Prevalence of Anti-leptospiral IgM and Detection of Pathogenic *Leptospira* Species DNA in Neonates Presenting With Clinical Sepsis in Southwestern Uganda

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Research Article

Abstract

Background

Leptospirosis is an emerging neglected zoonotic disease that presents with nonspecific signs/symptoms and it can be mistaken for other diseases. Due to limited diagnostic capacity and unawareness, data on human leptospirosis particularly in neonates is scarce in many sub-Saharan countries. It has been underreported hindering preventive and control measures in place. The study aimed at determining prevalence of leptospirosis as a cause of febrile illness in neonates using a commercially available IgM ELISA and a quantitative real-time PCR (qPCR).

Methods

This was a descriptive cross-sectional study that included 103 neonatal sepsis cases whose parents/legal guardians gave informed consent. Data on demographic and clinical characteristics was collected using structured data collection form. EDTA whole blood sample was collected from the neonates by trained study nurses. From the samples, IgM ELISA was done using automated analyzers, DNA extracted and qPCR was performed using primers for LipL32, specific for the pathogenic leptospires.

Results

The prevalence of anti-leptospiral IgM among the neonates as determined by ELISA was 4.3%, where all of them presented with lethargy and poor feeding. No pathogenic *Leptospira* species DNA was amplified by qPCR.

Conclusions

Evidence of leptospirosis was demonstrated in neonatal sepsis cases in this study. The findings suggest considerations of leptospirosis in the differential diagnosis of neonates with sepsis. More data is needed on the real epidemiology, clinical features and burden of leptospirosis in neonates. There is need to include intermediate pathogenic species of *Leptospira* in the diagnostic qPCR assays.

Introduction

Leptospirosis is a significant emerging zoonotic infection of public health concern that has been underreported particularly in developing countries and it is recognized as a neglected tropical disease affecting vulnerable populations [1–3]. The clinical presentation mimics that of other febrile illnesses often leading to misdiagnosis clinically [4, 5]. As such world health organization recommends confirmation of the disease by laboratory diagnosis in conjunction with clinical findings and exposure status [6]. For effective treatment, antibiotic therapy has to be initiated early thus necessitating early diagnosis of the infection. Leptospirosis is caused by the spirochaete of the genus *Leptospira* and the pathogen is divided into the pathogenic (P1) and intermediary pathogenic (P2) group [7–9]. Infections are reported to be caused mostly by the P1 group, explaining why commercial kits and diagnostic qPCR

assays widely used detect only the P1 group. However, there are increasing reports of detection of P2 in clinical samples including in the current study site [10–12]. The reservoirs for the leptospires include rodents particularly rats, domestic animals mainly pigs but also dogs and cattle [5], all of which are common in the current study region. Seroprevalence of leptospirosis in cattle and African buffalo in the study region, Southwestern Uganda was shown to be 29.35% and 42.39% respectively [13]. Humans get infected by direct or indirect contact with infected animals where the spirochetes penetrate the humans through breaks in the skin, mucous membrane or intact conjuctival mucosa [4, 5, 14]. Another route of infection noted is through congenital transmission [6, 12, 15]. Leptospirosis is more common in risk groups that are exposed to animal reservoirs or contaminated environment such as abattoir workers, farmers (rice and sugar cane workers), ranchers, veterinarians, sewage workers, and amongst individuals partaking in water sports and recreation [4, 5].

