The utility of a real-time PCR to detect *Leptospira* in a routine diagnostic setting

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SUMBMITTED TO THE UNIVERSITY OF CAPE TOWN

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE - MASTER IN MEDICINE MEDICAL MICROBIOLOGY (MMED)

FACULTY OF HEALTH SCIENCES
UNIVERSITY OF CAPE TOWN

DATE OF SUBMISSION:11-02-2022

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PART A: DECLARATION

I, **Raphael Chanda**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been/is being or is to be submitted for another degree in this or any other university. This work has not been reported or published prior to registration for the above-mentioned degree.

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ACKNOWLEDGEMENT

Author Contributions

PN initiated the project, LVP created constructs, NC tested constructs and did testing, Dr Clinton Moodley has intellectual contribution and edited manuscript. The authors want to thank Dr Alice Yamanya Tembo for data analysis. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by NHLS Research Trust Fund number 94558.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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Leptospirosis is a neglected zoonotic infection with world-wide distribution. A paucity of leptospirosis data from the African continent exists, mainly due to limited access to diagnostics. The clinical presentation ranges from mild to severe disease with multi organ involvement, while the mild form mimics another common tropical disease i.e., malaria. The gold standard for diagnostic detection currently is an immunological test discerning the presence of specific antibodies present in the immune phase of the disease. The serological methods are hindered by the inability to distinguish past from current infection and utility limited to only the immune phase of the disease. Due to lack of sensitivity and specificity in serological methods, improved diagnostic methods are needed to aid early identification in the acute phase. Methods should also distinguish saprophyte and pathogenic species. To address this gap, we developed an in-house PCR assay targeting the microbe's *rrs* and *lipL32* genes, using primer sets previously reported in literature to be both sensitive and specific for pathogenic *Leptospira* spp. Using in-house constructed plasmids, we did a non-clinical, technical validation employing probe-based, real time polymerase chain reaction assays and a locally available commercial kit. Although our assay needs further optimization, we demonstrated that the PCR reliably detected 100 copies and 1000 copies of *Leptospira rrs* and *lipL32* targets respectively. To test specificity, we did real-time PCR with pure DNA from a selected set of pathogens known to be prevalent in bacteremia's in local settings and observed that the rrs target was amplified with Group B streptococci as template but no other tested pathogens, while no non-specific amplification for *lipL32* was observed. The non-specific amplification had been reported previously in the literature, suggesting the rrs gene is not a good target to use, even when primers are specifically designed to only detect *Leptospira* rrs. Future work using the assay should include optimizing assay performance using DNA extracted from the ideal clinical samples to detect *Leptospira*, namely urine and blood of patients clinically suspected to have leptospirosis. However, the assay demonstrated potential for use as a diagnostic PCR using the constructed plasmid, but further optimization to improve PCR efficiency and assessing its performance in clinical setting is required

PART D: Structured literature review

1. Introduction

Leptospirosis is an emerging zoonotic disease caused by spirochetes from the genus *Leptospira.* According to a 2015 report, the disease has a global incidence estimated at 1 million severe cases and approximately 60 000 deaths(2). This incidence is not a reflection of the true burden, it includes minimal data from African countries due to lack of technical and diagnostic capacity to conduct surveillance. The significance of *Leptospira* is due to observed increased incidence of the disease, emergence in new locales and it's a significant association with socio-economic conditions such as informal settlements, slums and declining infrastructure(3). Additionally, outbreaks have been documented during weather related phenomena (such as floods)(5). All these factors warrant measures to improve clinical and diagnostic tools to diagnose affected persons in a timely manner. Improved surveillance in countries where it is underreported due to lack of, or inadequate tools are needed(6).

2. Epidemiology

As stated before, human leptospirosis global burden rests at about one million severe cases and 60,000 deaths per year(2), excluding data from geographical areas lacking proper surveillance. In high income countries within the temperate region, the risk for human transmission depends

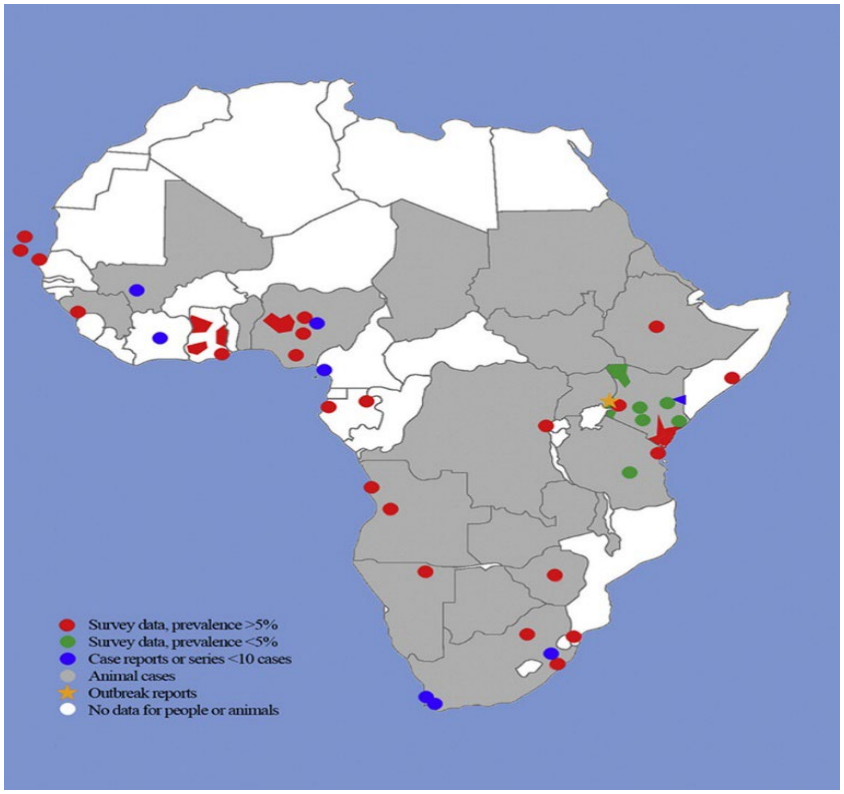

Figure 1.Leptospirosis in sub-Saharan Africa. Figure courtesy of Sophia G. de Vries et.al.(1)

on the interaction of humans with animal and the environment. Factors contributing to high-risk exposure include occupation (i. e. livestock and dairy farmers) and recreational activities which include water rafting and kayaking have recently been identified as important risk factors(7, 8). Transmission is exacerbated during periods of increased rainfall due to environmental dissemination of the bacteria(9) and is associated with outbreaks following

natural disasters. In developing countries with poor social economic status, high exposure is due to poor sanitation and inadequate drainage systems which contribute to increase exposure to zoonotic reservoirs. (10)

The World Health Organization (WHO) estimates the median incidence in Africa to be approximately 95.5 per 100,000 persons. In Africa seroprevalence data demonstrates widespread exposure to *Leptospira spp*. in both humans and animals.(1) However, limited studies have been conducted in this region thus the incidence might be underestimated(1). In South Africa, the incidence of infections in humans is largely unknown, with sporadic reports of cases of which the most recent documented exposure occurred at a correctional facility in the Western Cape Province in 2015 (11) .However, evidence from a retrospective study on human leptospirosis conducted 2010-2019 in the western cape province indicates 254 cases reported in the time period with the highest number of cases recorded in 2015 additionally the annual incidence ranged between 0.15 and 0.66/100,000 population with 10 year average incidence of 0.40/100,000 population(12). Furthermore, animal surveys have shown *Leptospira* seroprevalence rates of 19.4% in cattle in KwaZulu- Natal(KZN)(13) and 5% in dogs in coastal region of south Africa. Seropositivity rates of 19.8% has also been reported in a rodent related zoonosis study in people conducted between 2003-6 in KZN region(14)

3. Aetiology and description of the causative organism

The genus *Leptospira* comprises of pathogenic and non-pathogenic spirochetes (Table 1). Previously, it was divided into 35 species, 25 serogroups and more than 250 serovars(15, 16). However, recent genomic studies on New World species indicates that the number of species is underestimated, with one study reporting 30 new species when only 90 isolates from diverse locales were subjected to whole genome sequencing and phylogenetic studies(17).

Table 1. classification of Leptospira species

Pathogenic spp.	Intermediate spp.	Non-pathogenic spp.
Leptospira interrogans, *Leptospira kirschneri* *Leptospira borgpetersenii* *Leptospira santarosai* *Leptospira noguchii* *Leptospira weilii* *Leptospira alexanderi* and *Leptospira alstoni*	*Leptospira inadai* *Leptospira broomii* *Leptospira fainei* *Leptospira wolffii* and *Leptospira licerasiae*	*Leptospira biflexa* *Leptospira wolbachii* *Leptospira kmetyi* *Leptospira meyeri* *Leptospira vanthielii* *Leptospira terpstrae* *Leptospira yanagawae*

Leptospira are distributed globally, with most mammals chronically infected with leptospirosis, either asymptomatically, or mild clinical manifestation or asymptomatic carriers (18). In chronically infected animals, *Leptospira* colonizes the kidneys and contaminates the environment through shedding in urine. In the environment, the bacteria persist for weeks to months in contaminated water or soil(15). Humans are considered as dead-end hosts and are susceptible to various serovars acquiring infection due environmental exposure the exposure to animal exposure presents varying risk of infection, with exposure to rodent excrement presenting the highest risk(10).

Leptospira's are helical in structure with hook shaped ends that allow distinction from other spirochaetes and exhibit motility due to presence of flagella. They are thin and measure about 0.1-0.15µm and 6 -20 µm in length. The organism requires an aerobic environment and exhibits

slow growth under favorable conditions. Leptospires are highly susceptible to heat and hypertonic environmental conditions. The chromosomal DNA in *Leptospira* displays a uniform distribution along the length of the cell rather than central location(19)

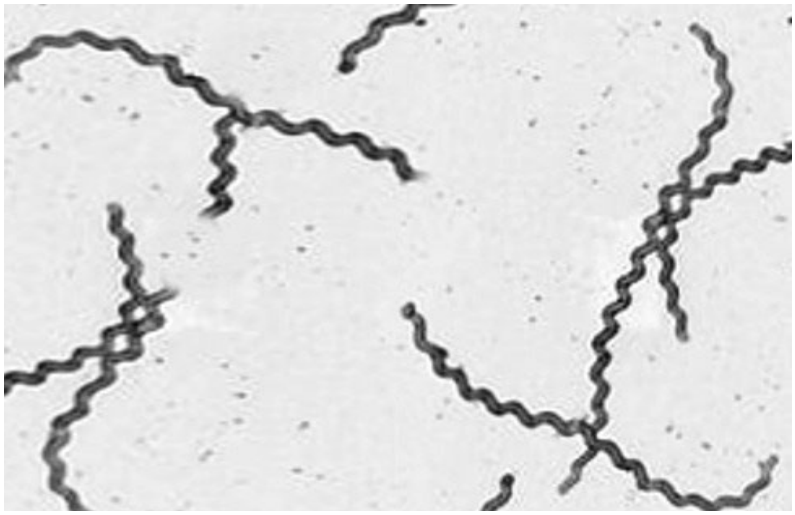

Figure 2. Spirochete cellular form of Leptospira. Leptospirosis on dark field microscopy. Image courtesy S Bhimji MD (Wang S, Stobart Gallagher MA, Dunn N(4).

4. Virulence factors

Currently, *Leptospira* are divided into 3 phylogenetic clusters, based on its virulence.
The composition of *Leptospira* structure includes presence of an outer membrane which is mainly composed of lipopolysaccharide (LPS), this structure shares characteristics similar to that of both gram negative and gram-positive bacteria, however the LPS possess lower endotoxin capacity compared to that of gram negative LPS(20). In addition to the LPS, the outer membrane contains transmembrane proteins whose main function is to act as diffusion barriers and facilitate uptake of nutrients required for growth.

Leptospira lipoproteins are major contributors aiding immune evasion(21). These proteins enable the organism to evade detection by the immune system and therefore contribute to virulence (22). Among the outer membrane proteins, the ones that are well documented to contribute to host immune evasion include lipoprotein L32(LipL32), lipopolysaccharide (LPS*),* *Leptospira* immunoglobulin like proteins (Lig), *Leptospira* endostatin like protein (Len) and *Leptospira* OmpA-like protein (Loa22)(20). A seen in Figure 3, depicting immune evasion mechanisms by spirochetes in general, certain *Leptospira* encode mechanisms to interact, modulate or evade host immune defenses. *Leptospira interrogans serovar Copenhageni* (LIC) encode LL11207 an antigenic surface protein, which has been shown in animal studies to be expressed during infection and interfering with apoptotic pathways in neutrophils(23) While the result of this interaction is not clear, it indicates interaction of invading *Leptospira* with the host immune system.

