys and the fact that 75% of the isolates originated from

abattoirs that slaughtered cattle. It has been documented that abattoir workers have a higher prevalence (serology and isolation) of leptospirosis and other zoonoses, compared to members of the general population, (Kurilung et al., 2017).

Of the risk factors (type of abattoir, throughput, animal species, sex and breed) investigated for isolation of *Leptospira* spp. from cattle and pigs, only sex in cattle had a significant (p=0.0209) effect. It is of potential clinical importance that the detection of the *LipL32* and *SecY* genes partial regions in this study is an indication of the presence of the pathogenic *Leptospira* virulence genes in the DNAs of the isolates and kidney tissues. Other researchers have used the detection of *LipL32* and the *SecY* genes partial region to determine the virulence of leptospires (Stoddard et al., 2009; Victoria et al., 2008; Wunder et al., 2016). Since the prior clinical status of the livestock was not determined pre-slaughter in our study, it will be prudent to assess the clinical significance of the virulence gene-positive isolates of *Leptospira* spp. in future studies. This is because the possession of virulence genes by leptospires or other pathogens does not always lead to the expression of virulence in susceptible hosts. Animal models, particularly hamsters, have been demonstrated to be very suitable for determining the virulence of *Leptospira* spp. (Agudelo-Flórez et al., 2013; Suepaul et al., 2010).

The genetic analyses of the cattle isolates and kidney tissue samples in our study for pathogenic *Leptospira* spp. used to determine their phylogeny were identified mostly through identical homology with *L. interrogans* and *L. borgpetersenii* sequences from GenBank. The 4 *SecY* partial gene sequences from Leptospira isolates from cattle samples were determined to belong to subclade G1 in **Figure 6.11** and were identical to *Leptospira interrogans* serovar Icterohaemorrhagiae A 20 (KU219597) sequence (**Figure 5.14** subclade G1) while almost identical sequences of the kidney samples clustered in subclade G2, which could be due to proofreading mistake by *Taq* polymerase during PCR since two of the *Leptopsira* isolates from cattle (*SecY* partial sequences (Clade G1) were detected in the kidney sample of the same animal (cow_5 and cow_177) in subclade G2.

The sequences of *SecY* from isolate from cattle (cow_iso245), kidney samples (cow_4 and sheep_329) were identical to *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 (EU365953) sequence **(Figure 6.11).** These findings suggest that the South African isolates might likely be *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 that may be circulating in the livestock population in South Africa. This suggestion is supported by the finding in Chapter 4, *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 antigen reacted with sera from livestock (cattle and pigs) using

the Microscopic Agglutination Test (MAT). It is also pertinent that despite the demonstration of presence of *L. borgpetersenii* serovar Hardjo bovis strain Lely 607, serologically and genetically, the ARC-OVR laboratory uses an eight-antigen panel for MAT which does not include *L. borgpetersenii* serovar Hardjo bovis strain Lely 607. The non-inclusion of this serovar in the diagnostic panel may therefore be contributing to the under-reporting of leptospirosis in South Africa. The *SecY* sequences from the isolate from pig (SADBB_pig_iso 290), pig kidney sample SADBB_pig_41 and sheep kidney SADBB_sheep_30 were identical and indicate that the same serovar might be circulating in the sheep and pig population in South Africa (**Figure 5.13** G3 subclade). In the combined phylogeny of the livestock (cattle, pigs and sheep) *SecY* sequences from isolates and kidney samples from cattle, sheep and pigs in this study were identical to *L. interrogan*s serovar Icterohaemorrhagiae A 20 (KU219597), *L. interrogan*s Lai 56601 (EU 358012), *L. interrogan*s serovar Copenhageni, (KU 219595) and *L. interogans* serovar Copenhageni strain Fiocruz LV 580 (KU219597) **(Figure 5.13 G1**) and *L. borgpetersenii* sequences from cattle and sheep had identical sequences to Genbank *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 (EU365953), *L. borgpetersenii* serovar Hardjo 105A and *L. borgpetersenii* Tunis strain P 2 25 (EU 358064) sequences in Clade H, **Figure: 5:14.**

6.5.1 Conclusions

i). The isolation of pathogenic *Leptospira* spp. at a rate of 3.9% (12/305) by bacteriological assay and the detection of pathogenic *Leptospira* DNA by PCR in 27.5 % (84/305) in the kidneys of slaughtered livestock tested are indicative of the level of infection of livestock on farms in Gauteng province and other provinces in the country with *Leptospira* spp., and the potential exposure of abattoir workers to the pathogen.

ii). The data presented in this chapter provide the most current data on the status of leptospirosis in the last two decades in Gauteng province and South Africa at large, using bacteriological and molecular methods.

iii). The study presented the first documentation of molecular characterization studies on pathogenic *Leptospira* spp. in South Africa.

iv). The combination of the gold standard MAT, isolation and molecular methods (cPCR and qPCR) as a strategy for the detection and diagnosis of leptospirosis significantly increased the sensitivity and specificity of the strategy.

v). It was significant that although all the kidney samples from sheep cultured for *Leptospira* spp. were negative, with an isolation rate of 0.0% (0/45) but the same kidney tissues yielded *Leptospira* DNA at a detection rate of 42.2% (19/45) by qPCR. This is considered a significant finding as bacteriological assay grossly under-estimated the occurrence of *Leptospira* spp. in the sheep sampled.

vi). Finally, this chapter produced baseline data that can be built upon for active and passive surveillance of leptospirosis in the livestock industry. This will boost livestock farming as a contribution to economic development, job and food security in South Africa; as well to control and prevent the spill over of leptospirosis to humans.

6.5.2 Limitations/Recommendations of the study:

i) Sample size was not high enough as required to provide ample research information. There is the need for a large sample size on country-wide survey of leptospirosis in livestock and if possible, in humans working in close contact to these animals

ii) Sensitization of the public on leptospirosis in livestock and humans need to be conducted in South Africa.

iii) There is the need for improved technical-know-how on the diagnosis of leptospirosis using combination of the three (bacteriological culture, serological MAT and the PCR) methods for the diagnosis of leptospirosis in animals and humans in South Africa as recommended by the WHO for better control and prevention strategies.

iv). The inclusion of *L. borgpetersenii* serovar Hardjo bovis strain Lely 607 in the panel of antigens used to serotype the sera of animals for the occurrence of leptospirosis is recommended.

6.5.3 Connecting statement:

Following the findings in Chapter 6 on the occurrence of pathogenic *Leptospira* spp. in the kidneys of slaughtered livestock and the molecular and genetic characterization of the isolates of *Leptospira* spp., we now plan to link the data to Chapter 7 where an assessment of the risk of exposure of abattoir workers to leptospirosis through contact with slaughtered livestock. It is anticipated that the study will emphasize the zoonotic importance of leptospirosis using both the MAT, IgM ELISA and qPCR assay on sera and whole blood from the workers, as well as determining the risk factors for exposure.

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CHAPTER 7

Seroprevalence and molecular detection of *Leptospira* spp. and associated risk factors for abattoir workers in Gauteng province, South Africa

Manuscript in preparation

7.1 Abstract:

Globally, abattoirs play an important role in the active and passive surveillance for leptospirosis in livestock. The risk of exposure of abattoir workers to zoonoses, including leptospirosis, has been documented in several countries. To date, there is a dearth of information on the seroprevalence of leptospirosis in abattoir workers in any abattoir in the country. Therefore, this study was conducted to determine the prevalence of *Leptospira* spp. in abattoir workers in Gauteng province, South Africa, using two serological tests (IgM Enzyme-linked immuno-sorbent assay, ELISA, and the microscopic agglutination test, MAT) and the quantitative polymerase chain reaction (qPCR) assay to detect *Leptospira* DNA in the whole blood of abattoir workers. The MAT used a panel of 25 serovars. A total of 103 consenting workers at 6 abattoirs (3 high throughput-HT and 3 low throughput-LT) from whom a maximum of 5 mL of blood was collected from each worker. During the sampling visit, both a standardized questionnaire and consent form were administered to each worker. Of the 103 workers tested, 17 (16.5%) were positive for *Leptospira* spp. DNA; 11 (10.7%) and 8 (7.8%) were seropositive for antibodies to *Leptospira* spp. by the IgM ELISA and MAT, respectively. Overall, with the use of the three diagnostic tests, 30.1% (31/103) of the workers were positive for exposure to *Leptospira* spp. All the six abattoirs had workers positive for exposure to *Leptospira* spp. For the 8 abattoir workers positive by the MAT, the predominant serovar detected was Djasiman in 4 (50.0%) abattoir workers, followed by Wolffi in 2 (25.0%). Additionally, the 8 seropositive workers had titres ranging from 100 to 3,200 and 4 (50.0%) had significant titres of 800 to 3,200 even though all the workers sampled were apparently healthy. Of a total of 19 risk factors investigated, three (HT abattoirs, OR=3; farm animal contact, OR=3.44 and exposure to blood or water splash during slaughter, OR=2.27), were associated with seropositivity for leptospirosis. The fact that overall, 30.1% of the workers tested were positive for exposure to *Leptospira* spp. (antiserum and DNA), the relatively high titres detected in seropositive workers, and the finding that all six abattoirs had seropositive workers are indicative of the risk of leptospirosis posed to abattoir workers. It is recommended that for the abattoir-associated risk factors (HT abattoirs and blood and water

splashes), there is a need for intervention measures to reduce the risk of exposure to the disease at the abattoirs. The possibility of non-abattoir associated risk factors for leptospirosis also needs to be investigated.

Key words: Seroprevalence, molecular detection, Leptospirosis, abattoir workers, risks factors and South Africa.

7.2 Introduction:

Abattoirs, which are facilities for the slaughter of livestock, could also be invaluable for active and passive surveillance of zoonotic diseases, such as leptospirosis, for its control, prevention, early warning, and intervention programmes. Leptospirosis is an important bacterial zoonosis of international significance. It is referred to as a febrile illness (Manocha et al., 2004), an occupational and recreational disease, and a re-emerging zoonosis (Ko et al., 1999; Vinetz et al., 1996). The disease is known to have a worldwide distribution (Vinetz, 2001).

The transmission of leptospirosis from animals to humans occurs at the interface of the environment where animals and humans have contact (Petrakovsky et al., 2014). The disease has many host types including rodents (rats and mice), cattle, pigs, sheep, horses, dogs, cats, and wild life species (Levett, 2001).

Humans get infected by the pathogenic *Leptospira* spp. through direct contact with animal reservoirs, infected animal tissue, environmental surface water and soil contaminated with the urine from infected animals (de Vries et al., 2014). Following the exposure of humans to pathogenic *Leptospira* spp., the organism binds and enters the skin, mucous membrane and eventually gets into the bloodstream and spreads throughout the body causing febrile illness in the acute form of the disease (Levett, 2001).

