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Cultivation of *Leptospira interrogans* serovar Icterohaemorrhagiae out of spiked sow urine samples - influence of pH and pathogen concentration

Diploma Thesis

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submitted by
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1. Introduction

One of the major difficulties in diagnosing Leptospirosis is the direct isolation of the bacteria from clinical material and the lack of recommendations concerning sample collection (Unterweger et al. 2017). The recommended material for diagnostics are urine and abortive tissue (Strutzberg-Minder et al. 2018, Steinparzer et al. 2017). Contradictory to these recommendations is the fact that leptospires are rarely found in either (Pedersen et al. 2016). There are two ways of diagnosing leptospirosis: (indirect) detection of anti-leptospiral antibodies and direct detection of leptospires in animal tissues or body fluids. The chosen method depends on the availability of capable laboratories in the area and the aim of the testing. The aim could be to diagnose an acute outbreak or to determine if a herd is chronically infected (OIE 2018).

Direct diagnostic methods used are either molecular (commonly PCR-) methods for DNA detection or laboratory cultures of leptospires. Molecular diagnostic methods are recommended. Compared with the direct isolation, they are quick and convenient, but lack the ability to determine the exact serovar (Siti et al 2019, Hartskeerl et al. 2011). Differentiation between different pathogenic serogroups and serovars using PCR is not done in routine diagnostics, but is currently possible in a scientific research setting (Steinparzer et al. 2017). Isolating leptospires is one of the most definite ways of proving their presence in a sample but also the most difficult. The main difficulties being the overgrowth with other bacteria, unsuitable growing conditions and an inadequate initial concentration of leptospires in the samples (Adler 2015). The importance of direct isolation of leptospires lies in its capability of making serological and molecular typing methods possible, including next generation sequencing (NGS). A pure culture of leptospires, absent of any contamination, is needed for this to happen. Isolation is not used in routine diagnostics to determine the presence of leptospires (Steinparzer et al. 2017) but is incredibly valuable in a scientific and epidemiological research setting.

According to the World Organization for Animal Health OIE (2018), the gold standard for diagnosing leptospires continues to be the microscopic agglutination test (MAT), which is a test for antibodies against single serovars chosen for the test. The MAT has its own set of

problems that need to be considered. For acute infections, the MAT is a suitable diagnostic method, if two paired samples are taken with a set time of 2–4 weeks in between, but it finds its limitations when trying to diagnose chronic infections. The MAT titers of chronically infected animals might be below the generally accepted minimum significant titer of 1:100 at final dilution (Strutzberg-Minder et al. 2018). The process of evaluation is very subjective and the possibility that the serovars used in the MAT do not correspond to the prevalent serovars in the region, must also be taken into consideration. There are at least 260 different serovars of pathogenic leptospires, with the expectation that there are even more yet to be discovered (Strutzberg-Minder et al. 2011). Testing for them all is currently impossible.

As outlined above, optimized diagnostic methods for leptospires are lacking. Urine is proposed to be the sample material of choice, but leptospires are hardly found in this medium. Therefore, the aim of this thesis is to discuss, research and answer the question if pH-level and pathogen concentration of sow urine samples influence the cultivation of pathogenic leptospires. In order to do so, three stand-alone experiments were performed with the help of the Austrian Agency for Health and Food Safety (AGES), department for Serology and Virology, Division for Animal Health: First, 30 urine samples from 30 different sows were spiked with a pure culture of *Leptospira interrogans* serovar Icterohaemorrhagiae (*L.* Icterohaemorrhagiae) and bacterial growth was analyzed over a period of four weeks in order to find out if pH has an influence on the growth of the leptospires. Second, ten urine samples were inoculated with a pure culture of *L.* Icterohaemorrhagiae in a dilution series and analyzed over the course of seven weeks in order to determine the minimum concentration needed for leptospires to grow. Lastly, the influence of phosphate buffer on leptospire growth was evaluated using ten different inoculated urine samples in order to evaluate the influence on the pH-level of the urine samples and if the altered pH through the phosphate buffer influences the growth of the leptospires in comparison to the same ten samples without phosphate buffer. If the pH-level plays a role in leptospiral growth, this information could be used to increase the likelihood of diagnosing leptospirosis in pig urine samples. *L.*Icterohaemorrhagiae was used because of its rapid growth compared to other serovars (OIE 2018) as well as the fact that the affiliated laboratory had pure culture *L.*Icterohaemorrhagiae on hand and experience with this serovar.

These experiments will provide valuable insight with respect to optimization of diagnostic methods, namely laboratory cultures, for leptospirosis. Currently, only a few serovars are routinely tested for with the help of MAT because, as mentioned above, there are too many and it is not feasible to test for all. Therefore, if test results are negative, it is conceivable that a different, currently undetected serovar is prevalent in the region. Hence, investigating culture conditions to enable isolation and further typing of serovars present in urine of pigs from a certain area is an essential first step to improve diagnostics and choose the appropriate serovars for MAT, which ultimately will greatly benefit pig farmers.

2. Literature Review

leptospires are thin, tightly coiled bacteria (lepto = thin, small; spira = spiral). One or both ends of the bacteria are bent into a semicircular hook when looked at in liquid media (Steinparzer et al. 2017, Breed et al. 1957). They have a diameter of 0.1 µm and a length of 6–24 µm. Similar to other spirochetes, they have a double membrane structure, with lipopolysaccharides (LPS) located in the outer layer. The fact that leptospires have the LPS embedded in their outer layer differentiates it to other spirochetes (Steinparzer et al. 2017, Adler 2015). The bacterial genus *Leptospira* is split into three groups: pathogens *(Leptospira interrogans)*, non-pathogens (*Leptospira biflexia*) and a nameless intermediate group (Steinparzer et al. 2017). The groups are further subdivided into serovars, which are grouped into serogroups if they are antigenically related.

Table 1: Most common serogroups and their associated serovars with which pigs can be infected (based on data by Strutzberg-Minder and Kreienbrock 2011).

Serogroup	**Serovar**
Australis	Australis
	Bratislava
	Muenchen
Pomona	Pomona
Tarassovi	Tarassovi
Canicola	Canicola
Grippotyphosa	Grippotyphosa
Icterohaemorrhagiae	Copenhageni
	Icterohaemorrhagiae
Sejroe	Sejroe
	Hardjo

leptospires are spirochetes that can cause chronic or acute infection in animals or can be found in freshwater as saprophytes (Adler 2015). An important factor, which must be taken into consideration and which makes the diagnosis even more important, is the zoonotic potential of leptospirosis. They are the cause of Weil's disease in humans. Pigs usually develop a subclinical infection. If they are immunodeficient or have never been in touch with leptospires, an infection can lead to abortions, birth of dead or weak piglets or infertility in sows, leading to economic losses for the farmer (Ospina-Pinto et al. 2019, Adler 2015). Contact with water contaminated with infected urine from carrier animals is the main source of infections (Liegeon et al. 2018). The carrier animals can be infected pigs, cattle or, very often, rodents. Rats play a major role in spreading leptospirosis. In fact, according to the WHO Guidelines (Faine 1982), rats are common carriers of *L*. Icterohaemorrhagiae.

The prevalence of Leptospire infections in one region may be very similar, but the frequency of individual serovars can vary considerably (Strutzberg-Minder et al. 2018). During the MAT, the serum to be tested is in contact with an equal volume of a suspension of leptospires at specific conditions and evaluated microscopically. However, since this test is laborious, well-equipped laboratories with experienced staff are needed (OIE 2018). Using MAT, antibodies against the serovars Pomona, Bratislava and Tarassovi are most commonly used (Strutzberg-Minder et al. 2011). Although some studies also highlight the presence of serovar Icterohaemorrhagiae specific antibodies (Unterweger et al. 2018, Steinparzer et al. 2017, Soto et al. 2007). *L.*Icterohaemorrhagiae has been recognized by Bergey's Manual since 1948. A benefit of *L.*Icterohaemorrhagiae is its rapid growth compared to other serovars. Some serovars require 16 weeks. The OIE (2018) even suggests incubating samples for up to 26 weeks.

Because leptospires nestle in the kidney tubules, chronically infected animals allegedly discharge large amounts of leptospires into the urine (Steinparzer et al. 2017). Most available literature on leptospirosis in swine agree that leptospires are found in the urine of infected pigs (Jacobs et al. 2015) and that leptospires grow well under aerobic conditions, at temperatures between 28–30°C and a pH range of 7.2–7.6 (Strutzberg-Minder et al 2011, Faine et al. 1999). Establishing a culture from contaminated urine is nonetheless challenging. Time intervals for urine sampling are not standardized and collected urine has different pH-levels (Nervig et al. 1977). As seen in other sampling material, the slow growth of leptospires in urine also results in the overgrowth of other microorganisms (Chakraborty et al. 2011).

Conflicting reports on the survival of leptospires in undiluted urine can also be found. Soto et al. (2006), for example, infected six pregnant sows with *Leptospira interrogans* serovar Canicola. No leptospires were detected in the urine of the inoculated sows, not even by PCR, which is the most sensitive method available. However, leptospires were detected in the kidneys and liver of the euthanized sows by PCR methods. Rocha et al. (1992) inoculated three sows with *L*. Mozdok and were able to detect leptospires indirectly in the urine with the help of the MAT. Miraglia et al. (2008) found one out of 22 urine samples to contain leptospiral DNA with the help of PCR. The samples gathered in the latter study were collected at a slaughterhouse in Sao Paulo, Brazil. The leptospiral DNA found belonged to the serovar *L*. Pomona. A handful of sources claim that Leptospiruria happens intermittently and the timing in taking the urine samples is crucial but actual proof of this claim is lacking (Ospina-Pinto et al. 2019, Strutzberg-Minder et al. 2011, Bolt et al. 1995). One publication found validates the occurrence of *L*. Icterohaemorrhagiae antibodies using MAT in pig serum (Couto Roloff Padilha et al. 2019), but only one outdated publication could be found affirming the occurance of *L*. Icterohaemorrhagiae in urine of infected pigs (Hathaway et al. 1981).

The conclusion, subject to the information gathered, is that there is an unsatisfying current situation. There is contradicting information about leptospires being found in urine, few laboratories that routinely isolate leptospires for testing and most publications use PCR or serology for testing. The general knowledge is limited or outdated.

3. Materials and Method

3.1. First experiment: Urine Spiked with pure culture *L. interrogans* serovar Icterohaemorrhagiae

For this experiment, 30 spontaneous urine samples from sows of the University of Veterinary Medicine Vienna's pig farm as well as a private piglet-producing farm in lower Austria were collected. Samples 1–13 were taken from duroc x german landrace sows and samples 14–30 from german landrace x pedigree pig sows. The pH was measured for each urine sample at the time of collection (pH/Temperature Measuring instrument, testo). The samples were kept frozen at -20 °C until the start of the experiment. In tbl. 2 the pH at time of collection and at the start of this experiment can be seen in comparison.

Table 2: pH at time of collection and at the start of this experiment, after thawing.

Urine Sample	pH at Collection	pH after Thawing
1	7.53	7.87
2	7.50	7.84
3	6.70	6.36
4	7.75	8.02
5	7.40	7.64
6	7.60	7.71
7	7.50	7.86
8	7.36	7.66
9	7.35	7.63
10	7.42	7.78
11	8.10	8.35
12	8.07	8.54
13	7.42	7.66
14	7.02	7.03
15	7.44	7.47
16	7.10	7.17

17	7.13	6.86
18	6.80	6.86
19	6.61	6.48
20	6.64	6.65
21	6.90	6.93
22	6.93	7.03
23	7.01	7.07
24	6.98	7.01
25	6.43	6.38
26	6.67	6.74
27	7.01	7.52
28	6.78	6.76
29	7.05	7.28
30	6.77	6.71

Before spiking the urine with leptospires, the density of a suspension of pure culture *L.* Icterohaemorrhagiae was determined. Using a Helber counting chamber, a density of 10^9 leptospires/ml suspension was counted following the instructions of the protocol contributed by Richard L. Zuerner (2005). Meanwhile, the urine samples were thawed at room temperature. The pH of each sample was measured again as described above, determining the starting value of the experiment.