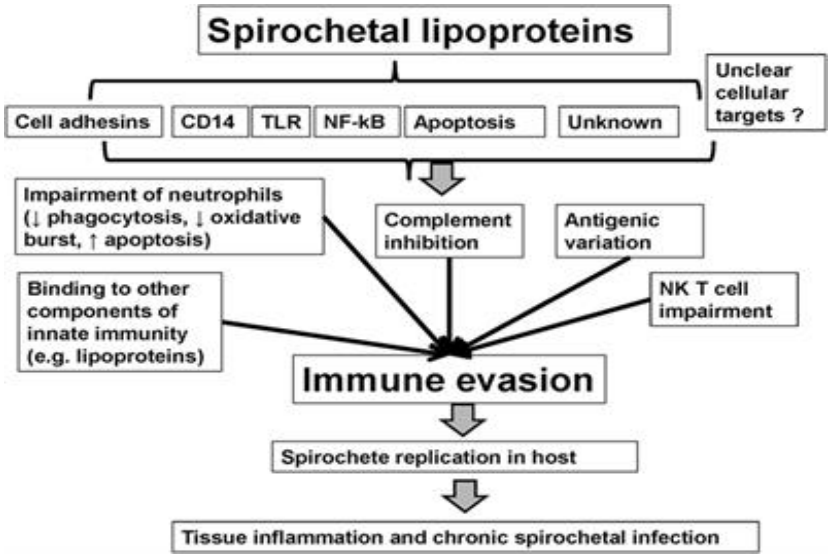

Figure 3. Interaction of spirochete surface proteins and the immune system

LipL32,or hemolysis associated surface protein, is highly conserved amongst pathogenic *Leptospira* spp and accounts for 75% of surface exposed outer membrane proteins, its presence has been considered to contribute to virulence in pathogenic spp (24). During acute lethal infections, higher levels of LipL32 are detected in comparison to levels expressed invitro cultures(25). The role of LipL32 as a virulence factor relates to its interaction with host immune system and ability to induce inflammatory response. Renal damage and development of tubulointerstitial nephritis results from production of large amounts of inflammatory mediators which include tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), RANTES and inducible nitric oxide synthase (iNOS). The inflammatory mediators that are produced in the vicinity of renal cells, lead to inflammatory response ultimately resulting in renal damage(26). Additionally, LipL32 also acts as a hemolysin which inducing proinflammatory cytokines via toll like receptor (TLR) signaling pathway main receptors for recognition being TLR2 and TLR4(27). There are several additional surface proteins which contribute to immune evasion these include: *Leptospira* immunoglobulin- like protein B (LigB) and *Leptospira*l endostatin like protein A (LenA). In the immune response pathway, factor H acts as a regulator, activating the host immune system alternative pathway. Pathogenic *Leptospira* bind factor H, inhibiting activation of complement via the alternative pathway and preventing pathogen complement mediated pathway. The gene sequence encoding LenA are highly conserved and are postulated to encode lipoprotein in pathogenic leptospires, which may be considered as a potential target in diagnostic assays (22) .

Immune evasion is vital for successful invasion *Leptospira* has evolved several strategies for it to evade the complement system (Figure 4) these strategies include:

I. Regulation of complement activation by acquisition of host soluble complement regulators: Factor H (FH)—AP regulator, C4b-binding protein (C4BP) —CP and LP regulator, and vitronectin (Vn) —terminal pathway regulator. FH and C4BP accelerate the decay of the C3 convertases which is required for further activation of the cascade.

II. Activation of host proteases: pathogenic *Leptospira* binds plasminogen, which in the presence of Urokinase-type plasminogen activator (uPA), is converted in the enzymatically active plasmin. Plasmin is serine protease cleaves C3b, C4b, and C5, promoting a downregulation of complement activation on the *Leptospira* surface.

III. Inactivation of complement proteins: Metalloproteases secreted by pathogenic *Leptospira* strains are able to cleave and inactivate the complement proteins which are important in formation of the membrane attack complex these include C3 (central complement molecule), Factor B (from AP), and C2 and C4 (CP and LP).

IV. The combination of these strategies enables pathogenic *Leptospira* to successfully evade the immune system and establish infection in target organs

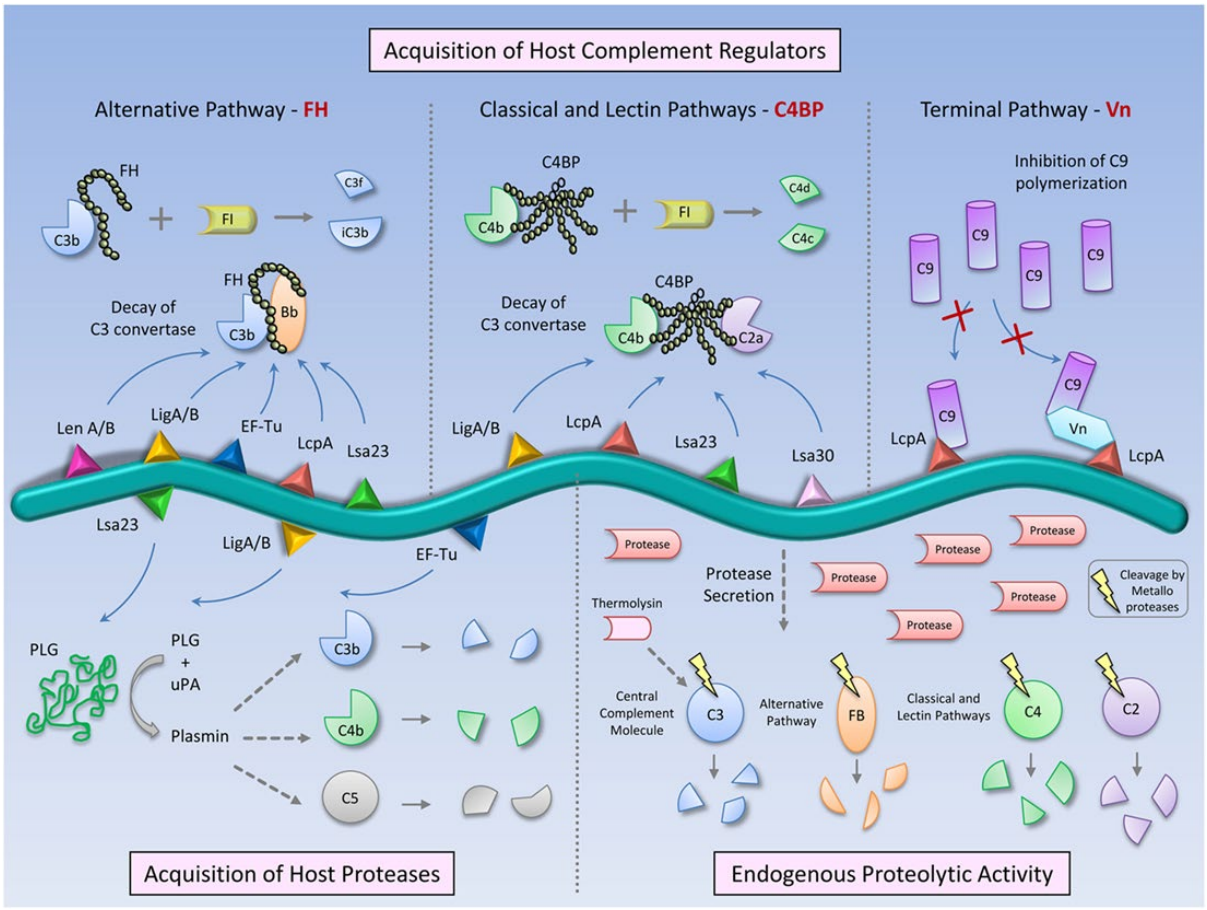

Figure 4. Complement evasion strategies of pathogenic Leptospira. Picture courtesy of Fraga et. al.(28).

5. Pathophysiology and Clinical presentation

Clinical presentation indistinguishable from other infections such as influenza, dengue, and malaria. The diagnosis of leptospirosis presents a challenge in settings with high prevalence of conditions with similar clinical presentation, often leading delayed diagnosis and unfavorable clinical outcome(29-31)

Table 2.Clinical presentation of leptospirosis

Clinical phases	Duration	Clinical manifestation
Incubation period	7 - 14 days	None
Acute phase	Approximately one week	• Fever, myalgia, • conjunctival suffusion, • nausea, vomiting • bacteremia • Leptospires present in blood and CSF
Immune phase	Recurrent cycle every 3-4 days	• Fever • Secretion of leptospires in urine

The spectrum of human disease caused by leptospirosis is diverse, ranging from subclinical infection to severe syndrome of multiorgan infection and subsequent death(32). The incubation period is approximately 7 - 14 days. Infection typically presents as a biphasic illness, with the acute phase being self-limiting and representing 90% of infections. In the acute phase the high levels of bacteraemia are in part due to poor recognition of *Leptospira* LPS by the innate immune system, this contributes to vascular damage by organism in this phase(16). As depicted in table 2 above, the most common manifestations in early phase include fever, myalgia, conjunctival suffusion, nausea, vomiting and bacteremia. This phase lasts up to a week and is followed by the immune phase associated with antibody production and excretion of *Leptospira*l in urine, the fever my recur after every 3-4 days(33, 34). In severe cases, the two phases are indistinguishable and patients commonly present with onset of the second phase of illness. Additionally, cytokine storm occurs in severe disease due to production of high level of interleukins and other inflammatory factors contributing to end organ damage. It has been observed that individuals with HLA DQ6 allele are at higher risk of disease which suggests a possible role for lymphocyte stimulation by *Leptospira* superantigen(35) In the acute phase, *Leptospira's* are isolated in blood and cerebral spinal fluid (CSF) despite lack of meningeal signs. *Leptospira* recovery from urine occurs 5-7 days after onset of symptoms and urinalysis may indicate mild proteinuria and pyuria, with or without hematuria.

The immune phase typically presents 7-10 days after symptom onset of the disease and can last up to 30 days. The phase coincides with the disappearance of *Leptospira* from blood and CSF and appearance of immunoglobulin M(IgM). It is associated with immunological organ damage and severe complications which include acute renal dysfunction, pulmonary hemorrhage, hepatic damage, coagulopathy, shock and neurological complications(32). Aseptic meningitis commonly occurs in 80% of patients and may occur with or without symptoms, with the CSF exhibiting a lymphocytic pleocytosis. Rarely severe neurological manifestations which include meningoencephalitis, transverse myelitis, Guillain-Barre syndrome or cerebral venous thrombosis may occur(15, 36). The most distinctive form of severe illness in the immune phase is called Weil's disease which is characterized renal and hepatic dysfunction(37)

6. Diagnosis

Leptospirosis remains a significantly underdiagnosed disease due to suboptimal performance of the current available diagnostic modalities. Laboratory diagnosis relies on microscopy, serology, culture from blood urine, or tissues, and/or by molecular methods using polymerase chain reaction (PCR). Below is a diagram illustrating diagnostic modality in relation to disease progression.

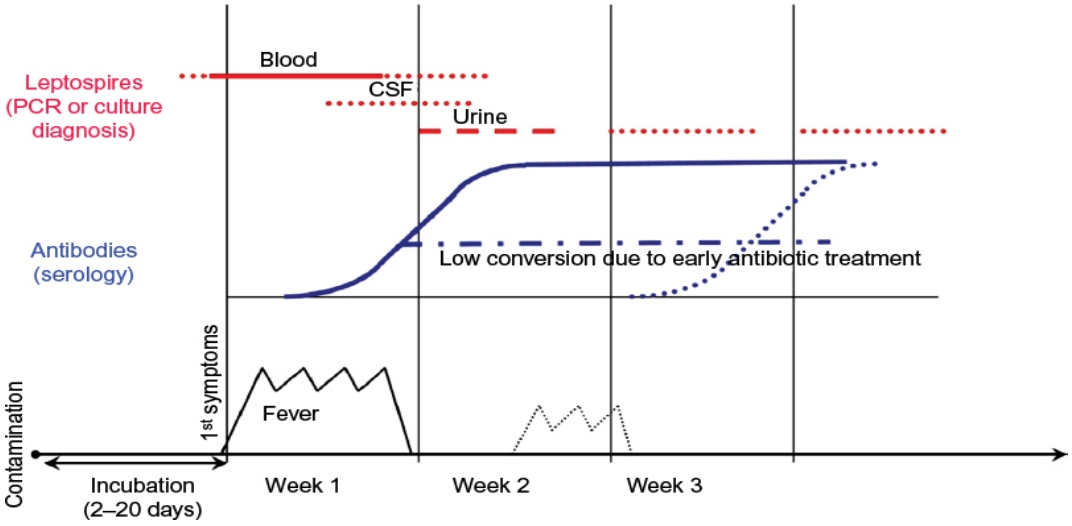

Figure 5 Diagnostic timeline of human leptospirosis Adapted from Turner(38).

