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Detection of Leptospira DNA by Real Time PCR (qPCR) in urine samples from clinically suspected patients

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Abstract

Leptospirosis is a widely distributed zoonotic disease with a significant public health threat globally. It is caused by 11 pathogenic species and five intermediate species of Leptospira belong to the order Spirochaetales, family Leptospiraceae, genus Leptospira. Human disease is acquired via direct or indirect contact with the infected urine from the reservoir hosts, mainly rats. The patients infected with Leptospira present with a wide range of severities, ranging from mild to severe life-threatening diseases. Due to the limited laboratory facilities available in the country, the usual practice is to suspect leptospirosis clinically and to manage it accordingly. The retrospective laboratory confirmation is obtained via serology. The present study aims to assess the presence of leptospirosis DNA in urine samples collected from ten clinically suspected patients of leptospirosis as an acute phase diagnostic test. The study was done on previously collected, de-identified, and preserved urine samples from patients who managed leptospirosis, and samples retained in the MMU, Faculty of Medicine, Ragama. QIAamp viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract DNA from urine samples. The extracted DNA samples were then tested for Leptospira positivity using Real Time PCR. A targeted DNA molecule was amplified and simultaneously quantified using real-time PCR (Polymerase Chain Reaction). A total of ten urine samples were tested and Leptospira DNA was detected in two samples indicating the potential for using urine samples as a specimen for the acute phase diagnosis of leptospirosis.

Key words: Leptospirosis, Molecular diagnosis, urine-based testing, Real-Time PCR, Public health

1 Introduction

Leptospirosis is a widely distributed zoonotic bacterial infection with a significant global health threat. The disease accounts for 2.9 million disability-adjusted life years (DALYs) annually, and the world reports about 1.03 million new cases each year with an average of 58,900 deaths. The disease is more prevalent in tropical and subtropical regions, where the resources are limited for patient management and diagnosis. Leptospirosis is caused by a spiral shape 11 pathogenic and 5 intermediate pathogenic bacteria belonging to the genus Leptospira, family Leptospirosis. Figure 1 depicts Leptospira from an electron microscope. Infection affects both animals and humans. Rodents are the main animals affected by the disease, although other animals such as dogs, cattle, pigs, and wildlife can harbor bacteria. These kinds of animals also act as the reservoir hosts. The infected animals excrete pathogenic Leptospira in their urine for a prolonged time. The first step in the transmission of an infection to humans is the contamination of soil and water by

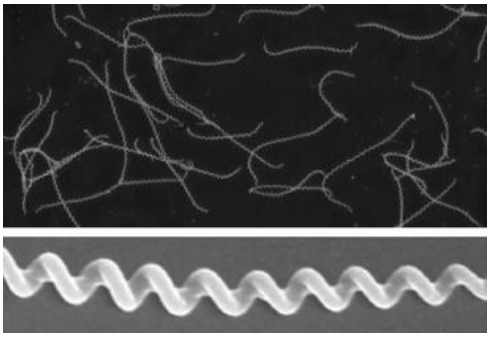


Figure 1: Leptospira bacteria[2]

an infected animal's urine. The bacteria find its way to human body through direct or indirect contact with contaminated water or soil, more easily through skin breaches and contact with unbroken mucous membranes (such as those in the mouth, nose, or eyes) and conjunctiva.1 Clinical manifestations of leptospirosis range from minor flu-like symptoms to severe illness affecting the kidneys, liver, lungs, and nervous system. Respiratory distress syndrome, liver

failure, and kidney damage are evident in the affected patients.