The Ellinghausen, McCullough, Johnson and Harris (EMJH) semi-solid medium with and without antibiotics was chosen for cultivation. The medium with antibiotics was used to evaluate the leptospiral growth. The medium without antibiotics was used to assess the amount of growth of other unwanted secondary bacterial strains, which commonly overgrow leptospire cultures. According to Faine et al. (1999), using a filter with a pore diameter of 0.2 µm or greater prior to cultivation will allow the leptospires to pass through but keep unwanted bacteria out. For this, one part of suspension of urine and leptospires was filtered in order to investigate whether this could replace antibiotics in the culture medium or not.

Controls were processed exactly as described below with liquid medium instead of using urine. Two controls were performed for each of the three conditions. Additionally, two tubes of medium with and without antibiotics with no leptospires added were kept to ensure that the medium was not contaminated.

A total of 900 µl of each urine sample was aliquoted three times into Nunc^{TM} disposable round-bottom test tubes (30 tubes each for EMJH without antibiotics, EMJH with antibiotics and for filtered samples in EMJH without antibiotics) (fig.1). The EMJH with antibiotics contains 100 µg/ml 5-Fluorouracil, 40 µg/ml EMJH Sulfmethoxazole, 20 µg/ml EMJH Trimethoprim, 5 µg/ml EMJH Amphotericin B, 200 µg/ml EMJH Fosfomycin, which is the standard formula used in the laboratory where this experiment was conducted (Chakraborty et al. 2011).

Subsequent steps involved living *L.* Icterohaemorrhagiae isolates and required that work be carried out in a vertical laminar airflow cabinet (BH-2000 S/D, Faster). 100 µl of the pure culture was added to each urine sample (fig.1), except for the samples dedicated for filtering (see fig. 3), resulting in a 1:10 dilution (i. e. 10^8 leptospires/ml). After mixing well by pipetting up and down at least 5 times and gently swirling the test tube, the mixture was left at room temperature for 30 minutes to allow possible pH-dependent effects to develop. The reasoning behind the 30 minute time interval is that fact that sows often excrete urine every 30 minutes, especially if they have a bladder infection (Cortus et al. 2005). This means the leptospires are in contact with urine for 30 minutes before they leave the pig's body. Then, 100 µl of urine/Leptospire suspension was added to 10 ml liquid medium with or without antibiotics (except for samples set aside for filtering), yielding a 1:100 dilution (i. e. 10^6 leptospires/ml). For the filtered samples, 100 µl of the 10^8 leptospires/ml suspension was added to 900 µl of urine (i. e. 10^7 leptospires/ml). After mixing well and further 30 minutes incubations, the complete 1000 µl of the suspension was added to the respective medium through the Acrodisc Syringe Filters (with 0.8/0.2 µm Supor Membrane), using Codan single use syringes and needles by Luer. 50% of the initial volume passes the filter. The diagrams below (fig. 1,2 and 3) provide a helpful tool in understanding the steps involved in this experiment.

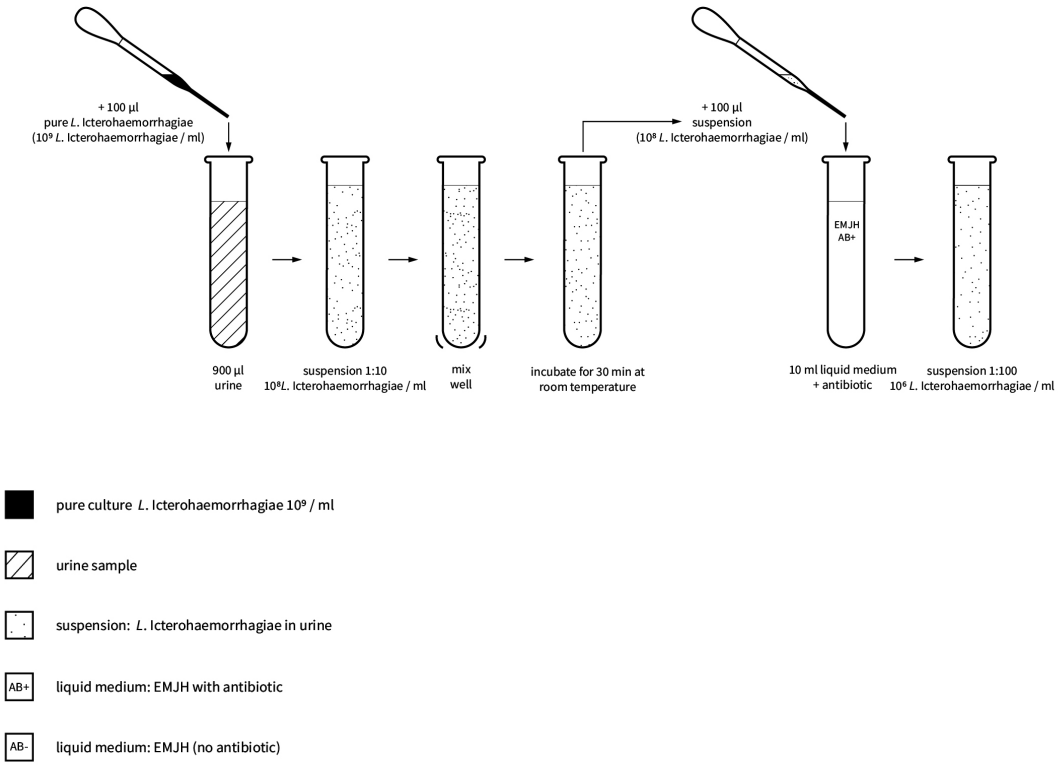

Figure 1: Method for creating a urine + leptospire suspension in EMJH with antibiotics and its legend.

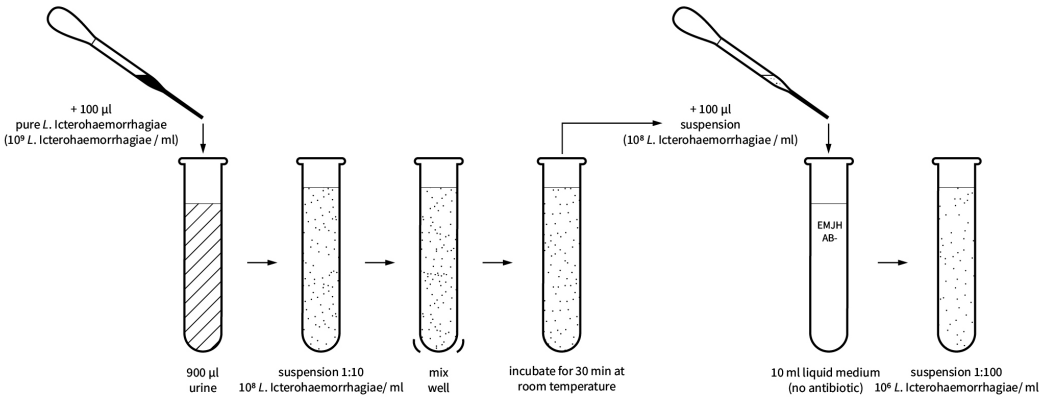

Figure 2: Method for creating a urine + leptospire suspension in EMJH without antibiotics. Refer to fig. 1 for legend.

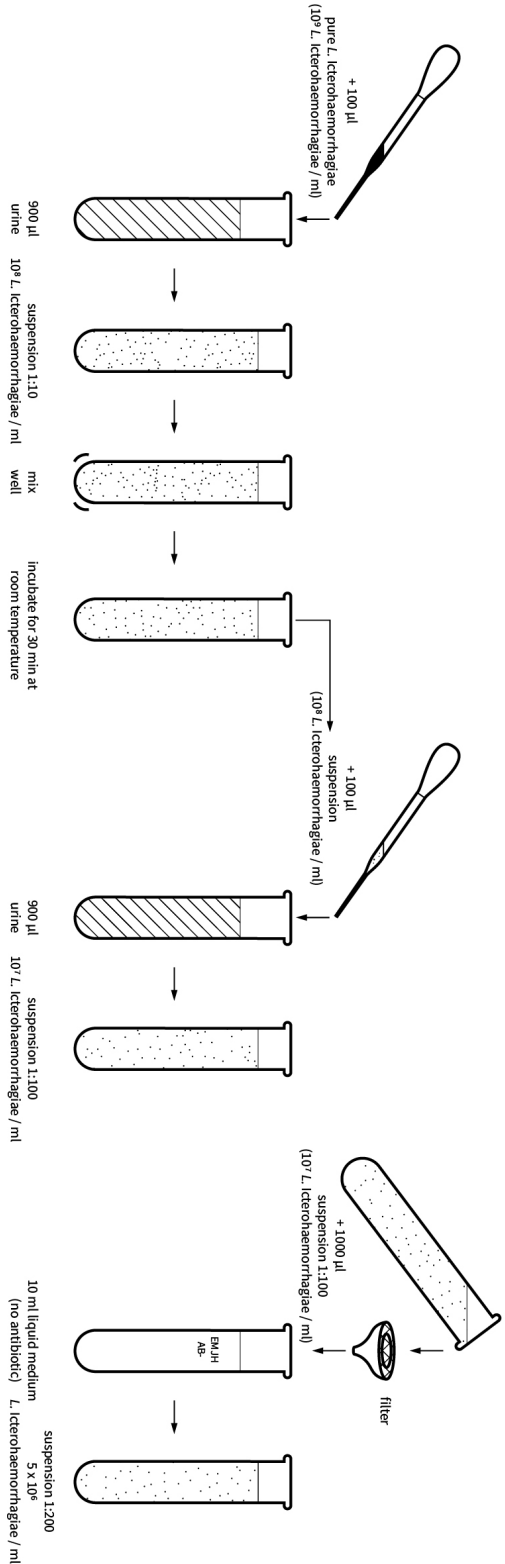

Figure 3: Method for creating a urine + leptospire suspension in EMJH without antibiotics by filtering the leptospire + urine mixture. Refer to fig. 1 for legend.

Finally, all samples were incubated in the ESCO Isotherm Forced Convection laboratory Incubator (Isocide^{TM}), which operated at 29 °C, for one month. Samples were checked visually for turbidity every workday, by placing the sample in front of a strong light source and gently shaking it. Small swirls of white substance can be seen if the sample is turbid. Turbidity is a sign for growth of bacteria. After every seven days, samples were assessed by dark-field microscopy for either: only leptospires, leptospires with contamination, contamination only or no growth (See fig. 4 A-D for reference). 10 µl of each sample was carefully placed on to a microscope slide and a cover glass was gently put on top. Each slide was labeled and then looked at underneath the dark field microscope at 40 x resolution. In order to standardize the evaluation, microscopy slides were checked five times from one side to another in five horizontal lines altogether. This procedure was done every seven days over the course of four weeks.

The amount of Leptospira was categorized into three groups:

Table 3: Categories of evaluation underneath the dark field microscope

Category	Amount of leptospires in one field of vision
+	1–5
++	5–100
+++	> 100

For illustration of these categories, fig. 4 A–C show dark field microscopy images as reference. Fig. 4 D depicts contamination by unknown bacteria.