There are many high risk individuals in the population resulting from their occupation (abattoirs workers, veterinarians, sewer workers, dairy farmers, sugarcane farmers, mine workers, etc.), practices and habits (Levett, 2004; Meites et al., 2004).

Leptospirosis in humans may be misdiagnosed as malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, melioidosis and other diseases (Ellis et al., 2008). The pathogenesis of leptospirosis involves an early leptospiraemic phase and later stage causing severe multisystem manifestations in the form of hepatic dysfunction, jaundice, acute renal failure,

pulmonary haemorrhage syndrome, myocarditis, eye vitreous humour and meningoencephalitis (Ko et al., 2009). Humans serve as accidental hosts considering that they are unable to serve as reservoirs because they cannot spread a high number of the pathogen in the urine or blood during the bacteraemia phase.

Generally, leptospirosis is believed to be an under-reported disease in human and animal populations, primarily due to similar clinical signs and symptoms shared with other diseases. The diagnosis of leptospirosis is usually confirmed by the Microscopic Agglutination test (MAT) (OIE, 2014) and it is considered the gold standard (WHO, 2003). The disadvantages of the MAT include the occurrence of false-positive and false-negative results (resulting in lower accuracy), the serogroups identified do not always correlate with the strains isolated, cross -reactivity with vaccine strains and other serovars, and it is laborious (Picardeau, 2013; WHO, 2003). Other disadvantages of the MAT are that it is serogroup-specific and cannot identify the serovar, it requires paired samples two weeks apart to diagnose acute leptospirosis, it needs expertise to read results microscopically, it requires vigilance in keeping the live antigen strains free of contamination (Picardeau, 2013; Smythe et al., 2009). MAT results do not always correlate with DNA analyses (Levett, 2011). However, the MAT has been used in epidemiological studies of a given population or herd with good specificity (OIE, 2014). Sensitivity and specificity of the MAT was determined to be 95.7% and 55.3%, respectively (Niloofa et al., 2015). Values may vary depending on the stage of infection or level of exposure in animals or humans in a location. Other studies have found values of 98.2% and 96.4% for sensitivity and specificity, respectively for MAT (Bajani et al., 2003).

Enzyme-linked immunosorbent assay (ELISA) is another test that is used for the diagnosis of leptospirosis and it can detect IgG and IgM classes of immunoglobulins (Hartskeerl et al., 2011; Levett and Branch 2002). The sensitivity and specificity of the IgM ELISA are known to vary depending on the region, and values range from 50.0% to 90.0% for sensitivity (Blacksell et al., 2006; Wagenaar et al., 200; Bajani et al., 2003; Levett and Branch, 2002) and from 46.5% to 93.0% for specificity (Agampodi et al., 2014; Levett, 2002). The IgM ELISA has been used to diagnose leptospirosis in suspected clinical or acute cases in diagnostic laboratories (Agampodi et al., 2014; Bajani et al., 2003; Levtt and Branch 2002) and also to screen for the disease in apparently healthy humans (James et al., 2013) and animals (Simpson et al., 2018). A major disadvantage of the ELISA in the diagnosis of leptospirosis is that, it does not identify antibodies to *Leptospira* spp., to serovar/serogroup level, since it is primarily genus-specific (Picardeau, 2013).

The IgM ELISAs are cheap in relation to the MAT and are not cumbersome (Agampodi et al., 2014). The World Health Organization advised that the IgM ELISA should be used in poor resource settings as a leptospirosis diagnostic tool (WHO, 2010).

Other serological tests reported to be used for the diagnosis of leptospirosis include the latex agglutination test, IgM Dipstick assay (LSD); IgM dot-ELISA dipstick (DST); Indirect hemagglutination (IHA), amongst others. These tests are sensitive and specific but are yet to be validated using the MAT as the gold standard (Levett et al., 2001; Smits et al., 1999; Levett and Whittington 1998).

The Polymerase Chain Reaction (PCR) has also been used to detect the presence of *Leptospira* DNA in fluids of abattoir workers or other high-risk groups. The method is highly recommended (WHO, 2003) for its efficiency in the diagnosis of pathogenic *Leptospira* spp. from the *Leptospira* DNA in body organs/tissues and fluids, such as kidneys and blood (Picardeau, 2013).

Other genes for the pathogenic leptospires identification by the PCR use the *LipL32* partial gene region for screening (Wunder et al., 2016) and through sequencing *SecY* gene region (Victoria et al., 2008) and *LipL41, rpoB* and *LigB,* to discriminate pathogenic leptospires (Cerqueira et al., 2010).

The advantages of qPCR are that it is fast, reduces chances of contamination, and is specific and sensitive, especially with the use of the hydrolysis probes. It also has a higher throughput than the conventional PCR (Wunder et al., 2016; Espy et al., 2006). The disadvantages of the qPCR are that it is very expensive, requires good skills and cannot identify leptospires to serovar level (Picardeau, 2013). The level of detection of the qPCR assay has been reported to be as low as 10^1 and 10^2 bacteria/ml of pure culture, whole-blood, plasma, urine and serum targeting the *LipL32* and *SecY* gene regions (Bourhy et al., 2012; Stoddard et al., 2009).

Leptospirosis in abattoir workers has been reported in several studies conducted globally. Although there are some regions with limited data on both human and animal leptospirosis, for example in Africa (Allan et al., 2015).

In New Zealand, according to Benschop and co-workers (2009), a seroprevalence of leptospirosis among workers in slaughterhouses was 9.5% (23/242) using the MAT. The seroprevalence in male and female workers was 13.1% (19/145) and 4.1% (4/97) respectively, with the detection of antibodies to *Leptospira interrogans* serovar Pomona and *Leptospira borgpetersenii* serovar Hardjo.

The study determined, based on odds ratio, that male workers were 3.51 times more likely to be exposed to leptospirosis. In the same study, the median age for positive workers was 54 years (47-59 years) and for negative workers it was 48 years (35-56 years). The seroprevalence of *Leptospira* antibodies was carried out in abattoir workers, comprising four sheep abattoirs where it was found to be 10.0 % to 31.0 %; two cattle abattoirs where it was 5.0 % and two deer abattoirs where it was 17.0% to 19.0% (Dreyfue et al., 2014). Another study in New Zealand conducted on dairy farm workers reported a seroprevalence of 66.6% (2/3) for antibodies to *L. borgpetersenii* serovar Hardjo, with the highest risk factors being shedding during milking and splashes of urine from cattle infected with *Leptospira* spp. (Mclean et al., 2014). With the use of the MAT, the seroprevalence was the same for both veterinary students (7.1%; 8/113), with the serovars identified being Icterohaemorrhagiae and Copenhageni) and non-veterinary students (7.1%; 7/99 (James, et al., 2013).

In Iran, the seroprevalence of leptospirosis, using the MAT, for abattoir workers was 34.7% (34/98) with the detection of serovars being, Harjo (47.8%), Grippotyphosa (15.2%) and Sejroe (4.3%) (Majd et al., 2012).

In the African continent, the disease has been reported according to de Vries et al (2014), but with minimal data on both human and animal leptospirosis available (WHO, 2011). In Tanga City of Tanzania, a cross-sectional study was conducted on 199 subjects for leptospirosis using the MAT, detected a seroprevalence of 15.1% (30/119). In the study, among the occupational high-risk group individuals, the most pre-disposed were livestock/farmers (19.4%), veterinary inspectors (18.1%) and abattoir workers (17.1%). The serovars detected were Icterohaemorrhagiae (30.0%), Bataviae (20.0%), Hardjo (6.6%), Tarassovi, and Ballum and Pomona (3.3%) (Schoonman and Swai, 2013).

A study in the Caribbean revealed that out of 3,455 human sera tested, 13.1% (452) were seropositive for IgM antibodies to leptospirosis using the ELISA. A statistically significant difference ($P<0.05$) was found among countries and years of sample collection (Adesiyun et al., 2011). The seropositivity of leptospirosis within the patients was 23.1% with age groups 1–20 years and 31–40 years having a significance difference in male patients (72.1%) compared with female patients (19.7%) ($P< 0.05$).

In Trinidad and Tobago, a review of 278 confirmed cases of human leptospirosis conducted by Mohan et al., (2009) reported that, 80.0% (222/278) of males and 10 % (53/278) of females were seropositive. The difference was statistically significant ($P<0.05$). The highest seropositivity (25%) in

the males was in the age group, 10–19 (n=51), and the lowest frequency (2%) was in the age group, 0–9 (n=3). In contrast , in the females the highest seropositivity was in the age group of 30–39 (36%; n=18) and the lowest frequency was in the age group 0–9, (0.0%, n=0) (Mohan et al., 2009).

The IgM ELISA also determined the seroprevalence of leptospirosis among veterinary students as 26.5% (30 /113) and among non-veterinary student as 13.1% (13/99) in Trinidad and Tobago (James et al., 2013).

In Kenya, the commercial Panbio *Leptospira* IgM ELISA kit was used and 13.4 % (41/737) of the abattoir workers were seropositive (Cook et al., 2017). The risk factors determined in their study included abattoir workers with wounds (OR 3.1; 95% CI 1.5 to 6.1), workers eating in between working hours (OR 2.1; 95% CI 1.2 to 3.6), workers cleaning offal (OR 5.1; 95% CI 1.8 to 15.0) and those workers with boreholes used as a personal source of water (OR 2.3; 95% CI 1.1 to 4.7).

The use of molecular methods in the diagnosis of leptospirosis is very important, especially for early detection of the disease. In Nan Province of Thailand, 2.7 % of human urine samples, using nested PCR, were positive for *Leptospira* DNA (Kurilung et al., 2017). The PCR detected *Leptospira* DNA from blood of patients in Thailand in 19.0% (81/418) of cases (Boonsilp et al., 2011). However, in a study conducted in Ecuador, *Leptospira* DNA was detected in the blood of 2.0% (8/394) of human cases of leptospirosis and from 15.6% (70/449) of urine samples. At a second site, human sera yielded amplified DNA in 3.7% (8/219) of patients, while in human urine, *Leptospira* DNA was detected in 8.8% (14/159) of subjects, according to Barragan and co-workers, (2016).

In South Africa, data on human leptospirosis are limited (Saif, 2012; Taylor et al., 2008), but a seroprevalence of human leptospirosis of 19.0% (41/217) using the IgM ELISA was reported. In a prevalence study on zoonotic pathogens in the Mpumalanga Province, antibodies to *Leptospira* spp. were detected in 6.8% (5/74) of febrile subjects using the ELISA IgM (Simpson et al., 2018). The Special Bacterial Pathogens Reference Unit tested for IgM *Leptospira* antibodies in suspect clinical cases of leptospirosis across the country and reported seropositivity rates that ranged from 6.5% (14/215) to 12.5% (12/96) (Saif, 2012). Most recently, cases of human leptospirosis were reported in Western Cape and Mpumalanga provinces between 2015 and 2016 according to the National Institute of Communicable Diseases (NICD) communique (http//www.nicd.ac.za).