6.1 Direct examination of blood: Dark field microscopy

In the acute phase of the disease, with high bacterial load can be diagnosed by darkfield microscopy, the limit of detection was determined to be 10^4 per milliliter of either blood or urine(39). In addition to requirement of darkfield microscopy, this method has presents other limitations which includes false positives in blood due to presence of artifacts resembling *leptospires*(40)

6.2 Culture based methods

Pathogenic species of leptospires are fastidious in nature and require specialized media for isolation. Media used in isolation include Ellinghausen McCullough Johnson Harris media (EMJH; BD Bioscience) and Fletchers media which is beef extract based and made in most clinical laboratories. *Leptospires* are isolated from blood and CSF within 7-10 days after infection and usually isolated in urine after 2-3 weeks later. Blood and CSF collected in heparin and sodium oxalate for transportation at room temperature. Isolation requires prolonged incubation of several weeks and exhibits poor sensitivity even in serologically confirmed cases(41)

Under optimal conditions, the organism grows slowly and negative culture results can only be confirmed after 6-8 weeks but preferably extended to 4 months (42). Cultures require weekly examination by darkfield microscopy before discarding as negative.

Due to culture requirements, prolonged incubation and reduced sensitivity, this method of identification is impractical for purposes of definitive diagnosis or as method to guide therapy.

6.3 Antibody based diagnostic methods: Serological tests

6.3.1 Microscopic agglutination test (MAT)

Also known as Martin and Pettit test, it was developed at Pasteur institute almost a century ago(43) The test is still employed as the reference standard in the diagnosis of leptospirosis and employs the use of dark field microscopy assessment of live *leptospires* by patient serum. MAT requires maintenance of live culture organisms which act as source of antigen. The collection includes strains representative of the main serogroups. The method is a specific for serogroups and not serovars.

The sensitivity of method depends on the size of the panel and timing of specimen collection, positive results obtained day 8 to 10 post infection(34). The principle of MAT involves serial incubation of patient serum with various strains of *leptospires*. Serum results are considered positive if at a given dilution there is presence of at least more than 50% agglutination in comparison to control.

The method has several limitations which include its subjective nature, cost, requirement of expertise and maintenance of live organisms. As an epidemiological tool, MAT lacks precision and does not always correlate with results obtained following isolation of strain(44). Antibody levels to *leptospires* decrease with time and residue levels may persist for several years. For reliable analysis it is therefore important to clinical documentation of disease onset and date of specimen collection. Early administration of antibiotics may also affect the result.

6.3.2 IgM enzyme linked immunosorbent assay (ELISA)

Several IgM assays are commercially available on the market, kits are mainly based on detection of antibodies against a total extract of *leptospires* with some ELISA kits containing extract of local epidemic strains or use of recombinant proteins(15). Most assays use lysates of nonpathogenic *Leptospira biflexa* whose antigen cross reacts with most l*eptospires*(45). The sensitivity and specificity of assay has shown variability, which my demonstrate differences in studied populations with variation in exposure to *Leptospira*. Despite poor performance, several reports indicate earlier detection of *leptospires* with this assay compared to MAT(15, 46, 47). Antileptospire IgM detection occurs within 5 days of symptom onset, before development of IgG and agglutinating antibodies and may persist up to 5 months(48)

The sensitivity of serological tests varies from 60% to 100% in some studies. However, test specificity may be low particularly in endemic areas this may be attributed to IgM antibodies that remain detectable for years(49-51). Problems associated with correct timing of *Leptospira* serology, labour intensive culturing methods, a requirement for live organisms, as well as need for paired patient samples, results in this test not being performed in many laboratories, especially since culture sensitivity were found to be as low as 10.5%(34, 46, 51, 52).

A combination of MAT serological testing and microbial culturing increased sensitivity and specificity up to 55.5% and 98.8% respectively(46).Other serological methods include microagglutination, immunofluorescence, latex agglutination, lateral flow assays and IgM dipstick(53)

7. Nucleic acid based diagnostic methods

7.1 Polymerase chain reaction

Improvement of sensitivity is achieved when *Leptospira* DNA is targeted in polymerase chain reactions. The combination of PCR and an IgM lateral flow assay (Leptotek, BioMerieux) produced highest sensitivity (93.2%) compared with culture and MAT as the gold standard(sensitivity of 66.2%)(46). Nucleic acid based methods provided improved detection and diagnosis of leptospirosis within the first 5 days of illness(16). The period of *Leptospira* detection also extends up to 15 days(39). Various *Leptospira* specific genes can be targeted, such as *lipL32,* encoding a major outer membrane lipoprotein or *ligAB* genes or pan bacterial genes such as *rrs* (16S r RNA gene). LipL32 is a virulence factor associated only with pathogenic species of *Leptospira* (54), thus enable distinction between pathogenic and non-pathogenic leptospires. Comparison of *rrs* and *lipL32* PCR assays reported overall respective sensitivities of 56% and 43% for rrs and *lipL32*, with specificity of 90% and 93%(52). Another study evaluating the diagnostic accuracy of the real time *lipL32* and 16S PCR assays (for blood cultures evaluated in duplicates) reported sensitives of 86% and 100% respectively(50). The latter study also showed that the *lipL32* target was useful for urine samples, but the *rrs* target had lower specificity for urine (91.5%) and blood (97%), while no false positives were reported with the *lipL32* assay. When the two targets were combined in multiplex PCR, specificity, and sensitivity increase(33, 54). In a recent review of the current diagnostic methods for leptospirosis, the approximate cost of real time PCR equates that of the conventional PCR and may be cost effective compared to some commercial IgM ELISAs(34) . PCR based diagnostics are lacking in most facilities and introduction of either commercial or in house assays would improve the diagnosis of leptospirosis.

7.2 Isothermal methods

These methods include loop mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification, helicase dependent amplification and strand displacement methods. Some of these methods have been applied in the diagnosis of leptospirosis, with LAMP presenting an alternative to PCR based methods(55). In resource limited setting, LAMP presents an attractive option due to lack of requirement for thermocyclers and results can be assessed by visual inspection without requiring a gel(55). The detection of LAMP products is done by visual detection for turbidity, centrifugation at 14,000 rpm for 1 min for pellet formation and gel electrophoresis using a 2% agarose gel containing ethidium bromide (10 µg/ml)(56). The product visualisation is conducted using a gel documentation system (Gel Doc, Bio-Rad Laboratories, Hercules, CA, USA).

8.0 Clinical case management

8.1 Treatment

Treatment options for leptospirosis include doxycycline, ceftriaxone, ampicillin, or penicillin. In cases of Severe disease treated with intravenous penicillin is recommended. Supportive treatment in severe cases will be necessary to maintain renal function which is a common complication.

8.2 Prevention

Preventative measures include the following:

- Avoid direct and indirect contact with rodents by ensuring that environment does not support rodent infestation.
- Use of protective equipment i.e., gloves, eye wear in occupations which have high risk to exposure.
- Avoid swimming in potential contaminated water in rodent infested areas.

Summary

Table 3. Summary of diagnostic methods for leptospirosis

TEST	SENSITIVITY	SPECIFICITY	ADVANTAGES	DISADVANTAGES	REFERENCE
Culture	5%-50%	100%	Definitive evidence and is applicable to animal and human	Slow and requires expertise	Levett(15) WHO(57)
Dark field microscopy	10^4bacteria/mL	Low confusion with protein fibres	Quick and early diagnosis, applicable to both animal and humans	Unreliable, requires confirmation	Levett(15) WHO(57)
Microscopic agglutination test (MAT)	90%	>90%	Gold standard	Requires live strains, expertise and laborious	Levett(15) WHO(57)
IgM enzyme linked immunosorbent assay	>90%	88-95%	Cost effective and relatively rapid	Needs confirmation by MAT	WHO(57)
Real time polymerase chain reaction	100%	93%	Early detection, applicable to both animal and human	Few validated assays, sophisticated, expensive equipment, and expertise	Ahmed et.al.(58)

WHO leptospirosis Epidemiology Reference Group (LERG) has the follow recommendations for diagnosis of leptospirosis(59)
Laboratory confirmed cases defined as follows:
Clinical signs and symptoms consistent with leptospirosis and any of the following

- Fourfold increase in MAT titre in acute and convalescent serum samples
- MAT titre ≥ 1:400 in single or paired serum samples.
- Isolation of pathogenic *Leptospira* species from normally sterile sites.

- Detection of *Leptospira* species in clinical specimen by histological or immunostaining technique.
- Pathogenic *Leptospira* species DNA detected by PCR.

Probable cases of *Leptospira* defined as follows:
Clinical signs and symptoms consistent with leptospirosis and any of the following

- Presence of IgM or a fourfold increase in IFA antibody titre in acute and convalescent samples.
- Presence of IgM antibodies by enzyme linked immunosorbent assay(ELISA) or dipstick
- MAT titre ≥1:100 in a single acute phase serum sample in non-endemic regions

The method currently in use for the diagnosis of leptospirosis in South Africa is an ELISA targeting IgM (PanBio, Alere) this assay is performed at two sites in South Africa: Tygerberg hospital and The Special Bacterial Pathogens Unit (SBPU) at the National Institute of Communicable Diseases(NICD). Molecular testing is not routinely done. A rapid molecular has the potential to offer early diagnosis in patients suspected of leptospirosis and complement the current available serological methods.

Conclusion

The true prevalence of human leptospirosis in Sub-Saharan Africa is unknown due to challenges in availability of diagnostic methods. Early diagnosis presents an opportunity for intervention, prevention of severe complications and may aid in public health response. In South Africa leptospirosis is not a notifiable medical condition, contrary to other countries with improved diagnostics.
Currently serology is the most widely used diagnostic modality. However, with its many limitations there is need for better diagnostics for improved early identification of pathogen. Molecular diagnostics have demonstrated better sensitivity and specificity hence validation of these assays would contribute to clinical diagnosis and surveillance of *Leptospira* in Sub-Saharan Africa.

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PART E: Journal Manuscript

Journal: Frontiers in microbiology
Impact factor: 6.064
UCT citation index: DOAJ 2022

Journal format

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The utility of a real-time PCR to detect *Leptospira* in a routine diagnostic setting

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Keywords: *Leptospira,* PCR, *lipL32*, *lep16*

ABSTRACT
Leptospirosis is a neglected zoonotic infection with world-wide distribution. A paucity of leptospirosis data from the African continent exists, mainly due to limited access to diagnostics. The clinical presentation ranges from mild to severe disease with multi-organ involvement, while the mild form mimics another common tropical disease i.e., malaria. The gold standard for diagnostic detection currently is an immunological test discerning the presence of specific antibodies present in the immune phase of the disease. The serological methods are hindered by the inability to distinguish past from current infection and utility is limited to only the immune phase of the disease. Serological methods lack sufficient sensitivity and specificity, thus improved diagnostic methods are needed to aid early identification in the acute phase. Methods should also distinguish saprophyte and pathogenic species. To address this gap, we developed an in-house real time PCR assay targeting the 16S ribosomal RNA (*rrs*, here referred to as *lep16*) and *lipL32* genes, using primer sets previously reported in literature. These are reported to be both sensitive and specific for pathogenic *Leptospira* spp. Using constructed plasmid vectors containing a synthetically constructed *lep16* and *lipL32* internal fragments, we performed a non-clinical, technical validation. Amplicon detection was probe-based, using real time polymerase chain reaction assays and a locally available commercial kit. Although our assay needs further optimisation, we demonstrated that the PCR reliably detected 1000 copies and 100 copies of *Leptospira lep16* and *lipL32* targets respectively. To test specificity, we tested the PCR assay using DNA from a selection of pathogens known to be prevalent in bacteremias in local settings and observed that the *rrs* target detected the *Leptospira* target and DNA from Group B streptococci, but none of the other pathogens tested. No cross-reactivity was observed for *lipL32*. This *rrs* non-specific amplification had been reported previously in the literature, suggesting the *rrs* gene is not a good target to use, even when this *rrs* primer set was designed specifically to only detect *Leptospira*. This study only did a non-clinical evaluation, thus future work should include optimising assay performance using DNA extracted from appropriate clinical samples to detect *Leptospira*, namely urine and blood of patients clinically suspected to have leptospirosis. In conclusion, the assay demonstrated potential for use as a diagnostic PCR using a plasmid construct in a technical validation, but further optimization to improve PCR efficiency and assessing its performance with patient samples and within a clinical setting is required.