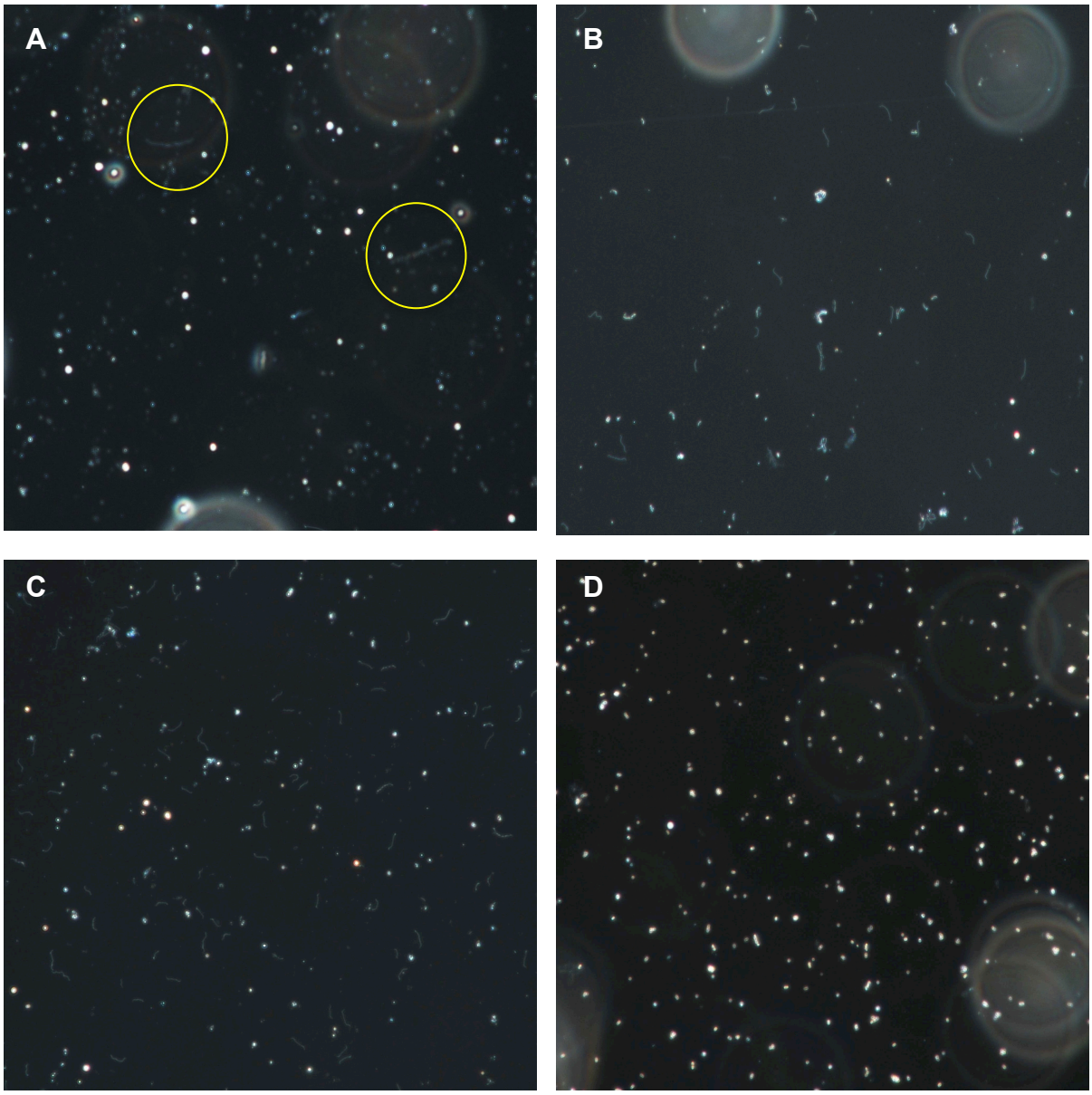

Figure 4: A–C illustrate the different categories from tbl. 3. (A) Category „+“: 1–5 leptospires found in one field of vision using a dark field microscope. (B) Category „++“: 5–100 leptospires and (C) Category „+++“: > 100 leptospires. (D) Illustrated contamination with unknown bacteria.

3.2. Second experiment: Dilution Series

Ten urine samples from the 30 pigs used above were selected based on rapid leptospire growth as determined in the experiment described above. The pH was re-measured as described earlier and listed in tbl 4.

Table 4: overview of the ten urine samples used for the dilution series and their respective ph.

Urine Sample	pH
1	8.32
2	8.19
6	7.94
7	8.03
8	7.95
9	8.34
13	8.04
14	7.16
15	7.72
27	7.67

As illustrated in fig. 5, 4950 µl of each urine sample was aliquoted into 15 ml tubes (1). 500 µl each were put into 3 Nunc^{TM} disposable round-bottom test tubes (2, 4, 6), and 900 µl into another three of these tubes (3, 5, 7) in preparation for a dilution series. EMJH with antibiotics was used in the final samples due to the results gained in experiment one. The leptospires grew better with less contamination in EMJH with antibiotics.

Before inoculating the dilution series, the density of a pure culture of *L.* Icterohaemorrhagiae had to be measured as described above, yielding 8.6 x 10^8 leptospires/ml. 50 µl culture was added to tube 1 and thoroughly mixed. From there, 100 µl were placed into tube 3, pipetting up and down at least five times to assure a well-mixed suspension. Using the same pipette tip, 100 µl were placed from tube 3 into tube 5 and finally to tube 7, where the pipette tip is emptied and discarded.

For tubes 2, 4 and 6, 500 µl were transferred from tube 1 into tube 2, mixed thoroughly, discarding the tip afterwards. From tube 3, 500 µl were placed into tube 4. From tube 5, 500 µl were placed into tube 6. The complete dilution series was incubated for 30 min in order for the pH to take effect on the leptospires. The last step was to transfer 100 µl of each urine sample and each dilution step into liquid media containing antibiotics. All cultures were placed into the incubator at 29 °C. For control samples, the same steps were performed. All steps containing living leptospires were carried out in the vertical laminar airflow cabinet. As described above for experiment one, the samples were checked every workday for turbidity and by dark-field microscopy once per week over the course of seven weeks.

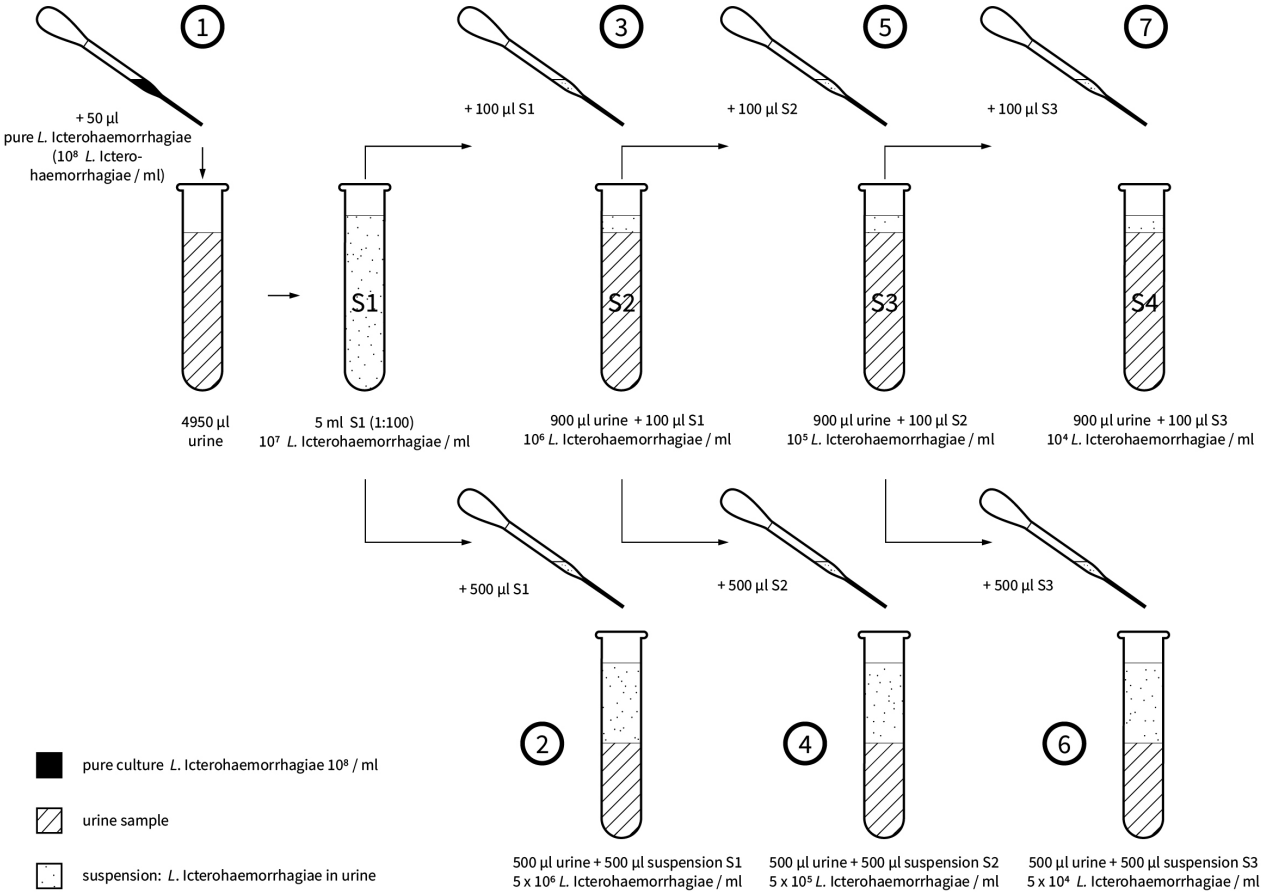

Figure 5: Method for creating the dilution series.

3.3. Third experiment: Adding Phosphate Buffer to influence the pH-level

The pH of ten urine samples, which showed very little growth in experiment one, was measured. 5 ml of phosphate buffer (1000 ml contains 8.50 g NaCl, 0.49 g KH_2PO_4, 1.14 g Na_2HPO_4 $2H_2O$, und Aqua bidest; pH 7.3) was added to 5 ml of each sample. The pH was measured again. The samples were left at 4 °C overnight in order to imitate the transportation of samples from the place of collection to the laboratory. The pH was measured again the next day. Refer to tbl. 7 in the results chapter for the pH measurements. Continuing the experiment, 900 µl of each urine sample was placed into a Nunc™ disposable round-bottom test tube. 900 µl of EMJH with antibiotics was also added into a Nunc™ disposable round-bottom test tube, which acted as our control sample. 500 µl of Phosphate buffer was added to another ten Nunc™ disposable round-bottom test tubes.

The following steps were performed in the vertical laminar airflow cabinet. As before, the density of the pure leptospiral culture was measured ($6x10^8$ leptospires/ml). 100 µl of pure leptospire culture was added to the urine samples and the control sample. Each sample is vortexed and thoroughly mixed by pipetting up and down at least 5 times. From the urine and leptospire mixture, 500 µl was added to the tubes with the phosphate buffer. The control sample was processed in the same way. To imitate the transportation of urine samples from the place of collection to the laboratory, the samples containing leptospires and buffer and the samples containing leptospires but no buffer, were stored overnight at 4 °C.

The next day, 100 µl of each urine and leptospire mixture was added to ten tubes of EMJH medium without antibiotics and ten tubes EMJH medium with antibiotics. The same step is done a second time but with the urine, leptospire and buffer mixture. Before pipetting each sample into the medium, the samples were mixed well, either by using a vortexer or pipetting up and down. For four weeks, the samples are checked for turbidity each day, and once per week, each sample is checked by dark-field microscopy as described above.

3.4. Genomic DNA extraction

From a pure culture of *L.* Icterohaemorrhagia, genomic DNA was extracted and quantified. First, the culture's density was measured with the help of the Helber Counting Chamber.

DNA extraction was performed from 1 ml of pure culture stock using the MagAttract® HMW DNA Mini Kit according to manufacturer's instructions and eluted in 100 µl. The concentration was measured spectrophotometrically and fluorometrically on the Denovix DS-11 FX. For comparison and double checking, the same sample was measured using the Qbit® assay kit according to manufacturer's instructions.

3.5. Real-Time quantitative PCR

1 ml of each spiked urine sample from experiment one was set aside for rt-qPCR at the beginning and at the end of the experiment. These samples were boiled at 70°C to inactivate the leptospires and were then stored at -20 °C. Before starting the rt-qPCR, the samples were thawed to room temperature.

The Rt-qPCR was conducted using the QuantiTect Multiplex PCR noROX Kit using the C1000 Touch™ Thermal Cycler (Bio-Rad) to manufacturer's instructions.

QuantiTect Multiplex PCR NoROX Master Mix includes:

- Lip32-Mix-FAM
- Bactin-Mix2-HEX/BHQ1
- 50X ROX Passive Ref. 1:10
- RNase free water

The PCR protocol was (45 cycles of steps 1-3):

1. 95 °C for 15 minutes
2. 94 °C for 1 minute
3. 60 °C for 1 minute

3.6. Statistical Methods

For experiments 3.1. and 3.3., a chi-squared-test (X^2-Test) was performed comparing the different groups. Results are considered statistically significant when $p < 0.05$. Furthermore, descriptive methods were used in order to analyse the results visually.

