The natural reservoir of *Neorickettsia* spp.

Investigating the presence of *Neorickettsia* in different potential reservoir hosts

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Preface & acknowledgments

This research focuses on the analysis of DNA from various potential natural reservoirs in the life cycle of *Neorickettsia risticii* and *Neorickettsia findlayensis*, the causative agents of Potomac Horse Fever (PHF). The study is performed at the Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada. Thanks to the funding from Boehringer Ingelheim, I have been given the opportunity to conduct this research in Guelph instead of pursuing another project at the Faculty of Veterinary Medicine, University of Utrecht, the Netherlands. This project contributes towards earning credits for the master's program in "Farm Animal Health" of the University Utrecht through the completion of a Master Thesis.

Research on Potomac Horse Fever at the Ontario Veterinary College has been ongoing for several years. This summer, the focus has been specifically on investigating the natural reservoir of the bacterium. The findings from this research contribute to a better understanding of the complete lifecycle of *Neorickettsia risticii* and *Neorickettsia findlayensis*, which is valuable information for epidemiologists, clinicians, and horse owners.

In this study, I have analyzed liver samples from various vertebrates, while another student has investigated samples from water sources where horses with PHF were found. By combining our results, we aim to enhance understanding of the complete life cycle of *N. risticii.*

I would like to express my gratitude to Boehringer Ingelheim for making this adventure abroad possible. Additionally, I want to extend my thanks to Brandon Lillie and Luis Arroyo Castro for their guidance throughout the research process, reviewing my assignments, and providing valuable feedback. I am also thankful to Jutta Hammermueller for her excellent explanations and assistance in the laboratory. I want to sincerely thank Brian Stevens and Claire Jardine for providing the valuable samples for this research. I wish Olivia Anderson success in completing the rest of the research, and I also want to thank her for her contributions. Furthermore, I would like to acknowledge Cornélie Westermann for facilitating the trip to Guelph and supporting the research from Utrecht.

Florance van Dam
Guelph, August 2, 2023

Abstract

Potomac horse fever (PHF), caused by *Neorickettsia risticii* and the more recently discovered *N. findlayensis,* can induce primarily colitis and other clinical signs in horses. The bacteria live in a commensal or mutualistic relationship within various species of trematodes. These trematodes utilize snails as their first intermediate host and aquatic insects as the second intermediate host. Horses act as dead-end-host by ingesting the aquatic insects. The full extent of potential definitive and reservoir hosts remains poorly understood.

In this study, a polymerase chain reaction (PCR) was performed on liver samples obtained from different wildlife species including bats, muskrats, raccoons, opossums, and skunks as well as several invertebrates to investigate the presence of *Neorickettsia* spp.

Neorickettsia spp. DNA was identified in 4/133 (3 %) vertebrates sampled (two bats, one skunk, and one muskrat). Additional research is currently underway to investigate *Neorickettsia* spp. DNA in invertebrates.

The findings of this study suggest that *Neorickettsia* spp. circulate among different wildlife species, indicating a broader natural reservoir for these bacteria than previously recognized. These results contribute to the understanding of the life cycle, epidemiology, and potential transmission options for these bacteria. Further research is warranted to clarify the role of wildlife species in the maintenance and spread of *Neorickettsia* spp.

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1. Introduction

Potomac horse fever (PHF), also referred to as equine neorickettsiosis and equine monocytic ehrlichiosis, can be a cause of equine colitis in areas where it is endemic. The disease was initially identified near Maryland's Potomac River in 1979, and since then, cases have been reported across North and South America.[1,2,3,4,5] The causative agent for PHF is *Neorickettsia risticii* (formerly *Ehrlichia risticii*), but recently another species called *Neorickettsia findlayensis* which also causes PHF was found.[6,7] These *Neorickettsia* species are intracellular, gram-negative bacteria of trematodes that parasitize snails and aquatic insects.[8,9] The snails and aquatic insects behave as intermediate hosts, whereas at least horses are a dead end host. While some studies suggest that animals like bats, birds, and muskrats may act as definitive hosts for *Neorickettsia* spp., the full range of potential definitive and reservoir hosts remains poorly understood.[10,11,12]

Considering the complexity of PHF transmission and the potential involvement of various mammalian species, this study aims to elucidate the prevalence and distribution of *Neorickettsia* spp. in liver samples obtained from different mammals. By exploring the prevalence of *Neorickettsia* spp. in various wildlife species, including bats, muskrats, raccoons, opossums, and skunks, using nested PCR, this research seeks to contribute to a better understanding of the disease dynamics and its potential reservoir hosts.

Before delving into the specifics of the research methodology and findings, it is essential to provide background information on the epidemiology of PHF, its clinical manifestations, prevalence in horses, and the life cycle of *Neorickettsia* spp. In addition, there is some background information on different diagnostic methods to determine the best method to study the available samples. This context will help readers grasp the significance of the study and its potential implications for equine health.

1.1. Pathogenesis and clinical presentation

Horses get the infection by ingesting *Neorickettsia*-infected trematodes present in aquatic insects or free-living trematodes.[13,14] Once inside the horse's gastrointestinal tract, the bacteria are released from the trematodes and invade the cells lining the colon and cecum, as well as tissue macrophages. The bacteria then spread into the bloodstream, infecting monocytes.[15,16,17] The most reported clinical signs in horses with PHF include diarrhea, fever, anorexia, lethargy, and colic.[7,15]

1.2. Prevalence in horses

As mentioned before, PHF was discovered along the Potomac River in Montgomery County, Maryland, USA.[5,22] The disease was found in 43 states across the United States, as well as in three Canadian provinces (Nova Scotia, Ontario, Alberta), South America (Uruguay, Brazil), Europe (The Netherlands, France), and India.[7,23,24] However, sources were not mentioned and how this data was collected is therefore unknown. The authors also specified that reports documenting the isolation or detection of the causative agent using conventional cell culture or molecular detection techniques such as polymerase chain reaction (PCR) were limited to 13 states within the United States. (California, Illinois, Indiana, Kentucky, Maryland, Michigan, New York, New Jersey, Ohio, Oregon, Pennsylvania, Texas, Virginia), along with Nova Scotia, Uruguay, and Brazil.[23]

In regions where PHF was endemic, clinical cases showed a significant correlation with the proximity to rivers, lakes, or other aquatic environments. The risk of PHF increases notably for horses that graze in pastures along waterways such as freshwater rivers, streams, ponds, and irrigation ditches. Additionally, horses originating from areas with high PHF prevalence or farms with a history of the disease, as well as those traveling to regions with a high incidence of PHF, are at an elevated risk.[25,26,27]

Since *Neorickettsia* species rely on intermediate hosts such as aquatic insects, the disease is primarily observed during the summer and autumn months.[7,28] Specifically, in the USA and Canada, the peak of clinical PHF cases was observed between late June and early September, but the disease has been reported from May to November.[7,23,25,26]

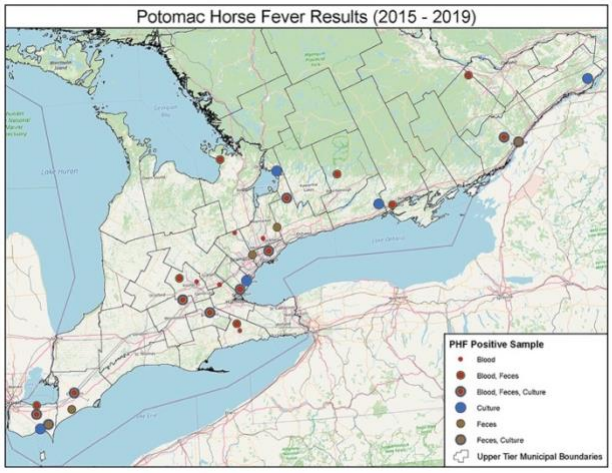

Figure 1. *Map of southern Ontario showing the geographic origins of Potomac Horse Fever cases. The colored dots depict the sample tested and the method of diagnosis.*[7]

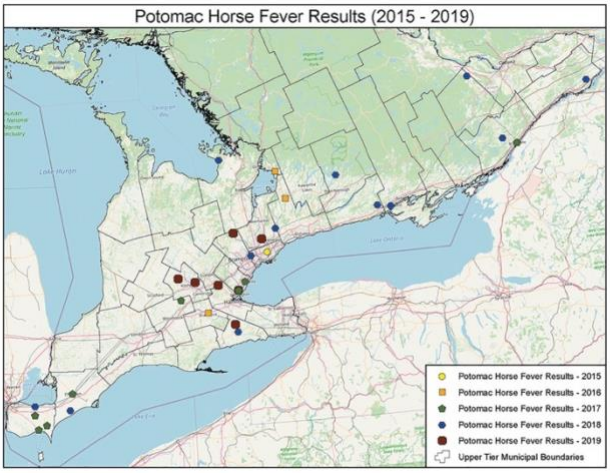

Figure 2. *Map of southern Ontario showing the geographic origins of Potomac Horse Fever cases. The colored dots depict the sample tested and the method of diagnosis.*[7]

1.3. Life cycle of *N. risticii*

The disease's seasonal occurrence and its geographical spread, suggest the potential involvement of a vector in PHF transmission.[23] Pretzman et al. (1995) made a significant discovery, revealing the involvement of digeneans, a class of trematodes/flukes, in the life cycle of *N. helminthoeca*. This finding prompted the notion that parasites might also play a role in the life cycle of *N. risticii*.[31]

N. risticii and *N. findlayensis* are known to live in a commensal or parasitic relationship within various species of flukes. These flukes utilize freshwater snails as their first intermediate host, while different types of aquatic insects, like caddisflies (order *Trichoptera*), mayflies (*Ephemeroptera*), damselflies (*Odonata, Zygoptera*), dragonflies (*Odonata, Anisoptera*), and stoneflies (*Plecoptera*), serve as the second intermediate host for both the flukes and the bacteria. Insectivorous birds (e.g. swallows) and/or bats are now seen as the definitive hosts (Fig 3).[8,9,10,17,23,25,26,30]