1.INTRODUCTION

Leptospirosis is a common zoonotic disease with a worldwide distribution. The disease was recently recognised by the World Health Organisation(WHO) as a neglected disease (1). The genus *Leptospira*, the causative agent, currently contains 35 species that are grouped into saprophytic, intermediate, and pathogenic species (2). To date, 13 pathogenic *Leptospira* spp., 7 intermediate spp. Namely *L. weilli, L. interrogans, L borgpetersenii, L. kirschneri, L. noguchii, L. alexanderi,* and *L. santarosai* are considered as important causative agents in both human and animal disease (3). However, sequencing of *Leptospira* from the so called "New World" revealed many new species previously not reported in the literature(4). This implies that knowledge gaps exist on species diversity and potentially could impact diagnostic detection of species not covered by current serological and molecular diagnostic methods.

A 2017 systematic review of data representing 80 published studies from 34 countries revealed an annual incidence of approximately 1 000 000 severe cases, approximately 60,000 associated deaths and the highest prevalence amongst adult men between of 20–49 years of age (1) (5). Tropical regions of the different continents experience the highest disease burden, while leptospirosis is also hyperendemic in specific geographic locales such as Sri Lanka (6). In Africa, WHO estimates a median incidence of approximately 95.5 per 100 000 persons (1). However, serological prevalence data suggests widespread exposure to leptospirosis in both animals and humans (7). The incidence of leptospirosis in Africa may be underestimated due to paucity of studies conducted in the region. In South Africa, animal seroprevalence surveys conducted in KwaZulu Natal in cattle showed rates of 19.4%, while similarly studies in rodents conducted in same region showed rates of 19.8% (8, 9). However, the true incidence of human disease is unknown, with only sporadic cases documented, mostly in high population settings. More recently, a 2015 prison outbreak of leptospirosis occurred amongst immune-compromised inmates in a Cape Town, overcrowded prison(10) A retrospective study on human leptospirosis recorded for period 2010-2019 in the Western Cape Province indicated 254 cases reported in the time period with the highest number of cases recorded in 2015 additionally the annual incidence ranged between 0.15 and 0.66/100,000 population with 10 year average incidence of 0.40/100,000 population(11). Additionally, a systematic review conducted by Allan et al. (7) reported a prevalence of human infection ranging from 2.3-19.8% in the African region.

Application of the One Health approach with identification and mapping of circulating serotypes in both animal and human population would contribute to the understating of disease distribution in our setting. Understanding circulating serovars in the local animal population is key to determining source and transmission routes for human infection (12). This was demonstrated in a Tanzanian study, where inclusion of local serovars using microscopic agglutination test (MAT) detection resulted in a 10-fold increase in leptospirosis prevalence, ranging from 1.9% to 16.9% in rodents and 0.26% to 10.75% in humans (13). This type of information is important, as *Leptospira* spp. affect a variety of animal spp., with the association with rodents presenting the highest risk of transmission (14). Infection in animals occurs early and often leads to chronic infection with colonization of the kidneys. Environmental contamination occurs with urinary shedding of the organism that could persist in contaminated water or soil for weeks to months(15).

Humans are dead-end hosts and infection occurs accidentally through interaction with environmental factors. Activities related with increased risk of exposure include occupations i.e., farmers, recreational activities i.e., kayaking and water rafting (16, 17). In South Africa, the incidence of leptospirosis in Western Cape is higher amongst males compared to females, with the 18-44 year old cohort having the highest incidence(11). In general, a global trend of

transmission is also associated with seasonal variation, with increased cases observed during periods of increased rainfall or following natural disasters (18). However, in Western Cape Province, retrospective data collected over a 10 year period did not show statistically significant season differences(11). In Tanzania, exposure to cattle and rice farming had been identified as significant risk factors to contract the disease (19).In resource limited settings, overcrowding, poor water and sanitation are also contributing factors (20, 21).

Clinical signs of human leptospirosis are not pathognomonic and require laboratory confirmation (22). Unfortunately, infections such as malaria, influenza, and dengue share similarity with the clinical presentation of leptospirosis. Thus, in settings with high prevalence of these conditions, the diagnosis of leptospirosis is often challenging, leading to delayed diagnosis, and associated poor clinical outcomes (23).

Leptospirosis presentation is diverse, ranging from subclinical infection to severe syndromes associated with organ dysfunction. The incubation period ranges from 7 to 14 days and the infection commonly presents as a biphasic illness. The initial or acute phase of the disease is self-limiting and is the most common presentation in 90% of cases. In the acute phase common manifestations include myalgia, fever, conjunctival suffusion, nausea, and vomiting. The initial phase lasts up to one week and is followed by the secondary or immune phase, which is associated with antibody production. The immune phase is associated with severe disease due to multi-organ immunological damage, commonly involving hepatic, respiratory or renal systems.

The laboratory diagnosis of leptospirosis depends on the clinical stage of disease. Available diagnostic modalities include direct and indirect detection methods (24). The Macroscopic Agglutination Test (MAT), an indirect method is considered as gold standard for serological diagnosis, however ELISA-based methods have demonstrated comparable sensitivity and specificity in several studies (25). The use of serological methods has several limitations which include poor sensitivity and specificity, while *Leptospira* culture is only performed at reference centres and not in routine diagnostic settings. The use of culture is limited by slow growth rate of the organism, poor yield and risk of contamination (3, 26). Other additional methods include Immunofluorescence assays targeting specific surface exposed proteins. False negative results are associated with these assays and may arise due to modification of surface proteins (27).

In contrast with phenotypic tests, molecular methods have demonstrated the highest sensitivity and specificity(3). Real time polymerase reaction (qPCR) using different clinical specimens including whole blood (28), serum, urine, and tissue samples (kidneys, liver, placenta) had been described previously (29). The early detection of *Leptospira* in blood increases the diagnostic window and presents an opportunity for early and targeted clinical intervention. PCR targets that have been validated include the *lipL32* and *lfb*1 genes; these are unique to pathogenic *Leptospira* (30, 31). In order to detect pathogenic species of *Leptospira* in the intermediate group, several assays have been developed targeting regions of the 16S RNA gene (30, 31).

In this report, we describe PCR assays targeting the *lipL32* gene, a marker specific to pathogenic *Leptospira* group (31, 32), and a PCR targeting the *Leptospira lep16* region. The study aimed to evaluate the performance of the previously described primers using a commercially available PCR mix and a real-time PCR instrument and plasmid DNA containing relevant targets. In clinical settings, molecular assays in addition to improved diagnostics can also be applied in epidemiological studies.

2. MATERIALS AND METHODS

2.1 Construction of control vectors and confirmation of targets

Previously described primer sets (Table 1) were analysed using the Basic Local Alignment Search Tool (BLAST) to confirm that it indeed bind to the *lipl32* and *rrs* genes(*lep16*) (33). Confirmation of the expected products was done by extraction of the relevant gene sequences from the genome of *L. interrogans* serovar *Hardjo* (GenBank accession number MT645311.1) and subsequent mapping showed that primers bind to fragments internal of the *Leptospira lipL*32 (PCR product size 243 bp) and 16s ribosomal RNA (PCR product size of185bp) genes. The nucleotide sequences of the specific fragments were used to purchase corresponding chemically synthesised G-Blocks DNA fragments (https://eu.idtdna.com/).

G-Blocks fragments were resuspended as per manufacturer's instruction to generate a DNA concentration of 10ng/ul and used in conventional PCR to amplify sufficient target for cloning. PCR reactions contained 1x GoTaq Buffer (Promega), 1.5mM $MgCl_2$, 0.2mM of each dinucleotide, 0.2uM of each primer, 1,25U of Taq polymerase and 60ng of DNA template. Conventional PCR conditions are as described in Table 2. Amplicons were cleaned-up (to remove residual PCR ingredients) using the Wizard PCR purification kit and associated instructions (https://www.neb.com/-/media/catalog/datacards-or-manuals/manualt1030.pdf).

Purified amplicons were cloned individually into a ready-to use, linearised commercial vector, pTZ57R/T (Instaclone PCR Cloning Kit, Thermo Scientific), as described by the manufacturers, except that incubation occurred at room temperature. Ligations were done for 2 hours, and reaction mixtures prepared as described in the kit and 3ul of respective PCR products (concentrations not determined).

Ligation mixtures were transformed into chemically competent One Shot™ TOP1 *Escherichia coli* (available from Thermo Fischer) cells and blue-white selection used to identify colonies containing putative plasmids with relevant inserts. This was done using Luria agar containing X-Gal and IPTG as per the Instaclone kit insert user guide. Colony PCR was used to verify the presence of target DNA in recombinant white *E. coli* colonies, whereafter plasmids extracted from selected PCR positive colonies. Pure plasmids were subjected to conventional PCR using the *lipL32* and *rrs* primers and PCR conditions as described above. The presence of amplicons was verified with gel electrophoresis before plasmids were subjected to Sanger sequencing by commercial service provider Inqaba Biotec (Pretoria), usingM13Forward and Reverse primers to confirm that inserts were correct(34).

2.2 Ethics

The project described here did not involve the use of any clinical information or material, as we used synthetically constructed DNA fragments as our target. Nevertheless, it is part of an ethically approved project, with HREC reference number 239/2014.

2.3 Data Availability Statement

All datasets presented in this study are included in the article (Appendix 2-4).

TABLE 1 Primer and probe sequences used in this assay

Target	Primer	Sequence (5' – 3')	Species	Reference
*lip*32A	LipL32-45F	AAG CAT TAC CGC TTG TGG TG	Pathogenic *Leptospira spp*	(35)
	LipL32-286R	GAA CTC CCA TTT CAG CGA TT		
	Probe	FAM-ZEN-AA AGC CAG GAC AAG CGC CG-3′-IBFQ		
Rrs	Lep16F	CGGGAGGCAGCAGTTAAGAA	*Leptospira spp*	(36)
	Lep16R	AACAACGCTTGCACCATACG		
	Probe	HEX-ZEN–GCRATGTGATGATGGTACCTGCCT-IBFQ		
GFP	GFPF	CCTGTCCTTTTACCAGACAACCA	Internal Amplification Control (IAC)	(37)
	GFPR	GGTCTCTCTTTTCGTTGGGATC		
	GFPP	CY5-ZEN-TACCTGTCCACACAATCTGCCCTTTCG-IBQ		
M13	M13F	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing Primers	(34)
	M13R	CAGGAAACAGCTATGAC		

2.2 *Leptospira* Realtime PCR

Real time PCR was done using published *LipL32* and 16 rRNA (*rrs*, here named *lep16*) targets, the selected target probes were labelled with FAM and HEX respectively(35, 36). Additionally, an internal amplification control, a fragment internal to the Green Fluorescent protein (GFP) gene, was added each reaction. Reaction preparations of 20µl included primers and probes, an internal amplification control, and LightCycler® 480 Probes Master (Roche, Germany) mixed as described in Table 2. Real time PCR reactions were performed using the CFX96 instrument (BioRad) with reactions run using Hard-Shell 96-Well 480 PCR Plates Kit (BioRad) Initial primer and probe titrations were tested, but the final concentrations of primers and probes used were as previously described(38). Precision and sensitivity were defined as previously described(https://www.fws.gov/aah/PDF/PrinValDiagAssayforInfDis%20-%20JACOBSON.pdf). Finally, in this study, the two constructs were tested in separate assays and not as a multiplex, to determine performance.

Previously published real-time PCR conditions were used to conduct the experiments. Amplification conditions were 95°C for 3 mins, followed by 45 cycles of 95°C for 10 mins and 60°C for 30 sec(35). Additional reactions in PCR runs included a non-template control and while each reaction contained an internal amplification control in order to monitor potential PCR inhibition(39)

Table 2. Master mix preparation for real time PCR

Reagents	starting (µM)	Final (µM)	V1(µl)	Number	Final master mix volume(µl)
NF H2O			225		22.5
Probe master	10	0.4	12.5		125
Forward Primer	10	0.4	1		10
Reverse Primer	10	0.4	1		10
GFPF	10	0.4	1	10	10
GFPR	10	0.4	1		10
Target probe	10	0.05	0.125		1.25
GFPP	10	0.05	0.125		1.25
pTZGFP (IAC)*	10	0.05	1		10
Total					200

*Construct provided by Dr. C. Moodley (Division of Medical Microbiology, UCT), contains gene coding for green-fluorescent protein.