The non-specific initial clinical manifestations are challenging to clinicians as they mimic other common infective and non-infective diseases in the country. Leptospirosis is diagnosed by a combination of laboratory testing, clinical features, and epidemiological risk factors.3 The facilities for the acute phase diagnosis are limited in the country, especially in disease prevalent areas. The usual practice is to manage patients clinically and to obtain laboratory confirmation retrospectively mainly by serological tests such as the enzyme-linked immunosorbent assay (ELISA) and the microscopic agglutination test (MAT) which detect antibodies against Leptospira in a patient's serum. Due to limited availability of acute phase testing and the disease severity on presentation, patients with leptospirosis are likely to receive broad spectrum antibiotics instead of targeted therapy. Therefore, establishing acute phase testing will improve the care minimizing adverse effects. When diagnostic facilities are available and leptospirosis is confirmed, it can be treated with narrow spectrum antibiotics such as penicillin or ceftriaxone, which efficiently eliminate the bacteria from the bloodstream.4

Leptospira DNA can be detected in clinical samples such as blood and urine, using polymerase chain reaction (PCR) assays. The disease must be confirmed in a laboratory. For the acute phase of disease diagnosis, real-time PCR is employed (qPCR).5 This molecular biology technique measures and amplifies specific DNA sequences in real time. Scientists can accurately determine the amount of DNA in a sample by using Real Time PCR, which detects DNA amplification as happens (in real time) during the PCR process. Adding fluorescent dyes or probes to the reaction mixture is a real-time PCR technique. A fluorescence dye or probe is used for the detection of amplified DNA. The fluorescence signal emitted is directly proportional to the concentration of DNA amplified. When comparing the fluorescence signals to those generated by standards or controls, researchers can determine with precision the initial concentration of DNA present in the sample.

Establishing diagnostic facilities will help to prevent disease transmission. Integrating environmental management, public health initiatives, and personal protective measures together is considered as the most effective way to prevent leptospirosis. Some preventive measures include staying out of water sources where animal feces may be present and refrain from swimming, wading, or submerging yourself, wearing protective gear, such as waterproof boots and gloves. However, preventing occupational exposure is challenging in the country. The main risk groups include paddy cultivators, chana cultivators, manual workers and people involved in gem mining. Given that rodents frequently carry Leptospira, health authorities must take action to control rodent populations.6

The main aim of this project is to establish a real time PCR (qPCR) as a method to detect Leptospira nucleic acid in acute phase urine samples collected from patients clinically suspected of having leptospirosis. Specific objectives, to establish QIAamp Viral RNA Mini Kit, to extract leptospirosis nucleic acid from urine samples; to assess the day of urine nucleic acid positivity for leptospirosis in compared to days of illness; to evaluate the urine qPCR result in compared to renal function of the patient.

2 Methodology

Study site: The laboratory work was carried out at MMU, Faculty of Medicine, Ragama.

Study samples: The study was done on retained urine specimens in the MMU, samples were collected from patients admitted to the hospital with clinically suspected Leptospirosis. The ten samples were collected from the patients during the acute stage of the disease and two positive controls, and one negative control also used in this project.

Study design: The study was carried out as a prospective descriptive study to detect Leptospira DNA in urine samples collected from patients clinically managed as leptospirosis by a pre-validated real time PCR.

Samples processed were collected from the patients clinically managed as leptospirosis and the duration of illness was less than 7 days.

Method of sample collection and transport: A urine sample was collected into a sterile container and was transported into the laboratory in ice.

DNA extraction from urine specimens. Total DNA from urine (200µl) were extracted using QIAamp viral RNA Mini Kit according to the manufacturer's instructions.7,8

2.1 Primer design. Pathogenic Leptospira specific primers were selected from the fragment of the secY gene homologous to the Leptospira interrogans S10-spc-a locus (Genbank accession number AF115283). The primers amplified a 202 bp fragment between the locus positions 15 744 and 15 946 of secY gene that is located within the S10-spc-α locus containing genes for ribosomal proteins and it encodes preproteintranslocase for Leptospira (Table 1).9

Table 1: Optimal reaction conditions for real time PCR

Reagents	Optimized concentration range	Final concentration
Double distilled water		
Forward primer SecY IV F	80-250 nM	2.5 mM
Reverse primer Sec Y IV R	80-250 nM	2.5 mM
SYBR Green qPCR Master mix	1X-5X	1X
Template DNA	<100 ng	variable

