EXAMINATION OF THE MOLECULAR DIVERSITY OF *KLEBSIELLA PNEUMONIAE* AND *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*: TWO PATHOGENS ASSOCIATED WITH THE DAIRY INDUSTRY IN NEWFOUNDLAND

by

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Abstract

Klebsiella pneumoniae and *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) are two important pathogens of cattle causing clinical mastitis (CM) and Johne's disease (JD), respectively. Information regarding the molecular diversity of these two pathogens is lacking from Newfoundland. The aim of this study was to evaluate the ability of different molecular techniques for bacterial species identification and strain discrimination, within and between dairy farms from Newfoundland. For CM, results demonstrate that molecular approaches were able to detect *K. variicola* and *Enterobacter cloacae*, which were misidentified as *K. pneumoniae* by standard biochemical/phenotypic tests. In the case of *Map*, fragment analysis of 4 short sequence repeats (SSRs) enhanced the capability to accurately differentiate between apparently identical isolates. Polyclonal infection patterns were observed for *K. pneumoniae* and *Map* in the current study. Therefore, the molecular identification of bacteria, along with precise genotyping analysis using contemporary and improved methods will be useful in future epidemiological studies. **(149 words)**

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List of Abbreviations and Symbols

Acronyms that are used commonly in molecular biology are not included in this list.

AFLP	Amplified fragment length polymorphism
CD	Crohn's disease
CLSI	Clinical and Laboratory Standards Institute
CM	Clinical mastitis
CMT	California mastitis test
DTP	Days to positive (for detecting *Map* growth in cultures)
ELISA	Enzyme-linked immunosorbent assay
FAM	Fluorescein amidite
*gyr*A	DNA gyrase subunit A
IMViC	Indole-Methyl red- Voges-Proskauer-Citrate
JD	Johne's disease
LJ	Lowenstein-Jensen medium
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
Map	*Mycobacterium avium* subsp. *paratuberculosis*
MLST	Multilocus sequence typing
MST	Minimum spanning tree
NL	Newfoundland and Labrador
*par*C	Subunit of topoisomerase IV
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplified polymorphic DNA
*rpo*B	Beta subunit of RNA polymerase
SCC	Somatic cell count
SSR	Short sequence repeat
TCAG	The Centre for Applied Genomics
TMP-sulfa	Trimethoprim sulfamethoxazole
UPEI	University of Prince Edward Island
VNTR	Variable number tandem repeat

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Chapter 1: General Introduction

1.1 The importance of the dairy industry in Newfoundland

The dairy industry, which includes dairy farms and dairy processing plants, is a major contributor to the economy. Dairy farms are involved in the production of raw milk, whereas dairy processing plants produce a variety of dairy products (such as processed milk, cheese, butter, yogurt, ice cream, etc.). Based on farm cash receipts, the dairy industry ranks third in the Canadian agriculture sector with Newfoundland and Labrador (NL) having the fewest number of dairy farms of all the provinces [1]. Although Quebec and Ontario are the major dairy producing provinces in Canada, NL has the highest number of dairy cows per individual farm and some of the largest farms in the country [1]. The close contact between large numbers of animals within dairy farms can make them prone to the spread of infectious diseases, which can cause substantial financial losses to the agricultural industry. Knowledge regarding the causative agents of these diseases can help implement better prevention and control programs, which can help to control and reduce disease occurrence.

1.2 Bacterial pathogens associated with the dairy industry

In addition to fungal, parasitic and viral agents, many diseases associated with bovine dairy animals are caused by bacterial pathogens from the genera *Staphylococcus*, *Streptococcus*, *Escherichia*, *Corynebacterium*, *Klebsiella*, *Pseudomonas*, *Mycobacterium* and *Mycoplasma*, to name a few [2-4]. Differences in herd management practices, environmental factors and host animal immune status can contribute to an increase in the occurrence of bacterial diseases [5]. Furthermore,

some diseases that infect dairy animals are zoonotic in nature and can therefore be transmitted to humans [6], highlighting the importance of understanding them better. The work described in this thesis will focus on two bacterial pathogens affecting dairy animals: (i) *Klebsiella* species, one of the causative agents of environmental clinical mastitis (CM, cases where the cow displays definitive symptoms of inflammation of the mammary glands and udder tissue) and (ii) *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), which causes Johne's disease (JD), a contagious bacterial disease of the intestine which is associated with inflammation.

1.3 Mastitis and *Klebsiella* species

Mastitis in dairy animals is caused by a number of microorganisms, with the major ones being *Streptococcus uberis*, *Staphylococcus aureus* and *E. coli* [7]. Other pathogens include *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, *Mycoplasma* spp.,other *Staphylococcus* spp., *Klebsiella* spp., *Citrobactor* spp., *Enterobacter* spp., *Pseudomonas aeruginosa*, *Pasteurella* spp. and *Bacillus* spp. [7]. Sometimes fungi, yeasts and molds are also present in the infected tissues [7]. Major and minor pathogens can be distinguished by the difference in somatic cell counts (SCCs, such as white blood cells) and clinical signs such as reduced milk production and occasionally by animal mortality [5]. It has been reported that reduction in milk production depends on the specific pathogen causing CM, and that Gram negative bacteria are responsible for greater losses than Gram positive bacteria and other microorganisms [8,9]. Presently, *K. pneumoniae* is emerging as a pathogen of concern associated with veterinary and human medicine

due to its ability to acquire and disseminate antibiotic resistance capabilities [10,11]. In a previous study, CM caused by *Klebsiella* spp. was responsible for significant reduction in milk yields in multiparous animals (animals having experienced more than one parturition) [12]. Therefore, precise knowledge of the infectious agent is important for making decisions regarding treatment and control of the disease.

Mastitis-causing pathogens can spread either from one animal to another (classified as contagious pathogens), or can be acquired from the environment (classified as environmental pathogens, which includes *K. pneumoniae*) such as animal bedding, manure and soil [13,14]. Contagious pathogens are usually present on the udder and teats, and are transferred from infected to uninfected animals during milking by the equipment or the handler. Environmental pathogens differ from contagious pathogens as they do not normally adhere to the udder or the teats [7]. Animals infected with environmental pathogens usually have lower SCCs as compared to those infected by contagious pathogens [15].

Klebsiella are rod-shaped Gram negative facultative anaerobes [16]. *K. pneumoniae* and *K. oxytoca* are closely related opportunistic pathogens [7] which are usually shed in the feces and milk of infected cows [10,17]. Generally cows with mastitis (especially *Klebsiella* associated mastitis) do not regain their full milk production levels post recovery, which leads to considerable economic losses [18]. Animals with *Klebsiella* associated mastitis are more likely to undergo mortality or to be culled as compared to animals with other types of mastitis [14,16,19]. Vaccination decreases the severity and occurrence of the clinical cases of coliform mastitis, but it cannot reduce the total number of new CM cases caused by *Klebsiella* spp. [14,20].

Therefore, it is important to know the source of the mastitis infection (as it can infect both lactating and dry cows) for effective prevention or control programs.

1.3.1 *K. pneumoniae* transmission and diagnosis

In the majority of cases, signs of CM include abnormal milk (flakes, clots and watery milk) and udder swelling [7]. Animals can also have a fever and display other symptoms which include lack of appetite, sunken eyes, diarrhea, dehydration and reduction in mobility [7]. There are also subclinical cases where the milk appears normal and animals have no clinical signs of mastitis. These subclinical cases can be a source of infection where transmission happens largely through fecal shedding [21]. CM is usually diagnosed through cultures of milk samples [10] as fecal shedding is not associated with active/clinical infections [14]. Major outbreaks of CM have been reported from numerous countries, mostly within the initial two weeks of lactation in animals [22]. These infections were linked to environmentally contaminated wood shavings or sawdust used in bedding and stalls [14]. In the environment, *K. pneumoniae* can survive as an endophyte (an organism that lives within a plant without causing any apparent disease) of wheat, corn and alfalfa, and aids in nitrogen fixation. Consequently, plant parts that are consumed by animals can be a source of infection even in the absence of contaminating animal feces [14]. Therefore, *K. pneumoniae* can be acquired by animals through crops used for feed, or by fecal contamination of the environment.

1.3.2 Economic impact of CM

Worldwide economic losses due to mastitis are immense, though actual losses vary from country to country [23]. The presence of animals with CM on Canadian dairy farms, especially lactating animals, is of major concern to the dairy industry. The deteriorating physical condition of infected animals and the decrease in milk quality and production levels cause significant economic losses to farmers and the dairy industry [7]. Other factors influencing financial losses are medication expenses, removal of contaminated bulk milk, culling and replacement of infected cattle and penalties for not meeting milk quality standards [7,24,25]. This has a huge impact not only on the dairy farmers, but also on consumers [23].

In Canada, *S. aureus* is the main cause of contagious CM, whereas *Klebsiella* associated environmental CM is not as prevalent [26]. However, when compared to other Gram negative pathogens such as *E coli*, mastitis caused by *Klebsiella* spp. is responsible for greater losses due to reduced milk production and longer periods of infection [16]. According to a previous report on Canadian dairy farms, CM caused by *Klebsiella* spp. occurs more frequently in animals housed in free stalls as compared to those housed in tie-stalls (animal tied in by a neck-chain) [27]. Therefore, farm practices can play an important role in the prevalence of diseases caused by particular pathogens, highlighting the importance of identifying an agent and its source.

1.3.3 Pathogenesis and pathology

Diverse groups of bacteria can cause CM, which include different or multiple isolates within the same species. The teat canals of dairy animals have physical and chemical barriers that inhibit the entrance of pathogens, but become vulnerable during calving and lactation [7,28]. During this period, CM causing pathogens release toxins that are recognized by the host immune system, which in response recruit more specialized immune cells to the site of the infection to combat the invading bacteria, causing inflammation and reduced milk production [7,28]. This leads to the manifestation of noticeable signs characteristic of CM, such as the reddening of the udder and the production of milk with watery appearance, which sometimes contains clots and flakes [7,28]. In subclinical mastitis, there are no visible changes in the milk or the udder, despite having inflammation in the mammary glands, and in cases of chronic mastitis, inflammation progression can continue for months and may persist from one lactation period to another [7].

1.3.4 Diagnostic and molecular tools for research on *Klebsiella* spp. associated with CM

To reduce the cost of mastitis treatment, early and accurate detection of the pathogen is very important. Assays used for CM diagnosis and pathogen identification include measurement of SCCs in milk, immunoassays, multiplex polymerase chain reaction (PCR), quantitative PCR, culture based tests, mass spectroscopy based identification of the pathogen, electrical conductivity tests and infra-red thermography of milk [7,28], most of which are expensive and labour

intensive. Currently, culture based techniques, along with biochemical tests, are considered the gold standard methods to detect mastitis-causing pathogens, but the search for more robust methods continues.

Although molecular detection techniques are more complex and cumbersome than culture based methods due to the involved equipment, protocols and reagents, in many instances they provide faster and more accurate results. Numerous nucleic acid sequence based testing methods such as multiplex PCR and quantitative PCR have been developed for the detection of mastitis pathogens in milk [7,29], which are more sensitive and faster than other diagnostic/molecular tools. Moreover, quantitative multiplex PCR can detect up to 11 bovine mastitis pathogens within a few hours [30]. In addition, sequencing of the 16S rRNA, *rpo*B (beta subunit of RNA polymerase) and other genes is commonly used to identify/type bacteria, mainly for research purposes [19,31,32]. Nucleic acid sequence based amplification assays are being perfected using information available from the whole genome sequences of pathogenic bacteria, as they have the capability to distinguish between dead, spore-forming, dormant and actively growing microorganisms in milk in a short period [28,33].

Despite the prevalence of *Klebsiella* mastitis (CM caused by *Klebsiella* spp. only) in dairy cattle [19], information regarding the transmission and molecular diversity of the pathogen is still insufficient for successful control programs [34]. Molecular typing methods can differentiate strains and are able to track the transmission of isolates between and within farms. Genotyping techniques for *Klebsiella* spp. include pulsed-field gel electrophoresis (PFGE), random amplified

polymorphic DNA (RAPD) analysis, repetitive DNA sequence PCR, ribotyping, multilocus sequence typing (MLST), *gyr*A (DNA gyrase subunit A) and *par*C (subunit of topoisomerase IV) gene sequencing and amplified fragment length polymorphism (AFLP) analysis [31,32,35,36]. These methods can enhance the discriminatory power for molecular typing analysis to provide deeper insight into strain prevalence and relatedness.

1.3.5 Drugs used for the treatment of CM

At present, the following 11 drugs are used for treating CM caused by Gram negative and Gram positive bacteria: oxytetracycline, trimethoprim-sulphadoxine, ceftiofur, cephapirin, erythromycin, pirlimycin, procaine, penicillin G, streptomycin, novobiocin, polymyxin B and cortisone [37]. Tetracycline (41%), cephalosporin (78%) and most importantly penicillin (86%) are the most frequently used antibiotics for CM treatment in dairy farms [7]. Trimethoprim sulfa or tetracycline is widely used for treating calves, whereas ceftiofur (80%), tetracycline (31%) and penicillin (32%) are used for adult cows [7,38]. In addition, cephalothin, ceftiofur, pirlimycin, novobiocin, streptomycin, tetracycline, trimethoprim sulfamethoxazole or TMP-sulfa are used for the treatment of CM cases in Newfoundland (Table 1.3.5.1) (Animal Health Laboratory, Animal Health Division, Department of Natural Resources, Newfoundland and Labrador, Canada).

Treatment of CM caused by *Klebsiella* spp. has a lower rate of success when compared to infections caused by Gram positive pathogens [16]. Schukken *et al.* (2011) reported that a third generation cephalosporin (ceftiofur hydrochloride) was

successful in treating uncomplicated cases of *Klebsiella* associated CM in dairy animals [38]. However, the majority of studies regarding antibiotic treatment outcomes on *Klebsiella* mastitis demonstrated the inadequate effectiveness of standard drug regimens [16]. Therefore, it is important to ensure that antibiotic resistance does not arise for any agents showing even low to moderate levels of efficacy; otherwise even the slightest hopes of treatment possibilities would be lost.

1.4 *Map* and JD

Map is the etiological agent of JD, which is associated with chronic inflammation of the small intestine of cattle, sheep, goats, farmed deer and other ruminants [39-44]. *Map* is an acid-fast, intracellular pathogenic bacterium [45], and requires specific conditions for replication *in vitro*. However, *Map* can survive in a dormant or non-replicating state under unfavorable conditions as well, which creates severe problems for eradicating *Map* from herds with JD [46]. JD shares some clinical and pathological features with human Crohn's disease (CD), making JD a cause for public concern [47]. In addition, *Map* has also been isolated from some, but not all patients with CD [48,49], HIV-AIDS [50-53] and type 1 diabetics [54], causing some concern regarding its association with humans having abnormal immune systems. Therefore, JD has become a major topic of discussion within the agricultural industry due to the potential link between *Map* and public health [43].

JD causes reduced milk production in dairy animals, and severe cases can lead to premature culling [55]. Owing to the economic impact of *Map* on the dairy industry, it has been studied extensively [56-58]. In Canada, the assessed yearly

losses caused by JD are CDN$ 15 million nationwide, and CDN$ 0.84 million for Canadian Maritime Provinces [59]. However, actual losses can be underestimated due to the lack of proper identification/diagnosis of infected animals and the absence of clinical symptoms in many infected animals [59]. In addition, there is no report regarding economic losses caused due to JD in NL to date.

1.4.1 *Map* transmission

Map transmission can occur through contaminated soil (such as pastures), contaminated water and infected animal feces. *Map* can survive for more than two years in the soil due to dormancy, which is responsible for bacterial survival under unfavorable conditions until the bacteria is taken up by a susceptible host [46]. The main animal to animal transmission pathways for *Map* are the fecal-oral route [60], consumption of infected milk by calves [61], transmission during birth and/or exposure to an infected animal [60,62]. Some epidemiological studies suggested that transmission of *Map* occurs early in a calf's life [60]. Young calves are more susceptible to infections than the adults, as adults require a substantial amount of bacterial load in order to become infected [43,61]. Calf management has been proposed for farm level *Map* eradication [63], but this was shown to be unsuccessful due to the other routes of transmission. Bioaerosols have also been suggested as a transmission route [64], as dust containing *Map* can be ingested and inhaled by animals leading to infection and spread of the disease [62,65]. *Map* spreads between and across species with no constraints, making it difficult to control. Moreover, wild animals can act as reservoirs and may transfer the bacteria to and between farms [66].