Leptospirosis in humans is under-reported in South Africa, primarily due to the limited technical know-how, particularly in the application of more sensitive and specific diagnostic tools such as PCR as recommended by the World Health Organization (WHO, 2003). To date, data on human leptospirosis, including abattoir workers, using PCR is lacking in the country. Furthermore, information is unavailable on the use of a three-test strategy (MAT, IgM ELISA and quantitative PCR) to diagnose leptospirosis in animals or humans in the country.

This study was therefore conducted with the following specific objectives:

7.2.1 Specific objectives:

i. To determine the seroprevalence of *Leptospira* antibodies in abattoir workers in Gauteng province.
ii. To determine the frequency of detection of the DNA of pathogenic *Leptospira* in the blood samples of abattoir workers in Gauteng province.
iii. To compare the positivity for *Leptospira* antibodies and *Leptospira* DNA in abattoir workers using two serological tests and a molecular method.
iv. To determine the risk factors that are important for the exposure of abattoir workers to leptospirosis.

7.3 Materials and Methods:

7.3.1 Brief overview of the study area/abattoirs

7.3.1.1 Study area:

a. South Africa

South Africa is in the southern part of the African continent **Figure 7.1.**

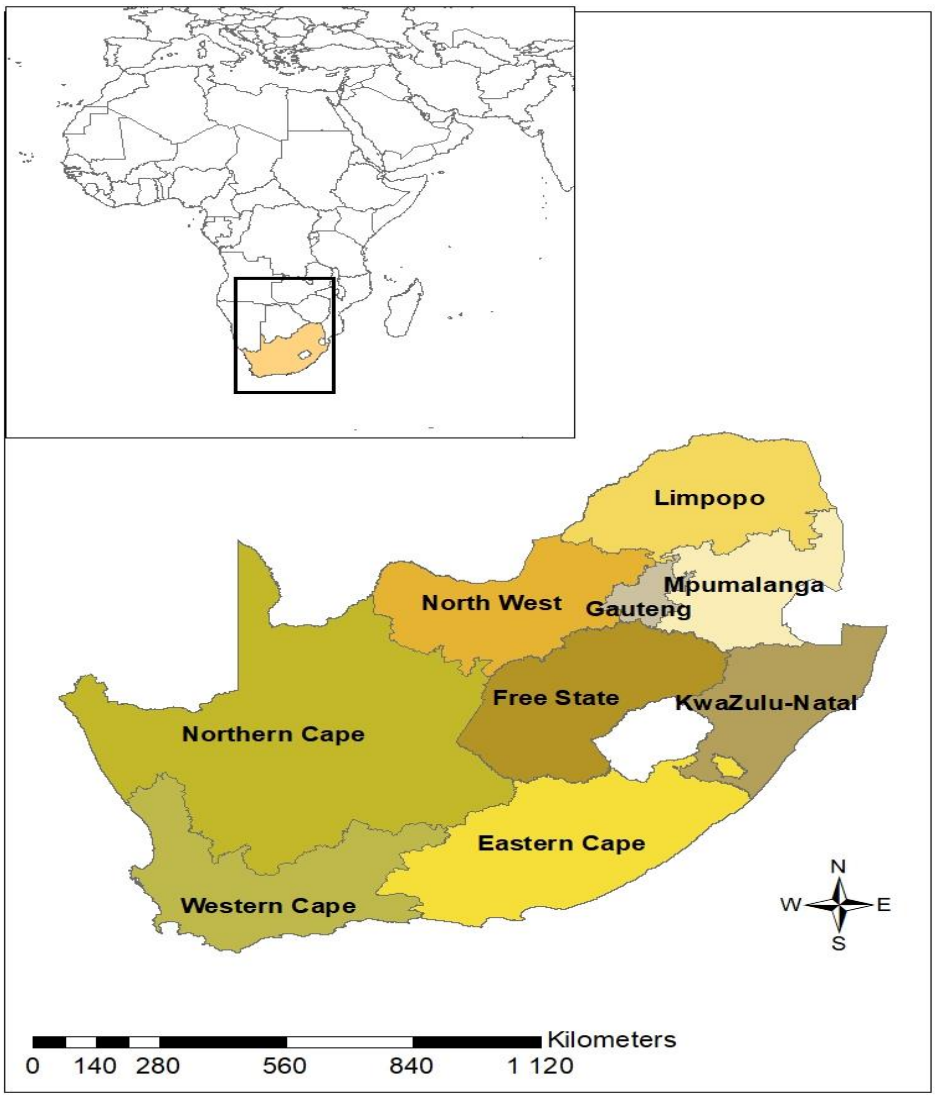

Figure 7.1: Map of South Africa, the country of study showing the 9 provinces Including Gauteng province, the study area.

b. Gauteng Map:

The map of Gauteng Province is shown in **Figure 7.2**.

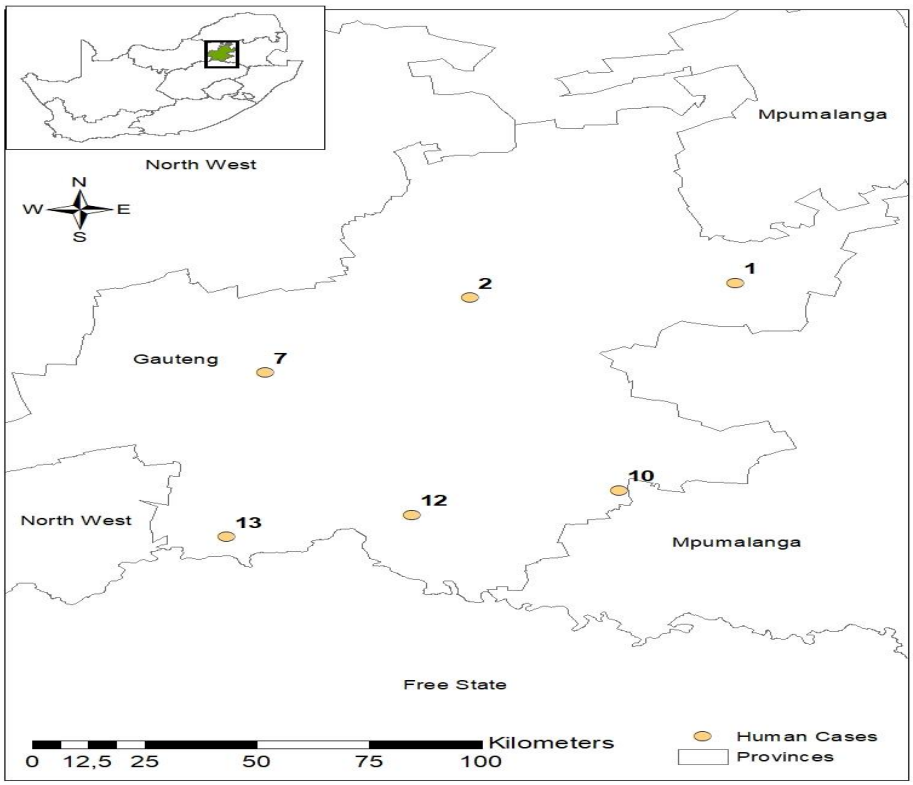

Figure 7.2: Map of Gauteng province showing the locations of the six abattoirs from where human samples were collected

The Gauteng province

The name Gauteng means, ‘‘place of gold’’. The name was used for Johannesburg (the city founded on the discovery of gold on the Witwatersrand and regarded as the financial capital of South Africa) and surrounding areas long before it was adopted in 1994 as the official name of a province. It is in the Highveld and is the smallest province in South Africa. It has only 1.5% of the land area (Stats in brief, 2006). Nevertheless, it is well urbanised, having Johannesburg as the largest city in the country, Pretoria as the administrative capital, Midland and Vanderbijlpark as other large areas.

As of 2018, Gauteng province was the most highly populated province in South Africa, with approximately, 14.7 million people according to estimates by the Mid-year population estimated report (2018). Gauteng province has 3.9 million households, with a population density of 680/km according to the South African Mid-year population estimated reported, (2017). The province's median age is 27 years. For every 100 females there are 101.2 males. Out of this population of

humans, many people work in abattoirs as the province also has the highest number of abattoirs (multi- and mono- species) comprising high throughput (HT) and low throughput (LT) abattoirs.

7.4 Type of study

This was a cross-sectional study by design.

7.4.1 Estimation of sample size for the study and study design

The minimum sample size for the study was determined using the formula $n = [t^2 \times p\ (1-p)]/m^2$ (Ciaran and Biswas, 2013) where n = minimum required sample size, t = confidence level at 95% (standard value of 1.96), and p=estimated at 50% and m=10%. The estimated minimum sample size was therefore 96. The study design was to recruit a minimum of 96 consenting workers at 6 of the 14 abattoirs where the work force was high, and the abattoir owners approved the study to be conducted at their facilities. Therefore, a total of 103 workers were sampled for the study over a period of 8 weeks (March 27, 2018 to May 27, 2018).

7.4.2 Selection of abattoirs

Following completion of the study which determined the seroprevalence of leptospirosis in livestock slaughtered in pigs and cattle abattoirs in Gauteng province **(Chapters 4 and 5)**, HT abattoirs with high work force were targeted for the study. The owners were contacted about the proposed study to solicit their support, consent and the participation of their workers in the study. Based on the feedback received, six abattoirs were selected for the study.

7.4.3 Study population

The study population comprised the workers at six abattoirs in Gauteng province performing different types of duties at the facilities which ranged from office work with no animal contact pre- or post-slaughter (for example office staff) to minimal contact (pre-slaughter contact) and to maximum contact (during and post slaughter contact). A spectrum of exposure to animals and possibly leptospirosis was therefore captured by these groups of workers. The study group included workers who were employed by the abattoirs and at work during the sampling visits.

7.4.4 Types of abattoirs

The study was conducted in six HT abattoirs comprising mono-species types, slaughtering only one livestock species (cattle or pig or sheep or goat) or multi-species type slaughtering more than one species of livestock. These six abattoirs were also among the 14 used in the seroprevalence study (**Chapters 4** and **5**) and isolation and characterization study (**Chapter 6**).

7.4.5 Type of sampling

Convenience sampling was applied at the six abattoirs selected for the study from consenting abattoir workers, owners and managers.

7.4.6 Collection of samples

A qualified phlebotomist, who was a member of the research team, collected peripheral whole blood samples from each of the 103 consenting, apparently healthy abattoir workers recruited for the study. From each worker, two tubes of whole blood were aseptically collected: one 5 mL sterile acid citrate dextrose (ACD) vacutainer tube containing clot and gel activator and another 5 mL ethylene-diamine-tetra-acetic acid (EDTA) Vacutainer tube. The ACD tube was used for serology and the EDTA tube for molecular studies. These samples were properly identified and transported in a cold chain to the National Institute for Communicable Diseases (NICD), Special Bacterial Pathogens Laboratory Unit in Johannesburg, South Africa. The NICD laboratories serve as diagnostic facilities in which human samples are processed and tested. At the NICD laboratory facilities leptospirosis is diagnosed serologically only, using the IgM ELISA for serum samples of suspected clinical human cases from hospitals and clinics across the country.

