A Phenotypic and Molecular Comparison of Community-Associated and Hospital-Associated Methicillin-Resistant *Staphylococcus aureus* in Canada

by

Christine Siemens

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

Master's of Science

Department of Medical Microbiology

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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i. Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) was first described in 1961 only one year after methicillin was introduced clinically. MRSA was initially restricted to the healthcare settings, but recently has become prevalent in the community setting. Community-associated MRSA (CA-MRSA) is generally sensitive to most non-β-lactam antimicrobials and commonly causes skin and soft tissue infections however, CA-MRSA has been linked to severe infections such as necrotizing pneumonia. CA-MRSA strains typically contain Staphylococcal Cassette Chromosome *mec* (SCC*mec*) type IV and the Panton-Valentine leukocidin (PVL) toxin.

One hundred sixteen methicillin-sensitive *S. aureus* (MSSA) and 26 MRSA were collected from east-central Saskatchewan as part of an on-going case control study. Previously characterized MRSA from the region were also included in antimicrobial resistance investigations. Newly collected strains were characterized by antimicrobial susceptibility testing, DNA fingerprinting using pulse-field gel electrophoresis (PFGE) and PCR to detect the genes responsible for PVL. PCR was used to detect erythromycin resistance genes and a D-test was used to confirm inducible clindamycin resistance in all erythromycin resistant strains. PCR was also used to detect the presence of the *mupA* mupirocin resistant gene. MSSA strains possessed a low level of antimicrobial resistance overall with 52% of strains sensitive to all antimicrobials tested. However, erythromycin resistance was of notable levels. Among the erythromycin resistant MSSA, PCR results revealed an even distribution of *ermA*, *ermC* and *msrA* resistance determinants, which is reflective of the large amount of diversity of PFGE patterns. Two MSSA strains were determined to be PVL positive, which is not unexpected, as PVL is known to exist in a

small number of MSSA. In both the new and previously characterized MRSA from the region, erythromycin resistance was high being present in 53% of strains. Of the erythromycin resistant MRSA, 61% were constitutively or inducibly clindamycin resistant due to the presence of *ermC*. The remaining erythromycin resistant strains contained *msrA* and were sensitive to clindamycin. When erythromycin resistance in MRSA from Saskatchewan was compared to that of MRSA strains collected from Canadian Nosocomial Infection Surveillance (CNISP) sites, the level of erythromycin resistance was found to be lower in strains from Saskatchewan. Among the CMRSA2 from Saskatchewan *ermC*, was the most prevalent resistance gene while in CMRSA2 from CNISP sites *ermA*, was the most prevalent. CMRSA7 from CNISP sites was found to have a lower amount of erythromycin resistance as well as a lower amount of inducible clindamycin resistance. A high rate (55%) of mupirocin resistance was observed in all MRSA from the region. Mupirocin resistance in MRSA was found to be due to *mupA*, which was shown to be carried on at least two distinct plasmids through plasmid restriction analysis and Southern blotting.

During routine investigation of PVL in CA-MRSA from CNISP sites, PVL was found to be absent in 31 of 76 CA-MRSA strains that had indistinguishable PFGE patterns. The strains were Canadian PFGE type 0142, which is indistinguishable from MW2/USA400 CA-MRSA strain. It was found that a homologous phage that does not contain PVL was inserted into the same site. There does not appear to be a difference in the number of infections versus colonizations or in the types of infections among the PVL positive and PVL negative strains suggesting that PVL may not be as important in milder infections as previously thought.

In the course of a separate investigation of infections in Canadian intensive-care units (ICU), seven CA-MRSA were found among 126 MRSA isolated. Six of the CA-MRSA strains were isolated from the western region of Canada. There was a lower amount of antimicrobial resistance in CA-MRSA than in hospital-associated MRSA (HA-MRSA). Additionally a PVL positive EMRSA15, a common strain from Europe, was found and is the first reported case in Canada.

The results of the three separate studies have shown that CA-MRSA is quite capable of adapting to several situations. CA-MRSA stains are not only able to acquire additional resistance genes but are also adaptable to the health care setting. As CA-MRSA have been known to cause very serious infections, it is important to control these strains in the health-care setting where they are immunocompromised patients who would be at risk for a serious infection.

ii. Acknowledgements

First and foremost I would like to thank my supervisor Dr. Michael Mulvey for his support and ideas that he provided me with throughout the course of my studies. I would also like to thank my committee members, Dr. John Wylie and Dr. Teresa deKievit for their advice and support. A big thank you to all the members of the nosocomial group at the National Microbiology Laboratory, especially David Boyd for his advice with all of my molecular work. Also I would like to thank Jennifer Campbell and Dave Spreitzer for their assistance with pulse-field and analysis. I would also like to thank all of the graduate students, Erica, Melissa and Sara, who were very helpful with their advice and help with course work and presentations.

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I would like to thank my family who provided me with the emotional support to fight through the hard times. I would like to give a big thank you to my mother, Michele Siemens, who spent countless hours baby-sitting. Thank you to George Golding who not only provided me with emotional support but also provided me with some very good advice for many of the frustrating experiments.

Last, and definitely not least, I would like to thank Emma Siemens, my beautiful babe, whose patience while Mom was studying or working in the lab or writing a thesis was unwavering. This was all for you babe. Love Mom.

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vi. List of Abbreviations

Ala Alanine

ATP Adenine Tri-Phosphate

bp Base Pair

CA-MRSA Community-Associated MRSA

CAN-ICU Canadian Intensive Care Unit

CLIS Clinical and Laboratory Standards Institute

CMRSA Canadian Epidemic MRSA

CNISP Canadian Nosocomial Infection Surveillance Program

EMRSA European Epidemic MRSA

Gly Glycine

Glu Glutamic acid

h Hour

HA-MRSA Hospital Associated MRSA

HSC Health Sciences Center

ICU Intensive Care Unit

kb Kilobase

Lys Lysine

MIC Minimum Inhibitory Concentration

min Minute

MRSA Methicillin-Resistant *Staphylococcus aureus*

MSSA Methicillin-Sensitive *Staphylococcus aureus*

NAG N-Acetylglucosamine

NAM N-Acetylmuramic acid

NCCLS National Committee for Clinical and Laboratory Standards

PBP Penicillin Binding Protein

PVL Panton-Valentine Leukocidin

SCC*mec* Staphylococcal Cassette Chromosome *mec*

sec Second

UDP Uracil Di-Phisphate

1 Introduction

1.1 *Staphylococcus aureus*

Staphylococci are Gram-positive cells that appear as grape-like clusters under the microscope (76). This unique feature lead to the naming of the genus by Ogston in 1883 based on his observations of the cells (76). Initially only two species, *S. aureus* and *S. albus*, were included in the genus which today is comprised of 28 species and seven subspecies.

S. aureus are non-motile, non-spore forming, catalase positive facultative aerobes with an optimum growth temperature of 37°C (145). *S. aureus* is coagulase positive and produces hemolysins along with several other exotoxins and enterotoxins (76).

S. aureus is capable of colonizing humans, most commonly the anterior nares (23, 31, 37, 53, 81). *S. aureus* colonization is a well known risk factor for infection and a recent report of *S. aureus* carriage rates estimates that up to 32.4% of Americans were carriers in 2001-2002 (33, 81). While *S. aureus* is typically a human pathogen it is capable of colonizing and infecting domestic animals (73). Transfer between animals and humans has been known to occur (161).

The range of diseases that *S. aureus* is capable of causing is vast and occurs either through toxin-mediated action or through a variety of virulence mediated mechanisms (73, 145). The most notable diseases that are toxin mediated are food poisoning, toxic shock syndrome and scalded skin syndrome. Virulence mediated infections include skin and soft tissue infections (SSTI), pneumonia, and bacteremia.

1.2 Antibiotic Resistance in *S. aureus*

S. aureus is highly capable of adapting to the pressure of antibiotics; resistance to virtually every clinically available antibiotic has been reported (136). Antibiotic resistance was first observed to β-lactam antibiotics due to an enzyme called β-lactamase (1, 9, 73). Later, resistance to β-lactams due to the altered penicillin binding protein (PBP) 2a was observed after the introduction of methicillin (discussed in section 1.3.4.2.2) (8, 46, 47, 57, 73). Some examples of resistance to non-β-lactam antimicrobials include resistance to aminoglycosides, which can result from mutations in the target site, reduced uptake or aminoglycoside modification (136). Resistance to fluoroquinolones can develop through a mutation in DNA gyrase, the target site of fluoroquinolones. More recently resistance to glycopeptides, such as vancomycin, has been observed and is thought to be due to thickening of the cell wall. Additionally the acquisition of the vancomycin resistance genes, theoretically transferred from *Enterococcus faecalis*, has been observed in a few MRSA strains (32, 153). Resistance has even been observed to the newest class of antibiotic, the oxazolidinones, which only has one member available for clinical use, linezolid (178). Linezolid has good activity against Gram-positive organisms however, *S. aureus* has been able to overcome its effects through mutations in the target site of the ribosome and mutations in the L4 region of the ribosome (118).

1.3 Methicillin Resistance in *S. aureus*

Methicillin is a semi-synthetic β-lactam antimicrobial that was first synthesized in the 1950's (8). Methicillin, which is unaffected by β-lactamase, was a promising antimicrobial with good activity against Gram-positive organisms that were resistant to

penicillin. To the surprise of many, resistance to methicillin was observed in less than a year following its introduction for clinical use. It was later discovered that resistance to methicillin was due to the acquisition of an altered PBP, which enabled the synthesis of peptidoglycan in the presence of methicillin (58) (discussed in section 1.3.4.2.2).

The first methicillin-resistant *S. aureus* (MRSA) was observed in England and quickly spread through hospitals in many parts of Europe (16). MRSA did not appear as an important pathogen in North America until the 1970's when MRSA rapidly spread among hospitals across the United States. MRSA was first observed clinically in Canada in 1981 and has quickly spread through Canadian hospitals (143). MRSA not only affects patients, it provides a huge burden of cost to the health care system. It is estimated that between 42 and 59 million dollars are spent every year on hospital-associated MRSA (HA-MRSA) in Canada (75).

In 1995, surveillance of MRSA was conducted through the Canadian Nosocomial Infection Surveillance Program (CNISP) to monitor MRSA in Canadian hospitals (143). A Canadian naming system for epidemic clones of MRSA was established (144). A clone is determined to be epidemic if it is clinically or epidemiologically relevant in five or more hospitals or from more than three geographic regions. All epidemic strains must be characterized by standardized methods. Currently epidemic strains are defined and classified based on their *Sma*I pulse-field gel electrophoresis pattern. Ten epidemic strains have been described to date (20, 143). CMRSA1-6 and CMRSA8 and 9 are typical nosocomial strains. CMRSA7 and CMRSA10 are classified as community-associated MRSA (CA-MRSA) and are indistinguishable from the newly described community-associated USA400 and USA300, respectively (20, 100). Initially CMRSA1

was the most prevalent clone but has now been replaced by CMRSA2, which accounted for approximately 55% of all strains in 2004 (20). CMRSA7 and CMRSA10 strains have both increased in frequency over the past few years however, CMRSA10 has become the more prevalent of the two. This has also been observed in the United States where USA400 was the first CA-MRSA strain of concern in North America but has now been overtaken by USA300 as the more prevalent of the two among community-associated infections (22, 60, 152). All CMRSA10 indistinguishable from the Canadian type strain (PFGE designation 0473) contain both of the Panton-Valentine leukocidin toxin genes, *lukF*-VP and *lukS*-PV (20). However, a large number of PVL negative strains indistinguishable from the PVL positive CMRSA7 type strain (Canadian type 0142) have been identified. PVL has also been detected in 67 and 90% of strains closely related to CMRSA7 and CMRSA10 respectively.

1.3.1 **Hospital-Associated MRSA**

MRSA in the hospital poses a very serious risk to patients, especially in the critically ill (29, 56). The risk factors for MRSA have been well documented and include such things as colonization, length of hospital stay, admission to intensive care unit, severity of illness, the use of intravascular devices and antibiotic use (122, 175). Colonization seems to be an important factor in acquiring an MRSA infection and has been shown to lead to wound infections, bacteremias and pneumonias in patients (33). It is because of this there has been a significant attempt to eradicate colonization in patients (89, 99). MRSA typically colonizes the anterior nares of patients (23, 31, 37, 53, 81). Use of intranasal antimicrobials, such as mupirocin, has been used to attempt to decolonize patients (99, 135, 169). This has been shown to help reduce the amount of

MRSA infection in surgical patients with relatively low rates of resistance seen to date, however, it can result in resistance to these agents (99, 100, 135, 169).

MRSA is endemic in many hospitals and has been known to account for up to 80% of *S. aureus* in hospitals (135). Infection control measures have been put into place in many hospitals with varying success however, studies have shown that even with strict hand-washing and contact isolation guidelines, MRSA is still capable of spreading (15, 90). For instance, even after proper hand washing and glove usage, MRSA could still be found on the hands of health-care workers (90). Lack of education and poor compliance of the health-care workers might be contributing factors. Most infection control measures only require gloves if the health-care worker is in contact with the infected site, however MRSA is present on areas of the body other than the infected site and on objects surrounding the patient which may contribute to the spreading of MRSA (40).

There is hope however, as practices in countries such as Denmark and the Netherlands have maintained very low numbers of infections due to MRSA as well as low rates of transmission (15, 78). In these countries, the guidelines for MRSA infection control are very strict and include the isolation of the patient, gloves, caps, masks and gowns upon entering the room of every patient colonized or infected with MRSA (whether or not contact with patient is anticipated). They also have a very effective screening program of all patients and healthcare workers who had contact with an infected patient. These guidelines, although being time consuming and costly, have proven to be very effective in controlling the spread of MRSA (15).

1.3.2 **Community-Associated MRSA**

Currently the definition of CA-MRSA varies greatly between groups leading to misclassification (42). CA-MRSA emerged in the late 1980s in patients lacking any of the risk factors for HA-MRSA (146). The first definitions of CA-MRSA were based on the identification of an MRSA infection from an out-outpatient or from a patient less than 24-72 h after hospital admission with no risk factors for hospital-associated MRSA infection (19, 41, 103, 151). MW2 (also known as USA400 and indistinguishable from CMRSA7) was the first CA-MRSA associated with serious infections to be identified in North America (22, 60). A second CA-MRSA clone, USA300 (indistinguishable from CMRSA10), has also been identified and is currently more prevalent than the MW2 clone (20, 96, 152). While CA-MRSA have been observed in severe necrotizing pneumonias, necrotizing fasciitis and severe furnuculitis, it is typically isolated from minor SSTIs (6, 7, 14, 19, 22, 26, 34, 35, 36, 37, 43, 44, 45, 55, 59, 71, 79, 95, 96, 97, 98, 100, 110,116, 117, 131, 132, 138, 173, 174). Various CA-MRSA strains have been reported in several parts of the world, including Europe and Australia (95, 162, 167), and do not appear to be related to many of the strains identified elsewhere in the world. However, several European strains have been observed in Canada while CA-MRSA strains from North American have been identified in Europe (20, 155, 156).

Both MW2 (USA400) and USA300 have very different PFGE patterns than any of the known HA-MRSA and are also sensitive to most non-β-lactam antimicrobials (22, 60, 100, 111, 152). Both PVL and SCC*mec* type IV (discussed in section 1.3.4.2.2.1) have been shown to be highly associated with CA-MRSA (6, 7, 14, 19, 20, 22, 26, 34, 35,

36, 37, 43, 44, 45, 55, 59, 71, 79, 95, 96, 97, 98, 100, 103, 110, 116, 117, 131, 132, 138, 152, 173, 174).

Initially CA-MRSA appeared to be present only in the community but has now spread to the health-care setting (17, 79, 109, 131, 138). CA-MRSA strains have been reported in maternity wards, nurseries and orthopedic wards (17, 79, 131). Several studies have reported isolation of CA-MRSA from bloodstream infections, post-operative infections and SSTIs from patients in hospital (17, 79, 109, 131, 138).

Risk factors for CA-MRSA are poorly characterized however, it is noted that CA-MRSA is typically observed in young (<65 yrs of age) previously healthy patients (22, 36, 43, 60, 100, 103, 104, 143, 174). This is unlike HA-MRSA infected patients who are generally elderly (>65 yrs of age) with underlying diseases (103, 143). Socio-economic status has also been identified as a possible risk factor for acquiring a CA-MRSA infection (55, 103, 132).

1.3.3 **CA-MRSA in Canada**

Two recent studies of CA-MRSA in Canada were both focused in the prairie provinces of Manitoba and Saskatchewan (100, 173). Six major clones were described in Manitoba, two of which appeared to be highly associated with community-acquired infections, Clone C and Clone E. Clone E was determined to be part of the CMRSA1 epidemic cluster which is typically a HA-MRSA clone, and was PVL negative (20, 142, 173). Clone C was determined to be indistinguishable from CMRSA7 (USA400/MW2) and was determined to be PVL positive. CMRSA1 showed low levels of antimicrobial resistance to trimethoprim-sulfamethoxazole, erythromycin, clindamycin, ciprofloxacin and gentamicin. CMRSA7 had a high level of erythromycin resistance and a low level of

resistance to the other four antimicrobials. CMRSA7 was found to have an increase in numbers between 1995 and 2000, which was reduced in the following years. Infections occurred more frequently in patients less than 30 years of age in both CMRSA7 and CMRSA1 than the health-care associated strains, which were more frequently greater than 31 years of age.

MRSA was first introduced into east-central Saskatchewan in April of 1999 (100). While this was not the first case of MRSA in the area it was the index case in an outbreak that occurred over the following three years. Six clone types were observed in the area from April 1999 to April 2002. The initial dominant clone in the area was Clone A (part of CMRSA2 epidemic cluster), which spread rapidly throughout the health-care setting in the area and spread into the community. A second dominant clone, Clone B (part of CMRSA7 epidemic cluster), appeared in July of 2000 and quickly became the predominant clone in the region resulting in a number of community-acquired infections. CMRSA2 exhibited a high amount of resistance to ciprofloxacin, erythromycin, gentamicin, tetracycline, mupirocin, and fusidic acid. CMRSA2 strains were generally sensitive to cefazolin, clindamycin, rifampin and trimethoprim-sulfamethoxazole and all CMRSA2 were sensitive to linezolid and vancomycin. CMRSA7 had a large number of strains resistant to both erythromycin and mupirocin however, most remained sensitive to cefazolin and clindamycin and all were sensitive to ciprofloxacin, gentamicin, rifampin, tetracycline, trimethoprim-sulfamethoxazole, linezolid and vancomycin. The high amount of mupirocin resistance in both clones (58 and 55.6%, respectively) appears to be unique to the region, as nationally mupirocin rates in MRSA are approximately 17% (80). All of the CMRSA7 isolates contained PVL (100).

The two clone types differ vastly in the age distribution; CMRSA2 was found in older patients and CMRSA7 was typically found in younger patients (100). However, when CMRSA2 was identified in the community, the patients tended to be younger in age.

1.3.4 **Molecular Genetics of Methicillin Resistance**

1.3.4.1 **Cell Wall Synthesis**

The cell wall of an organism has several functions; protection of the organism from the surrounding environment, selective entry of substances into and out of the cell, and it provides protection against changes in osmotic pressure (137). The cell wall of *S. aureus* is made up of polysaccharides, proteins and peptidoglycan, which comprise up to 60% of the cell wall. Peptidoglycan is formed during a three stage process involving several proteins.

The first stage of peptidoglycan synthesis occurs in the cytoplasm and begins with the formation of uracil-diphosphate-N-acetylmuramic acid (UDP-NAM) from N-acetylglucosamine (NAG) (137). L-Ala, D-Glu, L-Lys, and a D-Ala-D-Ala dimer are added to UDP-NAM in an ATP-dependent manner. During the second stage of synthesis UDP-NAM-pentapeptide is metabolized at the cytoplasmic membrane to form Lipid I (166). NAG is attached to Lipid I with a β-1-4 linkage, forming Lipid II. A pentaglycine bridge is added to the L-Lys of the NAM portion of the molecule forming a pre-peptidoglycan subunit, which is transported across the cytoplasmic membrane. The pre-peptidoglycan subunits are joined together through the action of PBPs in a transglycosylation reaction resulting in the final peptidoglycan subunit (N-

acetylmuramic-acid-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala-(Gly_5)-N-acetylglucosamine) (137, 166).

The cross-linking of pentapeptide side chains of neighboring NAM involves D-D carboxypeptidases, which are membrane-bound PBPs. The PBPs catalyze transpeptidation of D-Ala to Gly of the penta-Gly bridge (137). Acylation of Ser403 on the PBP (46) with the non-terminal D-Ala causes the removal of the terminal D-Ala (166). The terminal Gly is cross-linked to the D-Ala releasing the PBP, forming a bridge that allows for stability of the peptidoglycan (137).

1.3.4.2 **β-Lactams and Resistance**

1.3.4.2.1 **Penicillin Resistance**

Penicillin is a β-lactam antibiotic containing a β-lactam ring along side a sulfur-containing 5 membered ring (Figure 1) (166). The β-lactam ring is able to acylate Ser403 of PBPs which forms a stable complex and prevents transpeptidation reactions (46). Resistance to penicillin in *S. aureus* is due to an enzyme called β-lactamase, originally termed penicillinase, which was discovered in 1940 (1). β-lactamases are PBPs that bind to certain β-lactams and cause hydrolytic cleavage of the β-lactam ring rendering the drug inactive (166) (Figure 1). β-lactamase is released and is free to inactivate another β-lactam molecule.

The β-lactamase resistance gene, *blaZ*, is typically plasmid-encoded and is inducible in the presence of β-lactams (47, 92). *blaZ* is regulated by BlaI (repressor) and BlaR1 (sensor/transducer) which are encoded on the same operon as *blaZ* (47, 128). BlaR1 is activated by β-lactams which catalyzes the removal of BlaI, allowing synthesis of all three genes (47).