Waterborne *Map* is also known to be a source of infection and there are some reports where large quantities of *Map* were washed into the rivers from farm lands in the Midwest United States, which then reached some provinces of Canada (Manitoba, Saskatchewan and Alberta) [67]. Therefore, it is important to investigate the source of *Map* and its transmission in order to devise effective control programs.

1.4.2 Cell structure and metabolism

Mycobacterial species usually produce a secreted lipophilic siderophore called mycobactin, which is involved in the binding of extracellular iron for transport into the cell [68]. Iron is an essential nutrient for growth, and *Map* is unique amongst the mycobacteria because it is not capable of producing mycobactin [69,70]. *In vivo*, infected host macrophages usually provide the iron necessary for *Map* growth and replication, but mycobactin is essential for culturing *Map* under *in vitro* conditions [71]. However, the requirement for mycobactin can differ based on the media used for culture purposes. In a previous study, isolates collected from clinical cases of sheep paratuberculosis required mycobactin for growth on a Lowenstein-Jensen (LJ) medium, but the same *Map* isolates did not require mycobactin when cultured on Middlebrook 7H11 agar [72]. The reason for the differences in mycobactin requirement might be due to strain variation or specific components present in the different media [71,73]. *Map* is also unable to use iron and multiply in soil or water environments [46,61].

Similar to other mycobacteria, the cell wall of *Map* has a highly impermeable lipid rich barrier comprised of mycolic acids, which enables it to survive extreme

environmental conditions [74]. In addition, the cell wall enables *Map* to escape from host defenses and to survive within the phagosome of the host cell for up to two weeks [45]. In bovine macrophages, *Map* cell wall components modulate host responses for bacterial survival, such as intracellular multiplication and initiation of pathogenesis [45,75]. Up to 60% of the *Map* cell wall is comprised of complex lipids, due to which it exhibits the following properties: acid-fastness, increased lipophilicity, and resistance to a number of adverse conditions/reagents (low pH, ultraviolet light, high temperature, pasteurization, certain chemicals, hydrophilic antibiotics, etc.) [61,75]. The growth rate of *Map* is one of the slowest of all mycobacteria, which is partly attributed to the thick hydrophobic cell wall that acts as a barrier for the flow of nutrients [74]. The cell wall characteristics of *Map* along with its metabolism are important factors in diagnosis and for cultivating the bacterium.

1.4.3 Pathogenesis and pathology

Macrophages have the capability to kill a wide range of bacterial pathogens. *Map* can avoid this killing activity, and can grow and replicate inside macrophages [61]. This phenomenon is not only due to the chemically distinct cell wall of *Map*, but is also attributed to effector molecules secreted by the pathogen that neutralize the antibacterial chemicals produced by macrophages, thereby suppressing immune reactions [76,77]. Therefore, pathology and host cell dysfunction due to *Map* occurs because of the direct action of bacterial products combined with the host's immune response to the pathogen.

JD development can be divided into four stages: silent infection, subclinical, clinical and advanced clinical disease [55]. Following ingestion, *Map* initially establishes itself in the lymphoid tissue of the gastrointestinal tract of the animal (silent infection) [45]. Clinical signs and symptoms of the disease will not be noticeable during the initial years following infection, which can take up to ten years [47]. During this time, some of the *Map* cells are engulfed by macrophages, which initiate immune reactions such as lymphocyte instigation and clonal proliferation (subclinical stage). In subclinical cases, infected cows shed *Map* without exhibiting any clinical signs of the disease [43,57]. In addition, certain animals may never display any clinical signs of JD due to the host's immune system, such that they remain undetected and continue to infect other animals in the same herd [55,78]. After the initial immune response, infected animals start to display signs of the disease (clinical stage) [55], which include diarrhea, weight loss, decline in milk production and fatigue [3,43,79]. During this period the intestinal wall thickens due to inflammation, and after some time intestinal cells stop functioning, leading to malabsorption and enteropathy (advanced clinical stage) [61]. The pathology of JD can provide information about the infected animal's disease history and status, which when combined with analysis of *Map* isolates can provide a clearer picture on the molecular epidemiology of the disease.

1.4.4 Diagnosis

As for any infectious disease, prompt and proper diagnosis is necessary for the early detection and treatment of infected animals to prevent culling, along with the

spread of JD. Diagnosis has been proven to be more successful when an entire herd is examined rather than screening only suspected infected animals [43]. Unfortunately, tests for inspecting an entire herd are costly, laborious and time consuming [47,80], and vaccination can only reduce the rate of disease spread and not prevent JD occurrence [61,67]. There are a number of test methods available for diagnosing JD, such as: histopathology, microscopy, culture, PCR, agar gel immune-diffusion, enzyme-linked immunosorbent assay (ELISA), complement fixation test, delayed-type hypersensitivity and gamma interferon release assay [59,81-87].

Microscopy can be performed on fecal matter, fecal culture, and intestinal mucosal smears after Ziehl-Neelsen staining [88]. However, microscopy cannot distinguish *Map* from other mycobacterial species since all of them have acid-fast properties. In addition, specific sample smears on a slide might not contain *Map* at detectable levels. So, microscopy is not useful for confirmation of a negative case based upon a single fecal sample.

Cultivation of *Map* from a live animal specimen is considered 'the gold standard' for diagnosis [88, 89]. Although *Map* requires a long time to grow in culture media and is prone to contamination, culture results provide 100% specificity and are regarded as the 'definitive diagnosis of infection' [88]. The true infection status of an individual animal can be determined through culturing multiple (up to 100 sites) samples of intestinal tissue [90]. Fecal culture is the best non-invasive and most inexpensive diagnostic method for JD, but the rate of detecting *Map* in feces from infected animals is not consistent [84]. When successful, fecal culture can detect all the four stages of disease progression in an infected animal [88].

Since fecal samples contain many microorganisms in addition to *Map*, they require special treatments to enrich *Map* and to prevent the growth of contaminating microorganisms during subsequent culturing. There are two main methods for sample decontamination before *Map* culture: the first method uses oxalic acid combined with sodium hydroxide, and the second method uses hexadecylpyridinium chloride [88]. Increasing treatment times during the decontamination process can prevent the growth of unwanted bacteria and fungi, but it may also interfere with the subsequent growth of *Map* [91,92].

Previous studies have suggested that certain media might be better suited for cultivating and isolating colonies of *Map*. Various types of liquid, egg-based, and agar-based culture media have been used for *Map* cultivation, some of which include: Herrold's egg yolk medium, LJ medium, Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook 7H11 agar, liquid BACTEC^{TM} 12B or Middlebrook 7H12 supplemented with egg yolk, Trek-ESP II liquid culture, Kirchner liquid medium, Dubos Broth-base medium, Sauton's liquid medium, and the Proskauer and Beck liquid medium [53,71,90,93,94]. On solid media, small colonies of *Map* become visible after two to six months and liquid cultures show growth within 3-6 weeks, when incubated at the optimal temperature (37°C) and under aerobic conditions [3,75,88], although the time required for the appearance of isolated colonies varies according to media and the specific isolate [71]. As mentioned previously, *Map* generally requires exogenous mycobactin such as mycobactin J for growth in culture medium. However, *Map* sub-cultures can grow in a medium that has no mycobactin added because of iron carry-over from the primary culture [71,88]. For selective

growth of mycobacteria, media supplements, such as oleic acid, albumin, dextrose and catalase (OADC or ADC) are added to the medium, along with glycerol. Contamination can be reduced by adding antimicrobial agents, such as polymyxin B, amphotericin B, carbenicillin, trimethoprim lactate, nalidixic acid and azlocillin, to the media [53]. The addition of detergents such as Tween 80 helps to prevent the clumping of *Map* cells in liquid cultures so that dispersed growth can be attained [53]. Liquid supplements, such as sodium pyruvate that can function as a source of energy and protects against toxic hydrogen peroxide, can have beneficial effects, but they can also inhibit the growth of some *Map* strains [71,95]. Therefore, the proper selection of growth media and supplements is crucial for successful *Map* cultivation in the laboratory.

1.4.5 Molecular tools used for diagnosis and research

IS*900*, an insertion sequence (transposable DNA fragment) specific for *Map*, is used as the target for PCR amplification to identify *Map* in milk and fecal samples from infected animals [88,96]. Additional insertion sequences which are specific for *Map* include: IS*1311*, IS*Mav2*, f57 and IS*Map02* [88,97,98]. However, IS*900* is commonly used as it can be easily detected due to the presence of 17 copies of the sequence per *Map* cell [99]. PCR provides more rapid results when compared to most other diagnostic assays, but it has lower sensitivity as compared to fecal culture due to the presence of PCR inhibitors and low template DNA yields from samples [55,100,101]. Therefore, IS*900* PCR is commonly used for *Map* detection and

identification in Canada in combination with culture [55]. Quantitative PCR has also been reported to perform well for detecting *Map* from environmental sources [102].

The selection of appropriate sample collection, processing and DNA extraction procedures are important factors when conducting PCR on samples from animals infected with *Map* [102]. There are a number of methods for extracting high quality DNA from *Map* to conduct PCR, which include rapid lysis, organic extraction, silica particle-based and magnetic particle-based technologies [101]. The particle-based technologies give better yields and higher quality *Map* DNA in a short period of time for PCR analysis [101]. In addition, homogenization of samples by bead-beating prior to DNA extraction improved PCR performance [43], a factor that will be considered while performing the analysis described in chapter three of this thesis.

1.4.6 Epidemiological aspects of *Map*

Studying the epidemiology of JD is extremely important due to the financial losses caused by the disease [49,101,103] and the probable association between *Map* and CD in humans [3,104,105]. In a number of studies, viable *Map* was detected in certain dairy products such as pasteurized milk and cheese [106,107], which could have implications on the health of consumers [49,108]. Molecular techniques have been developed and applied to understand the transmission of *Map* and for identifying predominant strains [3,99]. In many dairy herds, distinct strains of *Map* were found to cause JD in different animals [3], but there are also reports where single or related strains were responsible for infections [43]. Therefore, to implement successful

control programs, it is necessary to find the source of the strain(s) in question. It is important to determine whether the detected cases of JD are due to new infections (acquired by introducing subclinically infected animal from another herd), or if the responsible strain type was already present in that herd (infected resident animal or environmental contamination) [43]. Therefore, there is value in conducting molecular epidemiological studies on *Map*.

1.4.7 Strain typing method for analyzing *Map* strain diversity

Phenotypic diversity is described based on the culture characteristics (colour/ pigmentation duration of growth) of *Map* strains. *Map* strains have been divided into two categories: sheep and cattle types [109,110]. Colonies of the sheep-types are pigmented and slow growing, whereas cattle-types are non-pigmented and fast growing [71]. Moreover, sheep-type strains are harder to cultivate than the cattle-types strains [71]. Standard tests such as ELISA cannot discriminate between *Map* isolates collected from different hosts [109], which requires more detailed molecular analysis.

Various molecular techniques are currently able to identify different *Map* strain-types with high confidence. *Map* epidemiological studies have been significantly impacted by genotyping techniques such as: restriction fragment length polymorphism, PFGE, multiplex PCR for IS*900* loci, representational differentiating analysis (RDA)-PCR and PCR-restriction endonuclease analysis (PCR-REA) [109]. These methods can successfully differentiate between *Map* isolates belonging to the sheep- and cattle-type strains [109]. One caveat is that the above mentioned

molecular genotyping techniques are not capable of further resolving *Map* isolates into subgroups, because of limited variations in the genomes of different *Map* isolates [3]. More detailed methods used for further subtyping isolates include: variable number tandem repeat (VNTR) analysis, large sequence polymorphism typing, single nucleotide polymorphism typing, short sequence repeat (SSR) sequencing, mycobacterial interspersed repetitive unit (MIRU) analysis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry [3,99,111,112]. These tools are useful for analyzing *Map* isolates from different geographical locations for analyzing isolates sources and dispersal patterns [109], and also for differentiating between the different strain types (sheep *vs.* cattle) [113].

At present, sequencing of multiple SSRs (short repeating sequence of DNA) is widely used for genotyping *Map* isolates [99]. There are 11 physically distinct SSR loci present in the *Map* genome [3]. Among them, four SSR loci are highly polymorphic but stable, making them good candidates for typing *Map* isolates [3,50,114]. Determining the sequences of the four loci enables discrimination between different *Map* isolates by assigning a specific SSR combination to each isolate. Therefore, the development of SSR worldwide databases for analyzing molecular diversity of *Map* isolates is possible through the use of this method. The main disadvantage of multilocus SSR sequencing is the inaccurate assigning of *Map* genotypes due to problems associated with determining the DNA sequences of repeats using conventional methods [3]. However, this problem can be sometimes overcome by repeating the sequencing multiple times or by sequencing both the DNA strands [3]. Another cost effective method of genotyping *Map* isolates is to perform

fragment analysis using instrumentation that has single nucleotide resolution capability on target SSR regions instead of sequencing [115, 116]. This makes fragment analysis a reliable method for accurately determining the sizes of the DNA fragments, which in turn enables the calculation of the exact lengths of the SSRs that they contain [116]. Therefore, SSR analysis using different molecular methods can effectively provide valuable information regarding the source of infection, host specificity, and inter-/intra-species transmission capabilities [3,43,50,114,117-119].

1.4.8 *Map* and CD

CD occurs due to abnormal immune responses within the gastrointestinal mucosa in humans [120]. Previous studies have reported similarities between the pathologies of CD and JD, including the presence of *Map* in breast milk [121] and intestinal tissues of some CD patients [122]. In spite of pathological similarities between the two diseases and similar responses to anti-microbial drugs in animals with JD and humans with CD, argument regarding the association of *Map* with CD still exists [120]. In previous sections, the risk of humans acquiring *Map* from animal products has been discussed, but further research for substantiating transmission through dairy products is warranted as there is a possibility that *Map* is zoonotic in nature [123]. Collaborative multidisciplinary endeavors can further facilitate epidemiological studies regarding the association between these two intestinal diseases [123].

1.5 Purpose of thesis

The purpose of the research conducted here was to evaluate fast, reliable and inexpensive molecular methods for typing bacterial pathogens associated with the dairy industry. Different methods were used for sub-typing *K. pneumoniae* and *Map* isolates, the two pathogens that are the focus of the described work. Through the use of these molecular tools, information was gathered for investigating the molecular diversity of the two pathogens from the island of Newfoundland, which has not been reported previously.

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Table 1.3.5.1: Brief description of antibiotics used for treating CM cases in Newfoundland

Antibiotic	Antibiotic class	Mode of action	Effective against	Common mode of resistance
Cephalothin/ Cefalotin	β-lactam (first generation cephalosporin)	Disrupts bacterial cell wall synthesis	*Staphylococcus* isolates	Enzymatic deactivation of antibiotic molecule
Ceftiofur	β-lactam (third generation cephalosporin)	Disrupts bacterial cell wall synthesis	Broad spectrum activity against Gram negative organisms	Enzymatic deactivation of antibiotic molecule
Pirlimycin	Lincosamide	Prevents protein synthesis in prokaryotes	Gram positive bacteria	Eliminates or reduces binding of antibiotic to cell target
Novobiocin	Aminocoumarin antibiotic	Inhibits the chromosome replication	Gram positive bacteria	Point mutation in the DNA gyrase subunit
Streptomycin	Aminoglycoside	Inhibits the protein synthesis of bacteria	Gram negative bacteria	Enzymatic deactivation of antibiotic molecule
Tetracycline	Polyketide	Inhibits the protein synthesis of bacteria	Gram negative bacteria	Active efflux from the cell
Trimethoprim sulfamethoxazole	Combination of a sulfonamide antibiotic and a methoprim	Inhibits the biosynthesis of essential tetrahydrofolic acid	Bacterial, fungal and prokaryotic infections	Alteration of metabolic pathway

Chapter 2: *Klebsiella* species associated with bovine mastitis in Newfoundland

Co-authorship statement

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Abstract

Klebsiella spp. is a common cause of bovine mastitis, but information regarding its molecular epidemiology is lacking from many parts of Canada. By using mass spectrometry and partial sequencing of the *rpo*B gene, it was found that over a one year period, *K. variicola* and *Enterobacter cloacae* were misidentified as *K. pneumoniae* in a small number of clinical mastitis (CM) cases from Newfoundland. Results suggest that the currently used standard biochemical and phenotypic tests lack the sensitivity required to accurately discriminate among these three Gram negative bacteria. In addition, a single strain of *K. variicola* was associated with CM from one farm as demonstrated by random amplified polymorphic DNA (RAPD) PCR. To the best of our knowledge, *K. variicola*, which is normally found in the environment, has not been isolated previously from milk obtained from cows with CM. Therefore, it is possible that *K. variicola* was not detected in milk samples in the past due to the inability of standard tests to discriminate it from other *Klebsiella* species. **(168 words)**

2.1 Introduction

Klebsiella pneumoniae is an important opportunistic human pathogen predominantly affecting immunocompromised or elderly human patients. Recently a hypervirulent *K. pneumoniae* strain was reported to be capable of causing fatal infections in healthy individuals [1]. Drug resistant forms of *K. pneumoniae*, especially those resistant to the β-lactam family of antibiotics are also a cause for concern due to the limited therapeutic options available for the treatment of such infections and the ability of these strains to rapidly spread and transfer the resistance phenotype [2-4]. In the dairy industry, *K. pneumoniae* is one of the known causes of primarily environment derived *Klebsiella* mastitis and has been the subject of numerous studies [3,5-7]. Clinical mastitis (CM) is classified as the condition where an animal displays the physical symptoms of mastitis [8] and milk production and quality is also affected [9]. Whilst most studies show limited impact of treatment, Schukken *et al.* [10] reported a significant increase in bacteriological cure after the use of antimicrobials for treating non-severe cases of *Klebsiella* associated CM. Mastitis adversely affects milk production and generally cows do not regain full production levels post recovery [11], leading to considerable economic losses. It has also been reported that the amount of decrease in milk production depends on the specific pathogen causing the infection and that Gram negative bacteria are responsible for greater reduction than Gram positive bacteria and other non-bacterial organisms [11-14]. Although, not routinely performed for diagnostic purposes, further characterization of bacterial isolates from infected animals helps to better identify the sources of the infection and to determine if herd infections are primarily clonal or polyclonal in nature [3,5,7]. These data allow clinicians to understand the nature of

transmission within herds and to implement targeted prevention strategies. Therefore, there is value in identifying the causative agent for quick action to prevent losses and also for surveillance purposes.