2.3 Limit of detection studies

To determine the limit of detection (LOD), tenfold serial dilutions of the vectors were prepared and used as template in PCR reactions. The copy number was determined by measuring the amount of DNA in nanograms (using the Biodrop Duo (BioDrop Duo+ - Biochrom), and the calculation:

number of copies = (amount * $6.022x10^{23}$) / (length * $1x10^{9}$ * 650), where
number = (ng * number/mole) / (bp * ng/g * g/ mole of bp)

Initially, the serial dilutions contained DNA template in 10-fold dilutions ranging from 1 copy to 100 000 copies of the target. Following initial runs, replicate runs were done, on 3 independent days. These runs were done with each concentration run in 9-fold replicates and at least five different concentrations of template, the concentrations were based on previously reported LoD in literature(40). Obtained Ct values were correlated to the number of DNA target copies present per well. The LOD was determined using at least 20 replicates of each of the selected concentrations and 95% confidence interval.

2.4 Repeatability and Reproducibility

The determination of assay repeatability and reproducibility was done using LOD experiments, thereby assessing intra and inter assay variability respectively.

2.5 Amplification efficiency

Amplification efficiency € was calculated by the Bio-Rad CFX-96 instrument, using the formula E = 10–1/slope.

2.6 Specificity

The specificity of the primers used in this assay had been described previously (36), thus we only assessed non-specific amplification of DNA from microbes prevalent in our clinical

setting. For this, purified genomic DNA (20ng per reaction) from selected organisms were used. These were *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and Enterococcus *faecalis.*

2.7 Data analysis

Quantitative data from respective runs was exported from CFX into Microsoft Excel (https://www.microsoft.com/en-us/microsoft-365/excel) for analysis. Parameters calculated included mean, median and standards deviation. Efficiency calculations were calculated on the Biorad CFX Manager software (Biorad, Hercules, California).

3.0 Results

3.1 Confirmation of cloned templates

The presence of inserts in the recombinant plasmids were verified using conventional PCR and gel electrophoreses, as illustrated in Figure 1, and the identity of inserts verified using Sanger sequencing (Appendix figure 1) and the BLAST algorithm, confirming matches with relevant genes from *Leptospira interrogans* serovar *Hardjo* and serovar *Copenhageni* respectively.

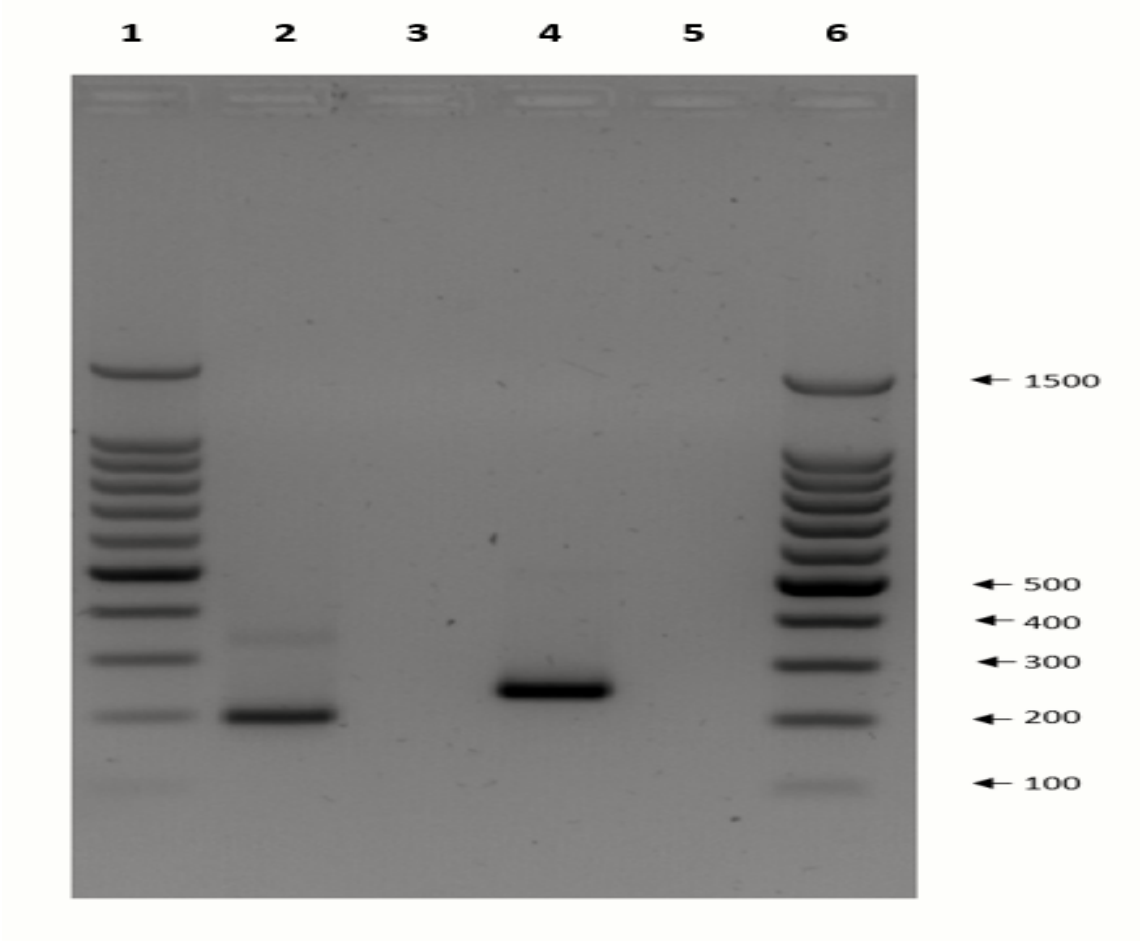

Figure1. Gel electrophoreses of PCR products obtained using conventional PCR as described in this document, using plasmid constructs as template. Lanes 1 and 6 100Bp DNA Ladder (Promega, cat nr G210A), Lane 2, *pLP16* as template, Lane3, No template control, Lane4, *pLip32* as template, Lane5, No template control.

3.2 Limit of detection studies

Using the data collected from the experiments, repeated on separate days and using 5 concentrations, the limit of detection (LOD)was determined. This analysis included a total of 27 data points in all copy numbers except 10^2 and 10^1 for *Lipl32* and 10^1 for *lep16*. The analysis excluded 10^0 as only a single run for *LipL32* had amplification with Ct value of 42.67 in a single well (Appendix, Table 2)._The lowest copy number with > 95 % detectable targets by *lipL32* was 1000 copies while *lep16* had a lower limit of 100 copies. The corresponding ranges of CT values associated with these copies were 30.55-35.27 and 31.74-36.75 for *lipL32* and *Lep16* respectively. Therefore, in this experiment it was established that the limit of detection for *lipL32* (Fig 2A) is between 1000 and 100 copies while for *Lep16* (Fig 2B) the LOD is between 100 and 10 copies. However, further experiments should be done to determine the true LOD.

Ct values are dependent on the concentration or copy numbers of target DNA present in the specimen. A tenfold dilution of target DNA, using known is expected to correspond with an increase of Ct value of 3(39) per dilution. This is demonstrated in Fig 2 below, with *Lep16*(Fig2B) presenting well distributed Ct values, compared to *LipL32* (Fig 2A) this difference is due to difference in data points.

Additionally, at low concentration greater variation in Ct values was noted, reflecting inconsistency in target amplification at low concentration of template DNA. The internal amplification control (IAC) demonstrated wide variation for *Lep16*(Table 3) with mean Ct range between 28 and 33, while *lipL32*(Table 3) demonstrated less variation. The variation in *Lep16* may indicate non-optimal PCR conditions. For experiments, a master mix containing primers and the IAC (pGFP) was prepared on the day.

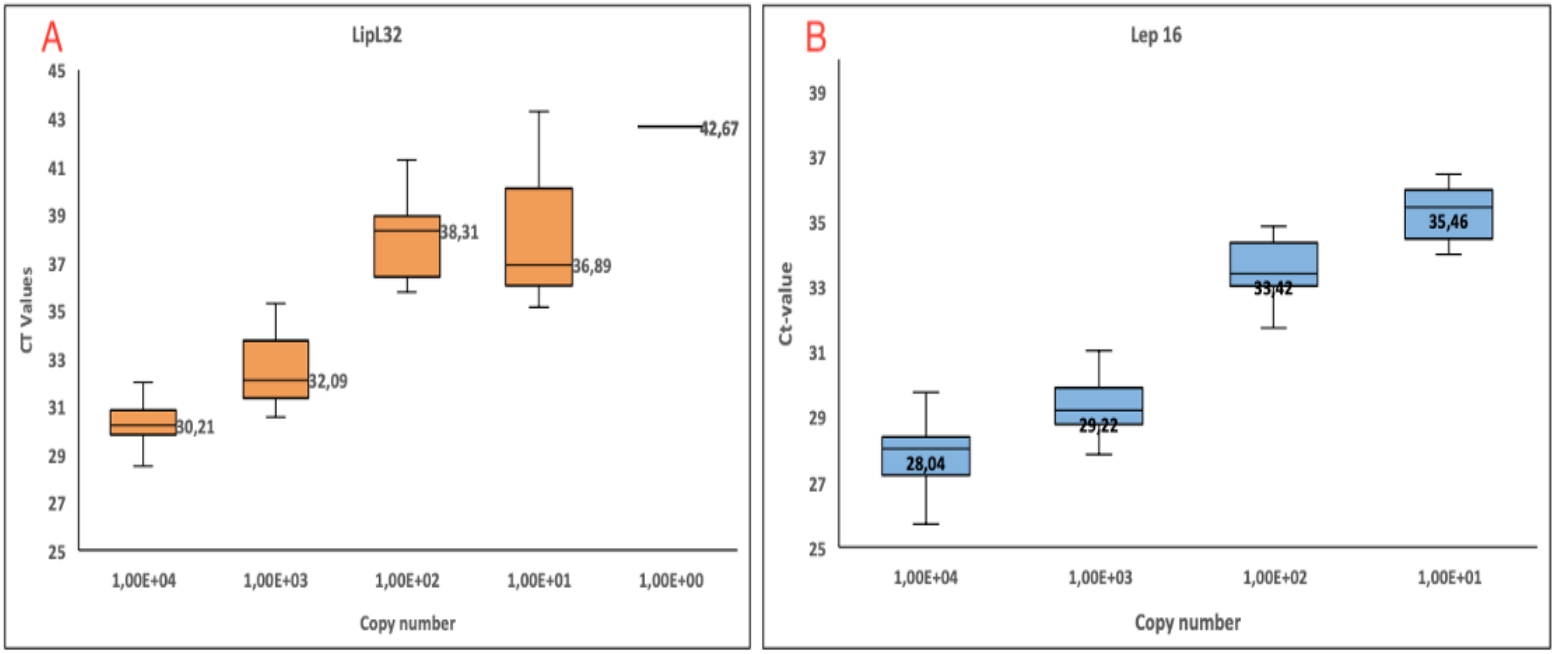

Figure 2. Limits of detection (LOD) determination for *lipL32* and *Lep16*. The number of data points are: lipL32: 10^4, 10^3 =27, 10^2= 11 data points 10^1 =4 data points; *Lep16*: 10^4,10^3,10^2 =27 data points and 10^1 =15 points. *Lep16* target is the rrs gene.

Thus, it is unlikely that the lower Ct values in the *Lep16* runs was due to differing amounts of template in individual wells. Further optimisation of primer concentration and temperature might aid in this case, where a commercial PCR mixture are used and only primers and template are to be added. Of course, technique proficiency by technical personnel might also influence reproducibility. Also, even when using previously described primers and real-time conditions, one must optimise when you are using different real-time instruments and PCR reagents.