4. Results

4.1. Urine spiked with pure culture *L. interrogans* serovar Icterohaemorrhagiae

Tbl. 5 gives an overview of the number of urine samples showing leptospiral growth in different media during the experimental setting of four weeks. It is split up into urine samples with a pH below seven and above seven. Two interesting findings could be shown: The presence of leptospires in the medium without antibiotics (AB-) peaked in week one and decreased each week thereafter due to contamination by other bacteria. Secondly, the growth of leptospires in the medium with antibiotics (AB+) had its peak in week two while also having a higher growth percentage all together. No growth could be seen using the filtered samples throughout the four-week period, including in the controls. All these results can be seen best in fig. 6.

Table 5: The growth of leptospires is categorized into +, ++ and +++. "+" is equivalent to one to five leptospires visible underneath the microscope, "++" five to 100 leptospires and "+++" 100 or more leptospires.

		< pH 7				**contamination only**	**> pH 7**			
		0	+	++	+++		0	+	++	+++
Week 1	AB-	1	3	0	2	12	1	3	0	8
	AB+	7	2	0	1	0	4	5	2	9
	F	10	0	0	0	0	20	0	0	0
Week 2	AB-	3	0	1	3	13	2	0	0	8
	AB+	7	2	0	1	0	3	3	4	10
	F	10	0	0	0	0	20	0	0	0
Week 3	AB-	2	0	2	1	15	2	0	1	7
	AB+	9	0	0	1	0	5	1	4	10
	F	10	0	0	0	0	20	0	0	0
Week 4	AB-	2	0	1	2	16	2	0	2	5
	AB+	9	0	0	1	0	5	2	1	12
	F	10	0	0	0	0	20	0	0	0

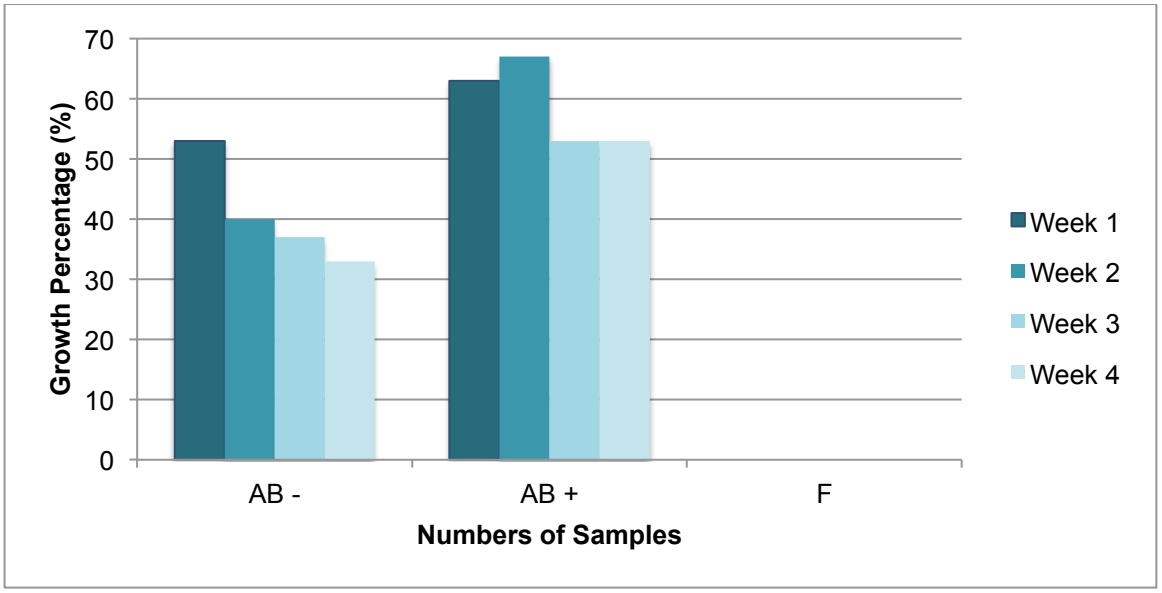

Figure 6: Four-week comparison of the presence of leptospires of samples without antibiotics (AB-), with antibiotics (AB+) and samples that have been filtered (F). The "Growth Percentage" represents the percentage of urine samples with successful leptospiral growth.

The control groups of AB- and AB+ both showed presence of leptospires after the first week and both were contaminated with unidentified bacteria after the four-week period. The empty controls, meaning only the liquid medium without anything added, showed no growth of any kind of bacteria throughout this experiment. Furthermore, the process of freezing and thawing the urine had little influence on the PH of the urine sample, as can be seen in tbl. 2.

The most important information that can be gathered from this data is shown in fig. 7. In these bar graphs, the percentage of growth of leptospires in medium with and without antibiotics from urine samples with pH levels less than or greater than pH 7 were compared each week. It becomes visually obvious that there is a higher percentage of growth in urine with a pH above 7, regardless of the presence of antimicrobials in the medium. The filtered samples were omitted because no growth of any kind of bacteria was found.

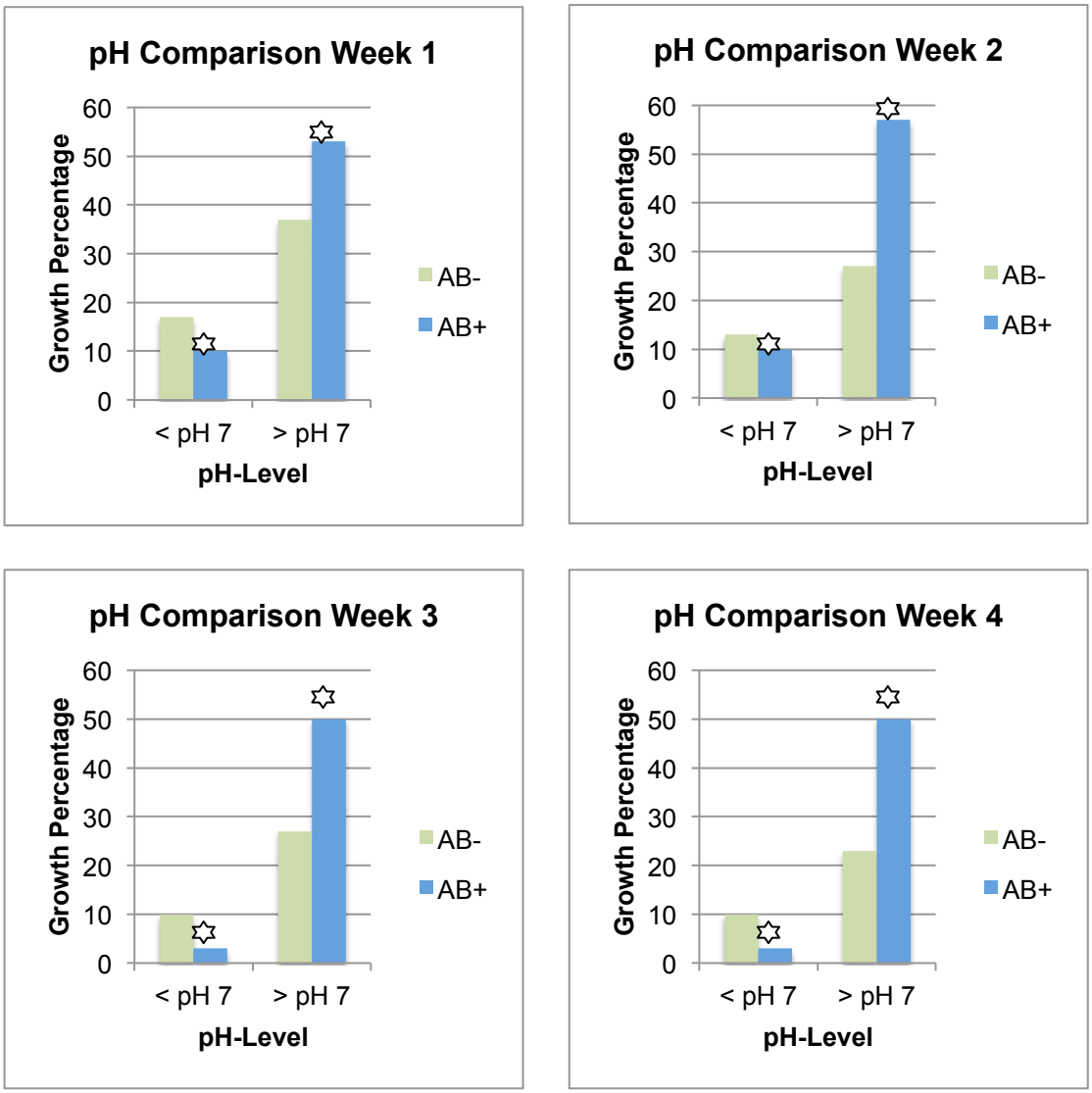

Figure 7: Comparison of the growth of leptospires in samples with pH below and above seven throughout the four-week period. AB- stands for medium without antibiotics and AB+ stands for medium with antibiotics. ☆ = $p < 0.05$.

Apart from visually evaluating the data, a chi-square test was conducted, which validated the hypothesis that leptospires showed better growth in neutral to alkaline urine in medium with antibiotics. Results were considered significant, when $p < 0.05$. Samples without antibiotics showed similar results visually but they were not statistically significant.

4.2. Dilution Series

In tbl. 6, the percentage of leptospiral growth each week and dilution step is shown. The higher the dilution of the suspension the longer the time needed for growth and the lower the percentage of samples showing growth. It also becomes visible, that at dilution step six and seven the biggest increase in growth was shown between weeks five and six. Fig. 8 portrays the information shown in tbl. 6 in graph form so as to easier grasp the concept.

Table 6: Growth of leptospires in percentage (%) for each week and dilution step. The left column is the weeks and top row are the dilution steps. Refer to figure 9 for the respective concentrations. The individual cells are the percentage of samples where leptospires grew.

	Dilution Steps						
Week	1	2	3	4	5	6	7
0	0	0	0	0	0	0	0
1	90	70	50	30	0	0	0
2	90	80	60	40	0	0	0
3	90	80	70	80	50	0	0
4	90	80	80	80	50	10	10
5	100	90	80	80	70	30	10
6	100	90	80	80	80	60	50
7	100	90	90	90	80	60	60

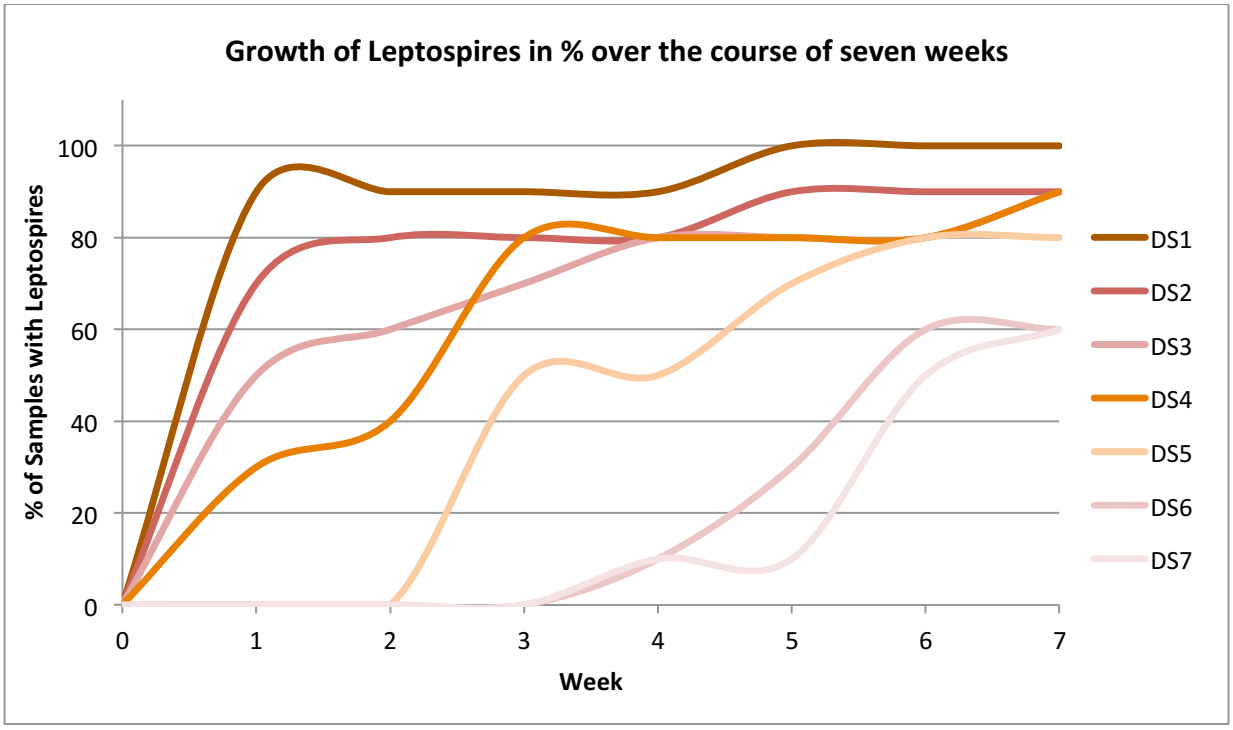

Figure 8: Presentation of the growth of leptospires in percentage over the course of seven weeks. DS: dilution steps.