In the current study, we used multiple methods to identify and analyze Gram negative coliforms associated with bovine CM from Newfoundland, Canada. Our results suggest that the currently used standard biochemical laboratory identification techniques were not sensitive enough to accurately identify the etiological agent in certain cases. In addition, we found that all CM cases from one farm were associated with *K. variicola*, which had been misidentified as *K. pneumoniae* using routine testing procedures.

2.2 Materials and Methods

2.2.1 Ethics statement

This study was carried out in cooperation with dairy farmers in the province and formalized by an agreement between their representative organization, the Dairy Farmers of Newfoundland and Labrador (NL), and the Chief Veterinary Officer for the Province of NL (HGW). Endangered species were not involved in the study and all samples were collected from the island of Newfoundland. The report is not intended to be a field study; instead it describes the application and accuracy of laboratory and molecular tests for the identification of coliform bacteria associated with bovine mastitis. Therefore, coordinates and details regarding the geographical origins of the samples are not included. Approval from an Institutional Animal Care and Use Committee (IACUC), or equivalent animal ethics committee, was not obtained as the samples used in the current study were obtained from routine veterinary diagnostic submissions unrelated to this research. The report is focused on the microbiological analysis following the isolation of bacteria from the milk samples, and did not directly involve any animals.

2.2.2 Standard phenotypic/biochemical testing for pathogen identification

Milk samples from animals with symptoms of CM were collected from 11 farms by the Animal Health Division, Department of Natural Resources, Government of NL between October, 2011 and October, 2012. Sample collection from dairy cattle was done through the expression of milk from the teats into a sterile collection container for submission to the diagnostic laboratory. For initial confirmation of CM status of the

animal, milk samples originating from infected quarters of the udder were subjected to the California mastitis test (CMT, Dairy Research Products Co., Canada). For isolation and phenotypic identification of CM associated bacteria, milk samples were streaked on tryptic soy agar with 5% sheep blood, MacConkey 3 agar with crystal violet and urea agar (Oxoid Ltd., Canada) as recommended (National Mastitis Council, 1999). To further characterize the isolates and to distinguish between members of Enterobacteriaceae, the IMViC test was performed using tryptic soy broth (I, Oxoid Ltd., Canada), methyl red (M), Voges-Proskauer (V) test (Becton Dickinson, Canada) and citrate test (C, Becton Dickinson, Canada). The M test differentiates *Klebsiella* spp. from *Enterobacter cloacae* and the I test differentiates between *K. pneumoniae* and *K. oxytoca*. Atypical results were reanalyzed using the API 20E identification kit (bioMérieux Canada, Inc.). Later on, the isolates were submitted to the Public Health Laboratory of the Government of NL (PHL-NL, St. John's, NL, Canada) for classification using the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) Biotyper System (Bruker Corp. USA). Certain isolates were also tested for their ability to metabolize adonitol using the API 50 CHE kit according to the manufacturer's instructions (bioMérieux Canada, Inc.).

2.2.3 Antibiotic susceptibility testing

All isolates were subjected to routine drug susceptibility testing using cephalothin, ceftiofur, streptomycin, tetracycline and trimethoprim sulfamethoxazole (TMP-sulfa) using the Kirby Bauer Disc Diffusion method. Minimum inhibitory concentrations were also determined for cephalothin, ceftiofur and tetracycline using the

Sensititer microdilution system (Trek Diagnostic Systems Inc.). All analyses were conducted according to the Clinical and Laboratory Standards Institute (CLSI) standards [15].

2.2.4 Genotyping of isolates

The isolates were successfully grown in nutrient broth (Becton Dickinson, Sparks, Md.) for molecular typing. Chromosomal DNA was extracted from 45 isolates and used for direct amplification and sequencing of the *rpo*B gene as described in a previous study [5] using the Thermo Scientific™ Phusion™ High-Fidelity PCR Kit. PCR products were subjected to DNA sequencing at the Centre for Applied Genomics, University of Toronto, Canada and the nucleotide sequences obtained were used to search the public database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The same 45 isolates were used for strain typing by random amplified polymorphic DNA (RAPD) PCR as described in previous studies [5,16]. The same primer pair (ERIC-2/1026) was designed to analyze different species of *Klebsiella* [17]. Images of DNA banding patterns obtained after agarose gel electrophoresis were analyzed using PyElph software [18] to prepare a presence/absence band matrix which was subsequently used to prepare a dendrogram using the neighbor joining method. Reproducibility of banding patterns for 45 isolates was also evaluated by repeating the entire gel analysis twice.

2.3 Results and Discussion

2.3.1 Identification of *K. pneumoniae* and other isolates

In the current study, *Klebsiella* spp. were detected in 61 milk samples that were routinely collected from cows with symptoms of CM from 11 farms in Newfoundland over a one year period. From these original 61 isolates that were identified as *K. pneumoniae* based on standard biochemical and phenotypic tests, only 45 could be re-cultured for further examination. Subsequent analysis of all the isolates by MALDI-TOF Biotyper mass spectrometry reclassified two of the 45 isolates as *Enterobacter cloacae* and the remaining 43 as *K. pneumoniae* (Table 2.3.1.1). To determine/confirm genus and species identity, partial sequencing of the *rpo*B gene was carried out as it has been shown to be a good candidate to discriminate between coliforms associated with CM in previous studies [5]. The DNA sequences obtained were aligned after trimming and were used to build a dendrogram (Figure 2.3.1.1). In this third round of analysis, all CM associated samples from a single farm (farm 9) were identified as *K. variicola* and not *K. pneumoniae*. In addition, two isolates from separate farms were confirmed to be *E. cloacae*, as suggested previously by MALDI-TOF analysis (Table 2.3.1.1). Therefore, *K. pneumoniae, K. variicola* and *E. cloacae* were identified to be associated with CM cases from 11 farms in the current study.

Only *K. variicola* was isolated from cows with CM from farm 9, originating from the left hind quarter of the udders of two animals. One of the strains (KM-49) was re-isolated from the same quarter during a second sampling conducted after a one month period. *K. variicola* was originally described to be unable to metabolize/ferment adonitol [20]. When the two *K. variicola* isolates from the current study were used in carbohydrate

fermentation assays, results demonstrated that both isolates could ferment adonitol (Table 2.3.1.2). Therefore, in the current study, *K. variicola* could only be identified based on *rpo*B sequencing, as all other methods, including the adonitol fermentation test failed to identify it.

2.3.2 Molecular typing by RAPD analysis

Many diverse *K. pneumoniae* strains are known to be present in dairy cattle feces and infections are normally linked to contaminated organic bedding material [16]. Genotyping techniques such as RAPD, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) have been used in the past to successfully analyze *K. pneumoniae* strain diversity associated with CM [5,21,22]. Of these, RAPD has many advantages as it is fast, relatively inexpensive and technically less demanding as compared to the other methods of analysis and was therefore chosen for the current study. Limited strain clustering was observed between the *K. pneumoniae* strains subjected to RAPD analysis (Figure 2.3.2.1A,B), which has also been reported in previous studies, suggesting an environmental source of the infection [16,19,21], with the exception of farm 6, where the two CM cases were associated with the same *K. pneumoniae* strain (Figure 2.3.2.1A-C). Therefore, it is possible that there was either direct or indirect animal to animal transmission on farm 6 or that a single environmental strain infected the two animals independently. Chromosomal DNA from the laboratory *K. pneumoniae* ATCC 15380 strain, was used as a control for RAPD analysis on two occasions, giving identical profiles, showing that the assay used is reliable enough for strain typing and all results were reproducible (Figure 2.3.2.1C). Examination of the two *K. variicola* isolates

from farm 9 demonstrated that they were identical, which could again suggest animal to animal transmission or that a single strain infected the two animals independently (Figure 2.3.2.1A-C). Similar results were also reported in previous studies where RAPD demonstrated clear difference between *Raoultella* spp. and *K. pneumoniae* using the same pair of primers as used in the current study [5]. The RAPD assay was repeated to check for reproducibility and verify the DNA banding patterns between identical isolates (Figure 2.3.2.1C). In addition, the two *E. cloacae* strains identified in the current study from two different farms were not the same (Figure 2.3.2.1A and 2.3.2.1B).

2.3.3 Antimicrobial activity and resistance profile

All 45 isolates were also subjected to antimicrobial susceptibility testing using five drugs commonly used to treat Gram negative infections in veterinary medicine (cephalothin, ceftiofur, streptomycin, tetracycline and TMP-sulfa). CLSI guidelines were used for determining the breakpoint concentration for each antibiotic. Cephalothin resistance (a first-generation cephalosporin β-lactam) was only observed in the *E. cloacae* isolates. Combined results from these analyses are superimposed on the dendrogram shown in Figure 2.3.2.1B and 2.3.1.1, and are also included in Table 2.3.3.1. Varying profiles/degrees of resistance against streptomycin and tetracycline were observed for the *K. pneumoniae* isolates. In addition, three isolates from farm 1 (F1-1, F1-17 and F1-33) were resistant to TMP-sulfa and the two *K. variicola* isolates were sensitive to all the drugs tested (Figure 2.3.2.1B and Table 2.3.3.1).

2.3.4 Conclusion

The insular nature of Newfoundland, its location and maritime climate pose unique challenges and environments for the management of dairy herds. In this study, bacteria were isolated from confirmed CM dairy animals from Newfoundland and were initially identified as *K. pneumoniae* using standard phenotypic laboratory testing protocols. MALDI-TOF finger printing further reclassified two isolates as *E. cloacae*, suggesting that this method is more sensitive than phenotypic biochemical analysis in discriminating between coliforms associated with CM [23]. MALDI-TOF has been used for proteomic analysis in several studies related to bovine mastitis [24,25]. MALDI-TOF is a rapid, precise and cost-effective method for bacterial identification compared to conventional phenotypic/biochemical techniques [26], but its sensitivity is dependent on the database used for matching the obtained spectra. Finally, partial *rpo*B gene sequencing revealed the presence of *K. variicola* from one farm, which could not be discriminated using the two previous methods. Similar results were also observed in previous studies where *Klebsiella oxytoca*, *Klebsiella variicola* and *Raoultella planticola* were isolated from environmental samples associated with CM [19]. Therefore, it is possible that the prevalence of *K. variicola* associated with CM might be under-reported, as the results suggest that routinely used identification tests are not sensitive enough to discriminate it from *K. pneumoniae*.

K. pneumoniae is an opportunistic, environmental pathogen causing CM in dairy cattle [16] and it is very rare to see a dominant strain associated with a herd [5]. Farm 1, which had the highest incidence of CM over the period included in the study, displayed large amounts of variation in *K. pneumoniae* strains and only farm 6 had infections

caused by a single strain (Figure 2.3.2.1). Results also showed that both cases from farm 9 were associated with a single strain of *K. variicola* (Figure 2.3.2.1) as it was the only bacterium cultured from the submitted milk samples (Table 2.3.3.1). The isolation of *K. variicola* from milk samples from cows with CM has not been reported previously as this organism is normally found in the environment [19]. In addition, *K. variicola* has also been previously isolated from plants and certain hospital settings [19,20]. Other reports have questioned the adonitol negative fermentation test for its ability to discriminate between *K. variicola* and other coliforms [27], which was also demonstrated by our results. The pathogenic potential of *K. variicola* is not well understood and there has been some concern in the ability to accurately detect it in humans. In a recent report, an incorrectly diagnosed *K. variicola* strain was responsible for human patient mortality, even though antibiotics were administered to which the isolate was sensitive under laboratory conditions [28]. To the best of our knowledge, this is the first report with evidence that an isolate of *K. variicola* can cause CM in dairy cattle, as it is normally found in soil and feed [19], and not in milk from infected animals. The relevance of the finding that one adonitol positive strain of *K. variicola* was responsible for both CM cases from a single farm will be investigated in future studies.

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Table 2.3.1.1: Details of genus and species level identification of 45 coliform isolates obtained from milk samples from 11 Newfoundland dairy farms using the different identification methods described in the current study

Farm of origin and number of isolates [a]	Identification [b]
Farm 1 (26)	All *K. pneumoniae*
Farm 2 (1)	All *K. pneumoniae*
Farm 3(5) [c]	4 *K. pneumoniae and* 1 *E. cloacae* [d]
Farm 4 (2)	All *K. pneumoniae*
Farm 5(3) [c]	2 *K. pneumoniae and* 1 *E. cloacae* [d]
Farm 6 (2)	All *K. pneumoniae*
Farm 7 (1)	All *K. pneumoniae*
Farm 8 (1)	All *K. pneumoniae*
Farm 9 (2) [e]	All *K. variicola* [f]
Farm 10 (1)	All *K. pneumoniae*
Farm 11 (1)	All *K. pneumoniae*

[a] Number of pure culture isolates from CM cases from each farm are shown in parenthesis

[b] Final identification using a combination of the methods (biochemical/phenotypic tests, MALDI-TOF and *rpo*B sequencing) are shown

[c] Farms showed the presence of cephalothin-resistant *E. cloacae*

[d] *E. cloacae* could be identified using both MALDI-TOF and *rpo*B sequencing, but not by standard biochemical/phenotypic methods

[e] All isolates from this farm were identified as adonitol fermenting *K. variicola*

[f] *K. variicola* could be only identified by *rpo*B sequencing, but not by standard biochemical/phenotypic methods or by MALDI-TOF