Leptospirosis has a worldwide distribution with epidemic potential. It is endemic in humid tropical and subtropical regions of the developing world [3, 6, 16]. It is regarded as disease of the poor where resource-poor regions are associated with highest burden of disease [1, 3]. The precise number of human leptospirosis cases worldwide is unknown. A systematic review in 2015 revealed that there are 1.03 million cases and 58,900 deaths each year with 2.9 million disability adjusted life years lost per annum [1, 17], placing it as a leading zoonotic cause of morbidity and mortality. The prevalence of leptospirosis in Africa in patients with non-specific febrile illness from a systematic review of studies that did serodiagnosis ranged from 2.3%-19.8% [18]. Due to limited diagnostic capacity and unawareness, data on human leptospirosis as a cause of febrile illness is scarce in many Sub-Saharan countries [16], hindering prevention and control measures. Results from a recent study in Uganda reported seroprevalence of 35% in rural western part of the country, but the study only included adults [19]. Studies conducted in Mbarara regional referral hospital (MRRH) and elsewhere in Uganda on neonatal sepsis (NS) reveal failure to isolate microorganisms in more than half of the NS cases [20, 21]. Leptospires are not routinely diagnosed or cultivated in MRRH microbiology laboratory. A study involving sequencing of 16s rRNA (an expensive technique) in Mbarara regional referral hospital (MRRH) showed that 40% of blood samples collected from neonates with suspected sepsis had *Leptospira* [12].

The current study sought to determine the prevalence of anti-leptospiral antibodies as an evidence of leptospirosis as a cause of febrile illness in neonates presenting with clinical sepsis using IgM ELISA and detection of pathogenic *Leptospira* species DNA by quantitative real-time PCR (qPCR). Merits of IgM ELISA over other serodiagnostics include detection of antibody in early phase of the disease, use of single genus-specific antigen, no need to maintain live leptospires through culture, less cumbersome and being able to be standardized [6].

Materials And Methods

Study design and setting

This was a descriptive cross-sectional study conducted in paediatrics ward of Mbarara Regional Referral Hospital (MRRH) and Holy Innocent Children's Hospital (HICH), Mbarara in southwestern Uganda. MRRH receives referrals from health facilities in the neighboring districts in the region and also receives patients from neighboring countries like Tanzania, Democratic republic of Congo and Rwanda. HICH is exclusively a children's hospital, the only one in the region. The population in the study site visits MRRH/HICH whenever they experience an illness or when referred from peripheral health units. The majority of the people in the study area live in rural areas and they are subsistence crop farmers and practice livestock farming.

Study population

The study included neonates who; presented with suspected clinical sepsis at the study sites, were from exposed contaminated environment and whose parent or legal guardian consent to participate in the study. In the current study, a neonate was defined as a newborn less than one month (30 days) old. Neonates who presented with fever/hypothermia and at least any one of the following; lethargy, poor feeding, full fontanel, vomiting, seizures, diarrhea, respiratory dysfunction and jaundice were enrolled in the study. Exposure status was defined by residence near a stream of water and or its use as source of water, presence of livestock or rodents at home and parents/guardian or caretaker's occupation that predisposes to contact with contaminated water and or animals. Participants with known underlying etiology such as malaria or any other cause of febrile illness at inclusion or done at bedside as determined by the clinician were excluded because of the possibility of cross reaction in the IgM ELISA analysis.

Sampling and data collection

A total of 103 participants were recruited through convenient sampling method from August, 2018 to November, 2018. An experienced and trained research nurse from each site with the guidance of a clinician noted the clinical presentation and exposure history of neonates admitted at MRRH/HICH and sought consent from parents/caretakers of neonates who fell in the inclusion criteria. Clinical and demographic data was recorded using the predesigned questionnaire from those who consented to participate in the study. The data collected included but not limited to; sex and date of birth (age of the baby, days), duration of fever, source of water for domestic use, presence of animals at home, and signs and symptoms of the infection. From the neonates whose parents/caretakers gave informed consent, about 0.5 mL of blood was drawn aseptically by venipuncture from the arm into EDTA microtube. The blood sample was later used to obtain plasma. Plasma was chosen because of the slightly higher sensitivity for PCR assays compared to whole blood and serum [22] and would later be used for IgM ELISA.