2.2 Optimal reaction conditions for real time PCR. The amplification process was carried out after an initial denaturation at 950C for 10 minutes. Thermal process of amplification for 40 cycles was carried out including steps of denaturation at 950C for 15 seconds, annealing at 540C for 30 seconds, extension at 720C for 30 seconds and with an extended final incubation at 720C for 8 minutes. After cooling at 300C for 1 minute, melting curve (Tm) analysis from 65-940C with readings every 0.5 0C was performed according to the manufacturer's instructions. The cut off for the analysis was set at Threshold Cycle (Ct) value 35 and the approximate total time taken to run the entire program was about 2 hours. All samples were tested twice for maximum reproducibility. All the data was analyzed using the software provided by the Esco e Swift Spectrum 48 fluorescence qPCR detection system (Table 2).9,10

Table 2: Optimal thermal conditions for real time PCR

Program	Cycles	Target Temperature	Incubation time
Initial Denaturation		95^{0}C	15minutes
Denaturation	40 Cycles	95^{0}C	15 seconds
Annealing		54^{0}C	30Seconds
Extension		72^{0}C	30Seconds
Final Extension		72^{0}C	8 Minutes
Melting Temperature		65^{0}C-95^{0}C	

3 Results

Table 3: Table of Fluorescence Values

Fluorescence												
A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13
24.06	11.66	11.14	13.90	22.16	7.68	16.56	15.44	8.52	21.96	22.66	14.08	12.14
24.00	11.64	11.08	13.94	22.16	7.68	16.56	15.50	8.50	21.98	22.66	14.08	12.14
23.96	11.62	11.04	13.98	22.16	7.70	16.56	15.50	8.50	21.98	22.66	14.08	12.14
23.92	11.60	11.00	14.02	22.16	7.74	16.56	15.50	8.50	21.98	22.68	14.08	12.14
23.90	11.58	10.98	14.06	22.16	7.80	16.56	15.50	8.50	22.00	22.72	14.08	12.14
23.88	11.56	10.96	14.10	22.16	7.86	16.56	15.50	8.50	22.04	22.80	14.10	12.14
23.86	11.56	10.94	14.14	22.16	7.86	16.56	15.50	8.52	22.08	22.94	14.14	12.14
23.86	11.56	10.94	14.16	22.16	7.86	16.56	15.50	8.54	22.12	23.08	14.20	12.14
23.86	11.56	10.94	14.20	22.16	7.88	16.56	15.50	8.56	22.16	23.28	14.28	12.14
23.84	11.54	10.94	14.24	22.16	7.90	16.58	15.50	8.56	22.18	23.62	14.42	12.14
23.84	11.54	10.94	14.30	22.16	7.92	16.60	15.50	8.56	22.20	24.22	14.70	12.14
23.86	11.54	10.94	14.34	22.16	7.94	16.60	15.50	8.56	22.22	25.06	15.18	12.14
23.88	11.54	10.94	14.36	22.18	7.96	16.60	15.50	8.56	22.24	26.32	15.88	12.14
23.88	11.54	10.94	14.38	22.20	7.96	16.60	15.50	8.56	22.22	27.86	16.90	12.14
23.88	11.54	10.94	14.40	22.18	7.96	16.60	15.50	8.56	22.20	30.12	18.14	12.12
23.88	11.54	10.94	14.40	22.16	7.96	16.60	15.50	8.56	22.18	32.58	19.94	12.12
23.88	11.54	10.94	14.40	22.16	7.96	16.60	15.50	8.56	22.16	35.04	21.94	12.12
23.88	11.54	10.94	14.40	22.16	7.96	16.60	15.50	8.56	22.14	37.22	23.98	12.12
23.88	11.54	10.94	14.42	22.16	7.96	16.60	15.50	8.58	22.12	39.16	26.02	12.12
23.88	11.54	10.94	14.44	22.16	7.96	16.60	15.50	8.60	22.12	41.04	27.88	12.12
23.86	11.54	10.94	14.46	22.16	7.96	16.60	15.50	8.64	22.12	42.80	29.60	12.12
23.86	11.54	10.94	14.50	22.16	7.96	16.60	15.50	8.68	22.16	44.42	31.18	12.12
23.86	11.54	10.94	14.56	22.16	7.96	16.62	15.50	8.72	22.20	45.84	32.70	12.12
23.86	11.54	10.94	14.62	22.16	8.02	16.64	15.50	8.74	22.24	46.94	34.08	12.12
23.86	11.54	10.94	14.68	22.16	8.08	16.66	15.50	8.76	22.26	47.78	35.20	12.12
23.86	11.54	10.94	14.72	22.16	8.08	16.66	15.50	8.76	22.28	48.52	36.10	12.12
23.86	11.54	10.94	14.76	22.16	8.08	16.66	15.52	8.76	22.30	49.14	36.92	12.12
23.86	11.54	10.94	14.80	22.16	8.08	16.68	15.54	8.76	22.32	49.68	37.62	12.12
23.84	11.54	10.94	14.82	22.16	8.08	16.70	15.56	8.78	22.34	50.18	38.16	12.12
23.82	11.54	10.94	14.82	22.16	8.08	16.70	15.60	8.80	22.36	50.68	38.62	12.12
23.80	11.54	10.94	14.82	22.16	8.08	16.70	15.64	8.82	22.38	51.02	39.08	12.12
23.80	11.54	10.94	14.84	22.16	8.08	16.72	15.64	8.84	22.38	51.30	39.54	12.12
23.78	11.56	10.94	14.86	22.16	8.08	16.74	15.64	8.84	22.38	51.54	40.00	12.12
23.78	11.58	10.94	14.90	22.16	8.08	16.76	15.64	8.84	22.38	51.78	40.40	12.12
23.78	11.68	10.96	14.94	22.14	8.08	16.78	15.64	8.84	22.38	52.00	40.76	12.12
23.76	11.94	10.98	14.96	22.14	8.08	16.80	15.66	8.84	22.38	52.20	41.06	12.12
23.76	12.34	11.00	14.98	22.16	8.08	16.86	15.74	8.86	22.38	52.38	41.38	12.14
23.76	13.02	11.04	15.02	22.18	8.10	16.92	15.88	8.88	22.38	52.56	41.70	12.16
23.74	14.08	11.10	15.06	22.20	8.16	16.98	16.10	8.92	22.42	52.64	42.04	12.20
23.76	15.34	11.18	15.10	22.24	8.22	17.06	16.54	8.98	22.52	52.70	42.38	12.26