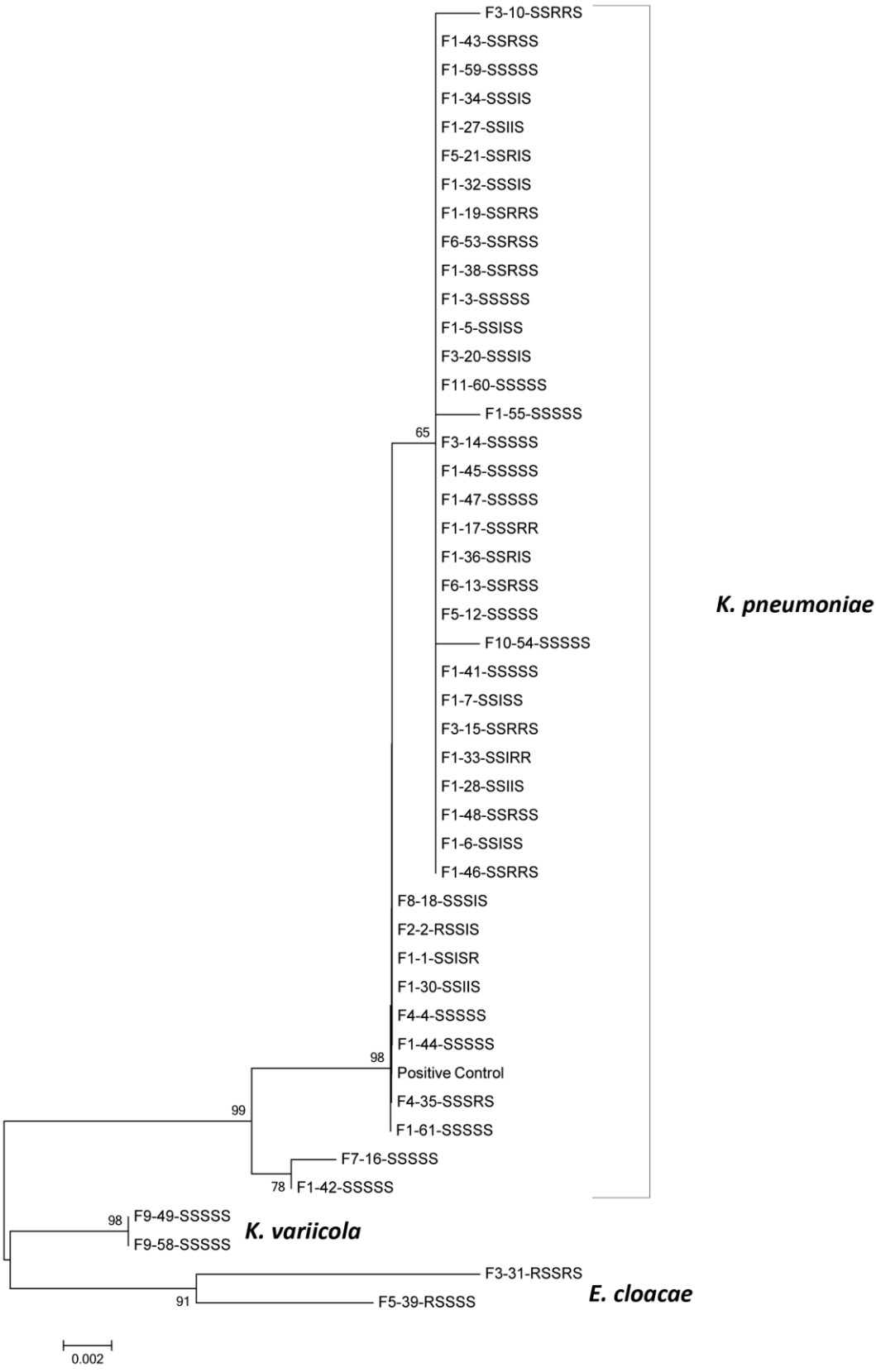
F3-10-SSRRS
F1-43-SSRSS
F1-59-SSSSS
F1-34-SSSIS
F1-27-SSIIS
F5-21-SSRIS
F1-32-SSSIS
F1-19-SSRRS
F6-53-SSRSS
F1-38-SSRSS
F1-3-SSSSS
F1-5-SSISS
F3-20-SSSIS
F11-60-SSSSS
F1-55-SSSSS
65
F3-14-SSSSS
F1-45-SSSSS
F1-47-SSSSS
F1-17-SSSRR
F1-36-SSRIS
F6-13-SSRSS
F5-12-SSSSS
F10-54-SSSSS
F1-41-SSSSS
F1-7-SSISS
F3-15-SSRRS
F1-33-SSIRR
F1-28-SSIIS
F1-48-SSRSS
F1-6-SSISS
F1-46-SSRRS
F8-18-SSSIS
F2-2-RSSIS
F1-1-SSISR
F1-30-SSIIS
F4-4-SSSSS
F1-44-SSSSS
98
Positive Control
F4-35-SSSRS
99
F1-61-SSSSS
F7-16-SSSSS
78
F1-42-SSSSS
98
F9-49-SSSSS
F9-58-SSSSS
K. variicola
F3-31-RSSRS
91
F5-39-RSSSS
E. cloacae
K. pneumoniae
0.002

Figure 2.3.1.1: Phylogenetic tree derived from the *rpo*B partial gene sequences of all 45 isolates built using the neighbour joining method in the Molecular Evolutionary Genetic Analysis (MEGA) package (version 6.06). Grouping of sequences of all the isolates were based on % confidence obtained by using a boot-strap value of 1000. The isolates were assigned labels based on the farm or origin (F1 to F11) followed by a number to identify the infected animal. The results of antibiotic susceptibility testing are also shown beside each isolate. S: sensitive, I: intermediate and R: resistant, based on Clinical and Laboratory Standards Institute (CLSI) interpretation. The S/I/R designations for each antibiotic are in the following order: cephalothin, ceftiofur, streptomycin, tetracycline and trimethoprim sulfamethoxazole, respectively. The isolates identified as *K. variicola*, *E. cloacae* and *K. pneumoniae* are also indicated.

Table 2.3.1.2: Results of tests using *K. variicola* isolates for their ability to metabolize select carbohydrates including adonitol, using the API 50 CHE kit after 24 hours of incubation (bioMérieux, Inc.)

Sample		*K. pneumoniae* (cont)[a]			F9-49			F9-58		
No.	**Test**	**Colour[b]**	**Result[c]**	**Bubbles[d]**	**Colour**	**Result**	**Bubbles**	**Colour**	**Result**	**Bubbles**
0	0	R	−	A	R	−	A	R	−	A
1	GLY	Y	+	P	Y	+	P	Y	+	A
2	ERY	R	−	A	R	−	A	R	−	A
3	DARA	R	−	A	R	−	A	R	−	A
4	LARA	Y	+	P	Y	+	P	Y	+	P
5	RIB	Y	+	A	Y	+	P	Y	+	A
6	DXYL	Y	+	P	Y	+	P	Y	+	P
7	LXYL	R	−	A	R	−	A	R	−	A
8	**ADO[e]**	**Y**	**+**	**P**	**Y**	**+**	**A**	**Y**	**+**	**A**
9	MDX	R	−	A	R	−	A	R	−	A
10	GAL	Y	+	P	Y	+	P	Y	+	P
11	GLU	Y	+	P	Y	+	P	Y	+	P
12	FRU	Y	+	P	Y	+	P	Y	+	P
13	MNE	Y	+	P	Y	+	P	Y	+	P
14	SBE	Y	+	P	Y	+	P	Y	+	P
15	RHA	Y	+	A	Y	+	P	Y	+	A
16	DUL	R	−	A	Y	+	P	Y	+	P
17	INO	Y	+	P	Y	+	A	Y	+	P
18	MAN	Y	+	P	Y	+	P	Y	+	P
19	SOR	Y	+	P	Y	+	A	Y	+	P
20	MDM	R	−	A	R	−	A	R	−	A
21	MDG	Y	+	A	Y	+	P	Y	+	A
22	NAG	O	+	P	O	+	P	O	+	A

Sample		*K. pneumoniae* (cont)[a]			F9-49			F9-58		
No.	**Test**	**Colour[b]**	**Result[c]**	**Bubbles[d]**	**Colour**	**Result**	**Bubbles**	**Colour**	**Result**	**Bubbles**
23	AMY	R	−	A	R	−	A	R	−	A
24	ARB	Y	+	P	Y	+	P	Y	+	P
25	ESC	B	−	A	B	−	P	B	−	A
26	SAL	Y	+	P	Y	+	P	Y	+	P
27	CEL	Y	+	P	Y	+	P	Y	+	P
28	MAL	Y	+	P	Y	+	P	Y	+	P
29	LAC	Y	+	P	Y	+	P	Y	+	P
30	MEL	Y	+	P	Y	+	P	Y	+	P
31	SAC	Y	+	P	Y	+	P	Y	+	A
32	TRE	Y	+	P	Y	+	P	Y	+	P
33	INU	R	−	A	R	−	A	R	−	A
34	MLZ	R	−	A	R	−	A	R	−	A
35	RAF	Y	+	P	Y	+	P	Y	+	P
36	AMD	Y	+	P	R	−	A	R/O	V	A
37	GLYG	R	−	A	R	−	A	R	−	A
38	XLT	R	−	A	R	−	A	R	−	A
39	GEN	Y	+	P	Y	+	P	Y	+	A
40	TUR	R	−	A	O	+	A	R	−	A
41	LYX	R	−	A	O	+	A	R	−	A
42	TAG	Y	+	P	Y	+	P	Y	+	P
43	DFUC	R	−	A	R	−	A	R	−	A
44	LFUC	Y	+	P	Y	+	P	Y	+	A
45	DARL	Y	+	P	Y	+	P	Y	+	P
46	LARL	R	−	A	R	−	A	R	−	A
47	GNT	O	+	P	O	+	P	R	−	P
48	2KG	O	+	A	O	+	P	R/O	V	P
49	5KG	R	−	A	Y/O	V	P	O	+	P

[a] Laboratory strain *K. pneumoniae* ATCC 15380 was used in the analysis as a control
[b] Colour: R = red and Y = yellow
[c] Result: '+' = positive, '-' = negative and V = variable
[d] Bubbles: A = absent and P = present
[e] ADO = adonitol
All interpretations are based on the manufacturer's recommendations

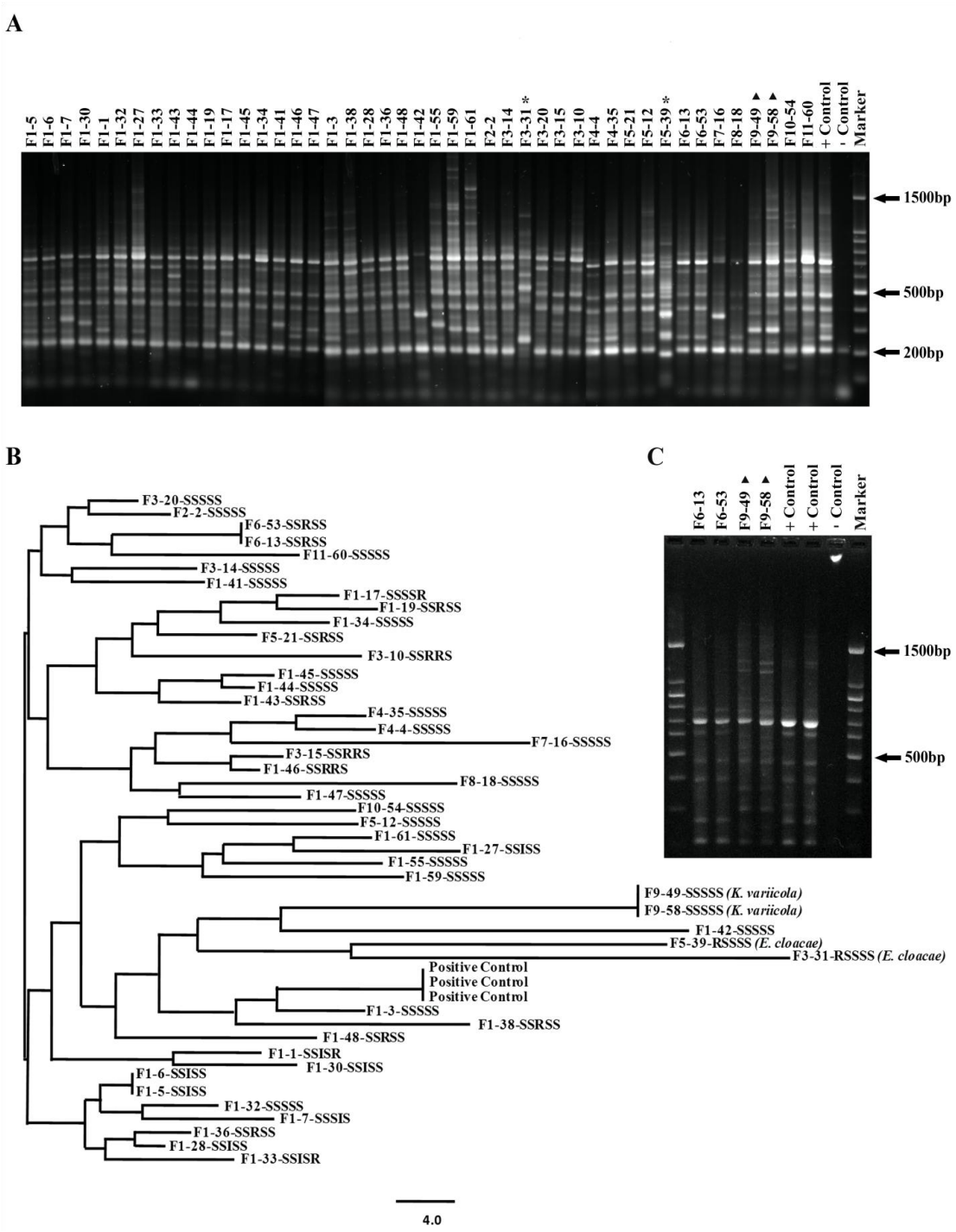
A
F1-5
F1-6
F1-7
F1-30
F1-1
F1-32
F1-27
F1-33
F1-43
F1-44
F1-19
F1-17
F1-45
F1-34
F1-41
F1-46
F1-47
F1-3
F1-38
F1-28
F1-36
F1-48
F1-42
F1-55
F1-59
F1-61
F2-2
F3-14
F3-31 *
F3-20
F3-15
F3-10
F4-4
F4-35
F5-21
F5-12
F5-39 *
F6-13
F6-53
F7-16
F8-18
F9-49 ▲
F9-58 ▲
F10-54
F11-60
+ Control
- Control
Marker
1500bp
500bp
200bp
B
F3-20-SSSSS
F2-2-SSSSS
F6-53-SSRSS
F6-13-SSRSS
F11-60-SSSSS
F3-14-SSSSS
F1-41-SSSSS
F1-17-SSSSR
F1-19-SSRSS
F1-34-SSSSS
F5-21-SSRSS
F3-10-SSRRS
F1-45-SSSSS
F1-44-SSSSS
F1-43-SSRSS
F4-35-SSSSS
F4-4-SSSSS
F7-16-SSSSS
F3-15-SSRRS
F1-46-SSRRS
F8-18-SSSSS
F1-47-SSSSS
F10-54-SSSSS
F5-12-SSSSS
F1-61-SSSSS
F1-27-SSISS
F1-55-SSSSS
F1-59-SSSSS
F9-49-SSSSS (K. variicola)
F9-58-SSSSS (K. variicola)
F1-42-SSSSS
F5-39-RSSSS (E. cloacae)
F3-31-RSSSS (E. cloacae)
Positive Control
Positive Control
Positive Control
F1-3-SSSSS
F1-38-SSRSS
F1-48-SSRSS
F1-1-SSISR
F1-30-SSISS
F1-6-SSISS
F1-5-SSISS
F1-32-SSSSS
F1-7-SSSIS
F1-36-SSRSS
F1-28-SSISS
F1-33-SSISR
4.0
C
F6-13
F6-53
F9-49 ▲
F9-58 ▲
+ Control
+ Control
- Control
Marker
1500bp
500bp

Figure 2.3.2.1: Molecular epidemiology of CM associated coliform bacteria isolated over a one year period from 11 dairy herds in Newfoundland. **(A)** Results from RAPD analysis employing 1.5% agarose gels to separate PCR products. Chromosomal DNA from a laboratory strain (*K. pneumoniae* ATCC 15380) was included as the positive control and sterile water functioned as negative control in the PCR assays. A 100 bp ladder (GeneDirex, USA, Cat No. DM001-R500) was used as the molecular weight marker. **(B)** Dendrogram based on RAPD results showing relatedness between strains was subjected to analysis in the current study. The dendrogram was generated by the neighbor joining method with the PyElph software. **(A, B and C)** The isolates were assigned labels based on the farm or origin (F1 to F11) followed by a number to identify the infected animal. The isolates determined to be *E. cloacae* (*) and *K. variicola* (▲) are also indicated. **(B)** The results from antibiotic susceptibility testing are also shown next to each isolate. S: sensitive, I: intermediate and R: resistant designations are assigned based on established break points. The S/I/R designations for each drug are in the following order: cephalothin, ceftiofur, streptomycin, tetracycline and trimethoprim sulfamethoxazole, respectively. The isolates identified as *K. variicola* and *E. cloacae* on further analysis are also indicated. **(C)** Re-analysis of strains by RAPD PCR to verify the accuracy of the assay and to confirm identities. PCR products generated independently for the second time using DNA from identical strains along with the positive control were reanalyzed, which confirmed results shown in **A**.