7.4.7 Detection of antibodies to *Leptospira* spp.

7.4.7.1 Serological techniques

i. Enzyme Link immunosorbent assay (ELISA)-IgM.

The blood samples were processed, and the human sera were collected into 2 ml screw cap cryovials. These sera were then tested for IgM antibodies to *Leptospira* spp. using the PanBio human IgM® Enzyme Link immunosorbent assay (ELISA)-IgM kits (Alere Company, South Africa), according to the manufacturer's instructions.

The results were interpreted by using the index and the PanBio units which classified a sample as positive when the index was >1.1 and PanBio units was >11; negative when the index was <0.9 and

PanBio units was <9, and equivocal when a sample index was 0.9 - 1.1 and the PanBio units is 9 - 11, according to the manufacturer's guideline.

Furthermore, aliquots of the serum samples used for the ELISA IgM were packed and shipped to Yale University according to the South African Department of Health regulations and with an import permit (**Number 2017-07-155**) (**Figure 7.3**)**,** from the Yale University, School of Public health Department of Epidemiology and Microbial Diseases Laboratory (Prof. Ko's I. Albert, Laboratory), New Haven, Connecticut, USA, for the shipment of infectious diseases category B, where the MAT was performed on the serum samples.

Figure 7.3: Permit to Import Infectious Biological Agents, Infectious Substances, and Vectors by the Department of Health and Human Services Public Health Service and Centers for Disease Control and Prevention Office of Health and Safety (CDC), MS A-46 Atlanta, Georgia, USA

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

Centers for Disease Control and Prevention
Office of Health and Safety, MS A-46
Atlanta, Georgia 30333
TEL: 404-718-2077; FAX: 404-718-2093; Email: importpermit@cdc.gov

SAFER • HEALTHIER • PEOPLE

Permit to Import Infectious Biological Agents, Infectious Substances, and Vectors

In accordance with 42 CFR Section 71.54 of the Public Health Service Foreign Quarantine Regulators, cited on the bottom of this permit, permission is granted the permittee to import into any port under control of the United States, or to receive by transfer within the United States, the material described in Item 1 below.

PHS PERMIT NO.	**2017-07-155**	
DATES	**ISSUED: Friday, July 28, 2017**	**EXPIRES: Saturday, July 28, 2018**
1. DESCRIPTION OF MATERIAL	**HUMAN, BOVINE, CANINE, AND RODENT BLOOD/BLOOD PRODUCTS, OTHER BODY FLUIDS, AND TISSUES THAT MAY CONTAIN LEPTOSPIRA SPECIES.** **LABORATORY CULTURES OF LEPTOSPIRA SPECIES.** **NOT APPROVED FOR RODENT MATERIAL FROM AFRICA AS DESCRIBED IN THE EMBARGO "AFRICAN RODENTS PRAIRIE DOGS AND OTHER ANIMALS THAT MAY CARRY MONKEY POX."**	
2. PERMITTEE (NAME, ORGANIZATION, ADDRESS AND CONTACT INFORMATION)	**ELSIO AUGUSTO WUNDER JUNIOR** **YALE SCHOOL OF PUBLIC HEALTH** **60 COLLEGE STREET** **NEW HAVEN, CT 06510**	**TEL: 203-785-3927** **FAX: 203-785-6193**
3. SOURCE OF MATERIAL (NAME, ORGANIZATION, ADDRESS, COUNTRY)	**WORLDWIDE**	
4. TYPE OF PERMIT AND INSTRUCTIONS FOR USE	As the permittee, your facility will be subject to inspection at some time in the future to confirm that the importer's biosafety measures are commensurate with the hazard posed by the items to be imported and the level of risk given its intended use. ☐ Single Importation into the U.S. ☑ Single Transfer Within the U.S. ☑ Multiple Importation into the U.S. ☐ Multiple Transfer Within the U.S. A. Record of each importation shall be maintained on permanent file by permittee. B. Enclosed label(s) must be forwarded to the shipper(s). C. One label shall be affixed to shipping container. Enclosed labels may be photocopied.	
5. CONDITIONS OF ISSUANCE ITEMS APPLICABLE WHEN CHECKED	☐ A. Subsequent distribution, within the U.S., of the material described in this permit is prohibited without prior authorization by the Public Health Service. ☑ B. All material is for laboratory use only - Not for use in the production of biologics for humans or animals. ☑ C. All material is free of tissues, serum and plasma of domestic and wild ruminants, swine and equines. ☑ D. Additional Requirements: ☐ IATA Packaged to preclude escape. ☑ USDA permit may be required (Telephone: 301-851-3300). ☑ E. Work with the agent(s) described shall be restricted to areas and conditions meeting requirements in the CDC/NIH publication "Biosafety in Microbiological and Biomedical Laboratories." ☑ F. Packaging must conform to 49 CFR Sections 171-180.	
	6. Signature of Issuing officer Samuel S. Edwin Samuel S. Edwin, Ph.D. Director, Division of Select Agents and Toxins	

CDC 0728 (F 13.40) REV. 4-13

42 CFR 71.54. Permit to Import Biological Agents, Infections Substances, and Vectors
A person may not import into the United States any infectious biological agent, infectious substance, or vector unless: It is accompanied by a permit issued by the Centers for Disease Control and Prevention (CDC). The possession of a permit issued by the CDC does not satisfy permitting requirements placed on materials by the U.S. Department of Agriculture that may pose hazards to agriculture or agricultural production in addition to hazards to human health.

ii. Microscopic agglutination test (MAT)

At the Yale University School of Public Health laboratories, the MAT was performed (OIE, 2014). Briefly, serum samples were diluted at 1:50 and first screened for the presence of antibodies to selected serovars of *Leptospira* spp. Thereafter, sera that were positive by the screening test were further serially diluted from 1:100 to 1: 3,200. The test was conducted using 25 sub-cultured *Leptospira* serovars which were live culture antigens of approximately, 2×10^8 leptospires per ml (OIE, 2014). The end point observed under the Dark Field Microscope (DFM) was at the dilution of serum samples that showed 50% agglutination, leaving 50% free leptospires compared with the control culture diluted at 1:2 in phosphate buffered saline (OIE, 2014). The standard international *Leptospira* spp. antigens used for the MAT are shown in Table 7.1**.**

Table 7.1: Species, serogroups, serovars and strains of *Leptospira* spp. used for MAT

Species	Serogroup	Serovar	Strain
L. interrogans	Djasiman	Djasiman	Djasiman
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
L. interrogans	Icterohaemorrhagiae	Copenhageni	M 20
L. weilii	Javanica	Coxi	Cox
L. noguchii	Louisiana	Louisiana	LSU 1945
L. biflexa	Semaranga	Patoc	Patoc 1
L. interrogans	Sejroe	Hardjo	Hardjoprajitno
L. borgpetersenii	Tarassovi	Tarassovi	Perepelitsin
L. borgpetersenii	Ballum	Castellonis	Castellon 3
L. interrogans	Bataviae	Bataviae	Van Tienen
L. interrogans	Sejroe	Wolffi	3705
L. interrogans	Pyrogenes	Pyrogenes	Salinem
L. borgpetersenii	Ballum	Ballum	Mus 127
L. interrogans	Pomona	Pomona	Pomona
L. weilii	Celledoni	Celledoni	Celledoni
L. interrogans	Autumnalis	Autumnalis	Akiyami A
L. interrogans	Canicola	Canicola	H. Ultrecht IV
L. kirschneri	Cynopteri	Cynopteri	3522C
L. kirschneri	Grippotyphosa	Grippotyphosa	Duyster
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. santarosai	Shermani	Shermani	1342 K
L. interrogans	Australis	Bratislava	Jez Bratislava
L. interrogans	Icterohaemorrhagiae	Copenhageni	L1 130
L. santarosai	Grippotyphosa	Canalzonae	CZ 188
L. borgpetersenii	Javanica	Poi	Poi

7.4.8 Polymerase chain reaction (PCR)

7.4.8.1 Quantitative Polymerase chain reaction (qPCR)

i. Standard Curve

A standard positive control *Leptospira* interrogans, serovar Copenhageni strain Fiocruz L1-130 (Nascimento et al., 2004), was used for a standard curve calibration of the genomic DNA prior to the qPCR of the extracted DNA.

ii. Extraction of DNA

In a Biosafety Cabinet Class II, DNA was extracted from 200 µl of whole blood from each subject (n=103) using the QIAamp DNA minikit (QIAGEN, Valencia, CA) according to the manufacturer's guidelines. The extracted DNA was then subjected to a qPCR targeting the *LipL32 gene* for pathogenic *Leptospira* spp. (Wunder et al., 2016). The 25 µL final volume reaction contained 12.5 µL Platinum Quantitative PCR Super mix Rox-UDG (Invitrogen®), 1.25 µL for each primer (*LiPL32*-45F and *LiPL32*-286R) at final concentration of 10 µM, 0.5 µL of TaqMan probe (*LipL32*-189P) of final concentration of 5 µM, 5 µL of extracted DNA and finally 4.5 µL of ultra-pure water. The cycling conditions were as previously described in ***Chapter 6, Section 6.3.10.3*** with a holding stage of 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute in a Real-time PCR ABI 7500.

7.4.9 Analysis of data

The data were collated, filtered and descriptively analyzed using Microsoft Excel 2010 and further analyzed using R Console version 3.2.1 (R Core Team, 2017) at 95% level of significance and Excel Microsoft software's 2010. Cohen's kappa (*k*) test was used to determine the level of agreement (Landis & Koch, 2019) among qPCR, MAT and IgM test results for exposure to *Leptospira* species. During the analyses, 30 risks factors (**Appendix 1**) were analyzed.

7.4.10 Human Ethics Committee approval:

Human ethics approval was granted according to the South African Department of Health Rules and Regulations *(Ref. No.: **519/2017**)*, (**Figure 7.4**).

Figure 7.4: Human ethics approval certificate by the Faculty of Health Sciences Research Ethics Committee

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

23/11/2017

Approval Certificate
New Application

Ethics Reference No: 519/2017

Title: Prevalence and characterization of selected pathogens in slaughter animals in Gauteng Province abattoirs: Food safety implications for meat consumers and zoonotic hazards posed to abattoir workers

Dear Dr Francis FB Kolo

The **New Application** as supported by documents specified in your cover letter dated 18/10/2017 for your research received on the 18/10/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 22/11/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**519/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- 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.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Room 4.59 / 4.60.*

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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7.5 Results:

7.5.1 Serological and molecular detection of Leptospira antibodies and DNA in blood samples of abattoir workers

For the 103 abattoir workers sampled from the 6 abattoirs in Gauteng province, the seroprevalence of antibodies to *Leptospira* spp. was 10.7% (11/103) and 7.8% (8/103) by IgM ELISA and MAT (IgG and IgM) respectively (**Table 7.2**). The difference was not statistically significant (P=0.4701). Molecular method (qPCR) detected the presence of DNA for pathogenic *Leptospira* in 16.5% (17/103) of the workers. Overall, the prevalence of antibodies to *Leptospira* spp. using the MAT and IgM ELISA (individually or in combination) was 13.4% (14/103). The difference in the prevalence of antibodies to *Leptospira* spp. and *Leptospira* DNA was not statistically significantly different (P=5232).