4.3. Adding Phosphate Buffer to influence the pH-level

The difference in pH after the addition of buffer can be seen in tbl 7. Due to the buffer, which has a pH of 7.3, the pH of the urine samples inched closer to a pH of 7.3. Storing them overnight increased the pH of all samples. A clear difference between the samples with added phosphate buffer and the samples without could be shown. Leptospires grew faster in the samples with added phosphate buffer. The samples with antibiotic free medium showed a faster growth, both with and without buffer, than the samples with added antibiotics. However, the massive decrease in the slope of the samples, when looking at the graph, without antibiotics and buffer, showed a high contamination only within one week and a consequent elimination of leptospires in 20 % of the samples. It must be noted however, that the results are not statistically significant ($p < 0.05$). This is likely due to the small number of samples used. The leptospires in the samples with phosphate buffer grew faster and at a

higher concentration, which is important information for practical use. Tbl. 8 and fig. 9 show the percentage of growth in leptospires over the course of four weeks.

Table 7: Comparison between pH of urine samples before and after the addition of phosphate buffer and after storing the samples overnight at 4 °C.

Urine Sample	pH	pH + Buffer	pH + Buffer + Storage Overnight
4	8.39	7.86	8.04
10	8.21	7.62	7.91
17	7.04	7.36	7.53
18	6.94	6.99	7.17
19	6.39	6.74	6.98
20	6.72	7.02	7.21
21	6.86	7.05	7.23
25	6.50	6.85	6.94
27	7.71	7.46	7.52
30	6.91	7.08	7.31

Table 8: Summary of the percentage of samples that showed growth of leptospires. AB- stands for medium without antibiotics, AB+ for medium with antibiotics. Buffer- means that no phosphate buffer was added while Buffer+ means that phosphate buffer was added.

	AB -		AB +	
Week	Buffer -	Buffer +	Buffer -	Buffer +
0	0	0	0	0
1	60	80	30	50
2	60	60	40	50
3	60	60	60	60
4	60	70	60	60

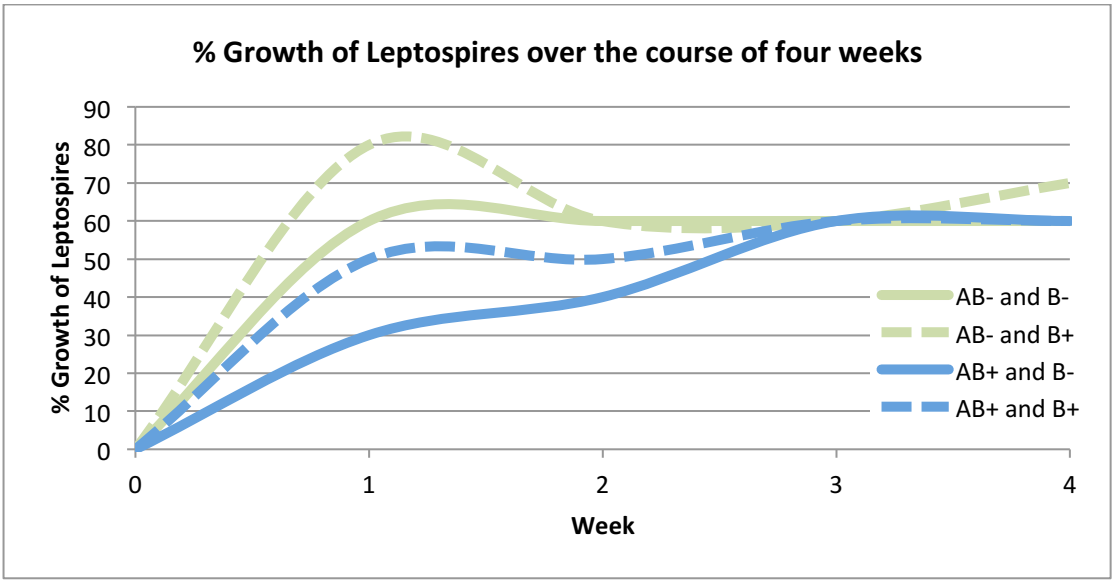

Figure 9: Percentage of samples that showed growth of leptospires over the course of four weeks. The green lines represent medium without antibiotics while the blue lines represent medium with antibiotics. The dashed lines are the samples where phosphate buffer was added.

4.4. Genome Quantification

In order to double check our results, all samples were checked by rt-qPCR to see if there are leptospires in the samples or not. To be able to have a point of reference, the genome of the pure culture *L.* Icterohaemorrhagiae was quantified.

Firstly, using the Helber counting Chamber, a density of 8.8 x 10^8 leptospires/ml was evaluated from the pure culture. The same sample was measured three times using a spectrophotometer and a fluorometer.

Table 9: Measurements of pure culture *L.* Icterohaemorrhagiae using a spectrophotometer.

Measurement	Results in ng/µl
1	12.58
2	13.05
3	12.99

Table 10: Measurements of pure culture *L.* Icterohaemorrhagiae using a Fluorometer

Measurement	Results in ng/µl
1	5.39
2	5.45
3	5.83

The average result of the measurements taken with the Fluorometer is 5.56 ng/µl, which is 0.0056 g/ml. The DNA size *L.* Icterohaemorrhagiae in base pairs is 4698134. With these two measurements, the number of copies per µl stock can be calculated. The result is 1.09 + 10^6 copies/µl Stock. By multiplying it with 10^2 and thus backtracking it to the 1ml Stock, the result is 1.0^9 x 10^8 copies/ml stock.

The graphs below offer a visual representation of the genome. Fig. 10 shows the amplification of the DNA and the dilution steps of the sample. The higher the concentration, the less cycles are needed for the PCR to detect leptospiral DNA. Fig. 11 shows the same results as a standard curve.

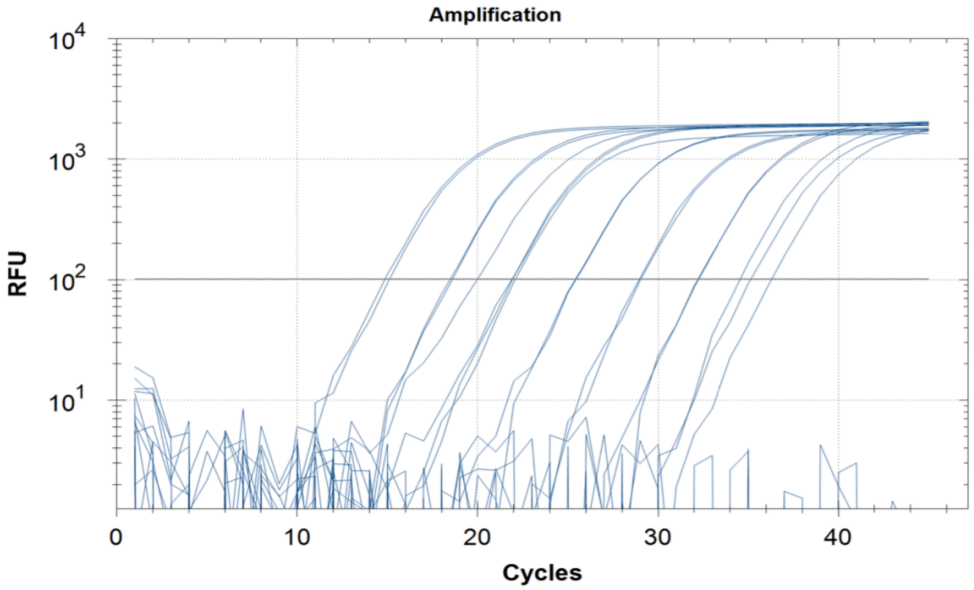

Figure 10: Amplification of pure culture *L.* Icterohaemorrhagiae with real time PCR. RFU stands for relative fluorescence units

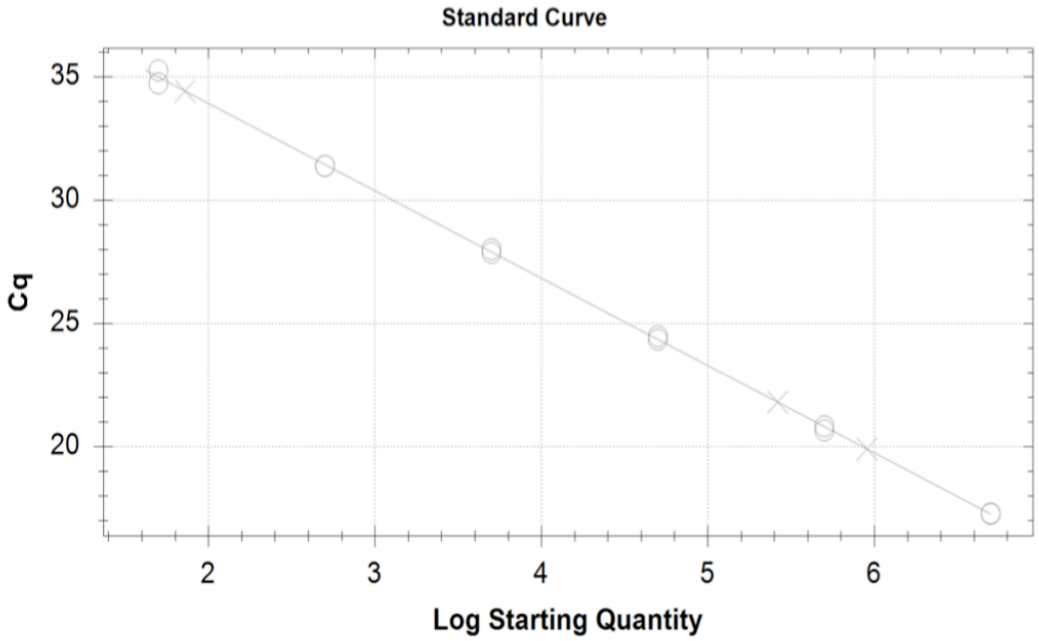

Figure 11: Standard Curve of Genome Quantification of pure culture *L. Icterohaemorrhagiae*. Cq stands for quantification cycle.

4.5. Real-Time qPCR

The aim of including rt-qPCR is to be able to validate the results gathered in experiment one. Using rt-qPCR, all pure urine samples and the spiked urine samples collected at the start and end of the first experiment, were checked for leptospires.

All pure urine samples were negative, meaning there were no leptospires in any sample. The filtered samples were also negative, both at the beginning of the experiment and at the end of the experiment, validating our results. In all remaining samples, the growth of leptospires could be confirmed by rt-qPCR .

All detailed results are summarized in fig. 12–23.