Laboratory procedures

The samples were analyzed at Epicentre research laboratory at Mbarara University of science and technology (MUST). The EDTA whole blood was centrifuged at 3000 rpm for 5 minutes to obtain about 0.2ml (200μl) of plasma. IgM ELISA was done on the plasma (10μl) to detect anti-leptospiral antibodies and the remaining plasma was used for DNA extraction and subsequent detection. ELISA was done using the automated set of analyzer Washer 470 and Reader 270 (BioMérieux) and the Leptospira IgM ELISA kit, EIA-4715 (DRG International, INC). Nine (9) plasma samples were excluded from ELISA analysis either because of anticipated interference due to sample properties (cloudy lipemic samples) or too little volume to proceed with DNA extraction. The procedure was performed and interpreted according to manufacturer's instructions. For IgM ELISA, positive and negative controls were included for quality control (QC) and the test was considered valid when the QC passed. All the samples were run in duplicates, and checked whether the replicates gave the same cutoff result. Besides the reader, the wells were checked visually with reference to positive and negative control against a white background and the intensity of color formed graded according to the kit insert.

DNA was extracted from the plasma using the QIAamp® DNA Mini, 250 (Qiagen, German), following manufactures protocol. Internal positive and negative control samples were included in each batch of DNA extraction procedure and PCR. A real-time PCR assay using the probe-specific 'BactoReal *Leptospira* spp, LipL32' kit (Ingenetix, Austria) was run for the detection of the LipL32 gene found in pathogenic leptospires with the Rotor-Gene Q real-time PCR instrument according to kit instructions. The sensitivity of the initial PCR protocol was 10 target copies/PCR reactions and Ct of 24, 3. A repeat PCR was done with in-house optimized reaction. The mastermix 5x HOT FIREPol EvaGreen qPCR Supermix (Solis Biodyne, Estonia), Positive DNA controls from Institut Pasteur, and Primers; LipL32F, 5'-AAGCATTACCGCTTGTGGTG-3' & LipL32R, 5'-GAACTCCCATTTCAGCGATT -3' (Inqaba biotec, South Africa) with sensitivity of 1 target copies/PCR reaction were used in the optimization of the quantitative PCR. The primers used were designed and described by Picardeau (Institut Pasteur) and colleagues[22]. The final reaction considered had 4μl of mastermix, 0.4μl each of forward and reverse primers (10pmol/μl), 10.2μl molecular grade water and 5μl of template. The temperature profile consisted of initial denaturation at 95°C for 12 minutes and 40 cycles of; 95°C for 15 seconds, 65°C for 30 seconds and 72°C for 30 seconds (acquisition at Green channel). Analysis was done using quantitative, endpoint and melt curve analysis at the Green channel.

Data management and analysis

Data was entered using Epidata and imported to and analyzed using STATA version 12. It was presented by use of pie charts and tables. Continuous variables were presented as mean ± standard deviation. Prevalence was determined as the proportion of positive samples.

Results

Demographic, clinical characteristics and exposure risk

A total of 103 neonates were included in the study based on clinical presentation and risk of exposure, of which 67 (65%) were from HICH and 36 (35%) were from MRRH. Out of the total, 53 (51.5%) were females (Table 1). The age ranged from 1 to 29 days with mean of 6.5±7.4. The weight ranged from 1.7 to 6 kg with mean of 3.2±0.66. The duration of illness from onset to review as reported by the mothers ranged from 4 hours to 336 hours (14 days) and the mean was 52.1±59.8. The most common sign and symptoms included fever, lethargy, poor feeding, respiratory dysfunction, seizures and jaundice (Table 1). Also rash, vomiting hypothermia, bleeding among others were observed. Information on the mothers of the neonates indicated that 51 (49.5%) reported to have experienced fever during pregnancy. On exposure risk, 92 (89.3%) and 77 (74.8%) had rats and livestock respectively at home with goats (56.3%) followed by cattle (31.1%) forming majority of livestock. Tap water was the main source of water, as well as springs, wells, streams, tanks and swamps among others (Table 1).

