UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias Biológicas y Ambientales

Optimization of a DNA extraction method from complex samples (pig´s kidneys) to detect *Leptospira* spp.

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RESUMEN

La leptospirosis es una enfermedad zoonótica causada por *Leptospira* spp. En Ecuador esta enfermedad es un problema latente que puede afectar tanto a humanos como a animales. La relevancia del monitoreo de *Leptospira* spp. patógena, específicamente en animales de traspatio, radica en la posible exposición del ser humano y otros animales a este patógeno y contribuye a la comprensión epidemiológica de esta enfermedad. La detección molecular, a pesar de que es costosa, es una herramienta mucho más fiable para la detección de *Leptospira spp*. que otro tipo de técnicas. Para que esta herramienta sea confiable, se neesita tener un protocolo de extracción de ADN eficiente y que ademas sea de bajo costo. Este estudio busca identificar un protocolo de extracción de ADN basado en el uso de *Chelex 100*. Para esto se realizó tres variaciones de este protocolo: la primera utiliza proteinasa K y *Chelex 100* (método A), la segunda únicamente *Chelex 100* (método B) y la tercera consititió en la adición de un paso de purificación de ADN al primer método (método C) . La comparación entre el método A y B se realizó utilizando 5 muestras de riñones de cerdo. A continuación se determinó la presencia de especies patógenas de *Leptospira* mediante un PCR en tiempo real con tecnología TaqMan que amplifica el gen *lipL32.* Los resultados de esta comparación mostraron que el método B permite detectar un mayor número de positivos: el ADN de 5 muestras extraído con esta variación reveló 5 positivos, frente a 3 positivos con el método A. El método C dio resultados similiares al método A. Es así que el método B fue utilizado para extraer el ADN de un total de 76 riñones de cerdo y posteriormente detectar en ellos Leptospira. El porcentaje de positividad encontrado (43.42%) coincide con estudios previos sobre la presencia de *Leptospira* spp. en cerdos de la misma localidad. Nuestros resultados proveen evidencia de que el método de extracción de ADN que utiliza únicamnte *Chelex 100* permite detectar *Leptospira spp.* en muestras de riñones de cerdo, eficazmente. Este estudio abre las puertas a futuras investigaciones de monitoreo y diagnóstico de *Leptospira* en tejido animal.

Palabras clave: Extracción de ADN, *Leptospira*, riñones de cerdo, *Chelex 100*, Purificación de ADN, *Sus crofa*.

ABSTRACT

Leptospirosis is a zoonotic disease caused by *Leptospira* spp. In Ecuador this disease is a latent problem that can affect both humans and animals. The relevance of monitoring *pathogenic Leptospira* spp.*,* specifically in backyard animals, lies in the possible exposure of humans and other animals to this pathogen and contributes to the epidemiological understanding of this disease. Molecular detection, although expensive, is a much more reliable tool for the detection of *Leptospira spp.*than other types of techniques. To ensure reliability of this tool, an efficient and low-cost DNA extraction protocol is needed. This study seeks to identify a DNA extraction protocol based on the use of *Chelex 100.* Therefore, three variations of this protocol were made: the first one uses proteinase K and *Chelex 100* (method A), the second varition uses only *Chelex 100* (method B) and the third consists in the addition of a DNA purification step to the first method (method C). The comparison between method A and B was made using 5 samples of pig kidneys. The presence of pathogenic species of *Leptospira* was then determined using a TaqMan real-time PCR with that amplifies the *lipL32 gene.* The results of this comparison showed that method B allows to detect a greater number of positives: the DNA of 5 samples extracted with this variation revealed 5 positives, compared to 3 positives with method A. Method C gave similiar results to method A. Thus, method B was used to extract DNA from a total of 76 pig kidneys and subsequently detect Leptospira. The percentage of positivity found (43.42%) coincides with previous studies on the presence of *Leptospira* spp. in pigs in the same locality. Our results provide evidence that the DNA extraction method which uses *Chelex 100* alone allows to detect *Leptospira spp.* in swine kidney samples, effectively. This study opens the door to future research on monitoring and diagnosing *Leptospira* in animal tissue.

Key words: DNA extraction, *Leptospira,* swine kidneys, *Chelex 100,* DNA purification, swine, *lipL32,* qPCR.

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INTRODUCTION

Leptospirosis is a zoonotic disease widely spread in tropical and sub-tropical regions that affects approximately 1 million people and causes 60.000 deaths per year (CDC, 2018). This disease is produced by pathogenic bacteria infection from the genus *Leptospira*, which belongs to the Spirochaetales order. The genus *Leptospira* is classified in 65 species organized in two clades and four different phylogenetic sub-clades , according to their genome sequence. This classification shows that the two major clades correspond to saprophytic and pathogen species. Pathogenic species may infect animals, mostly mammals. On the other hand, there is no evidence that saprophytic species may cause an infection nor disease (Vincent, et al., 2019).