The transmission to horses is believed to happen when they inadvertently ingest insects containing metacercariae. This can occur through various pathways, including ingesting insects while grazing, consuming hay contaminated with insects, or drinking water containing insects attracted to lights at night, which may have fallen into the water. Consequently, it appears that digenean metacercariae do not require reaching the adult stage of development to pass the infection to horses.[30,32]

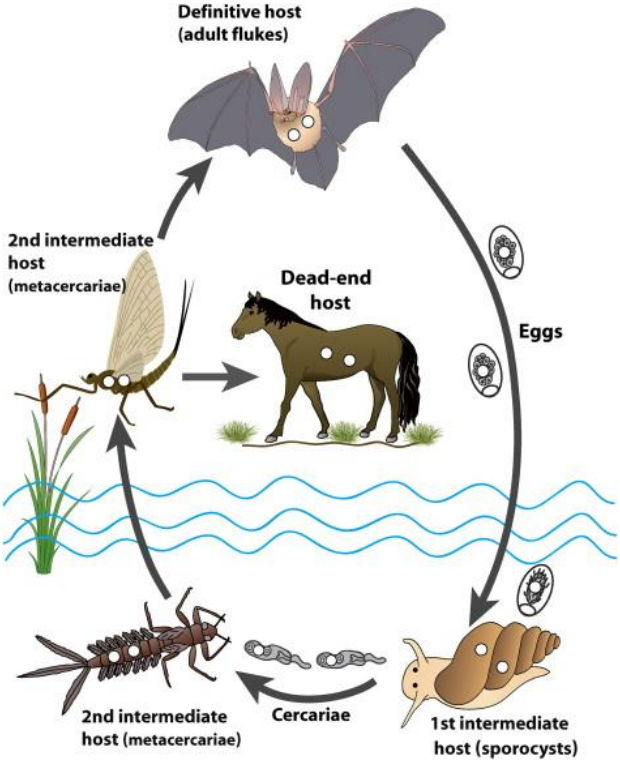

Figure 3. *Circulation of Neorickettsia risticii (white dots) involving digeneans (e.g. Acanthatrium* spp. *in this diagram) and horses as a dead-end host.*[17]

Other potential definitive or reservoir host

Several years in the past, dogs, cats, and cattle were subjected to inoculation with *Ehrlichia risticii* (former *N. risticii*). The seroepidemiological studies yielded seropositive outcomes across all these animal groups, without any clinical symptoms. However, when a horse was inoculated with *E. risticii* isolated from the previously inoculated animals, clinical signs of Potomac horse fever (PHF) manifested in the horse.[36,37,38] In a separate study, seropositivity was similarly discovered in 8 out of 48 cats (across 2 farms), as well as in 3 out of 14 pigs (from a single farm). These farms were situated approximately 3 km away from the location where a seropositive goat was identified.[39] This could indicate that species other than bats, birds and muskrats may also play a role in the life cycle of *N. risticii*. Notably, among the tested animals, which included 79 dogs, 75 cattle, and seven sheep, none displayed antibodies against *E. risticii*.[39]

1.4. Diagnostic Tests

Bacterial culture of *N. risticii* or *N. findlayensis* remains the gold standard for diagnosis, but Polymerase Chain Reaction (PCR) on whole blood combined with fecal testing gives a relatively high sensitivity and specificity.[15,19] PCR testing on whole blood is more likely to detect the presence of *N. risticii* than fecal samples and can do so for a longer period of time compared with feces. In experimental infections, positive PCR results from whole blood were observed approximately 7 to 21 days after infection, while fecal PCR positivity was detected from around 11 to 16 days post-infection.[8,18]

A recent advancement includes the development of a real-time PCR test specifically designed to identify *N. findlayensis*. This test utilizes two real-time PCR assays targeting the *Neorickettsia* ssa2 gene, along with sequencing of the *Neorickettsia* 16S rRNA gene, which detects both *Neorickettsia* species in horse samples. Through this approach, *N. findlayensis* can be distinguished from *N. risticii*. [6,7] Additionally, besides the 16S rRNA gene and the ssa2 genes, other genetic markers such as P51, ssa1, and ssa3 can also be used to detecting *Neorickettsia* species like *N. risticii* and *N. findlayensis.*[6,11,12,21]

Detection of *Neorickettsia* spp. in different organs (e.g. liver and blood) of bats is performed by a study which used nested PCR with the 16S rRNA and p51 genes.[11] Therefore the expectation is that using the same method (PCR utilizing the 16S gene) would be a good fit for this study.

1.5. Aims and hypothesis

After discussing the causative agents and epidemiology of Potomac horse fever, it becomes evident that further investigation into the presence of *Neorickettsia* spp. in various mammalian species is warranted. Therefore, this study aims to explore the prevalence of *Neorickettsia* spp. in liver samples from different mammals, including bats, muskrats, raccoons, opossums, and skunks by using PCR testing. It is expected that *N. risticii* DNA will be detected in at least some of the samples provided.

2. Methods

To explore the presence of *Neorickettsia* spp. DNA in potential definitive or reservoir hosts, liver samples were collected and subjected to PCR analysis. The 16S rRNA gene was chosen as the target due to its wide applicability in bacterial identification and phylogenetic analysis.[11]

The frozen liver samples, collected over several years by the Department of Pathobiology at the Ontario Veterinary College, University of Guelph, Ontario, Canada, were accompanied by location data from Ontario, with most samples also including collection dates. A total of 133 livers from various mammals, including 25 bats, 9 skunks, 4 opossums, 15 raccoons, and 80 muskrats, were analyzed. Detailed sample information is provided in appendix 7.1.

Liver tissue was selected for DNA extraction because it serves as a filter organ, potentially capturing a higher concentration of *Neorickettsia* DNA. Additionally, liver tissue typically contains abundant DNA, facilitating the extraction process. The E.Z.N.A.® Tissue DNA Kit[a] was used to isolate the DNA from the frozen liver tissue. In appendix 7.2 a summary made by the lab assistant of the Department of Pathobiology (Ontario Veterinary College) is shown.

After DNA extraction from frozen liver samples, each sample was assessed for DNA quantity and quality using the Thermo Scientific Nanodrop 2000[b]. The spectrophotometer determined the DNA concentration in each sample after a 1µL drop of the DNA extraction mixture was applied. These concentrations are detailed in appendix 7.1. Most samples yielded sufficient DNA (>10-100 ng/µL), with only 5 out of 141 samples falling below this threshold. Despite this, PCR analysis was conducted on all samples, with attention paid to the lower DNA concentrations.

The next step involved preparing a mastermix, which included a forward and reverse primer. The composition of the TopTaq™ master mix[c] is provided in *Table 1*. The information about the *Neorickettsia* 16S rRNA primers is provided in *Table 2*. Thereafter, 2 microliters of DNA sample and 23 microliters of the TopTaq™ master mix were combined and loaded into the T professional TRIO Thermocycler[d] for amplification. The PCR product is run through a 1 % agarose gel in a 1x agarose electrophoresis buffer in the Horizon 11.14[d]. The full protocol utilized for this process can be found in appendix 7.3.

Negative controls were prepared using pure mastermix, while the first batch of positive controls comprised PHF positive samples from The Animal Health Laboratory, 419 Gordon St, Guelph, ON N1G 2W1, Canada. Subsequently, samples that had previously tested positive multiple times been used as positive controls, labeled as 'PCR product'. Gel imaging was performed using the BioRad Chemidoc XRS+[e], using the Image lab Faint bands protocol and filter 1 from the settings.

If a sample tested positive, it underwent multiple rounds of PCR analysis for increased certainty. Furthermore, an additional DNA extraction was performed using the original liver sample to reduce the risk of cross-contamination. The positive DNA samples also underwent additional analysis using The LightCycler 480[f] by the lab assistant to confirm the results. The full protocol conducted by the lab assistant can be found in appendix 7.4. Furthermore, the positive DNA samples were sent in for additional sequencing to the lab of Ohio State University[g] for further validation.

[a] Omega Bio-Tek, Norcross, Georgia, United States
[b] Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States
[c] Qiagen, Hilden, Germany
[d] Biometra GmbH, Gottingen, Germany
[e] Bio-Rad Laboratories Inc, Hercules, California, United States
[f] Roche Diagnostics, Basel, Switzerland
[g] Molecular, Cellular, and Environmental Rickettsiology Laboratory, Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University.

Table 1. *Mastermix.*

Component	µl Per Tube (25 µl rx)	Final Concentration	# Tubes	Master Mix (µl)
DEPC water	18.875		10	188.75
10 X TopTaq buffer w $MgCl_2$	2.5	1 X	10	25
dNTP (10 mM)	0.5	0.2 mM	10	5
Forward primer (10 uM)*	0.5	0.2 uM	10	5
Reverse primer (10 uM)*	0.5	0.2 uM	10	5
TopTaq	0.125	1 Unit	10	1.25
Mix contents of master mix, aliquot 23 µl into each PCR tube then add template				
Template DNA	2			

Table 2. *Neorickettsia primers.*

			Primer (5' to 3')	TM °C	Prod Size (bp)
***Neorickettsia* 16S rRNA**	Neorick16S_F	Forward	GTGTGAAATCCTTGGGCTTAACC	66.4	
	Neorick16S_R	Reverse	AACACTCATCGTTTACAGCGTGG	67	226

2.1. Explanation of the terms

Single PCR amplification (single amp)
A polymerase chain reaction (PCR) was conducted using a DNA template (sample) along with specific primers (16S) targeting the desired genetic region. The reaction mixture underwent a series of thermal cycles, including denaturation, annealing, and extension, in a thermal cycler. As a result of the amplification process, the target DNA region underwent exponential replication, producing a detectable amount of the desired DNA product. The amplified DNA fragments were then immediately analyzed through gel electrophoresis to confirm the success of the PCR reaction and the presence of the amplified genetic material. Utilizing 2 microliters of the initial DNA sample is called a single PCR amplification (single amp in the tables in chapter 3).