Table 3: Amplification of the internal amplification control

Template: *Lep16*			
Copy number	**Replicates**	**Median**	**SD**
10^4	27	28.79	4.16
10^3	27	30.02	0.34
10^2	26	33.41	1.91
10^1	22	35.05	3.44
10^0	9	30.14	0.23
Template: *lipL32*			
Copy number	**Replicates**	**Median**	**SD**
10^4	27	34.89	7.0
10^3	27	34.60	1.52
10^2	27	34.66	1.30
10^1	27	34.50	1.61
10^0	27	34.63	1.38

3.3 Repeatability and Reproducibility

Inter and intra-assay variability (repeatability) data were obtained from replicates samples and repeat runs (9 replicates per copy number tested)., while inter- run variation (reproducibility) was assessed for the three consecutive runs performed for each target. In both assays there was less variability at high copy numbers, while lower copy number input, most notably for *lipL32*, exhibited poorer repeatability and reproducibility. *Lep16* assays demonstrated less variation at various concentration conducted in 3 runs compared with *lipL32* (Fig 3)

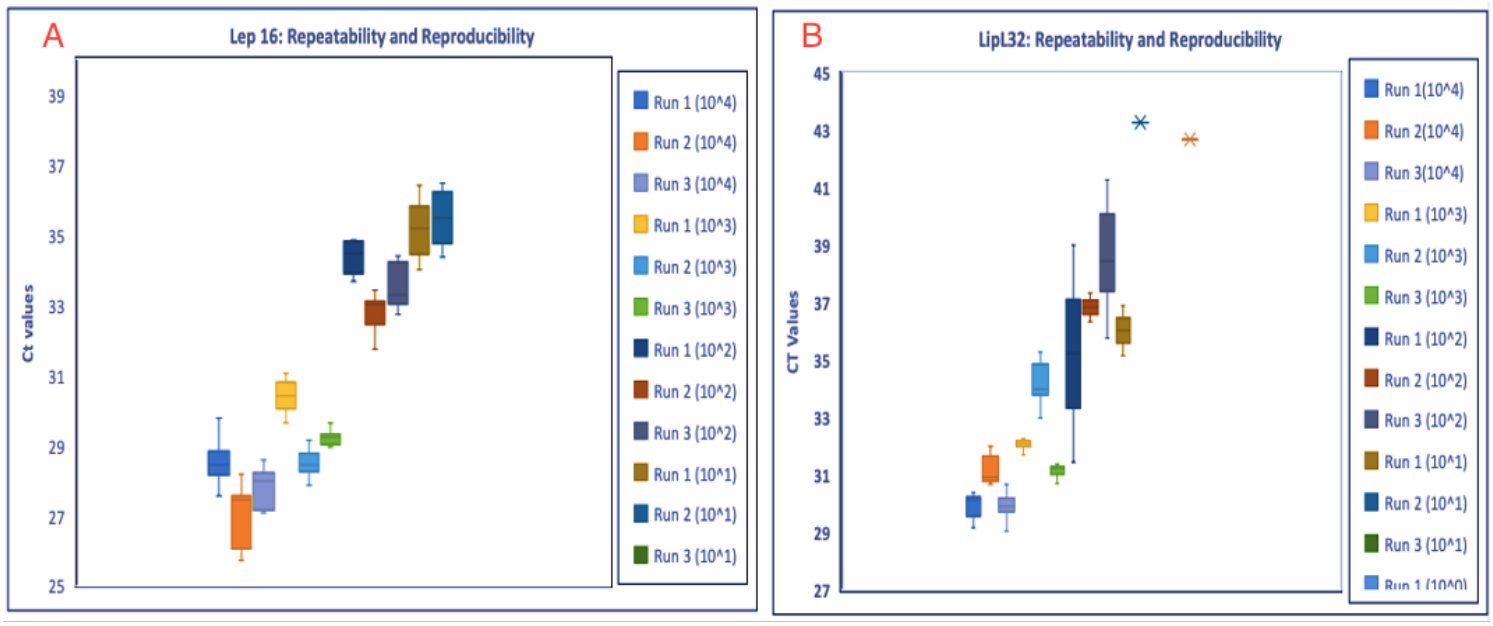

Figure 3 Reproducibility and repeatability data for the reported assays. Ct values reported on the y-axis, while the figure legend and different colours represent the repeated runs and copy number input. The number of replicates plotted are

3.4 Efficiency

As per MIQE standards, optimised reactions have efficiencies between 90% and 110% corresponding with a slope between -3.6 and -3.3(39). PCR efficiency of 100% corresponds to a slope of -3.32 at this point CT vales of 10-fold dilutions will be approximately 3.3 cycles apart. As illustrated in Table 4, the calculated efficiency range for the two targets were 104.9% to 159.6% and 94.1% to 112.8% for *lep16* and *lipL32* respectively. For *Lep16*, only run 2 satisfied the recommended parameters with efficiency 104.9% and slope -3.209, while for *lipL32* target run 1 and 3 with efficiencies 100.9% and 94.1% and slopes -3.299 and -3.471 were within range. This indicates that technical issues contributed to less efficient PCR in the last run. These could be due to poor pipetting technique, poorly calibrated pipette or potential compromise to the integrity of reagents used. Freshly made primer stocks as well as repeated freezing and defrosting of the commercial PCR mixture might contribute to technical issues. In a routine diagnostic setting, this could be circumvented by preparing master mixes, aliquot into amounts sufficient for 8 reactions (PCR strips contain 8 wells) if request for the specific test is low, or larger volumes to do a 96-well plate if the test demand is large. In this case, the optimal time that master mixes could be stored frozen, before it influences assay outcomes, should be determined.

R^2 is a measure of reproducibility with acceptable values >0.985, all runs performed did not satisfy the requirement. However, for *lep16* run 2 and 3 showed better reproducibility with R^2 0.9 and 0.93 respective while for *lipL32* run 1 had a value close to acceptable target (Table 4).

The lack of reproducibility is also evident from Figure 3 which shows the variability of mean CT values at defined concentrations in the 3 runs for each target,

Table 4. Amplification efficiency for *16S rRNA gene* and *lipL32*

Target		Efficiency %	Slope	R^2
Lep 16	Run 1	159.6	2.414	0.537
	Run 2	104.9	3.209	0.9
	Run 3	141.4	2.613	0.931
Lipl32	Run 1	100.9	3.299	0.931
	Run2	112.8	3.049	0.702
	Run3	94.1	3.471	0.737

The number of replicates for each run is reported in Table3.

3.5 Specificity

As illustrated in Figure 4 triplicate reactions with the primers targeting *lipL*32 did not amplify any of the bacterial DNA tested (20ng DNA tested per isolate), only the *lipL32* positive control is amplified (Fig 4A) confirming its specificity as previously described in the literature (41). For the *lep16* gene, duplicate samples (Fig 4B) clearly demonstrated amplification with DNA from Group B *Streptococcus* with CT 31.03 and 32.25 (table 5). In addition, other pathogens included had off target amplification at higher CT, of note is the amplification of *Staphylococcus epidermidis* with CT 37.93 and 37.24. The Ct values for this off target amplification is between 37-45 cycles; it is known that some non-specific amplification might occur in PCRs in the late stages of PCR, especially if not optimised fully, or due to poor primer sets (41).

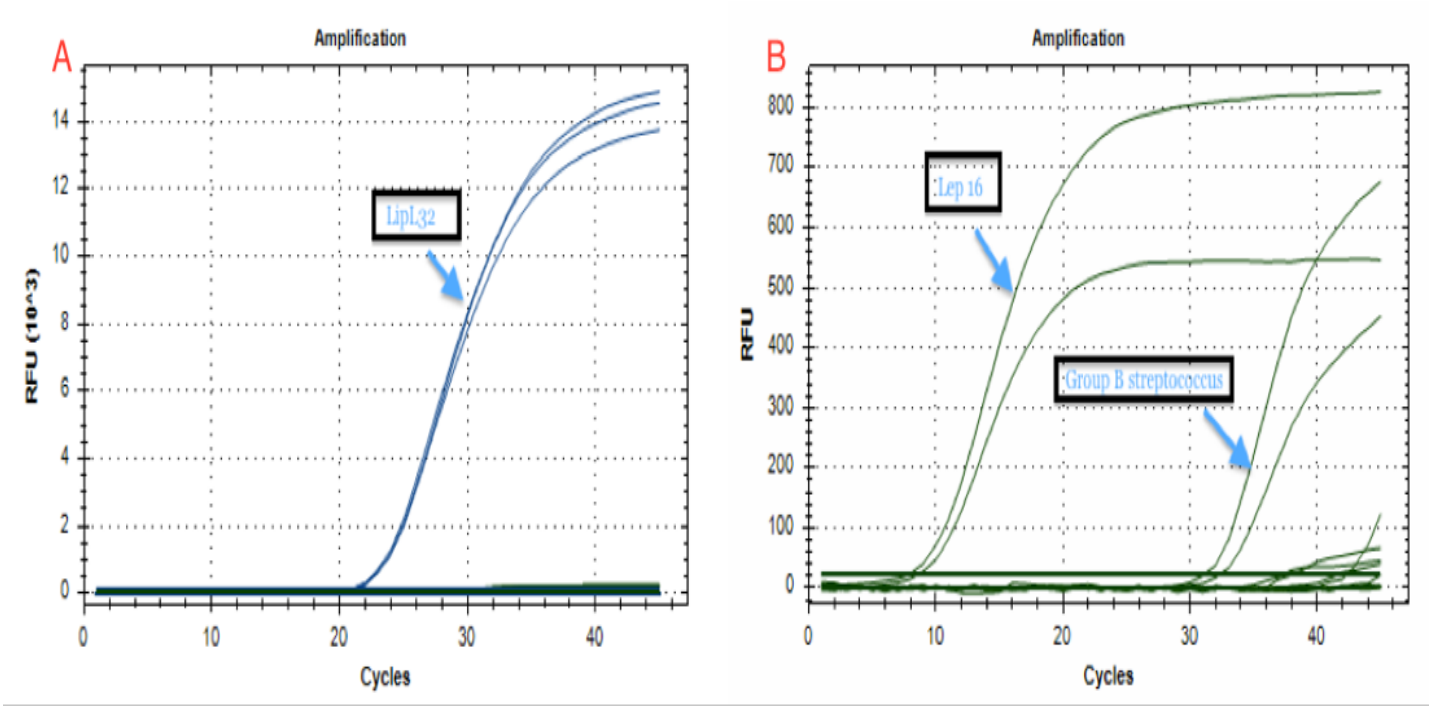

Figure 4. Specificity of *lipL32* and *lep16* target genes with *Lep16* showing non- specific amplification of group B streptococcus extracted DNA.

Table 5. *Lep16 gene* non-specific amplification

DNA	*Lep16*	Group B Streptococcus	E. coli	Pseudomonas	Staphylococcus epidermidis	Staphylococcus aureus	E.faecalis	NTC
CT	8.46	31.03	41.49	42.28	37.93	-	44.97	-
CT	7.71	32.25	-	-	37.24	-		-

4. Discussion

Two plasmid constructs harbouring the *lep16 gene* (names *lep16* in here) and *lipL32* PCR targets were successfully constructed and used to evaluate the real-time PCR described in this report. The sensitivity and specificity of a real time PCR are influenced by design of primers and probes, their selection criteria and optimal parameters. Additionally, optimisation of concentration and reaction conditions may have an effect. The primers used here are previously described and well characterised, thus just needs to be evaluated for performance using the different reaction mixtures available on the market. In our case, target DNA fragments carried on two plasmids were used as template for PCR reactions after confirmation of presence of inserts. In these experiments, commercially prepared PCR premix was used, and the two targets were assessed individually and not in a multiplexed assay.

Although not reported in the data presented here, we checked whether the two primer sets would generate non-specific amplification. The primer pair for *lipL32* did not generate amplicon when the *lep16*-containing plasmid was used as target, neither did the *lep16* containing plasmid resulted in amplicons when using *lipL32* primers. This indicate multiplexing could be done if necessary.

Using extracted DNA from common pathogens which included *Escherichia coli*, *Pseudomonas Aeruginosa*, Group B *Streptococcus*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*, the *lipL32* target demonstrated 100% specificity. However, PCR reactions with the *lep16* plasmid had non-specific amplification against group B streptococci as illustrated in Figure 4 and Table 5 with mean Ct values of 31.03 and 32.25 in the respective duplicates. This unexpected cross reactivity has been reported in other studies especially when performed on urine samples(28, 41) and also when used on blood amplification of non-pathogenic species of *Leptospira*(42),. Non-specific amplification had been described in a recent study reporting on one patient where the primers optimised to only detect *Leptospira lep16* (*rrs*) produced an amplicon that was subsequently identified, via Sanger sequencing, as *Atopobium vaginae (41)*. The PCR reported here, as well as published reports, thus suggests that the rRNA gene is not a good target to use, even if the primers are reportedly optimised to only detect *Leptospira*. If included in real-time assays, its utility will be to confirm at least the presence of bacterial DNA. There are a commercial *Leptospira* kit targeting this gene, but our result and those of other reports, suggests that the use of this gene as a target could result in false positive tests and needs to be used in combination with a more reliable target such as *lipL32*. If a PCR run amplifies only the *lep16* target and not the other *Leptospira* specific target in the assay, that would need to be interpreted carefully, as it could be a false positive result (meaning not pathogenic *Leptospira* or not *Leptospira* at all.)(41).