People that life in rural and periurban localities are usually very exposed to leptospirsosis, specially in developing countries from tropical and subtropical regions. In this places, extreme weather, poor sanitary conditions, and nearness to animals increase risk for contracting leptospirosis (Goarant, 2016). *Leptospira* is spread in the environment via urine from infected animals (Romero & Falconar, 2016). Humans exposed to urine of infected rats and livestock, and to contamined environent are in risk for contracting leptospirosis, especially when animals live near houses (Sarkar, et al., 2002). Rats are abundant in rural and periurban localities, these animals are asymptomatic hosts and excrete pathogen through urine (Agudelo, et al., 2010). In these places, other animals, including humans, are exposed to *Leptospira* by direct contact to urine or to contaminated soil and water. Infection occurs when *Leptospira* penetrates through mucosae or abraded skin (Ko, Goarant, & Picardeu, 2009). Clinical manifestations of leptospirosis vary from subclinical forms (fever, jaundice, and headache) to multisystemic affection (Weil syndrome) that derive in renal or hepatic failure, and even pulmonary hemorrhage (Carrada, 2005). (Tovar, 2013).

Pigs are domestic animals that can transmit leptospirosis to humans, speccially when these animals are raised in the owner houses backyard (Hernandez, Gomez, & Villamil, 2017). Indeed, in Ecuador, the monthly income of some families is based on agricultural activities that include raising pigs. It have been estimated that the amount of swine meat produced in this conditions represents more than 30.000 metric tons per year (Benitez, 2003). Another important fact to consider about swine backyard rearing is that in this conditions rodents, the main animal vectors in *Leptospira* cycle, are very common. (Ospina, Rincon, Soler, & Hernandez, 2017).

Leptospirosis in pigs is usually diagnosed by serological methods: ELISA or microagglutination tests (MAT). MAT is currently the gold standard in leptospirosis diagnosis (Singh & Krishnakumar, 2006). However, Polymerase Chain Reaction (PCR) technique is also a good choice, as it allows to distinguish between pathogen and saprophityc species (Castellar, Arrieta, Caraballo, Torres, & Rios, 2016). Thus, PCR has emerged as an innovative and useful tool that allows detection of this and other pathogens (Arya, Shergil, Williamson, & Gommersall, 2005). A useful variation of conventional PCR is Real-Time PCR which allows the quantification of bacteria by detecting the presence of a specific gene (Stoddard, Gee, Wilkins, McCaustland, & Hoffmaster, 2009). TaqMan technology is a type of Real-Time PCR which uses probes to improve the accuracy and specificity of detection. TaqMan probes work by binding to the sequence right after the forward primer. When amplification starts, the probe unbinds of the target sequence and reporter fluorophore loosens emiting luminescence which is detected by the Real-Time Thermal Cycler. (Sedano, Pinto, Siuce, & Calle, 2016). Indeed, a Taqman assay that amplifies *lipl32* is widely used for pathogenic *Leptospira* species detection (Deepak, et al., 2007). Lipoprotein 32 is an antigen that is located under the membrane surface of pathogenic *Leptospira* spp. (Hernandez, Baquero, Santander, & Gomez, 2015).

One of the most important steps in *Leptospira* spp. detection is DNA extraction as it infers difectly on DNA quality and concentration (Sedano, Pinto, Siuce, & Calle, 2016). DNA quality is directly related to detection efficiency as low purity, and high proteins and fenolic compunds concentration inhibit the reaction. Likewise, DNA concentration influences the success of a PCR reaction as low concentrations difficult target DNA amplification and high concnetrations could inhibit reaction. (Pachchigar & Khunt, 2016). One of the protocols currently being used for DNA extraction is the *Chelex 100* method. *Chelex 100* is a styrene-divinylbenzene that works as an anion exchanger with a high affinity for metal ions such as Magnesium, which is a cofactor of DNases (Singh, Kumari, & Iyengar, 2018). It has been widely used to succesfuly extract DNA from different sorts of samples. Previous articles have reported using it to extract DNA from semen, bucall cells, blood, bacterial and virical isolates, plant and algal tissues, sediment and soil, human liver, sputum and even cigarette butts (Miller, Bryant, Madsen, & Ghiorse, 1999) (HwangBo, Son, & Lee, 2010) (Nagdev, et al., 2010) (Hochmeister, et al., 1991) (de Lamballerie, Chapel, Vignoli, & Zandotti, 1994) (Walsh, Metzger, & Higuchi, 1991) (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992) (Phillips, McCallum, & Welch, 2012).

In our study, three DNA extraction protocols using *Chelex 100* were tested in swine kidney samples. Presence of pathogenic *Leptospira* DNA was evaluated by a Taqman PCR assay. Our aim was to establish one DNA extraction protocol that allows efficient and low cost detection of *Leptospira* in swine kidneys.