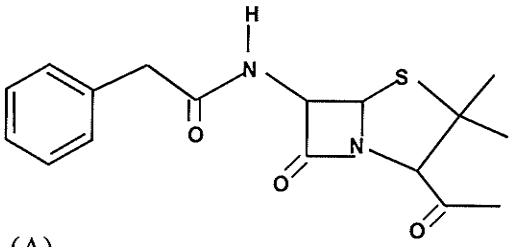
1.3.4.2.2 **Methicillin Resistance**

In *S. aureus*, methicillin resistance is due to the presence of an altered PBP (58), PBP2a, a 78kDa membrane bound transpeptidase with a low affinity for certain β-lactams due to conformational protection of Ser403 (46). PBP2a is able to replace cell wall synthesis when native PBPs are affected by β-lactams (88). While PBP2a is essential for survival of *S. aureus* in the presence of methicillin, the native PBPs may still play a role in peptidoglycan synthesis (119). It is hypothesized that PBP2a carries out transpeptidation while the native PBP2 is still required for transglycosylation.

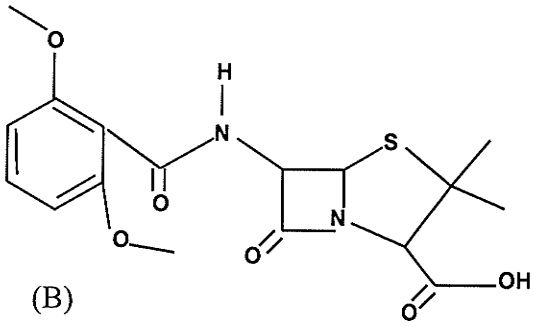
1.3.4.2.2.1 **SCC*mec***

mecA, the gene that codes for PBP2a, is located on the Staphylococcal Cassette Chromosome *mec* (SCC*mec*), a mobile genetic element that inserts at a specific site (*orfX*) near the origin of replication (27, 68, 72, 73). To date there have been five SCC*mec* types described (type I-V) along with several subtypes (68). Several different regions surrounding *mecA* have been described including: 1) class A *mec* region containing *mecA*, *mecR1* and *mecI*, 2) class B *mec* region containing *mecA*, Δ*mecR1* and Ψ*ISI1272*, and 3) class C2 *mec* region containing *mecA* and Δ*mecR1*. SCC*mec* types differ both in the classes of *mec* and Cassette Chromosome recombinase (*ccr*) regions. SCC*mec* type I possesses a class B *mec* region and type 1 *ccr*. SCC*mec* type II contains a class A type *mec* and type 2 *ccr*. SCC*mec* type III is largest of all of the known SCC*mec* types and possesses a class A *mec* region and type 3 *ccr*. SCC*mec* type IV possesses a

Figure 1. Schematic structure of (A) penicillin G and (B) methicillin. (C) Inactivation of penicillin G by β-lactamase hydrolysis of the β-lactam ring (adapted from 166).



(A)



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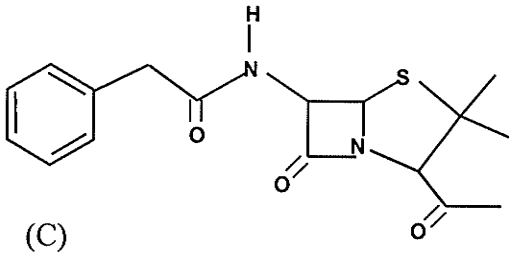
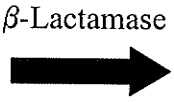
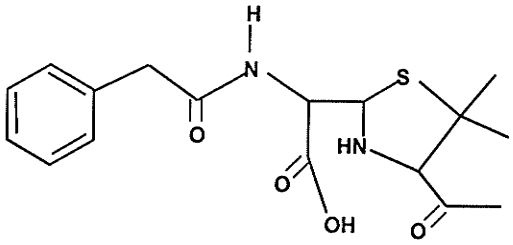


(C)





class B type *mec* and a type 2 *ccr*. The newest SCC*mec* to be described, type V, contains a class C2 *mec* region and a type 5 *ccr* (68).

SCC*mec* types I-III are typically associated with HA-MRSA, while types IV and V are associated with CA-MRSA. In addition to the *mecA* gene, SCC*mec* types II and III carry antibiotic resistance genes to non-β-lactams including *ermA*, which confers resistance to macrolides, lincosamides and streptogramins, and *tetK*, which confers resistance to tetracycline (86, 112). These additional resistance genes are due to an insertion of either a transposon or plasmid into the SCC*mec* region and provide a selective advantage in hospital settings where there is a high rate of antibiotic use (86). On the other hand, the energy expenditures associated with antibiotic resistance genes and the larger sizes of SCC*mec* types I-III may cause slower replication (111). The large sizes of SCC*mec* types I-III (34-67kb) may also make it more difficult for transfer between species and strains (86, 111). In contrast SCC*mec* types IV and V lack any antibiotic resistance genes other than *mecA*, making SCC*mec* types IV and V much smaller (21-25kb). The smaller size might allow for a faster rate of replication, possibly allowing the strains to be more competitive with normal flora and therefore more competitive in a community setting (86, 111, 131).

Another advantage of the smaller SCC*mec* type IV is that it may be transferred between species and strains more readily than the larger SCC*mec* types I-III (86). It has been speculated that SCC*mec* type IV might be capable of being packaged into a phage and subsequently transduced into different strains. This is not as likely with SCC*mec* types I-III as they are thought to be too large to fit into a phage. The theoretical ease of ability of SCC*mec* type IV to transfer between strains could lead to greater diversity in

the CA-MRSA strains than is seen in the HA-MRSA strains, where it is thought that horizontal transfer is rare (86, 111). Additionally, it is thought that SCC*mec* type IV has a larger range of genetic backgrounds that are capable of supporting it, while SCC*mec* types I-III have a limited number of strains able to maintain the larger SCC*mec* regions (113). This could explain why only a few HA-MRSA clones are responsible for infections worldwide.

1.3.4.2.2.2 *mecA* Regulation

In strains containing SCC*mec* type II and III, *mecA* transcription may be regulated by a signal transduction pathway involving MecI, a homodimer repressor that binds to a specific region of the *mecA* promoter preventing transcription of *mecA*, *mecR1* and *mecI* (47), together with MecR1 (sensor/transducer), a cytoplasmic bound PBP (88). The extracellular C-terminal domain of MecR1, binds β-lactams inducing a conformational change in MecR1 (47, 50). The altered MecR1 in turn inactivates MecI through a proteolysis reaction allowing transcription of all three genes (88). The synthesis of PBP2a, MecR1 and MecI is halted when there is no longer cleavage of MecI, that is, when β-lactams are no longer present in the environment (47, 88).

In SCC*mec* types I and IV, where MecI is not present and there is no functional MecR1, regulation of *mecA* may be governed by BlaI and BlaR1 from the penicillin-resistance *bla* operon (92). BlaI repressor is highly homologous to MecI repressor in function and structure which may allow the two to work interchangeably (92, 128). BlaR1 and MecR1 while being functionally similar have a low degree of sequence homology. Consequently, they are specific to their respective repressors, BlaI and MecI, and can not be used interchangeably (92).

1.4 Other Antibiotic Resistance in *S. aureus*

1.4.1 Macrolide, Lincosamide and Streptogramin B Resistance

Erythromycin, a member of the macrolide class of antibiotics, was first used clinically in 1953 (168). Erythromycin inhibits protein synthesis by causing the dissociation of peptidyl tRNA from the ribosome (94). Resistance to macrolides was first observed in *S. aureus* shortly after their introduction (24). Three mechanisms of resistance to macrolides are known in *S. aureus* including: alteration of the target site, efflux of the antibiotic and enzymatic modification of the antibiotic (83, 129, 172).

Alteration of the target site occurs through dimethylation of an N^6-adenine residue (A2058) in the ribosome and is caused by a ribosome methylation protein encoded for by an erythromycin ribosome methylation (*erm*) (83). Several *erm* genes are known to exist; in *S. aureus*, including *ermA*, *ermB* and *ermC* (66, 170). *ermA* is carried on a transposon, Tn*554*, which inserts into the *S. aureus* genome, including regions within SCC*mec* types II and III (113). *ermB* is found on Tn*551* and integrates into the chromosome of both MSSA and MRSA (11). *ermC* is found on small plasmids (139). Efflux of macrolides is caused by an ATP-dependent pump encoded by the plasmid-borne *msrA* gene (129). In *S. aureus*, alteration of the antibiotic occurs through hydrolysis of the lactone ring of erythromycin which is caused by an unknown enzyme that is homologous to EreA and EreB in *Escherichia coli* (172).

Cross resistance to two other distinct, but functionally similar classes of antibiotics, the lincosamides and streptogramins B was observed in erythromycin resistant *S. aureus* (54). This is thought to be due to the overlapping functional sites of the antibiotics on the ribosome (163, 164). ErmA, ErmB and ErmC are all capable of

providing cross resistance to both lincosamides and streptogramins, referred to as an MLS_B phenotype (168). However, resistance to lincosamides is not always constitutive. *S. aureus* isolates possessing MsrA without any of the Erm methylases only provides cross-resistance to streptogramins, which is referred to as an MS_B phenotype (129).

1.4.2 **Mupirocin Resistance**

Mupirocin (pseudomonic acid) is a naturally occurring antimicrobial produced by *Pseudomonas fluorescens* that possesses good activity against many Gram-positive organisms, including *S. aureus* (25, 150). Mupirocin is used as a topical antibiotic for skin and soft tissue infections (SSTIs) as well as intranasally for decolonization of MRSA (123). Mupirocin's activity inhibits protein synthesis by interacting with isoleucyl-tRNA synthetase, thereby preventing RNA synthesis (67).

Multiple mechanisms of resistance to mupirocin have been reported (4, 39, 49). High-level resistance is due to the acquisition of *mupA*, a homologue of the native isoleucyl-tRNA that is unaffected by mupirocin (64). While *mupA* is typically found on plasmids of varying sizes, it has also been reported to be integrated into the chromosome (10, 64, 125, 158, 159, 160). The presence of *mupA* in the chromosome has been shown to produce low- and high-level mupirocin resistant phenotypes (4-256μg/ml and >512μg/ml, respectively) (100, 125, 158). Additionally, low-level resistance to mupirocin has also been reported to be due to mutations in the native isoleucyl-tRNA gene (4).

1.5 Panton-Valentine Leukocidin Toxin

Panton-Valentine leukocidin (PVL) is a cytolytic exotoxin that is found in some strains of *S. aureus* (5, 7, 34, 130). The products, LukF-PV and LukS-PV, combine to form a hetero-hexameric pore in human leukocytes, allowing the influx of Ca^{2+} which ultimately leads to cell death (48, 69, 115). The *lukF*-PV and *lukS*-PV PVL genes can be detected through various PCR techniques including real-time PCR and traditional PCR, both of which have been proven effective in detecting the PVL toxin genes (84, 91).

Although not very common in methicillin-susceptible *S. aureus* (MSSA) (<10%) or HA-MRSA (0-1%) (84, 120), PVL is a common feature of CA-MRSA (up to >95%) (34, 36). PVL toxin is most commonly found in *S. aureus* strains that are involved in primary infections (84). The ability of the PVL positive CA-MRSA to cause primary infections may allow for CA-MRSA to disseminate in the community more readily than HA-MRSA which is more commonly associated with secondary infections (84).

The genes for PVL toxin are carried on at least 3 lysogenic phages, ΦPVL, ΦSLT and ΦSa2mw (34, 69) which insert into the *S. aureus* genome at a specific *att* site that is present in 6 of the *S. aureus* genomes sequenced (5, 69). The ΦSa2mw phage exists in several homologous forms that insert into the same *att* site however, only ΦSa2mw contain the PVL toxin genes (5). PVL containing phages are able to transfer PVL to different strains of *S. aureus* (69, 70). Once the phage has inserted into the *S. aureus* genome, both *lukF*-PV and *lukS*-PV are transcribed and translated (69).

1.6 Techniques used for Surveillance of MSSA and MRSA

Bacterial typing systems fall into one of two categories, phenotypic or genotypic (176). Phenotypic typing methods such as antimicrobial susceptibilities are typically

easy to perform and interpret however, they often lack the discriminatory power and reproducibility required for either outbreak or long term surveillance. Genotypic typing methods, such as PFGE, PCR and sequencing, are advantageous over phenotypic systems and offer a higher degree of discrimination and reproducibility (148, 176).

1.6.1 Antibiotic Resistance Surveillance

Antibiotic resistance surveillance has recently become a major part of infection epidemiology studies (126). The SENTRY Study Group in North America currently tracks antibiotic resistance for several important pathogens against a wide variety of antibiotics (51, 62, 63, 126). This provides valuable information to hospitals and research laboratories, giving insight into how the levels of antibiotic resistance in certain smaller areas compare to Canada and North America.

1.6.2 SCC*mec* Typing

SCC*mec* typing is a multiplex polymerase chain reaction (PCR) that discriminates between the different SCC*mec* types using primers specific to each SCC*mec* (112, 177). The first protocol described was useful for distinguishing between SCC*mec* types I-IV (112). However, since the discovery of SCC*mec* V and several subtypes of SCC*mec* type IV (68, 141), a new protocol has been designed to accommodate for some of these additional SCC*mec* types (177).

1.6.3 Pulse-Field Gel Electrophoresis

Pulse-field gel electrophoresis (PFGE) has the ability to resolve large segments of DNA on a gel (12). This is useful for the comparison of restricted whole genomes. Software programs are used to analyze and compare the PFGE patterns. A 1.0-2.0%

band tolerance is allowed due to run to run variations that occur (21, 101). Isolates can be compared using the criteria outlined by Tenover et al (1995) or by using cluster analysis. According to Tenover's rules (1995), two isolates are considered to be indistinguishable if the two organisms have the same number of bands of the same size. Organisms are considered to be closely related if the difference in the bands is due to a single genetic event which corresponds to a two to three band difference. Possibly related organisms have a four to six band difference due to 2 independent genetic events. Organisms are considered to be unrelated if there is a seven or more band differences due to three or more independent genetic events. Using these criteria a dendrogram can be created showing the relatedness of organisms. Cluster analysis using DICE coefficients is commonly used to show the relatedness of the organisms and is based on the number of band differences and similarities (21).

Band differences between strains are due to a mutation in the restriction site causing a site to be missed by the enzyme or due to a mutation that creates a new restriction site (154). Additionally large deletions and insertions can also be detected. If a strain has acquired a large deletion or insertion, the number of bands remains the same but the size of a particular band will be smaller or larger than the corresponding band in a closely related strain.

PFGE offers a high degree of discrimination between strains, allowing bacteria to be clustered into smaller groups (101). In addition PFGE is reproducible and accurate. The use of software to compare multiple samples has allowed PFGE typing to be a useful tool in outbreak and long term surveillance as multiple labs can compare samples to large

databases (12, 21). The databases can then be used to track organisms through large geographic areas (101).

PFGE has a disadvantage in long term surveillance of organisms due to the high frequency of mutations which leads to too many small groupings (12). Mutations may evolve too rapidly in large segments of DNA making it difficult to compare strains over a long period of time. The use of small bands for analysis in long term surveillance may help to resolve this problem as mutations occur less rapidly in small segments of DNA. However, the analysis of small bands by PFGE has proven to be difficult due to a low amount of resolution.

1.6.4 **Multilocus Sequence Typing**

Multilocus sequence typing (MLST) is a genotypic typing method that uses the sequences from seven essential genes to determine the relatedness between strains of an organism (87). In *S. aureus* carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acteyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*ypiL*) are sequenced and assigned an allele number (38). A sequence type (ST) number is assigned based on the mutations, which is determined by an internet-based sequence typing program available at www.mlst.net. The ST profile for many isolates allows for the creation of a dendrogram and the relatedness of the organisms can be compared.

MLST is an appropriate technique for creating a library for comparing large numbers of isolates (87). Sequence libraries provide the most accurate data about an isolate due to the unambiguous results of the sequence products. The results from MLST can be readily compared between laboratories accurately and quickly. MLST is an

appropriate tool for long-term surveillance or for the study of population genetics. It is however, inappropriate for outbreak surveillance due to the low discriminatory power of the technique (28).

While there are several other *S. aureus* typing schemes such as phage (124) and *spa* typing (140), most MRSA surveillance programs use a combination of one or more of the following: antimicrobial resistance patterns, PFGE, SCC*mec* typing and MLST (6, 20, 23, 26, 31, 36, 37, 38, 41, 44, 55, 71, 81, 87, 100, 101, 102, 104, 108, 112, 138, 142, 144, 152, 162, 171, 173).

1.7 Thesis Rational

In Canada, MRSA has only recently been observed to cause community-acquired infections. Few publications comparing antimicrobial resistance between CA-MRSA, MSSA and HA-MRSA strains in Canada are available. Strains were collected from east-central Saskatchewan in an attempt to gain a better understanding of the amount of antimicrobial resistance in *S. aureus* in this region. We speculate that the differences or similarities in antimicrobial resistance patterns among MSSA, HA-MRSA and CA-MRSA would provide more valuable information for the treatment of *S. aureus* infections in the region.

Despite increasing reports of CA-MRSA in hospitals on a global scale, there have been no reports of CA-MRSA circulating among Canadian hospitals. Surveillance of infections among Canadian ICUs is therefore important to determine if CA-MRSA was: 1) present, 2) circulating throughout the units, 3) becoming a competitive organism in the health-care setting and 4) replacing the HA-MRSA or if it will just add to the continually growing number of antibiotic resistant organisms. To accomplish this, surveillance of

infections in several Canadian ICUs will provide an overview of 1) the total amount of MRSA, 2) the total amount of CA-MRSA and 3) the amount of antibiotic resistance among MRSA strains. Phenotypic and molecular characterization of identified MRSA would provide a good comparison of any CA-MRSA found to other MRSA strains that are detected in ICUs.

PVL is commonly associated with CA-MRSA infections however, during routine investigation of CA-MRSA, several PVL negative CMRSA7 strains were discovered. Interestingly, these strains were indistinguishable by PFGE to CMRSA7 strains containing the PVL toxin genes. These strains appear to be unique as, to the best of my knowledge, there are no reports of this occurring elsewhere. To determine why these strains are indistinguishable, the region containing the PVL carrying phage will be molecularly characterized through several specific PCR reactions, as well as sequencing and Southern blotting. Additionally, any differences or similarities among the PVL positive and PVL negative strains in the types of infections may provide an argument to the necessity of PVL in minor CA-MRSA infections.

2 Methods

2.1 Strain Isolation and Storage

2.1.1 MRSA and MSSA from east-central Saskatchewan Case Control

MSSA and MRSA strains were collected between Sept 1, 2004 and Nov 30, 2005 from east-central Saskatchewan, Canada as part of an on-going case control. MSSA strains were defined as a laboratory confirmed MSSA infections from a patient residing in east-central Saskatchewan at the time of infection. MRSA cases were defined as a laboratory confirmed MRSA infection from a patient who resided in east-central Saskatchewan at the time of infection and who had not been hospitalized within the 48 hours prior to infection. Furthermore, the patient had not received dialysis or surgery, was not a resident of a long-term care facility, required no hospitalization within 12 months prior to infection and had no percutaneous device or in-dwelling catheter. MRSA were confirmed at the Saskatchewan Provincial Laboratory using standardized techniques.

2.1.2 MRSA from east-central Saskatchewan Surveillance

MRSA isolated from east-central Saskatchewan between January 1, 1999 and March 31, 2002 were previously characterized and described as part of Saskatchewan's routine diagnostic protocol (100).

2.1.3 MRSA from CNISP Hospital sites

MRSA strains were collected as part of an on-going surveillance study from 39 sentinel sites in 9 provinces across Canada as part of the Canadian Nosocomial Infection Surveillance Program (CNISP). MRSA cases were defined as a new MRSA infection

collected from any body site 24 hours after the patient was admitted to hospital. Epidemiologic data was collected and submitted by each sentinel site. A strain was determined to be MRSA using standardized techniques at each hospital. Once a case was identified the strain was sent to the National Microbiology Laboratory (Winnipeg, Manitoba) for molecular typing. Collection of MRSA began in 1995 and is on-going.

2.1.4 MRSA from Intensive Care Units

MRSA from intensive care units was collected as part of the ongoing Canadian Intensive Care Unit (CAN-ICU) Infection Study by Dr. G. Zhanel at the University of Manitoba (Winnipeg, MB). Specimens were collected from 19 hospitals in 8 different provinces from patients receiving care in an intensive care unit (ICU). Specimens were accepted from respiratory, urine, wound and blood sites. Strains were collected and identified in the participating hospitals and sent to Health Sciences Center (HSC) for phenotypic and genotypic typing. More than one isolate may have been collected from each patient however, only one isolate per infected site was accepted. For example, if a patient had respiratory and wound infections, one specimen from each infection may have been submitted. General patient demographic data was attached with each specimen.

Identification of MRSA was performed using standardized protocols at HSC. Antimicrobial resistance data for cefazolin, cefepime, ceftriaxone, ciprofloxacin, levofloxacin, gentamicin, meropenem and piperacillin/tazobactam was performed according to CLSI guidelines at HSC.

2.2 Storage of Strains

Samples were streaked for isolation on T-Soy agar (TSA) containing 5% sheep's blood and incubated overnight at 37°C. A single isolated colony was re-streaked for isolation for a second overnight growth. A loopful of growth was added to the liquid of Microbank™ (Pro-Lab Diagnostics, Richmond Hill, ON) storage beads. The tubes was inverted several times and allowed to sit for a minimum of 30 sec before the liquid was removed. The cultures were stored at -80°C.

2.3 Antimicrobial Susceptibilities

2.3.1 Antibiograms

Antimicrobial susceptibilities were determined using microbroth dilution. Microbroth panels were kindly provided by Dr. G. Zhanel, University of Manitoba. Media was prepared according to Clinical and Laboratory Standard Institute (CLSI) guidelines (formerly National Committee on Clinical Laboratory Standards) using cation adjusted Mueller Hinton broth. Oxacillin, clindamycin, vancomycin, erythromycin, trimethoprim-sulfamethoxazole, cefazolin, tetracycline, ciprofloxacin, rifampin, fusidic acid, linezolid, gentamicin and mupirocin antibiotics were prepared according to recommended guidelines and added to the media in doubling dilutions and placed in a 96 well plate. The range for each of the antimicrobials included on the panels is given in Table 1.

Table 1. Range of dilutions for antimicrobials used in antibiogram panel to determine MICs for MRSA and MSSA.