4.5.1. Samples with antibiotics – Start of Experiment

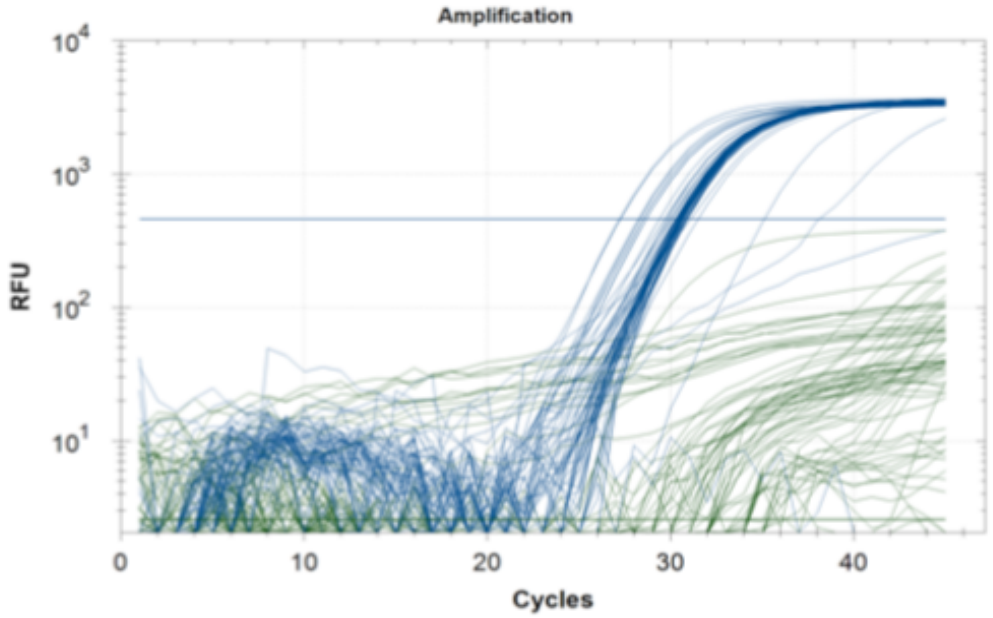

Figure 12: Amplification of the samples with antibiotics using real time PCR at the start of the experiment. RFU stands for relative fluorescence units

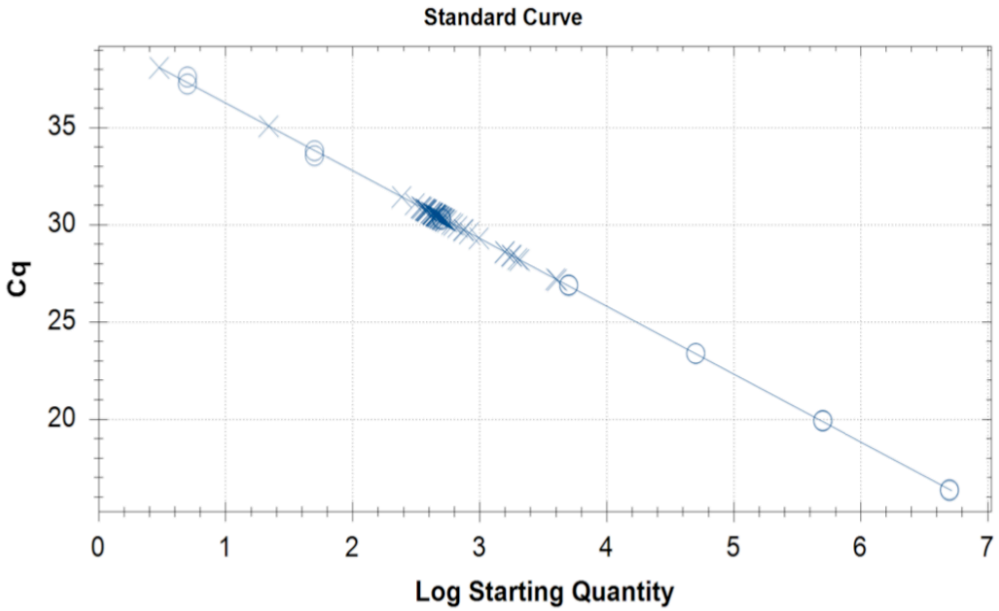

Figure 13: Standard Curve of the amplification of the samples with antibiotics at the start of the experiment. Cq stands for quantification cycle.

4.5.2 Samples with antibiotics – End of experiment

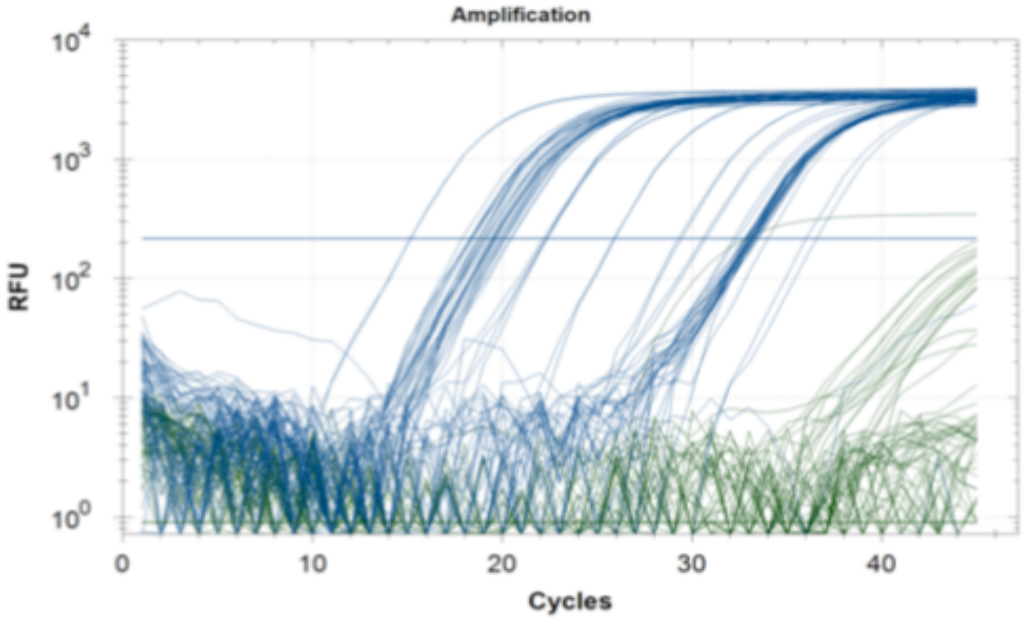

Figure 14: Amplification of the samples with antibiotics using real time PCR at the end of the experiment. RFU stands for relative fluorescence units.

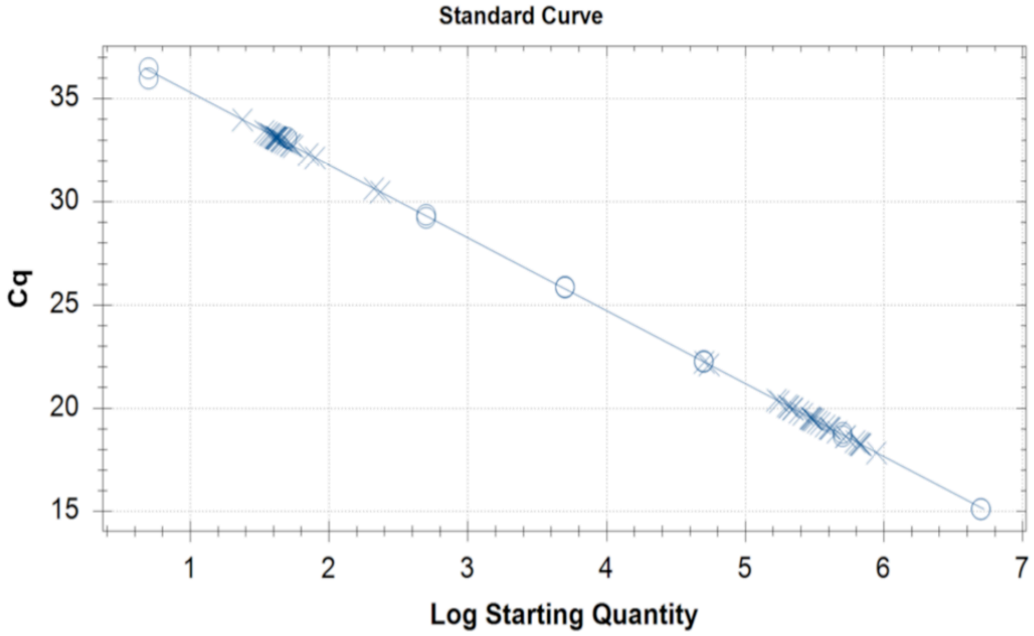

Figure 15: Standard Curve of the amplification of the samples with antibiotics at the end of the experiment. Cq stands for quantification cycle.

4.5.2. Samples without antibiotics – Start of experiment

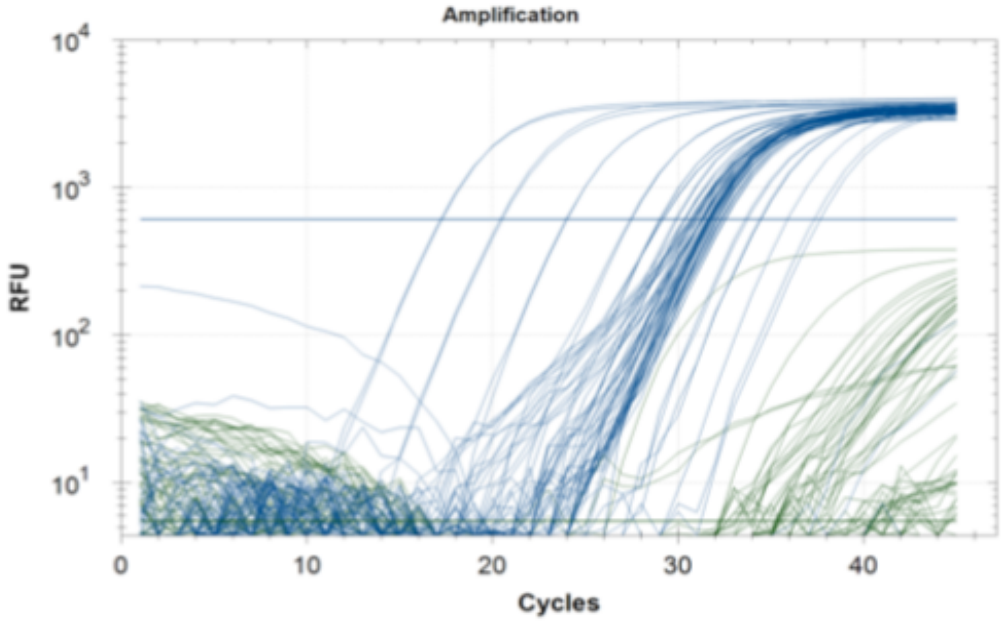

Figure 16: Amplification of the samples without antibiotics using real time PCR at the start of the experiment. RFU stands for relative fluorescence units.

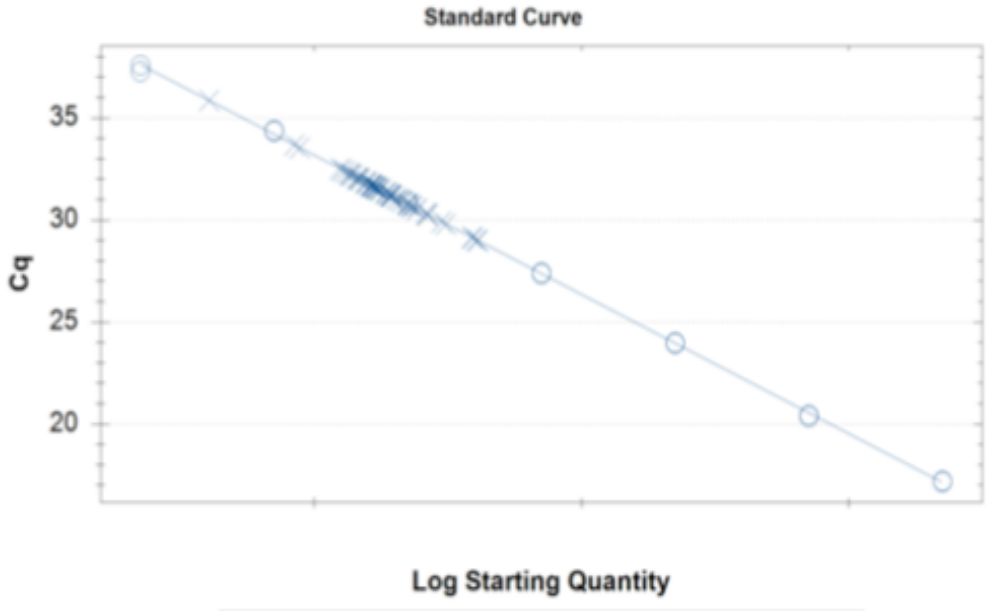

Figure 17: Standard Curve of the amplification of the samples without antibiotics at the start of the experiment. Cq stands for quantification cycle.