METHODS

Sampling

Kidney samples were collected at the Portoviejo local slaughterhouse (*Matadero Municipal del Cantón de Portoviejo*) from 76 pigs with caracteristics that suggest backyard rearing , this included stain layer, long hair, complete tail, uncastrated males, or sign of being tied up. Five cm^2 of the kidney were extracted from each pig after sacrifice and conserved in 90% ethanol. Samples were stored at -20°C. The molecular analysis was performed under the permit MAE-DNB-CM-2018-0106.

DNA extraction.

Three variations of *Chelex 100* protocol were tested: the first one was described by Bruzzese and uses proteinase K and *Chelex 100* (method A), the second varition was described by Singh *et. al.* and uses only *Chelex 100* (method B). The third variation consists on the addition of a DNA purification step, described by Singh *et. al.,* to the method B (method C). In this last method, after the precipitation step with ammonium acetate , a resting step for 24 hours at -20°C in 75% ethanol was added to the original protocol. Table 1 details differences between these three methods of extraction. To compare method A with B we used 5 kidneys, the method with better performance was chose for downstream comparisions (method B, see results for details). Comparison between method B and C was performed on 24 kidney samples. Differences between methods were assesed by DNA concentration, DNA quality, and by *Leptospira* detection in the samples.

Pathogenic *Leptospira spp.* detection

To discart the possibility of false negative results due to PCR ihibitors, we amplify a fragment of β-actin gene (Du Breuil, Patel, & Mendelow, 2019). β-actine is present in all vertebrate cells, therefore, it is expected to be found in all samples, even if they are not positive for *Leptospira* spp. (Selvey, et al., 2001). For β-actin PCR we used a final volume of 10 µL according to the conditions and concentrations reported by Du Breuil *et al.* in a Bio-Rad T100 Thermal Cycler. PCR products were visualized through electrophoresis in a 1.5% (w/w) agarose gel.

A total of 76 pig kidney samples were tested for the presence of pathogenic species of *Leptospira* using a TaqMan real-time PCR that amplifies the *lipL32* gene (Stoddard, Gee, Wilkins, McCaustland, & Hoffmaster, 2009) . As a positive control in the PCR assays, we used whole genome *Leptospira interrogans* DNA, kindly donated by Dr. Mercy Falconi. PCR reactions were performed in a final volume of 10 µL according to the conditions reported by Stoddard, et al. PCR products were visualized through Bio-Rad CFX96 Touch Real-Time PCR Detection System.

Data analysis

Statistical analysis to compare the different methods were performed using Minitab 17 Software (Minitab 17 Statistical Software, 2010). Levene´s test was conducted to compare variances of values obtained for DNA concentration, 260/280, and 260/230. Data was arranged in two sets: Method A vs Method B and Method B vs Method C. A confidence interval of 95% was used.

The null hypothesis was established as follows: the parameters are equal between Method A and B, and Method B and C. Meanwhile, the alternative hypothesis states that

those methods are different in the three parameters considered. This statistical comparison is corroborated by P-value , which will allow us to confirm the null hypothesis or to reject it. Levene´s test was used to compare the variances between two or more groups. A P-value $< \alpha$ indicates that the null hypothesis is confirmed, meanwhile a P-value $> \alpha$ indicates that the null hypothesis is rejected (BioStats, 2020).

RESULTS

Comparing DNA extraction methods

The comparison between methods A and B showed that method B has higher quality. However, method A showed higher DNA concentration. According to P-value, which is lower than alpha (0.05), it can be said that concentration and quality are significantly different between both methods (Table 2). For β-actinPCR, all samples were positive, amplifying a 300 bp fragment. This indicates there is no inhibition in the sample´s amplification, as shown in Appendix A . Non-template control did not amplify any fragment.

For *lipL32* gene PCR, FAM and ROX curves of DNA extracted by method A showed inconsistencies. Either FAM curves did not show an exponential behavior, that would confirm those samples as positives, or ROX curve showed an exponential amplification rather than a basal fluorescence as it should be. An improvement was observed in *lipL32* gene PCR ran with DNA extracted by method B; concretely in amplification curves and Cq values (Figures 1, Appendix B) between doubtful, negative, and positive samples. Figure 1 show the differences in amplification curves of *lipL32* gene Real-Time PCR for sample P162 extracted with methods A and B. Also, method B allows to detect *Leptospira* more precisely as shown in Table 3.

Based on *lipL32* gene PCR results, the optimal extraction method for the detection of pathogenic *Leptospira* was method B. This method was chosen to be compare to method C.

The concentration values obtained for DNA extracted with methods B and C showed that purified samples have lower DNA concentration (Appendix D). Likewise, 260/230 ratio values show that purified samples have higher quality than non-purified samples (Appendix D). Difference in concentration values and 260/230 ratio is statistically validated by F test ($p=0.00$) and Levene´s test ($p=0.00$) . Values for 260/280 ratio show that DNA extracts from

methods B and C are similar (Appendix D) and statistically equal according to Levene´s test (0.269) .

Leptospira spp. was not amplified in samples extracted with method C. In contrast, DNA extracts obtained by method B amplified in a 29.16% (Table 4)

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Method B was used to ampify the totality of samples (n=76). PCR for *lipL32* gene showed 43.42% of positivity (Table 5).