Table 1: Demographic/Clinical characteristics of neonates and mothers, exposure risk and water sources

Variables			
Variable name	Category	Frequency* (%)	Mean (SD)
Neonates			
Sex	Female	53 (51.5)	
	Male	50 (48.5)	
Age (days)	1-10	82 (79.6)	6.5 (7.37)
	11-20	9 (8.7)	
	21-30	11 (10.7)	
Weight (kg)	1.6-2.5	11 (10.7)	3.2 (0.66)
	2.6-3.5	70 (68)	
	3.6-4.5	19 (18.4)	
	4.6-5.5	2 (1.94)	
	5.6-6.5	1 (1)	
Clinical presentation*	Fever	87 (85.3)	
	Lethargy	61 (59.8)	
	Poor feeding	92 (90.2)	
	Seizures	21 (20.6)	
	Respiratory dysfunction	53 (52)	
	Jaundice	17 (16.7)	
Time from onset of illness (fever) to sample collection (hours)†	4-48	77 (74.8)	52.1 (59.8)
	49-96	10 (9.7)	
	97-144	1 (1)	
	>144	9 (8.7)	
Visible bruises	Yes	58 (56.3)	
	No	43 (41.7)	
Mothers			
Fever at pregnancy	Yes	51 (49.5)	
	No	37 (35.9)	

Exposure risk and water sources		
Rats	Yes	92 (89.3)
	No	11 (10.7)
Livestock	Yes	77 (74.8)
	No	26 (25.2)
Water source	Tape water	57 (55.3)
	Well	17 (16.5)
	Streams/river	4 (3.9)
	Springs	5 (4.9)
	Others	20 (19.4)

†Duration of illness at sample collection as reported by mothers/caretakers.

*Information on clinical presentation of one participant was missing. Some variables had a few missing values as well either because mothers/caretakers had no response or such data was missing on the medical form.

ELISA results

The results of IgM ELISA showed that 4 out of 94 tested samples were reactive, all of which were from HICH. All replicates had the same cutoff result when compared with first run. And according to the set cutoff definition, clinical picture and exposure risk, these were considered positive giving anti-leptospiral antibody prevalence of 4.3% by IgM ELISA (Figure 1). The samples which tested reactive for anti-leptospiral IgM on ELISA reader were all graded as + (plus one) on visual interpretation. There were four other samples with very little color development according to visual observation but they were considered non-reactive not only basing on visual interpretation but also because they were below the set cutoff definition on the reader. These positive cases had rats and/ or livestock and the duration of illness from onset (fever) to review as reported by the mothers/caretakers was 48 hours for two cases, 24 hours and 96 hours for the other two. The age for two of the positive cases was 7 days while the others were 2 and 3 days old. Sex was equally distributed in the positive cases. All the positive cases had the clinical presentations lethargy and poor feeding. Two (2) out of 4 had respiratory dysfunction and one (1) had pustular rash.

PCR results and analysis

Real-time PCR was run on all the samples (103) to detect the pathogenic LipL32 gene. Out of all the analyzed samples, no pathogenic *Leptospira* was detected. Analysis was done using quantitative, melt

curve, and endpoint analysis on the green channel altogether to rule out any non-specific amplification. Using the above analyses collectively it can be seen that PCR was negative for the samples (Figures 2 a-c).