Double PCR amplification (double amp)
To enhance the positive outcome in the gel electrophoresis, the PCR reaction was amplified using 2 microliters of the product derived from the initial amplification (single amp) round as a template. This is known as 'double PCR amplification' (double amp). The goal is to intensify the positive lines in the imaging.

PCR product
Frequently, a single PCR amplification sample that showed positive results in the earlier PCR round and then stored, was utilized in a following gel electrophoresis round to guarantee the presence of a strong positive control. This is indicated using the term 'PCR product'. The PCR product is a simple amplification product, stored in the freezer and thawed before use.

Gel electrophoresis
Gel electrophoresis was used to separate and analyze the DNA based on their size and charge. After a single or double PCR amplification, the PCR product is loaded onto a gel matrix made of agarose. An electrical field is then applied across the gel, causing the charged molecules to migrate through the gel matrix at different rates.

Due to the porous nature of the gel, smaller molecules move more quickly through the pores, while larger molecules move more slowly. As a result, the molecules become separated into distinct bands or zones along the length of the gel. The log ladder is used in gel electrophoresis as a reference standard for estimating the size of DNA fragments being analyzed.

Imaging system
The separated molecules can be visualized using the BioRad Chemidoc XRS+, and the resulting pattern of bands provides valuable information about the molecular size and abundance of the analyzed molecules. In this paper, the result of this image is referred to as the image of the gel electrophoresis.

Positive samples
All images with a positive sample are displayed in chapter 3, accompanied by relevant information. The table indicates the lane of the gel electrophoresis (Gel#), the corresponding original sample number (Sample#), the associated sample information (species & info), the DNA concentration of the product ([DNA]), and whether it involves a single or double amplification (single/double amp) of the utilized product. Most of the time, a previous strong positive PCR product is used as a positive control. Samples that were previously identified as positive underwent repeated testing more frequently. Additionally, a negative control was employed. See 7.1 for the full information of the samples.

The LightCycler 480 / Real-time PCR
When samples tested positive, the LightCycler 480 was used to confirm the results. The LightCycler is a type of real-time polymerase chain reaction (real-time PCR) instrument utilized for rapidly and efficiently amplifying and quantifying DNA. It employs fluorescence-based detection to monitor DNA amplification during each cycle of the PCR reaction in real-time, enabling continuous measurement of DNA accumulation. This allows researchers to analyze the reaction's progress and determine the amount of DNA present in the sample.

3. Results

Out of the 133 distinct liver samples obtained from various mammals, four animals tested positive for *Neorickettsia* DNA: one vesper bat, one muskrat, one striped skunk, and one big brown bat. These positive results are highlighted in bold in chapter 3.1, where the meaningful results from these samples are presented.

Further PCR runs were conducted in addition to these positive identifications. However, they are not included in this chapter as they involved samples that were deemed non-meaningful, as they showed no positive results except for the positive control or encountered issues with the negative/positive controls. To complement the findings, results from an external laboratory at Ohio State University are also included in chapter 3.2. This collaboration provides valuable insights and strengthens the overall interpretation of the results.

Chapter 3.3 provides comprehensive information on each of the positive samples, including their origin and the number of PCR cycles performed on The LightCycler 480. This detailed analysis sheds light on the diversity and distribution of *Neorickettsia* infections across different mammalian species.

3.1. Information tables and images of the gel electrophoresis

Table 3. *Information about the samples in the Gel electrophoresis run of 6 July 2023 15:00 (Figure 4)*

Gel#	Sample#	Species	Info	[DNA] in µg	Product	Positive/ Negative
1	Log					
2	29	SFB	2-163-09	228,2	Single amp	Negative
3	30	Vesper bat	2-19-04	916,6	Single amp	Negative
4	31	Vesper bat	2-109-04	725,8	Single amp	Negative
5	**32**	**Vesper bat**	**2-175-03**	**647,5**	**Single amp**	**Positive**
6	33	Muskrat	MSKRT 23-001	436,1	Single amp	Negative
7	34	Muskrat	MSKRT 23-002	425,2	Single amp	Negative
8	35	Muskrat	MSKRT 23-003	441	Single amp	Negative
9	36	Muskrat	MSKRT 23-004	646,6	Single amp	Negative
10	37	Muskrat	MSKRT 23-005	1217,9	Single amp	Negative
11	38	Muskrat	MSKRT 23-006	225,9	Single amp	Negative
12	39	Muskrat	MSKRT 23-007	684,5	Single amp	Negative
13	40	Muskrat	MSKRT 23-008	394,1	Single amp	Negative
14	41	Muskrat	MSKRT 23-009	392,3	Single amp	Negative
15	**42**	**Muskrat**	**MSKRT 23-010**	**148,9**	**Single amp**	**Positive**
16	43	Muskrat	MSKRT 23-011	462,4	Single amp	Negative
17	44	Muskrat	MSKRT 23-012	284,3	Single amp	Negative
18	Positive control	PHF+ horse	From AHL lab		PCR product	Positive
19	Negative control		Mastermix + elution buffer		Single amp	Negative
20	Positive control	Pondsnail	Olivia's sample #10		Single amp	Positive

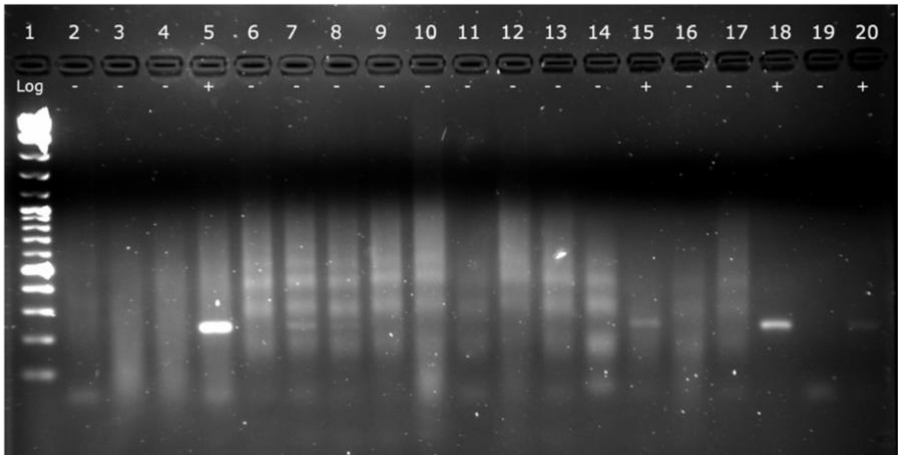

Figure 4. *Image of the gel electrophoresis of PCR products on 6 July 2023 at 15:00.*

Table 4. *Information about the samples in the Gel electrophoresis run of 7 July 2023 15:00 (Figure 5)*

Gel#	Sample#	Species	Info [DNA] in µg	Product	Positive/ Negative	
1	Log					
2	45	Muskrat	MSKRT 23-013	197,4	Single amp	Negative
3	9	Big brown bat	W0140-20	631,3	Single amp	Negative
4	**16**	**Striped skunk**	**W0235-20**	**8,9**	**Single amp**	**Positive**
5	46	Muskrat	MSKRT 23-014	149,8	Single amp	Negative
6	47	Muskrat	MSKRT 23-015	277,1	Single amp	Negative
7	48	Muskrat	MSKRT 23-016	486,9	Single amp	Negative
8	49	Muskrat	MSKRT 23-017	586,3	Single amp	Negative
9	60	Muskrat	MKRT 22-001	264,6	Single amp	Negative
10	61	Muskrat	MKRT 22-004	897,3	Single amp	?
11	62	Muskrat	MKRT 22-005	401,7	Single amp	Negative
12	63	Muskrat	MKRT-22-007	660,1	Single amp	Negative
13	64	Muskrat	MKRT-22-008	526,7	Single amp	Negative
14	65	Muskrat	MKRT-22-009	600,3	Single amp	Negative
15	66	Muskrat	MKRT-22-017	248,9	Single amp	Negative
16	67	Muskrat	MKRT-22-040	497,4	Single amp	Negative
17	68	Muskrat	MKRT-22-041	1324,9	Single amp	Negative
18	Negative control					Negative
19	Positive control	Pondsnail	Olivia's sample #10		Single amp	Positive
20	Positive control; 42	Muskrat	Positive before; MSKRT 23-010	148,9	PCR product	Positive

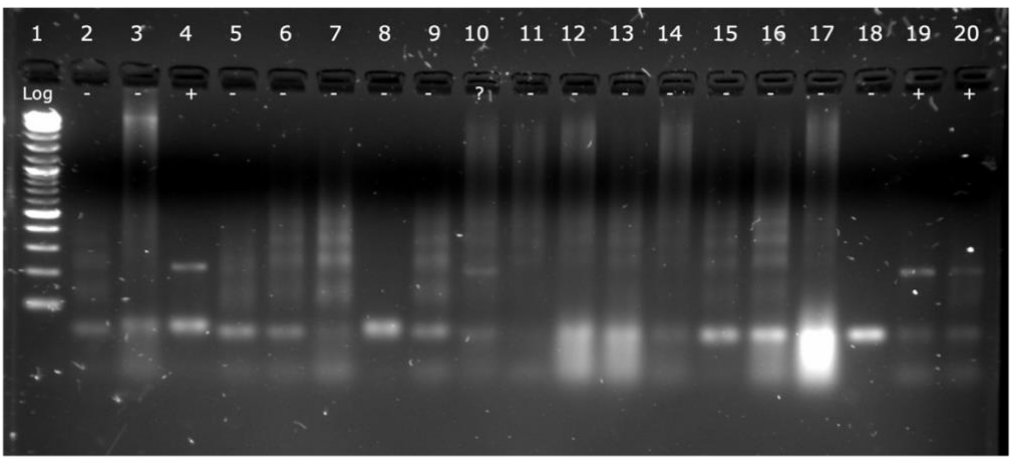

Figure 5. *Image of the gel electrophoresis of PCR products on 7 July 2023 at 15:00.*