Antimicrobial	Range (μg/ml)
Oxacillin	0.06-128
Clindamycin	0.25-8
Vancomycin	0.25-8
Erythromycin	0.25-8
Trimethoprim-sulfamethoxazole	0.25-8
Cefazolin	0.25-32
Tetracycline	2-16
Ciprofloxacin	0.06-8
Rifampin	0.25-4
fusidic acid	0.06-8
Linezolid	0.25-32
Gentamicin	0.5-16
Mupirocin	0.12-128

Organisms were plated on TSA containing 5% sheep's blood and grown at 37°C for 16-18h. An isolated colony was streaked for isolation on TSA with 5% sheep's blood for a second overnight growth at 37°C. Two to three isolated colonies were suspended in 2ml sterile distilled water and adjusted to a 0.5 McFarland Equivalence Turbidity Standard (Remel Inc. Lenexa, KS USA) according to CLSI guidelines (107). 400μl of the cell suspension was placed in an inoculator plate (Dynex Technologies, Chantilly, VA USA) and 39.6ml of sterile distilled water was added and mixed using a sterile wooden stick. An MIC-2000 disposable hand inoculator (Dynex Technologies) was used to inoculate 95 of the 96 wells with 10μl of inoculum. To verify the purity of the inoculum each sample was streaked on TSA containing 5% sheep's blood. American Type Culture Collection (ATCC) (Manassas, VA USA) *S. aureus* strain 29213 and *Enterococcus* strain 29212 were used as control strains to verify the quality of the antimicrobials every time a susceptibility test was performed.

The MIC for the antibiotic was determined by observing the well of the lowest concentration that contained no growth (106). Strains were determined to be susceptible, intermediate or resistant based on CLSI guidelines (107). A breakpoint of </=4μg/ml was used for *S. aureus* sensitive to mupirocin. Low level and high level *S. aureus* mupirocin resistant breakpoints were >/=4 to <256μg/ml and >/=258μg/ml respectively, as previously described (100). Fusidic acid breakpoints for *S. aureus* were sensitive </=1μg/ml and resistant >/=2μg/ml (30).

2.3.2 **D-Testing**

D-testing is a double-disk diffusion antimicrobial susceptibility test that allows for detection of inducible clindamycin resistance in the presence of erythromycin. D-tests were performed according to CLSI guidelines (105) on strains that exhibited resistance to erythromycin while appearing to be susceptible to clindamycin (107). Strains were plated on TSA, containing 5% sheep's blood agar and incubated at 37°C overnight. A second overnight growth was performed to allow for the cells to have optimal expression of their antibiotic resistance genes. Cells were adjusted to a 0.5 McFarland standard in sterile distilled waster as described above in section 1.3.1. The cell suspension was plated on a cation-adjusted Mueller Hinton agar plate using a sterile cotton swab. A 15μg erythromycin disc (Med-Ox Diagnostics, Ottawa, ON) and a 2μg clindamycin disc (Med-Ox Diagnostics) were placed 15mm apart, edge to edge, on the plate. The plates were incubated at 37°C for 18h when the results were recorded as D positive, D negative, sensitive to both, resistant to both or hetero-resistant as previously described (147).

2.4 DNA Isolation

Phenol-chloroform extraction was adapted from previously described protocols (133).

Strains were streaked for isolation and grown overnight at 37°C on TSA containing 5% sheep's blood. A single isolated colony was transferred to 2ml of brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD USA) and grown overnight. One ml of broth was centrifuged at 13,000 rpm for 2 min in a 1.5ml tube and the supernatant was removed. The pellet was re-suspended in 600µl phosphate buffered saline (PBS) containing 50µg/ml lysostaphin (Sigma, Saint Louis, MO USA) and incubated at 37°C for 15-30 min or until clearing was observed. Following this 600µl of phenol-chloroform-isoamyl (25:24:1) (Sigma, St. Louis MO USA) was added and the tube was vortexed for 30 sec or until the solution became homogeneous. The solution was centrifuged at 14,000 rpm for 5 min, the top layer was added to a new tube and 600µl phenol-chloroform-isoamyl was added a second time. The solution was vortexed for 10 sec and centrifuged at 14,000 rpm for 3 min. The aqueous top layer was again removed and transferred to a new tube where 600µl of chloroform-isoamyl (24:1) was added. The tubes were inverted several times and then centrifuged at 14,000 rpm for 3 min. The top layer was transferred to a new tube and 500µl of isopropanol (Fisher Scientific, Fair Lawn, NJ USA) was added. The tubes were inverted approximately 25 times and centrifuged at 13,000 rpm for 5 min. The supernatant was removed and the pellet was washed in 500µl of 70% ethanol (Commercial Alcohols Inc., Brampton, ON) and spun at 13,000 rpm for 1 min. The ethanol was removed and the pellet was air-dried for 15 min. The pellet was re-suspended in 100µl water and the concentration of the

DNA was measured using a NanoDrop (NanoDrop Technologies, Wilmington, DE USA). DNA was diluted to 5ng/µl for all PCR reactions.

2.5 PCR

2.5.1 Conventional PCR

All PCR, with the exception of *ermA*, was performed in a total volume of 50µl with a master mix containing 0.5µM forward and reverse primers, 1.5mM $MgCl_2$, 200µM of each dATP, dCTP, dTTP and dGTP, 1X PCR buffer (diluted from 10X PCR buffer) (Applied Biosystems, Branchburg, NJ USA), AmpliTaq Gold (final concentration of 1.25U) (Applied Biosystems) and 12.5ng of template DNA. PCR for *ermA* was performed using the above conditions only using $MgCl_2$ at a concentration of 3.0mM (149).

The PCR cycling parameters included a denaturing step at 94°C for 5 min for one cycle followed by 30 cycles of 94°C for 30 sec, primer annealing temperature (see Tables 2-6) for 30 sec, and 72°C for 1 min. A final extension was performed at 72°C for 7 min.

All PCR products were visualized on a 1.5% agarose gel (Fisher Scientific, Fair Lawn, NJ USA) in 0.5X Tris-Borate-EDTA (TBE) buffer (Sigma, St. Louis, MO USA) run for 1h at 100volts/cm. The gel was stained in 50µg/ml ethidium bromide (Bio-Rad Laboratories, Hercules, CA USA) and visualized under UV light using an AlphaImager™ 2200 (Alpha Innotech Corporation, San Leandro, CA USA). Images were saved as .tif files using AplhaEaseFC™ Software (Aplha Innotech Corporation).

Control strains N02 0570, N02 0590, N02 0642, 00S 0907, 01S 0012 and 04S 0073 were all clinical MRSA strains that were available from the collection at the National Microbiology Laboratory. *S. aureus* RN1389, *S. pyogenes* AC1 (pAC1) and *S. aureus*

RN4220 (pE194) were kindly provided by Dr. Lai King Ng (National Microbiology Laboratory, Winnipeg, MB). Control strains for SCC*mec* typing were kindly provided by Christine Watt (Sunnybrook Health Science Center, Toronto, ON).

All primers used in the course of all experiments are described in the subsequent six tables. Primers were designed using Primer3 design program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for PVL experiments were designed using sequence from MW2 (GenBank accession number BA000033) and MRSA252 (GenBank accession number BX571856). Primers for promoter region of *mupA* were designed using the complete 3kb sequence of *mupA* (GenBank accession number DQ102365). PCR primers were synthesized by DNA Core at the National Microbiology Laboratory.

2.5.2 **Real-time PCR**

Real-time PCR was performed on CAN-ICU strains to detect the presence of PVL, *nuc* and *mecA* genes using primers previously described (91) (Table 7). Confirmation of PVL was performed using conventional PVL PCR as previously described. Reactions were performed on an ABI Prism 7900HT Sequence Detector System (Applied Biosystems, Foster City, CA USA). Cycling conditions were as follows, one cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 sec and 55°C for 1 min. Samples were held at 4°C once cycling was complete. Results were analyzed using ABI Prism SDS v2.1 software (Applied Biosystems). A result was considered to be positive when the CT value was less than 35 using a threshold of 0.2.

For real-time PCR reactions *Staphylococcus epidermidis* ATCC 14990 was used as a negative control for *mecA*, *nuc* and PVL, *S. aureus* ATCC 29213 was used as a

positive control for *nuc* and a negative control for *mecA* and PVL and *S. aureus* strains 00S 0907 and 04S 0073 were used as positive control strains for all genes. Taqman™ probes were purchased from Applied Biosystems.

Table 2. PCR primers used to detect mupirocin resistance genes in *S. aureus* strains.

Primer Name	Primer Sequence 5'→3'	Expect Product Size	Annealing Temperature (°C)	Gene	Reference	Positive Control Strain
MupA	TATATTATGCGATGGAAGGTTGG	458	58	*mupA*	(3)	N02 0570
MupB	AATAAAATCAGCTGGAAAGTGTTG		58	*mupA*	(3)	N02 0570
MupRF	CAAAGATTACGAAGTTAATCAACC	475	58	*mupA* promoter	This study	N02 0570
MupRR	GCAACTAAATCCTTGAATACT		58	*mupA* promoter	This study	N02 0570

Table 3. PCR primers used to detect erythromycin resistance genes in *S. aureus* strains.

Primer Name	Primer Sequence 5'→3'	Expect Product Size	Annealing Temperature (°C)	Gene	Reference	Positive Control Strain
ErmAfor	TCTAAAAAGCATGTAAAAGAA	645	50	*ermA*	(149)	*S. aureus* RN1389
ErmArev	CTTCGATAGTTTATTAATATTAGT			*ermA*	(149)	
ErmBfor	GAAAAGGTACTCAACCAAATA	639	50	*ermB*	(149)	*S. pyogenes* AC1 (pAC1)
ErmBrev	AGTAACGGTACTTAAATTGTTTAC			*ermB*	(149)	
ErmCfor	TCAAAACATAATATAGATAAA	642	48	*ermC*	(149)	*S. aureus* RN4220 (pE194)
ErmCrev	GCTAATATTGTTTAAATCGTCAAT			*ermC*	(149)	
MsrAfor	GCAAATGGTGTAGGTAAGACAACT	399	50	*msrA*	(149)	N02 0590
MsrArev	ATCATGTGATGTAAACAAAAT			*msrA*	(149)	

Table 4. PCR primers used to detect PVL toxin genes and integration of phage into the *S. aureus* genome.

Primer Name	Primer Sequence 5'→3'	Expect Product Size	Annealing Temperature (°C)	Gene	Reference	Positive Control Strain	Notes
LukPVLF	ATCATTAGGTAAAATGTCTGGACATGATCCA[1]	433	55	*lukF*-PV/*lukS*-PV	(84)	N02 0642	
LukPVLR	GCATCAAATGTATTGGATAGCAAAAGC						
LukSF	GCTGCAACATTGTCGTTAGG	658	55	*lukS*-PV	This study	N02 0642	
LukSR	GGTAATTCATTGTCTGGCAC						
LukFF	CAAATCCAAAAGACACTATTAG	409	55	*lukF*-PV	This study	N02 0642	
LukFR	ATTTAAGTTGCTTTGTCTTGAG						
Intfor	TCCATCGTTTGAATTGCTTG	244	55	*att* site	This study	00S 0907	
Intrev	GCAAATGGGAGCCTTTTCTT						
MW2_3'	TTAGGGTAACCACGTCTTAATTGAC	301	55	ΦSa2mw phage into CMRSA7 host	This study	00S 0907	Used with Intfor
MW2_5'	GCTTGAGAACACTGGCGTTA	173	55	ΦSa2mw phage into CMRSA7 host	This study	00S 0907	Used with Intrev
252phage_3'	GTTGGTCCCACAGGAGACAT	442	55	MRSA 252 phage into CMRSA7 host	This study	01S 0012	Used with Intfor

[1] A: adenine, C: cytosine, G: guanine, T: thymidine

Table 5. PCR primers used to determine SCC*mec* type in MRSA.

Primer Name	Primer Sequence 5'→3'	Expected Product Size	Annealing Temperature (°C)	Target	Reference
Type I-F	GCTTTAAAGAGTGTCGTTACAGG	613	55	SCC*mec* I	(177)
Type I-R	GTTCTCTCATAGTATGACGTCC				
Type II-F	CGTTGAAGATGATGAAGCG	398	55	SCC*mec* II	(177)
Type II-R	CGAAATCAATGGTTAATGGACC				
Type III-F	CCATATTGTGTACGATGCG	280	55	SCC*mec* III	(177)
Type III-R	CCTTAGTTGTCGTAACAGATCG				
Type IVa-F	GCCTTATTCGAAGAAACCG	776	55	SCC*mec* IVa	(177)
Type IVa-R	CTACTCTTCTGAAAAGCGTCG				
Type IVb-F	TCTGGAATTACTTCAGCTGC	493	55	SCC*mec* IVb	(177)
Type IVb-R	AAACAATATTGCTCTCCCTC				
Type IVc-F	ACAATATTTGTATTATCGGAGAGC	200	55	SCC*mec* IVc	(177)
Type IVc-R	TTGGTATGAGGTATTGCTGG				
Type IVd-F5	CTCAAAATACGGACCCCAATACA	881	55	SCC*mec* IVd	(177)
Type IVd-R6	TGCTCCAGTAATTGCTAAAG				
Type V-F	GAACATTGTTACTTAAATGAGCG	325	55	SCC*mec* V	(177)
Type V-R	TGAAAGTTGTACCCTTGACACC				
MecA147-F	GTGAAGATATACCAAGTGATT	147	55	*mecA*	(177)
MecA147-R	ATGCGCTATAGATTGAAAGGAT				

Table 6. PCR and sequencing primers used for *S. aureus* MLST.

Primer Name	Primer Sequence 5'→3'	Expected Product Size	Annealing Temperature (°C)	Gene	Reference
arc-Up	TTGATTCACCAGCGCGTATTGTC	456	55	*arcC*	(38)
arcC-Dn	AGGTATCTGCTTCAATCAGCG				
aroE-Up	ATCGGAAATCCTATTTCACATTC	456	55	*aroE*	(38)
aroE-Dn	GGTGTTGTATTAATAACGATATC				
glpF-Up	CTAGGAACTGCAATCTTAATCC	465	55	*glpF*	(38)
glpF-Dn	TGGTAAAATCGCATGTCCAATTC				
gmk-Up	ATCGTTTTATCGGGACCATC	429	55	*gmk*	(38)
gmk-Dn	TCATTAACTACAACGTAATCGTA				
pta-Up	GTTAAAATCGTATTACCTGAAGG	474	55	*pta*	(38)
pta-Dn	GACCCTTTTGTTGAAAAGCTTAA				
tpi-Up	TCGTTCATTCTGAACGTCGTGAA	402	55	*tpi*	(38)
tpi-Dn	TTTGCACCTTCTAACAATTGTAC				
yqiL-Up	CAGCATACAGGACACCTATTGGC	516	55	*ypiL*	(38)
yqiL-Dn	CGTTGAGGAATCGATACTGGAAC				

Table 7. PCR primers and probes used in triplex real-time PCR to detect PVL, *nuc* and *mecA* genes in MRSA from CAN-ICU.

Primer Name	Primer Sequence 5'→3'	Gene	5' Reporter Dye	Reference
mecA For	GGCAATATTACCGCACCTCA	*mecA*		(91)
mecA Rev	GTCTGCCSCTTTCTCCTTGT	*mecA*		(91)
mecA Probe	AGATCTTATGCAAACTTAATTGGCAAATCC		FAM[1]	(91)
nuc For	CAAAGCATCAAAAAGGTGTAGAGA	*nuc*		(91)
nuc Rev	TTCAATTTTCTTTGCATTTTCTACCA	*nuc*		(91)
nuc probe	TTTTCGTAAATGCACTTGCTTCAGGACCA		VIC[1]	(91)
PVL For	ACACACTATGGCAATAGTTATTT	*lukF-PV/lukS-PV*		(91)
PVL Rev	AAAGCAATGCAATTGATGTA	*lukF-PV/lukS-PV*		(91)
PVL Probe	ATTTGTAAACAGAAATTACACAGTTAAATATGA		TET[1]	(91)

[1]Repoter dye activated with 3'TAMRA

2.5.3 **Sequencing**

PCR samples were prepared for sequencing by applying a total of 40μl of PCR product to a Microcon™ YM-100 Centrifugal Filter Unit (Millipore, Etobicoke, ON). The samples were centrifuged in the columns for 10 min at 2300 rpm. The columns were inverted into a new tube and 50μl of sterile distilled water was added to each sample. The samples were centrifuged for 5 min at 2600 rpm and the DNA concentration was measured. Samples were adjusted to a DNA concentration of 50μg/ml and primers were adjusted to a 1μM concentration. Primers used for sequencing were the same primers used in PCR reactions (Tables 2-7). Sequencing was kindly provided by DNA Core Facilities at the National Microbiology Laboratory.

Sequences were analyzed using Seqman™II expert sequence analysis software (DNASTAR, Madison, WI USA) and compared using a Blast search (2). *mupA* sequences were compared to the *mupA* gene sequence (GenBank accession number DQ102365) obtained from www.ncbi.nlm.nih.gov/BLAST. *lukF*-PV and *lukS*-PV sequences and products of MW2intfor/SaW2int1 primers and MW2intrev/SaW2int2 were compared to the MW2 genome (GenBank accession number BA000033), products of MW2intfor/MW2intrev primers were compared to the N315 genome (GenBank accession number BA000018), and products of MW2intfor/252SaW3 were compared to the MRSA252 genome (GenBank accession number BX571856) obtained from www.ncbi.nlm.nih.gov/BLAST.

2.5.3.1 Multi-locus Sequence Typing

Multi locus sequencing typing (MLST) was performed using primers for carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) (38). Primers (Table 7) were used to PCR amplify the target genes using the conditions described in section 1.5. Determination of the sequence type (ST) was performed using the data analysis program supplied at www.mlst.net (Imperial College, London England).

2.6 Pulse-Field Gel Electrophoresis

Pulse-field gel electrophoresis of MRSA and MSSA was adapted from the Canadian Standardized Guidelines (101).

2.6.1 Preparation of PFGE Plugs

Cultures were grown overnight on TSA containing 5% sheep's blood at 37°C for 16-18 h. An isolated colony was transferred to 2ml of BHI broth and grown for 16-18 h at 37°C. The cells were then isolated by centrifuging 150μL of sample of overnight culture at 14, 000 rpm in a microcentrifuge for 1 min. The supernatant was removed and cells were resuspended in 150μl of cell suspension buffer (CSB) made up of 10mM Tris-HCl, pH 7.2 (Invitrogen, Carlsbad, CA USA), 20mM NaCl (Sigma), 50mM EDTA (Sigma) with 2μl of 1mg/ml lysostaphin. 150μl of melted 1.0% Bio-Rad pulsed-field grade agarose was added to the suspended cells, gently mixed, immediately dispensed into PFGE plug molds (Bio-Rad) and allowed to solidify for 15 min at room temperature. Cells were lysed by placing the hardened plugs into 500μl of cell lysis buffer (CLB)

(10mM Tris-HCl pH 7.2, 50mM NaCl, 50mM EDTA, 0.2% deoxycholate (Sigma), 0.5% N-lauryl-sarcosine (Sigma) and incubated for 1 h at 37°C. The lysis reaction was stopped by removing the CLB and adding 500μl of a proteinase K (Roche)/proteinase K buffer (PKB) (final concentration 50mg/ml proteinase K in 250mM EDTA pH 9.0, 1% N-lauryl-sarcosine) and incubated at 55°C for 1 h. Plugs were washed using MRSA wash buffer (10mM Tris-HCl pH 7.6, 0.1mM EDTA) two times immediately after removing proteinaseK/PKB followed by five more washes at intervals of 5, 10, 15, 20, and 20 min. The plugs could then be immediately digested or stored at 4°C for digestion at a later time.

2.6.2 **Preparation of Standards**

Standards were prepared from *Salmonella* serotype Braenderup H9812 after overnight growth on TSA containing 5% sheep's blood. A cell suspension was prepared in 2ml CSB (100mM Tris, 100mM EDTA, pH 8.0) and adjusted to 0.48-0.52 using Dade MicroScan Turbidity Meter (Dade Behring Inc., West Sacramento, CA USA). 190μl of cell suspension was transferred to a 1.5ml tube and 200μl of low melting point agarose (Bio-Rad) containing 1% sodium dodecyl sulfate (SDS) (Sigma) was added and mixed. The mixture was then dispensed into the PFGE plug molds and allowed to solidify. The hardened plugs were then transferred into 5ml of CLB (50mM Tris, 50mM EDTA, 1% N-lauryl-sarcosine) with 40μl of 20mg/ml proteinase K and placed at 55°C for 2 h. The lysis solution was removed and the plugs were washed with 25ml of 50°C water, the water was removed and plugs were washed two times with 5-10ml 50°C sterile distilled water at 50°C for 10-15 min. Plugs were then washed four times with 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).

2.6.3 Digestion of PFGE Plugs

Approximately 1/6th of a plug containing sample was equilibrated with 150μl of restriction enzyme buffer (REB) A (Roche) at 25°C for 10 min. REB A was removed and 100μL of REB A containing 40U of *Sma*I (Roche)/sample was added to the plugs and placed at 25°C overnight for digestion.

Restriction digest of standards was carried out using 1/8th of a slice. Standards were equilibriated in 150μl of REB H (Roche) at 37°C for 10 min. The REB H was removed and 100μl of REB H/40U *Xba*I (Roche) was added and placed at 37°C overnight for digestion.

2.6.4 Preparation and Loading of Gel

Gels were prepared using 100ml of 1% Bio-Rad pulsed-field grade agarose in 0.5X TBE and melted in a microwave for 1-2 min. The standards were loaded solid onto a 0.75mm comb corresponding to lanes 1, 5, 10 and 15. The samples were placed on the remaining spaces of the comb which was then placed into a casting tray and 100ml of BioRad pulse-field grade agarose was poured and allowed to solidify for 30-45 min. The gel was placed into an electrophoresis chamber with 2.0 L of 0.5X TBE. The run conditions on the Chef DR III (Bio-Rad) had an initial switch time of 5.3 sec, a final switch time of 34.9 sec, at 14°C with a voltage of 6.0V/cm at an included angle of 120° for 20 h. Gels were strained in ethidium bromide for 1 h followed by a 2-4 h destaining in distilled water. The gels were visualized under a UV light using an AlphaImager™ 2200 (Alpha Innotech Corporation, San Leandro, CA USA). Images were saved as .tif files using AplhaEaseFC™ Software (Aplha Innotech Corporation).