Table 4: Table of Temperatures and Time Used by Real Time PCR

Program	Cycles	Target Temp.(C) /Melting End Temp.(C)	Incubation Time	Temp. Transition Rate(C/s)	Secondary Target Temp.(C) /Melting Start Temp.(C)	Step Size(C)	Step Delay (Cycles)	Grad Temp.(C)	Sample Mode
Cycle/Temp.1	1	95.0	0:12:00	4.0	-	-	-	-	None
Cycle/Temp.2	40	95.0	0:00:15	4.0	-	-	-	-	None
		60.0	0:00:20	4.0	-	-	-	-	None
		72.0	0:00:20	4.0	-	-	-	-	Single
Cycle/Temp.3	1	72.0	0:05:00	4.0	-	-	-	-	None
Melting1	-	95.0	0:00:30	-	65.0	0.5	-	-	Step
End	-	4.0	-	4.0	-	-	-	-	-

Table 5: Table of Patient Details

SN	Sample	Group	Property	Date of illness	Diagnosis	Age in years	Gender	Serum creatinine (mg/dl)
A1	1	1	S	5	Leptospirosis	41	Male	1.4 (high)
A2	2	1	S	8	Leptospirosis	44	Male	2.6 (high)
A3	3	1	S	7	Leptospirosis	32	Male	0.9
A4	4	1	S	7	Leptospirosis	56	Male	2.2 (high)
A5	5	1	S	8	Leptospirosis	37	Female	3.1 (high)
A6	6	1	S	8	Leptospirosis	41	Male	0.8
A7	7	1	S	6	Leptospirosis	46	Male	1.0
A8	8	1	S	7	Leptospirosis	45	Male	1.1
A9	9	1	S	8	Leptospirosis	39	Female	2.0 (high)
A10	10	1	S	8	Leptospirosis	58	Female	1.65 (high)
A11	11	1	P	9	Leptospirosis	67	Male	1.0
A12	12	1	P	10	Leptospirosis	69	Male	1.4(high)
A13	13	1	N	4	Leptospirosis	41	male	2.3(high)