In assessing specificity, the limitation of this study is the non-inclusion of known pathogenic and non-pathogenic species in our determinations. However, other multiple studies with *lipL32* gene target against pathogenic and non-pathogenic *Leptospira spp* have demonstrated specificity for pathogenic *Leptospira* (41). Furthermore, genome analysis and comparisons between pathogenic and non-pathogenic *Leptospira* indicates this gene is unique to pathogenic species(31, 43). Additionally, unlike the *lep16* based PCRs designed for *Leptospira* specifically, thus far non-specific amplification with *lipL32* as target have not been reported.

The limit of detection of a PCR by consensus has been defined as the minimal concentration that yields a positive result with 95% confidence (44). Our data suggests that an in-house PCR is plausible, with ability to detect close to between 100-1000 copies for *lipL32* and between

100-10 copies for *Lep16*(Figure 2). In order to ascertain the exact limit of detection for both targets, follow up experiments of serial dilutions runs with initial concentration of 1000 copies and 100 copies for *lipL32* and *Lep16* will need to be conducted. Each standard concentration would require at least 20 replicates in order to discern reproducibility and repeatability, plus determine LoD (44). It has to be noted that in clinical samples the real LoD is affected by multiple factors which include extraction procedure, sampling presence of inhibitors and hence is usually higher (39). A case control study conducted in Thailand comparing the two targets and using MAT as gold standard also showed higher diagnostic sensitivity of *lep16* (the *rrs* gene referred to in this study as *lep16*) compared *to lipL32*(42). Bacteria in general have more than one copy of the *lep16* gene(*rrs*), enabling more sensitive detection of the target. *Leptospira* are known to encode for at least two copies of *rrs*, hence enabling more sensitive detection in PCR(45-47). This would not explain however, why this gene target, in this study, is detected more sensitively compared to the *lipL32* gene as we used cloned fragments on plasmids. If genomic DNA was used, increased sensitivity of *rrs* could be explain by the fact that it is a multi-copy target.

Efficiencies below 90% may be ascribed to the use of non-optimal reagent concentrations, non-optimal primer/probe design and quality and age of enzymes and other reagents. The presence of inhibitors produces efficiencies greater than 110%(48, 49). In this study, only the plasmid extraction kit and efficient extraction of pure DNA could be the reason for reduced efficiency, as we used a commercial PCR kit that was still within its expiry date. Human error and proficiency could also be a limiting factor. A combination of these factors may explain the variability in the efficiency of runs for both targets indicating the need for further optimisation of the assay. Additionally, reproducibility determined by R^2 for both targets demonstrated suboptimal values of less than ≤ 0.985 with recommended acceptable values ≥ 0.985, this may reflect inconsistency in reaction volume or lack of pipette calibration (the latter might not be applicable as pipettes in our setting are calibrated as per requirements).

Future optimisation should be done using mock clinical samples and real diagnostic samples. To discern the impact of inhibitors, present in blood and urine, DNA isolated from spiked blood and urine samples should be used to. For the latter, samples should be added to lysis buffer first, then the control plasmids added into lysed samples, shortly before extraction, as reported in the literature (35, 40). After optimisation with DNA extracted from mock clinical samples, clinical validation need to be done, using relevant clinical samples of persons suspected to have leptospirosis to evaluate the performance of the assay in real life PCR results should be compared to diagnostic outcomes for the tested samples, who should have been tested with the current gold standard (MAT)(41).Anticipated challenges during clinical validation may include the low concentration of bacterial DNA and presence of PCR inhibitors such as urea, crystals and beta human chorionic gonadotropin in the urine which may affect assay sensitivity and have to be addressed when developing validation protocol(50, 51).

In conclusion, the experiment demonstrates proof of concept on the practicality and performance of an in-house PCR. However, it requires further development and optimisation before proceeding to clinical validation. The data suggests that the in-house constructs, when used with the LightCycler® 480 Probes Master (Roche), could detect the *rrs* gene and a gene target unique to pathogenic *Leptospira*, i.e., *lipL32* gene. For laboratories equipped with real-time PCR machines, but where availability of commercial *Leptospira* kits is problematic, the use of in-house assays is an option to consider. Currently a commercial BactoReal kit (manufactured by Ingenetix) fit the requirements, as it contains both targets. This kit recommends similar temperature parameters to those used in our experiments and has a

multiplex design with both 16S rDNA (*rrs* aka *lep16*) and *LipL32* targets(52). Additionally, assay validation for the kit reports specificity for pathogenic and intermediately pathogenic *Leptospira* species and a limit of detection between 18 and 20 target copies/ reaction(52). A multiplex PCR with both targets would ensure better sensitivity and specificity leading to improved diagnostics especially in the acute phase of the disease. However, we showed the current primer set targeting *Leptospira*l *rrs* might generate false positive results and requires further evaluation. Finally, the utility of these assays in our setting may improve laboratory diagnosis of leptospirosis and additionally contribute to the growing body of *Leptospira* epidemiology in Sub Saharan Africa.

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APPENDIX: 1

VALIDATION OF REAL-TIME PCR FOR DETECTION OF LEPTOSPIROSIS IN CLINICAL SPECIMENS

Validation Protocol

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August 2020

Contents

BACKGROUND AND RATIONALE

Leptospirosis is a globally important zoonotic infection with clinical presentation indistinguishable from other infections such as influenza, dengue and malaria. The spectrum of human disease caused by leptospires is diverse, ranging from subclinical infection to a severe syndrome of multi organ infection and subsequent death. The incubation period is approximately 7-14 days. Infection typically presents as a biphasic illness, starting with an acute onset febrile illness presenting with fever, myalgia, conjunctival suffusion, nausea, vomiting and bacteremia. This phase lasts up to a week and is followed by an 'immune phase' associated with antibody production and the excretion of leptospires in the urine; the fever may recur after 3-4 days (1,2).

Leptospirosis remains a significantly under-diagnosed disease. The World Health Organisation (WHO) estimates the median incidence in Africa to be approximately 95.5 per 100, 000 persons. However, limited studies had been conducted in this region (http://apps.who.int/iris/handle/10665/44588, WHO 2011), thus the incidence might be underestimated. In South Africa, the incidence of infection in humans is largely unknown. Animal surveys showed seroprevalence rates of 19.4% in cattle in KwaZulu-Natal (KZN)[3] and 5% in dogs in the coastal regions of South Africa(4). Seropositivity rates of 19.8% have been reported in a rodent-related zoonosis study in people conducted between 2003-6 in the KZN region (5).

Another reason for the disease not being diagnosed optimally, relates to difficulties related to serology and culturing of the causative leptospires. Laboratory diagnosis relies on serology (such as the Macroscopic Agglutination Test (MAT test), culture from blood, urine or tissues or by molecular methods using polymerase chain reaction (PCR). Serology is dependent on the duration of the disease, as specific biomarkers, e.g. IgM, is only present from day 5 and onwards, of disease[6]. The correct timing of the leptospirosis diagnostic tests is thus important. The sensitivity of serological tests varies from 60% to 100% in some studies. However, test specificity may be low, particularly in endemic areas; this is attributed to IgM antibodies that remain detectable for years (7-10). Problems associated with correct timing of the *Leptospira* serology, labour-insensitive culturing methods, a requirement of live organisms, as well as a need for paired patient samples, results in this test not being done in many laboratories, especially since culture sensitivity were found to be as low as 10.5% (2,11-13). A combination of MAT serological testing and microbial culturing increased sensitivity and specificity up to 55.5% and 98.8% respectively (13).

Improvement of sensitivity is achieved when leptospire DNA is targeted in polymerase chain reactions, and that result, when combined with an IgM lateral flow assay (Leptotek, BioMerieux), produced highest sensitivity (93.2%) compared with culture and MAT as the gold standard (specificity of 66.2%) (13). Detection of leptospire DNA makes the detection of the organism in blood easier, and detection is possible within the first 5 days of illness. The period of *Leptospira* detection also extends up to 15 days (14). Various *Leptospira* -specific genes can be targeted, such as l*ip*L32, encoding a major outer membrane lipoprotein or *lig*A, B
genes, or pan-bacterial genes such as *rrs* (*lep16* gene). LipL32 is a virulence factor associated only with pathogenic species of *Leptospira* (9), thus enable distinction between pathogenic and non-pathogenic leptospires. Comparison of *rrs* and *lip*L32 PCR assays reported overall respective sensitivities of 56% and 43% for *rrs* and *lip*L32, with specificity of 90% and 93% (12). Another study evaluating the diagnostic accuracy of the real time *lip*L32 and 16S PCR assays (for two blood cultures tested in duplicate) reported sensitivities of 86% and 100% respectively (8). The latter study also showed that the *lip*L32 target was useful for urine

samples, but the *rrs* target had lower specificity for urine (91.5%) and blood (97%), while no false positives were reported with the *lip*L32 assay. When the two targets are combined in multiplex PCR, specificity and sensitivity increase (9,15). In a recent review of current diagnostic methods for leptospirosis, the approximate costs of real time PCR equates to that of conventional PCR and now may be cost effective compared to some commercial IgM ELISAs (16).

The method currently in use for diagnosis of acute leptospirosis in South Africa is an ELISA targeting IgM (PanBio, Alere); this test is performed at 2 sites in South Africa: Tygerberg Hospital and The Special Bacterial Pathogens Unit (SBPU) at National Institute of Communicable Diseases (NICD). Molecular testing is not done routinely. A rapid, molecular diagnostic test could improve laboratory demonstration of leptospires in patients with a clinical suspicion of leptospirosis and could complement current serological tests in use in local diagnostic laboratories.

STRATEGY

This will be a laboratory-based study, starting with a technical validation to assess the use of the specific targets for the assay. This genus specific assay will focus on 2 DNA targets from pathogenic *Leptospira interrogans,* i.e., internal fragments from *lep16* (*rrs*) and *lipL32* genes respectively. Initial assays will be done with pure plasmids to optimise PCR conditions whereby the two different DNA targets from the target organism can be amplified in one multiplexed reaction. This optimisation will also verify that the primers do not cross-amplify the two *Leptospira* targets or non-related microbes frequently found in blood and urine in our clinical setting, but only find its intended target. Following the initial optimisation, a purified plasmid containing two *Leptospira* gene targets (small, internal fragments of the *rrs* and *lipL32* genes) will be purchased from a commercial vendor and used for technical and clinical validation assays. These assays comprise determinations for sensitivity, specificity, reproducibility and repeatability optimised PCR conditions. A clinical validation will follow to complete the assessment of the clinical utility of the proposed assay, using residual clinical laboratory samples, as well as a small number of consented samples obtained from recruited patients with a suspicion of leptospirosis.

Technical validation

Experimental objectives for technical validation: to determine optimal PCR conditions, minimal DNA needed and assay validity. Plasmid DNA containing target inserts i.e., *Lep16* and lipL32 will be used as per manufacturer's instructions by trained laboratory personnel as per SOP VIRO243v7.

Test vectors

Two previously constructed plasmids, containing respective internal fragments from the *Leptospira rrs* and *lip*L32 genes (19) will be used for initial PCR assays, to optimise PCR conditions. The vector to use in the technical and clinical validation (and containing both

targets on one plasmid) will be purchased from a commercial provider, namely IDT (Integrated DNA Technologies, idtdna.com).

Primers and probes

Primers and probes used for this study is reported in Table 1.

Table 2. Primer and probe sequences used in this assay.