DISCUSSION.

Chelex 100 DNA extraction methods are widely used, however, its efficacy varies depending on the tissue. This fact generates the need to identify a protocol in which to rely on, for specific types of tissues. *Chelex 100* has shown to be a cheap and easy protocol that can be used to detect *Leptospira* from various tissues. In this study we focused on swine kidney, as it is the most common sample used for *Leptospira* spp. detection (Calderon, Mattar, Rodriguez, & G, 2013).

As leptospirosis is a zoonotic disease, the number of samples to analyse in order to track *Leptospira* in animals from a slaughterhouse can be massive. For example, a monitoring study of *Leptospira* spp. in cattle in Cordoba-Argentina, tested 282 samples. Also, a *Leptospira* spp. suvey from Brazil tested 742 samples of *Mus musculus*. Another study in Valdivia-Chile tested 106 wild rodents. Likewise, another research study in Chile tested 784 wild rodents in urban zones (Moreno, Trujillo, Maia, & Torres, 2015). Also, DNA extraction from complex tissues such as kidneys usually implies laborious work that requires hours of labor and expensive reagents and equipment (García, Rodrigo, Sanchez, Ramos, & Suarez, 2004). Therefore, to establish a cheap and effective DNA extraction protocol is crucial in epidemiological studies and for routine monitoring of the pathogen. Scarce studies have used *Chelex 100* for extracting DNA and detecting *Leptospira* spp. (Noda & Rodriguez, 2014) (Baquero & Revelo, 2016). Our results provide evidence that DNA extraction using *Chelex 100* can be used to detect pathogenic *Leptospira* spp. from swine kidneys effectively.

Comparing multiple *Chelex 100* DNA extraction methods

Chelex 100 have been used to extract DNA from a variety of tissues and organisms such as blood from zebra finches, Indian house crows, carcinomas, carcass from *Anopheles*, and forensic samples (Singh, Kumari, & Iyengar, 2018), (García, Rodrigo, Sanchez, Ramos,

& Suarez, 2004), (Musapa, et al., 2013) (Butler, 2007). However, in *Leptospira* spp. detection, *Chelex 100* methods have only been used to extract DNA from blood tissues and urine samples (Noda & Rodriguez, 2014) (Baquero & Revelo, 2016).

This study allowed us to find an optimal DNA extraction method using *Chelex 100* to detect *Leptospira* spp. in swine kidneys. To get to this result, three methods were compared in terms of quality, concentration, and *Leptospira* spp. detection by Real-Time PCR. Quality and concentration parameters allowed to have a better idea about the condition of DNA samples (Khare, Raj, Chandra, & Agarwal, 2014). Quality parameters include ratios of absorbance at 260/280 and 260/230. 260/280 ratio represents the quantity of proteins present in the sample. On the other hand, 260/230 ratio represents the quantity of phenolic compounds contained in the sample. Ideal values for 260/280 and 260/230 ratios vary from 1.8 to 2 and from 2 to 2.2 respectively (Koetsier & Cantor, 2019). Comparisons in 260/230, 260/280, and concentration parameters showed a clear difference between method A and method B. Method A allowed to get a higher DNA concentration. Extraction with method B resulted in higher 260/230 and 260/280 quality parameters, which approximate to the ideal quality values for DNA. DNA quality parameters have a bigger impact in *lipL32* Real-Time PCR results when concentration is significantly high and similar between methods. The improvement shown in DNA quality might be due to the pre-heating of *Chelex 100* solution, which allows *Chelex 100* to increase bindinding to Magnesium ions in the water and sample. Magnesium acts as a cofactor in deoxyribonucleases, preserving DNA integrity and probably allowing us to get higher DNA quality (Singh, Kumari, & Iyengar, 2018).

As concentration of DNA extracted by method C is low, it could be inferred that DNA is lost during the purification steps, where the main goal is to eliminate inhibiting compounds. Several articles have reported that a DNA purification can reduce DNA concentration but it will improve the quality (Lienhard & Schaffer, 2019). In our case it is

possible that the lost DNA belongs to *Leptospira* spp., generating a bias in detection and consequently, false negatives. Bias generetad by DNA loss is particulary important in this study, as TaqMan assays are more specific and sensitive than other PCR techniques (Trujillo, et al., 2006).

Our Real-Time PCR results show that amplification curves have a clear difference between methods A and B. Difference in amplification might be explained due to DNA quality. In method A DNA might not be clean enough for downstream protocols, inhibiting *Leptospira* spp. detection (Nolan, 2009). Method B allows to detect all 5 samples as positives meanwhile method A confirmed only 3 samples as positives, one sample as doubtful and one as negative.