Table 2.3.3.1: Details regarding animal/farm ID, sample collection date, infected quarters sampled and California Mastitis Test (CMT) results. Information regarding bacterial identification by various methods (culture/biochemical tests, *rpo*B sequencing and MALDI-TOF), and antibiotic susceptibility test results (by minimum inhibitory concentrations and Kirby Bauer disc diffusion method according to CLSI guideline) are also included. Other organisms that were present in the samples besides *K. pneumoniae* based on biochemical/phenotypic methods are also indicated

Lab ID	Farm /Animal ID	Collection Date	Qu[a]	CMT[b]	Growth on MacConkey 3 agar with crystal violet	Identification of isolates by various methods[c]			Antibiotic susceptibility Test result[e]					Other organisms present (if any)
						*rpo*B	Pheno -type	MALDI-TOF (Score value)[d]	CEF[f]	CF[g]	S[h]	TE[i]	TS[j]	
1	001-007	6-Oct-11	LH	3+	Heavy	+	+	+ (2.397)	S	S	I	S	R	*Streptococcus uberis*[k]
2	002-001	6-Oct-11	RH	NA	Very light	+	+	+ (2.526)	S	S	S	S	S	None
3	001-022	10-Nov-11	LH	3+	Light	+	+	+ (2.435)	S	S	S	S	S	Mixed[k]
4	004-001	12-Nov-11	RF	2+	Very light	+	+	+ (2.43)	S	S	S	S	S	None
5	001-001	21-Nov-11	NG	3+	Heavy	+	+	+ (2.18)	S	S	I	S	S	None
6	001-002	17-Nov-11	LF	3+	Heavy	+	+	+ (2.234)	S	S	I	S	S	None
7	001-004	15-Nov-11	RF	NA	Light	+	+	+ (2.456)	S	S	I	S	S	None
10	003-006a	13-Dec-11	LH	3+	Scanty	+	+	+ (2.382)	S	S	R	R	S	None
12	005-003	3-Jan-12	RH	3+	Heavy	+	+	+ (2.488)	S	S	S	S	S	None
13	006-004	28-Dec-11	RF	2+	Heavy	+	+	+ (2.446)	S	S	R	S	S	None
14	003-002	6-Jan-12	RH	NA	Heavy	+	+	+ (2.516)	S	S	S	S	S	None
15	003-005	6-Jan-12	RH	NA	Heavy	+	+	+ (2.504)	S	S	R	R	S	*E. coli*[k]
16	007-001	17-Jan-12	RH	NA	Very scanty	+	+	+ (2.334)	S	S	S	S	S	None
17	001-015	16-Feb-12	RH	NA	Heavy	+	+	+ (2.473)	S	S	S	S	R	None
18	008-001	28-Feb-12	NG	2+	Light	+	+	+ (2.236)	S	S	S	S	S	Mixed[k]

Lab ID	Farm /Animal ID	Collection Date	Qu[a]	CMT[b]	Growth on MacConkey 3 agar with crystal violet	Identification of isolates by various methods[c]			Antibiotic susceptibility Test result[e]					Other organisms present (if any)
						*rpo*B	Pheno -type	MALDI-TOF (Score value)[d]	CEF[f]	CF[g]	S[h]	TE[i]	TS[j]	
19	001-014	25-Feb-12	RH	NA	Heavy	+	+	+ (2.466)	S	S	R	S	S	None
20	003-004	7-Mar-12	1Q	3+	Moderate	+	+	+ (2.425)	S	S	S	S	S	None
21	005-002	9-Mar-12	LF	2+	Very light	+	+	+ (2.494)	S	S	R	S	S	None
22	006-001	23-Mar-12	NA	NA	Scanty	+	+	+ (2.385)	S	S	R	I	S	Mixed[k]
25	003-006b	28-Mar-12	LH	3+	Scanty	+	+	+ (2.444)	S	S	R	R	S	None
27	001-009	5-Apr-12	RF	NA	Moderate	+	+	+ (2.481)	S	S	I	S	S	None
28	001-024	6-Apr-12	LF	NA	Moderate	+	+	+ (2.485)	S	S	I	S	S	None
29	001-011	15-Apr-12	RF	NA	Moderate	NA	+	+ (2.528)	S	S	I	I	S	None
30	001-006	10-May-12	RF	NA	Heavy	+	+	+ (2.426)	S	S	I	S	S	None
31	003-003	29-May-12	LH	3+	Heavy	-	+	- (2.027)	R	S	S	S	S	None
32	001-008	16-May-12	RH	NA	Very light	+	+	+ (2.39)	S	S	S	S	S	None
33	001-010	20-May-12	LH	NA	Heavy	+	+	+ (2.477)	S	S	I	S	R	None
34	001-017	22-May-12	RF	NA	Very light	+	+	+ (2.507)	S	S	S	S	S	None
35	004-002	21-Jun-12	LH	3+	Light	+	+	+ (2.448)	S	S	S	S	S	Mixed[k]
36	001-025	21-Jun-12	RH	NA	Scanty	+	+	+ (2.481)	S	S	R	S	S	None
38	001-023	23-Jul-12	RF	NA	Very scanty	+	+	+ (2.443)	S	S	R	S	S	None
39	005-004	2-Aug-12	NG	3+	Moderate	-	+	- (2.348)	R	S	S	S	S	coagulase Neg. *Staphylococcus*[k]
41	001-018	14-Aug-12	RF	NA	Very light	+	+	+ (2.483)	S	S	S	S	S	None
42	001-027	13-Sep-12	RF	NA	Heavy	+	+	+ (2.396)	S	S	S	S	S	None
43	001-012	13-Sep-12	RF	NA	Scanty	+	+	+ (2.487)	S	S	R	S	S	None
44	001-013	13-Sep-12	LH	NA	Scanty	+	+	+ (2.481)	S	S	S	S	S	None
45	001-016	13-Sep-12	LH	NA	Moderate	NA	+	+ (2.423)	S	S	S	S	S	*E.coli*[k]
46	001-019	13-Sep-12	RF	NA	Heavy	+	+	+ (2.358)	S	S	R	R	S	Mixed[k]
47	001-020	13-Sep-12	RF	NA	Scanty	+	+	+ (2.499)	S	S	S	S	S	None

Lab ID	Farm /Animal ID	Collection Date	Qu[a]	CMT[b]	Growth on MacConkey 3 agar with crystal violet	Identification of isolates by various methods[c]			Antibiotic susceptibility Test result[e]					Other organisms present (if any)
						*rpo*B	Pheno -type	MALDI-TOF (Score value)[d]	CEF[f]	CF[g]	S[h]	TE[i]	TS[j]	
48	001-026	13-Sep-12	RH	NA	Scanty	+	+	+ (2.475)	S	S	R	S	S	None
49	009-001a	20-Sep-12	LH	3+	Scanty	-	+	+ (2.385)	S	S	S	S	S	None
50	005-001	25-Sep-12	RH	1+	Heavy	+	+	+ (2.447)	S	S	S	S	S	None
53	006-006	13-Aug-12	LH	NA	Scanty	+	+	+ (2.508)	S	S	R	S	S	None
54	010-001	3-Oct-12	NG	3+	Light	+	+	+ (2.422)	S	S	S	S	S	None
55	001-029	1-Oct-12	LF	NA	Moderate	+	+	+ (2.446)	S	S	S	S	S	None
56	009-001b	16-Oct-12	LH	3+	Light	+	+	+ (2.379)	S	S	S	S	S	None
58	009-004	27-Oct-12	LH	2+	Moderate	-	+	+ (2.372)	S	S	S	S	S	None
59	001-030	10-Oct-12	LF	NA	Light	+	+	+ (2.521)	S	S	S	S	S	None
60	011-001	26-Oct-12	LH	3+	Scanty	+	+	+ (2.449)	S	S	S	S	S	None
61	001-031	19-Oct-12	RF	NA	Heavy	+	+	+ (2.48)	S	S	S	S	S	None

[a] Infected quarter of the udder sampled: RF = right forward, LF = Left forward, RH = right hind, LH = Left hind, 1Q = one quarter and NG = not given

[b] California Mastitis Test result: N/A = not applicable (as milk samples were frozen upon arrival), 2+/ 3+ = positive result. The reaction of CMT is scored on a scale of 0 (mixture liquid, no precipitate) to 3 (almost-solid gel forms) where 2+ means distinct gel formation and 3+ is strong gel formation that tends to adhere to paddle

[c] Identification of isolates by various methods: + = positive for *K. pneumoniae* and - = negative for *K. pneumoniae*

[d] MALDI-TOF (Range of score value): 2.3-3.00 = highly probable species identification, 2.00-2.99 = secure genus identification, probable species identification

[e] Antibiotic susceptibility Test result: S = sensitive, I = intermediate and R = resistant

[f] CEF: ceftiofur

[g] CF: cephalothin

[h] S: streptomycin

[i] TE: tetracycline

[j] TS: trimethoprim sulfamethoxazole

[k] In some cases other organisms were also detected in the milk samples, which could not be identified in certain instances

Chapter 3: Typing of *Mycobacterium avium* subspecies *paratuberculosis* isolates from Newfoundland using fragment analysis

Co-authorship statement

Study design and development: Drs. Kapil Tahlan (Department of Biology, Memorial University of Newfoundland), Hugh G. Whitney (Animal Health Division, Newfoundland and Labrador Department of Natural Resources) and Greg P. Keefe (Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island). Initial diagnosis and establishment of *Map* cultures for subsequent analysis were performed in the laboratories of Drs. Hugh G. Whitney and Greg P. Keefe. Experiments were conducted by: Milka P. Podder (Department of Biology, Memorial University of Newfoundland) and Susan E. Banfield (Department of Biology, Memorial University of Newfoundland). Data was analyzed by: Milka P. Podder. Reagents/materials/analysis tools were contributed by: Dr. Hugh G. Whitney and Dr. Greg P. Keefe. Manuscript was written by: Milka P. Podder and Dr. Kapil Tahlan with input from Drs. Hugh G. Whitney and Greg P. Keefe.

Portions of this chapter will be included in a manuscript currently under preparation.

Abstract

Short sequence repeat (SSR) typing of *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) isolates is one of the most discriminatory methods available for genotyping this pathogen. Currently used techniques have challenges in analyzing mononucleotide repeats >15 bp, which include some of the *Map* SSRs. Fragment analysis is a relatively simple technique, which can measure the size of DNA fragments and can be used to calculate the repeat length of the target SSR loci. In the present study, fragment analysis was used to analyze 4 *Map* SSR loci known to provide sufficient discriminatory power to determine the relationship between *Map* isolates. Eighty-eight *Map* isolates from 18 animals from the island of Newfoundland were successfully genotyped using fragment analysis. To the best of our knowledge, this is the first report on *Map* SSR diversity from Newfoundland dairy farms. In addition, multiple *Map* SSR-types were isolated from a single animal in many cases, which is not a common finding. **(154 words)**

3.1 Introduction

Mycobacterium avium subspecies *paratuberculosis* (*Map*) is a slow growing bacterium and is the cause of Johne's disease, which is associated with chronic debilitating granulomatous enteritis that affects the small intestine of cattle, sheep, goats, farmed deer and other ruminants [1-6]. Johne's disease is a major cause of concern to the dairy industry and there is also some concern regarding the association of *Map* with Crohn's disease in humans [7-9]. Treatment of dairy animals infected with *Map* is impractical because it can be only achieved by using a combination of antibiotics, many of which are very expensive, not licensed for food animals and require long term dosing [10]. Therefore, infected animals are culled, which is also a part of the Johne's disease control/management practice [5]. Because diagnosis is very challenging early on in the disease process, animals can still get infected by *Map* through exposure to shedding asymptomatic animals and environmental contamination [11,12]. The long incubation period of *Map* and the non-specific clinical symptoms in infected animals makes the diagnosis, management and control of Johne's disease difficult. To decrease the spread of Johne's disease, surveillance programs are being established throughout the world for determining the sources of infections, the prevalence of the causative agent and the relationship(s) between *Map* isolates from dairy farms. Studies are also being conducted to examine the role of host genetics in determining the susceptibility of individual animals and their clinical course once infected. Such programs are vital for devising effective control strategies against this devastating disease [13].

More recently, there have been a number of reports on the molecular epidemiology of *Map*. In previous studies, single or combined molecular methods have

been used to obtain epidemiological data regarding *Map* strain types [14-16]. Most of the previously used strain typing methods are expensive, time consuming, and lack discriminatory capabilities [14,15,17]. Despite these limitations, the information obtained from such studies is essential for identifying sources and transmission routes more accurately. When combined with information on host genetics, strain typing studies can also be used to determine strain pathogenicity and host resistance. Molecular techniques, especially DNA-based short-sequence-repeat (SSR) analysis, have been shown to be powerful tools for discriminating between *Map* isolates at the genetic level [5,14,15,16,18]. Due to differences in the numbers of nucleotide repeats associated with SSRs from different *Map* isolates, the relatedness and prevalence of *Map* strains can be monitored within/between farms and the environment [14,18]. One major problem with conventional methods for SSR analyses such as the use of Sanger sequencing, is that they are prone to artifacts and failure due to challenges associated with determining the DNA sequences of the repeats, with the most recent technology being capable of analyzing repeats up to 15 bp using a mass spectrometry based approach [17]. Therefore, there is a need to develop cheap, reliable and reproducible methods for *Map* SSR analysis, which can accurately measure repeats totaling over 15 bp in length.

Recently, DNA fragment analysis of PCR products obtained using fluorescently labeled primers was used for typing *Map* SSRs [16]. There is significant movement of animals within the Newfoundland dairy industry, with new animals being brought onto the island for entry into the production chain. In addition, some heifers are also shipped to other Atlantic Canada provinces on the mainland for rearing, before they return as adult cows, as it is economically more feasible to do so in some situations. Therefore,

there is considerable interest in analyzing the diversity *Map* isolates infecting animals from the island for comparison to those found elsewhere in North America. To achieve this, in the current study we used fragment analysis to analyze *Map* isolates from five Newfoundland dairy farms.

3.2 Materials and Methods

3.2.1 Ethics statement

The described study was carried out under a formal agreement between the Dairy Farmers of Newfoundland and Labrador (NL) and the Chief Veterinary Officer for the Province of NL (HGW). The study was approved by the Institutional Animal Care Committee (IACC, Memorial University of Newfoundland) as an 'A' rated protocol because the samples used in the study were obtained from routine veterinary diagnostic submissions unrelated to this research. The report describes laboratory microbiological analysis and did not directly involve any animals.

3.2.2 Media, reagents and culture conditions

All reagents and media used in the study were purchased from Sigma Aldrich, Fisher Scientific or VWR International, Canada, unless otherwise mentioned. DNA oligonucleotide primers were purchased from Integrated DNA Technologies (USA). Fecal samples from 18 animals displaying varying clinical symptoms of Johne's disease or suspected of being infected (Table 3.2.2.1) were collected by the Animal Health Division, Department of Natural Resources, Government of NL and were sent to the Atlantic Veterinary College, UPEI for diagnosis. Trek-ESP II liquid culture using Trek ESP Para-JEM media (Thermo Scientific, Canada) was used to culture *Map* from bovine fecal samples as described previously [19], and mycobacteria were verified by acid-fast staining. *Map* cultures were grown at 37°C. To confirm the presence of *Map* in the cultures, chromosomal DNA was isolated using the Tetracore *Map* extraction system and was used as a template along with the Tetracore VetAlert^{TM} Johne's Real-Time PCR kit

as per the manufacturer's instructions (Tetracore, USA). After the described analysis, the culture samples were stored as frozen glycerol stocks and were sent to the Memorial University of Newfoundland for further analysis.

The culture samples from UPEI were streaked out onto Middlebrook 7H11 agar plates supplemented with oleic acid-albumin-dextrose-catalase (OADC) and mycobactin J (2 mg/L, Allied Monitor, USA) to obtain isolated *Map* colonies as described previously [14]. The PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) antibiotic mixture was also added to the medium to prevent the growth of other contaminating microorganisms [20]. The plates were incubated for 4-6 months until minute colonies were observed, which were confirmed to be *Map* by acid-fast staining. Three to five isolated colonies from each plate (corresponding to each animal) were then used to inoculate separate 5 mL Middlebrook 7H9 broth cultures supplemented with albumin-dextrose-catalase (ADC) and mycobactin J (2 mg/L). To avoid the clumping of cells, culture tubes contained sterile glass beads and were incubated with agitation. Growth was observed for 88 isolates (3-5 isolates sampled from each animal) after 2-3 months of incubation based on an increase in the turbidity of the cultures, which were then used to prepare glycerol stocks for storage and for chromosomal DNA isolation as described below. Acid-fast staining was performed at different stages to ensure that the cultures were axenic.