2.6.5 Gel Analysis

Gels were analyzed using BioNumerics version 3.0 (Applied-Maths, Saint-Martens-Latem, Belgium). Gels were normalized using the molecular weight standards. Similarities of banding patterns were determined based on the DICE coefficient. Cluster analysis was performed using the unweighted pair-group method based on arithmetic (UPGMA) with 1% optimization and a 1% band tolerance (21, 101).

MRSA strains were compared to the Canadian National database provided by the National Microbiology Laboratory. MSSA strains were compared to each other and the relatedness of the strains was determined using rules outlined by Tenover et al (1995).

2.7 Plasmid Isolation

Plasmid isolation was performed using the Qiagen® Plasmid Purification Midi Kit with a few modifications to the protocol as described below.

All strains for plasmid purification were grown overnight in 100ml BHI broth containing 16μg/ml mupirocin as mupirocin resistant strains were being examined (AppliChem Chemical Synthesis Service, Ottoweg, Darmstadt Germany). Cells were harvested by centrifugation in 50ml conical tubes at 6000g and resuspended in 4ml P1 resuspension buffer with RNase A. To this suspension, 1ml of 50μg/ml of lysostaphin was added and incubated at 37°C for 10 min, to aid in the lysis of *S. aureus* which was poor with buffer P2. Four ml of Buffer P2 was added, gently mixed, and allowed to sit at room temperature for 5 min. Subsequently, 4ml of buffer P3 was added, the solution was gently mixed, and placed on ice for 15 min. The entire mixture was gently mixed again before centrifuging for 30 min at 20,000 g. The supernatant was transferred to a new tube and centrifuged at 20,000 g for an additional 15 min.

A Qiagen-tip 100 was equilibrated with 4ml buffer QBT. The supernatant resulting from the second spin was applied to the tip after equilibration was complete and allowed to flow through. The tips were washed 2 times with 4ml buffer QC. Finally 5ml of buffer QF was added to the tip and the fraction was collected in a 12ml tube.

Plasmid DNA was precipitated using 3.5ml room temperature isopropanol which was then centrifuged at 20,000 g for 30 min. The supernatant was removed and pellets were washed in 2ml of 70% ethanol by centrifuging at 20,000 g for 10 min. Ethanol was removed and the pellets were air dried for 5-10 min and resuspended in 70µl of sterile distilled water.

Five µl of product was run on a 0.8% agarose gel in 0.5X TBE, stained in ethidium bromide and visualized under UV light using an AlphaImager™.

2.8 **Plasmid Restrictions**

*Eco*RI and *Cla*I plasmid restrictions were carried out in a 30µl reaction volume containing 1X NEBuffer 4 (New England Biolabs, Ipswich, MA USA), 40U of either *Eco*RI or *Cla*I (New England Biolabs), 500ng DNA. Restricted plasmids were run on a 0.8% Seakem® Gold agarose (Rutherford, NJ USA) gel in 0.5X TBE at 80V/cm for 5 h, stained in ethidium bromide and visualized under UV light using an AlphaImager™.

2.9 Southern Blotting

2.9.1 Gel Preparation

2.9.1.1 PFGE Gel Preparation

PFGE was performed using the protocol described in section 2.6 with the exception that low weight lambda marker (New England Biolabs) was used in place of H9812 *Salmonella* serotype Braenderup standard.

To aid the transfer of the DNA from the gel to the nylon membrane, depurination, denaturation and neutralization was performed. The PFGE gel was put in approximately 100ml of 0.25M HCl (Sigma) for 30 min, with shaking for depurination. The gel was moved to a denaturation solution made up of 1.5M NaCl and 0.5M NaOH (Fisher) again with shaking for 30 min. Finally the gel was placed into a neutralizing solution containing 1.0M Tris (Invitrogen) and 1.5M NaCl for 30 min with shaking.

2.9.1.2 Preparation of 0.8% Gel

Plasmids were isolated (section 2.7), digested and run with a 1kb standard (Invitrogen) on a 0.8% SeaKem® Gold agarose gel (section 2.8). The gel was depurinated, denaturated and neutralized in the same manor as described for the PFGE gel in section 2.9.1.1.

2.9.2 Gel Blotting

Gel blotting was performed as previously outlined (133). A TurboBlotter™ (Schleicher and Schuell Bioscience, Keene, NH USA) was used to blot gels. Twenty pieces of GB004 Gel Blot Paper (Schleicher and Schuell Biosciences) were cut to the approximate size of the gel and placed in the stack tray followed by 4 pieces of GB002

(Schleicher and Schuell) and one GB002 which was pre-wet using the 0.4M NaOH transfer buffer. The Hybond™-XL (Amersham Biosciences UK Ltd) nylon membrane was pre-wet in distilled water and placed on the stack. After depurination, denaturation and neutralization were complete the gel was placed directly on top of the nylon membrane along with 3 pieces of GB002 wet with transfer buffer. A buffer wick (GB002) pre-wet with transfer buffer was placed over the stack along with a wick cover. Approximately 200ml of 0.4M NaOH transfer buffer was added to the TurboBlotter™ tray, the wick was placed into the buffer tray and the blot was left for 18 h. To confirm that DNA transfer had occurred, the gel was stained again in ethidium bromide for 1 h and visualized under UV light using the AlphaImager™.

2.9.3 **Probing of Nylon Membrane**

The ECL™ protocol for Southern blot probing and signal detection was performed as follows.

2.9.3.1 **Prehybridization of Nylon Membrane**

Solid NaCl was added to the hybridization buffer (provided in ECL™ kit) to a final concentration of 0.5M and the blocking reagent (provided in ECL™ kit) was added to a final concentration of 5% (weight per volume). The solution was mixed at room temperature for 2-4 h. After blotting was complete, the nylon membrane was placed in a hybridization tube with the prepared hybridization buffer and placed in a hybridization oven (VWR International, Mississauga, ON) at 42°C with constant rotation for 1 h. While the membrane was pre-hybridizing, the probes were labeled.

2.9.3.2 **Probe Labeling**

The probes were prepared using PVL PCR products from N02 0642 generated with previously described primers (84) and using the *mupA* PCR products from strain N02 0570 using previously described primers (3). The PCR products (probes) were purified using Microcon™ YM-100 Centrifugal Filter Unit (Millipore, Etobicoke, ON) and diluted to 10ng/µl. For the probing of PVL, 300ng (in 30µl) of the purified product and 200ng (in 20µl) of *Hin*dIII lambda DNA (provided in ECL™ kit) were denatured in a boiling water bath for 5 min followed by 5 min on ice. *Hin*dIII-digested lambda DNA was used as a probe for the lambda molecular marker also run on the gel as a size marker. For probing of *mupA*, 300ng (in 30µl) of purified product and 200ng (in 20 µl) of 1kb standard (Invitrogen) was denatured for 5 min and put on ice for 5 min. To each probe an equivalent volume of DNA labeling reagent (provided in ECL™ kit) was added and mixed gently. An equivalent volume of gluteraldehyde (provided in ECL™ kit) was added and the mixture was mixed gently and briefly spun down. The labeled DNA was placed at 37°C for approximately 30 min. Once the incubation period was complete the labeled probes were added to the hybridization buffer. The membrane was probed at 42°C for approximately 16-18 h.

2.9.4 **Membrane Wash**

To wash the membrane the hybridization buffer was removed and replaced with an excess of pre-warmed primary wash buffer containing 0.4% (SDS) and 0.5X SSC (diluted from 20X SSC: 0.3M sodium citrate, 3M sodium chloride) (Sigma), for 10 min at 55°C. The wash buffer was removed and a second wash with primary wash buffer was performed for 10 min at 55°C. The buffer was removed and the membrane was placed

into a wash tray where an excess of secondary wash buffer (2X SSC) was added. The membrane was washed for 5 min two times at room temperature.

2.9.5 **Signal Detection**

To detect the probe signal, approximately 1ml of detection reagent 1 was mixed with 1ml of detection reagent 2 on a piece of plastic wrap. The membrane was placed DNA side down in the mixture for 1 min. The excess detection reagent was removed and the membrane was wrapped in a fresh piece of plastic wrap. The membrane was then placed in a film cassette and exposed to Hyperfilm™ (Amersham Biosciences) for 10 min. The film was developed using a Feline™ 14 Automated Quick-Cleaning X-ray film processor (Fischer Industries Inc., Geneva, IL USA).

2.10 **Statistical Analysis**

Chi-squared and Fisher's Exact tests were performed using EpiInfo™ (Centers for Disease Control and Prevention, Atlanta, GA).

3 Results

3.1 *S. aureus* from East-Central Saskatchewan

3.1.1 MSSA from East-Central Saskatchewan Case Control

One hundred and sixteen MSSA samples were collected between Sept. 2004 and Nov. 2005 as part of an on-going case control. MICs were determined using antibiogram panels described in section 2.3.1. All strains were susceptible to oxacillin (</=2μg/ml), vancomycin (</=4 μg/ml), trimethoprim-sulfamethoxazole (</=2 μg/ml), cefazolin (</=8 μg/ml), tetracycline (</=4 μg/ml), rifampin (</=1 μg/ml) and linezolid (</=4 μg/ml). The greatest resistance was to erythromycin (>/=8 μg/ml) and mupirocin (>/=4 μg/ml) with 27 and 22% of strains exhibiting resistance, respectively (Table 8) (100, 107). Resistance to fusidic acid (>/=2 μg/ml), gentamicin (>/=16 μg/ml), ciprofloxacin (>/=4 μg/ml) and clindamycin (>/=4 μg/ml) was observed in 15, 12, 9 and 4% of strains, respectively (30, 106). A total of 52% of strains were sensitive to all antimicrobials tested and an additional 25% were resistant to only one antimicrobial tested. Approximately 10% of strains were resistant to 3 or more classes of antimicrobials. To determine the relatedness of MSSA, PFGE was performed on all strains. A total of 62 unique PFGE patterns were found with several strains falling into one of 4 clusters comprised of 10 or more indistinguishable or related strains (Figure 2). To further characterize the strains, DNA was extracted from all 116 strains and tested for the presence of PVL. Two strains were PVL positive by PCR.

Figure 2. PFGE dendrogram of all unique MSSA PFGE types isolated in east-central Saskatchewan. Numbers indicate number of times a pattern was observed. Boxed areas represent major clusters (>/=80% similarity). Dendrogram was created using BioNumerics 3.0.

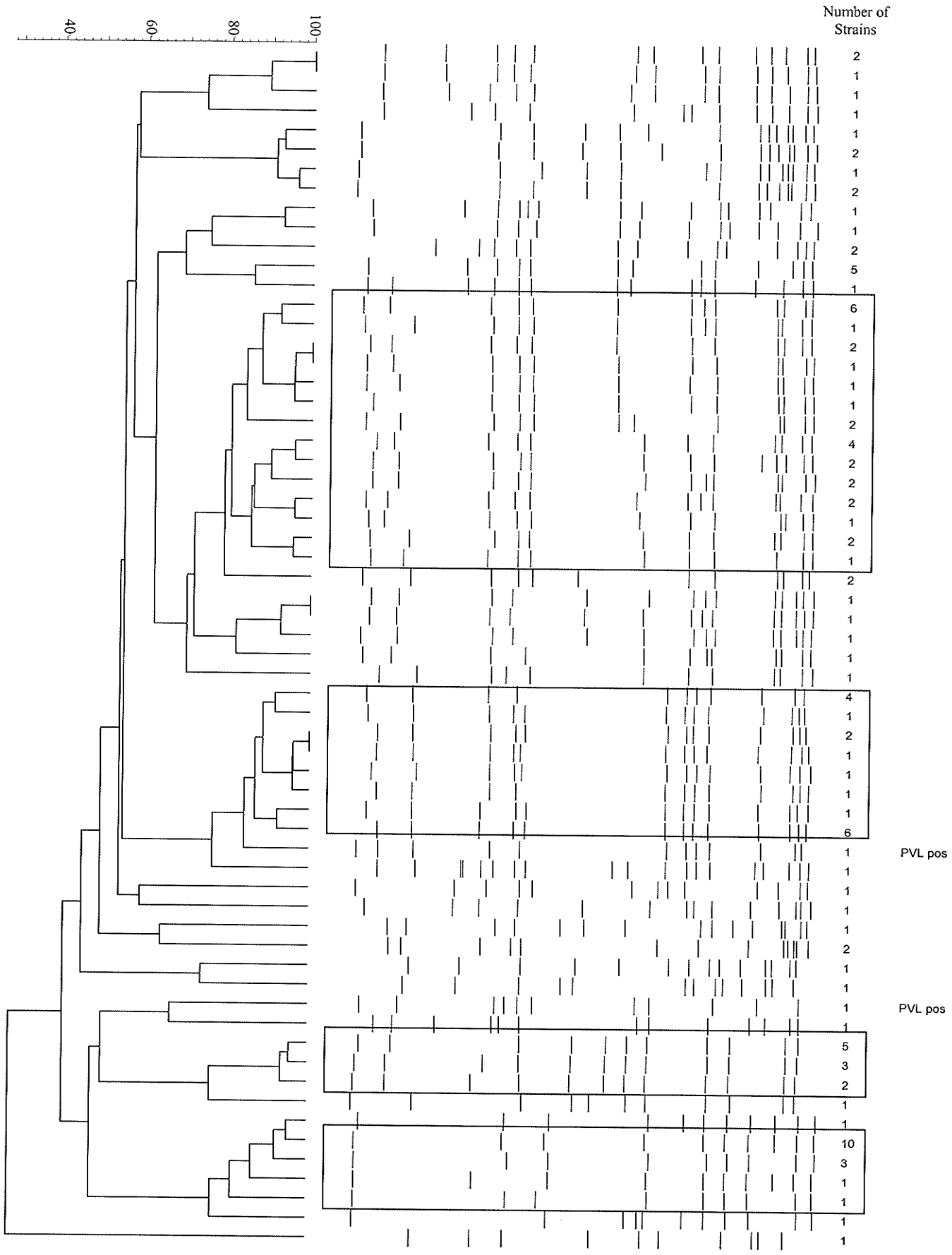

Percent Similarity
40
60
80
100
Number of Strains
2
1
1
1
1
2
1
2
1
1
2
5
1
6
1
2
1
1
1
2
4
2
2
2
1
2
1
2
1
1
1
1
4
1
2
1
1
1
1
6
1
PVL pos
1
1
1
1
2
1
1
1
PVL pos
1
5
3
2
1
1
10
3
1
1
1
1

Table 8. Number of MSSA resistant to various antimicrobials.

Antimicrobial	Number of Resistant Strains (%)		
	MSSA n=116	CMRSA2 n=11	CMRSA7 n=14
Oxacillin	0 (0)	11 (100)	14 (100)
Clindamycin	5 (4)	2 (18)	3 (21)
Vancomycin	0 (0)	0 (0)	0 (0)
Erythromycin	31 (27)	9 (82)	4 (29)
Trimethoprim-sulfamethoxazole	0 (0)	0 (0)	0 (0)
Cefazolin	0 (0)	1 (9)	0 (0)
Tetracycline	0 (0)	0 (0)	0 (0)
Ciprofloxacin	10 (9)	7 (64)	0 (0)
Rifampin	0 (0)	0 (0)	0 (0)
Fusidic acid	17 (15)	10 (91)	0 (0)
Gentamicin	14 (12)	4 (36)	0 (0)
Linezolid	0 (0)	0 (0)	0 (0)
Mupirocin	26 (22)	3 (27)	10 (71)

3.1.2 **MRSA from East-Central Saskatchewan Case Control**

A total of 25 MRSA were identified from east-central Saskatchewan. MIC data was collected and PFGE (Figure 3) and PVL testing was performed on all strains. All 25 strains were sensitive to vancomycin, trimethoprim-sulfamethoxazole, tetracycline, rifampin and linezolid (Table 8). PFGE revealed 10 different patterns that fell in one of two clonal groups. The first group, comprised of 11 strains, had 9 strains that were related to CMRSA2 and 2 strains that clustered with the epidemic clonal group. The remaining 14 strains were related to the CMRSA7 epidemic cluster, of which 11 were indistinguishable from CMRSA7 type 0142. A higher amount of antimicrobial resistance was observed in CMRSA2 than in CMRSA7 strains with 10 of the 11 strains being resistant to 3 or more antimicrobials and 3 of the 14 strains being resistant to 3 or more antimicrobials, respectively (Figure 4). CMRSA2 strains had a high amount of resistance

to fusidic acid (91%), erythromycin (82%), and ciprofloxacin (64%). However, resistance to gentamicin (36%), high-level mupirocin (27%), clindamycin (18%) and cefazolin (9%) was also observed. CMRSA7 strains had a higher level of mupirocin resistance (71%) than CMRSA2 while erythromycin resistance was lower (29%) and clindamycin resistance was similar (21%). CMRSA7 remained sensitive to cefazolin, ciprofloxacin, fusidic acid and gentamicin. Most strains clustering with CMRSA7 were PVL positive however, two strains with a unique PFGE pattern were PVL negative. None of the strains related to CMRSA2 were PVL positive.

None of the MSSA strains had indistinguishable PFGE patterns from any of the MRSA strains. There were however, two unique MSSA strains that had similar patterns to strains in the CMRSA2 cluster, each differing by only one band of approximately 40-60 kb from the respective MRSA patterns.

Figure 3. PFGE patterns and dendrogram of MRSA from east-central Saskatchewan with the Canadian PFGE designation, Canadian epidemic type and the number of strains of each type observed.

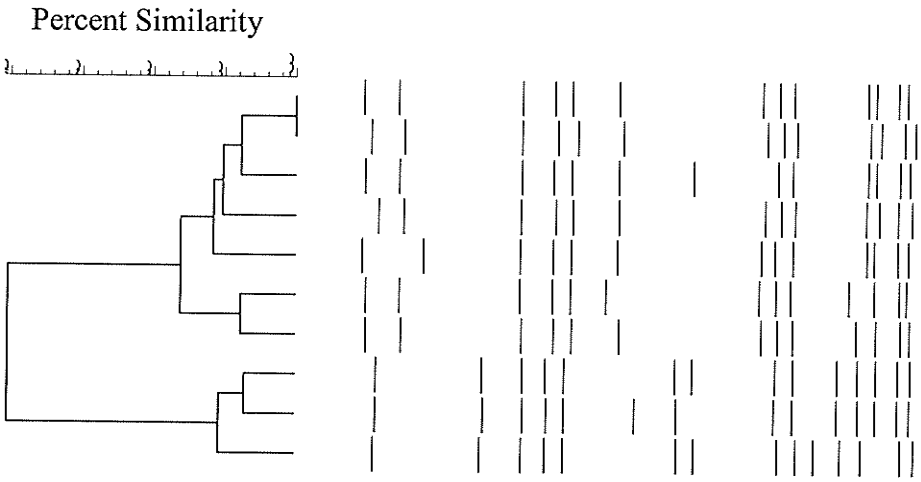
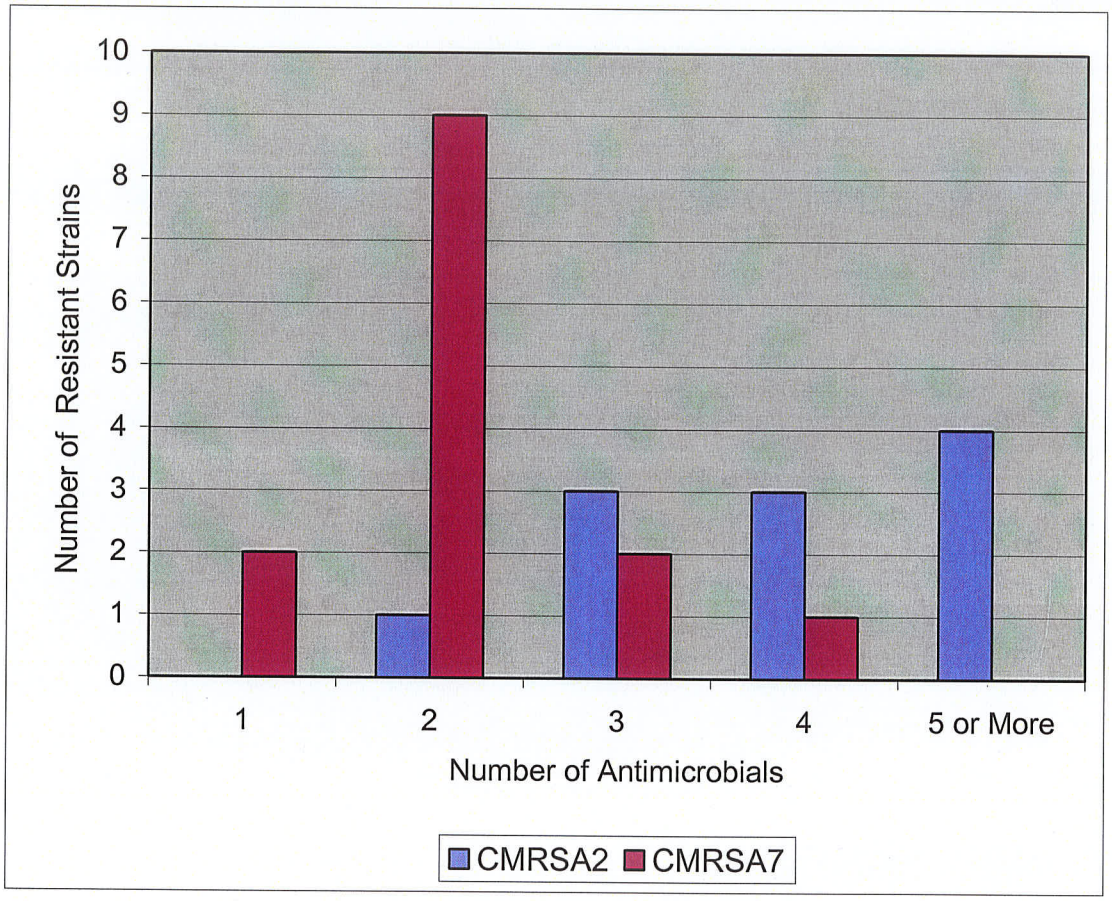

Percent Similarity
PFGE Designation
Epidemic Type
No. of Strains
PVL
0417 CMRSA2 1 neg
0695 CMRSA2 2 neg
0696 CMRSA2 1 neg
0914 CMRSA2 4 neg
unique CMRSA2 1 neg
unique CMRSA2 1 neg
unique CMRSA2 1 neg
0142 CMRSA7 11 pos
0418 CMRSA7 1 neg
unique CMRSA7 2 pos

Figure 4. Distribution of CMRSA2 and CMRSA7 from east-central Saskatchewan resistant to varying number of antimicrobials.