Comparison of the results obtained in Real-Time PCR is highly relevant as they relate directly to the aplicability of de DNA extraction methods. Molecular detection turns to be more sensitive than serological detection. Therefore, molecular tools allow to detect more accurately than other techniques and tools, making this technique more trustworthy than others (Thomas, 2006). Also, Real-Time PCR detection provides results much faster than other techniques such as serological detection, microagglutination tests, and dark-field microscope, allowing to detect *Leptospira* spp. easier and with no need for bacteria isolation (Stoddard, Gee, Wilkins, McCaustland, & Hoffmaster, 2009).

Positivity of pathogenic Leptospira in backyard reared pigs

Our findings show that pigs reared near owners houses in Portoviejo (Manabí-Ecuador) have high positivity of pathogenic *Leptospira* (43.42%). A previous study in the same locality (Santa Ana-Portoviejo) reported similar restuls (26.9%), amplicon sequencing in this study showed high positivity and multiple species of pathogenic *Leptospira*

circulating in the study area (Barragán, et al., 2016). Positivity of *Leptospira* differs among localities. For instance in Brazil, *Leptospira* detection from female swine kidneys showed 8% (n=137) of positivity (Miraglia, et al., 2008). In Colombia, 34% of urine samples (n=337) were positive for *L. interrogans* (Bolivar, Lagares, Varela, & Vergara, 2012). These differences may be due to the sampling location. Portoviejo is a city with high precipitation levels during the year, therefore, environmental conditions benefit high *Leptospira* spreading in the land (Barragán, et al., 2016).

The high percentage found in this study matches information reported in previous investigations conducted in Portoviejo, which indicate leptospirosis is an endemic disease in the area (Barragán, et al., 2016). Moreover, according to Instituto Nacional de Investigación en Salud Publica (INSPI), Portoviejo is the city with the highest percentage of *Leptospira* spp. human cases (Pinargote, 2012), and the high positivity found in pigs may suggest an important roll of this animal in the epidemiology of the disease. Animal positivity and the tropical location of Portoviejo makes it a place with high risk of human leptospirosis.

In our knowledge, this is the first study that reports *Leptospira* spp. detection by TaqMan Real-Time PCR using DNA extracted with *Chelex100* from swine kidneys. Accurate detection of pathogenic *Leptospira* for monitoring in backyard swine is important due to the closeness of these animals with other animals and human beings. *Leptospira* in rural Portoviejo may be circulating easily among animals and infecting people living near them. The DNA extraction method used in this study has reduced processing time and total processing cost. We think that this protocol can be used to monitor *Leptospira* in massive amounts of samples from local slaughterhouses at a low cost.

CONCLUSIONS.

Leptospira spp. detection from swine kidneys amplifying *lipL32* gene by real-time PCR can be performed successfully with a DNA extraction method based on *Chelex 100.* Our results show that this technique can be used in screening assays to confirm the presence of *Leptospira.*

To support our results and in order to build a stronger conclusions, further assays may compare *Leptospria* positivity results of this work with positivity of the same DNA extracted with a commercial kit. Also, we recommend performing an Multi Locus Sequence Typing (MLST) should be performed in order identify the species circulatinig in the study location.

High *Leptospira* spp. positivity found in this study suggests a strong risk and probability of *Leptospira* spp. spreading into other animals or human beings. Thus it represents an urgent issue to focus research on.

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TABLES

Table #1

Main differences between DNA extraction methods

	Method A	Method B	Method C
Chelex Solution	7%	7%	7%
Mechanic Lysis	X	X	X
Proteinase K	X	-	-
Pre-heated Chelex	-	X	X
Incubation at 56°C	X	-	-
Incubation at 96°C	X	X	X
Incubation at 100°C	-	X	X
Purification process	-	-	X
Processing time	3 hours	40 minutes	2 days

Table #2

Comparison of quality and concentration for Methods A and B

Parameter	Method A (n=5)	Method B (n=5)	P-value
260/230	0.567 (C.I=0.364-1.498)	0.874 (C.I=0.057-1.805)	0.001
260/280	1.448 (C.I=0.517-2.379)	1.967 (C.I=1.036-2.898)	0.000
Concentration	2240.88 (C.I=2239.94-2241.81)	1126 (C.I=1125.07-1126.93)	0.001

All values are showed as means. Also, confidence intervals are included.

Table #3

Comparison of positivity for pathogenic Leptospira in DNA extracts from methods A and B

	Method A	**Method B**
Positive	3	5
Doubtful	1	0
Negative	1	0

Table #4.