3.2.3 Chromosomal DNA isolation, SSR sequencing and fragment analysis

The QIAamp DNA Mini kit (Qiagen, Canada) was used for isolating chromosomal DNA from the remaining 3.5 ml 7H9 cultures from above using 0.1 mm

zirconia silica beads and a SpeedMill PLUS homogenizer (Analytik Jena, Germany) according to the manufacturer's recommendations. All PCR reactions were performed using the Phusion High-Fidelity PCR Kit along with 3% DMSO and the GC buffer (New England Biolabs, Inc.). PCR products were visualized by agarose gel electrophoresis, purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic, Canada) and were sent for DNA sequencing or fragment analysis to The Centre for Applied Genomics (TCAG), University of Toronto, Canada. The four SSR loci (L1-L4) previously shown to provide good discriminatory power for subtyping *Map* isolates were chosen for analysis [5,14]. The DNA sequences of the four SSR repeats were determined for three *Map* isolates obtained as part of a separate study as described previously [5,14]. The sequences of the 4 loci for the *Map* K10 (genome sequenced strain) were obtained from the database [21]. Sequences were obtained to determine the exact numbers of the SSR repeats for each of the strains, for the strains to be used as standards (S) during fragment analysis.

For fragment analysis, four primer pairs were designed which were specific for each locus to give PCR products ranging from 127 to 255 bp, and one primer from each pair was labeled with 6-fluorescein amidite (6-FAM). Primers that have the 6-FAM dye next to a guanine base near the 5' end can have decreased fluorescence. Therefore, the most appropriate of either the forward or the reverse primer from each pair was labeled to avoid any complications. The DNA sequences of the primers used for obtaining PCR products for fragment analysis for each locus were as follows: L1 (F: GGTGTTCGGCAAAGTCGTT/R: TTGACGATCACCAGCCCG), L2 (F: TCGCCTCAGGCTTTACTGAT/R: CACGTAGGTCCGCTGATGA), L3

(AGGCCTTCTACGTGCACAAC/R: GAGATGTCCAGCCCTGTCTC) and L4 (F: CTCGTGGAAACCCTCGAC/R: GGTGCTGAAATCCGGTGT). Unpurified PCR products were sent to the TCAG facilities for fragment analysis using the ABI 3730XL or 3100 capillary electrophoresis instruments using the GeneScan™ 500 ROX™ Size Standard, which is capable of accurately sizing DNA fragments ranging from 35 to 500 bp (http://www.tcag.ca/facilities/geneticAnalysis.html). The Peak Scanner software v1.0 (Applied Biosystems) was used to analyze the fragment profiles/peaks to determine the sizes of the DNA fragments, which were used to calculate SSR copy numbers. Comparison of the fragment sizes from Newfoundland isolates with those from the sequenced standards, which were included in every fragment analysis run, enabled the determination of the exact copy number of each repeat at the target SSR loci. If fragment analysis showed a difference in 1 bp for L1 and L2 (mononucleotide repeats), and 3 bp for L3 and L4 (tri-nucleotide repeats) between a fragment from an isolate and a standard, it implied that there was a difference in one copy of the repeat between the two strains (Table 3.2.3.1). In total 44 SSR-types were assigned on the basis of the combinations of alleles for each locus and the information was used to build a dendrogram using the BioNumerics 7.1 program (Applied Maths, Inc., USA). The unweighted pair group method with arithmetic mean was used to create a minimum spanning tree using the same program; this portrays the level of divergence between strains utilizing pairwise genetic distances [22]. It was possible to directly compare SSRs up to 14 bps from the current study with those reported previously. Longer SSRs identified in the current study were deemed to be similar, but not the same as those from previous studies which were reported as >14 bp.

3.3 Results and Discussion

3.3.1 Fragment analysis on *Map* isolates

The sequencing of DNA repeats using conventional methods is often challenging and is prone to artifacts, which is further exacerbated by repeats with high GC content such as those found in *Map*. In addition, currently available technologies can only determine the lengths of repeats up to ~15 bp accurately and often longer repeats cannot be measured [17]. In the case of *Map*, it has been previously reported that the analysis of 4 SSRs (L1/L2: mononucleotide, and L3/L4 trinucleotide) provides enough sequence information for strain discrimination, and that one of the mononucleotide SSRs (L1) can be >14 bp in length depending on the isolate [5,14]. In our own studies we found that the sequencing of PCR products containing SSRs ~14 bp using the Sanger method was not straightforward (Figure A.1). Previous reports also describe similar problems where the L1 SSR had sequencing errors during analysis, leading to the misinterpretation of repeat lengths [17]. Therefore, to overcome issues associated with the analysis of *Map* SSRs, we adapted fragment analysis as a method to analyze *Map* isolates from 5 dairy farms from Newfoundland [16]. Fecal samples were collected from 18 animals, some of which showed clinical signs of Johne's disease, displayed an immune response against *Map* in milk samples collected during previous surveys or were suspected of being infected (Table 3.2.2.1). The samples were processed to obtain 18 primary fecal cultures, which were then used to establish 88 axenic cultures for use in the current study.

Before using the fragment analysis based approach, three *Map* isolates (referred to as control strains from hereon) that were obtained as part of another study were subjected to SSR sequencing using the Sanger method as described previously [5,14,15,18].

Multiple sequencing runs were carried out until we could reproducibly sequence across the SSRs (Figure A.1). This was done to determine the exact numbers of repeats at the four SSR loci for the respective isolates for subsequent use as standards for comparisons during fragment analysis. Again, we could only obtain accurate and reliable sequences for SSRs smaller than 14 bp using Sanger method (Figure A.1). The lengths of the SSRs for the K10 strain were already known (L1:19, L2:10, L3:5 and L4:5) because its genome has been sequenced [21]. Next, the four loci specific sets of fluorescently labeled primers were used in separate PCR reactions along with chromosomal DNA as template from the control strains described above. Since the exact numbers of SSRs present in each PCR product were known from Sanger sequencing for the control strains, they were included as size standards in all future fragment analysis experiments.

Chromosomal DNA was isolated from 88 axenic *Map* cultures established using samples from Newfoundland, and were used as template to obtain fluorescently labeled PCR products for fragment analysis. Comparison of each SSR PCR product with the respective standards described above provided accurate data regarding the copy number of the repeats at each locus for all 88 isolates in a short period of time (Table 3.2.3.1). In the current study we were also able to analyze L2, which was not possible in a previous report that also used fragment analysis [16]. Control strains were reanalyzed by fragment analysis to rule out ambiguities. After excluding *Map* isolates from the same animal with identical SSR profiles, a total of 70 isolates with 44 different SSR-types (M1-M44) were identified from 18 animals from 5 different Newfoundland farms (Table 3.3.1.1). In addition, in many cases *Map* with multiple SSR-types were isolated from the same animal (Table 3.2.3.1).

3.3.2 Comparison with other epidemiological data

The most predominant SSR-types were M1 and M4, which were isolated from 5 separate animals each from different farms (Table 3.3.1.1 and Figure 3.3.2.1). One reason for this observation could be the random distribution of *Map* SSR-types within farms following animal movement between farms, which is known to increase the probability of detecting multiple and/or similar strains on the farms involved [23]. Most SSR-types in the population were closely related to M4, differing from it by only 1-2 SSR loci (Figure 3.3.2.2). Overall, some farm based clustering of isolates was observed (Figure 3.3.2.1). A high level of diversity was seen in isolates from farm A, which alone had 23 different SSR-types out of the 44 detected. Eight SSR-types present on farm A were also present on other farms (C, E, F; Figure 3.3.2.1) suggesting possible inter herd transmission or a common source of infection. SSR-types from farm D were not detected on other farms, although they showed some level of genetic similarity with isolates from farm C (Figure 3.3.2.1). Animals from farm F did not exhibit any clinical signs of Johne's disease, but tested positive for *Map* with unique SSR-types, in addition to SSR-types found on other farms also (Figure 3.3.2.1).

3.3.3 Conclusions

The resolution of long SSRs by fragment analysis and the recent report showing that the technique can be multiplexed for analyzing multiple SSRs [16] further demonstrates the power and versatility of this technique for typing *Map* isolates. Future studies using techniques with better resolution capabilities and samples from other regions of North America will help to explain if the previously unidentified *Map* SSRs-

types reported in the current study are unique to Newfoundland or if they were not detected due to technical limitations. In addition, results from the current study also indicated *Map* co-infection with multiple genotypes within a single animal, which has been reported as a rare event [18]. The isolation of multiple genotypes from the same animal could be due to evolving SSR-types as a result of the instability associated with long DNA repeats [24]. Alternatively they might represent true co-infections due to the movement of animals within Newfoundland and between Atlantic Canada, which is important in terms of source tracking and the status of the animals involved. As part of another study we were also able to obtain *Map* isolates with different SSR copy numbers from single animals from a farm in Atlantic Canada (unpublished data). Therefore, studies are currently underway to address the significance and implications of these findings.

3.4 References

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Table 3.2.2.1: Details of 18 animals from Newfoundland regarding assigned identification numbers (IDs) and status of the animal from which the primary Trek-ESP II liquid cultures were derived

Farm/animal ID[a]	Animal status/symptoms[b]
A-001	Clinical signs
A-002	Clinical signs
A-005	Clinical signs
A-006	Milk Positive for *Map*
A-007	Milk Positive for *Map*
A-008	Milk Positive for *Map*
A-009	Milk Positive for *Map*
A-010	Milk Positive for *Map*
A-011	Clinical signs
C-001	Clinical signs
C-002	Clinical signs
C-003	Clinical signs
C-004	Clinical signs
C-005	Clinical signs
D-001	Clinical signs
E-001	Clinical signs
F-001	Normal
F-002	Normal

[a] The first letter denotes the farm of origin followed by a identification number assigned to each respective animal (ID: Identity)

[b] The status of each animal sampled for Trek-ESP II liquid culture for *Map* analysis is included. Animals were either asymptomatic (normal), showed clinical signs of Johne's disease or had milk samples which showed a positive immune response against *Map* during previous surveys

Table 3.2.3.1: Fragment analysis results for the 4 SSR loci of all 88 *Map* isolates derived from 18 Newfoundland animals. Strain types were designated as M1-M44 based on their unique SSR combinations

Farm/animal/isolate ID[a]	DTP[b]	L-1 (G)[c]	L-2 (G)[c]	L-3 (GGT)[c]	L-4 (TGC)[c]	SSR-Type
A-001-1	27	21	10	5	5	M20
A-001-2	27	14	10	5	5	M1
A-001-3	27	19	11	5	5	M21
A-001-4	27	16	11	5	5	M2
A-001-5	27	20	9	5	5	M22
A-003-1[d]	32	17	11	5	5	M3
A-003-2[d]	32	17	11	5	5	M3
A-003-3[d]	32	17	11	5	5	M3
A-003-4	32	11	10	5	5	M16
A-003-5	32	11	11	5	5	M4
A-005-1[d]	13	11	12	5	5	M5
A-005-2	13	20	10	5	5	M23
A-005-3	13	16	11	5	5	M2
A-005-4[d]	13	11	12	5	5	M5
A-005-5	13	15	10	5	5	M11
A-006-1	28	12	10	5	5	M15
A-006-2	28	16	12	5	5	M10
A-006-3	28	7	10	5	5	M13
A-006-4	28	13	11	5	5	M14
A-006-5	28	16	12	5	5	M10
A-007-1[d]	22	11	11	5	5	M4
A-007-2	22	10	10	5	5	M33
A-007-3	22	15	10	5	5	M11
A-007-4	22	14	10	5	5	M1
A-007-5[d]	22	11	11	5	5	M4
A-008-1	46	18	11	5	5	M12
A-008-2	46	16	10	5	5	M19
A-008-3	46	20	11	5	5	M34
A-008-4	46	7	10	5	5	M13
A-008-5	46	7	11	5	5	M35
A-009-1[d]	36	13	11	5	5	M14
A-009-2	36	11	11	5	5	M4
A-009-3	36	14	11	5	5	M36

Farm/animal/isolate ID[a]	DTP[b]	L-1 (G)[c]	L-2 (G)[c]	L-3 (GGT)[c]	L-4 (TGC)[c]	SSR-Type
A-009-4	36	18	11	5	5	M12
A-009-5[d]	36	13	11	5	5	M14
A-010-1[d]	49	12	10	5	5	M15
A-010-2	49	15	9	5	5	M37
A-010-3[d]	49	12	10	5	5	M15
A-010-4	49	12	9	5	5	M17
A-010-5	49	16	11	5	5	M2
A-011-1[d]	8	15	11	5	5	M18
A-011-2	8	11	11	5	5	M4
A-011-3[d]	8	15	11	5	5	M18
A-011-4	8	10	11	5	5	M44
A-011-5	8	15	10	5	5	M11
C-001-1[d]	28	14	10	5	5	M1
C-001-2[d]	28	14	10	5	5	M1
C-001-3	28	15	11	5	5	M18
C-001-4[d]	28	11	11	5	5	M4
C-001-5[d]	28	11	11	5	5	M4
C-002-1	28	5	11	4	4	M6
C-002-2[d]	28	14	10	5	5	M1
C-002-3[d]	28	14	10	5	5	M1
C-002-4	28	6	12	4	4	M24
C-002-5	28	5	11	4	4	M6
C-003-1[d]	17	15	14	5	5	M7
C-003-2	17	13	13	5	5	M8
C-003-3[d]	17	15	14	5	5	M7
C-003-4	17	6	13	5	5	M25
C-003-5	17	13	13	5	5	M8
C-004-1	32	8	11	4	5	M26
C-004-2[d]	32	9	12	4	5	M9
C-004-3	32	9	11	4	5	M27
C-004-4[d]	32	9	12	4	5	M9
C-004-5[d]	32	9	12	4	5	M9
C-005-1	40	11	10	5	5	M16
C-005-2	40	10	13	4	5	M28
C-005-3	40	11	10	5	5	M16
C-005-4	40	10	12	4	5	M29
C-005-5	40	11	10	5	5	M16

Farm/animal/isolate ID[a]	DTP[b]	L-1 (G)[c]	L-2 (G)[c]	L-3 (GGT)[c]	L-4 (TGC)[c]	SSR-Type
D-001-1	13	5	15	4	4	M30
D-001-2	13	7	15	4	4	M31
D-001-3	13	7	14	4	4	M32
E-001-1	18	11	11	4	5	M38
E-001-2	18	18	11	5	5	M12
E-001-3	18	15	10	5	5	M11
E-001-4	18	11	9	5	5	M39
E-001-5	18	7	11	6	5	M40
F-001-1	19	18	9	5	5	M41
F-001-2	19	19	9	5	5	M42
F-001-3	19	16	7	5	5	M43
F-001-4	19	12	9	5	5	M17
F-001-5	19	12	10	5	5	M15
F-002-1	35	16	10	5	5	M19
F-002-2	35	14	10	5	5	M1
F-002-3	35	16	10	5	5	M19
F-002-4	35	16	11	5	5	M2
F-002-5	35	17	11	5	5	M3

[a] The first letter denotes the farm of origin followed by a number assigned to the animal and the last number corresponds to the single colony/isolate from 7H11 plates that were used in the analysis. In every case, 3-5 colonies were picked for analysis from each 7H11 plate based on colony morphology and acid fast staining results

[b] DTP: Days to positive, days of incubation after which growth was detected in the automated Trek-ESP II liquid culture system

[c] Genotyping of SSRs (mononucleotide or trinucleotide) for the four loci (L): locus 1 (G repeats), locus 2 (G repeats), locus 3 (GGT repeats) and locus 4 (TGC repeats). The copy numbers of each SSR for all isolates are shown

[d] *Map* with identical SSR-types isolated from the same animal are indicated and were treated as duplicates during subsequent analysis

Table 3.3.1.1: Details of the 44 *Map* SSR-types that were isolated from five Newfoundland farms in the current study and were analyzed using fragment analysis

L-1 (G)[a]	L-2 (G)[a]	L-3 (GGT)[a]	L-4 (TGC)[a]	SSR-type[b]	No. of Animals with SSR-type[c]	Farm ID[d]
14	10	5	5	M1	5	A(2), C(2), F(1)
16	11	5	5	M2	4	A(3), F(1)
17	11	5	5	M3	2	A(1), F(1)
11	11	5	5	M4	5	A(4), C(1)
11	12	5	5	M5	1	A(1)
5	11	4	4	M6	2	C(2)
15	14	5	5	M7	1	C(1)
13	13	5	5	M8	1	C(1)
9	12	4	5	M9	1	C(1)
16	12	5	5	M10	1	A(1)
15	10	5	5	M11	4	A(3), E(1)
18	11	5	5	M12	3	A(2), E(1)
7	10	5	5	M13	2	A(2)
13	11	5	5	M14	2	A(2)
12	10	5	5	M15	3	A(2), F(1)
11	10	5	5	M16	2	A(1), C(1)
12	9	5	5	M17	2	A(1), F(1)
15	11	5	5	M18	2	C(1), A(1)
16	10	5	5	M19	2	A(1), F(1)
21	10	5	5	M20	1	A(1)
19	11	5	5	M21	1	A(1)
20	9	5	5	M22	1	A(1)
20	10	5	5	M23	1	A(1)
6	12	4	4	M24	1	C(1)
6	13	5	5	M25	1	C(1)
8	11	4	5	M26	1	C(1)
9	11	4	5	M27	1	C(1)
10	13	4	5	M28	1	C(1)
10	12	4	5	M29	1	C(1)
5	15	4	4	M30	1	D(1)
7	15	4	4	M31	1	D(1)
7	14	4	4	M32	1	D(1)

L-1 (G)[a]	L-2 (G)[a]	L-3 (GGT)[a]	L-4 (TGC)[a]	SSR-type[b]	No. of Animals with SSR-type[c]	Farm ID[d]
10	10	5	5	M33	1	A(1)
20	11	5	5	M34	1	A(1)
7	11	5	5	M35	1	A(1)
14	11	5	5	M36	1	A(1)
15	9	5	5	M37	1	A(1)
11	11	4	5	M38	1	E(1)
11	9	5	5	M39	1	E(1)
7	11	6	5	M40	1	E(1)
18	9	5	5	M41	1	F(1)
19	9	5	5	M42	1	F(1)
16	7	5	5	M43	1	F(1)
10	11	5	5	M44	1	A(1)

[a] The number/copies of repeats for each SSR detected in the current study are indicated.