The following frequency of detection of *Leptospira* antibodies and/or DNA in the 103 workers was as follows: MAT only, (3.9%), IgM ELISA only (7.8%), qPCR only (13.6%), MAT and IgM ELISA (1.9%), MAT and qPCR (1.9%) and IgM and qPCR (1.0%).

With the use of the three tests, a total of 31 (30.1%) of the 103 had been exposed to *Leptospira* spp. while 72 (69.9%) had not had any exposure experience of the pathogen.

Table 7.2: Seroprevalence of antibodies to *Leptospira* using the ELISA IgM and MAT and the detection of DNA of *Leptospira* by qPCR in abattoir workers

Type of test	Parameter detected	No. tested	No. (%) positive	P-value
ELISA	IgM antibodies	103	11 (10.7)	0.1380
MAT	IgM and IgG antibodies	103	8 (7.8)	
qPCR	DNA of *Leptospira* spp.	103	17 (16.5)[a]	

[a]Based on the CT values

The CT values detected during qPCR assays are detected in Table 7.3. The CT values ranged from 26.47167 (Sample #14) to 39.14591 (Sample #7).

Table 7.3: CT values for the 17 human samples positive by qPCR.

S/no.	Sample ID	CT value
1	1sK	32.78531
2	3SK	28.82298
3	8SK	34.13245
4	11SK	38.19641
5	14SK	36.88227
6	1SR	37.29918
7	4SR	39.14591
8	2SC	30.04613
9	4SC	38.28095
10	6SC	35.98036
11	7SC	35.20235
12	10SC	37.80003
13	1SB	34.96934
14	2SP	26.47167
15	5SP	37.67214
16	10SP	34.56234
17	13SP	38.09241

7.5.2 Comparison of qPCR, MAT and IgM ELISA test results

Table 7.4 shows the comparison of the test results from qPCR, MAT and ELISA IgM assays for *Leptospira* species among abattoir workers in Gauteng Province, South Africa. Quantitative PCR had the highest detection rate for *Leptospira* species among the abattoir workers (16.5%, 17/103), followed by ELISA IgM (10.7%, 11/103) and MAT (7.8%, 8/103). The test for agreement between the test methods showed that there was only slight agreement between qPCR and MAT [*kappa* = 0.06 (*p*=0.5), 95% CI: -0.14] and between ELISA IgM and MAT [*kappa*= 0.13 (*p*=0.17), 95% CI:-0.13, 0.39], and poor agreement between qPCR and ELISA IgM (*kappa*= -0.07 (*p*=0.48), 95% CI: -0.22, 0.08]. Two workers tested positive by qPCR and MAT, two workers by both ELISA IgM and MAT, and only one worker showed a positive result by qPCR and IgM.

Table 7.4: Comparison of quantitative polymerase chain reaction (qPCR), microscopic agglutination test (MAT) and ELISA immunoglobulin M (IgM) assays in the detection of *Leptospira* species among abattoir workers in Gauteng Province, South Africa

		MAT*				**IgM***			
		Positive	Negative	Total	**Cohen's kappa (*k*) and 95% CI**	Positive	Negative	Total	**Cohen's kappa (*k*) and 95% CI**
qPCR*	Positive	2 (1.9)	15 (14.6)	17 (16.5)	**0.06*** (-0.14, 0.26), *p*=0.5**	1 (1.0)	16 (15.5)	17 (16.5)	**-0.07** (-0.22, 0.08), *p*=0.48**
	Negative	6 (5.8)	80 (77.7)	86 (83.5)		10 (9.7)	76 (73.8)	86 (83.5)	
	Total	8 (7.8)	95 (92.2)	103		11 (10.7)	92 (89.3)	103	
ELISA IgM	Positive	2 (1.9)	9 (8.7)	11 (10.7)	**0.13*** (-0.13, 0.39), *p*=0.17**				
	Negative	6 (5.8)	86 (83.5)	92 (89.3)					
	Total	**8 (7.8)**	**95 (92.2)**	**103**					

*A total of 103 sera from workers at 6 abattoirs were tested by each test

CI, confidence interval; Cohen's kappa, proportion of agreement over and above the agreement expected by chance (range -1 to +1)

Interpretation of kappa value was done as described by Landis and Koch (1977):

**poor agreement; 0.0-0.20, ***slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; 0.81-1.00, almost perfect agreement. The measure of agreement was not statistically significant in all the three comparisons (p>0.05).

7.5.3 Seroprevalence of leptospirosis in abattoir workers by gender and age

The prevalence of leptospirosis as determined by the three tests used according to gender was 29.9**% (**26/87) and 31.3% (5/16) in male and female workers respectively. The difference was not statistically significant (*p*=1.00). The distribution of seroprevalence by age of abattoir workers was as follows: 18-30 years, 25.0 % (8/32); 31-40 years, 38.9 % (14/36); 41-50 years, 27.3 % (6/22) and 51-60 years, 23.1 % (3/13), with no statistically significant difference (*p*=0.57). The group with the highest seroprevalence of antibodies to leptospirosis was 31-40 years, (28.9 %).

The prevalence of leptospirosis using the three tests (ELISA, MAT and qPCR) was 31 (30.1%) and the distribution was 29.9% (26/87) and 31.3% (5/16) in male and female workers respectively. The difference was not statistically significant (*p*=1.00).

7.5.4 Serogroups/serovars of *Leptospira* spp. detected by MAT from abattoir workers

The MAT was used to serotype the *Leptospira* spp. antibodies from the abattoir workers sampled from the six HT (high throughput) abattoirs. Among the eight abattoir workers seropositive for antibodies to *Leptospira* spp., two were females while six were males. The age range of seropositive workers was from 27 to 57 years. The titres ranged from 100 to 3,200. Serovars Woffi and Djasiman were detected in both abattoir workers and livestock slaughtered in abattoirs 1 and 7 respectively. It is important to note that, out of the six abattoirs where abattoir workers were sampled, only two abattoirs, these were 1 and 7 in **Table 7.5** had all the eight (8) seropositive antibodies to abattoir workers (**Table 7.5**).

Table 7.5: Serovars of *Leptospira* spp. detected in abattoir workers by gender, age and duties performed by abattoir workers

Abattoirs	**Abattoir workers**				**Serotyping data**		
ID of Abattoir*	ID of Worker	Gender	Age (year)	Duty	Serogroup	Serovars	Titre
1	CH_AB1	Female	33	Slaughter	Djasiman	*Djasiman*	800
1	CH_AB2	Female	27	Washing offal	Djasiman	*Djasiman*	3,200
1	CH_AB3	Male	37	Slaughter	Djasiman	*Djasiman*	400
1	CH_AB4	Male	32	Cleaner	Wolffi	*Wolffi*	200
1	CH_AB5	Male	46	Slaughter	Sejroe	*Semaronga Patoc*	800
1	CH_AB6	Male	49	Washing offal	Sejroe***	*Wolffi*	100
7	CM_AB7	Male	41	Slaughter	Icterrohaemorrhagiae	*Copenhageni L1-130*	400
7	CM_AB8	Male	57	Washing offal	Pomona***	*Djasiman*	800

*The eight abattoir workers were working in multi species (slaughtering cattle, sheep, pigs, etc) and high throughput (HT) abattoirs.
**These serogroups were detected in abattoir workers and in livestock slaughtered in abattoir 1 and 7, respectively. Of the seven (7) abattoirs where leptospires were isolated from livestock kidneys, two abattoirs (1 and 7), were among those abattoirs where abattoir workers were seropositive

7.5.5 Risk factors associated with the presence of *Leptospira* spp. antibodies in abattoir workers

Out of the thirty (30) risk factor predictors investigated (**Appendix 1**) only three showed an association with seropositivity for *Leptospira* antibodies as measured by the odds ratio (**Table 7.6**). Workers at HT abattoirs were 3 times (OR=3) more likely to be exposed to leptospirosis while workers who interacted with farm animals outside of the abattoirs were 3.44 times (OR=3.44) more likely exposed to leptospirosis and those subjected to blood and water splashes during slaughter at the abattoirs had a 2.27-time greater likelihood of exposure to the disease. However, for the three risk factors, statistically significant differences were not detected (P>0.05).

Table 7.6: Association between three important risk factor predictors and positive abattoir workers

Risk factor (variable)	Odds ratio	Standard Error	(P>|z|)	[95% Conf. Interval]
High throughput abattoirs	3	3.19	0.31	0.3576975 - 24.45716
Farm animals*	3.44	2.29	0.06	0.9363117 - 12.66461
Splash of blood/water	2.27	2.04	0.36	0.3927211 - 13.16086

7.6 Discussion

In the current study conducted in six abattoirs where cattle, pigs and sheep were slaughtered, each had at least one worker positive for leptospirosis with the use of three diagnostic tests (MAT, ELISA-IgG and qPCR). With the use of MAT, the gold standard, 7.8% of the apparently healthy abattoir workers were seropositive for leptospirosis. This seroprevalence is higher than reported by others who also used the MAT as documented in livestock abattoirs where the seroprevalence of leptospirosis was 1.2% in New Zealand (Dreyfus et al., 2015a), 4.0% in Brazil (Gonçalves et al., 2006) and 5.0% in New Zealand (Dreyfus et al., 2014). Other researchers have however reported seropositivity higher than found in the current study such as 9.5% in New Zealand (Benschop et al., 2009), 10.0%, 13.4% and 18.1% in Tanzania (Mirambo et al., 2018; Cook et al., 2017 and Schooman and Swai, 2013) respectively, and 87.7% in Nigeria (Abiayi et al., 2015). The differences in the seroprevalence of leptospirosis in several studies in many countries may be due, in part, to the seroprevalence of leptospirosis in the livestock slaughtered, vaccination status of slaughtered livestock, duties performed by abattoir workers at the abattoirs, sanitary practices of abattoir

workers, environmental factors, flood, rodent population and the MAT regarding the number and types of antigens in the panel and the cut-off titres used for diagnosis of leptospirosis (Picardeau, 2013; Dechet et al., 2012; Dorjee et al., 2008; Vinetz, 2001; Gummow et al., 1999; Ko et al., 2009).