Target	Primer	Sequence (5' – 3')	Fluorescent tags	Species	Reference
*lip*32A	LipL32-45F	AAG CAT TAC CGC TTG TGG TG		*Leptospira spp*	17,19
	LipL32-286R	GAA CTC CCA TTT CAG CGA TT			
	Probe	FAM-ZEN--AA AGC CAG GAC AAG CGC CG-3′- IBFQ			
Rrs	Lep16F	CGGGAGGCAGCAGTTAAGAA		*Leptospira spp*	18,20
	Lep16R	AACAACGCTTGCACCATACG			
	Probe	HEX-ZEN – GCRATGTGATGATGGTACCTGCCT-IBFQ			
GFP	GFPF	CCTGTCCTTTTACCAGACAACCA		Internal Amplification Control (IAC)	23
	GFPR	GGTCTCTCTTTTCGTTGGGATC			
	GFPP	TACCTGTCCACACAATCTGCCCTTTCG	(HEX-ZEN - IBFQ)		

Interpretation of results

The guide in Table 2 will be used to interpret the results obtained from the PCR assay.
Table 3. PCR Interpretation guide.

Sample	Target	Identification and Result
pLep16L32	*rrs*	PCR positive CT <35: DNA from *Leptospira* present PCR negative: PCR fail or target absent; interpret in context of positive control- if latter also negative, then PCR failed.
pLep16L32	*pLipL32*	PCR positive CT<35: DNA from *Leptospira* present PCR negative: PCR fail or target absent; interpret in context of positive control- if latter also negative, then PCR failed.
Extraction control	None	Negative: Indicates extraction contamination free, no PCR cross contamination Positive: Indicates contamination occurred either during DNA extraction or during PCR preparation, or that one/more PCR reagents are contaminated.
pGFP	GFP	PCR positive CT<35: included as control to verify that sample do not contain inhibitory substances. Ct value within range for known DNA concentration used – no inhibition. If Ct value much later than usual – sample contains inhibitory substances

Analytical Sensitivity

The analytic sensitivity of the assay evaluates the limit of detection (LOD), that is the minimum amount of DNA present in a sample that will be amplified successfully. This will be accomplished by conducting initial PCRs using serial dilutions of the recombinant vector (pLep16L32). Once the lower limit of detection is determined in initial PCR reactions, the assay will be repeated using a dilution series consisting of at least 5 concentrations, including a concentration one value below the observed LOD. The LOD will be determined with a 95% confidence interval by repeated testing, at least 20 replicates of each of the selected concentrations, done over a period of 3 days.

Analytical Specificity

Selected microbes commonly found in blood and urine cultures, in our clinical setting, will be used to determine the specificity of the primer set to only detect *Leptospira* DNA. These organisms are listed in Table3. PCRs will be done using DNA from the organisms listed in Table3.

Table 4. Organisms to be tested for cross-reactivity with the new assay.

Bacteria		Fungi
Escherichia coli	*Streptococcus agalactiae*	*Candida albicans*
Staphylococcus aureus	*Haemophilus influenzae*	
Klebsiella pneumoniae	*Pseudomonas aeruginosa*	
Enterococcus spp	*Enterobacter cloacae complex*	

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APPENDIX 2

A. **Fragment internal to 16s Ribosomal RNA gene of *L. interrogans***

ACGGGAGGCAGCAGTTAAGAATCTTGCTCAATGGGGGGAACCCTGAAGCAGCGACGCCGCGTGAACGATGAAGGTC
TTCGGATTGTAAAGTTCAGTAAGCAGGGAAAAATAAGCAGCAATGTGATGATGGTACCTGCCTAAAGCACCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTATGGTGCAAGCGTTGTT

B. **Confirmation of identity as 16s ribosomal RNA gene of *Leptospira***

Leptospira interrogans serovar Hardjo strain untyped 16S ribosomal RNA gene, partial seque

Sequence ID: MT645311.1 Length: 1502 Number of Matches: 1

Range 1: 339 to 536 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
366 bits(198)	3e-97	198/198(100%)	0/198(0%)	Plus/Plus

```
Query  1    ACGGGAGGCAGCAGTTAAGAATCTTGCTCAATGGGGGGAACCCTGAAGCAGCGACGCCGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  339  ACGGGAGGCAGCAGTTAAGAATCTTGCTCAATGGGGGGAACCCTGAAGCAGCGACGCCGC  398

Query  61   GTGAACGATGAAGGTCTTCGGATTGTAAAGTTCAGTAAGCAGGGAAAAATAAGCAGCAAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  399  GTGAACGATGAAGGTCTTCGGATTGTAAAGTTCAGTAAGCAGGGAAAAATAAGCAGCAAT  458

Query  121  GTGATGATGGTACCTGCCTAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  459  GTGATGATGGTACCTGCCTAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG  518

Query  181  TATGGTGCAAGCGTTGTT  198
            |||||||||||||||||
Sbjct  519  TATGGTGCAAGCGTTGTT  536
```

C. **Fragment targeting pathogenic specific *Leptospira* gene l*ipL32***

TTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAA
TCCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAG
GACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTG
AAATGGGAGTTC

D. **Confirmation of identity as 16s ribosomal RNA gene of *Leptospira***

Leptospira interrogans serovar Copenhageni strain SK1 chromosome I, complete sequence

Sequence ID: CP048830.1 Length: 4279936 Number of Matches: 1

Range 1: 1666746 to 1666991 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
455 bits(246)	3e-128	246/246(100%)	0/246(0%)	Plus/Minus

```
Query  1        TTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTG  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1666991  TTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTG  1666932

Query  61       TTCTGAGCGAGGACACAATCCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1666931  TTCTGAGCGAGGACACAATCCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACG  1666872

Query  121      GATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1666871  GATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCG  1666812

Query  181      ATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGG  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1666811  ATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGG  1666752

Query  241      GAGTTC  246
               ||||||
Sbjct  1666751  GAGTTC  1666746
```

Figure 2. DNA fragments targeted in this study. A and B represents 16s rRNA target while C and D represents lipL32 target. C and D shows BLAST results obtained following Sanger sequence to confirm the presence of inserts in plasmid constructs. Fragments were chemically synthesised (G Blocks, Integrated DNA technologies).

APPENDIX 3

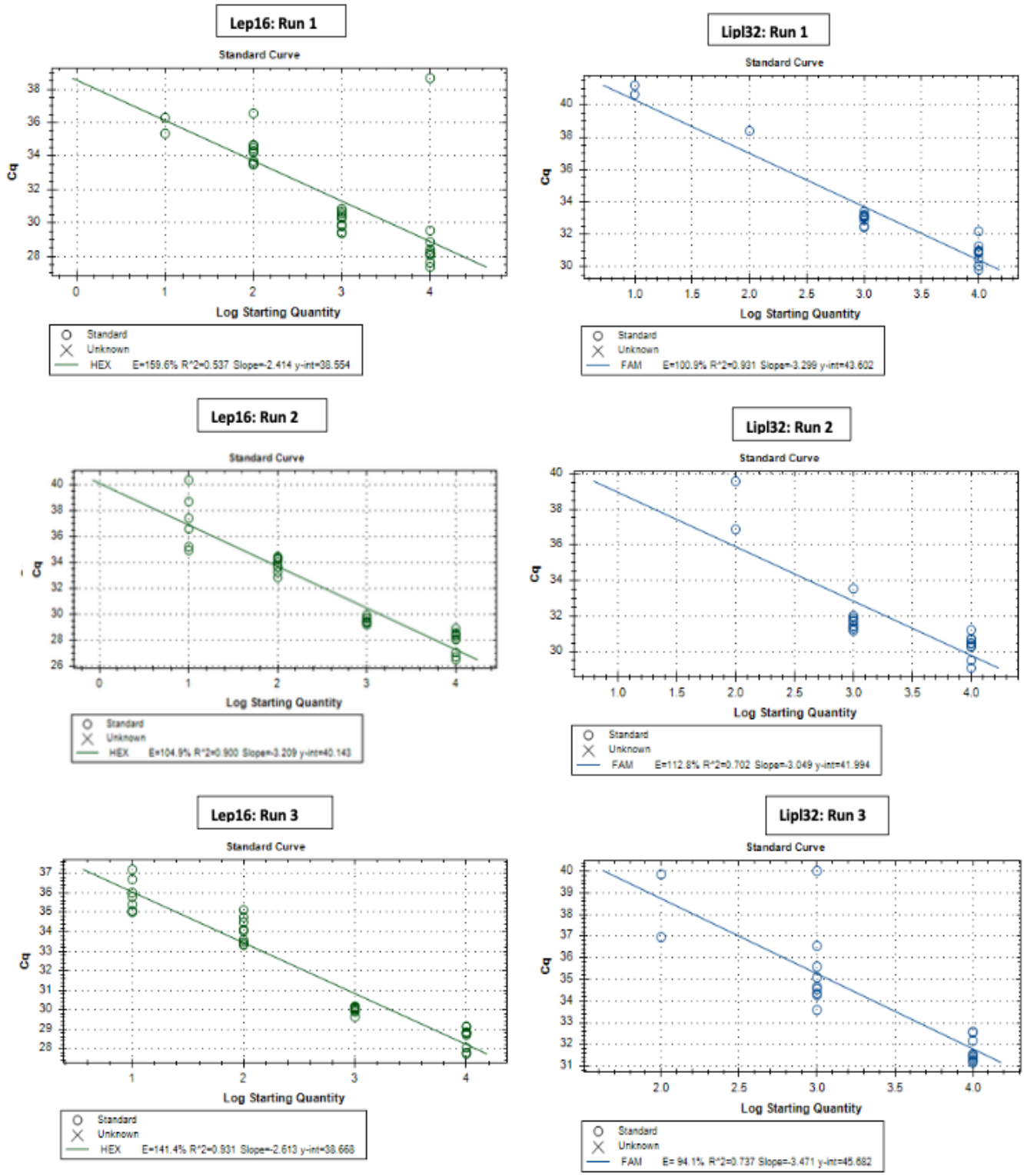

Figure 5: Amplification efficiency for *lipL32* and *Lep16* demonstrating assay performance.

APPENDIX 4

Table 1. lipL32 Descriptive analysis summary

Conc	Mean CT	Standard Erro	Median	Mode	Standard Devi	Sample Varian	Kurtosis	Skewness	Range	Minimum	Maximum	Sum	Count	Largest(1)	Smallest(1)	Confidence Level(95,0%)
1,00E+04	30,3155556	0,16833298	30,21	30,66	0,87468383	0,76507179	-0,2378188	0,11394949	3,47	28,52	31,99	818,52	27	31,99	28,52	0,346013397
1,00E+03	32,5085185	0,26824251	32,09	#N/A	1,39382899	1,94275926	-0,8820539	0,53303058	4,72	30,55	35,27	877,73	27	35,27	30,55	0,551380385
1,00E+02	37,9845455	1,0057827	38,31		3,33580384	11,1275873	1,78783547	0,11409437	13,26	31,42	44,68	417,83	11	44,68	31,42	2,241023514
1,00E+01	38,4333333	2,47053525	36,89	#N/A	4,27909258	18,3106333	#DIV/0!	1,4118841	8,13	35,14	43,27	115,3	3	43,27	35,14	10,62985526
1,00E+00	42,67	0	42,67	#N/A	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	42,67	42,67	42,67	1	42,67	42,67	#NUM!

Table 2. lipL32 Runs performed on three consecutive days

				lipL32 Run 1					
CONC	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04
CT	31,45	29,85	30,38	30,11	30,21	29,15	30,2	29,17	29,55
CONC	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03
CT	31,99	32,09	32,26	32,18	31,68	32,07	31,6	32,74	31,96
CONC	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02
CT	0	31,42	0	0	38,99	0	0	0	0
CONC	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01
CT	0	0	0	36,89	0	35,14	0	0	0
CONC	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00
CT	0	0	0	0	0	0	0	0	0
				lipL32 Run 2					
CONC	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04
CT	30,77	31,99	30,9	31,14	30,65	31,95	31,64	30,87	30,66
CONC	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03
CT	34,84	33,96	33,78	34,52	35,27	33,82	32,97	33,68	34,88
CONC	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02
CT	0	36,32	37,33	0	0	0	0	0	0
CONC	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01
CT	0	0	0	0	0	0	43,27	0	0
CONC	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00
CT	0	0	0	0	0	42,67	0	0	0
				lipL32 Run 3					
CONC	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04	1,00E+04
CT	29,02	28,52	30,23	30,17	29,69	29,89	29,78	30,66	29,92
CONC	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03	1,00E+03
CT	33,08	30,55	31,24	31,03	31,28	31,37	30,67	31,22	31
CONC	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02
CT	44,68		35,75		41,26	36,46	38,44	38,87	38,31
CONC	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01	1,00E+01
CT	0	0	0	0	0	0	0	0	0
CONC	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00
CT	0	0	0	0	0	0	0	0	0