# Molecular epidemiology and clinical impact of heteroresistant vancomycin-intermediate coagulase negative staphylococci in the NICU

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## List of Abbreviations

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| CoNS | Coagulase negative staphylococcus |
| hVICoNS | Vancomycin-intermediate heteroresistant coagulase negative staphylococcus |
| VSCoNS | Vancomycin susceptible coagulase negative staphylococcus |
| hV | Vancomycin-intermediate heteroresistance |
| MRSA | Methicillin resistant Staphylococcus aureus |
| hVISA | Vancomycin-intermediate heteroresistant *Staphylococcus aureus* |
| hVISE | Vancomycin-intermediate heteroresistant *Staphylococcus epidermidis* |
| VSSE | Vancomycin susceptible *Staphylococcus epidermidis* |
| NICU | Neonatal intensive care units |
| STJ | Centre Hospitalier Universitaire Ste-Justine |
| MCH | Montreal Children's Hospital |
| BSI | Bloodstream infections |
| CLABSI | Central line associated BSI |
| HAI | Healthcare-associated infections |
| CLSI | Clinical and Laboratory Standards Institute |
| PFGE | Pulsed-field gel electrophoresis |
| MLST | Multi-locus sequence typing |
| GRD | Glycopeptide Resistance Detection |
| PAP-AUC | Population analysis profile-area under the curve ratio method |
| BMD | Broth microdilution |
| CC | Clonal complex |
| ST | Strain types |

## Abstract

BACKGROUND: Coagulase negative staphylococci (CoNS) have emerged as a leading cause of bloodstream infections (BSIs) in ICUs, particularly in premature neonates. Methicillin resistance is widespread in clinical CoNS isolates; therefore vancomycin is often used as the first-choice antimicrobial therapy. Vancomycin-intermediate heteroresistance – well described in clinical isolates of *Staphylococcus aureus* – has been reported in clinical CoNS isolates. Heteroresistance exists when a vancomycin-intermediate resistant subpopulation of cells is detected in an otherwise susceptible microbial population. Yet, unlike with *S. aureus,* CoNS vancomycin-intermediate heteroresistance is not yet clinically widely accepted. The data collected from this study will be used to help elucidate the burden and severity of vancomycin-intermediate heteroresistant coagulase negative staphylococci (hVICoNS) central line associated BSI (CLABSI) in two tertiary-care NICUs in Québec. The first aim was to describe the molecular epidemiology of the hVICoNS outbreak in a single tertiary care NICU in Québec, and compare it to a second tertiary care NICU without an outbreak. The second aim of this study was to determine whether the duration of bacteremia, as well as risk of thrombocytopenia, differed between patients with a CLABSI due to a hVICoNS compared a vancomycin susceptible CoNS.

METHODS: Between November 2009 and April 2014, all CoNS causing CLABSI were identified in two tertiary care NICUs in Québec through the laboratory information system and the infection control databases. Vancomycin-intermediate heteroresistance (hV) was determined by both the Macro E-test and E- test GRD. Antibiotic susceptibility to daptomycin and linezolid was determined by E-test, following the manufacturer's

recommendations. Clonal relationships were determined using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).

Categorical variables were analyzed using the $X^2$ or the Fisher exact test, and continuous variables were analyzed using the Student *t*-test or the Mann-Whitney test. Simple and multiple linear regression were used to evaluate the association between hVICoNS CLABSI and duration of bacteremia, while adjusting for identified confounding variables. Additionally, univariate and multivariable logistic regression were used to evaluate the association between hV and risk of thrombocytopenia.

RESULTS: Decreased vancomycin susceptibility (hV) was identified in about 88% of all collected strains. All strains were susceptible to linezolid, and few isolates demonstrated daptomycin resistance. Great genetic diversity was observed within the collection, with thirty-one PFGE types identified. The outbreak strains were all confirmed to be heterogeneously vancomycin-intermediate and were polyclonal. The study identified two major clones, PGFE types E and G (ST2), which were found in both NICUs across the five-year study period. This suggests the persistence of highly successful clones that are well adapted to the hospital environment.

For the second aim, 111 patients were included; 98 had an hVICoNS infection. Median length of bacteremia was 4 days (range; 0-33) for patients with hVICoNS, and 4 days (range; 2-8 days) for patients without hVICoNS. The duration of bacteremia was not significantly different between those with and those without an hV infection (*B*: -0.56; 95% CI: -2.76 to 1.65). Further, the risk of thrombocytopenia for patients with and without an hV infection was not significantly different (OR: 0.42, 95% CI: 0.076 to 2.72).

CONCLUSIONS: hVICoNS seems more common than currently realized in the NICU, and certain hVICoNS clones can become endemic to the NICU. The reservoirs for these clones remain unknown at this time, and identification of the reservoirs is needed to better understand the impact of hVICoNS in the NICU and inform infection prevention strategies. Moreover, hVICoNS does not seem to be associated with more prolonged bacteremia or with a higher risk of thrombocytopenia. hVICoNS may not have a significant clinical impact compared to vancomycin susceptible CoNS.

