<u>Genetic Studies of *Mycobacterium avium* subspecies *paratuberculosis* and associated *Klebsiella*
mastitis research</u>

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Submitted by: Dr. Hugh Whitney

Chief Veterinary Officer

Forestry and Agrifoods Agency

Executive Summary

The bacterium *Mycobacterium avium* subspecies *paratuberculosis* (Map) causes Johne's disease (JD) in cattle as well as other domestic and wild ruminant species such as sheep, goats and moose (1). Map can be shed in the feces of both symptomatic and asymptomatic infected animals, making it difficult to control its dissemination in the environment. This study aims to build up a molecular epidemiological profile of Map from the provinces of Atlantic Canada starting with Newfoundland and Labrador. In order to build an epidemiological profile of Map from Atlantic Canada, six farms were chosen from Newfoundland and four farms from New Brunswick and Nova Scotia. From these samples, Map was then isolated and a pure culture was obtained for genetic analysis. Fragment analysis on short sequence repeat (SSR) loci was determined to be the best strain typing method due. The team found that in most cases more than one Map strain could be isolated from a single infected animal, suggesting that the animals can be infected with multiple strains/isolates at the same time. Once whole genome sequencing has been accomplished, the comparative bioinformatics will be performed to compare genetic diversity and further expand the epidemiological profile of Map in this province. Future research is aimed at genotyping isolates to determine if the bacterial load corresponds with growth rate, as well as a developing a model to potentially eradicate MAP in its dormant state. The total amount of funding to date dedicated to these efforts is approximately $130,000.00 from various sources.

Clinical mastitis (CM) is characterized by visible abnormalities in the milk or the udder. Mastitis adversely affects milk production and cows may not regain full production levels post recovery, leading to considerable economic losses (2). It is important to determine which pathogens cause CM in NL dairy cattle. Upon identification of the bacteria, the genomes will be sequenced, virulence factors will be assessed and degree of antibiotic resistance will be a focus of the research. This research will give insight into effective biosecurity among dairy farmers and veterinarians, as well as better knowledge of how to care for dairy cows with CM. Milk samples routinely submitted to the Animal Health Laboratory from cows with CM for determination of the bacteria responsible for the illness from October 2011 to October 2012 were the primary source of isolates. All *Klebsiella pneumoniæ* (Kpn) isolates from that time period were provided for this project. The sample size was 96 bacterial isolates, each originating from a single animal from 15 dairy farms in Newfoundland. Isolates were then used for strain typing using Random Amplified Polymorphic DNA (RAPD), a PCR based fingerprinting method for strain discrimination. Results of RAPD showed that two bacterial isolates from a small number of CM animals that misidentified as Kpn were actually *Enterobacter cloacae* and *K. variicola*. The literature suggests that *K. variicola* is an emerging pathogen contributing to human infections around the world, many of which are fatal. To the best of our knowledge, this is the first report with evidence of *K. variicola* associated with CM in dairy cattle. Therefore, the prevalence of *K. variicola* may be under-reported in CM samples. The researchers aim to obtain better insight into the emergence of a *K. variicola* isolate pathogenic to animals and to determine if it has the same virulence factors as the human isolates, or is it truly an animal specific emerging pathogen.

Background

Mycobacterium avium subspecies *paratuberculosis* (Map)

The bacterium *Mycobacterium avium* subspecies *paratuberculosis* (Map) causes Johne's disease (JD) in cattle as well as other domestic and wild ruminant species such as sheep, goats and moose (1). Map can be shed in the feces of both symptomatic and asymptomatic infected animals, making it difficult to control its dissemination in the environment. In addition, Map may also be associated with some cases of human Crohn's disease (CD), a very significant cause of human illness in NL. In cattle, JD can cause significant economic losses which may be minimized through the identification of animals carrying the disease, their segregation or eventual elimination from the herd, and the implementation of on-farm biosecurity measures to reduce the introduction and spread of this and other diseases.