10
9
8
7
6
5
4
3
2
1
0
Number of Resistant Strains
1
2
3
4
5 or More
Number of Antimicrobials
CMRSA2
CMRSA7

3.1.3 **Mupirocin Resistance**

Mupirocin resistance in MRSA from east-central Saskatchewan was seen in 115 of the 209 case control and surveillance strains tested. All mupirocin-resistant MRSA had an MIC >128μg/ml and contained *mupA*. In addition one CMRSA2 that had a mupirocin MIC of 0.25μg/ml and one CMRSA7 with an MIC of </=0.12μg/ml also contained *mupA*. Neither strains produced a product when PCR amplification was attempted using MupRF and MupRR primers specific to promoter region of *mupA*; a positive result was observed in N02 0570, a *mupA* positive strain suggesting that there was a deletion in the promoter region of the strains with a sensitive MIC.

CMRSA2 appeared to have a large plasmid (>50kb) and CMRSA7 appeared to have 2 large plasmids (~40-50kb and >50kb) (Figure 5A). Plasmids from CMRSA2 and CMRSA7 were restricted with *Cla*I and *Eco*RI and were determined to be different based on the banding patterns (Figure 5A). *mupA* was determined to be present on the large plasmid in CMRSA2 by Southern blot analysis and in both of the large plasmids in CMRSA7 (Figure 5B). When plasmids were restricted with *Eco*R1, which does not cut within *mupA*, *mupA* hybridized with a ~4kb plasmid fragment in both CMRSA2 and CMRSA7. *mupA* was present on a 2kb and a 2.5kb fragment in CMRSA2 and on a 2kb and an 8kb fragment in CMRSA7 when restricted with *Cla*I which cuts once within *mupA* at position 1527.

A total of 26 MSSA were mupirocin resistant. Of these, 10 were low-level resistant. All 16 high-level resistant strains contained *mupA*. None of the low-level resistant strains contained *mupA*.

Figure 5. (A) 0.8%TBE gel. Lane 2 unrestricted plasmid isolated from N02 0258 (CMRSA2); Lane 3 unrestricted plasmid isolated from N02 0274 (CMRSA7); Lane 5 N02 0258 plasmid digested with *Cla*I; Lane 6 N02 0274 plasmid digested with *Cla*I; Lane 8 N02 0258 plasmid digested with *Eco*RI; Lane 9 N02 0274 plasmid digested with *Eco*RI. Lane 1, 4, 7 and 10: 1kb standard. (B) Southern blot of (A) using PCR probe made with primers described by Anthony et al (1999), Lanes correspond to lanes described for (A).

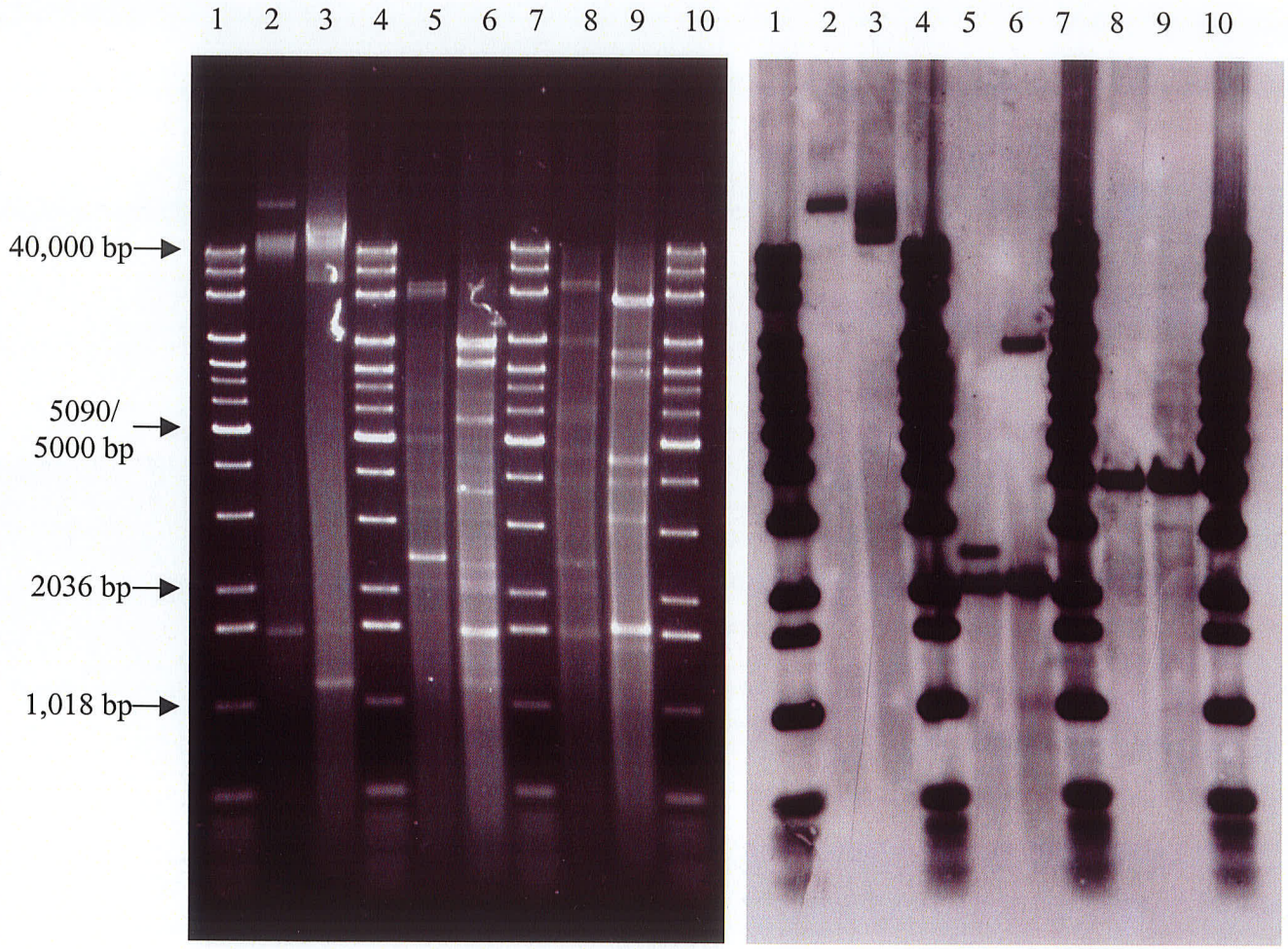
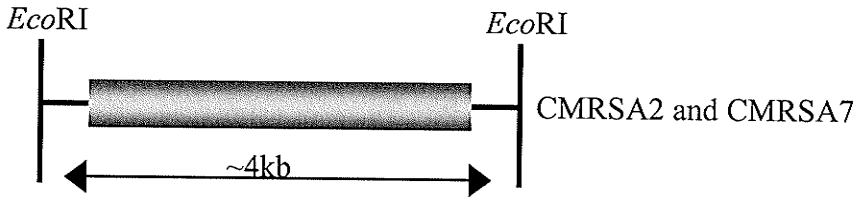
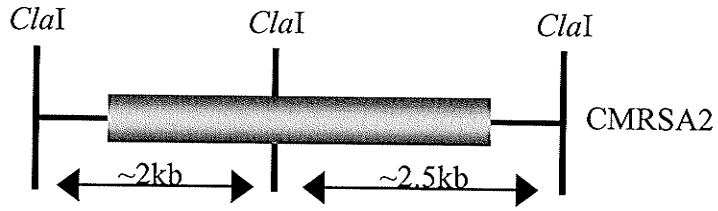

1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
40,000 bp
5090/
5000 bp
2036 bp
1,018 bp
A
B

Figure 6. Schematic diagram of *mupA* gene on: (A) 4kb *Eco*RI fragment which was the same in both CMRSA2 and CMRSA7, (B) on the 2 and 2.5kb *Cla*I fragment of CMRSA2, (C) 2 and 8kb fragments of *Cla*I restricted plasmid of CMRSA7. Grey boxes represent the 3kb *mupA* gene. Not drawn to scale.

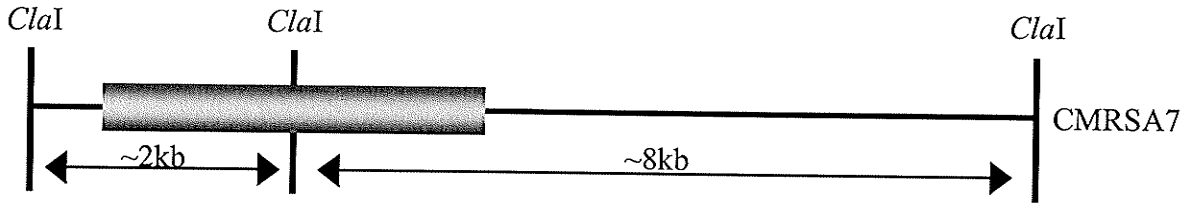
A


*Eco*RI
*Eco*RI
CMRSA2 and CMRSA7
~4kb

B


*Cla*I
*Cla*I
*Cla*I
CMRSA2
~2kb
~2.5kb

C


*Cla*I
*Cla*I
*Cla*I
CMRSA7
~2kb
~8kb

3.2 Erythromycin and Clindamycin Resistance

3.2.1 Erythromycin and Clindamycin Resistance from East-Central Saskatchewan

A total of 304 MRSA and MSSA strains from east-central Saskatchewan had MIC data. Of the 188 MRSA strains, 163 had MIC and PFGE data provided by the Nosocomial Group at the National Microbiology Laboratory. The remaining MRSA and all MSSA MICs and PFGE were performed as part of the current study.

Of the 188 MRSA, 56 were part of the CMRSA2 epidemic cluster and 132 were part of the CMRSA7 epidemic cluster (Table 9). Forty-eight (86%) of the CMRSA2 were resistant to erythromycin, of which 7 were constitutively clindamycin resistant and 41 were inducibly clindamycin resistant and all possessed the *ermC* resistance determinant (Table 10). CMRSA7 strains also had an elevated level of erythromycin resistance with 52 (39%) of strains resistant to erythromycin. However, only 22/52 (44%) strains were constitutively or inducibly resistant to clindamycin which was significantly lower ($p</=0.001$) than the CMRSA2 strains. Twenty-two of the 23 clindamycin resistant strains contained *ermC* and 1 strain contained *msrA*. The strains that were not constitutively or inducibly clindamycin resistant all contained *msrA*. Of the 116 MSSA, 30 (26%) were resistant to erythromycin, of which 5 were constitutively resistant to clindamycin and 16 were inducibly resistant to clindamycin. Nine of the 21 clindamycin resistant strains contained *ermA* and 12 contained *ermC*. Eight of the 9 *ermA* containing strains were related by PFGE while most of the 12 strains containing *ermC* were unrelated (Figure 7).

Table 9. Comparison of erythromycin and clindamycin among MRSA and MSSA from Saskatchewan and from CNISP

Saskatchewan				
Part A-1	CMRSA2 n=56 (%)	CMRSA7 n=132 (%)	Odds Ratio[b] (95% CI[c])	P-Value[b]
Erythromycin Resistance	48 (85.7)	52 (39.4)	9.23 (3.82-23.09)	<0.001
Clindamycin Resistance	7 (12.5)	4 (3.0)	4.57 (1.09-22.05)	0.018
Inducible Clindamycin Resistance[a]	41 (73.2)	19 (14.4)	16.26 (7.10-37.87)	<0.001
Part A-2	CMRSA2 n=56 (%)	MSSA n=116 (%)	Odds Ratio (95% CI)	P-Value
Erythromycin Resistance	48 (85.7)	30 (25.9)	16.6 (6.6-43.18)	<0.001
Clindamycin Resistance	7 (12.5)	5 (4.3)	3.17 (0.96-12.22)[d]	0.098
Inducible Clindamycin Resistance	41 (73.2)	16 (13.8)	17.08 (7.24-41.16)	<0.001
Part A-3	CMRSA7 n=132	MSSA n=116 (%)	Odds Ratio (95% CI)	P-Value
Erythromycin Resistance	52 (39.4)	30 (25.9)	1.86 (1.05-3.33)	0.034
Clindamycin Resistance	4 (3.0)	5 (4.3)	0.69 (0.13-3.32)[d]	0.738
Inducible Clindamycin Resistance	19 (14.4)	16 (13.8)	1.22 (0.57-2.64)	0.717
CNISP				
Part B-1	CMRSA2 n=32 (%)	CMRSA7 n=90 (%)	Odds Ratio (95% CI)	P-Value
Erythromycin Resistance	32 (100)	62 (68.9)	Not defined	<0.001
Clindamycin Resistance	27 (84.4)	10 (11.1)	43.2 (12.1-166.8)	<0.001
Inducible Clindamycin Resistance	5 (15.6)	49 (54.4)	0.15 (0.05-0.47)	<0.001
Part B-2	CMRSA2 n=32 (%)	CMRSA10 n=96 (%)	Odds Ratio (95% CI)	P-Value
Erythromycin Resistance	32 (100)	88 (91.7)	Not defined	0.206
Clindamycin Resistance	27 (84.4)	13 (13.5)	34.48 (0.16-125.71)	<0.001
Inducible Clindamycin Resistance	5 (15.6)	5 (5.2)		
Part B-3	CMRSA7 n=90 (%)	CMRSA10 n=96 (%)	Odds Ratio (95% CI)	P-Value
Erythromycin Resistance	62 (68.9)	88 (91.7)	0.2 (0.08-0.5)	<0.001
Clindamycin Resistance	10 (11.1)	13 (13.5)	0.8 (0.3-2.08)	0.779
Inducible Clindamycin Resistance	49 (54.4)	5 (5.2)	21.75 (7.57-67.29)	<0.001

[a]strains that were phenotypically sensitive to clindamycin by broth dilution but had inducible resistance as observed by D-test. [b]Odds Ratio and p-value calculated using 2X2 Chi-squared Statistical analysis. [c]CI: confidence interval. [d]Calculated using Fisher's Exact test.

Table 10. Number of erythromycin resistant isolates from east-central Saskatchewan containing an *erm* or *msrA* gene.

	Gene								
	ermA			*ermC*			*msrA*		
Clone Type	R[a]	I[b]	S[c]	R	I	S	R	I	S
CMRSA2	0	0	0	7	41	0	0	0	0
CMRSA7	0	0	0	3	19	0	1	0	29
Unique MRSA	0	0	0	1	11	0	0	0	9
MSSA	4	5	0	1	11	0	0	0	9
Total	4	5	0	12	82	0	1	0	47

[a]R: constitutively clindamycin resistant; [b]I: strains that were phenotypically clindamycin sensitive by broth dilution but had inducible resistance as observed by D-test; [c]S: strains that were phenotypically clindamycin sensitive by broth dilution and did not have inducible clindamycin resistance as observed by D-test.

3.2.2 Erythromycin and Clindamycin Resistance from CNISP Hospital Sites

Using the MRSA strains described in section 3.2.1 and strains isolated from CNISP hospital sites, the differences in erythromycin and clindamycin resistance between community and hospital isolated strains was investigated. A total of 218 MRSA strains from CNISP hospital sites had MIC and PFGE data provided by the Nosocomial Group at the National Microbiology Laboratory. Of the 218 MRSA, 32 were part of the CMRSA2 epidemic cluster, 90 belonged to CMRSA7 cluster and 96 to the CMRSA10 cluster (Table 11). All 32 (100%) CMRSA2 strains were resistant to erythromycin, all of which were either constitutively or inducible clindamycin resistant. Twenty-seven CMRSA2 were constitutively resistant to clindamycin, while only 5 expressed the inducible phenotype. Twenty-one CMRSA2 strains contained *ermA* alone and 11 contained *ermA* and *ermC* (Table 12). A total of 62 (69%) of CMRSA7 strains were erythromycin resistant. Of these 10 were constitutively resistant to clindamycin and 49 were inducible for clindamycin resistance. Four of the clindamycin resistant strains contained *ermA* alone, 1 contained *ermA* and *ermC* together, 52 contained *ermC* alone and 2 had no

detectable *erm* gene or *msrA*. The 3 non-inducible clindamycin sensitive strains contained *msrA*. Eighty-eight of the 96 (92%) CMRSA10 were resistant to erythromycin. Only 13 of which were constitutively resistant to clindamycin and an additional 5 strains expressed clindamycin resistance in the presence of erythromycin. Of the inducibly or constitutively clindamycin resistant CMRSA10 strains, 6 contained *ermA* alone, 7 contained *ermA* and *ermC*, 1 contained *ermC* alone and 2 contained *msrA* alone.

Table 11. Comparison of erythromycin and clindamycin resistance in Saskatchewan to CNISP

Part C-1	CMRSA2 (Sask) n=56 (%)	CMRSA2 (CHEC) n=32 (%)	Odds Ratio[b] (95% CI[c])	P-Value[b]
Erythromycin Resistance	48 (85.7)	32 (100)	0.0 (0.00-0.96)[d]	0.469
Clindamycin Resistance	7 (12.5)	27 (84.4)	0.03 (0.01-0.10)	<0.001
Inducible Clindamycin Resistance[a]	41 (73.2)	5 (15.6)	14.76 (4.32-53.85)	<0.001
Part C-2	CMRSA7 (Sask) n=132 (%)	CMRSA7 (CNISP) n=90 (%)	Odds Ratio[b] (95% CI[c])	P-Value
Erythromycin Resistance	52 (39.4)	62 (68.9)	0.29 (0.16-0.54)	<0.001
Clindamycin Resistance	4 (3.0)	10 (11.1)	0.25 (0.06-0.91)	0.031
Inducible Clindamycin Resistance	19 (14.4)	49 (54.4)	0.14 (0.07-0.28)	<0.001

[a]strains that were phenotypically sensitive to clindamycin by broth dilution but had inducible resistance as observed by D-test. [b]Odds Ratio and p-value calculated using 2X2 Chi-squared Statistical analysis. [c]CI: confidence interval. [d]Calculated using Fisher's Exact test.

Table 12. Number of erythromycin resistant MRSA strains from CNISP Hospital Sites containing an *erm* or *msrA* gene.

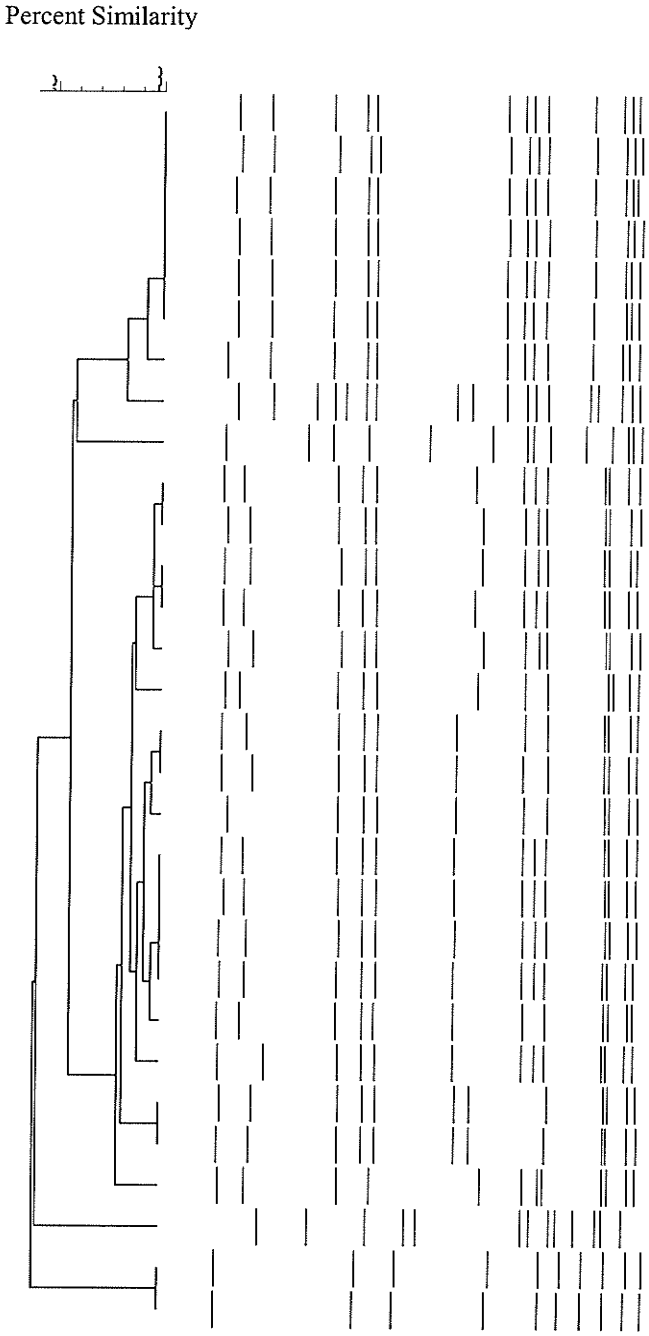
Clone	Gene														
	ermA			*ermC*			*ermA* and *ermC*			*msrA*			*msrA* and *erm gene*		
	R[a]	I[b]	S[c]	R	I	S	R	I	S	R	I	S	R	I	S
CMRSA2	16	5	0	0	0	0	11	0	0	0	0	0	0	0	0
CMRSA7	4	0	0	3	49	0	1	0	0	0	0	3	0	0	0
CMRSA10	4	2	0	0	1	0	7	0	0	2	0	70	0	2	0
Total	24	7	0	3	50	0	18	0	0	2	0	73	0	2	0

[a]R: constitutively clindamycin resistant; [b]I: strains that were phenotypically sensitive to clindamycin by broth dilution but had inducible resistance as observed by D-test; [c]S: strains that were phenotypically sensitive to clindamycin by broth dilution and did not have inducible clindamycin resistance as observed by D-test.