Table 5. *Information about the samples in the Gel electrophoresis run of 12 July 2023 16:04 (Figure 6)*

Gel#	Sample#	Species	Info	[DNA] in µg	Product	Positive/ Negative
1	Log					
2	136	Big brown bat	WO0037-21	1025,1	Single amp	Negative
3	**137**	**Big brown bat**	**W0340-20**	**493,5**	**Single amp**	**Positive**
4	138	Silver haired bat	W0418-21	690	Single amp	Negative
5	147	Striped skunk	WO321-21 A2	30,8	Single amp	Negative
6	**42 + positive control**	**Muskrat**	**MSKRT 23-010**		**Single amp**	**Positive**
7	Negative control					Negative
8	**150**	**Striped skunk**	**W0235-20**	**79,6**	**Single amp**	**Positive**
9	**151**	**Muskrat**	**MSKRT 23-010**	**18,3**	**Single amp**	**Positive**
10	152	Big brown bat	W0013-21	1690,3	Single amp	Negative
11	153	Muskrat	MKRT-22-029	514,6	Single amp	Negative
12	**32 + positive control**	**Vesper bat**	**2-175-03**	**647,5**	**Single amp**	**Positive**
13	Negative control					Negative
14	Empty					

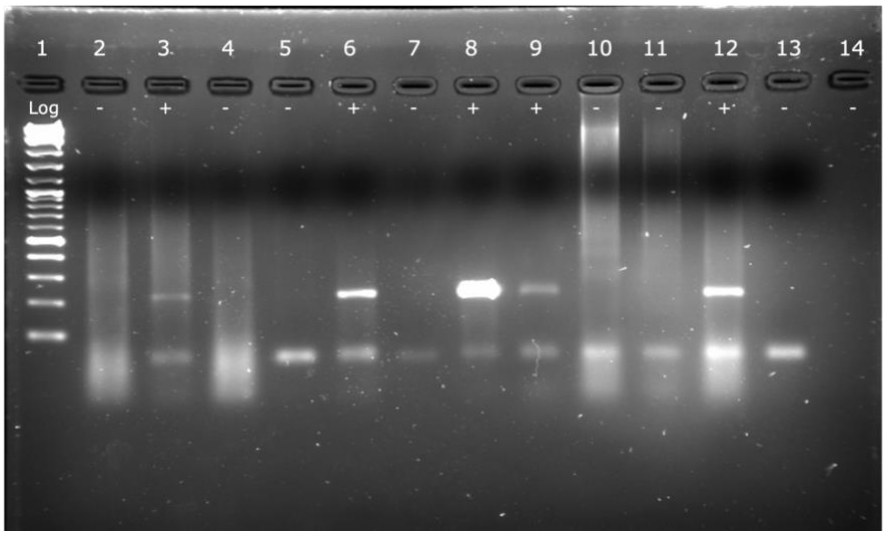

Figure 6. *Image of the gel electrophoresis of PCR products on 12 July 2023 at 16:04.*

Table 6. *Information about the samples in the Gel electrophoresis run of 17 July 2023 18:06 (Figure 7), using half amount of the mastermix with primers.*

Gel#	Sample#	Species	Info	[DNA] in µg	Product	Positive/ Negative
1	Log					
2	16	Striped skunk	W0235-20	8,9	Single amp	Negative
3	**16**	**Striped skunk**	**W0235-20**	**8,9**	**Double amp**	**Positive**
4	**16**	**Striped skunk**	**W0235-20**	**8,9**	**Double amp**	**Positive**
5	**150**	**Striped skunk**	**W0235-20**	**79,6**	**Single amp**	**Positive**
6	**150**	**Striped skunk**	**W0235-20**	**79,6**	**Double amp**	**Positive**
7	32	Vesper bat	2-175-03	647,5	Single amp	Negative
8	32	Vesper bat	2-175-03	647,5	Single amp	Negative
9	**32**	**Vesper bat**	**2-175-03**	**647,5**	**Double amp**	**Positive**
10	**32**	**Vesper bat**	**2-175-03**	**647,5**	**Double amp**	**Positive**
11	42	Muskrat	MSKRT 23-010	148,9	Single amp	Negative
12	**42**	**Muskrat**	**MSKRT 23-010**	**148,9**	**Double amp**	**Positive**
13	**42**	**Muskrat**	**MSKRT 23-010**	**148,9**	**Double amp**	**Positive**
14	151	Muskrat	MSKRT 23-010	18,3	Single amp	Negative
15	**151**	**Muskrat**	**MSKRT 23-010**	**18,3**	**Double amp**	**Positive**
16	137	Big brown bat	W0340-20	493,5	Single amp	Negative
17	137	Big brown bat	W0340-20	493,5	Single amp	Negative
18	**137**	**Big brown bat**	**W0340-20**	**493,5**	**Double amp**	**Positive**
19	**137**	**Big brown bat**	**W0340-20**	**493,5**	**Double amp**	**Positive**
20	Negative control					Negative

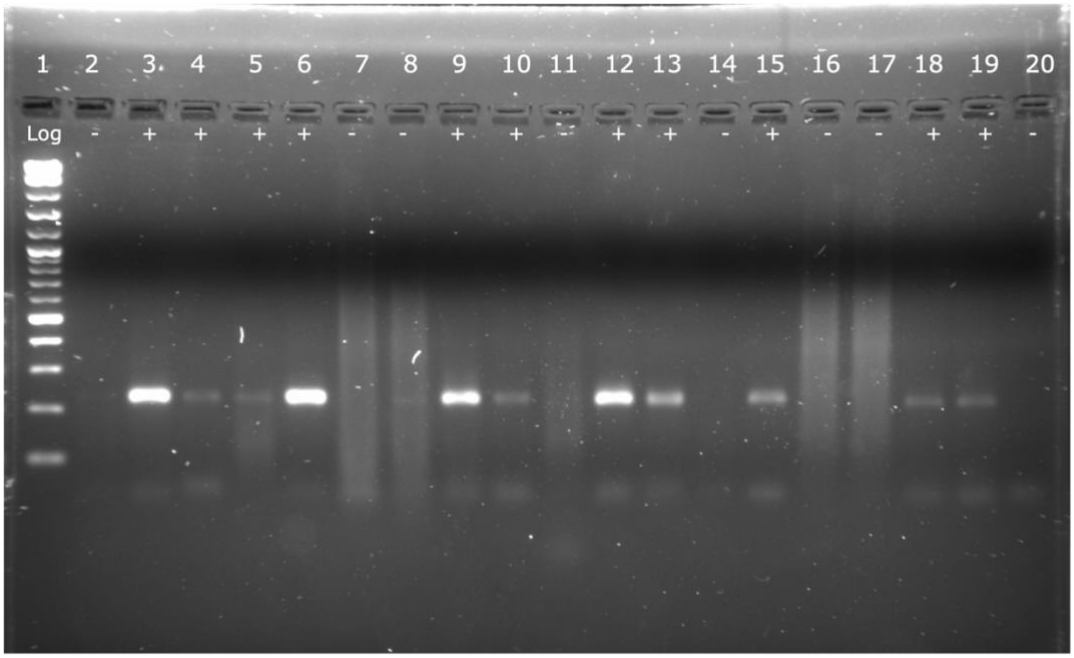

Figure 7. *Image of the gel electrophoresis of PCR products on 17 July 2023 at 18:06.*

3.2. Results from the laboratory of Ohio State University

To verify the accuracy of the positive results, some samples were sent to the laboratory at Ohio State University for confirmation. At this facility, differentiation between *N. findlayensis* and *N. risticii* was achieved using the ssa2 gene. All samples were identified as *N. risticii*. Notably, Sample 5, inadvertently included in the batch, also tested positive.

Table 7. *Results of the laboratory of Ohio State University.*

No	Names on tube	DNA Concentration	*Neorickettsia* 16S rRNA gene	*N. findlayensis ssa2*	*N. risticii ssa2*
1	16 SSK Liver	14.6 ng/ul	Positive	Negative	Positive
2	42 MSKR Liver 10	116.3 ng/ul	Positive	Negative	Positive
3	32 V.Bat Liver	525 ng/ul (1/5 diluted as template)	Positive	Negative	Positive
4	150 SSK Liver	81.4 ng/ul	Positive	Negative	Positive
5	60 MSKT Liver 22-001	250 ng/ul	Positive	Negative	Positive
6	151 MSKT Liver	21 ng/ul	Positive	Negative	Positive

3.3. Positive results

In this section, all the information on the samples that were at least one time positive are outlined below. This information can also be found in appendix 7.1. All of the samples had undergone different runs, and when there was enough liver left to make a second sample, those samples were tested with PCR again. When the origin and sample date were available, they are listed below. Besides that, the number of cycles in The Lightcycle 480 is showed. The lower the number, the more *Neorickettsia* DNA is available in the sample.

3.3.1. Vesper bat: 2-175-03

- Sample number: 32
    - Run 36: Positive
    - Run 84: Positive
    - Run 121: Positive
    - Run 165: Positive
    - Run 179: Negative
    - Run 180: Negative
    - Run 181 (= Double #36): Positive
    - Run 182 (= Double #84): Positive
- Second sample number: not enough liver left
- Origin & sample date: Unknown
- Number of cycles in LightCycler: 35
- Positive on *Neorickettsia* 16S rRNA gene by the lab of Ohio State University
- Negative on *N. findlayensis* ssa2 gene by the lab of Ohio State University
- Positive on *Neorickettsia risticii* ssa2 gene by the lab of Ohio State University

3.3.2. Muskrat: MSKRT 23-010

- Sample number: 42
    - Run 46: Positive
    - Run 86: Negative
    - Run 102: Positive
    - Run 139: Positive
    - Run 159: Positive
    - Run 183: Negative
    - Run 184 (= Double #102): Positive
    - Run 185 (= Double #159): Positive
- Second sample number: 151
    - Run 161: Positive
    - Run 177: Negative
    - Run 178 (= Double #161): Positive
- Origin: Ball road, Lot 17 Concession 1, Hinchinbrooke Twp, Central Frontenac
- Sample date: 2023
- Number of cycles in LightCycler: 31
- 2 samples positive on *Neorickettsia* 16S rRNA gene by the lab of Ohio State University
- 2 samples negative on *N. findlayensis* ssa2 gene by the lab of Ohio State University
- 2 samples positive on *Neorickettsia risticii* ssa2 gene by the lab of Ohio State University

3.3.3. Striped skunk: W0235-20

- Sample number: 16
    - Run 16: Positive
    - Run 53: Positive
    - Run 174: Negative
    - Run 175 (= Double #16): Positive
    - Run 176 (= Double #53): Positive
- Second sample number: 150
    - Run 161: Positive
    - Run 177: Positive
    - Run 178 (= Double #161): Positive
- Origin: Port McNicoll, Ontario
    - Coordinates: 44.75 -79.81
- Sample date: September 1-2020
- Number of cycles in LightCycler: 28
- 2 samples positive on *Neorickettsia* 16S rRNA gene by the lab of Ohio State University
- 2 samples negative on *N. findlayensis* ssa2 gene by the lab of Ohio State University
- 2 samples positive on *Neorickettsia risticii* ssa2 gene by the lab of Ohio State University

3.3.4. Big brown bat: W0340-20

- Sample number: 137
    - Run 146: Positive
    - Run 188: Negative
    - Run 189: Negative
    - Run 190 (= Double #146): Positive
    - Run 191 (= Double #146): Positive
- Second sample number: not enough liver left
- Origin: Peterborough, Ontario
    - Coordinates: 44.31 -78.34
- Sample date: November 10-2020
- Number of cycles in LightCycler: 40

3.4. Map

Combining the results of this study with the map of southern Ontario showing the geographic origins of Potomac Horse Fever (positive PCR and/or culture) cases in horses from Arroyo LG, et al. (2021), the following map is created. Unfortunately, one of the positive samples (vesper bat) had no information on the origin.

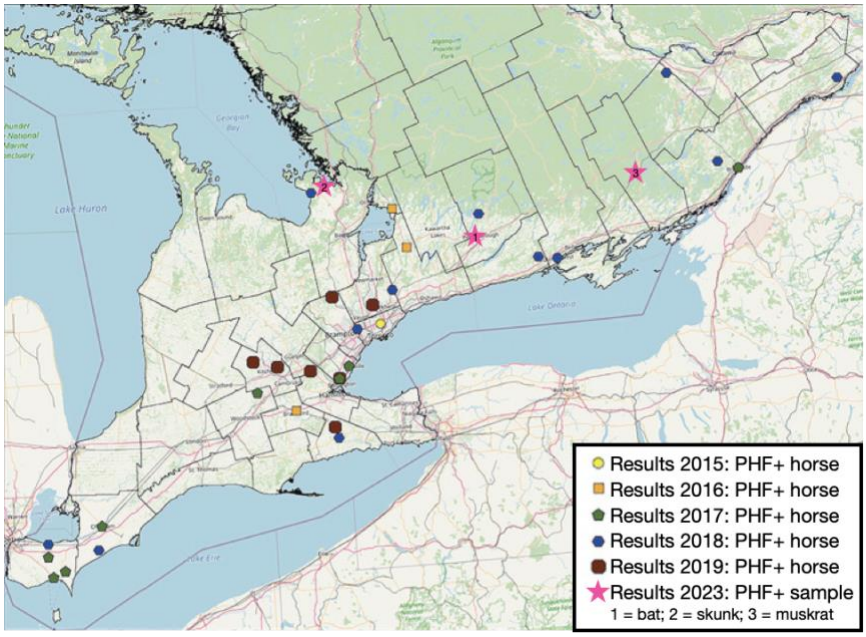

Figure 8. *Map of southern Ontario showing the geographic origins of Potomac Horse Fever (positive PCR and/or culture) cases in horses[7], supplemented with the results from this research (PCR on Neorickettsia) in pink.*

4. Discussion

The present study aimed to investigate the prevalence of *Neorickettsia* spp. DNA in potential reservoir hosts in Ontario, Canada. The findings of this research contribute significantly to the understanding of the bacteria's life cycle, epidemiology, and potential transmission routes. This discussion section will delve into the implications of the results, their alignment with existing knowledge, potential limitations, and future directions.

The identification of positive samples in two bats, one skunk, and one muskrat underscores the wider range of wildlife species that harbor *Neorickettsia risticii.* This wider range of hosts suggests a more extensive ecological interaction involving these bacteria than previously recognized. The detection of *Neorickettsia risticii* in wildlife species raises questions about the role of these species in the maintenance and dissemination of these bacteria, particularly given their interaction with invertebrate vectors.

While the identified wildlife hosts indicate a more expansive reservoir for *Neorickettsia risticii*, there are certain limitations to be considered. The specific mechanisms of interaction between the bacteria and wildlife hosts remain unclear, as this study primarily focused on detection. Further research involving experimental infections, studies into the potential excretion of the bacterium and studies into the potential impact of *Neorickettsia* spp. on these wildlife hosts could provide a more comprehensive understanding. By only detecting the bacteria in the liver of these host, the role as a dead-end-host, definitive host or reservoir host is unclear.

The confirmed positivity of the samples using both conventional and real-time PCR (LightCycler) methods ensures the robustness of the results. False positives may arise due to contamination of samples with DNA from other sources, cross-contamination between samples during handling or processing, or non-specific amplification of unintended DNA sequences. Measures were employed to minimize the risk of false positives, including the use of stringent laboratory protocols, proper handling techniques, and inclusion of negative controls to monitor for contamination. This is why the possibility of a false positive is minor and especially using different kind of PCRs and labs further reinforces the validity of the findings.

There is a potential for false negative samples, as the approach involved retesting only those samples identified as positive through conventional PCR in the subsequent real-time PCR analysis. This was due to a lack of time. False negatives can occur in PCR due to various factors such as low DNA concentration, PCR inhibitors, primer mismatches, or technical errors during sample preparation or amplification. There are probably several false negatives present, due to contamination, technical errors, and degradation. This may be what happened with the samples that were labeled positive but were occasionally negative (see chapter 3.3). Sample processing, methodological variations and reagents used in different labs influenced the rate of detection for PHF.[7] That is why another lab with probably another method of handling was involved to confirm some of the results.

The implications of this study extend to veterinary health perspectives. The broader range of wildlife hosts could potentially lead to a reevaluation of strategies for managing and controlling the spread of *Neorickettsia* spp. and therefore controlling Potomac Horse Fever, especially in areas where wildlife-horse interactions are common.

The ongoing research on invertebrates is expected to provide additional insights into the dynamics of *Neorickettsia* spp., aiding in the assessment of bacterial distribution and potentially shedding light on the endemic status.

In conclusion, this study significantly contributes to the existing knowledge of *Neorickettsia* spp. infections in potential definitive or reservoir hosts. The identification of positive samples in various wildlife species implies a more complex ecological scenario involving these bacteria. As future research unfolds, a more comprehensive picture of the role of these wildlife species in the transmission and maintenance of *Neorickettsia* spp. is expected to emerge, guiding future preventive and control measures.

5. Conclusion

The findings of this study suggest that *Neorickettsia* spp. circulates among different wildlife species in Ontario, Canada, indicating a broader natural reservoir for these bacteria than previously recognized. Out of 133 samples tested, 2 bats, 1 muskrats, and 1 skunk were found positive for *Neorickettsia* spp. DNA. However, it is important to acknowledge the potential for false negatives, so there could be more positive samples.

Further research is warranted to clarify the role (definitive/reservoir host or dead-end-host) of wildlife species in the maintenance and spread of *Neorickettsia* spp., including investigations into ecological interactions between wildlife hosts and the bacteria. Future studies should consider experimental infections, ecological studies, and investigations into the impact of *Neorickettsia* spp. on wildlife hosts to gain a more comprehensive understanding of their role in the epidemiology of these bacteria.

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7. Appendices

7.1. Sample information sheet

Sample number	Spieces	Organ	Origin	Time	Extra information	Concentratie	Figuur	PCR sample	Pos/Neg
1	Horse	Cecum		2021	Positive on PHF	100,3		X	X
2	Horse	Colon		2021	Positive on PHF	168,9		X	X
3	Horse	Colon		2021	Positive on PHF	144,3		X	X
4	Horse	Small intestine		2021	Positive on PHF	296,8		X	X
5	Horse	Liver		2021	Positive on PHF	1242,3		X	X
6	Horse	Spleen		2021	Positive on PHF	1316	Geen mooie	X	X
7	Big brown bat	Liver		August 21-2020	W0128-20	793,1		1	Negative
8	Big brown bat	Liver		August 21-2020	W0139-20	1085,7		2	Negative
9	Big brown bat	Liver		August 21-2020	W0140-20	631,3		52	Negative
10	Big brown bat	Liver		August 21-2020	W0141-20	693,7		3	Negative
11	Big brown bat	Liver		Januari 22-2021	W0008-21	254,7		4	Negative
12	Big brown bat	Liver		Januari 28-2021	W0013-21	1448,4	lost		X
13	Skunk	Liver		July 20-2020	W0133-20	164,6		5	Negative
14	Skunk	Liver		July 20-2020	W0135-20	70,7		14	Negative
15	Skunk	Liver		July 20-2020	W0144-20	756,6		15	Negative
16	Striped skunk	Liver	Port McNi	September 1-2020	W0235-20	8,9	Geen mooie	16; 53; 174;	Positive; Positive; negative; positive; positive
17	Opossum	Liver		July 20-2020	W0145-20	955,7		17	Negative
18	Raccoon	Liver		Januari 5-2021	W001-21	376,5		18	Negative
19	Raccoon	Liver		Januari 25-2021	W0011-21 A	465,5		19	Negative
20	Raccoon	Liver		Januari 25-2021	W0011-21 B	49,8		20	Negative
21	Seba bat	Liver		December 3-2010		2232,7		21	Negative
22	Seba bat	Liver		December 3-2010		347,7		22	Negative
23	Seba bat	Liver		December 3-2010	Adult female	1770,7		23	Negative
24	Seba bat	Liver		Januari 25-2011	Adult male	1823,3		24	Negative
25	SCF bat	Liver			2-002-10; HVER	413,7		25	Negative
26	SCF bat	Liver			2-195-06	553,1		26	Negative
27	SFB (slit-faced bat	Liver			2-008-08; HVER	1487,9		27	Negative
28	SFB (slit-faced bat	Liver			2-023-08	927,2		28	Negative
29	SFB (slit-faced bat	Liver			2-163-09	228,2		33	Negative
30	VB (vesper bat)	Liver			2-19-04	916,6		34	Negative
31	VB (vesper bat)	Liver			2-109-04	725,8		35	Negative
32	VB (vesper bat)	Liver			2-175-03	647,5		36; 84; 121;	Positive; Positive; Positive; Positive; Negative, Negative, Positive; Positive
33	Muskrat	Liver		2023	MSKRT 23-001	436,1		37	Negative
34	Muskrat	Liver		2023	MSKRT 23-002	425,2		38	Negative
35	Muskrat	Liver		2023	MSKRT 23-003	441		39	Negative
36	Muskrat	Liver		2023	MSKRT 23-004	646,6		40	Negative
37	Muskrat	Liver		2023	MSKRT 23-005	1217,9		41	Negative
38	Muskrat	Liver		2023	MSKRT 23-006	225,9		42	Negative
39	Muskrat	Liver		2023	MSKRT 23-007	684,5		43	Negative
40	Muskrat	Liver		2023	MSKRT 23-008	394,1		44	Negative
41	Muskrat	Liver		2023	MSKRT 23-009	392,3		45	Negative
42	Muskrat	Liver	Ball road,	2023	MSKRT 23-010	148,9	Rechter staa	46; 86; 102;	Positive; Negative; Positive; Positive; Positive; Negative; Positive; Positive
43	Muskrat	Liver		2023	MSKRT 23-011	462,4		47	Negative
44	Muskrat	Liver		2023	MSKRT 23-012	284,3		48	Negative
45	Muskrat	Liver		2023	MSKRT 23-013	197,4		49	Negative
46	Muskrat	Liver		2023	MSKRT 23-014	149,8		54	Negative
47	Muskrat	Liver		2023	MSKRT 23-015	277,1	Rechter staa	55	Negative
48	Muskrat	Liver		2023	MSKRT 23-016	486,9		56	Negative
49	Muskrat	Liver		2023	MSKRT 23-017	586,3		57	Negative
50	Horse	Blood 100 uL				2,5	Bijna plat, mi	X	X
51	Horse	Blood 80 uL				2,7	Bijna plat, mi	X	X
52	Horse	Blood 60 uL				0	Bijna plat, mi	X	X
53	Horse	Blood 50 uL				0,3	Bijna plat, mi	X	X
54	Horse	Blood 40 uL				-0,5	Bijna plat, mi	X	X
55	Horse	Blood 30 uL				0,7	Bijna plat, mi	X	X
56	Horse	Blood 25 uL				0,1	Bijna plat, mi	X	X
57	Horse	Blood 20 uL				0,8	Bijna plat, mi	X	X
58	Horse	Blood 15 uL				-1,3	Bijna plat, mi	X	X
59	Horse	Blood 11 uL				0,7	Bijna plat, mi	X	X
60	Muskrat	Liver			MKRT 22-001	264,6		58	Negative
61	Muskrat	Liver			MKRT 22-004	897,3		59; 103	Positive?; Negative
62	Muskrat	Liver			MKRT 22-005	401,7		60	Negative
63	Muskrat	Liver			MKRT-22-007	660,1		61	Negative
64	Muskrat	Liver			MKRT-22-008	526,7		62	Negative
65	Muskrat	Liver			MKRT-22-009	600,3		63	Negative
66	Muskrat	Liver			MKRT-22-017	248,9		64	Negative
67	Muskrat	Liver			MKRT-22-040	497,4		65	Negative
68	Muskrat	Liver			MKRT-22-041	1324,9		66	Negative
69	Muskrat	Liver			MKRT-22-042	186,5		67	Negative
70	Muskrat	Liver			MKRT-22-043	169		70	Negative
71	Muskrat	Liver			MKRT-22-044	343,5		71	Negative
72	Muskrat	Liver			MKRT-22-045	323,3		72	Negative
73	Muskrat	Liver			MKRT-22-047	282		73	Negative
74	Muskrat	Liver			MKRT-22-048	323,2		74	Negative
75	Muskrat	Liver			MKRT-22-049	177,7		75	Negative
76	Muskrat	Liver			MKRT-22-050	384,3		76	Negative
77	Muskrat	Liver			MKRT-22-051	237		77	Negative
78	Muskrat	Liver			MKRT-22-052	258,1		78	Negative
79	Muskrat	Liver			MKRT-22-053	269,7		79	Negative
80	Muskrat	Liver			MKRT-22-070	258,5		80	Negative
81	Muskrat	Liver			MKRT-22-071	366,2		81	Negative
82	Muskrat	Liver			MKRT-22-072	522,7		82	Negative
83	Muskrat	Liver			MKRT-22-073	444,4		83	Negative
84	Muskrat	Liver			MKRT-22-074	307,4		87	Negative
85	Muskrat	Liver			MKRT-22-075	1286,8		88	Negative
86	Muskrat	Liver			MKRT-22-076	664,8		89	Negative
87	Muskrat	Liver			MKRT-22-078	421,2		90	Negative
88	Muskrat	Liver			MKRT-22-079	316,8		91	Negative
89	Muskrat	Liver			MKRT-22-082	396,2		92	Negative
90	Muskrat	Liver			MKRT-22-083	509,2		93	Negative
91	Muskrat	Liver			MKRT-22-084	527		94	Negative
92	Muskrat	Liver			MKRT-22-002	19,1	Niet een hele	95	Negative
93	Muskrat	Liver			MKRT-22-003	409,3		96	Negative
94	Muskrat	Liver			MKRT-22-006	302,3		97	Negative
95	Muskrat	Liver			MKRT-22-010	1295,9		98	Negative
96	Muskrat	Liver			MKRT-22-011	908,4		99	Negative
97	Muskrat	Liver			MKRT-22-012	466,3		100	Negative
98	Muskrat	Liver			MKRT-22-014	910,6		101	Negative
99	Muskrat	Liver			MKRT-22-015	107,7		105	Negative

101	100	Muskrat	Liver			MKRT-22-016	281,1		106	Negative
102	101	Muskrat	Liver			MKRT-22-018	274,5		107	Negative
103	102	Muskrat	Liver			MKRT-22-019	355,6		108	Negative
104	103	Muskrat	Liver			MKRT-22-020	248,9		109	Negative
105	104	Muskrat	Liver			MKRT-22-021	471,5		110	Negative
106	105	Muskrat	Liver			MKRT-22-022	39,1	Klein beetje l	111	Negative
107	106	Muskrat	Liver			MKRT-22-023	722,3		112	Negative
108	107	Muskrat	Liver			MKRT-22-024	1265,3		113	Negative
109	108	Muskrat	Liver			MKRT-22-025	687		114	Negative
110	109	Muskrat	Liver			MKRT-22-026	719,8		115	Negative
111	110	Muskrat	Liver			MKRT-22-027	458,7		116	Negative
112	111	Muskrat	Liver			MKRT-22-028	272,1		117	Negative
113	112	Muskrat	Liver			MKRT-22-029		Lost		X
114	113	Muskrat	Liver			MKRT-22-056	106,6		118	Negative
115	114	Muskrat	Liver			MKRT-22-058	409,7		119	Negative
116	115	Muskrat	Liver			MKRT-22-059	995,5		120	Negative
117	116	Muskrat	Liver			MKRT-22-060	1246,5		123	Negative
118	117	Muskrat	Liver			MKRT-22-064	179,1		124	Negative
119	118	Muskrat	Liver			MKRT-22-066	596,6		125	Negative
120	119	Muskrat	Liver			MKRT-22-094	469,8		126	Negative
121	120	Muskrat	Liver			MKRT-22-097	778,3		127	Negative
122	121	Muskrat	Liver			MKRT-22-099	785,4		128	Negative
123	122	Muskrat	Liver			MKRT-22-101	1201		129	Negative
124	123	Muskrat	Liver			MKRT-22-102	936,2		130	Negative
125	124	Raccoon	Liver		Oktober 23-2020	WO308-20	825,9	2x BL buffer	131	Negative
126	125	Raccoon	Liver		November 9-2020	WO331-20	770,4	2x BL buffer	132	Negative
127	126	Raccoon	Liver		November 10-2020	WO332-20	814,2	2x BL buffer	133	Negative
128	127	Raccoon	Liver		November 9-2020	WO334-20	1488,4	2x BL buffer	134	Negative
129	128	Raccoon	Liver		November 10-2020	WO336-20	824,5	2x BL buffer	135	Negative
130	129	Raccoon	Liver		November 6-2020	WO339-20	1299,6	2x BL buffer	136	Negative
131	130	Raccoon	Liver		November 23-2020	WO341-20	1307,02	2x BL buffer	137	Negative
132	131	Raccoon	Liver		November 13-2020	WO391-20	947,4	2x BL buffer	138	Negative
133	132	Raccoon	Liver		November 8-2021	WO416-21	1175,6	2x BL buffer	141	Negative
134	133	Raccoon	Liver		November 29-2021	WO448-21	1131,4	2x BL buffer	142	Negative
135	134	Raccoon	Liver		February 4-2021	WO14-20	72,9	2x BL buffer	143	Negative
136	135	Raccoon	Liver		February 4-2021	WO15-21	1709,9	2x BL buffer	144	Negative
137	136	Big brown bat	Liver		February 26-2021	WO0037-21	1025,1	2x BL buffer	145	Negative
138	137	Big brown bat	Liver	Peterboro	November 10-2020	W0340-20	493,5	2x BL buffer	146; 188; 18	Positive; Negative; Negative; Positive; Positive
139	138	Silver haired bat	Liver		November 4-2021	W0418-21	690	2x BL buffer	147	Negative
140	139	Red bat	Liver		November 17-2021	W0432-21	122,2	2x BL buffer	148	Negative
141	140	Viop	Liver		Oktober 01-2020	W0284-20	1562,6	2x BL buffer	149	Negative
142	141	Viop	Liver		September 14-2021	WO324-21	750,2	2x BL buffer	150	Negative
143	142	Opossum	Liver		Oktober 23-2020	WO311-20	7	Eerst BL buff	151	Negative
144	143	Opossum	Liver		Oktober 30-2020	WO319-20	28,9	Eerst BL buff	152	Negative
145	144	Opossum	Liver		November 19-2021	WO431-21	15,1	Eerst BL buff	153	Negative
146	145	Skunk	Liver		Oktober 23-2020	WO310-20	0,5	Eerst BL buff	154	Negative
147	146	Striped skunk	Liver		September 15-2021	WO321-21 A1	9,5	Eerst BL buff	155	Negative
148	147	Striped skunk	Liver		September 15-2021	WO321-21 A2	30,8	Eerst BL buff	156	Negative
149	148	Striped skunk	Liver		September 15-2021	WO321-21 B	29,1	Eerst BL buff	157	Negative
150	149	Skunk	Liver		November 9-2020	WO330-20	9,7	Eerst BL buff	158	Negative
151	150	Striped skunk	Liver		September 1-2020	W0235-20	79,6	New sample,	161; 177; 17	Positive; Positive; Positive
152	151	Muskrat	Liver		2023	MSKRT 23-010	18,3	New sample,	162; 186; 18	Positive; Negative; Positive
153	152	Big brown bat	Liver		Januari 28-2021	W0013-21	1690,3	New sample,	163	Negative
154	153	Muskrat	Liver			MKRT-22-029	514,6	New sample,	164	Negative
155										

7.2. Summary of DNA extraction protocol

May 23 2023

Before Starting:

- Turn on 55°C and 70°C heating blocks
- Add elution buffer to 70°C heating block
- Prepare DNA and HBC Wash Buffer according to the directions on the bottles

Omega Tissue .,/DNA Extraction Protocol

1. Add metal bead and **200µL of TL Buffer** to 2 mL *flatbottom*microcentrifuge tube.
2. Mince up 30 mg of tissue and transfer to tube.
3. Add **25µL OB Protease** Solution. Vortex to mix thoroughly.
4. Set tissuelyser to 30 Hz and run for 4 minutes. If not using 48 samples, use counter balances.
5. Incubate at 55°C on ThermoMixer (min. 30 min – 1 hour). Lysis can proceed overnight. If ThermoMixer is not available, vortex sample every 20-30 minutes or place in heating block on orbit shaker.
6. Add **220µL BL Buffer** and vortex immediately.
7. Incubate at 70°C for 10 minutes.
8. Add **220µL 100% ethanol** and vortex immediately.
9. Transfer entire sample (~700µL) from step 8 to DNA Spin Column.
10. Centrifuge at maximum speed for 1.5 minutes and discard flow through.
11. Add **500µL HBC Buffer (make sure isopropanol has been added to bottle)**.
    a. Centrifuge at 10,000 rpm for 1 minute and discard flow through.
12. Add **700µL DNA Wash Buffer (make sure alcohol has been added to the bottle)**.
    a. Centrifuge at 10,000 rpm for 1 minute and discard flow through.
13. Repeat step 12.
14. Move DNA Spin Column to new2 mL Collection Tube. Centrifuge at 10,000 rpm for 2.5 minutes to dry the column.
15. Transfer DNA Spin Column to sterile 1.5mL *pointed bottom* microcentrifuge tube.
16. Add **100- 200 µL Elution Buffer** heated to 70°C. Let sit at room temperature for 2 minutes.
17. Centrifuge at 10,000 rpm for 1.5 minutes.
18. Store flow through in new 1.5 mL *pointed bottom* microcentrifuge tube and store at -20°C.
    a. Best to nanodrop samples immediately following this step.

7.3. PCR protocol

TopTaq DNA Polymerase from Qiagen
Potomic Horse Fever Primers

Kit is stored in freezer. Remove items needed for thawing but leave enzymes in freezer or on ice until required

1. In a thin walled PCR tube set up a Master Mix to contain all items that are the same for all samples (ie. typically the DNA is the only item being varied)

				TM	Prod Size
Neorickettsia 16S rRNA	Neorick16S_F	Forward	GTGTGAAATCCTTGGGCTTAACC	66.4	
	Neorick16S_R	Reverse	AACACTCATCGTTTACAGCGTGG ccacgctgtaaacgatgagtgtt	67	226

Add the following to a 0.2 ml or 0.5 ml thin walled PCR tube:

Component	Per Tube (25 ul rx)	Final Concentration	# Tubes	Master Mix
DEPC water	18.875		10	188.75
10 X TopTaq buffer w $MgCl_2$	2.5	1 X	10	25
dNTP (10 mM)	0.5	0.2 mM	10	5
Forward primer (10 uM)*	0.5	0.2 uM	10	5
Reverse primer (10 uM)*	0.5	0.2 uM	10	5
TopTaq	0.125	1 Unit	10	1.25
Mix contents of master mix, aliquot 23 ul into each PCR tube then add template				
Template DNA	2			

* Primers come from Sigma lyophilized at approximately 30-100 nmol

- Centrifuge the tubes before opening to prevent loss of pelleted oligos
- Reconstitute the primers in DEPC water using the volume indicated for 100 uM (10 ul / nmole)
- Prepare a Stock dilution of primers at 10 uM for use in setting up PCR Master Mix
  ⇨ 1:10 dilution in 10 mM Tris pH 8
  ⇨ typical PCR reaction mix contains primers at a final concentration of 0.2uM which is a 1:50 dilution of Stock primers

2. Add 2*3* ul to each sample PCR thin walled reaction tube. Some will be left in the tube that you made the Master Mix in. This can be used as your negative control. If doing more than 10 samples set the Master Mix for **n+1** tubes rather than multiplying the reagent volumes by **n.n**.

3. Add 2 ul of DNA to each sample PCR reaction tube

4. Mix

5. Incubate at 94°C for 3 minutes then do 30-40 cycles of PCR
   Sample PCR cycle:
   94°C for 30 seconds
   60°C for 30 seconds (annealing temp. ~ 5 degrees below primer Tm)
   72°C for 1 min/kb product
   Follow 30-40 cycles with a final extension at 72°C for 5-7 minutes

6. Analyze 8 ul of PCR product on a 1% agarose gel in TBE buffer

7.4. LightCycler protocol + results

PHF PCR using Neorickettsia 16s r RNA primers on light cycler in 3852 (previously in Sharif's lab)

Samples previously run on regular thermocyclers – strong, medium and variable results

Light cycler 480 SYBR Green I Master Mix			
Component	**10 ulrx**	**# wells**	**Master Mix**
2 X Master Mix	5	35	175
Fwd Primer (10 x)	0.5	35	17.5
Rev Primer (10 x)	0.5	35	17.5
DEPC water	1.5	35	52.5
Total vol	7.5		
Mix, aliquot 7.5 ul / well			
diluted cDNA template	2.5		2.5

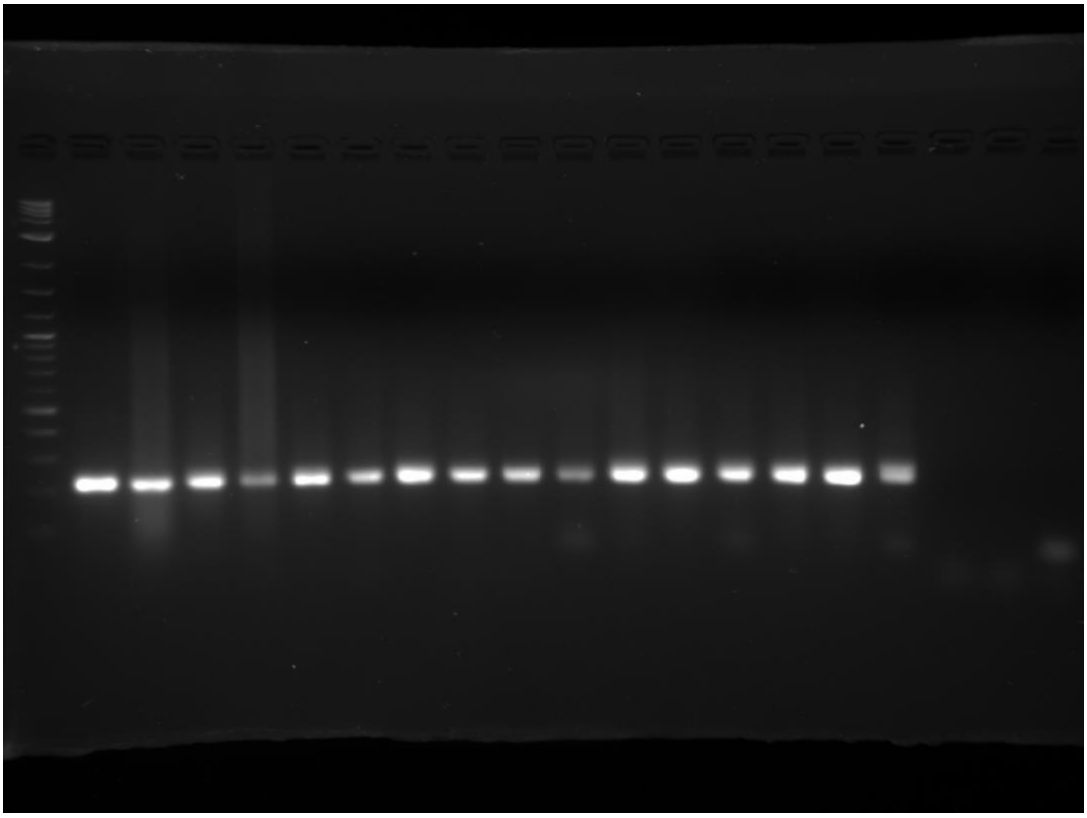
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	16 SSK liver	"	43 Nith P	"	**neg**	"						
B	32 VBat liver	"	13 Nith Crickett	"								
C	42 MSK liver 10	"	80	"								
D	137 BBbat Liver	"	9 Nith P Crickett	"								
E	57	"	10 Nith P Cricket	"								
F	63	"	68	"								
G	29 Nith P	"	77	"								
H	24 Nith P	"	54	"								

Thermocycler: 480 light cycler 3852
Computer Login: operatorPassword: LC480
LightCycler software Login: adminPassword: Roche1
96 well block, white plate, 10 ulrx, Detection format SYBR Green 1

Program name	**Cycles**	**Analysis**	**Temp °C**	**Time**	**Aquisition**
Preincubation	1		95	for 5 min	
Amplification	45	Quanitfication analysis	95	for 10 sec	
			60	for 10 sec	
			72	for 15 sec	single acquisition
Melting Curve	1	Melting Curve analysis	95	for 5 sec	
			65	for 1 min	
			97		continuous acquisition
Cooling	1		40	for 30 sec	

Samples
2 % Agarose in L.A.B. buffer 100V 45 min

1	2 log ladder
2	16 SSK liver
3	32 VBat liver
4	42 MSK liver 10
5	137 BBbat Liver
6	57
7	63
8	29 Nith P
9	24 Nith P
10	43 Nith P
11	13 Nith Crickett
12	80
13	9 Nith P Crickett
14	10 Nith P Cricket
15	68
16	77
17	54
18	Negative
19	Negative
20	



Subset Name	PHF

Results

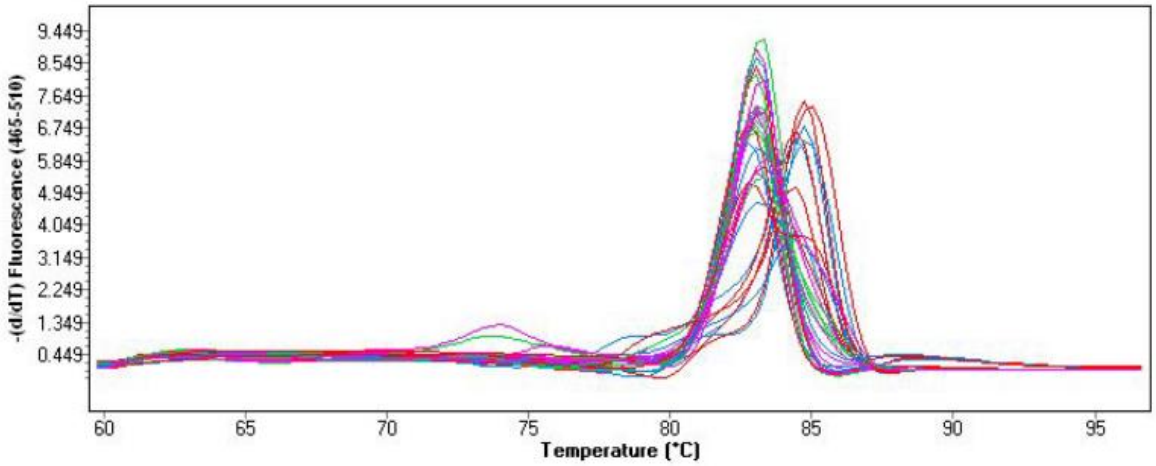
Inc	Pos	Name	Type	CP
☑	A1	16SSK liver	Unknown	28.79
☑	A2	16SSK Liver	Unknown	28.48
☑	A3	43 Nith Pond	Unknown	34.37
☑	A4	43 Nith Pond	Unknown	34.63
☑	B1	32 V Bat liver	Unknown	35.47
☑	B2	32 V Bat liver	Unknown	35.55
☑	B3	13 Nith P Crickett	Unknown	30.42
☑	B4	13 Nith P Crickett	Unknown	30.43
☑	C1	42 MSK liver 10	Unknown	31.11
☑	C2	42 MSK liver 10	Unknown	32.08
☑	C3	80	Unknown	30.56
☑	C4	80	Unknown	30.85
☑	D1	137 BBbat liver	Unknown	40.00
☑	D2	137 BBbat liver	Unknown	40.00
☑	D3	9 Nith P Crickett	Unknown	34.29
☑	D4	9 Nith P Crickett	Unknown	34.69
☑	E1	57	Unknown	35.58
☑	E2	57	Unknown	35.97
☑	E3	10 Nith P Cricket	Unknown	31.20
☑	E4	10 Nith P Cricket	Unknown	31.53
☑	F1	63	Unknown	37.62
☑	F2	63	Unknown	37.19
☑	F3	68	Unknown	33.79
☑	F4	68	Unknown	34.07

Inc	Pos	Name	Type	CP	C
☑	G1	29 Nith P	Unknown	30.97	
☑	G2	29 Nith P	Unknown	31.09	
☑	G3	77	Unknown	29.10	
☑	G4	77	Unknown	29.64	
☑	H1	24 Nith P	Unknown	38.26	
☑	H2	24 Nith P	Unknown	35.98	
☑	H3	54	Unknown	32.86	
☑	H4	54	Unknown	32.45	

Late Cp call (last five cycles) has higher uncertainty

Inc	Pos	Sample Name	Peak 1				Peak 2				Status
			Tm	Area	Width	Height	Tm	Area	Width	Height	
☑	A1	16SSK liver	84.64	18.74	2.92	6.42					
☑	A2	16SSK Liver	84.81	20.87	2.79	7.47					
☑	A3	43 Nith Pond	83.00	18.30	2.44	7.49					
☑	A4	43 Nith Pond	83.01	19.80	2.76	7.19					
☑	B1	32 V Bat liver	84.69	14.64	2.21	6.64					
☑	B2	32 V Bat liver	84.68	15.95	2.19	7.30					
☑	B3	13 Nith P Crickett	82.93	15.63	2.29	6.82					
☑	B4	13 Nith P Crickett	73.89	2.24	2.76	0.81	82.99	14.10	2.51	5.62	
☑	C1	42 MSK liver 10	84.52	12.32	1.90	6.50					
☑	C2	42 MSK liver 10	84.30	18.29	2.82	6.49					
☑	C3	80	82.91	18.69	2.27	8.25					
☑	C4	80	83.02	20.28	2.23	9.08					
☑	D1	137 BBbat liver	84.05	12.25	3.80	3.22					
☑	D2	137 BBbat liver	84.38	9.54	1.94	4.92					

Inc	Pos	Sample Name	Peak 1				Peak 2				Status
			Tm	Area	Width	Height	Tm	Area	Width	Height	
☑	D3	9 Nith P Crickett	83.10	19.55	2.91	6.71					
☑	D4	9 Nith P Crickett	83.01	17.81	2.42	7.37					
☑	E1	57	83.51	18.62	3.91	4.77					
☑	E2	57	83.41	18.76	3.29	5.70					
☑	E3	10 Nith P Cricket	83.14	18.95	2.78	6.83					
☑	E4	10 Nith P Cricket	83.28	19.89	2.79	7.13					
☑	F1	63	82.73	15.88	2.47	6.42					
☑	F2	63	82.75	16.78	2.39	7.02					
☑	F3	68	83.10	18.56	2.59	7.16					
☑	F4	68	83.08	18.41	2.63	7.00					
☑	G1	29 Nith P	83.05	19.66	2.23	8.83					
☑	G2	29 Nith P	82.99	19.25	2.25	8.55					
☑	G3	77	83.11	21.45	2.29	9.35					
☑	G4	77	83.14	18.39	2.25	8.16					
☑	H1	24 Nith P	83.05	17.35	2.79	6.22					
☑	H2	24 Nith P	83.38	20.51	4.06	5.05					
☑	H3	54	83.27	18.26	3.52	5.19					
☑	H4	54	83.59	20.87	3.64	5.73					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