A national program, the Canadian Johne's Disease Initiative, has been started by the Dairy Farmers of Canada to minimize the impact of JD in the dairy cattle population. This initiative aims to directly benefit the economy by reducing infection rates, the associated reduction in other contagious diseases (through enhanced biosecurity) and the consumer confidence perspective of reducing the possible risk of human illness. In Atlantic Canada, the Atlantic Johne's Disease Initiative (AJDI) is currently funded through partnerships with the four Atlantic dairy producer groups, federal granting agencies, the four Atlantic Agri-Adapt Councils and the Atlantic Veterinary College (AVC), with the cooperation of the veterinary profession. As there was no planned research component, the AJDI project stops at the identification of Map positive farms and animals, with subsequent advice being provided to farm owners concerning disease management.

Clinical Mastitis

Bovine mastitis is an inflammation of the mammary glands and the udder tissue of dairy cattle. Mastitis-causing pathogens can either spread from cow to cow (contagious pathogens) or are picked up from the environment (environmental pathogens) such as animal bedding, manure and soil. Clinical mastitis (CM) is characterized by visible abnormalities in the milk or the udder. Mastitis adversely affects milk production and cows may not regain full production levels post recovery, leading to considerable economic losses (2). One of the CM causing environmental pathogens is *Klebsiella pneumoniae* (Kpn) which causes *Klebsiella* mastitis.

Similar to the above detailed Map research, genetic research was initiated on Kpn. Kpn is of particular concern due to its ability to acquire broad resistance to antibiotics in both humans and animals. Dairy Farmers of NL specifically requested that research be carried out in this province on *Klebsiella* mastitis. This study was also intended to describe the application and accuracy of laboratory and molecular tests for the identification of coliform bacteria associated with bovine mastitis (2).

Rationale for the Investigation

This study aims to build up a molecular epidemiological profile of Map from the provinces of Atlantic Canada starting with Newfoundland and Labrador. Such genetic analysis is commonly used with other micro-organisms to be able to follow the pattern of movement of the pathogen and to determine if there are strains that are more important than others in causing disease.

In addition, it is important to determine which pathogens cause CM in Newfoundland dairy cattle. Upon identification of the bacteria, the genomes will be sequenced, virulence factors will be assessed and degree of antibiotic resistance will be a focus of the research. This research will give insight into effective biosecurity among dairy farmers and veterinarians, as well as better knowledge of how to care for dairy cows with CM.

Funding and Partnerships

The total budget awarded from the Provincial Agriculture Research and Development Program for the 2014/15 fiscal year was $32,667.00, broken down as follows:

Professional Services	$19,667.00
Supplies	$13,000.00

The total contribution by the Agriculture Research Initiative and Provincial Agriculture Research & Development Program since the commencement of this research project has been:

Year	Amount
2014/15	$32,667.00
2013/14	$45,028.77
2012/13	$29,000.00
2011/12	$7,500.00

Additional funding has been awarded to this project and is detailed in the table below:

Source	Amount	Year	Purpose
Animal Health Division	$3,333.00	2014/15	Supplement professional services budget
Memorial University	$11,737.00	2014/15	Stipend for Susan Banfield

Methods and Implementation

Mycobacterium *avium* subspecies *paratuberculosis* (Map)

In order to build an epidemiological profile of Map from Atlantic Canada, six farms were chosen from Newfoundland and four farms from New Brunswick and Nova Scotia. In total, 85 pure cultures of Map were isolated from Newfoundland and over 100 more fromNB/NS. All samples were further classified into 3 categories based on the DTP (days to positive).

Analyzing genetic variability is commonly used with other micro-organisms to be able to follow the pattern of movement of the pathogen, to determine if there are strains that are more important than others in causing the disease and potentially other research uses. To achieve this, samples obtained from farms were plated on three separate occasions to determine the optimal growing conditions for Map. Map was then isolated and a pure culture was obtained for genetic analysis.

PCR conditions were optimized for multilocus short sequence repeat (MLSSR) sequencing for four target loci. These four loci gave the highest genetic diversity indices and they were the most discriminatory, stable and informative. Therefore, they were selected as targets for strain

discrimination. In addition, fragment analysis is another technique used to discriminate between the different MAP genetic loci. This technique uses fluorescently labeled PCR fragments of DNA that are separated by capillary electrophoresis for comparison to a size standard. This system can detect a one base-pair difference, making it a reliable method for measuring DNA fragment size and discriminating between different loci. Fragment analysis is less costly than sequencing each locus and does not require as much manipulation when compared to MLSSR for strain discrimination. The best method will be determined.