It is pertinent to mention that other serological tests have been used to diagnose leptospirosis in abattoir workers. As obtained in this study, the seroprevalence of 10.7% (11/103) with the use of IgM ELISA is considerably higher than detected in Trinidad, 1.0% (Adesiyun et al., 2011), Nigeria, 3.5% (Ngbede et al., 2012) but much higher seroprevalence of leptospirosis have been reported in Mexico, 17.7% (Alvarado-esquivel et al., 2016), 81.0% in Nigeria (Abiayi et al., 2015) and in Iran, 23.4% (Esmaeili et al., 2016). It is known that the use of ELISA (IgG and IgM) is usually more sensitive than the MAT (Cumberland et al., 1999).

The risk of leptospirosis posed to abattoir workers by the different prevalences of infection in the slaughter animals have been reported to be an important epidemiological factor (Benschop et al., 2009). In the current study, within eight months of the animal seroprevalence study in the same six abattoirs where the human samples originated, the overall seroprevalence of leptospirosis in the livestock sampled (pigs and cattle) was 22.7% (32/141) as shown in ***Chapters 4*** and ***5***. The possibility of abattoir-acquired leptospirosis in abattoir workers has been reported by others (Abiayi et al., 2015; Benschop et al., 2009; Cook et al., 2017; Dorjee et al., 2008; Ngbede et al., 2012).

It is important to mention that this was a cross-sectional study to determine the prevalence of leptospirosis in apparently healthy workers. Unlike in clinical cases or settings where paired serum samples are used or required to arrive at serological diagnosis as recommended (OIE, 2014; WHO, 2003), high MAT titres (800 to 3,200) of antibodies to *Leptospira* in a single sampling strategy have been considered significant diagnostic (OIE, 2014). The only disadvantage with this approach is that the titres detected may not reflect current acute infection but titres of apparently healthy workers during convalescence after experiencing leptospirosis. It is however instructive that 50% (4/8) of the workers who were detected to be seropositive for leptospirosis by the MAT had titres ranging from 800 to 3,200 and were apparently healthy at the time of sampling. Furthermore, none of these seropositive workers reported having experienced confirmed episodes of leptospirosis based on the questionnaire data obtained in this study. In our study, we reported high titres by MAT (1:800 and 1:3,200), at a single sampling, which is considered as a significant diagnosis according to OIE (2014). These findings might be an indication that these abattoir workers were once infected but later recovered from leptospirosis and became apparently healthy abattoir workers.

The public health and zoonotic significance of detecting antibodies to L*eptospira* in abattoir workers cannot be over-emphasized. This is because exposure of abattoir workers to leptospires in the body fluids of infected animals during and after slaughter has been documented as a potential means for exposure to the pathogen (McCLean et al., 2014). Our studies showed that the workers who were exposed to blood and water splashes were 2.27 times more likely to be exposed to *Leptospira* than workers without such an exposure at the abattoir, although there was no statistical significance. This finding agrees with published reports where exposure of abattoir workers to leptospirosis during slaughter was a significant risk factor (Dreyfus et al., 2014, 2015; Esmaeili et al., 2016; Ngbede et al., 2012). Additionally, the throughput of the abattoir was determined to be an important risk factor for the workers exposure to leptospirosis as it was determined that, workers at HT abattoirs were 3 times more likely to be exposed to leptospirosis at the facilities where they slaughtered animals. To our knowledge, from the findings of this risk factor, the high throughput abattoirs play an important role in the exposure of abattoir workers to leptospirosis. The fact that abattoir workers may have been exposed to leptospirosis through other activities outside of the abattoirs was confirmed by our finding that in our study population, workers who had farm animals or had contact with farm animals were 3.44 times more likely to be exposed to leptospirosis than those without such an exposure. This is an indication of non-abattoir related exposure amongst some of the abattoir workers (Schooman and Swai, 2013). Animal contact is known to increase exposure to leptospirosis as reported by Simpson et al. (2018). The authors had conducted a survey for several zoonoses, including leptospirosis, among veterinary staff, farmers and herders associated with 'dip tanks' in Mpumulanga province in South Africa where animals from several herds congregated weekly for ectoparasite control and to monitor for Foot-and-Mouth Disease (FMD). They found a seroprevalence of leptospirosis higher than found in patients with acute fever at local clinics. However, home slaughter, farming or hunting were found not be significantly associated with leptospirosis (Dreyfus et al., 2014). Several other risk factors have been reported to be significant risk factors for the exposure of abattoir workers to leptospirosis including removal of high risk materials such as bladder, kidneys and offal (Dreyfus et al, 2015b), eating at work and having wounds (Cook et al., 2017), and lack of personal protective gears (Brown, et al., 2011).

It is interesting to have detected in our study that the individual risk factors, specifically age and gender of the abattoir workers, did not significantly affect the seroprevalence of *Leptospira* spp. Regarding the age of abattoir workers, the seropositivity ranged from 25.0 % (18 to 30 years) to 23.1 % (51 to 60 years). Although our findings of no association between age and seropositivity in abattoir workers agree with the report of Alvarado-Esquivel et al., (2016), others have documented

an association between increasing age and leptospirosis in abattoir workers (Brown et al., 2011; Benschop et al., 2009). Similarly, the seroprevalence of leptospirosis in the abattoir workers was not significantly affected by gender with the finding of 29.7% (26/87) and 31.0 % (5/16) in male and female workers respectively. Male abattoir workers have been reported to have a higher probability of exposure to leptospirosis (Benschop et al., 2009). This may be related to the types of duties performed by male workers, such as, slaughtering and dressing of carcasses which have been documented to pose a higher risk of exposure to leptospirosis (Dreyfus et al., 2015b; Brown et al., 2011). The failure to detect a significant effect of age and gender of abattoir workers on their seropositivity of leptospirosis in this study may also have been confounded by factors such as the disparity in the sample sizes, abattoir-related factors such as types of duties performed, personal hygiene, seroprevalence of leptospirosis in slaughtered animals amongst other factors.

Of the 4 infecting serovars detected in the eight seropositive workers, serovar Djasiman was detected in 50% of them. Since vaccination against leptospirosis is not routinely practised for abattoir workers in South Africa, it could be concluded that this finding was because of natural exposure to the pathogen, which may have taken place during their activities in the abattoirs. It is evident that all the eight seropositive abattoir workers in this study performed high risk jobs such as slaughtering of animals, washing offal, and cleaning duties in the abattoirs. Different serovars of *Leptospira* spp., usually isolated from livestock and abattoir workers or humans in general, have been documented in different countries. Unlike the predominance of serovar Djasiman detected in this study, serovar Hardjo in New Zealand (Benschop et al., 2009), Pomona also in New Zealand (Pittavino et al., 2017; Drysfus et al., 2014), Canicola, Hardjo and Pomona amongst other serovars in Nigeria (Onyemelukwe et al., 1993), Sekoine in Tanzania (Mirambo et al., 2018) have been detected. Serovars detected in animals and humans in different geographical locations reflect the endemic serovars in the animal population, particularly the reservoirs of this zoonosis.

It is significant that both the IgM ELISA and MAT classified 67 workers as being sero-negative for leptospirosis, and there was an overlap of seropositivity by the MAT and IgM ELISA (MAT and ELISA positive) in only 1 (1.0%) sample in the current study. In this study, the Cohen's kappa (*k*) at 95% CI comparison of the three test between MAT, 7.8% (8/103) and qPCR, (16.5 %; 17/103), had slight agreement [0.06 (-0.14, 0.26), p=0.5], while the MAT, 7.8% (8/103) and IgM ELISA, 10.7% (11/103) comparison also had a slight agreement [0.13 (-0.13, 0.39), p=0.17] and finally, the IgM ELISA, 10.7% (11/103) and qPCR, 16.5% (17/103) when compared had a poor agreement [-0.07 (-0.22, 0.08), p=0.48]. This disparity in the results obtained from ELISA and MAT was also observed in a study

conducted on veterinary and non-veterinary students in a university in the Caribbean to determine the seroprevalence of leptospirosis (James et al., 2013). In that study, the use of the IgG ELISA test kits detected statistically significantly ($P<0.05$) higher seropositivity in veterinary students, 26.5% (30/113) compared with non-veterinary students, 13.1% (13/99). On the other hand, the use of the MAT on the same serum samples detected a seropositivity that was not statistically significant ($P>0.05$) different between veterinary students, 7.1% (8/113) and non-veterinary students, 7.1% (7/99). Disparity in the detection rates of leptospirosis between the MAT and ELISA (IgM or IgG) have also been reported by others (Levett and Whittington, 1998), which may reflect the differences in the sensitivity and specificity of both tests, in addition to the class and quantity of immunoglobulins they detect.

Unlike both serological (MAT and ELISA) tests used in the current study which are based on the detection of antibodies (IgM, IgG or both) produced in the abattoir workers in response to exposure and infection by *Leptospira* spp., we also used a molecular method (qPCR) to detect the presence of pathogenic *Leptospira* spp. by assaying for their DNA in whole blood. This is considered the first diagnostic strategy in South Africa to apply a combination of both serological and molecular methods on the same subjects for the diagnosis of human leptospirosis.

The frequency of detection of pathogenic *Leptospira* DNA in the blood samples from 103 abattoir workers tested was 17 (16.5%). It is pertinent to mention that there was only one, 1 (1.0%, 1/103) overlap of positivity between the MAT and qPCR (i.e. MAT-positive and qPCR-positive). It was also of interest that there was no agreement in the positive results by the IgM ELISA and qPCR results. This was not unexpected because the qPCR detects DNA from dead and live leptospires while the serological tests (MAT and ELISA) are designed to detect antibodies only.

The advantages of molecular methods in the early diagnosis of leptospirosis are their higher sensitivity and specificity, throughput, fast, with low chances of contamination (Espy et al., 2006; Wunder et al., 2016). It is important to note that during the awareness sessions with abattoir workers at several abattoirs when the questionnaires on leptospirosis were administered it was evident that most of the abattoir workers, including abattoir managers, were ignorant of leptospirosis. Most of them mentioned that when they had symptoms such as fever, headaches, cough and cold, they were unable to go for diagnosis. These responses made it difficult to ascertain whether or not they were infected or exposed to *Leptospira* spp. All these led us to collect blood samples and to subject them to a sensitive and specific qPCR using probes (Wunder et al., 2016).

Another objective of this chapter was to use qPCR to test the belief by veterinarians and other researchers in South Africa that leptospirosis is not a problem in the country to know the current status of the diseases as recommended by WHO on the use of three methods of leptospirosis diagnoses, specifically, bacteriological culture, serological and molecular (WHO, 2003). In addition, another rationale for the qPCR on blood of abattoir workers was the indication we had from the high titres of MAT (1:400 and 1:3,200), which is an indication that, these abattoir workers might be considered as convalescence apparently healthy workers and could be shedding the *Leptospira* spp. to other workers at the abattoirs.