Comparison of positivity for pathogenic Leptospira in DNA extracts from methods B and C

	No purification method	Purification method
Tested samples	24 samples	
Positives	7 samples	0 samples
Positive percentage	29.16%	0%

Table #5

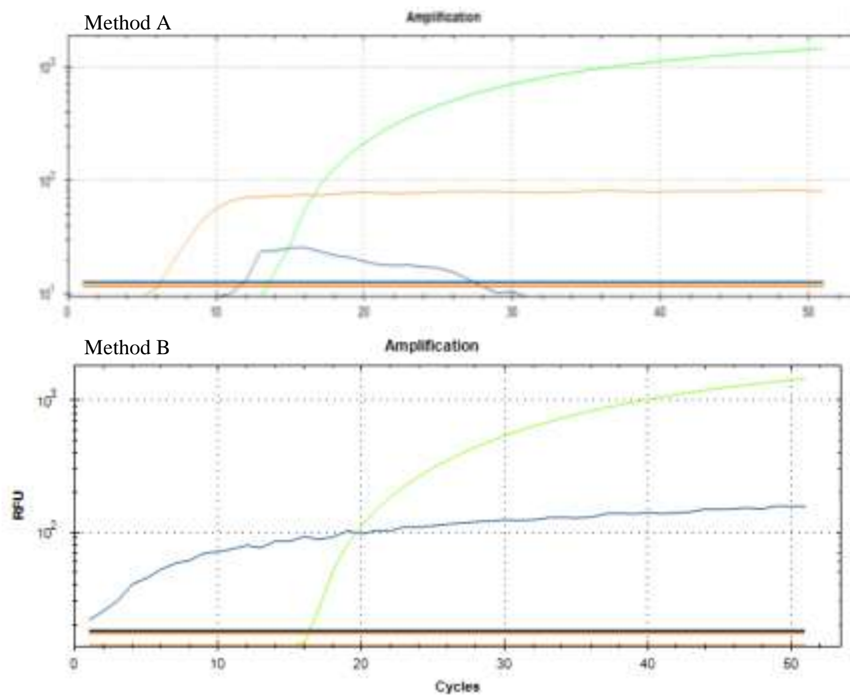
Overall positivity for Leptospira spp.

Total samples	76 samples
Total samples positive to *Leptospira spp.*	33 samples
Positive percentage	43.42%

FIGURES

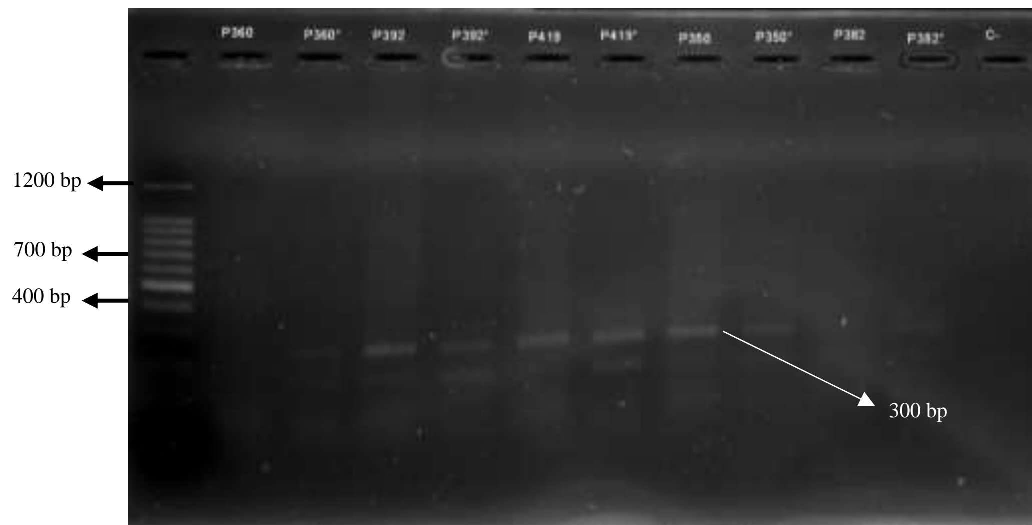
Figure #1.

Real-Time PCR amplification plot of sample P162 using DNA extraction method A and B.