Klebsiella mastitis

Milk samples were collected from animals with signs of CM by the Animal Health Division between October 2011 and October 2012. The sample size was 96 bacterial isolates, each originating from a single animal from 15 dairy farms in Newfoundland. After initial species determination in the Animal Health Laboratory, isolates were sent to the Public Health Laboratory of Eastern Health (St. John's) for genus and species confirmation using a mass spectrometry based approach. Chromosomal DNA was extracted from 45 isolates, and species level identification was achieved by determining the DNA sequence of the *rpoB* gene and comparing it to those present in a public database. These isolates were then used for strain typing using Random Amplified Polymorphic DNA (RAPD); a PCR based fingerprinting method for strain discrimination. DNA banding patterns were used to produce dendrograms using the PyElph software.

Results and Discussion

Mycobacterium avium subspecies *paratuberculosis*

From the fecal culture samples, collected from NB, NS and NL dairy farms, 200 pure cultures of Map were grown, a necessity for further molecular studies. A majority of the samples could not be cultivated due to contamination. Initial work on the genetic characterization of Map isolates from NL has also been completed and a publication on the results has been accepted for publication in PLOS ONE "Typing of *Mycobacterium avium subspecies paratuberculosis* isolates from Newfoundland using fragment analysis".

Fragment analysis on short sequence repeat (SSR) loci was determined to be the best strain typing method due to its high resolution capacity, discriminatory power, reproducible pattern, time and cost effectiveness. Fragment analysis was conducted on 88 isolates from Newfoundland based on SSRs of 4 loci. From these 88 isolates, 40 distinct strains were successfully genotyped and differentiated. One reason for this finding could be the insular nature of Newfoundland, which could allow for the emergence of unique SSR-types on the island. Alternatively, animals could have acquired Map with the respective SSR-types from other herds in Atlantic Canada and these unique SSR-types could be circulating in other provinces. Unfortunately, SSR analysis has not been reported for the majority of herds in NS or NB for comparison. In addition, these SSR-types could exist in other parts of the world, but are not yet published, as there are only a few published Map epidemiology studies using SSR analysis (1).

The team found that in most cases more than one Map strain could be isolated from a single infected animal. This suggests that the animals can be infected with multiple strains/isolates at the same time, which is not a common finding. The team is following-up on the implications of their findings to see if similar patterns are also seen in farms from other provinces in Atlantic Canada.

Klebsiella Mastitis

Results of RAPD showed that two bacterial isolates from a small number of CM animals that misidentified as Kpn were actually *Enterobacter cloacae* and *K. variicola*. Originally, *K. variicola* was described to be unable to ferment adonitol, which was a test used for identification. Upon species confirmation based on *rpoB* sequencing, it was determined that both *K. variicola* isolates could metabolize/ferment adonitol and the original test failed in identification. Examinations of the two *K. variicola* isolates demonstrated that they were identical. Therefore, infection could have resulted in animal to animal transmission, or a single strain infecting both animals independently. The *E. cloacae* strains observed from the two different farms were not the same.

In addition, all 45 strains were tested for antimicrobial susceptibility using 5 drugs that are commonly prescribed to treat gram-negative infections in veterinary medicine; cephalothin, ceftiofur, streptomycin, tetracycline and TMP-sulfa. *E. cloacae* isolates showed resistance to cephalothin, *K. pneumonaie* isolates showed varying degrees of resistance to streptomycin, tetracycline and TMP-sulfa. *K. variicola* isolates were sensitive to all drugs tested.

Communications and Outreach

In 2014 the team published a peer reviewed article (Podder *et al*. 2014. *Klebsiella* species associated with bovine mastitis in Newfoundland. PLoS ONE 9(9): e106518. doi:10.1371/journal.pone.0106518), identifying that some of the CM cases were caused by *K. variicola*. The publication put out by the team was the first report on pathogenic *K. variicola* from dairy animals. Initial work on the genetic characterization of Map isolates from NL has also been completed and a publication on the results has been accepted for publication.