4.5.3. Samples without antibiotics – End of experiment

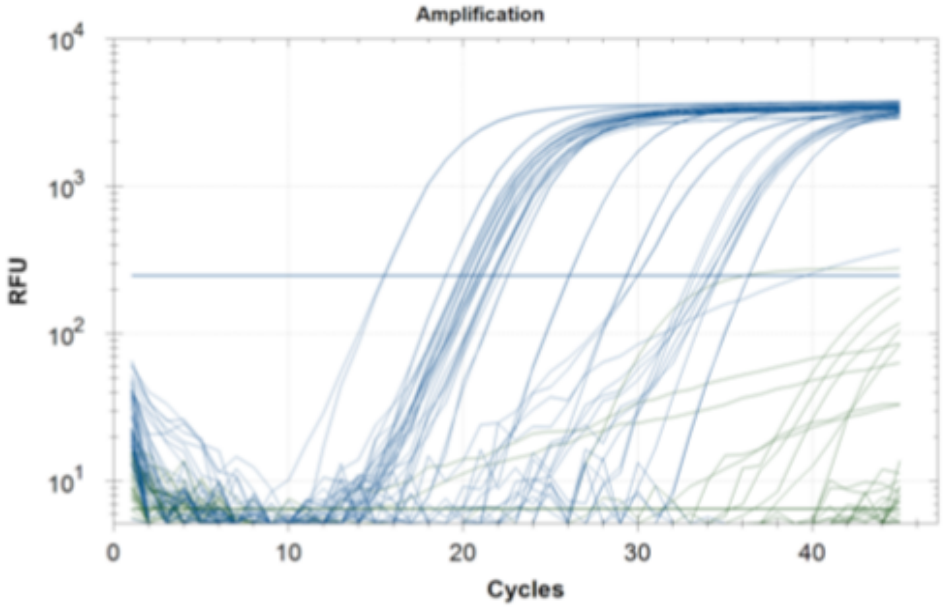

Figure 18: Amplification of the samples without antibiotics using real time PCR at the end of the experiment. RFU stands for relative fluorescence units.

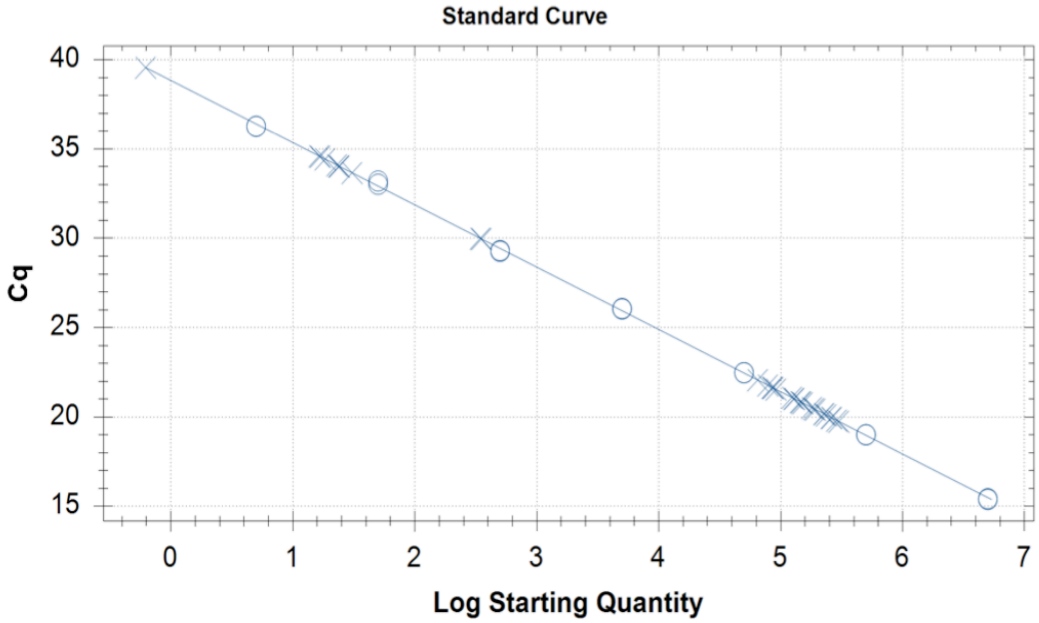

Figure 19: Standard Curve of the amplification of the samples without antibiotics at the end of the experiment. Cq stands for quantification cycle.

4.5.4. Filtered Samples – Start of experiment

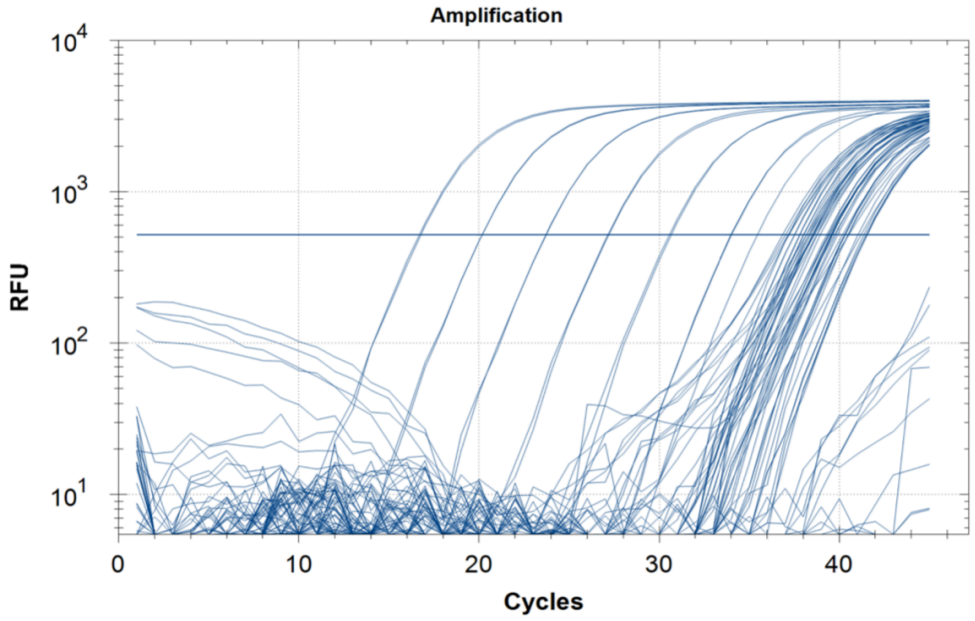

Figure 20: Amplification of the filtered samples using real time PCR at the start of the experiment. RFU stands for relative fluorescence units.

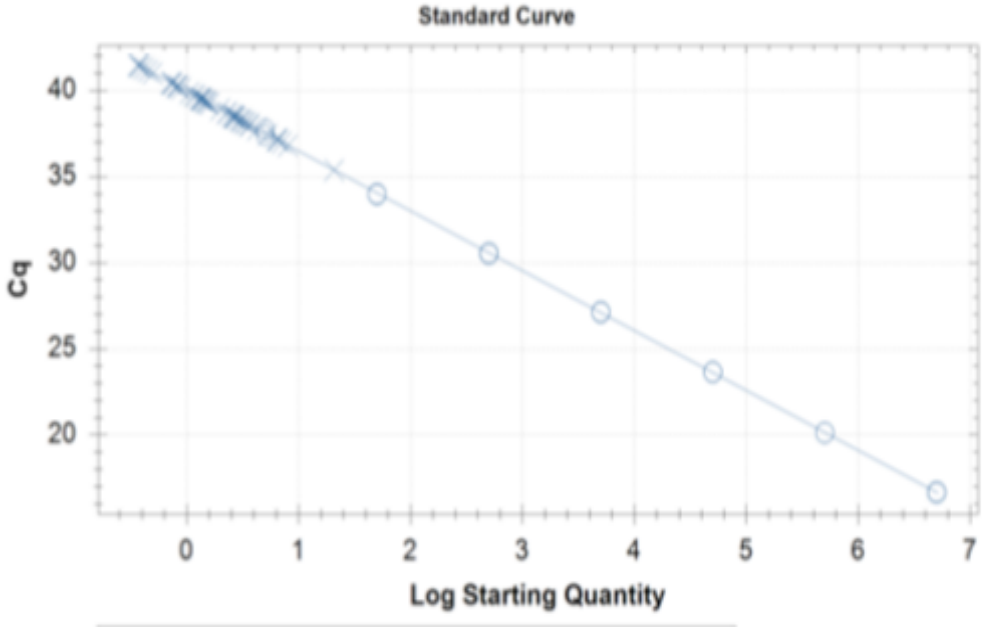

Figure 21: Standard Curve of the amplification of the filtered samples at the start of the experiment. Cq stands for quantification cycle

4.5.5. Filtered Samples – End of Experiment

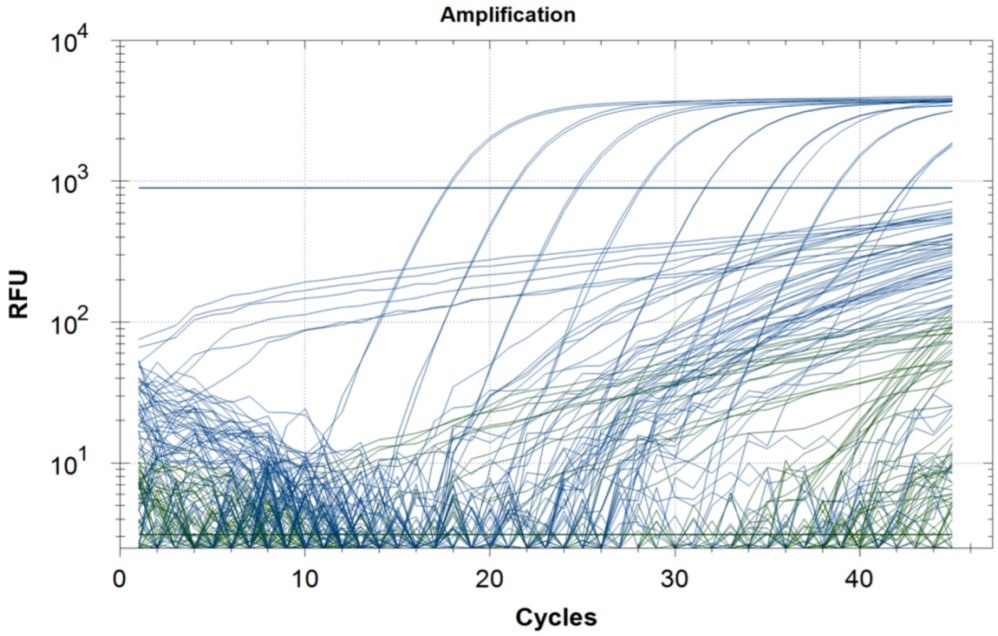

Figure 22: Amplification of the filtered samples using real time PCR at the end of the experiment. RFU stands for relative fluorescence units.