[b] SSR-types were designated as M1-M44 based on the copy number of the repeats for the 4 SSR loci used in the analysis.

[c] The total number of animals are indicated from which *Map* with the respective SSR-types (M1-M44) were isolated

[d] The assigned identity (ID) of each farm is indicated by capital letters followed by the number of animals from that farm from which Map with the specific SSR-type was isolated. For example, A(3) implies that 3 individual animals from Farm A had Map with the specific SSR-type

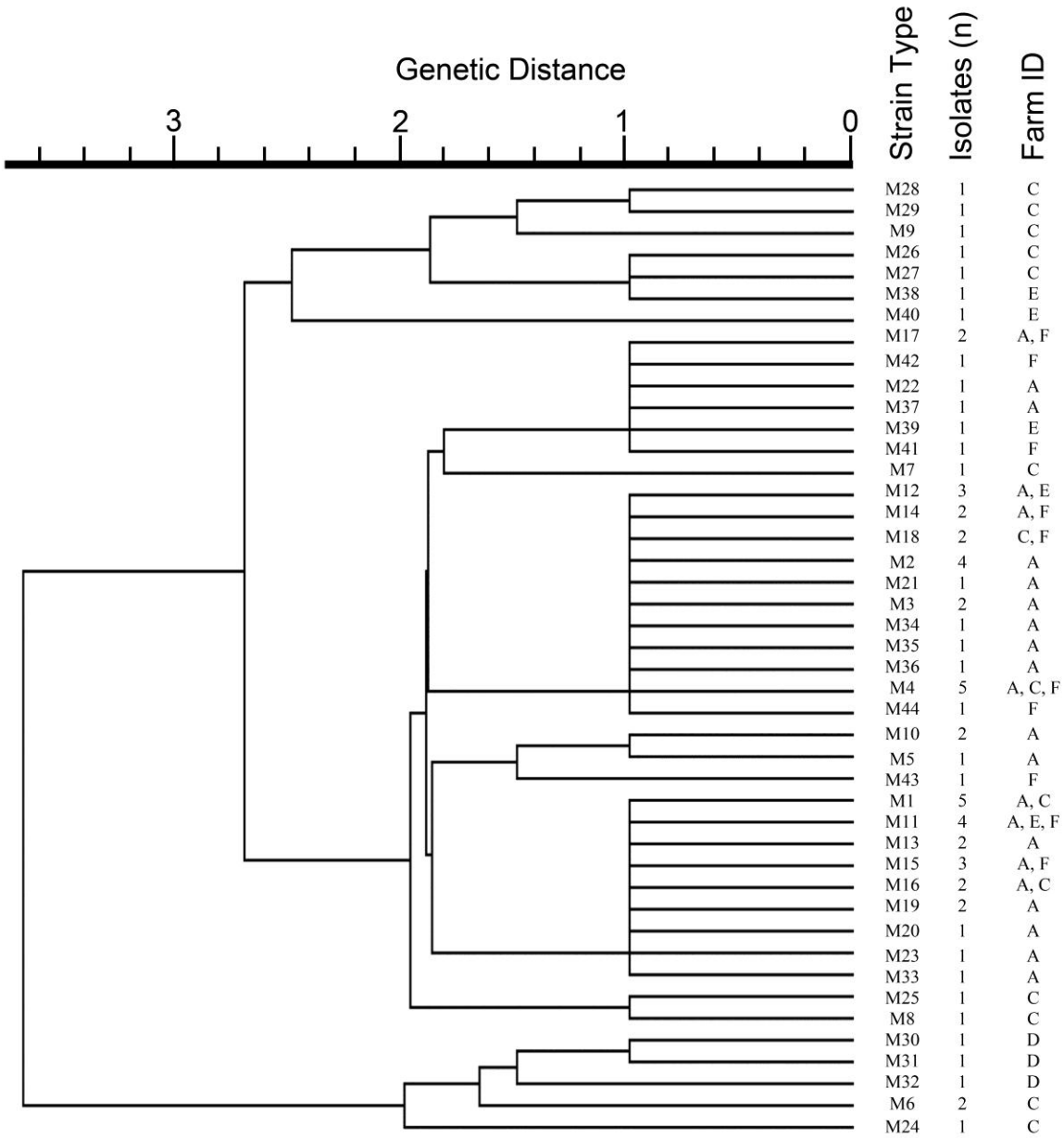

Figure 3.3.2.1: Dendrogram representing the genetic relationship between all *Map* isolates based on the 4 SSRs loci used in the analysis. The dendrogram was built using the unweighted pair group method with arithmetic mean (UPGMA) using the BioNumerics 7.1 multilocus sequence typing program. Genetic distance (Categorical coefficient) is indicated at the top of the dendrogram. SSR-types, number of isolates (n) and farm ID are displayed to the right side of the dendrogram.

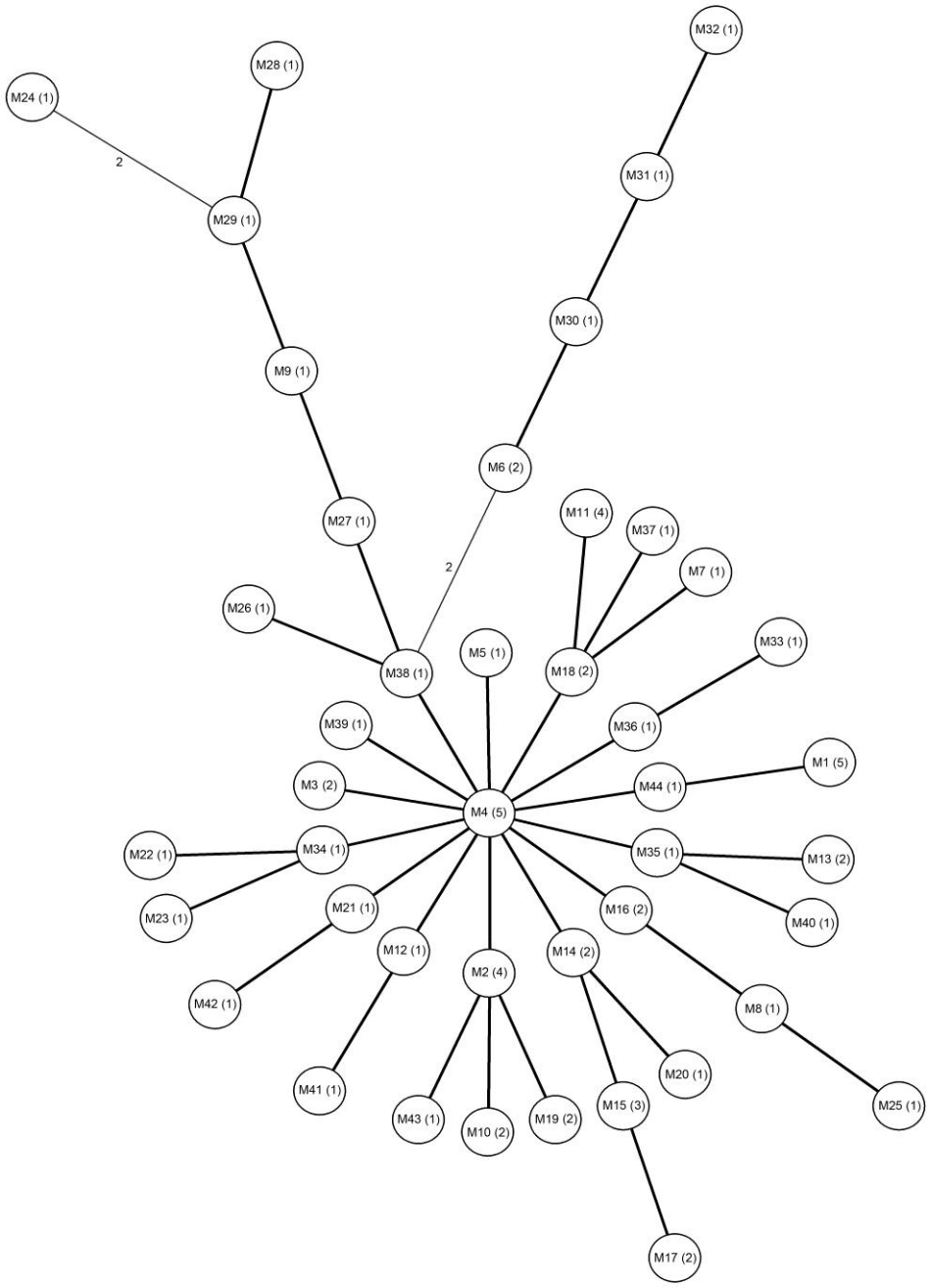

Figure 3.3.2.2: Minimum spanning tree (MST) based on the SSR profiles of the 4 loci for all 44 SSR-types identified in the current study. The circles represent the different strain types generated by the BioNumerics 7.1 multilocus sequence typing program. The number of *Map* isolates (after omitting duplicates from the same animal) belonging to each SSR-type is shown in parenthesis within the respective circles. Thick lines represent only one variation amidst the 4 loci, whereas thin lines represent 2 differences between the 4 loci, the latter of which is indicated.

Chapter 4: Summary

4.1 *K. pneumoniae*

The purpose of the research conducted in chapter two was to apply molecular methods for identifying and strain typing Gram-negative bacteria isolated from animals with CM, thus broadening our knowledge regarding the diversity of *Klebsiella* spp. present on a subset of Newfoundland dairy farms. Strain typing was performed using RAPD analysis, which is a quick and cost-effective method and provided valuable information regarding genetic relationship between different *Klebsiella* isolates. Furthermore, drug sensitivity profiles of the isolates were also determined and mapped on to the dendrograms, but relationships between resistance profiles/patterns and transmission were not observed.

Through the use of mass spectrometry and gene sequencing, it was found that *K. variicola* and *E. cloacae* were misidentified as *K. pneumoniae* in a small number of CM cases from Newfoundland, during the one year study. *K. variicola*, which is normally considered as an environmental and hospital-acquired opportunistic bacterium [1], had not been isolated previously from animals with CM. Therefore, the standard tests to discriminate *Klebsiella* species are not sensitive enough for detecting *K. variicola* and other Gram negative pathogens. Overall, polyclonal infection patterns were observed in the majority of farms, and a predominant or overrepresented strain was not observed in any of the cases. In the future, whole genome sequencing of the *K. variicola* isolates will be conducted, which will help to reveal the virulence factors and pathogenicity associated mechanisms present in this newly emerging pathogen [2].

4.2 *Map*

The goal of the research described in chapter three was to apply a cost effective molecular method (fragment analysis) for SSR typing of *Map* strains isolated from five dairy herds in Newfoundland. The information obtained using this method was useful for differentiating/subtyping *Map* strains and for analyzing molecular diversity.

SSR typing of *Map* isolates by mass spectrometry and Sanger sequencing are currently the most discriminatory genotyping methods used in epidemiological analyses, although both techniques fail to accurately resolve mononucleotide repeats longer than 15 bp [3-7]. There is also a need for an inexpensive, fast and reliable method for differentiating *Map* isolates, which could be fulfilled by fragment analysis. Fragment analysis was conducted on 88 isolates form Newfoundland dairy farms based on SSRs of 4 loci, and 44 distinct strains were successfully genotyped and differentiated. A polyclonal infection pattern was mostly observed between and within farms, and no predominant or overrepresented strains were identified. The total number of *Map* SSR-types found in animal isolates was higher in dairy herds from Newfoundland, compared to farms from other geographical regions in related studies [5,6,8]. Thus, a fragment analysis based approach for SSR-typing would be an improved strain typing method compared to other molecular methods due to its high resolution capability and discriminatory power. Thus, this method can be useful in generating and analyzing large quantities of molecular epidemiologic data within a short time frame. In the future, *Map* isolates from dairy herds from other provinces in Atlantic Canada (except Prince Edward Island) will be subjected to fragment analysis for comparison to the SSR-types that were identified in Newfoundland in the current study.

4.3 Conclusion

Overall goal of this thesis was to genetically type two animal pathogens that cause significant financial losses to the dairy industry in NL and other regions of the world. The two molecular methods described in this study were easily adaptable and were robust enough to differentiate among different isolates of *Klebsiella* and *Map*, respectively. Results from the described work showed that a single genotype could not be associated with large scale infections, suggesting a polyclonal pattern in the circulating isolates for each pathogen, based on the techniques used in the current analysis. Thus, in the future whole genome sequencing studies should be performed for more in-depth genetic analysis of the *Map* isolates so that the questions regarding co- or multiple- infections within/between animal(s) can be clarified, which is important in terms of source tracking and the status of the animals involved. Future research will contribute to the significance and implications of the described/current findings.

4.4 References

1. Rosenblueth M, Martinez L, Silva J, Martinez-Romero E (2004) *Klebsiella variicola*, A Novel Species with Clinical and Plant-Associated Isolates. Syst Appl Microbiol 27: 27-35.

2. Seki M, Gotoh K, Nakamura S, Akeda Y, Yoshii T, et al. (2013) Fatal sepsis caused by an unusual *Klebsiella* species that was misidentified by an automated identification system. J Med Microbiol 62: 801-803.

3. Amonsin A, Li LL, Zhang Q, Bannantine JP, Motiwala AS, et al. (2004) Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis* strains. J Clin Microbiol 42: 1694-1702.

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6. Sohal JS, Arsenault J, Labrecque O, Fairbrother J-H, Roy J-P, et al. (2014) Genetic structure of *Mycobacterium avium* subspecies *paratuberculosis* population in Quebec cattle herds revealed by using a combination of multi-locus genomic analysis. J Clin Microbiol 52: 2764-2775.

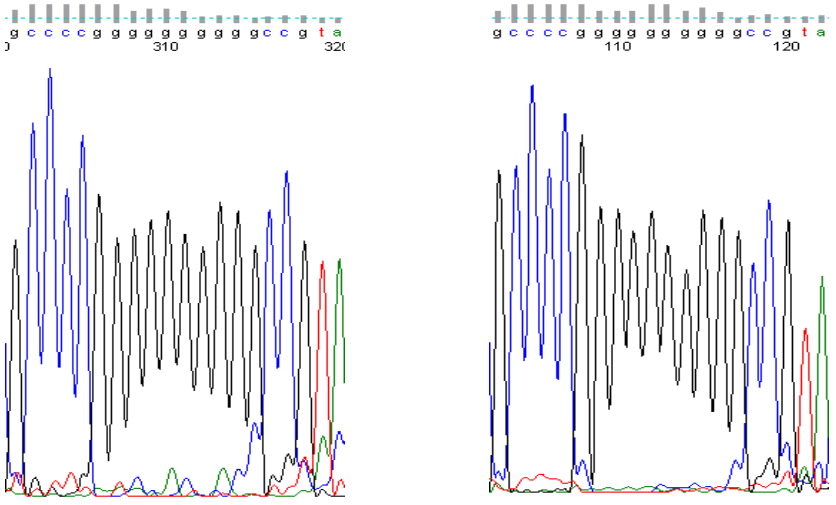
7. Ahlstrom C, Barkema HW, De Buck J (2014) Improved Short-Sequence-Repeat Genotyping of *Mycobacterium avium* subsp. *paratuberculosis* by Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. Applied and environmental microbiology 80: 534-539.