In addition, results were presented at national, international and provincial level meetings (Ottawa in 2013, Turkey in 2014, Banff in 2014 and Corner Brook in 2014); even though no travel funding was received for this purpose as part of previous submissions. Most of these were invited presentations, demonstrating the establishment of an internationally competitive research program here in NL.

Conclusions and Future Recommendations

Mycobacterium avium subspecies *paratuberculosis*

The team found that in most cases, more than one Map strain could be isolated from a single infected animal. This suggests that the animals can be infected with multiple strains at the same time, which is not a common finding. The team is following up on the implications of their findings to see if a similar pattern can be found on other farms in Atlantic Canada. Based on the fragment analysis, the 6 most diverse strains of Map will have their genomes sequenced and annotated in order to compare genetic diversity based on SSR loci strain typing based on genome analysis. Once whole genome sequencing has been accomplished, comparative bioinformatics will be performed to compare genetic diversity and further expand the epidemiological profile of Map in this province.

All samples were classified into 3 categories based on the DTP (days to positive). This measure is used for detecting Map growth in cultures by applying the automated growth procedure used at UPEI. It has been speculated that DTP information can be used as an indication of the

shedding loads from infected animals. One of the questions we are interested in is if the differences in DTP values are due to the growth rates of different Map isolates instead of initial shedding/loads numbers. Therefore, the cultures that test positive for growth in approximately less than 3 weeks are classified as fast growing with a higher bacterial burden, cultures that test positive for growth after approximately one month are classified as slow growing with a low bacterial burden, all others were classified as intermediate. Susan Banfield is currently genotyping isolates from the different classifications and will be determining and comparing their growth rates to address the validity of using DTP values to compare shedding loads.

Map can survive for prolonged periods in the body of a human or animal without causing any symptoms. This is referred to as a state of dormancy. The researchers are currently developing anaerobic and nutrient deprivation models for Map to determine the necessary conditions required to force the mycobacterium into dormancy under laboratory conditions and study the mechanisms involved in this transition. While in a metabolically active state, Map is susceptible to metals such as copper. Once Map enters a dormant state, it is very difficult to eradicate. This model will allow for metal toxicity and chemo susceptibility trials on dormant Map, giving insight on how to eradicate the bacteria in its asymptomatic state.

Klebsiella Mastitis

In the past year, it was determined that *K. variicola* and *E. cloacae* were misidentified as Kpn in a small number of CM cases in Newfoundland. *K. variicola* is usually found on plants and in the environment, not on the tissues of animals with CM. The literature suggests that *K. variicola* is an emerging pathogen contributing to human infections around the world, many of which are fatal. Results from the work conducted in NL are the first report showing that *K. variicola* is also associated with animal infections.

To the best of our knowledge, this is the first report with evidence of *K. variicola* associated with CM in dairy cattle as it was originally described to be unable to metabolize/ferment adonitol. Upon further analysis, the researchers determined that *K. variicola* was in fact able to ferment adonitol by identifying the bacteria through *rpo*B sequencing. Therefore, the prevalence of *K. variicola* may be under-reported in CM samples because current testing may not be sensitive enough to discriminate from *K. pneumonaie.* Epidemiologic analysis of the isolates was also completed. RAPD typing was done for all isolates and a dendrogram was built using the PyElph software.

The researchers aim to conduct WGS on *K.variicola* isolates from NL and compare with those of non-pathogenic strains and isolates from human patients, which have already been sequenced by other groups. The obtained genome sequence will also be analyzed for the presence of previously identified virulence factors. This will give the team better insight into the emergence of the animal pathogenic *K. variicola* isolate and to determine if it has the same virulence factors as the human isolates, or if it is truly an animal specific emerging pathogen. The team also plans to conduct WGS analysis on any new and atypical bacteria that are isolated from cases with CM, which is an ongoing process.

References
(1) Podder, M. P., Banfield, S. E., Keefe, G. P., Whitney, H. G., Tahlan, K. (2015).Typing of Mycobacterium avium subspecies paratuberculosis isolates from Newfoundland using fragment analysis. Draft.

(2) Podder, M. P., Rogers, L., Daley, P., Keefe, G. P., Whitney, H. G., Tahlan, K. (2014). *Klebsiella* species associated with bovine mastits in Newfoundland. *PLOS One, 9*(9), e1065518.