Discussions

This study aimed at determining prevalence of anti-leptospiral antibodies as evidence of leptospirosis using a commercilly available IgM ELISA and detection of pathogenic *Leptospira* DNA in neonates presenting with clinical sespsis in southwestern Uganda. Among the participants, 89.3% and 74.8% had rats and livestock, respectively, at home with goats (56.3%) followed by cattle (31.1%) forming majority of livestock. The positive cases as indicated by IgM ELISA had rats and/ or livestock. These animals have been known as natural maintenance host for leptospires, shedding the spirochetes in their urine and hence transmitting to humans [5]. Further still, leptospirosis has been demonstrated to be present in animals in southwestern Uganda [13]. In neigboring Tanzania, extensive contact with cattle has been associated with higher rates of seroprevalence [23]. Contact with animals increases risk of exposure and infection. Patients may get infected through direct or indirect contact with domestic animals where transmission may occur through breaks in the skin or intact conjuctival mucosa [5]. The study showed a prevalence of 4.3% with IgM ELISA. Data on prevalence of leptospirosis in neonates is lacking in sub-saharan Africa. Serological studies have been conducted in infants and children with febrile illness in the neigboring Tanzania with prevalence of 7.7% [24] and 6.2% (probable and confirmed leptospirosis) in infants and children alone [25]. Anti-leptospiral IgM antibodies are produced first during infection and may remain for months or years [6]. However for newborns who are only less than one month old, detection of IgM may be suggestive of recent or current infection. The neonates with reactive IgM ELISA ranged from 2 to 7 days old in age and the duration of fever on medical review as reported by the mothers ranged from 1 day to 4 days. This might suggest possible congenital transmission since the neonates had not been exposed considerably to the environment. Congenital vertical transmision is more likely to occur in the third trimester a stage when the IgG level transmitted to the fetus from the mother just begins to increase and at the sametime when fetal plasma cells are fully developed [26]. Antigen-specific antibody response can be mounted in both prenatal and neonatal life but at a lower intensity. The antibody response is better to proteins than polysaccharides and it is noteworthy that antibody production in leptospirosis is mainly directed against the lipopolysaccharide [14, 26]. There has been evidence that leptospirosis can be vertically transmitted though rarely [6, 12, 15]. Detection of IgM alone is not a diagnostic confirmation, but rather should be in conjuction and consideration with clinical findings, exposure history and other laboratory findings [6]. Neonates in the study were all from environment with increased risk of exposure. A nonreactive IgM ELISA may not necessary mean absence of leptospirosis but it could be due to poor immune response as noted in neonates [26] or in the early phase of the disease when antibody levels have not been attained to a detectable amounts, which normaly occurs 4-7 days after onset of the disease [5, 6]. Since antibody levels may not be detected during early disease, repeat 2-3 weeks later when antibody levels are significant is indicated for laboratory diagnosis. This will imply that there is need for a second convalescent sample, a point when patients will

have been discharged with empiric therapy hence not practical for patient management in the current practice. Serodiagnosis of leptospirosis using IgM ELISA in a systematic review was found to be sensitive for use as initial screen for leptospiral infections especially in endemic areas with sensitivity and specificity of 84% and 91 % respectively [27].

In the present study, leptospires were not detected on qPCR performed. This is in contrast to the 40% prevalence in a previous study on neonatal sepsis at MRRH [12]. This could be attributed to the fact that the previous study used 16s rRNA gene sequencing which detects the intermediary pathogenic species (P2) in addition to pathogenic species (P1). In this current study qPCR was done targeting LipL32 gene. Studies that conducted qPCR assays to amplify the LipL32 revealed failure to detect the intermediary pathogenic strains (P2) [22, 28]. Commercial kits and diagnostic qPCR assays for leptospiral lipL32 gene use primers specific for P1 group [22, 28, 29] hence detecting only the pathogenic strains which until recently are known to be responsible for much of the leptospirosis. There has, however, been increasing reports of the intermediate species been detected in clinical samples, including in the current study site [10–12]. These previous studies used 16s rRNA sequencing. It is noteworthy that the prevalence of *Leptospira* spp DNA in febrile humans in Ecuador showed that the percentage of intermediate cluster strains was higher than that of pathogenic cluster strains, that is 96% and 4% respectively [11], while a study in Uganda showed that of the 32 detected *Leptospira* spp, 31 were intermediary species [12]. These studies reveal the high prevalence of the intermediary pathogenic species in clinical samples that could not be detected with the current qPCR assays. Other possible reasons for negative qPCR results may include neutralization of leptospires by antibodies (IgG from the mother), elimination of leptospires from blood by administered antibiotics where 13.7 % of the patients in the current study received antibiotics at the time of sampling (see additional file 1) and possibility of PCR inhibition.