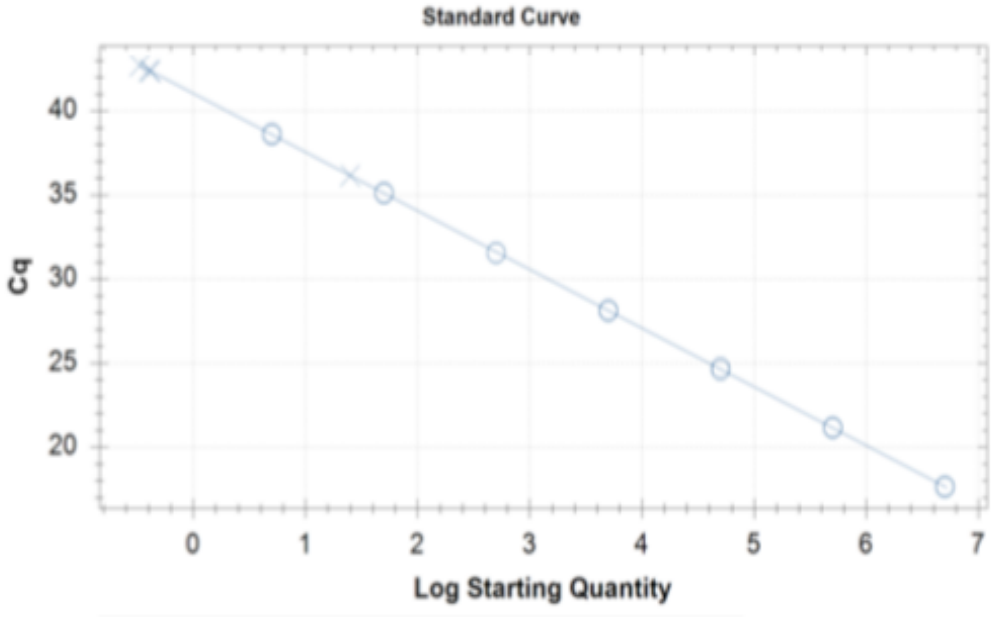

Figure 23: Standard Curve of the amplification of the filtered samples at the end of the experiment. Cq stands for quantification cycle.

5. Discussion

All three experiments were conducted to achieve a better understanding of the causative factors behind the difficulties in isolating leptospires from urine. We set out to answer questions such as what medium should be used, does pH influence growth, what initial concentration of leptospires is required and can the growth of these spirochetes be influenced by adding phosphate buffer.

L. Icterohaemorrhagiae grew best at pH-levels between 7.5 and 8.00. This is in accordance with Faine et al. (1991), who concluded that optimum leptospiral growth occurs in the pH range of 7.2–7.6. Our results show that *L.* Icterohaemorrhagiae isolates grow best at neutral to alkaline conditions and in medium with antibiotics. We presuppose that is most likely true for all *Leptospira* serovars. The antibiotics in the medium most likely inhibit the growth of other bacteria, allowing the leptospires to reproduce and flourish. Due to this finding, the same medium was used for the dilution series. Because of these results, AGES has decided to preferentially use EMJH with antibiotics for their cultivation of leptospires. Interestingly, when comparing the first experiment with the last experiment and only taking into consideration the samples without added phosphate buffer, in more samples leptospires were detectable in the medium without antibiotics in week one and two after storing them for 24 hours. Possible explanations for this could be the change of pH after thawing the urine samples again (tbl. 2 and tbl. 7) as well as the diluting affect of the condensation that is created by the process of freezing and thawing. Furthermore, urine might contain nutrients that support the survival or growth of leptospires.

Faine et al. (1991) also suggested the use of selective media when attempting to isolate leptospires from body fluids, such as urine. However, the study suggests different antibiotics including 5 fluorouracil, vancomycin, polymyxin-B-sulfate or rifampicin. Compared to the media used in this thesis, the only overlap is 5-fluorouracil, which we used in addition to Sulfamethoxazole, Trimethoprim, Amphotericin B and Fosfomycin. These are the antibiotics of choice in the laboratory where this experiment was conducted and have proven themselves capable of preventing the overgrowth of unwanted bacteria.

Using medium without antibiotics had its problems with microbial contamination. By the end of the four weeks in experiment one, 14 samples had to be discarded because their contamination with unidentified bacteria was too high and no more leptospires could be found. Therefore, fewer samples were available for statistical analysis compared to the condition containing antibiotics. Faine et al. (1991) and Adler (2015) both suggest using selective media when attempting to isolate leptospires from tissue or body fluids.

Contrary to the claims of Faine et al. (1991), filtrating did not work in this experiment. No leptospires or other bacteria grew in the span of four weeks. One must take into consideration that only *L*. Icterohaemorrhagiae was researched and therefore no overall assumption can be made or interpreted. Nevertheless, this result was rather surprising because filtration is a common method used for contaminated pure cultures to eliminate contamination at AGES (Austrian Agency for Health and Food Safety Ltd.), where this research was performed and although it does take up to eight days for the filtrated culture to reach a plateau (much longer than non-filtered cultures need) in the laboratory, there is leptospire growth. This is also true for *L.* Icterohaemorrhagiae. The results gathered in this experiment suggest that filtering urine possibly contaminated with leptospires is not advisable. A possible explanation is the initial volume that was being filtered into the EMJH medium. Only 1 ml was used, the standard procedure at the laboratory is to use as much as 10 ml.

Another aspect to be discussed is the time the culture needs to be given for the leptospires to grow and after how many weeks it would be beneficial to transfer the culture to a new medium. Studies suggest leaving the cultures for up to six months (Miraglia et al. 2008), although this depends on the serovar and cannot be considered practical for routine diagnostics. As mentioned at the beginning, *L*. Icterohaemorrhagiae is known to grow quickly, which could be validated with the gathered results. In our first experiment, four weeks presented a good time to transfer the samples in medium with antibiotics to fresh medium, if the aim was to keep the cultures alive, which can be seen in fig. 6. However, when looking at the dilution series, a time span of five to six weeks was required to obtain reasonable results (fig. 8). By the end of six weeks, even the lowest starting concentration of most samples showed a growth of leptospires, while after four weeks, only the higher starting concentrations showed growth.

Using four weeks as a cut off, the dilution series experiment suggests that a concentration of at least 10^6 leptospires per ml is required in order for them to grow. Continuing the experiment for three weeks further, it turned out that a starting concentration of 10^5 leptospires per ml can lead to substantial growth of leptospires, and even a starting concentration as low as 10^4 leptospires per ml will lead to growth. After seven weeks, most samples with strong growth were contaminated with unidentified bacteria. These results can aid in the decision as to whether trying to isolate leptospires from an infected urine sample is viable. It should not be forgotten that even though the variables in each experiment were limited to one or two, for example pH and starting concentration, it is likely that each urine sample contained different additional substances, which may or may not inhibit or help leptospires in their growth. Urine samples from different pigs will never be the same but the results indicate that pH-level has an influence on the growth of leptospires.

Lastly, the use of phosphate buffer in order to alter the pH-level must be discussed. In a clinical setting, it is difficult to determine which material should be sent to laboratories and how to test for leptospirosis. The aim was to determine if adding phosphate buffer to urine with different pH levels would affect the growth of the leptospires. Phosphate buffer was, for example, already used in the experiment done by Ospina-Pinto et al. (2019). This study did not have the aim to see how this buffer influenced the growth of leptospires but assumed that it would maintain proper growing conditions for leptospires. However, no statistically significant difference between samples with and without buffer could be determined during the course of this study. The experiment could be repeated with a different kind of buffer, since the pH of the urine does influence the survival of leptospires, as proven in experiment one of this thesis. Adding the buffer to the urine caused the pH to lean closer to a neutral pH of 7.3, which is the pH of the phosphate buffer itself. Furthermore, the dilution of the urine caused by adding the phosphate buffer may also have an affect on the growth of leptospires. More importantly, the experiment should be repeated with a larger number of samples because it is likely that a statistically significant result would be gathered. Referring to fig. 9 in the results section, it becomes visually obvious that the addition of phosphate buffer has an impact on the growth of leptospires. Therefore, although there is no statistical difference between adding buffer and omitting it, the results are important for practical use.

Successful cultivation of leptospires in urine would be essential to get information about the isolate and to perform further tests. The results suggest that it is likely that at least the

serovar *L.* Icterohaemorrhagiae can be found in urine of infected pigs and therefore the chance for isolation exists. Next generation sequencing could determine the exact serovar. It is not known how many or which serovars are currently prevalent in a given country or region, and through globalization, animal transportation across borders, new serovars may enter the sow herds. Serological tests, such as MAT, are limited to a few serovars (OIE 2018, laboratory experience). Therefore, the serologic diagnostic methods could be improved by including the most prevalent serovars of this region.

PCR is less laborious and much quicker than MAT (Hernándes-Rodríguez et al. 2011) and therefore has become an important diagnostic tool, but has its limitations. PCR is not used to identify the infecting serovar in routine diagnostics (Steinparzer et al. 2017) and it does not only count the living leptospires but also dead ones (Adler 2015). The PCR primer based on lipL32, which was also used in our experiment, is the most commonly used one, but a general agreement on which PCR primers should be used is missing, and most PCR primer sets are designed with human tissue or body fluid samples in mind. Finally, validation is one of the main bottlenecks concerning the use of PCR in diagnosing animal leptospirosis, since each individual laboratory is responsible for their validation (OIE, 2018, Galloway et al. 2015). However, genome sequencing, as was performed in this experiment, is becoming more widely available, making direct serovar identification in routine diagnostics possible in the near future.

6. Conclusion

In conclusion, the study showed that the pH-Level of the urine has an influence on the growth of *L. interrogans* serovar Icterohaemorrhagiae and that EMJH medium with antibiotics provides a better environment for the leptospires to grow. The concentration of leptospires in the urine can be as low as 10^4 for them to grow according to the results gained in the dilution series. PCR is a valid diagnostic method but it has its limitations. Being able to isolate leptospires from urine or tissue would be beneficial for diagnostics because samples could be tested for the serovar that is currently the most prevalent in the region instead of testing only for a few serovars.

7. Abstract/Zusammenfassung

English:

Leptospirosis in pigs is a disease with severe economic losses for farmers. It leads to reproductive problems and thus to a decrease in production. Diagnosing leptospirosis is difficult, especially considering the numerous different existing serovars. Isolating leptospires from infected sow urine would offer the possibility for further diagnostics and accelerate the development of reliable techniques for routine diagnostics. Therefore, in this study, the role of pH, the initial concentration of leptospires and the influence of added phosphate buffer was taken under scrutiny. Neutral to mildly alkaline pH leads to better growth of leptospires while acidic pH negatively affects growth. With the help of a dilution series, it could be found that concentrations as low as 10^4 leptospires/ml led to growth. Concerning the addition of phosphate buffer, a tendency towards a positive influence could be seen when looking at the results visually but no statistical significance could be determined between the samples with and without buffer. All three experiments aim to improve isolation of leptospires so as to be able to test for the serovar that is currently most prevalent in the area and improve diagnostics.

Deutsch:

Leptospirose bei Schweinen ist eine Krankheit, die mit erheblichen wirtschaftlichen Verlusten verbunden ist. Sie führt zu Reproduktionsstörungen und damit zu einem erheblichen Leistungsrückgang. Die Diagnose von Leptospirose ist schwierig, insbesondere angesichts der zahlreichen verschiedenen Serovare. Die Isolierung von Leptospiren aus infiziertem Sauenurin würde eine weitere Diagnosemöglichkeit darstellen und die Entwicklung zuverlässiger Techniken für die Routinediagnose beschleunigen. Daher wurden in dieser Studie die Rolle des pH-Werts, die Anfangskonzentration von Leptospiren und der Einfluss von zugesetztem Phosphatpuffer untersucht. Ein neutraler bis schwach alkalischer pH-Wert führte zu einem besseren Wachstum von Leptospiren, während ein saurer pH-Wert das Wachstum negativ beeinflusst hat. Mit Hilfe einer Verdünnungsreihe konnte festgestellt werden, dass Konzentrationen von nur 10^4 Leptospiren/ml zu einem Wachstum führten. In Bezug auf die Zugabe von Phosphatpuffer konnte bei visueller Betrachtung eine Tendenz zu

einer positiven Beeinflussung festgestellt werden, es konnte jedoch keine statistische Signifikanz zwischen den Proben mit und ohne Puffer festgestellt werden. Alle drei Experimente zielten darauf ab, die Isolierung von Leptospiren zu verbessern, um auf das derzeit im Untersuchungsgebiet am häufigsten vorkommende Serovar zu testen und die Diagnostik verbessern zu können.

8. Abbreviations

MAT	Microscopic Agglutination Test
OIE	World Organization for Animal Health
EMJH	Ellinghausen-McCullough-Johnsen-Harris
AGES	Austrian Agency for Health and Food Safety Ltd.
rt-qPCR	Real-Time quantitative PCR
NGS	Next Generation Sequencing
L. interrogans	*Leptospira interrogans*

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10. Tables and Figures