Finally, the findings of our study need to be further investigated in the context of detecting *Leptospira* DNA in workers considered to be apparently healthy. This is because it is generally believed that *Leptospira* DNA is normally detected only in the acute phase of leptospirosis (Boonslip et al., 2011). However, considering the CT values in our study confirmed the presence of *Leptospira* DNA in the human blood samples tested. The collection of blood samples from the abattoir workers by a qualified phlebotomist was carried out professionally. Furthermore, the fact that the assay was conducted in a laboratory at Yale University School of Public Health, USA where laboratory quality control is optimal. It is possible that the workers sampled may be in the early stage of active infection by *Leptospira* spp. without showing clinical manifestations. These are important factors to consider in interpreting the results, and a justification for a follow-up study.

The diagnostic application of the strategy used in the current study which utilized two serological tests and qPCR was the increase of its sensitivity to 30.1% (31/103). The implication is that unlike both serological tests which determined that 13.6% (14/103) workers had exposure experience of *Leptospira* spp., this frequency increased to 30.1% with the inclusion of qPCR. It is recognized that PCR is based on detecting genetic materials (nucleic acids) of microorganisms of interest but it is unable to determine whether the microorganism is dead or alive, which may be important for risk assessment regarding exposure.

To our knowledge, diagnosis of leptospirosis using the real-time PCR has not been documented in South Africa, especially using the qPCR of the outer membrane lipoprotein (*LipL32*) gene region, for the detection of pathogenic *Leptospira* spp. These data therefore represent the first application of qPCR to diagnose leptospirosis in apparently healthy humans from abattoirs and the identification of potential risk factors can be used to educate workers, to facilitate the introduction of preventive measures to reduce the transmission of leptospirosis in the high-risk group such as abattoir workers.

7.6.1 Limitations of the study:

i) The small sample size of the abattoir workers sampled was limited by the number of abattoirs owners, managers and individuals who agreed to participate in the study.
ii) No paired samples were collected from subjects for serological methods as recommended by the WHO.
iii) Sequences were not generated from the amplified DNA from the qPCR for further genetic analyses,
iv) Bacteriological culture was not attempted on the human samples.

7.6.2 Conclusions

a. The documentation for the first time of the prevalence and risk factors for leptospirosis in abattoir workers in South Africa has public health implications.
b. Working in HT abattoirs and exposure to blood and water splashes during slaughter are risk factors that should be managed to reduce the exposure of abattoir workers to leptospirosis.
c. Exposure to farm animals outside of the abattoir environment is a risk factor for exposure of abattoir workers to leptospirosis.
d. The fact that the predominant serovar of *Leptospira* spp., Djasiman, in seropositive workers, is not included in the vaccines used in livestock is an indication that the workers acquired infection through natural exposure.
e. The finding that only three (HT abattoirs, exposure to blood/water splashes and farm animals) of the 30 risk factors investigated were significantly associated with exposure of workers to leptospirosis suggests that there may be a need to conduct a larger study to include more abattoir workers and abattoirs to fully elucidate the status of leptospirosis in abattoir workers. The need for this is emphasized by the recent outbreaks of human leptospirosis in South Africa.
f. The use of the strategy to determine the seroprevalence of leptospirosis using two serological tests (MAT and IgM ELISA) and detection of the DNA of pathogenic *Leptospira* spp. using qPCR determined that 30.1% (31/103) of the abattoir workers have had exposure to the pathogen.
g. This study is the first to concurrently determine the seroprevalence (using MAT and ELISA IgM) and prevalence of *Leptospira* DNA (using qPCR) to identify the potential risk factors for leptospirosis in abattoir workers in South Africa.

7.6.3 Recommendations:

i) More sensitization of occupational high-risk individuals (abattoir workers, animal health and extension workers, farmers, students, veterinarians, and the public in general) on leptospirosis is needed.

ii) A larger sample size should be collected for future studies on leptospirosis in abattoir workers in South Africa to increase the chances of detecting more serovars and their distribution.

iii) Paired samples should be collected from subjects for serological methods as recommended by WHO in future studies

iv) More efforts should be made to sequence amplified DNA from humans in South Africa to compare the genetic relatedness between the South African sequences and sequences from other parts of the world.

v) An attempt should be made to isolate *Leptospira* spp. from high risk individuals in South Africa

7.6.4 Connecting statement:

Following the set objectives of this research project:

i) Retrospective analyses of the diagnostic laboratory data on samples submitted to the Onderstepoort Veterinary Research (OVR), between 2007-2017 (11 years).
ii) Seroprevalence of leptospirosis from slaughtered livestock at the Gauteng Province abattoirs, South Africa.
iii) Isolation, molecular detection and characterization of *Leptospira* spp.by culture and PCR from slaughtered livestock at Gauteng Province abattoir, in South Africa and
iv) Seroprevalence and molecular detection of *Leptospira* spp., using IgM, MAT and qPCR respectively, and its associated risk factors from abattoir workers at Gauteng in South Africa.
v) The implications of the above set objectives will be discussed in **Chapter 8** of this thesis.

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CHAPTER 8

8.1 General discussion

Leptospirosis, an important occupational and environmental zoonosis, is a known global disease which also has economic significance, particularly in animals due to the burden of the disease and mortalities caused. In South Africa, there is a widely held view among veterinarians, some livestock owners and researchers that leptospirosis is not an important animal disease in the country. This possibly erroneous view may have been due to a dearth of information on the status of the disease in the country and the lack of active and passive surveillance in both animal and human populations. There is also limited diagnostic capability in the country contributing to the under-diagnosis and under-reporting of the disease (Dupouey et al., 2014).

Considering the contribution of livestock to the economy of South Africa, the pivotal position of Gauteng Province in having a high livestock population, with the highest number of abattoirs in the country, the highest human population density in the country and finally, the location of the only national veterinary leptospirosis laboratory in the province, it was important to have conducted an investigation on leptospirosis in the province. Furthermore, the recent outbreak of human leptospirosis in Mpumalanga Province and several other provinces highlighted the recognition of leptospirosis as an important zoonosis in South Africa.

This investigation was therefore designed and conducted, firstly, to undertake a comprehensive, critical review of the literature on human and livestock leptospirosis globally, in Africa and South Africa (**Chapter 2**). This was followed by a retrospective analysis of diagnostic laboratory data (2007-2017) which assessed the past occurrence of leptospirosis in routine or suspect cases of livestock leptospirosis and to identify the infecting serovars of *Leptospira* spp. (**Chapter 3**).

In **Chapters 4** and **5**, a cross-sectional study was conducted in Gauteng abattoirs slaughtering livestock (pigs and cattle) from Gauteng Province and other provinces in South Africa, to determine the seroprevalence and risk factors for leptospirosis, and the predominant infecting serovars. Thereafter, the infecting strains of *Leptospira* spp. were isolated and characterized using molecular methods in **Chapter 6**. Finally, the zoonotic risk of leptospirosis posed to abattoir workers was determined in **Chapter 7**.

The retrospective analysis of laboratory data revealed an overall seropositivity for leptospirosis in livestock of 20.5% (1,425/6,945), using an eight-serovar panel in MAT at the national veterinary leptospirosis laboratory at ARC-OVR. The seropositivity according to species was 25.0% (1,293/5,168) in cattle, 16.2% (286/1,763) in pigs, and 0.0% (0/14) in sheep ($p<0.001$). It was important that only 14 sheep samples were submitted for leptospirosis testing. This may indicate that leptospirosis is not considered an important disease among sheep.

In the seropositive pigs and cattle, the predominant serovar was Australis (sv. Bratislava), with a frequency of 32.2% (94/292) and 29.4% (333/1133), respectively. This finding may be of clinical and diagnostic significant because the serovar was detected in clinical cases or suspect cases of leptospirosis and the infection was contracted through natural exposure because the serovar is not included in the vaccines used to prevent livestock leptospirosis in the country. The livestock vaccines sold in South Africa include five *Leptospira* spp. serovars: Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae and Pomona. This is the first documentation of review laboratory diagnostic data on leptospirosis in livestock in South Africa.

The main limitations of the study were that the animals from which the samples originated were mostly clinical cases and did not represent the apparently healthy livestock population in the country and the fact that the database available at the ARC-OVR laboratory did not contain any information on the risk factors such as age, sex and vaccination status of the animals. Notwithstanding these limitations, the data generated provided information on the circulating serovars in livestock populations in the country, which are invaluable in assessing their role in clinical leptospirosis.

The limitations identified in the retrospective analysis of data were addressed in the cross-sectional study conducted between September 2016 and April 2017, using a 25 and 26 antigen panel for abattoir workers and slaughtered livestock respectively, collecting information on the risk factors for leptospirosis in slaughtered animals and except the vaccination status of livestock in the current study that, was unavailable. In the study conducted on slaughtered livestock (n=85 for pigs and n=199 for cattle) in 14 abattoirs across Gauteng province, with the overall species-specific seroprevalence being, 24.7 (21/85) and 27.6 (55/199) for pigs and cattle, respectively.

It was of interest that in comparison with the retrospective data analyses using only the year 2016 of the submitted samples from across the country to ARC-OVR with the cross-sectional study we conducted in 2016 from slaughtered animals across South Africa at the Gauteng abattoirs. The

seroprevalences detected by animal species in the retrospective were also statistically significantly different. However, the seropositivity rates were similar for cattle in both the retrospective data for the year 2016 only, 21.6%, (101/466) and the cross-sectional study, 27.6% (55/199), with no statistically significant difference ($p<0.05$); while for the pigs there was a statistically significant difference for the retrospective data and the cross-sectional study, 100% (2/2) and 24.7% (21/85) respectively conducted 2016 at the Gauteng abattoirs slaughtered pigs, analysed with the use of only an 8 serovar MAT panel (retrospective study) and using a 26-serovar MAT panel for the cross-sectional study. Although the small samples size, especially (n=2) submitted for pigs in the year 2016 of the retrospective study is insignificant to draw a statistical conclusion. The detection of antibodies to only 9 of the 26 serovars in the MAT in the cross-sectional study compared with the detection of antibodies to the eight antigens in the retrospective data analysis livestock, support is given for the prudence of the ARC-OVR laboratory in using only the eight serovars determined to be prevalent in South Africa.

Equally of significance was the finding that the predominant serovar amongst seropositive cattle was Sejroe (sv. Hardjo), (36.4%) while in pigs it was Australis (sv. Bratislava), (90.5%) in our current study, compared to the study on retrospective analysis of data, a change in the pattern of the predominant serovar was observed in cattle from Bratislava to Hardjo in the cross-sectional study while Bratislava was unchanged in the pig samples in both studies.

Changing patterns of circulating serovars in animal species and geographical locations have been reported by Weekes et al. (1997). The seropositivity for antibodies to *Leptospira* spp. was 0.0% (0/14) in sheep Submitted in the retrospective study were seronegative. Considering that the numbers of sheep tested are low, it is difficult to draw any inference and therefore, these findings require further investigation with larger sample size within the country.