4 Discussion

Direct detection of *Leptospira* DNA by conventional or real-time PCR is done in clinical laboratories to facilitate diagnosis and management of leptospirosis in the acute phase of the illness. Such facilities are minimally available in the country even in disease-prevalent areas due to high cost, there is a need for laboratory facilities, and qualified, experienced human resources. However, establishing such facilities will result in better patient management with a reduction in mortality rate. The present study aimed to re-evaluate a pre-validated real-time PCR for the detection of *Leptospira* DNA in urine samples while obtaining hands-on experience to perform the technique.[7,8]

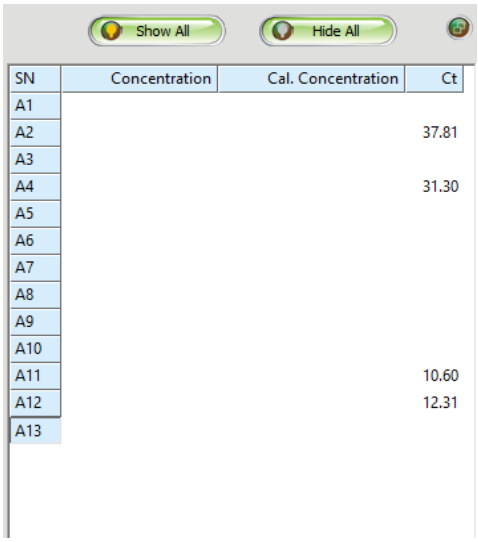
A total of ten samples were tested using real-time PCR. Two of the samples tested positive with a Ct value of 37.81(sample 2) which is not a higher detection of *Leptospira* DNA because the Ct value is above 35 and other sample's Ct value was 31.30 (sample 4) this Ct value is a higher detection of *Leptospira* DNA because the Ct value is below 35. The two positive controls have Ct values of 10.60 and 12.3 these Ct values are higher detection of *Leptospira* DNA because Ct values are below 35. The results for the remaining samples and the negative control were negative.

Two tested positive samples were collected on day 7 and day 8 of the illness. The tested negative samples were collected before day 10 of the illness. A possible explanation for negative results includes degradation of DNA while storing in the laboratory, inadequate sample volume, intermittent excretion of pathogen, and the presence of DNA in undetectable

Figure 2: Samples and Fluorescence curves

levels. DNA degradation might happen if the samples were not stored properly or are subjected to extremely high or low temperatures; The patient may have a concentration of the target organism or

nucleic acid below the PCR test's detection limit. This could happen if the patient is responding well to treatment or at an early or late stage of the infection; When collecting samples, it is crucial to consider how the infection is progressing. It is possible that the target nucleic acids will not be detectable if the sample is taken too early or too late during the infection. Furthermore, substances that can inhibit PCR reactions can be found in certain biological samples. The sample may naturally contain these inhibitors, or they may be added during collection or processing; False negative results can be caused by mistakes made during the PCR procedure itself, such as poor reagent quality, broken thermocyclers, or incorrect reaction setup by the operator. The test might not detect it if the patient has a slightly different strain or if the target sequence has mutated; A mismatch between the primers or probes used in the PCR test may arise from the considerable



genetic variation exhibited by certain pathogens;

And a false negative test result could result from the patient taking antibiotic medication prior to collection of the specimen which would lower the pathogen's detectable amounts.[11]

Figure 3: Concentration and Ct Values

High serum creatinine values were noted in the two patients from whom *Leptospira* DNA was tested positive. However, negative results were noted in 6 patients with impaired renal function as suggested by elevated serum creatinine. No statistical analysis was done to evaluate the significance of impaired renal function and the detection of *Leptospira* DNA in urine due to limited sample numbers.[9,10]

5 Conclusion

According to the test results obtained in the research, that urine samples can be used as specimen for diagnosing leptospirosis in the patients.

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