8. Bannantine J, Li LL, Sreevatsan S, Kapur V (2013) How does a *Mycobacterium* change its spots? Applying molecular tools to track diverse strains of *Mycobacterium avium* subspecies *paratuberculosis*. Lett Appl Microbiol 57: 165-173.

Appendix A: Sequence analysis of 4 SSR loci for multiple standards used in *Map* strain determination

A

S1 (L1): G10

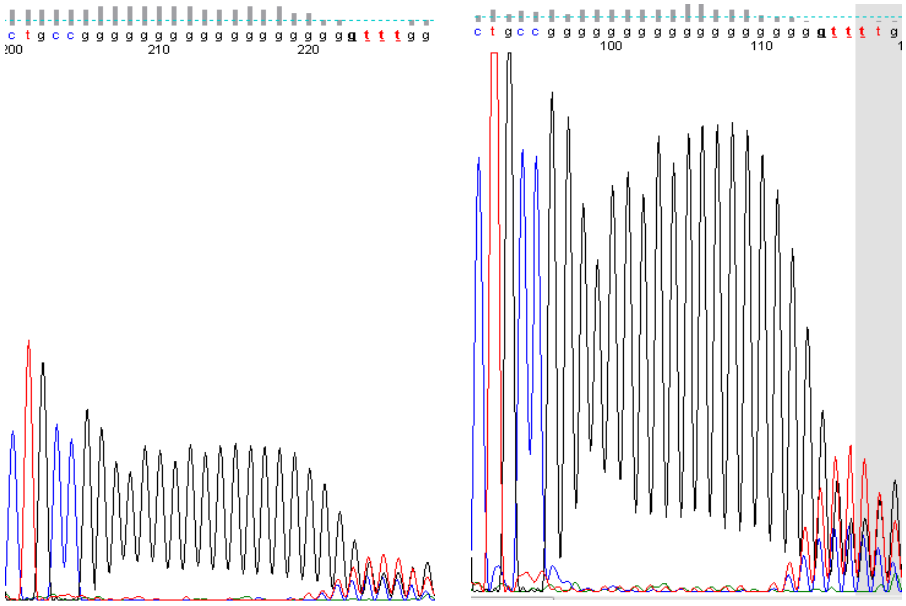


Forward sequencing

Reverse sequencing

B

S1 (L2): G19

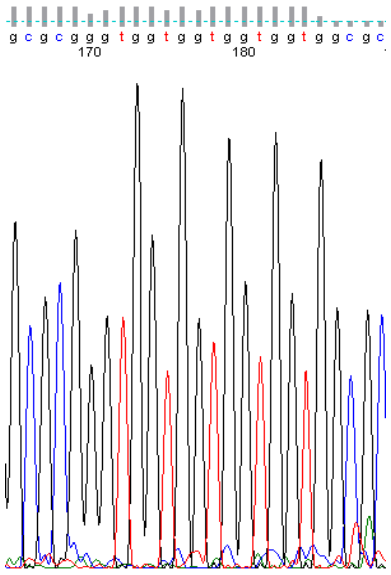


Forward sequencing

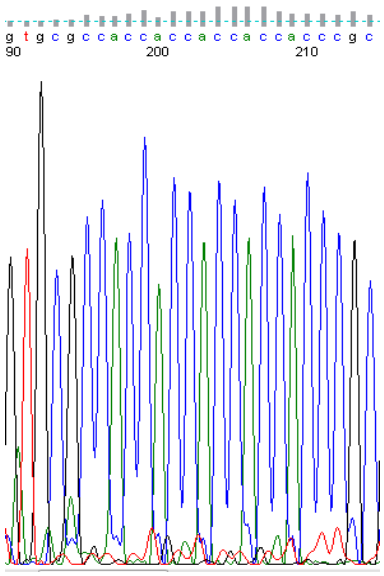
Reverse sequencing

C

S1 (L3): GGT5



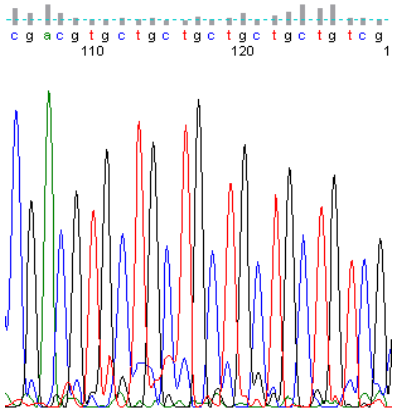
Forward sequencing



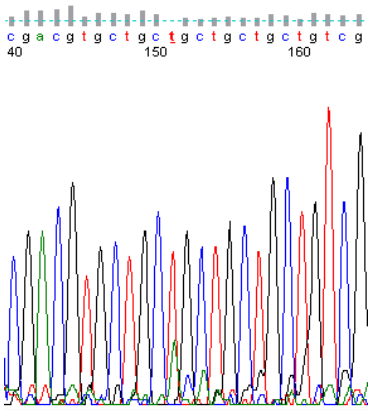
Reverse sequencing

D

S1 (L4): TGC5



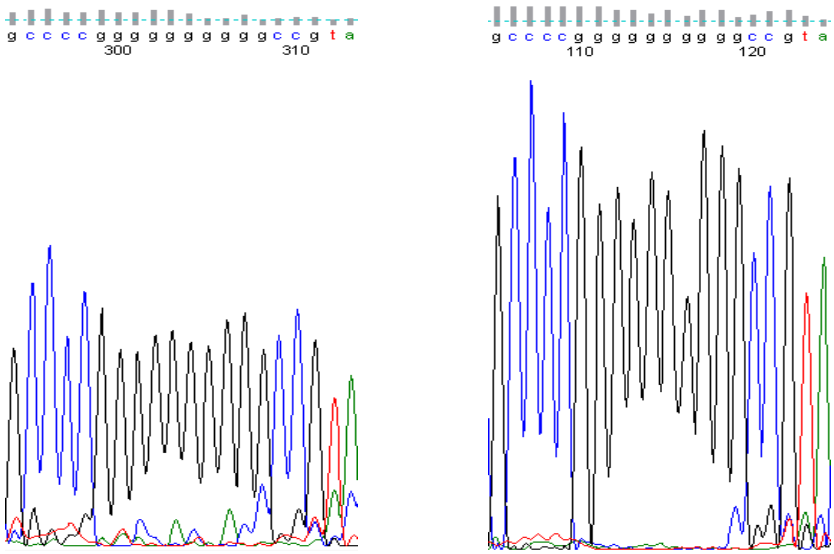
Forward sequencing



Reverse sequencing

E

S2 (L1): G10

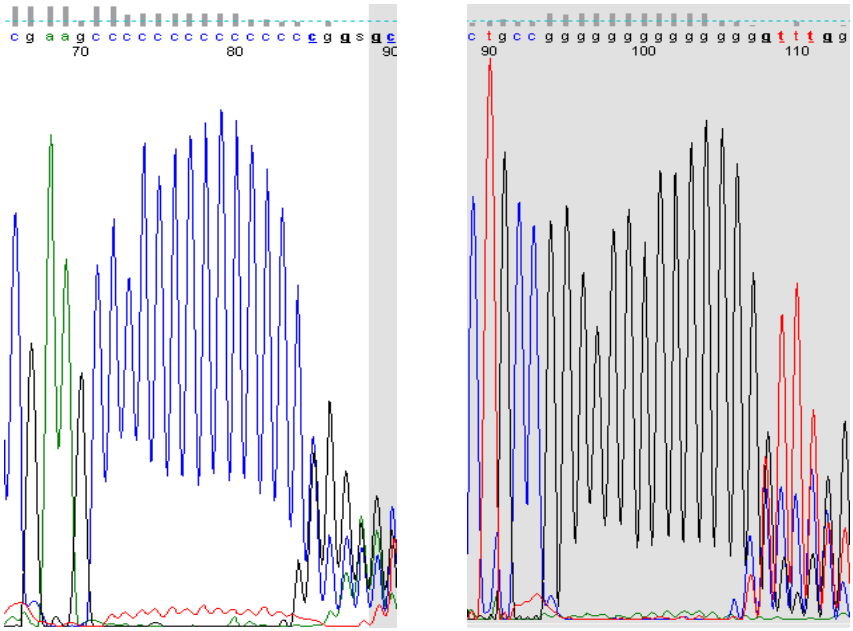


Forward sequencing

Reverse sequencing

F

S2 (L2): G15

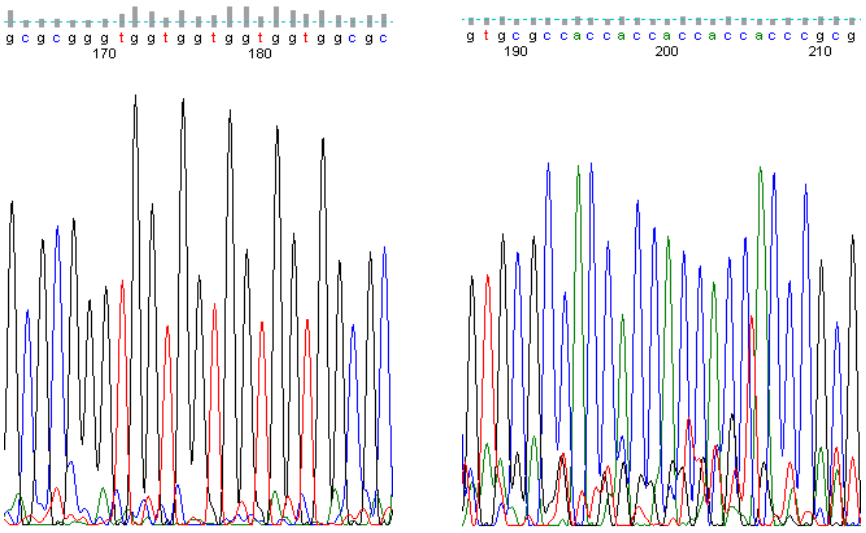


Forward sequencing

Reverse sequencing

G

S2 (L3): GGT5

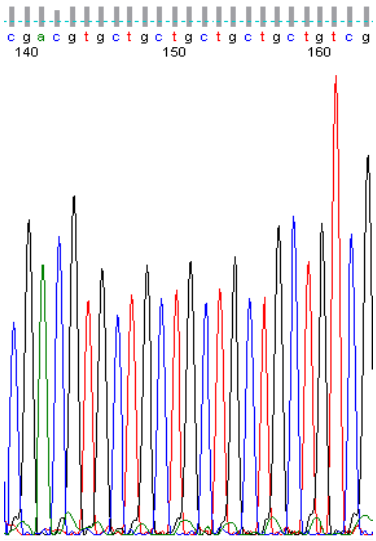


Forward sequencing

Reverse sequencing

H

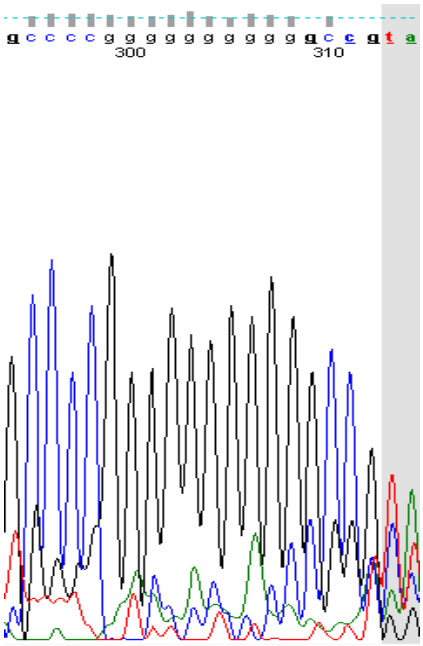
S2 (L4): TGC5

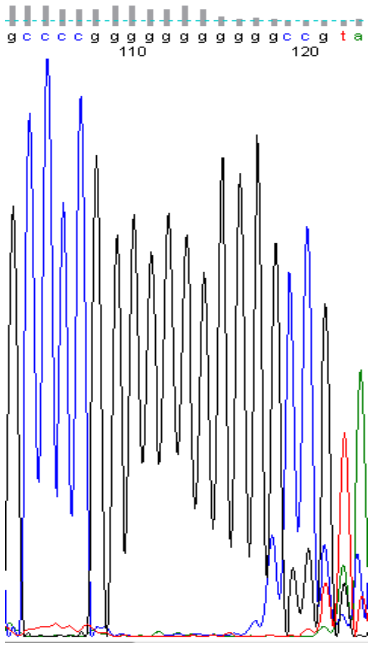


Reverse sequencing

I

S3 (L1): G11



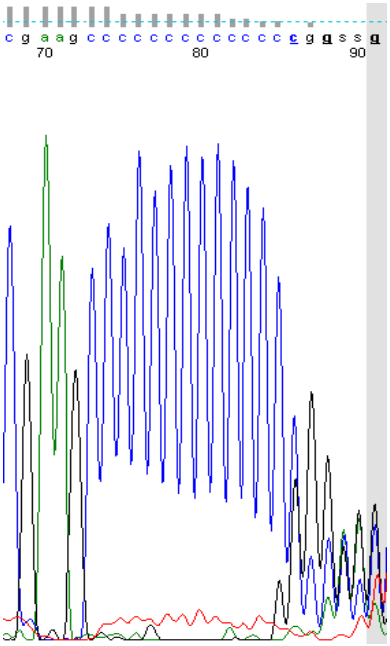


J

Forward sequencing

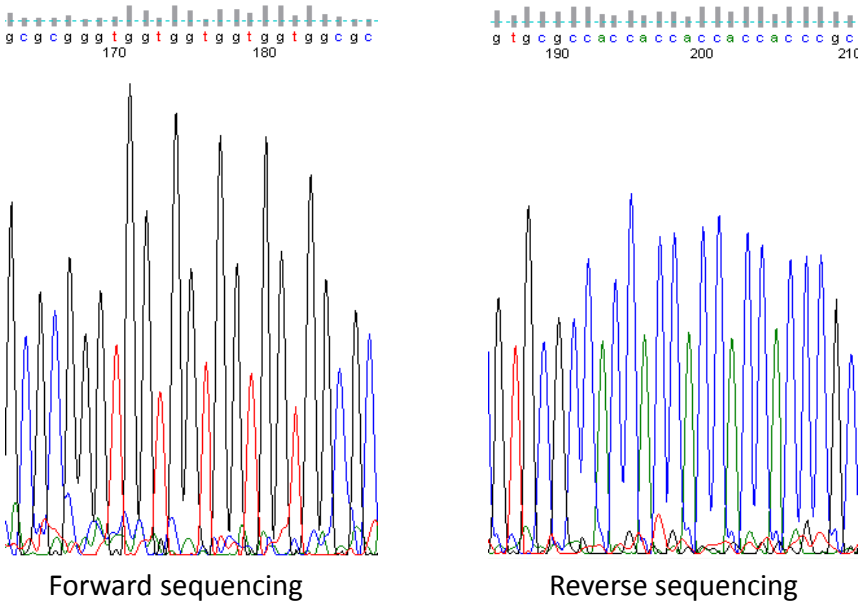
Reverse sequencing

S3 (L2): G14



Forward sequencing

K



L

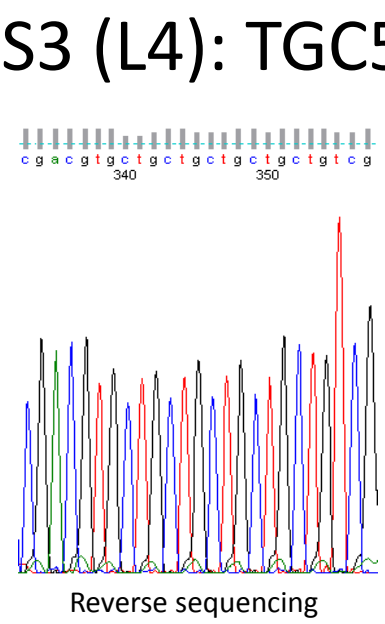


Figure A.1: Sequence analysis of multiple standards (S1, S2 and S3) based on the 4 SSR loci used in the present study. (A-L) Each SSR locus (L) is mentioned in parenthesis,

followed by mononucleotide or trinucleotide repeats. Sequencing errors were observed in either forward or reverse sequences of S2 and S3 for locus 4, and S3 for locus 2, respectively.