3.2.3 Statistical Comparison of Erythromycin, Constitutive Clindamycin and Inducible Clindamycin Resistance

To compare the differences in the number of strains among the various clonal groups that exhibited erythromycin resistance, constitutive clindamycin resistance and inducible clindamycin resistance, chi-squared statistical analysis was performed. Each of CMRSA2, CMRSA7 and MSSA from east-central Saskatchewan were compared to each other. Among the CNISP hospital site isolates, CMRSA7 was compared with CMRSA10. CMRSA2 from east-central Saskatchewan was compared with CMRSA2 from CNISP hospital site isolates. CMRSA7 from east-central Saskatchewan was compared with CMRSA7 from CNISP hospital site isolates. The chi-squared statistical comparison of erythromycin resistance, constitutive clindamycin resistance and inducible clindamycin resistance among various strain types are given in Tables 9 and 11.

Figure 7. PFGE dendrogram of MSSA erythromycin resistant strains from the east-central Saskatchewan. Dendrogram was created using BioNumerics version 3.0 with similarities based on the UPGMA. Date of isolation with erythromycin and clindamycin MIC data and resistance determinant are shown for each sample. Note nd: no date; rec: received at National Microbiology Laboratory; Erythro: erythromycin MIC; Clinda: clindamycin MIC.



Date	Gene	Erythro	Clinda	Clindamycin Phenotype
Oct 23/04	MsrA	>8	0.25	non-inducible
nd (rec Mar24/05)	MsrA	>8	0.25	non-inducible
nd (rec Mar24/05)	MsrA	>8	0.25	non-inducible
Mar 29/05	MsrA	>8	0.25	non-inducible
May 9/05	MsrA	>8	0.25	non-inducible
May 9/05	MsrA	>8	0.25	non-inducible
Sept 24/04	MsrA	8	0.25	non-inducible
Mar 31/05	MsrA	>8	0.25	non-inducible
Sept 27/05	ErmA	>8	0.25	inducible
nd (rec Mar24/05)	ErmC	>8	0.25	inducible
nd (rec Mar24/05)	ErmA	>8	>8	resistant
Oct 29/04	ErmA	>8	>8	resistant
nd (rec Mar24/05)	ErmC	>8	0.25	inducible
nd (rec Mar24/05)	ErmA	>8	>8	resistant
Jun 19/05	ErmC	>8	0.25	inducible
May 25/05	ErmC	>8	0.25	inducible
nd (rec Sep2/05)	ErmA	>8	0.25	inducible
May 25/05	ErmC	>8	0.25	inducible
nd (rec Mar24/05)	ErmC	>8	0.25	inducible
nd (rec May30/05)	ErmC	>8	0.25	inducible
Apr 2/05	ErmC	>8	0.25	inducible
July 6/05	ErmC	>8	0.25	inducible
Aug 8/05	ErmC	>8	0.25	inducible
July 6/05	ErmC	>8	0.25	inducible
nd (rec Mar24/05)	ErmA	>8	0.25	inducible
nd (rec May30/05)	ErmA	>8	0.25	inducible
nd (rec Mar24/05)	ErmA	>8	>8	resistant
Aug 16/05	MsrA	>8	0.25	non-inducible
nd (rec May30/05)	ErmC	>8	8	resistant
May 22/05	ErmA	>8	0.25	inducible

3.3 PVL in CMRSA7 type 0142

A total of 31 of the 76 CMRSA7 PFGE type 0142 (the major PFGE type of CMRSA7) from CNISP hospital sites were determined to be PVL negative by the nosocomial group at the National Microbiology Laboratory using primers previously described (91). A PFGE gel containing PVL positive CMRSA7 type 0142 and PVL negative CMRSA7 type 0142 showed that there was no detectable banding difference among the strains (Figure 8). An *in silico* investigation of the MW2 genome (GenBank Accession Number BA000033) showed that the ΦSa2mw PVL containing phage was present in a 573kb *Sma*I fragment. Presence of PVL in the large band (~600kb band) of PVL positive CMRSA7 type 0142 and absence in PVL negative CMRSA7 type 0142 was confirmed using Southern blot analysis (Figure 9A and 9B). None of the 31 PVL negative strains tested positive for *lukF*-PV and *lukS*-PV genes using LukFF/LukFR and LukSF/LukSR primer sets, respectively. Using primers Intfor and Intrev specific to the host integration site, a positive result was observed in all 31 CMRSA7 type 0142 PVL negative strains (Figure 10). However, a product of correct size was also observed in a PVL positive CMRSA7 type 0142 and a PVL positive CMRSA10 type 0473. The PCR result in all CMRSA7 and CMRSA10 strains, regardless of PVL presence, was considered to be a weak positive (see section 4.3) while a strong positive result was observed in 03S 1232, a CMRSA2 isolate known to be PVL negative (Figure 11). PCR using the Intfor and MW2_3' primers specific to the host integration site and ΦSa2mw phage end, respectively, produced a positive result in both the PVL positive CMRSA7 type 0142 and PVL positive CMRSA10 type 0142 strains, while none of the 31 PVL negative CMRSA7 strains had a detectable product. Products were detected in both PVL

positive and negative CMRSA7 type 0142 with Intrev and MW2_5' primers specific to the host integration site and the start of the ΦSa2mw phage genome. Using a primer (252phage_3') specific to a homologous phage (see section 1.5) and the primer specific to the host integration site (Intfor) produced a positive result only in PVL negative CMRSA7 type 0142.

The 404 bp sequence product of Intfor/252phage_3' primer set was analyzed and aligned with portions of the MW2 genome (GenBank Accession Number BA000033), which contains ΦSa2mw, and the MRSA252 genome (GenBank Accession Number BX571856), which contains a phage that is 82% homologous to ΦSa2mw. Of the 294 bp sequence past the attachment site in the PVL negative CMRSA7 type 0142, a 97% homology was found to the phage of MRSA252 and 58% homology to the PVL containing phage present in MW2.

There was no difference in the number of strains attributed to infections versus the number of colonizing strains between the PVL positive and PVL negative CMRSA7 type 0142 (p</=1) (Table 14). SSTIs were the most common type of infections observed in both PVL positive and PVL negative CMRSA7 type 0142.

Figure 8. PFGE of PVL positive and negative strains. Lanes 2 and 14 01S 0147 (PVL positive); Lanes 3-13 PVL negative CMRSA7 strains; Lanes 1 and 15 *Salmonella* Braenderup H9812 size markers.

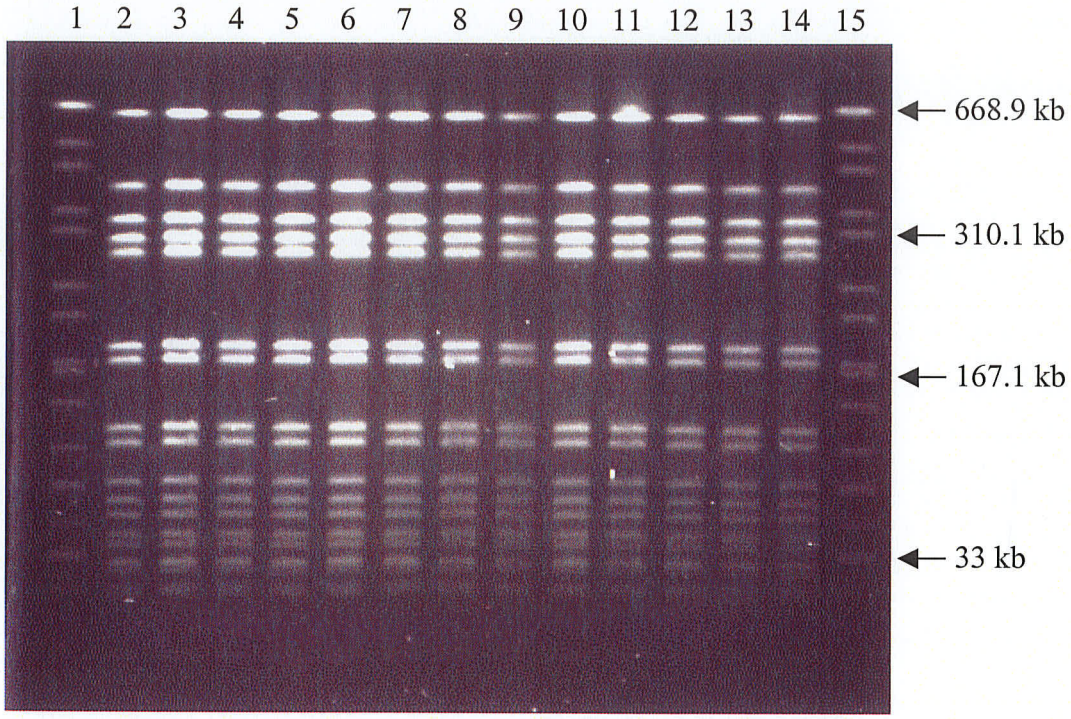

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
668.9 kb
310.1 kb
167.1 kb
33 kb

Figure 9. (A) PFGE of PVL positive and negative strains used for Southern blot to detect presence of PVL. Lane 2 00S 0907 (CMRSA7 type strain, PVL positive); Lane 3 03S 0338 (CMRSA7 PVL positive); Lane 4 03S 1222 (CMRSA7 PVL negative); Lane 6 04S 0073 (CMRSA10 type strain, PVL positive); Lane 7 04S 0616 (CMRSA10, PVL positive) Lanes 1, 5, 8 low molecular weight lambda marker. (B) Southern blot using PVL probe, lanes correspond to those in PFGE gel.

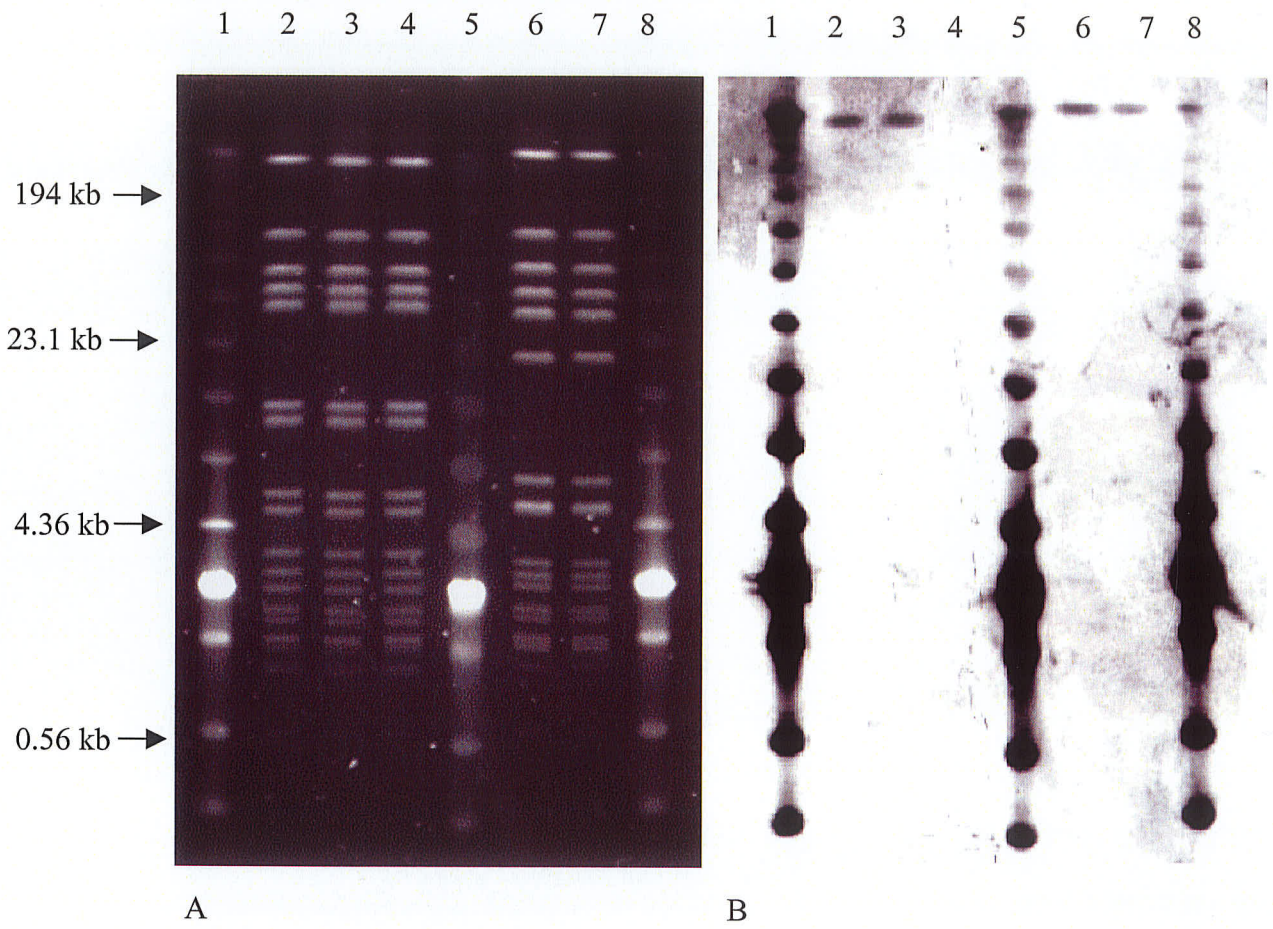
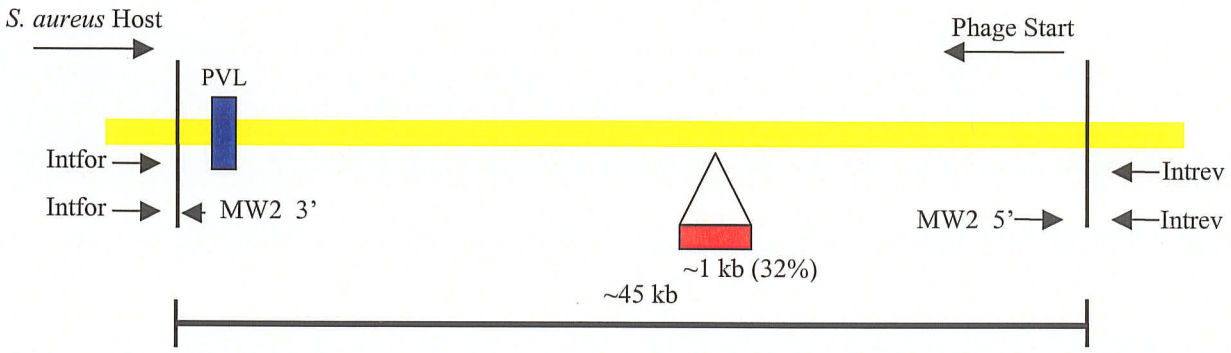
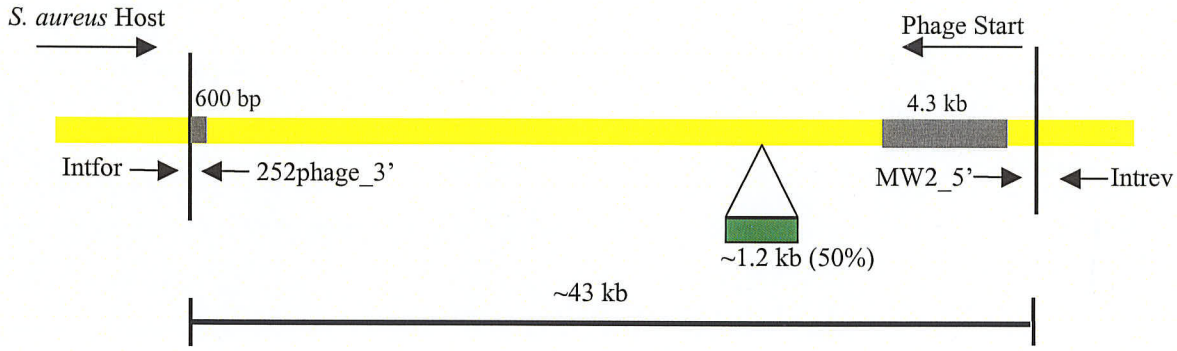

1 2 3 4 5 6 7 8
194 kb
23.1 kb
4.36 kb
0.56 kb
A
1 2 3 4 5 6 7 8
B

Figure 10. Schematic diagram: (A) the integration of the ΦSa2mw into MW2 genome with the primers used to detect the phage integration as well as the phage. PVL (*lukF*-PV and *lukS*-PV) is shown in blue. The red box is a 1 kb region that is present in ΦSa2mw that has additional bases and only 32% sequence homology to the phage present in MRSA252. (B) the integration of a ΦSa2mw homologous phage present in MRSA252. The 4.3kb and 600bp regions in grey have less than 50% homology to the same region on ΦSa2mw. The region in green is additional bases that are not present in ΦSa2mw. Primers used to identify phage are shown. (C) the integration site present in *S. aureus* without the presence of any phage. Shown is the expected product size of Intfor /Intrev primer set with no phage integration.

A


S. aureus Host
Phage Start
PVL
Intfor
Intfor
MW2 3'
Intrev
MW2 5'
Intrev
~1 kb (32%)
~45 kb

B


S. aureus Host
Phage Start
600 bp
4.3 kb
Intfor
252phage_3'
MW2_5'
Intrev
~1.2 kb (50%)
~43 kb

C

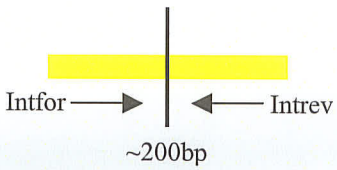

Intfor
Intrev
~200bp

Figure 11. PCR results for Intfor/Intrev primer set. Lane 2 negative control, Lane 3 00S 0907 (PVL positive CMRSA7); Lane 4 04S 0073 (PVL positive CMRSA10); Lane 5 03S 1222 (PVL negative CMRSA7); Lane 6 03S 1232 (PVL negative CMRSA2); Lanes 1 and 7 100bp standard.

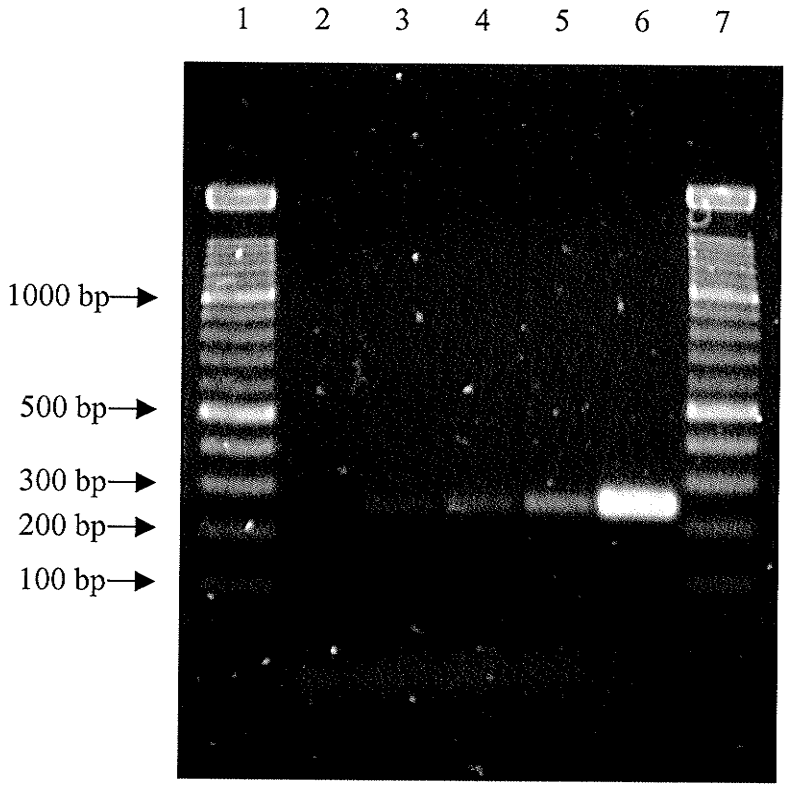

1
2
3
4
5
6
7
1000 bp
500 bp
300 bp
200 bp
100 bp

Table 13. PCR results for PVL and phage presence.

Primer Pair/Strain	CMRSA7 type 0142 PVL positive	CMRSA7 type 0142 PVL negative	CMRSA10 type 0473 PVL positive	CMRSA2 PVL negative
LukPVLF and LukPVLR	+[a]	-[b]	+	-
LukSF and LukSR	+	-	+	-
LukFF and LukFR	+	-	+	-
Intfor and Inrev	(+)[c]	(+)	(+)	+
Intfor and MW2_3'	+	-	+	-
Intrev and MW2_5'	+	+	+	-
Intfor and 252phage_3'	-	+	-	-

[a]+: positive; [b]-: negative, [c](+): weak positive.

Table 14. Number of strains that were attributed to infection and colonization.

PVL	Number Colonized (%)	Number Infected (%)	Unknown
Positive	13 (30)	31 (70)	1
Negative	10 (32)	21 (68)	0

Twenty-five of the 31 CMRSA7 type 0142 PVL negative were isolated in Alberta (Table 15). The PVL negative strains were isolated over a total of a 3 year time span with no indication of an outbreak at any given time (Figure 13). Approximately 71% of patients colonized or infected with PVL negative CMRSA7 type 0142 were between the ages of 18 and 65 and only 6% of patients were under the age of 18 (Figure 14). Approximately 42% of patients colonized or infected with PVL positive strains were between the ages of 18 and 65 while 39% of patients were under the age of 18 which was significantly different than the ages of patients who were infected or colonized with PVL negative strains ($p</=0.01$).

Table 15. Number of strains isolated from various provinces in Canada.

PVL	Alberta	Saskatchewan	Manitoba	Ontario	Quebec
PVL Positive	5	18	16	5	1
PVL Negative	25	1	4	2	0
p-value	p</=0.001	p</=0.001			

Figure 12. Sequence alignment of 110 bases from MRSA host and 294 bases of phages from 01S 0012 PVL negative CMRSA7, MRSA 252 (GenBank accession number BX571856, bases 1591516-1591916) and MW2 (GenBank accession number BA000033, bases 1529042-1529397). Differences in bases from 01S 0012 are shown, similarities are shown as ".". Highlighted in pink is the attachment site of the ΦSa2mw and homologous phages. The phage DNA starts 13 bp into the *att* site (which is a direct repeat found on both the 5' and 3' end of the phage).

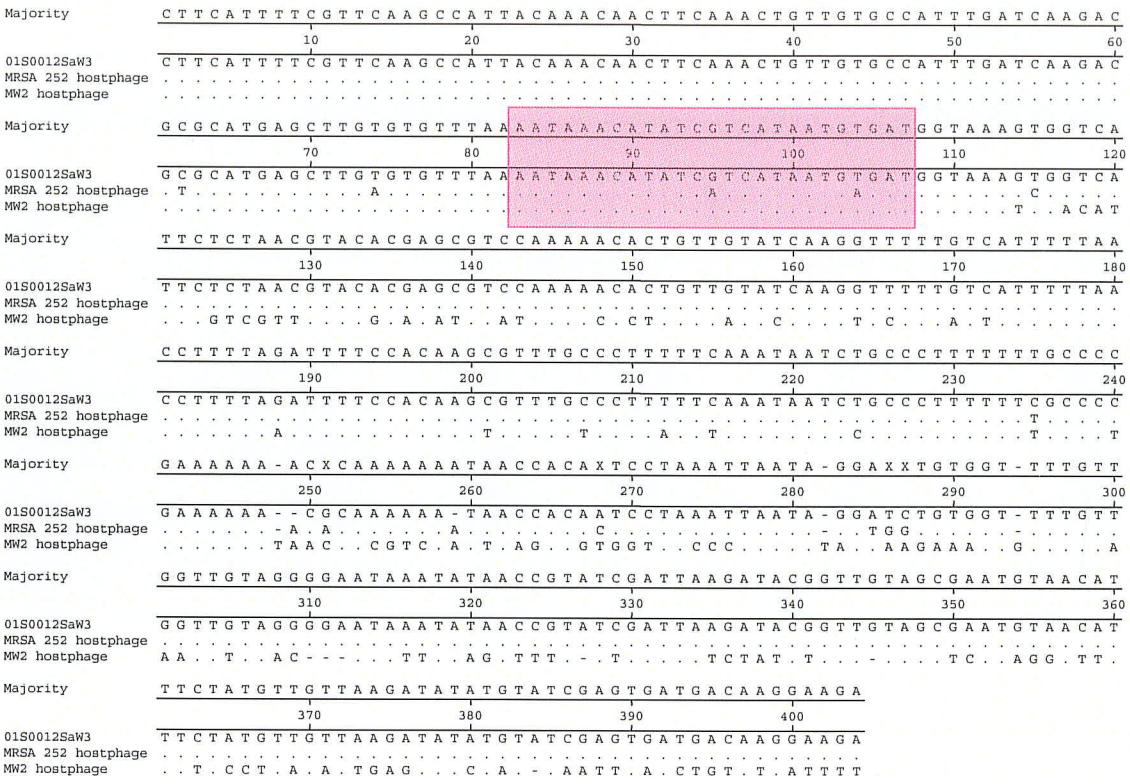

Majority
01S0012SaW3
MRSA 252 hostphage
MW2 hostphage

Figure 13. Graph of the number of strains with the dates of isolation of PVL positive and PVL negative CMRSA7 type 0142.

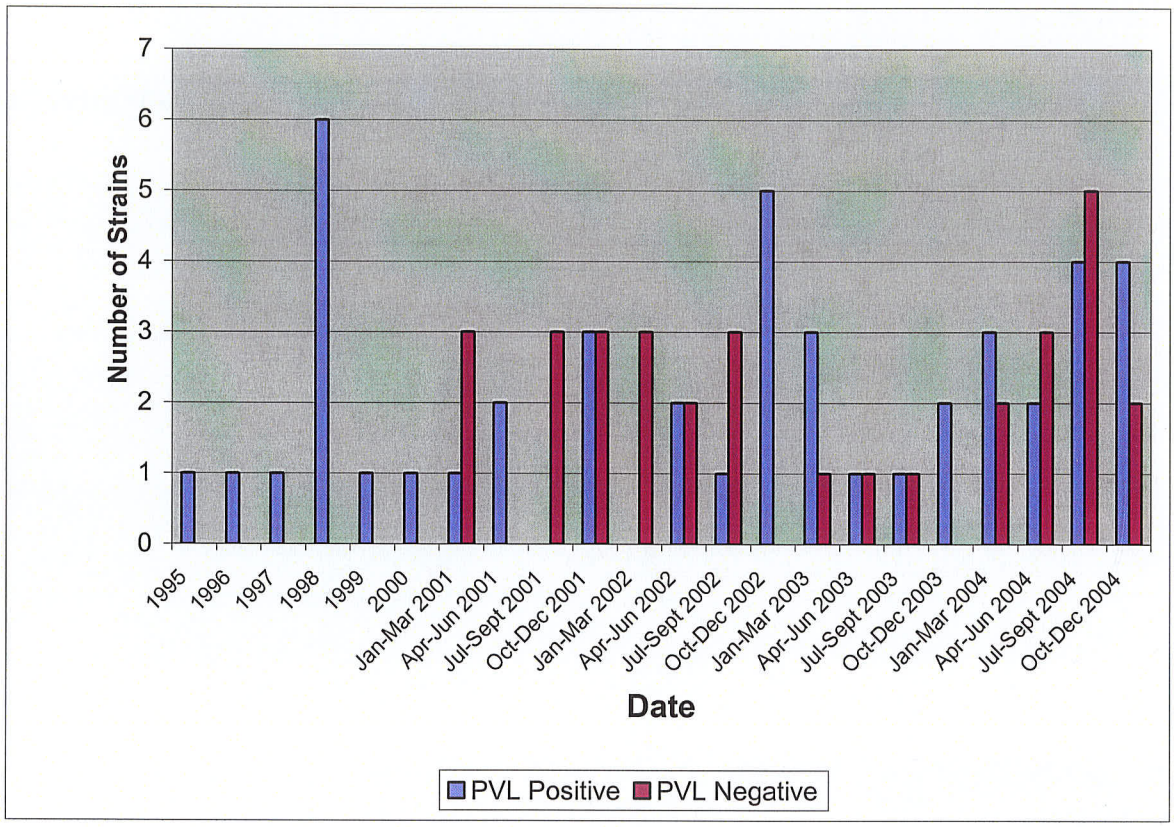
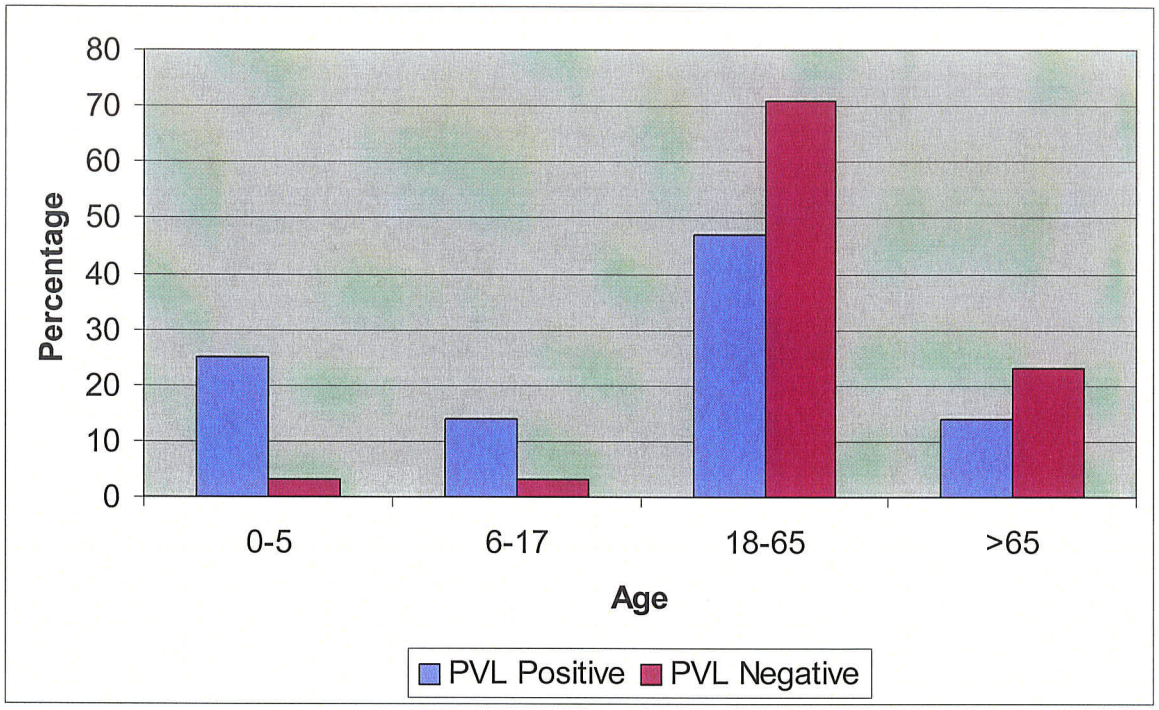

Number of Strains
0
1
2
3
4
5
6
7
1995
1996
1997
1998
1999
2000
Jan-Mar 2001
Apr-Jun 2001
Jul-Sept 2001
Oct-Dec 2001
Jan-Mar 2002
Apr-Jun 2002
Jul-Sept 2002
Oct-Dec 2002
Jan-Mar 2003
Apr-Jun 2003
Jul-Sept 2003
Oct-Dec 2003
Jan-Mar 2004
Apr-Jun 2004
Jul-Sept 2004
Oct-Dec 2004
Date
PVL Positive
PVL Negative

Figure 14. Graph showing the percentage of patient's age at time of infection with CMRSA7 type 0142 PVL positive or CMRSA7 type 0142 PVL negative.


80
70
60
50
40
30
20
10
0
Percentage
0-5
6-17
18-65
>65
Age
PVL Positive
PVL Negative

3.4 CAN-ICU

3.4.1 Phenotypic Characterization Provided by Dr. Zhanel

The initial finding and phenotypic characterization of all organisms in the CAN-ICU study was performed by Dr. Zhanel at the Health Sciences Centre in Winnipeg, MB. They identified 2260 organisms in Canadian ICU wards from 19 hospitals in 8 provinces across Canada between Jan. 1, 2005 and Oct. 14, 2005. 589 (26%) were identified as *S. aureus* of which 126 (21%) were determined to be MRSA. The antibiotic resistance profiles revealed that most MRSA strains (93%) were resistant to 3 or more antimicrobials while 1.2% of MSSA were resistant to 3 or more antimicrobials. 73 to 91% of MRSA specimens were resistant to cefazolin, cefepime, ceftriaxone, ciprofloxacin, levofloxacin, meropenem and piperacillin/tazobactam, while 86.5% of strains were sensitive to gentamicin (Table 16). The highest level of resistance in MSSA was 7.1% to ciprofloxacin. All 126 MRSA and 463 MSSA were sensitive to vancomycin.

A total of 82 MRSA were from respiratory samples, 27 from blood, 15 from wounds and 2 specimens isolated from urine accounting for 65, 21, 12 and 2% of MRSA infections, respectively. Of the 463 MSSA specimens, 72.4% were isolated from respiratory, 14.3% from wound, 10.4% blood and 3.0% in urine infections. In both MRSA and MSSA infections, there were a greater number of males infected, accounting for 66.7 and 59.4% of infections, respectively, which was not significantly different between MRSA and MSSA infections ($p</=0.2$). The average age of a patient with an MRSA or MSSA infection was 62.1 and 51.2 years of age, respectively.

Table 16. Distribution of strains that have a susceptible, intermediate or resistant phenotype to antimicrobials tested.

Antimicrobial	Sensitive[a] (%)		Intermediate (%)		Resistant (%)	
	MRSA n=126	MSSA n=463	MRSA n=126	MSSA n=463	MRSA n=126	MSSA n=463
Cefazolin	17 (13.5)	458 (98.9)	17 (13.5)	0 (0)	92 (73.0)	5 (1.1)
Cefepime	5 (4.0)	457 (98.7)	6 (4.8)	1 (0.2)	115 (91.2)	5 (1.1)
Ceftriaxone	0 (0)	455 (98.3)	19 (15.1)	3 (0.6)	107 (84.9)	5 (1.1)
Ciprofloxacin	11 (8.7)	425 (91.8)	0 (0)	5 (1.1)	115 (91.3)	33 (7.1)
Gentamicin	109 (86.5)	455 (98.3)	0 (0)	0 (0)	17 (13.5)	8 (1.7)
Levofloxacin	11 (8.7)	431 (93.1)	6 (4.8)	6 (1.3)	109 (86.5)	26 (5.6)
Meropenem	39 (31.0)	458 (98.9)	16 (12.7)	1 (0.2)	71 (56.3)	4 (0.9)
Piperacillin/tazobactam	15 (11.9)	456 (98.5)	NA[b]	NA	111 (88.1)	7 (1.5)
Vancomycin	126 (100)	463 (100)	0 (0)	0 (0)	0 (0)	0 (0)

[a]Susceptible, intermediate and resistant breakpoints were based on CLIS guidelines (107). [b]not applicable

3.4.2 **Molecular Characterization of MRSA from CAN-ICU**

To determine if there were CA-MRSA strains present in ICUs, PFGE and PVL testing was performed on all 126 MRSA. A total of 70 different PFGE patterns were observed within the MRSA collected in ICUs (Figure 13). Strains related to CMRSA1-4 and CMRSA6-10 were observed; no strains related to the CMRSA5 epidemic cluster were found (Table 17). Twenty-nine patterns not related to any of the 10 Canadian epidemic clones were found. CMRSA2 was the most predominant epidemic clone type observed accounting for 48% however, CMRSA6 was the most common strain encountered in the west. A total of 7 CA-MRSA strains were isolated, 4 CMRSA7 and 3

CMRSA10 and were all determined to be PVL positive using primers previously described (84). All but 1 of the CA-MRSA strains were located in the West.

CMRSA7 and CMRSA10 strains were resistant to at least 3 classes of antimicrobials. Five of the 7 CA-MRSA strains were isolated from bloodstream infections and 2 were isolated from respiratory infections. The average age of patients with a CA-MRSA infection was 32 years old (range 1 to 75 years of age).

Table 17. Number of CMRSA1 to 10 isolated in ICUs in Canadian regions.

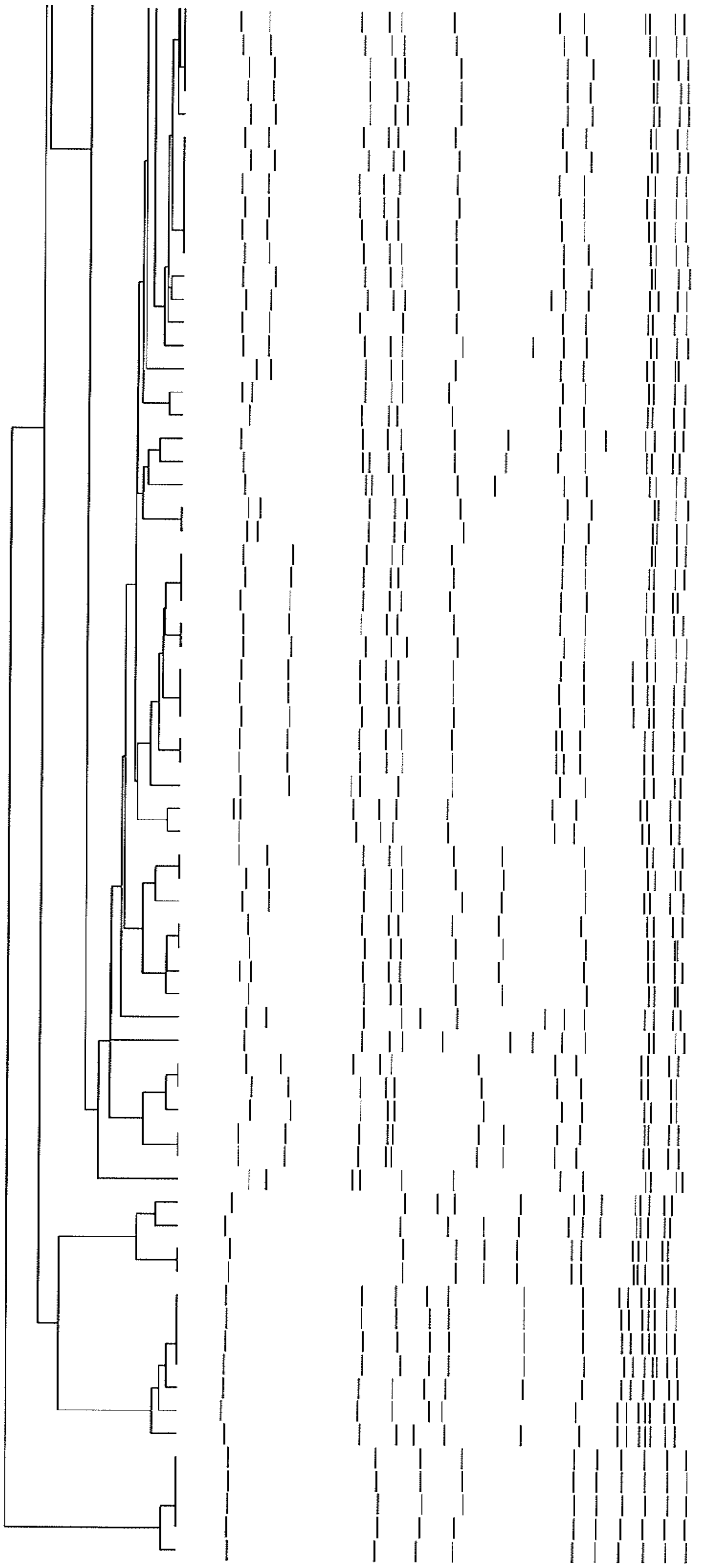
Region/Clone	1[a]	2[b]	3[c]	4[d]	6[e]	7[f]	8[g]	9[h]	10[i]	Unique[j]	Total
East (n=21)[k]	0	16	0	0	0	0	0	0	1	3	20
Central (n=64)[l]	7	39	0	0	0	0	2	1	0	15	64
West (n=42)[m]	0	6	2	5	11	4	1	0	2	11	42

[a]CMRSA1; [b]CMRSA2; [c]CMRSA3; [d]CMRSA4; [e]CMRSA6; [f]CMRSA7; [g]CMRSA8; [h]CMRSA9; [i]CMRSA10; [j]unique strains that are not related to the epidemic strains; [k]East provinces of New Brunswick and Nova Scotia; [l]Central provinces of Quebec and Ontario; [m]West provinces of Manitoba, Saskatchewan, Alberta and British Columbia.

One other strain (designated 59972) isolated in the West from a 62 year old male was found to be PVL positive by PCR. This strain was not related to any of the epidemic CA-MRSA strains found in Canada. Strain 59972 was related to EMRSA15 by PFGE (Figure 16) and was determined to have an MLST of ST22, the same ST as EMRSA15. SCC*mec* typing showed that 59972 contained SCC*mec* type IVa.

Figure 15. PFGE dendrogram and cluster analysis of MRSA collect from CAN-ICU study. Included: city, date of isolation, MRSA epidemic type, age in years and sex. Dendrogram and cluster analysis performed using BioNumerics v3.0. M: male; F: female.

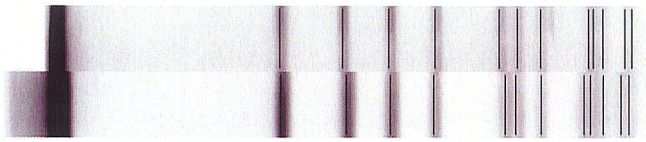
		Epidemic PFGE Type	Age	Sex
Winnipeg	Mar 17/05		60	M
Winnipeg	Apr 28/05		60	M
Winnipeg	Feb 28/05	CMRSA7	1	F
Saskatoon	Jun 8/05	CMRSA7	5	M
Regina	Jul 27/05	CMRSA7	25	F
Winnipeg	Sep 15/05	CMRSA7	58	M
Montreal	Apr 10/05	CMRSA9	79	M
Victoria	Apr 19/05	CMRSA6	63	M
Victoria	Jul 13/05	CMRSA6	65	M
Victoria	May 12/05	CMRSA6	64	M
Vancouver	Apr 3/05	CMRSA6	78	M
Edmonton	May 15/05	CMRSA6	72	F
Edmonton	May 16/05	CMRSA6	72	F
Vancouver	May 19/05	CMRSA6	49	M
Vancouver	Jun 14/05	CMRSA6	42	M
Victoria	Mar 27/05	CMRSA6	51	M
Victoria	Apr 23/05	CMRSA6	67	M
Edmonton	May 5/05	CMRSA6	78	M
Victoria	Apr 22/05	CMRSA 3	76	M
Victoria	Apr 17/05	CMRSA 3	75	M
Winnipeg	Mar 1/05		50	F
Vancouver	May 18/05	CMRSA10	39	M
Victoria	Aug 9/05	CMRSA10	21	F
Halifax	Sep 7/05	CMRSA10	75	F
Toronto	May 1/05		27	M
Hamilton	Jun 20/05		37	M
Toronto	Apr 30/05		74	M
Montreal	May 17/05	CMRSA2	73	F
Hamilton	Jul 12/05	CMRSA2	58	M
Hamilton	Jul 31/05	CMRSA2	76	F
Hamilton	Jul 15/05	CMRSA2	62	M
Regina	Aug 16/05	CMRSA2	78	F
London	Aug 12/05	CMRSA2	23	M
London	Sep 7/05	CMRSA2	77	F
London	Aug 21/05	CMRSA2	75	M
Moncton	Apr 15/05	CMRSA2	63	M
Moncton	Mar 29/05	CMRSA2	63	M
Toronto	Apr 11/05	CMRSA2	59	F
Montreal	Apr 9/05	CMRSA2	70	F
London	Aug 16/05		58	M
London	Apr 13/05	CMRSA2	77	M
Winnipeg	Aug 19/05	CMRSA2	56	M
Winnipeg	Aug 30/05	CMRSA2	56	M
Winnipeg	Sept 2/05	CMRSA2	72	M
London	Aug 15/05	CMRSA2	77	M
London	Aug 15/05	CMRSA2	77	M
London	Aug 29/05	CMRSA2	77	M
London	Sep 2/05	CMRSA2	60	F
London	Sep 23/05	CMRSA2	77	M
Winnipeg	Jun 23/05	CMRSA2	62	F
London	Sep 1/05		65	M
Montreal	Jun 6/05	CMRSA2	71	M
St John	Jul 28/05	CMRSA2	72	M
Moncton	Sep 13/05	CMRSA2	68	F
Montreal	Apr 18/05	CMRSA2	76	M
Montreal	Apr 17/05	CMRSA2	76	M
Montreal	Apr 24/05	CMRSA2	52	F
Hamilton	Jun 11/05	CMRSA2	79	M
Hamilton	Aug 18/05	CMRSA2	79	F



London	Aug 17/05	CMRSA2	49	M
London	Sep 1/05	CMRSA2	84	M
Moncton	Sep 13/05	CMRSA2	68	F
London	Sep 13/05	CMRSA2	76	M
St John	Aug 16/05	CMRSA2	64	M
St. John's	Jun 8/05		61	M
St John	Sep 17/05	CMRSA2	68	F
Montreal	Jul 9/05	CMRSA2	88	M
Montreal	Jun 6/05	CMRSA2	71	M
Halifax	Mar 23/05	CMRSA2	73	M
Ottawa	May 15/05	CMRSA2	2	F
London	Jul 5/05	CMRSA2	59	M
Hamilton	Sep 10/05	CMRSA2	70	M
Montreal	Apr 21/05	CMRSA2	77	M
Toronto	Jul 7/05	CMRSA2	33	M
Hamilton	Jul 30/05	CMRSA2	76	F
Montreal	Apr 9/05	CMRSA2	76	F
London	Aug 31/05	CMRSA2	77	M
Toronto	Jun 22/05		53	F
Montreal	Jun 13/05		82	F
Halifax	Apr 27/05		72	F
Vancouver	Jun 29/05		59	M
Montreal	May 10/05		88	M
Halifax	Jul 21/05	CMRSA2	54	M
Montreal	Sep 12/05	CMRSA2	90	F
Halifax	Jul 25/05	CMRSA2	54	M
Montreal	Apr 20/05	CMRSA2	88	F
Toronto	Jun 19/05	CMRSA2	76	M
Toronto	Apr 27/05		64	M
Toronto	May 14/05		64	F
Toronto	May 14/05	CMRSA2	64	F
Toronto	Jun 19/05		76	M
Toronto	Jun 20/05		76	M
Winnipeg	Jun 18/05		43	F
Winnipeg	Jul 5/05		66	M
Winnipeg	Jul 21/05		85	M
Hamilton	Oct 14/05	CMRSA2	69	M
Hamilton	Oct 14/05	CMRSA2	69	M
Toronto	Apr 17/05		66	M
Halifax	May 25/05	CMRSA2	73	M
Halifax	May 13/05	CMRSA2	84	M
Halifax	Aug 5/05	CMRSA2	84	M
Halifax	Jul 6/05	CMRSA2	35	F
Edmonton	Sep 3/05		68	F
Hamilton	Aug 4/05		78	M
Halifax	May 1/05		30	M
Halifax	Sep 13/05	CMRSA2	17	M
Halifax	Sep 13/05	CMRSA2	36	M
Winnipeg	Feb 28/05		1	M
Winnipeg	Mar 14/05		3	M
Edmonton	May 1/05	CMRSA2	65	F
Edmonton	Sep 2/05	CMRSA8	56	M
Vancouver	Apr 30/05		62	M
London	May 18/05	CMRSA8	32	M
London	May 24/05	CMRSA8	78	M
Hamilton	May 29/05	CMRSA1	72	M
Hamilton	Jun 11/04	CMRSA1	57	F
London	May 9/05	CMRSA1	35	F
London	May 27/05	CMRSA1	70	M
Hamilton	Jun 13/05	CMRSA1	74	M
London	Aug 18/05	CMRSA1	60	F
London	Aug 5/05	CMRSA1	76	F
Winnipeg	Apr 5/05	CMRSA4	75	F
Winnipeg	Apr 8/05	CMRSA4	75	F
Winnipeg	Apr 10/05	CMRSA4	75	F
Vancouver	Apr 16/05	CMRSA4	28	F
Victoria	Jun 26/05	CMRSA4	66	M

Figure 16. PFGE image of 59972 and EMRSA15 with bands identified. Image was generated using BioNumerics 3.0.

PFGE - SmaI



4 Discussion

4.1 MRSA and MSSA from East-Central Saskatchewan

4.1.1 MSSA from East-Central Saskatchewan

The large amount of diversity among the PFGE patterns in east-central Saskatchewan suggests that there is little clonal spread of MSSA, which was not surprising as this has been observed in other regions (23). The amount of antimicrobial resistance is much lower in MSSA than MRSA from the region (100), which has been observed in other areas (35). However, the level of mupirocin resistance was much higher than what has been observed in the rest of the country (80). Low-level resistance was present in the region along with high-level resistance which suggests that at least two resistance mechanisms exist within the region. It is possible that the high level of mupirocin use in the region is driving the high level of resistance (100). Erythromycin resistance was also of notable levels. However, most (52%) strains were sensitive to all antimicrobials tested and an additional 25% were resistant to only one of the antimicrobials tested. Two strains (1.7%) were PVL positive which is consistent with other reports of PVL in MSSA (84, 120, 174).

4.1.2 MRSA from East-Central Saskatchewan

While only a few strains were available for typing, there does not appear to be a difference from the strains that were first found circulating in the area (100). However, with so few strains available it is difficult to speculate. MRSA in east-central Saskatchewan were generally community-acquired (100). MRSA isolated had similar antimicrobial resistance patterns to the MRSA previously reported in the region with

CMRSA2 displaying resistance to a greater number of antimicrobials compared to CMRSA7. A high number of isolates showed resistance to mupirocin. All CMRSA7 type 0142 were PVL positive along with two related clones which was also previously observed (100).

4.1.3 **Mupirocin Resistance in East-Central Saskatchewan**

mupA confers resistance to mupirocin in *S. aureus*. While *mupA* has been found on the chromosome of *S. aureus*, it is typically encoded on 35 to 45kb plasmids (10, 64, 159, 160). In the mupirocin resistant strains in east-central Saskatchewan, *mupA* appeared to be present on a >50kb plasmid in both CMRSA2 and CMRSA7. While the plasmids carrying *mupA* were different, the region directly surrounding *mupA* appears to be similar as *mupA* was present on a 4 kb fragment in both clone types when the plasmids were restricted with *Eco*RI (a restriction enzyme that does not cut within *mupA*) (Figure 5). When restricted with *Cla*I (cuts once in the middle of *mupA*) one fragment was the same size (2kb) which again suggests that the region directly outside *mupA* is similar. However, the second fragment in CMRSA2 was 2.5 kb and an 8kb fragment was found in CMRSA7 (Figure 6). This again suggests that the plasmids are different. This also suggests that there is an approximately 4kb region surrounding *mupA* that is similar. However, it is possible that just past the 4kb region the area is no longer homologous. Given that *mupA* is present on two different plasmids in two different clone types and that the clones are in close proximity to each other, it is possible that *mupA* may be present on a transposon or other mobile element.

It is unclear if the two large plasmids observed in the undigested plasmid preparations from CMRSA7 were the same plasmid in different forms or if they were two

different plasmids. It is however, suspected that the two plasmid bands are different confirmations of the same plasmid with one being supercoiled and the other being a linear or nicked circular form.

As none of the low level mupirocin resistant MSSA contained *mupA*, a different mechanism of resistance is assumed to be present in these strains, which is unknown at this time. Sequencing of the native isoleucyl tRNA gene may provide an answer as it has previously been observed that mutations in the native gene confer low-level resistance to mupirocin (4). It is also possible that there is reduced uptake or efflux of mupirocin although this has not been previously observed (74).

It is possible that the increased use of mupirocin resistance in the region is driving more than one type of mupirocin resistance (100). It would be wise to implement strict control of mupirocin use until the levels of resistance decrease in the area which has previously been shown to reduce the levels of both high- and low-level resistance (165).

4.2 Erythromycin and Clindamycin Resistance

4.2.1 Erythromycin and Clindamycin Resistance in MRSA and MSSA from East-Central Saskatchewan

The amount of clindamycin resistance (43%) in east-central Saskatchewan was found to be relatively high. The amount of resistance in MRSA was much higher than that of MSSA (Table 9, Part A-2 and A-3) which is consistent with other recent reports in the United States (18, 35). Among MRSA in the region it was found that CMRSA2 had a higher amount of both erythromycin and clindamycin resistance (constitutive and inducible) than CMRSA7 (Table 9 Part A-1). This was not unexpected as CMRSA2 is

typically health-care associated and is generally resistant to a higher number of antimicrobials than CMRSA7, which is typically community-associated (100, 142).

ermC was the most prevalent resistant determinant among MRSA followed by *msrA*. All CMRSA2 possessed *ermC* while *msrA* was found in 60% of erythromycin resistant CMRSA7. It is surprising that none of the MRSA possessed *ermA* or *ermB* as some recent studies have found these as the most prevalent *erm* genes (26, 147). It was previously reported that the CMRSA2 strains from east-central Saskatchewan have an SCC*mec* type IIIb (100) a variant of SCC*mec* type III that does not contain a copy of Tn*554*, the transposon that carries *ermA* (113). The diversity of erythromycin resistance genes in MSSA is not surprising as there is a large amount of genetic diversity shown by PFGE. One CMRSA7 was clindamycin resistant yet did not contain any of *ermA*, *ermB* or *ermC*. Clindamycin resistance in this case may be due to a different mechanism such as a mutation in the ribosome which prevents erythromycin and clindamycin from binding to their respective target sites (121). Further studies would be required to confirm this hypothesis.

4.2.2 **Erythromycin and Clindamycin Resistance in MRSA from Hospital Sites**

The total amount of erythromycin resistance was found to be 83% which is not unusual for hospital isolates of MRSA (18, 35). CMRSA2 had the highest number of strains displaying erythromycin and clindamycin resistance (Table 9 Part B-1). CMRSA10 had a high number of strains exhibiting resistance to erythromycin resistance yet a very low number of strains resistant to clindamycin (Table 9 Part B-2). This has been observed in the United States among the USA300 strains (indistinguishable from CMRSA10 by PFGE) (26, 152). A large number of CMRSA7 strains (70%) were

erythromycin resistant. Of the clindamycin resistant strains *ermC* was more prevalent than *ermA* however, *ermA* was found in 100% of CMRSA2 and in all but 1 clindamycin resistant CMRSA10. In CMRSA2 the high prevalence of *ermA* is presumably due to the presence of SCC*mec* type II which carries *ermA* which was identified in at least 3 of the erythromycin resistant CMRSA2 strains tested (unpublished data, personal communication with Michael Mulvey).

4.2.3 Differences in Erythromycin Resistance among CMRSA2 and CMRSA7 Collected from Saskatchewan and Hospital Sites

There were a large number of CMRSA2 strains displaying resistance to erythromycin and clindamycin in Saskatchewan as well as in Canadian hospitals (Table 10 Part C-1). However, these findings were shown to be due to different resistance genes. *ermA* was prevalent in hospital site strains and *ermC* was prevalent in Saskatchewan strains. This was due to the presence of different SCC*mec* regions. It is unclear why CMRSA2 from Saskatchewan would carry *ermC* instead of *ermA*. However, it suggests that among the MRSA and MSSA strains tested, that CMRSA2 may be the most successful at acquiring and maintaining erythromycin and clindamycin resistance.

CMRSA7 from Saskatchewan had a lower number of strains resistant to erythromycin than CMRSA7 strains from hospital sites (Table 10 Part C-2). This may be due to the isolation of the communities in Saskatchewan where the strains were obtained. Additionally, among the erythromycin resistant CMRSA7 in Saskatchewan, there was a lower number of strains that had inducible clindamycin resistance than strains from hospital sites. This is due to the high number of strains in the region containing the *msrA*

erythromycin resistance gene as opposed to containing *ermC* which was most prevalent in the hospital isolates. This is probably reflective of clonal spread of MRSA in the region.

4.3 PVL Negative CMRSA7 Type 0142

PVL was first reported as a common toxin associated with community-acquired MRSA infections by Lina *et al* (1999). PVL has since been reported in 33 to 92% of CA-MRSA infection causing strains (6, 34, 130). PVL is highly associated with SSTIs as well as more severe infections such as necrotizing fasciitis and pneumonia (6, 7, 13, 14, 17, 19, 20, 22, 26, 34, 36, 37, 41, 43, 44, 45, 52, 55, 77, 79, 84, 95, 96, 97, 116, 117, 131, 138, 146, 162, 167, 171, 174).

Our group of PVL negative CMRSA7 type 0142 indistinguishable by PFGE from PVL positive CMRSA7 type 0142 (MW2 or USA400) (100) appears to be unique. To the best of my knowledge there are no reports of PVL negative strains indistinguishable from MW2/USA400 (130, 131). One report does state that only 63% of the USA400 strains were PVL positive however they did not state whether these PVL negative strains were of the same PFGE types as MW2 (152). It was also noted that not all USA300 strains contained PVL however, the strains lacking PVL were subtypes of the major clone and not indistinguishable.

PVL negative strains were confirmed to be lacking PVL by PCR and Southern blot. Both components of PVL, *lukF*-PV and *lukS*-PV, were confirmed to be absent based on PCR aimed at each gene. Initially we speculated that the phage carrying PVL may be absent due to the PCR amplification of the integration site. Here the primers were designed to produce a PCR product of approximately 200 bp if there is nothing integrated

into the attachment site (Figure 10). A negative product was expected if there was the presence of a phage. This reaction was positive in both PVL positive and PVL negative strains which, in both cases was only a faint product. It was not initially clear why PVL positive strains had a product however, it may be explained due to the ΦSa2mw phage having a lytic life cycle that is excised during the normal phage replication processes (69). This would explain the faint positive result in PVL positive strains as a small portion of cells within a culture may have the *att* site empty due to the phage excision. However, this did not fully explain the faint positive result in the PVL negative strains, which lead us to believe that there may be either a truncated phage lacking PVL or a different phage present at the site. Additionally, PCR directed at the 5' end of the phage was positive in all 31 PVL negative and 45 PVL positive CMRSA7 type 0142. This is indicative of the presence of a phage in the PVL negative strains. The 31 PVL negative strains were negative by PCR using primers specific to the 3' end of the phage genome while all PVL positive strains produced a product. Primers unique to a phage present in MRSA252 (EMRSA16) (GenBank accession number BX571856) were used to determine if a different phage is present (5). This reaction was positive in the PVL negative strains and negative in PVL positive strains indicating that a phage different from ΦSa2mw was present in the genome. The products were sequenced and found to be 97% homologous to the phage in EMRSA16 and only 58% homologous to ΦSa2mw (GenBank accession numbers BX571856 and BA000033, respectively) (Figure 11). This suggests that our PVL negative strains may be carrying a phage that is the same or similar to the phage present in EMRSA16, instead of just the loss of the PVL genes.

All of the strains were found within a relatively small region in Canada, with 25 of the strains being isolated in Alberta, 5 more being isolated in Saskatchewan and Manitoba and 2 strains isolated in Ontario over a 3 year time span. This suggests that there may be clonal spread of the strain and that this may be a subtype of the PFGE type 0142 lineage. It is unknown how and why these strains acquired a phage other than the ΦSa2mw. Sequence analysis of the entire region may reveal more about the phage that is present.

There did appear to be a difference among the ages of patients, however it is not known why. It is interesting to note that the PVL negative strains seemed to be present in patients ages 18-65 which suggest that the majority of PVL negative strains are quite capable of infecting healthy young patients. Because there was no difference among the PVL positive and PVL negative strains in the number of infections versus colonizations or the type of infections observed, PVL may not be as important of a determinant in milder infections as previously thought (5, 130). These results further confirm a recent report on the differential expression of PVL (130). It was previously shown that the presence of PVL does not mean that there is a sufficient amount of product expressed to cause lysis of leukocytes, as they found varying levels of expression of PVL among PVL containing strains. Additionally, the same group found that when human leukocytes were infected with PVL positive and PVL negative strains, there was little to no difference in destruction of the cells, in some cases the PVL negative strains appeared more virulent. Our unique strains provided further evidence for the argument that PVL may only compliment the vast array of toxins present in MW2 and is not the sole determinant of infection (5, 65). For example MW2 possess a genomic pathogenicity island υSaα,

which contains 11 putative staphylococcal exotoxins (*set*) genes (5). MW2 also contains a set of bacteriocin genes that may provide MW2 with a selective advantage over the natural flora. Additionally, MW2 contains a collagen adhesion protein that has been associated with osteomyelitis and septic arthritis (5). Further studies using PVL positive and PVL negative CMRSA7 *in vivo* and *in vitro* would be required to determine if PVL is important in the infection process of PVL positive strains. Sequencing of the entire phage and a genomic comparison using microarrays would be useful in determining the presence of other toxin genes in the PVL negative and PVL positive strains.

4.4 CAN-ICU MRSA

The ratio of MRSA to MSSA infections in Canadian ICU is lower than previous reports in ICUs in the United States (29, 127). There was a high level of antimicrobial resistance in MRSA strains which differed greatly from the MSSA strains. This is not unique as HA-MRSA is well known to be resistant to a high number of antimicrobials (152). Respiratory infections accounted for the majority of infections in both MRSA and MSSA. Patients with MRSA infections were significantly younger than patients with MSSA infections in Canadian ICUs which has been observed in other ICU MRSA studies as well as in MRSA infections in general (61, 143). In the present study, it was also observed that males accounted for approximately 60% of the total infections. A difference in gender was also observed in other MRSA ICU studies and MRSA infection studies, which may be a reflection of the male population to the female population in ICUs (93).

CMRSA2 was the most prevalent clone observed however, there was a large amount of diversity of strains which has been previously observed in many Canadian

hospitals (20, 142). This suggests that strains causing infections in the ICU reflect what is occurring in other areas of the hospital.

There was one unique strain that did not match any of the PFGE patterns found in the national database. The strain was PVL positive, an MLST ST22 and SCC*mec* type IVa. The strain had the same sequence type as EMRSA15 and was determined to be related by PFGE. EMRSA15 is the most common clone in England and PVL positive strains have been identified in Kuwait and the Netherlands (114, 157, 167). PVL positive MSSA related to EMRSA15 have been reported in England (65). It is possible that the patient may have recently traveled or may have had contact with a person that has recently traveled to Europe. As EMRSA15 strains have been found in other Canadian hospitals (20), it may also be possible that an EMRSA15 clone acquired PVL from a PVL positive strain.

Of the 7 CA-MRSA infections, 2 were respiratory and 5 were blood infections, both of which have previously been reported among CA-MRSA infections in hospitals (59, 138). CA-MRSA has also been reported to cause SSTIs and infections in prosthetic joints among hospital patients which was not observed in this study (79, 131). It appears that the patients infected with CA-MRSA strains were sporadic and that there was no spread of CA-MRSA among the units.

4.5 Thesis Conclusions

CA-MRSA isolated in Saskatchewan had antibiotic resistance profiles similar to previous reports (100). While it is difficult to speculate due to the low number of MRSA strains available, MSSA and CA-MRSA in the region appeared to have similar resistance profiles, with the highest levels of resistance observed to erythromycin and mupirocin.

The high number of MSSA and CA-MRSA resistant to a low number of antimicrobials is promising. This provides many treatment options for physicians however, erythromycin, clindamycin and mupirocin use should be limited due to the elevated levels of resistance in the region. Future work in the case control studies are currently under way, the results of which should reveal more about the level of antibiotic resistance in CA-MRSA infections as well as in community-associated MSSA. Additionally it is hoped that the case-control study will provide more information on the risk factors of CA-MRSA.

While CA-MRSA was isolated from ICUs in Canadian hospitals, it does not appear that CA-MRSA is circulating in the units involved in this study. It is possible that the patients were colonized or infected with CA-MRSA prior to admission to the ICU. The finding of typical CA-MRSA strains in ICUs is concerning, as regardless of how the patient acquired the infection, it provides CA-MRSA with the opportunity to spread among ICU patients although this has not been observed. It is also possible that the CA-MRSA strains may acquire more antibiotic resistance genes possibly allowing CA-MRSA to become endemic in hospitals. Additionally, if CA-MRSA retains a high level of virulence factors they may lead to many serious infections in already immuno-challenged patients causing an increase in the number of mortalities due to MRSA in hospitals.

The importance of PVL among CA-MRSA infections has yet to be determined. We have found PVL negative strains with similar genetic backgrounds to PVL positive strains. We are unsure if the PVL negative strains are less virulent than their PVL counterparts. The finding of these PVL negative strains brings up the question of what strains should be classified as community-associated MRSA. Our unique strains suggest

that both PFGE and PVL testing may be a minimum requirement to determine if a strain is community-associated and the PVL status of the strain. Additionally, this study has provided further evidence that PVL may not be as important as once thought in CA-MRSA infections. Even though PVL is present in many CA-MRSA strains the finding of PVL negative strains with a similar infection pattern to the PVL positive strains may suggest that PVL may not be as important among mild infections as once thought. Additionally the frequency and importance of other virulence factors should be investigated to determine if there are other factors that may play a role in the virulence of CA-MRSA.

4.6 **Concluding Remarks**

MRSA is a continuing problem in both the health-care setting and is a growing concern in the communities. It is becoming apparent that MRSA is quite capable of adapting to a variety of settings. While CA-MRSA still remains susceptible to many classes of antimicrobials, the possibility of severe infections still remains high. Due to the potential of CA-MRSA to be highly virulent, care should be taken with CA-MRSA in hospitals (14, 19, 43, 45, 95, 174). The close proximity to HA-MRSA may allow for transfer of resistance genes to CA-MRSA. This combined with the potential of higher virulence in CA-MRSA may make room for super-bugs that can kill rapidly with little treatment options. Strict infection control measures should be implemented with CA-MRSA infections as the hospital provides a setting with a high amount of selective pressure for antibiotic resistance.

CA-MRSA should not be taken lightly and with the high number of strains being isolated in communities and health-care settings, surveillance is very important. Due to

the potential of CA-MRSA acquiring antimicrobial resistance genes, infections should be handled quickly with appropriate treatments. While new treatments are currently being investigated, for example a new cephalosporin called ceftobriole (85) and a new vaccine targeted at a protein iron surface determinant B (82), *S. aureus* has proven itself very capable of adapting to the selective pressures of antimicrobials in very short periods of time. It leads to the conclusion that prevention is the best way to control *S. aureus* infections overall.

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