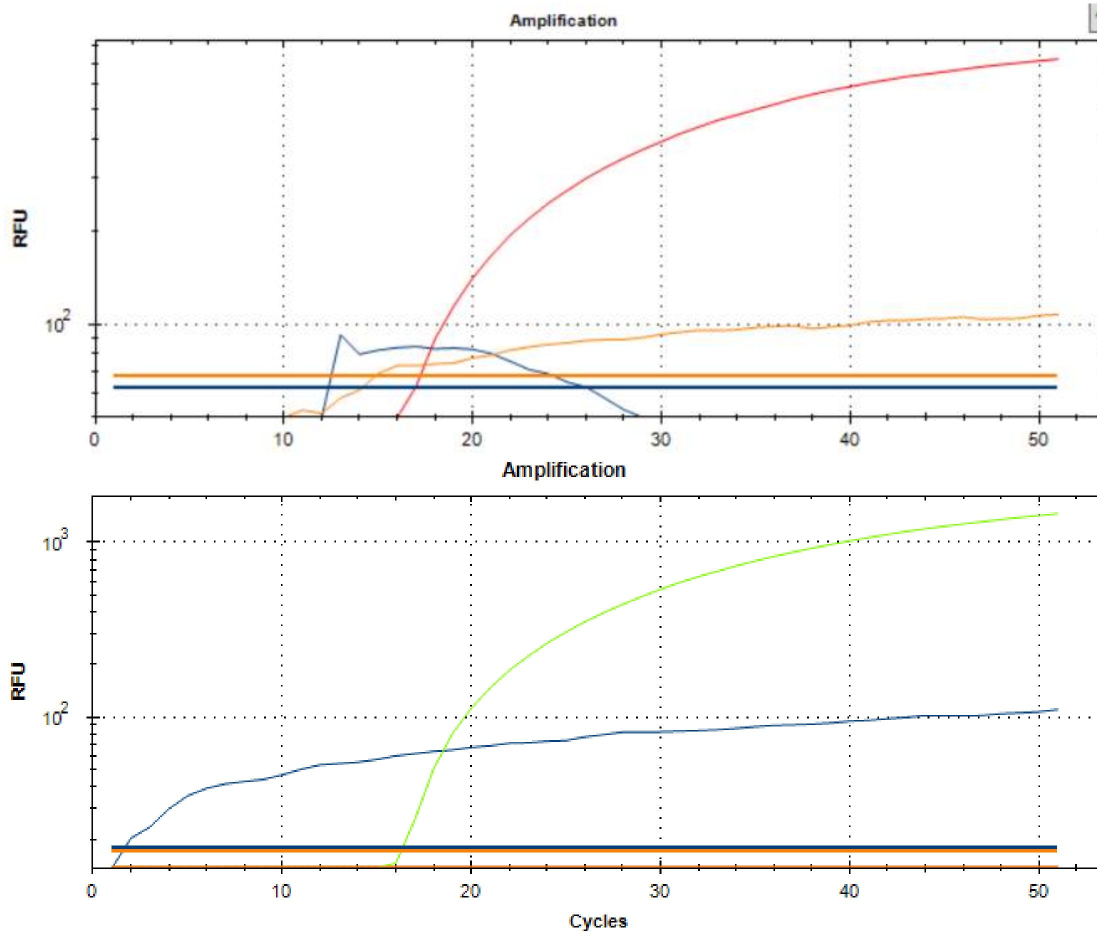


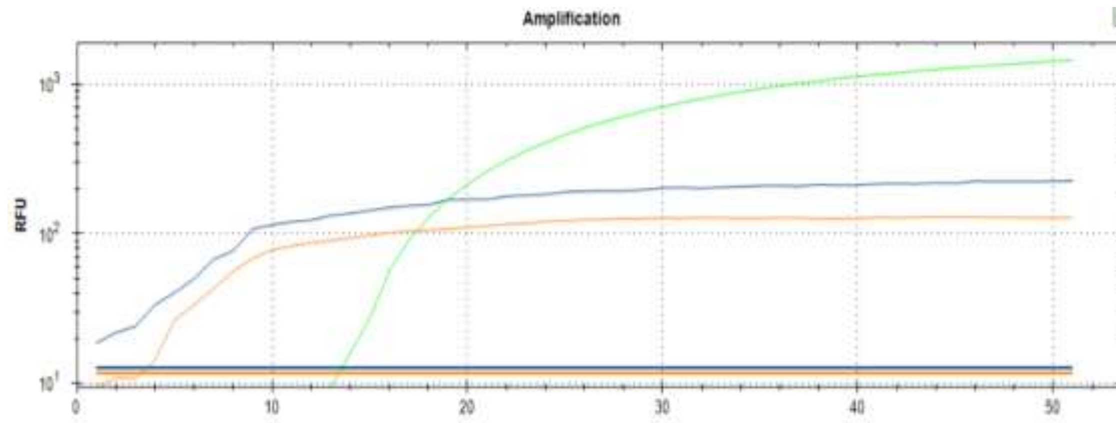
The blue line represents FAM, the orange line represents ROX, and the green or red line represents positive