Conclusions

The study revealed presence of leptospirosis in neonates with clinical sepsis as evidenced by the detection of anti-leptospiral IgM with prevalence of 4.3%. Pathogenic species of *Leptospira* DNA was not detected in any of the samples by qPCR. The findings suggest considerations of leptospirosis in the differential diagnosis of neonates with febrile illness in endemic areas. More data is needed to determine the real epidemiology and burden of leptospirosis in neonates and understand the clinical presentations, so as to draw preventive and control measures like screening of mothers in endemic area. Result from the study points towards a need to include detection of intermediary pathogenic species of Leptospira in the diagnostic qPCR assays.

List Of Abbreviations

HICH - Holy Innocent Childrens' Hospital

MRRH - Mbarara Regional Referral hospital

Declarations

Ethics approval and consent to participate

The research was approved by the Research Ethics Committee of MUST, reference number 18/05-18 and Uganda national council of science and technology, registration number HS 2753. Site clearance was sought from the Hospital Director (s) of MRRH and HICH. Written informed consent was obtained from the parents/legal guardians of the neonates. The study was conducted in accordance with existing ethical guidelines and regulations of declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DH and JB conceived and designed the study. DH was responsible for conducting investigations and analysis. SB and SK provided guidance and supervised the clinical component of the research. JB provided technical expertise on microbiological laboratory investigation. DH, SB, SK and JB participated in writing and critical reviewing of the manuscript for important intellectual content. All the authors read and approved the final manuscript.