In the cross-sectional study, the important risk factors for leptospirosis in cattle was only abattoir, with a statistically significant effect on the seroprevalence ($p<0.001$) by univariate analysis. However, using the multivariate analyses, it was not statistically significant but by logistic regression it was detected that pig abattoir category one (1) (Odds ratio: 8.72) and age (young) (Odds ratio: 3.86) were, indicative of increased risk of exposure to *Leptospira* spp. Similar findings were reported in a study conducted in Nigeria (Ngbede et al., 2012).

For the first time in South Africa, the study documented the use of 26 antigens panel and the occurrence of four serovars, namely Hardjo bovis strain lely 607 (which is not included in the panel used to diagnose leptospirosis in the country), Topaz, Hebdomadis and Medensis in slaughtered livestock populations. The vaccine used locally for prevention of livestock leptospirosis does not contain the three serovars (Topaz, Hebdomadis and Medensis) detected for the first time in South Africa.

The investigation on the isolation and characterization (conventional and molecular) of *Leptospira* spp., performed on 305 livestock kidney and 14 abattoir effluent samples revealed a bacteriological isolation rate of 3.9% (12/305) in kidney samples using Ellinghausen McCaullough Johnson Harris (EMJH) media. The isolation rate by animal species was 4.8% (9/186), 4.1% (3/74) and 0.0% (0/45) in cattle, pigs and sheep, respectively ($P>0.05$). In agreement with the sero-negativity for leptospirosis detected in sheep in both serological studies (retrospective data and cross-sectional), the isolation rate in the current study was also determined to be 0.0%.

The rate of detection of *Leptospira* spp. by PCR (27.5%) was statistically significantly ($P=0.00001$) higher than bacteriological assay (3.9%). The frequency of detection of *Leptospira* DNA in kidney tissues by animal species was 26.9% (50/186), 20.3% (15/74) and 42.2% (19/45) for cattle, pigs and sheep respectively. The implication of detecting *Leptospira* DNA in the kidney tissues of sheep at a statistically significantly ($P<0.05$) higher frequency than in cattle and pigs cannot be over-emphasized considering that all the sera and kidney tissues from sheep tested for antibodies and bacteriological isolation for *Leptospira* spp. were negative. Therefore, these findings may be evidential to conclude that, the sheep that were tested had been exposed to *Leptospira* spp. with the detection of the DNA of the pathogen in the kidneys but undetectable by serology and culture. These findings require further investigation.

The isolation of *Leptospira* spp. is a definitive diagnosis of leptospires (Picardeau, 2013) and the application of the molecular diagnosis is highly recommended for the diagnosis of leptospirosis (OIE, 2014; WHO, 2003) because of its high sensitivity and specificity (Espy et al., 2006; WHO, 2003). The molecular diagnostic method revealed the highest frequency of detection of *Leptospira* DNA in the kidney samples of slaughtered livestock from the abattoirs in this study.

It is pertinent to mention that although qPCR detected 27.5% of the kidneys to have had prior exposure to *Leptospira* spp., the DNA from the kidneys could have originated from dead or live

leptospires. The bacteriological isolation rate (3.9%) measures the risks to which the abattoir workers are exposed through the shedding of live leptospires in urine and contact with contaminated soil and water (Benschop et al., 2017; Adler & de la Peña Moctezuma, 2010) or from the blood of infected livestock (Campagnolo et al., 2000).

Sequencing of DNA from isolates of *Leptospira* spp. and kidney tissues from cattle identified 13 as *L. interrogans* and 2 as *L. borgpetersenii*), from pigs 4 as *L. interrogans* and from sheep kidney tissues 2 as *L. interrogans* and 1 as *L. borgpetersenii*. The phylogenetic tree analyses revealed that all the isolates and kidney samples grouped together with the pathogenic *L. interrogans* serovars icterohaemorrhagiae and *L. borgpetersenii* serovar Hardjo bovis strain lely 607 from the GenBank retrieved sequences. These findings have diagnostic implication because *L. borgpetersenii* serovar Hardjo bovis starin lely 607 is not included in the MAT panel of serovars used for the serological diagnosis of leptospirosis at the ARC-OVR leptospirosis laboratory. This study also reports for the first time, in the country the genetic analyses of the pathogenic *Leptospira* spp. in livestock.

The recent outbreaks of human leptospirosis reported in South Africa (Http//www.nicd.ac.za, NICD Communicaque, 2016; 2015; Saif, 2012), make it imperative to determine the prevalence and risk factors for leptospirosis in high risk individuals, such as abattoir workers. The study analyzed blood samples from 103 consenting abattoir workers in six HT, multi-species abattoirs for antibodies to *Leptospira* spp. using two serological tests (MAT and IgM ELISA) and one molecular method (qPCR) to detect *Leptospira* DNA. The seroprevalence of leptospirosis in the 103 abattoir workers was 10.7% and 7.8% by IgM ELISA and MAT respectively while the frequency of detection of *Leptospira* DNA in whole blood by qPCR was 16.5%. The predominant serovar detected in the seropositive workers was Djasiman (50.0%). Since abattoir workers are not routinely vaccinated against leptospirosis in South Africa, the study concluded that they were exposed to *Leptospira* spp. through natural exposure.

The abattoir-related risk factors for exposure of the workers to leptospirosis were working in HT abattoirs and exposure to blood and/or water splashes during slaughter. These factors should therefore be managed to reduce their exposure to leptospirosis.

The diagnostic strategy comprising the use of three tests (MAT, IgM ELISA and qPCR) increased its sensitivity from 13.6% (14/103) for both serological tests to 30.1% (31/103) when used in combination with qPCR and performed on the samples from the same individuals. This is the first

use of such a diagnostic strategy and the identification of the important risk factors for human leptospirosis in South Africa. The implication is that the use of any of the tests alone results in under-diagnosis and under-reporting of leptospirosis.

8.2 General conclusions

The data provided in the four studies **(*Chapters 3, 4, 5, 6 and 7)*** have provided information on the seroprevalence and risk factors for leptospirosis in apparently healthy livestock and clinical cases of leptospirosis, the seropositivity of leptospirosis in clinical and suspect cases and finally, the seroprevalence of *Leptospira* spp. and risk factors for leptospirosis in abattoir workers **(*Chapter 7*).** The risk factors identified in these studies will be invaluable for any intervention strategies to prevent leptospirosis in livestock and abattoir workers in the country.

It is imperative that a diagnostic laboratory, like the national leptospirosis laboratory at ARC-OVR, in South Africa, needs to consider the possibility of reviewing the type and number of serovars (antigens) used in the diagnostic MAT panel. This is because some of the serovars detected by serology, isolation and qPCR are not in the panel of antigens used for MAT diagnosis in the country.

To improve the potential application of the database maintained at the ARC-OVI Leptospirosis Laboratory, it is necessary to obtain basic information on the animals from which blood samples were collected and submitted for the diagnosis of leptospirosis. Some of the key information required in understanding the epidemiology and prevalence of leptospirosis should include the age, sex, reason for submission of samples and vaccination status, among others.

The increased capacity for isolation of *Leptospira* spp. on a more extensive basis at the National Leptospirosis Laboratory at ARC-OVR will add value to the diagnostic capacity and contribute to risk assessment of leptospirosis in animals and humans.

The addition of PCR as a diagnostic tool for leptospirosis in both animals and humans will increase the sensitivity of the diagnostic strategy.

Leptospirosis in sheep in the country needs to be further investigated using a larger sample size based on the findings of negative serological and bacteriological test results but a high frequency of detection of *Leptospira* DNA using qPCR in the same animals.

Abattoir workers tested in the current study had a high prevalence (30.1%) of leptospirosis prior exposure to *Leptospira* spp. and the identified abattoir-related risk factors should be used to facilitate interventions for preventing the disease in workers.

There is a need to use the data from the studies in this thesis to develop effective passive and active surveillance at the abattoirs in the country. This would result in more effective control and prevention of leptospirosis in the livestock industry to boost food production, economy of the country, wellbeing of livestock and livelihood of humans, especially for those in high risk occupations and the general public.

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APPENDIX

Appendix 1: Standard questionnaire used for human leptospirosis exposure risk factors.

A copy of the standard questionnaire constructed and used to capture abattoir workers data ***Appendix 1.***

Section 1: Background information of the individual [17 factors]

1. Address of the Abattoir _____ Abattoir type _____ Abattoir GPS coordinates N 0 . E 00 .
2. Sex of respondent Male ☐ Female ☐ Unknown ☐
3. Age of respondent in years ☐
4. Marital status of respondent Married ☐ Single ☐ Divorced ☐ Widowed ☐
5. Job description Butcher/slaughter man ☐ Inspector ☐ Transporter ☐
   Other: _____
6. How long have you worked in the abattoir? 1 year ☐ 2 years ☐ Three years and above ☐
7. How many days do you work in the abattoir per week? Everyday ☐ 5-7 days ☐ 3-4 days ☐ 1-2 days ☐
8. What animal section do you work in? Cattle ☐ Sheep/goat ☐ Pig ☐ Games ☐ All Sections ☐
9. Do you take care of animals at home or work on farms? Yes No
   If yes, Indicate the types of animals you take care of: _____
   _____
10. Do you slaughter animals at home? Yes No
11. Do you have personal protective gear? Yes No
12. Do you wear your personal protective gears? Yes No
13. Have you ever had any of the symptoms below in the abattoir?
    Fever ☐ Headache ☐ Tiredness ☐ Coughing ☐
    Diarrhoea ☐ Rashes ☐ Weakness ☐
    Itching of the eyes ☐ Nausea and vomiting ☐ Loss of appetite ☐
14. Where do you get your drinking water at the abattoir? Taps ☐ Dam/well ☐
    Other: _____
15. Where do you get your drinking water at home? Taps ☐ Dam/well ☐
    Other: _____
16. If not from the tap, how do you treat it? Bleach ☐ Boiling ☐
    Other: _____

17. Do you have health concerns working at the abattoir? Yes No

If 'yes', indicate your concerns: _____

_____

Section 2: Leptospirosis-specific [13 factors]

		Yes	No
1.	Do you know what leptospirosis disease is?	Yes	No
2.	Do you think you can get leptospirosis from abattoir?	Yes	No
3.	Do you see rats around the abattoir?	Yes	No
4.	Do water/blood splash unto your face in the abattoir?	Yes	No
5.	Do you think you can get sick from the abattoir	Yes	No
6.	Do you work with injuries in your hands in the abattoir?	Yes	No
7.	Do you use water bodies around the abattoir?	Yes	No
8.	Do you see dogs around the abattoir?	Yes	No
9.	If you are a meat inspector or involved in slaughtering, within the last 12 months have you observed any carcass and mucous membranes with jaundice (yellow carcass)?	Yes	No / NA
10.	Do you eat/drink with your hand gloves at the abattoir?	Yes	No
11.	Do you have dog/pets at home?	Yes	No
12.	Within the last 12 months, have you been diagnosed of having clinical leptospirosis?	Yes	No
13.	Within the last 12 months, have your dogs or cats been diagnosed of having clinical leptospirosis?	Yes	No

If 'yes' indicate the clinical signs observed. _____

_____