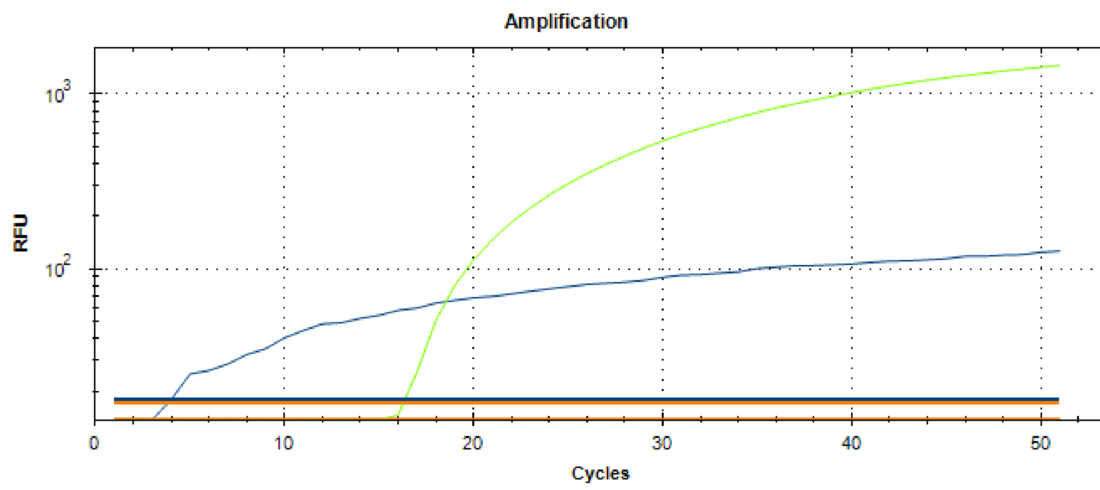
APPENDIX A: β-actin PCR



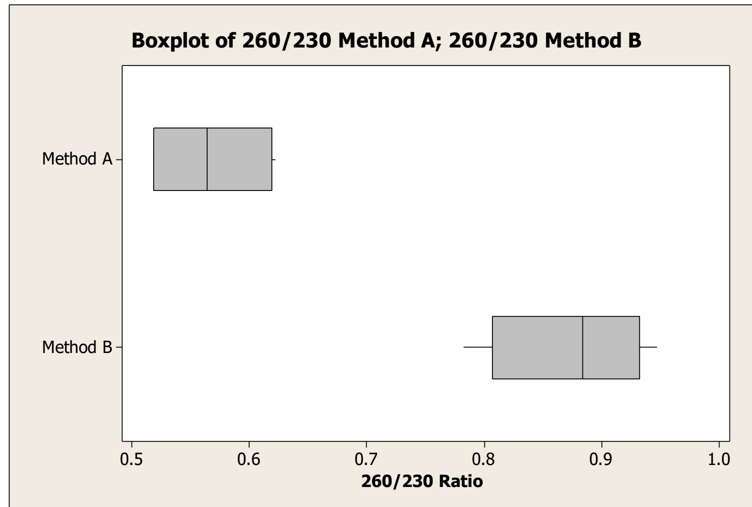
APPENDIX B: AMPLIFICATION CURVES

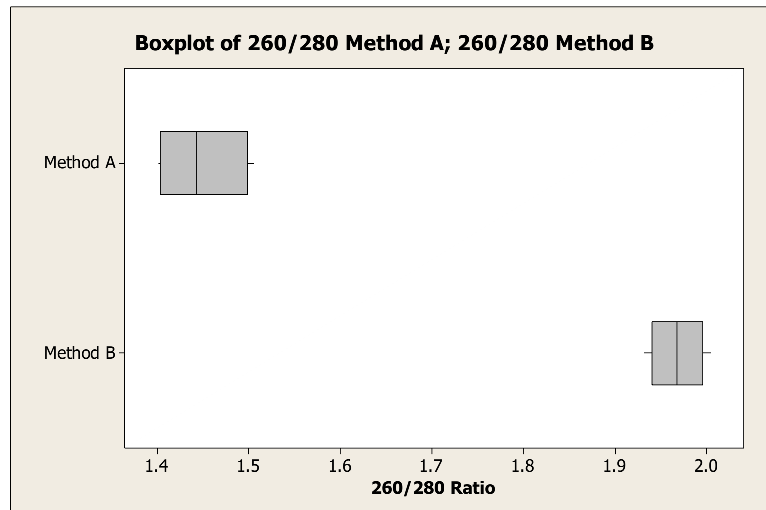


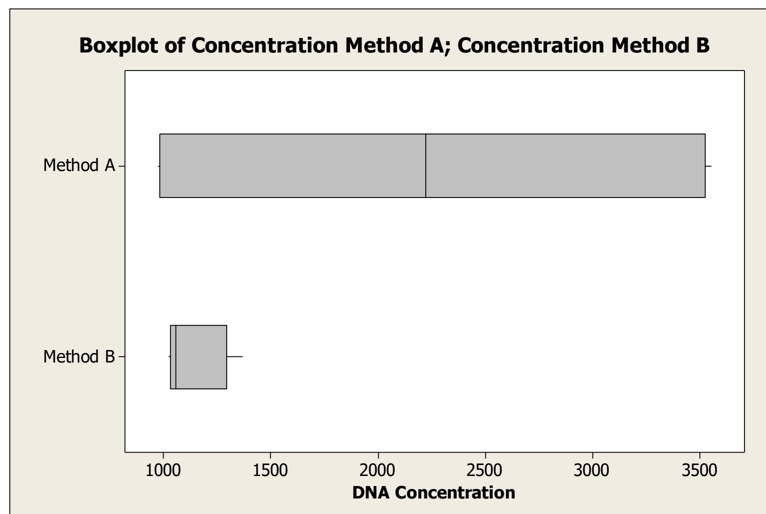

Amplification
RFU
0
10
20
30
40
50


Amplification
RFU
10^3
10^2
0
10
20
30
40
50
Cycles

APPENDIX C: CONCENTRATION AND QUALITY PARAMETERS (METHOD A VS METHOD B)







APPENDIX D: CONCENTRATION AND QUALITY PARAMETERS (METHOD B VS METHOD C)