Acknowledgement

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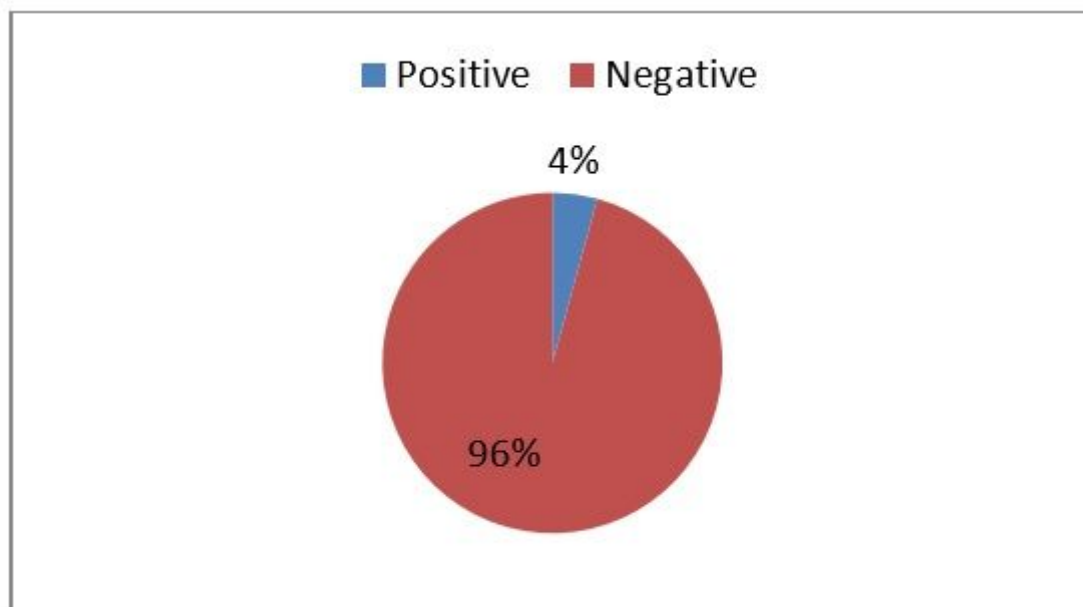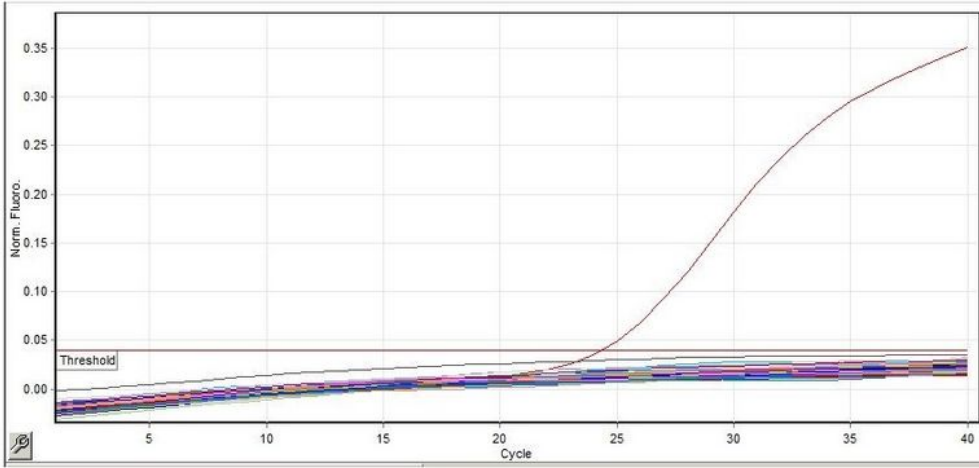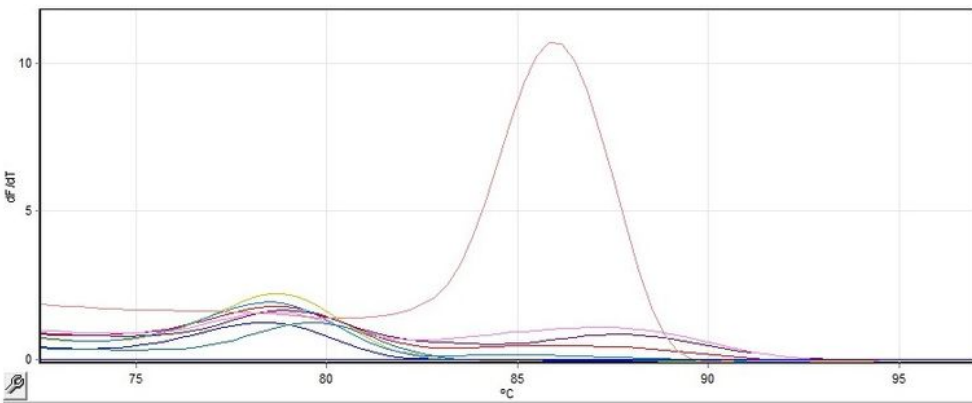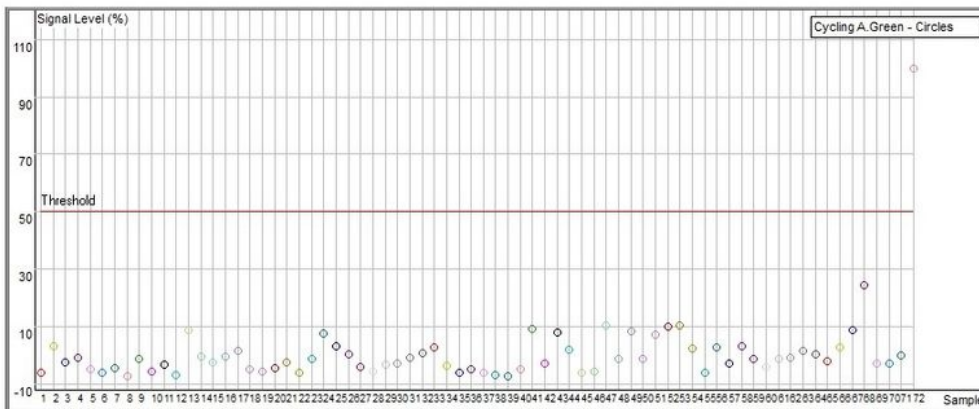
Figures

Figure 1

Prevalence of leptospirosis by IgM ELISA

2a

2b

2c

Figure 2

2 a: Quantitative analysis

2 b: Melt curve analysis

2 c: Endpoint analysis

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalfileLeptospiraRawdataA.xlsx