

Application of molecular methods and high-throughput sequencing to identify pathogens involved in
mink footpad necrosis disease

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Executive Summary

Footpad necrosis (FPN) manifests as a vascular disease with the formation of blisters around the margins of the footpads and surrounding skin and can also appear on the face (around the eyes and the nose). These lesions can progress to ulcers because of secondary bacterial infections. In addition to the negative health impacts for the individual mink, FPN affects mink farming activities through negative impacts on breeding and production.

Suitable samples have been taken from mink with early and late stages of the disease. A metagenomic analysis of the microorganisms (both viruses and bacteria) present in all kinds of collected samples will allow the unbiased identification of pathogens involved in the disease. In total, 12 obtained libraries (4 for bacterial analysis and 8 for viral analysis) have been included in the library preparation procedure for sequencing. A different sequence barcode was assigned to each library, which will allow samples to be pooled during sequencing.

The obtained libraries will be sequenced in the coming weeks and the acquired results will allow identification of the candidate pathogens associated with FPN infections. By comparing the microbial communities identified outside the surface of the feet (representing microorganisms resident in the environment) and the ones inside the wound, it will be possible to detect those microorganisms which grow specifically in infected areas (higher load inside the wound as compared to the external environment). This unbiased identification will allow us to design a focused epidemiological study to identify which candidate can be called the causative agent.

Background

FPN is a relatively novel disease which was first reported in 1996 in eastern and central Canada, affecting farmed mink with a largely unknown etiology. The disease shows a seasonal trend as it appears during the breeding season (February-March), and it also has a higher prevalence in males. FPN manifests as a vascular disease with the formation of blisters around the margins of the footpads and surrounding skin and can also appear on the face (around the eyes and the nose). These lesions can progress to ulcers because of secondary bacterial infections. In addition to the negative health impacts for the individual mink, FPN affects mink farming activities through negative impacts on breeding and production. Some veterinarians and farmers suspect that the disease has been artificially introduced from seals to mink, since the original reported cases coincided with the first attempts of using seal meat in the mink ration. However a clear connection between the use of seal meat and the disease has never been definitively proven.

Thus far, two distinct pathogens have been nominated as possible causative agents of FPN. The first is San Miguel Sea Lion Virus, a calicivirus that can cause vesicular disease characterized by fever, vesicle formation in the oral cavity and extremities, and reproductive failure in seals and pigs. Pigs can develop the vesicular disease if fed infected meat. The second is *Arcanobacterium phocae*, a Gram-negative bacterium that can be isolated from ulcerated lesions on the flippers of seals. This bacterium has been found on mink farms with a history of FPN in both healthy and infected footpads. However, insufficient studies about the etiology of FPN have been performed and no clear correlations with any known pathogen or environmental condition have been confirmed. An additional explanation for the formation of foot lesions could be irritation to the foot pads from the wire-mesh cages commonly used to house mink. Superficial damage to the foot could allow entry to any number of bacteria that would quickly infect these lesions. Secondary bacterial infection in foot pads could make it difficult to isolate and determine the pathogen causing the primary

infection in footpads. This possible non-pathogenic explanation of FPN is beyond the scope of this project.

Rationale for Investigation

Findings from this project will serve as preliminary data to plan and perform epidemiological studies to prove a causative link between identified pathogens and FPN in mink, with the end goal of eradicating the disease from mink farms. The research will allow identification of the source(s) of the infectious agents in farmed animals, and lead to better testing and biosecurity practices.

Funding and Partnerships

To date, the researchers have received \$3,500.00 total funding for their investigation into FPN etiology.

Source	Amount	Purpose
Forestry and Agrifoods Agency	\$3,500.00	Supplies

Future funding bodies may include the National Sciences and Engineering Research Council of Canada (NSERC) and the Joint Mink Research Committee (JMRC).

Methods and Implementation

Suitable samples have been taken from mink with early and late stages of the disease. Testing different types of samples were ideal because of the potential problems with each individual sample type. For example, during the early stage of disease the pathogen load might be below the detection limit whereas during the later phase the background from bacterial super-infections could mask the signal of the true causative agent. Also, the pathogen might not spread via the blood stream or originate from the suspected environmental source. A metagenomic analysis of the microorganisms (both viruses and bacteria) present in all kinds of collected samples will allow the unbiased identification of pathogens involved in the disease.

To study the characteristics of involved bacterial communities, DNA has been isolated from the samples by means of a commercial kit (Qiagen). A multiplex PCR was performed, which was able to simultaneously amplify 3 different fragments of the highly conserved region of bacterial ribosomal RNA (16s-V3, 16s-V6-7 and 16s-V9) (Library 1).

Concerning the viral metagenomics analysis, collected samples were subjected to a pre-treatment to remove intact cells (centrifugation at 10,000g for 10 minutes) and free DNA (DNase treatments). Total nucleic acids were isolated from the pre-treated samples with a commercial kit (Qiagen) and a reverse transcription (with the Enzyme Protoscript II, NEB), and a second strand synthesis (used enzymes: RNase H, DNA polymerase I, *E. coli* DNA ligase) reaction was performed to convert all nucleic acids to double stranded DNA. The obtained DNA was divided into 2 different aliquots. The first aliquot (Library 2) was divided into 3 sub-aliquots subjected to enzymatic fragmentation (DNA Fragmentase, NEB) for 5, 10 and 15 minutes and pooled back together. The second aliquot (Library 3) was used as input to perform a random amplification PCR with random octamers (Optimized reaction parameters: 20mM primer concentration, 2.5U/reaction Taq polymerase, 26°C annealing, 20 seconds extension, 40 PCR cycles).

The 3 different libraries were then subjected to DNA purification and then included in the pipeline intended for library preparation for Ion torrent. This pipeline includes: end repair reactions to generate blunt-ended DNA fragments, a ligation reaction to ligate proper (barcoded) adaptors to the obtained fragments, an amplification reaction for the enrichment of successfully ligated fragments and a dual size selection to select fragments of appropriate length for the subsequent sequencing process.

Obtained libraries were pooled and sequenced with Ion Torrent Technology, utilizing a 400nt insert sequencing pipeline, after performing a clonal amplification of obtained fragments on beads (emulsion PCR).

Results and Discussion

A total of 20 feet samples have been received from infected mink at various degrees of the disease, and four different samples have been collected from each foot. Samples preserved in PBS were used for bacteriological analysis and samples preserved in Viral Transport Media (VTM) were used for virological tests.

1. Outside surface swab preserved in PBS
2. Internal swabs after surgical cut preserved in VTM
3. Washing of the inside of the wound preserved in VTM
4. Washing of the inside of the wound preserved in PBS

Two initial samples were selected to perform bacteriological investigations: one sample presenting late stage disease manifestations (foot #7) and one sample presenting early disease manifestations (foot # 17). DNA isolated from all four samples (swab and wash collected from both feet) was subjected to 16S amplification by means of 2 different multiplex PCRs: the first one targeting regions V3, V6-7 and V9, and the second one amplified regions V2, V4 and V8. Since it allowed us to obtain a higher amplification yield, the first approach was considered more suitable for our analysis and therefore PCR products obtained with this method were selected for library preparation.

Regarding viral metagenomics analysis, since a very low DNA load was obtained, different optimization steps were required to select the best procedures that allow us to obtain high quality reads:

1. Different random amplification approaches have been tested. This allowed us to select the one resulting in a higher DNA load. That approach involved 2 different polymerases (Taq polymerase and Klenow Fragment), different primer concentrations (from 2 to 50mM) and length (octamers vs. decamers), various incubation conditions (different extension times (from 15 seconds to 1 minute) and number of cycles (from 30 to 45).
2. Different DNA purification procedures (column- and bead-based; different DNA:beads ratios: from 1:0.8 to 1:1.8) have been compared to select the one that minimized DNA loss;
3. Different fragmentation incubation times (from 5 to 25 minutes) were attempted to determine the method that allowed the generation of a DNA library with a suitable average length.

After identifying the best experimental conditions, one sample (foot # 7) presenting late stage FPN was selected to be tested singularly, and all other samples were tested as a pool. All 4 samples (swab and wash collected from foot #7 and from all other samples as a pool) were used

to prepare viral a metagenomics library in 2 different ways: via direct fragmentation or random amplification of the obtained double stranded DNA.

In total, 12 obtained libraries (4 for bacterial analysis and 8 for viral analysis) have been included in the library preparation procedure for sequencing. A different sequence barcode was assigned to each library, which will allow samples to be pooled during sequencing.

Communication and Outreach

Communication with the farm(s) supplying the samples has been continuous, with frequent updates throughout the study. A final report detailing results will be prepared and circulated once testing is complete.

Conclusion and Future Recommendations

Microbial metagenomic studies allow the simultaneous detection of every microorganism present in a sample. These methods permit the unbiased identification of candidate pathogens which might be responsible for a disease. These procedures are very powerful, but require a very accurate optimization of every experimental step since their targets are different kinds of organisms (bacteria, DNA viruses, RNA viruses) and they must be able to detect them all with the same efficiency.

Our preliminary study identified the best procedures to adopt to perform microbial metagenomics analysis in samples collected from minks affected with footpad necrosis. Several difficulties have been found during this process, primarily due to the low DNA level present in those samples, which delayed the preparation of the libraries.

The obtained libraries will be sequenced in the coming weeks and the acquired results will allow identification of the candidate pathogens associated with FPN infections. By comparing the microbial communities identified outside the surface of the feet (representing microorganisms resident in the environment) and the ones inside the wound, it will be possible to detect those microorganisms which grow specifically in infected areas (higher load inside the wound as compared to the external environment). This unbiased identification will allow us to design a focused epidemiological study to identify which candidate can be called the causative agent.