## Résumé

CONTEXTE : Les staphylocoques à coagulase négative (SCN) sont la plus fréquente cause d'infections nosocomiales (IN) dans les unités des soins intensifs (USI), et représente une préoccupation majeure en néonatologie. La résistance à la méthicilline est répandue chez les isolats cliniques de SCN; par conséquent, la vancomycine est souvent utilisée comme traitement de première ligne. L'hétérorésistance à la vancomycine (hV), bien décrite chez les isolats cliniques de *Staphylococcus aureus*, a été aussi rapportée chez les isolats cliniques de SCN. L'hétérorésistance se manifeste par la présence d'une sous population hétérogène de bactéries chez une même souche qui possèdent un niveau intermédiaire de résistance à la vancomycine parmi la population totale qui elle est sensible à la vancomycine. Ce phénomène est encore sujet à controverse chez les SCN. Les résultats recueillis à partir de ce mémoire seront utilisés pour aider à déterminer l'importance clinique des souches de SCN hétérorésistantes à la vancomycine (hVISCN) associées avec des bactériémies nosocomiales sur cathéters centraux (BACC) pour deux unités de soins intensifs néonatals (USIN) tertiaires au Québec. L'objectif principal était de caractériser une éclosion de hVICoNS dans une USIN au Québec, et de la comparer à une seconde USIN n'ayant pas eu d'éclosion. Le deuxième objectif de ce mémoire était de déterminer si la durée de la bactériémie, ainsi que le risque de thrombocytopénie diffère entre les patients avec une BACC à hVISCN comparé à une BACC à SCN sensible à la vancomycine.

MÉTHODOLOGIE: En utilisant le système d'information du laboratoire et les bases de données de la prévention des infections, tous les SCN d'origine nosocomiale causant une BACC ont été identifiés dans deux USIN au Québec entre novembre 2009 et avril 2014.

L’hétérorésistance à la vancomycine a été déterminée par le macro E-test et le E-test GRD. La sensibilité à la daptomycine et au linézolide a été déterminée par E-test, en suivant les recommandations du fabricant. Le niveau de clonalité des souches fut déterminé par électrophorèse sur gel en champs pulsé (EGCP) et par multi-locus sequence typing (MLST).

Les variables catégoriques ont été analysées en utilisant le test du chi-carré ou le test exact de Fisher, et les variables continues ont été analysées en utilisant le test t de Student ou le test de Mann - Whitney. Des régressions linéaires simples et multiples ont été utilisées pour évaluer l'association entre la présence de hVISCN et la durée de la bactériémie, ajustée pour les variables confondantes identifiées. De plus, les régressions logistiques univariées et multivariées ont été utilisées pour évaluer l'association entre hVISCN et le risque de thrombocytopénie.

RÉSULTATS: Une diminution de la sensibilité à la vancomycine a été identifiée dans environ 88 % de toutes les souches analysées. Toutes les souches étaient sensibles au linézolide, et certaines étaient résistantes à la daptomycine. Une grande diversité génétique a été observée parmi les souches, avec 31 types EGCP identifiés. Les souches épidémiques ont toutes été confirmées comme étant hV et étaient polyclonales. L'étude a identifiée deux clones majeurs, les types EGCP E et G (ST2), qui ont été retrouvés dans les deux UNSI tout au long la période de l’étude. Cela suggère la persistance de quelques clones particulièrement adaptés à l'environnement hospitalier.

Pour le deuxième objectif, 111 patients ont été inclus; 98 avaient une infection à hVISCN. La durée médiane de la bactériémie était de 4 jours (écart; 0-33) pour les patients atteints de hVISCN, et de 4 jours (écart; 2-8 jours) pour les patients sans hVISCN. La durée de la bactériémie ne différait pas significativement entre ceux avec et ceux sans infection hV (*B*: -0,56 ; IC à 95% : -2,76 à 1,65). En outre, le risque de thrombocytopénie chez les patients avec et sans infection hV n'était pas significativement différent (OR : 0,42, IC à 95% : 0,076 à 2,72).

CONCLUSIONS: Les hVISCN semblent plus fréquemment retrouvés dans les USIN et certains clones peuvent devenir endémiques. Les réservoirs de ces clones restent inconnus à ce jour, et l'identification de ces réservoirs est nécessaire pour mieux comprendre l'impact des hVISCN à l'UNSI et identifier les stratégies de prévention des infections. En outre, les hVICoNS ne semblent pas être associés à une prolongation des bactériémies ou avec un risque plus élevé de thrombocytopénie. Les hVISCN ne semblent pas avoir un impact clinique significatif par rapport aux SCN sensibles à la vancomycine.

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## Preface and contributions of authors

### Manuscript 1. Heteroresistant Vancomycin-Intermediate Coagulase Negative Staphylococcus in the NICU: A Systematic Review

*Jasmine Chong:* designed and executed the systematic review, drafted the initial manuscript, and approved the final manuscript as submitted.

*Chelsea Caya:* assisted in the design of the systematic review, screening of studies, data collection, and critically reviewed and approved the final manuscript as submitted.

*Simon Lévesque:* approved the design of the study and critically reviewed and approved the final manuscript as submitted.

*Caroline Quach:* approved the design of the study, assisted in the screening of papers, and critically reviewed and approved the final manuscript as submitted.

### Manuscript 2. Molecular epidemiology of vancomycin-intermediate heteroresistant coagulase negative *Staphylococcus* outbreak in the neonatal intensive care unit

*Jasmine Chong:* conducted microbiological and molecular tests, performed analyses, drafted and revised the manuscript.

*Caroline Quach:* oversaw the design and progress of the research project, provided guidance on the interpretation of results, and revised and edited the manuscript.

*Ana Blanchard:* prepared the database of CoNS CLABSI from one NICU, sent strains to the LSPQ, critically reviewed and approved the manuscript.

*Philippe Guillaume Poliquin:* prepared and performed the modified PAP-AUC, critically

reviewed and approved the manuscript.

*George Golding:* prepared and performed the modified PAP-AUC, critically reviewed and approved the manuscript.

*Céline Laferrière:* oversaw the development of the CoNS database from one NICU and transfer of strains to the LSPQ. Critically reviewed and approved the manuscript.

*Simon Lévesque:* oversaw the development and design of the research project, provided guidance on performing microbiological and molecular tests, analyses, and the interpretation of results, and revised and edited the manuscript.

**Manuscript 3. Central line associated bloodstream infections in the NICU: Does vancomycin-intermediate heteroresistance of coagulase negative staphylococci matter?**

*Jasmine Chong*: study design, performed data extraction, statistical analysis, drafted and revised the manuscript.

*Simon Lévesque:* oversaw study design, provided guidance on the interpretation of results, revised and edited the manuscript.

*Ana Blanchard:* prepared the database of CoNS CLABSI from one NICU, sent strains to the LSPQ, critically reviewed and approved the manuscript.

*Céline Laferrière:* oversaw the development of the CoNS database from one NICU and transfer of strains to the LSPQ. Critically reviewed and approved the manuscript.

*Caroline Quach:* oversaw the development of the study design, provided guidance on statistical analyses and interpretation of results, and edited and revised the manuscript.

# Chapter 1. Introduction

Coagulase negative staphylococci (CoNS) are the leading cause of bloodstream infections (BSIs) in neonatal intensive care units (NICU). Patients in the NICU are particularly at risk of healthcare-associated infections (HAI), given their immature immune systems, the acuity of care needed, and the frequency of invasive procedures performed (1, 2). CoNS are notoriously difficult to treat, due to multi-drug resistance in the majority of clinically relevant species. This resistance stems from the presence of antimicrobial resistant genes and biofilm formation (3). Further, methicillin resistance is widespread in clinical CoNS isolates; therefore vancomycin is often the first-choice antimicrobial therapy. However, vancomycin-intermediate heteroresistance, where there exists a vancomycin-intermediate subpopulation of cells in an otherwise susceptible microbial population, has been detected in clinical CoNS isolates (4-8). Vancomycin-intermediate heteroresistance is known to complicate treatment in *S. aureus*, but the clinical relevance of hV remains unknown in CoNS, particularly in the NICU.

## 1.1 CoNS Epidemiology

Coagulase negative staphylococci are a heterogeneous group within the genus *Staphylococcus*, broadly differentiated from species such as *S. aureus* based on the production of coagulase (of which *S. aureus* is positive)(9). CoNS are ubiquitous commensals of the human skin and mucosa, with a preference for areas of higher humidity including the axillae, head, nares, and groin (3, 10-12). *Staphylococcus epidermidis* and *S. haemolyticus* are the most commonly isolated species, with *S. epidermidis* accounting for nearly 75% of clinical isolates (13, 14).

Previously, CoNS were thought to be benign, and have even been shown to prevent colonization from more pathogenic bacteria such as *S. aureus* (3, 15). However, due to advances in medical technology, CoNS have emerged as clinically significant opportunistic pathogens. Further, they are the most common source of infection associated with indwelling medical devices. The National Healthcare Safety Network (NHSN) and the Centers for Disease Control and Prevention (CDC) reported that CoNS are responsible for 30% of nosocomial central-line associated bloodstream infections (CLABSI) in the United States (16). Additionally, CoNS have been shown to be the most frequently isolated organism in Canadian intensive care units (ICU) with regards to CLABSI (17). More specifically, according to a provincial surveillance of CLABSI in Québec, CoNS were responsible for 33.9% of cases from 2014-2015 (18).

### 1.2 CoNS Clinical Manifestations in NICU

Coagulase negative staphylococcus are not as pathogenic as *S. aureus*, and clinical manifestations of CoNS infections are sub-acute or even chronic, non-specific, and relatively mild (13, 19). As such, it is difficult to differentiate between infections caused by CoNS and infections caused by other microorganisms. Common symptoms of CoNS invasive infection include apnea, bradycardia, feeding intolerance, hypoxemia, lethargy, and temperature instability (13). However, more severe and at times lethal courses of infection occur in immunocompromised patients, such as premature infants or patients with leukemia and other cancers (19); those most at risk for CoNS infections being patients with indwelling medical devices. These infections are defined as either local (to the exit site of the device), or systemic. Depending on the type of device and its location in the human body, systemic device-associated infections may manifest as endocarditis,

meningitis, sepsis, and/or vertebral abscesses (9). Meanwhile, local exit site infections are characterized by erythema, purulent drainage, swelling, tenderness, and/or warmth at the exit site (9).

When clinical signs of sepsis are identified, it is important for clinicians to distinguish truly pathogenic CoNS from contaminants. One method commonly used to discriminate true CoNS infections is the "CDC/NHSN Surveillance Definitions for Specific Types of Infections" criteria for laboratory-confirmed primary bloodstream infections (20). According to the criteria, the patient must either i) have a recognized pathogen cultured from one or more blood cultures and the organism cultured from the blood must be unrelated to an infection at another site or ii) have at least one of the following signs/symptoms: fever (>38°C core), hypothermia (<36°C core), apnea, or bradycardia and positive laboratory results are unrelated to an infection at another site and the same common commensal (i.e. coagulase negative staphylococci) is cultured from two or more blood cultures drawn on the same of consecutive days and separate occasions.

Although CoNS BSIs are not as severe as infections with other pathogens, they lead to increased rates of morbidity, such as higher relative risk of bronchopulmonary dysplasia in premature infants with CoNS sepsis compared to premature infants without CoNS sepsis (21, 22). Additionally, research has shown associations between CoNS sepsis and adverse neurodevelopmental impairments, including cerebral palsy (23, 24). Infections with CoNS also lead to higher rates of antibiotic use, prolonged hospital stays, and higher healthcare costs (2, 25).

## 1.3 Host Risk Factors

Risk factors for CoNS infections in neonates in the NICU include low gestational age, low birth weight, use of total parental nutrition, and the total number of central venous catheters inserted since birth (6, 13, 26). A retrospective cohort study conducted by Healy et al. (27) found that determinants of true CoNS infection in neonates include birth-weight of <2000g, and gestational age <34 weeks. Additionally, they determined that an increasing number of central lines before infection positively predicted true CoNS infection (OR=3.5, 95% Confidence Interval (CI) [1.4-8.3] for each additional line). They highlighted that it was the number of breaches of the skin, and not solely the presence of a central line, that predicted infection. Furthermore, premature infants are particularly at risk of infection given their developmentally immature immune systems, and damaged skin/mucosal membranes (9). Extremely premature or very low birth-weight (VLBW) infants also have a high frequency of invasive procedures, such as the insertion of indwelling medical devices, which increases the risk of CoNS infection (9, 27).

## 1.4 Pathogenesis and Virulence Factors

The virulence mechanisms of CoNS are not as well-known as for *S. aureus* and, unlike *S. aureus*, CoNS lack aggressive virulence factors. Rather, the ability of CoNS to colonize polymer surfaces (such as medical devices) and form an adherent multilayered biofilm is critical to its pathogenicity. Biofilm formation can be broken down into three broad steps (9, 13, 28):

1. CoNS cells adhere and bind to the host via organic (skin/tissue) or inorganic matter.
2. Once bound, the cells grow and mature, aggregating into a thick biofilm layer.

3. Cells detach from the biofilm and disperse through the bloodstream to colonize and form biofilms at other sites

A major component of the thick biofilm, polysaccharide intercellular adhesion (PIA in *S. epidermidis*), aids the biofilm to act as a non-specific barrier to host defenses (28). Biofilm production has also been shown to lessen host inflammatory response and inhibit the penetration of antibiotics (13, 29). Another virulent factor thought to aid *S. epidermidis* to successfully colonize the human skin and mucosa is the production of lantibiotics. Lantibiotics are antibiotic-like peptides called bacteriocins, and are active against Gram-positive bacteria. This bactericidal agent allows *S. epidermidis* to eliminate competing microorganisms on the human skin/mucosa that are sensitive to lantibiotics (9, 30).

## 1.5 Clinical Microbiology and Molecular Typing Methods

Coagulase negative staphylococci are aerobic and facultative anaerobic Gram-positive cocci (13). Pasteur and Ogston first identified CoNS in 1880, when they were originally named *Staphylococcus albus* (31). There are over 40 species of CoNS, including *S. haemolyticus*, *S. capitis*, S. *hominis*, and *S. warneri* (28). For clinicians and microbiologists to fully understand disease caused by CoNS, it is paramount to have methods that are both accurate and reproducible to characterize bacterial isolates (32). In general, there are two categories of methods to characterize CoNS (33). The first are phenotypic methods, which aim to differentiate between coagulase-negative and coagulase-positive staphylococci, as well as to determine species-level identification. These methods consist of assessments such as antibiotic susceptibility pattern analyses, biochemical tests, phage typing, and physiological tests (9). These phenotypic methods

are straightforward, with identification kits and automated systems available for routine clinical use (28).

While phenotypic methods of characterization have broadly been used and provide a well-rounded description of the microorganism, they do not have a fast turn-around time and lack in discriminatory power (identification only to species level). For instance, an evaluation of various methods to type *S. epidermidis* determined that phenotypic methods had low discriminatory capacity, differentiating only 5 types among 16 unrelated strains (34). Further, commercially available systems are sometimes unable to identify uncommon isolates, or provide vague identification results (35). Instead, a novel technological approach, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), is now replacing these conventional methods for bacterial identification (36). It is a rapid and cost-efficient diagnostic tool that identifies microorganisms based on the mass of molecules inside the cells (such as biomarkers).

The second group of methods to characterize *S. epidermidis* is genotypic, which are DNA-based techniques that compare the genetic matter of bacterial variants. While phenotypic methods are easily and routinely performed in the majority of clinical microbiology labs, genotypic methods require more specialized equipment and are performed in reference or research laboratories, at this point in time. Genotypic methods comprise of those solely for bacterial identification, such as sequencing a region of the 16s ribosomal RNA, and methods for strain characterization, which subtype bacterial strains and aim to determine the relatedness of strains of the same species. Strain characterization methods include, among others amplified fragment length polymorphism (AFLP), pulse-field gel electrophoresis (PFGE), restriction fragment length

polymorphism (RFLP), and ribotyping (28). Of these, PFGE is the most widely used technique, and involves using restriction enzymes to cut the whole bacterial genome DNA at specific sites into fragments. These DNA fragments are then separated on a gel matrix using an electric field of alternating direction and/or intensity, which results in a DNA fingerprint unique to a single strain. By comparing the fingerprints, scientists can verify if the strains were genetically related (identical/nearly identical fingerprint), or not. PFGE has been shown to be highly discriminatory, and is useful for determining clonal relationships and short-term, local epidemiology (37, 38). For instance, PFGE can be used to describe outbreaks in a hospital or city by providing genetic fingerprints for each infected patient. PFGE can also be used to determine the source of an outbreak, whether from an animal, food, or water.

While PFGE is used to describe local transmission, it is not useful for explaining the population structure of a species (28). Instead, another method, multi-locus sequence typing (MLST), is used to describe the evolution and long-term global epidemiology of microbial species (38). MLST is also discriminatory, and involves sequencing the internal fragments of highly conserved genes, called housekeeping genes. The DNA sequences of different bacterial variants are then compared to evaluate genetic relatedness (38, 39). If the DNA sequences of the housekeeping genes are highly similar, then it is posited that the strains are related. In contrast, if the sequences are highly dissimilar, it is posited that the strains are very distantly related. Through using MLST, scientists were able to build a global population structure of *S. epidermidis*. They believe that clinical *S. epidermidis* disseminated from an original nine epidemic clones that have since undergone significant recombination and evolution (38).

## 1.6 Transmission

In a clinical setting, the most common source of CoNS is the patient's own bacterial microbiota. In addition, CoNS can be transmitted by health-care workers and between patients (9). For instance, in an American NICU, it was revealed that clonal strains of *S. epidermidis* were shared amongst infants and nurses (40). The authors highlighted that hand hygiene alone was not sufficient to prevent cross-transmission between neonates and health-care workers. Moreover, biofilm-producing and multi-drug resistant CoNS have demonstrated its capability to become endemic to the NICU over long periods of time. Huebner et al. (41) described two distinct *S. epidermidis* clones that prevailed in the NICU over a 10-year period. Additionally, Low et al. (42) identified a single *S. haemolyticus* strain endemic to the NICU that remained for 5 years. Therefore for clinicians to truly understand an outbreak in a clinical setting, it is necessary to determine the strains genetic identity. By characterizing the pathogens, clinicians are then able to determine if outbreaks were due to a clonal spread of a bacterial variant, and whereby appropriate measures can be taken to eliminate the clone.

## 1.7 Antimicrobial Treatment

Over 90% of clinical CoNS isolates carry the *mecA* gene, which is associated with beta-lactam antibiotic resistance (methicillin) (25, 43). Therefore, treatment options are limited and glycopeptides are used to treat CoNS infections, with vancomycin as the first-line antimicrobial agent. Apart from vancomycin, there are a limited number of antimicrobial therapies available to treat multi-drug resistant CoNS, including daptomycin and linezolid. Daptomycin is a novel cyclic lipopeptide, and is highly active against CoNS, with no cases of daptomycin-resistant CoNS reported (44). Linezolid is an antimicrobial in the

class of synthetic oxazolidinones, and is highly active against clinical CoNS isolates (reported 98% susceptibility from surveillance studies (45)). Currently, linezolid is only used in cases of vancomycin treatment failure (46, 47). Additionally, the most recent Infectious Diseases Society of America (IDSA) guidelines state that linezolid should not be used as an empirical therapy given the increased risk of mortality associated with Gram-negative BSIs (48). Alternatively, for methicillin-susceptible CoNS isolates, treatment options include first or second-generation β-lactamase-resistant penicillins and cephalosporins (9).

### 1.8 Vancomycin-intermediate Heteroresistance

Vancomycin is the main antimicrobial therapy used to treat both methicillin-resistant *Staphylococcus aureus* and CoNS infections. However, resistance to vancomycin has been identified in clinical *S. aureus* isolates (minimum inhibitory concentration, MIC, >8 mg/L) and reduced susceptibility to vancomycin, a phenomenon dubbed heteroresistance, has been well described in this species (49, 50). Vancomycin-intermediate heteroresistance (hV), where there exists a vancomycin-intermediate subpopulation of cells in an otherwise susceptible microbial population, has also been detected in clinical CoNS isolates (4-8). In terms of clinical impact, patients with vancomycin-intermediate heteroresistant *S. aureus* (hVISA), compared to patients with methicillin resistant *S. aureus* (MRSA), were more likely to have prolonged bacteremia, greater rates of complications such as endocarditis and osteomyelitis, and a higher frequency of rifampin resistance (51), but no difference in mortality (51-53). In contrast, the clinical impact of hVICoNS remains unknown, with no available studies addressing this concern.

The existence of vancomycin-intermediate heteroresistant CoNS strains is troubling as it may lead to the emergence of homogenously vancomycin resistant CoNS. Based on what has been reported with *S. aureus*, it is thought that continuous treatment with vancomycin of hV strains will select for resistant subpopulations. In this case, vancomycin therapy eradicates the susceptible population, leaving the resistant sub-population to proliferate. The mechanism for reduced vancomycin susceptibility in CoNS is not entirely understood. Gene transfer of *vanA* from vancomycin-resistant enterococci is partly responsible for the emergence of vancomycin-resistant *Staphylococcus aureus*. Further, hVISA strains are characterized by a thicker and reorganized cell wall, as well as a slower growth rate (51). Cell-wall thickening in response to glycopeptide resistance has also been demonstrated in CoNS (54). Some researchers posit that vancomycin-intermediate heteroresistance in CoNS is related to the pressure of antimicrobial usage in hospitals and the resulting selection of resistant subpopulations (5). Additionally, vancomycin-intermediate heteroresistance has been suggested to be an intrinsic property of *Staphylococcus capitis* (55). However, the clinical impact of hVICoNS sepsis in the NICU remains unknown, and this thesis seeks to fill this knowledge gap.

## 1.9 hV Detection Methods

According to the Clinical and Laboratory Standards Institute (CLSI) (56), the antimicrobial susceptibility testing standards for vancomycin in CoNS are:

i) Susceptible: ≤4 mg/L

ii) Intermediate: 8-16 mg/L

iii) Resistant: ≥32 mg/L

This means that a CoNS strain would be called resistant if its growth is not inhibited by 32 mg/L of vancomycin (MIC). Typically, broth microdilution and E-test can be used to measure the MIC. Broth microdilution consists of exposing the bacteria to a gradient of antibiotic concentrations on a microtiter plate. Following incubation, the MIC is evaluated by identifying the lowest concentration of antibiotic that stopped bacterial growth. Meanwhile, the E-test is a diffusion method where a defined suspension of bacteria is inoculated onto an agar plate. When it is dried, an E-test strip (strip with increasing gradient of antimicrobial) is added and the antibiotic immediately diffuses onto the plate. MICs are then read directly from the strip following incubation, at the point where the elliptical zone of inhibition intersects with the antimicrobial agent scale on the strip. The sensitivities and specificities of the broth microdilution and the E-test to detect reduced vancomycin susceptibility are 11% and 100%, and 82% and 93% respectively (57).

Vancomycin-intermediate heteroresistance in CoNS is largely undetected by standard laboratory measures such as the broth microdilution or E-test. As mentioned previously, vancomycin-intermediate heteroresistance is when there is a small intermediately resistant subpopulation within a much larger population of cells. The inoculum in standard procedures is thought to be too low to detect these small resistant subpopulations. Modified E-tests such as the GRD (Glycopeptide Resistance Detection) and Macro E-test are considered more reliable to detect heteroresistance, using higher inoculum and various methods to promote cell growth, including longer incubation periods and more nutritious media (50). However, the MICs determined via these methods do not represent the true vancomycin MIC (50). The GRD E-test consists of a double-sided strip with

vancomycin on one end and teicoplanin on the other, while the Macro E-test consists of separate vancomycin and teicoplanin strips. After the bacteria are plated on the medium and the strips are laid on top, technologists are able to determine MIC after incubation by evaluating the inhibition zone diameters. The sensitivities and specificities of the GRD and Macro E-tests are higher than the standard laboratory methods at 88% and 88%, and 96% and 97% respectively (57). Due to the greater discriminatory power of these methods to detect hV, it has been suggested to initially perform Macro/GRD E-tests on all samples and to then confirm positive results with a gold-standard (58).

Population analysis profile-area under the curve ratio method (PAP-AUC) is the most reliable method to determine vancomycin-intermediate resistance and is considered the gold standard for hV detection in *S. aureus*. This method involves exposing bacterial populations to a gradient of antimicrobials, and quantifying the growth of the bacteria at each concentration. The growth is then plotted against vancomycin concentration. To compare the area under the curve, the standard PAP graph of the test strain is compared to the graph of a control/referent hV strain. PAP-AUC ratios between the test and control are based on *S. aureus* and are as follows (58):

i) <0.9: Vancomycin susceptible

ii) 0.9-1.3: Vancomycin-intermediate heteroresistant

iii) >1.3: Vancomycin-intermediate resistant

Although the PAP-AUC method is much more discriminatory to determine hV, it is far too labour intensive and expensive for routine purposes (57, 59). Instead of using this method to screen for hV in all clinical isolates, it is far more efficient to use PAP-AUC to confirm hV determined via other methods (58). However, no standard criteria exist to test

for hV in CoNS, and studies have instead been using *S. aureus* standards. Thus, the clinical relevance of PAP-AUC hV determination in CoNS remains uncertain, and this thesis will seek to address this concern.

## 1.10 Epidemiology of NICU CLABSI in Québec

CoNS are the most common organisms associated with central line associated bloodstream infections (CLABSIs) in ICUs in Québec (17, 60). CLABSI incidence rates have continued to rise in Québec despite the implementation of preventive measures. In particular, one of Québec's largest NICUs has been struggling with an outbreak of CLABSI caused by strains of coagulase-negative *Staphylococcus* that developed heterogeneous intermediate resistance to vancomycin (hVICoNS). Local investigations so far have shown that the strains have demonstrated higher MICs to vancomycin each subsequent year following the outbreak onset. Further, the molecular epidemiology of the outbreak, in terms of CoNS species involved, clonality, and epidemic spread remains unknown. Moreover, clinicians in the NICU hypothesized that hVICoNS was associated with prolonged CLABSI and thrombocytopenia. These observations, and the clinical impact of an hVICoNS outbreak compared to a vancomycin-susceptible CoNS outbreak, are uncertain.

# Chapter 2. Rationale and Study Objectives

## 2.1 Rationale

Heterogeneous vancomycin-intermediate resistance has been well described in *Staphylococcus aureus* strains, with known associations to greater morbidity and prolonged bacteremia. However, much remains unknown in the case of hVICoNS, especially in terms of severity of infection and its clinical implications in the NICU. Potentially, hVICoNS could have a greater clinical impact than vancomycin-susceptible CoNS, meaning that constant surveillance of hV would be critical to avoid further morbidity. Alternatively, if hVICoNS proves to not alter the burden of vancomycin-susceptible CoNS, the expensive and time-consuming constant surveillance of hV would be unnecessary. Further, while PAP-AUC is the gold standard and is recommended to validate hV determination via other methods, the clinical relevance of this method in CoNS remains unknown. As PAP-AUC is much more laborious than GRD and Macro E-tests and are only available in research laboratories, determining whether this method's validations are clinically relevant are important to save both time and money. The hVICoNS CLABSI outbreak in a single NICU in Québec is therefore the perfect context to explore the burden and severity of vancomycin-intermediate heteroresistant CoNS. Additionally, the clonality and spread of the outbreak, as well as the current state of antibiotic susceptibilities in the NICU, can be evaluated.

## 2.2 Objectives

The clinical impact of heterogeneous vancomycin-intermediate coagulase negative staphylococci (hVICoNS) in the NICU remains unknown, therefore the objectives of my thesis are:

1) To conduct a systematic review of scientific literature to investigate the clinical impact of hVICoNS infections, particularly in the NICU.

2) To describe the molecular epidemiology of an outbreak of hVICoNS central-line associated BSI (CLABSI) in a single NICU in Québec, and compare it to a second tertiary care NICU that had not been associated with an outbreak.
    a. Investigate the clonality of hVICoNS strains and determine whether or not endemic clones caused the outbreak.
    b. Determine antibiotic susceptibility of hVICoNS strains to alternative therapies (daptomycin and linezolid).

3) To determine whether the risk of thrombocytopenia and duration of bacteremia is different in patients with an hVICoNS CLABSI compared to patients without a hVICoNS CLABSI.

# Chapter 3. Heteroresistant Vancomycin-Intermediate Coagulase Negative Staphylococcus in the NICU: A Systematic Review

The first manuscript of this thesis is a systematic review of the clinical impact of hVICoNS infections in the NICU. It summarizes the results of 5 cohort, surveillance, and laboratory-based studies, published between 1980-2014. The definition and method for the detection of vancomycin-intermediate heteroresistance varied between studies. Further, none of the studies addressed the clinical impact of hVICoNS, only the prevalence, which ranged from 2.3% to 100% among the included studies.

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## Abstract

**Context:** One NICU in the province of Québec has been struggling with an outbreak of hVICoNS.

**Objective:** To investigate the clinical relevance of hVICoNS on the course of infection, and to determine the impact of hVICoNS sepsis in patients in the NICU.

**Data Sources:** We searched MEDLINE, EMBASE, and PubMed from 1 January 1980 to 1 September 2014.

**Study Selection:** Both observational and interventional studies were considered eligible if they provided data on hVICoNS in the NICU population.

**Data Extraction:** Two investigators independently reviewed studies for data extraction. Data extracted includes: number of CoNS cultures, prevalence of hVICoNS, and clonality of strains.

**Results:** Of the 613 studies identified, 19 studies were reviewed, with 5 studies included in the final review. No studies addressed the clinical significance of hVICoNS in the NICU. The prevalence of hVICoNS in the NICU varied greatly, ranging from 2.3% to 100%.

**Limitations:** Publication bias could not be assessed, and risk of bias in some of the included studies due to small sample size and poor methods reporting. The quality of all included studies was low, and the inclusion criteria restricted to either English or French studies.

**Conclusions:** Our review suggests that vancomycin-intermediate heteroresistance is much more common than previously believed. Our search however did not identify any studies that explicitly assessed any clinical implications of hVICoNS infections, thereby highlighting the need for research to assess the true impact of hVICoNS infection and to determine its significance on patient mortality and morbidity in the NICU.

## Introduction

Coagulase-negative staphylococci (CoNS) have emerged as a leading cause of bloodstream infections (BSI) in intensive care units (ICU)(61, 62). Patients in neonatal intensive care units (NICU) are particularly at risk for healthcare-associated infections (HAI), given their immature immune systems, the acuity of care needed, and the frequency of invasive procedures performed (1, 2). Though CoNS BSI are not as severe as infections with other pathogens, they lead to increased morbidity, such as a higher relative risk of bronchopulmonary dysplasia in premature infants with CoNS sepsis compared to premature infants without CoNS sepsis (22, 63). Additionally, associations between CoNS sepsis and neurodevelopmental anomalies, including cerebral palsy have been observed (24, 64). Infections with CoNS also lead to higher rates of antibiotic use, prolonged hospital stays, and higher healthcare costs (2, 65).

Over 90% of clinical CoNS isolates carry the *mecA* gene, which is associated with beta-lactam antibiotic resistance (methicillin) (49, 66). Vancomycin is therefore often considered as the first-line antimicrobial therapy. Resistance to vancomycin however, has been identified in clinical isolates of *Staphylococcus aureus* (minimum inhibitory concentration, MIC, >8 mg/L), and reduced susceptibility to vancomycin, a phenomenon dubbed heteroresistance, has been well described in this species (hVISA)(67, 68). Vancomycin heteroresistance (hV), where there exists a vancomycin-intermediate subpopulation of cells in an otherwise vancomycin susceptible microbial population, has also been detected in clinical CoNS isolates (67, 69, 70).

Recently, some NICUs have been struggling with outbreaks of central line associated bloodstream infections (CLABSI) caused by strains of coagulase-negative *Staphylococcus* that developed heterogeneous intermediate resistance to vancomycin (hVICoNS). What remains unclear is the actual clinical relevance of hVICoNS on the course of infection. The objective of this systematic review was thus to determine the impact of hVICoNS sepsis in patients in the NICU.

## Methods

### *Search Strategy*

We searched MEDLINE, EMBASE, and PubMed from 1 January 1980 to 1 September 2014 to identify research studies on hVICoNS in the NICU. Details of the search strategy are available in Appendix 1.

Two reviewers (JC and CC) independently screened study titles and abstracts for inclusion. In case of disagreement between the two reviewers, a third reviewer was consulted (CQ). Our review was focused on neonatal populations admitted to the NICU with hVICoNS infections; we excluded studies in older children and adults, and studies that only reported hVISA. Studies written in languages other than English or French, and those presented solely as abstracts at scientific conferences were excluded. Studies were also excluded if they focused on colonization and not bacteremia. Both observational and interventional studies were considered eligible if they provided data on hVICoNS in the NICU population (any measure of prevalence). Finally, we accepted any definition of hospital-acquired bacteremia, all types of techniques used for specimen collection, and any approach to hVICoNS screening/detection.

### *Data Extraction*

Following screening, all relevant studies were independently reviewed by two investigators (JC and CC) for data extraction. The following data were extracted: first author, year of publication, country where the study was conducted, study design, study population, population characteristics (sex, age), number of CoNS cultures, number of patients enrolled, type of cultures used for hVICoNS confirmation, vancomycin heteroresistance criteria, and MIC testing method used. In addition, any reported measure of the prevalence of hVICoNS, clonality of hVICoNS strains, and any reported associations with mortality and morbidity were collected. The quality of each included study was assessed independently (JC & CC) using the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach.

## Results

### *Study selection*

Our search strategy identified 613 records, of which 502 were unique (Figure 1). Of these, 483 were excluded because they did not provide data on hVICoNS bacteremia in the NICU. After a review of 19 selected full-text articles and abstracts, five studies met our inclusion criteria and were included in the present systematic review (Table 1)(6, 7, 55, 68, 71). One included study also examined adult/older pediatric ICU patients, but we excluded that information from the data collection (6).

### *Characteristics of included trials*

Three studies were conducted in Europe (60%), 1 in Australia (16.6%), and 1 in Pakistan (16.6%). All studies were observational and designed as either prospective (2 of 5; 40%)

or retrospective (3 of 5; 60%). In all included studies, isolates of CoNS were collected from blood cultures, with one study also collecting samples from tracheal/bronchial aspirate, urine, cerebrospinal fluid, and purulent exudate (7). D'mello et al. (55) used 4 screening methods to determine vancomycin-intermediate heteroresistance, broth microdilution, E-test, VAN 4 screening, and population analysis profile-area under the curve ratio method (PAP-AUC). Following Wootton et al. (58), strains were determined hV if PAP-AUC ratios were between 1.0-1.3. Rasigade et al. (6) used the BHI screen agar method to determine vancomycin-intermediate heteroresistance, where strains were considered hV if ≥1 droplet plated on the BHI agar plate had ≥2 colonies. Van der Zwet et al.(68) used two methods to confirm vancomycin-intermediate heteroresistance, the first being BHI agar with vancomycin with an aztreonam disk. Strains with enhanced growth around these disks were considered candidates for hV. Further, representatives were confirmed for hV using the PAP method. The authors used a definition of subpopulations resistant to 4 mg of vancomycin per liter at frequencies of $2.8x10^{-5}$, $1.8x10^{-4}$, $3.0x10^{-5}$, $6.5x10^{-5}$, and $3.4x10^{-5}$ for determining vancomycin-intermediate heteroresistance. Villari et al. (7) also used the PAP method to investigate heteroresistance. Finally, Zubair et al. (71) determined vancomycin-intermediate heteroresistance using disk diffusion, following CLSI 2010 (M100-S20) guidelines.

***Quality assessment***

Four of five studies obtained a score of 'low' for study quality, while one received a score of 'very low' (Table 2). Methodological shortcomings in the paper that received a score of 'very low' included a very small sample size (n=9), unclear sampling methods, and possible risk of selection bias (55).

### Prevalence of reduced vancomycin susceptibility

None of the five studies included in this review addressed the clinical significance of hVICoNS in the NICU, particularly in terms of how the clinical course of hVICoNS bacteremia differs from vancomycin susceptible CoNS bacteremia. From these studies, only data on the prevalence of CoNS with reduced vancomycin susceptibility were found. D'mello et al. (55) assessed nine *S. capitis* isolates, all of which demonstrated heterogeneous vancomycin-intermediate resistance, detected through PAP analysis. Rasigade et al. (6) analyzed 40 methicillin-resistant *S. capitis* isolates collected from various NICUs in France, of which 62.5% were identified as hV, and 37.5% were determined resistant (following EUCAST 2010 recommendations). Van der Zwet et al. (68) screened 217 CoNS isolates and found that 22.1% of the strains were hV using the BHI agar method. Using the PAP method, Villari et al. (7) found that all 81 *S. epidermidis* isolates displayed heterogeneous intermediate resistance to vancomycin. Finally, Zubair et al. (71) assessed 388 CoNS isolates, of which 2.3% of the strains demonstrated hV using the disk diffusion method. It should be noted that the prevalence of hVICoNS varied across the studies owing in part to different NICU populations, methods of hV detection, and number/variety of strains collected.

## Discussion

### *Clinical impact*

The primary aim of this systematic review was to assess the literature on hVICoNS and determine its clinical impact in the NICU. Four of the five studies did not explicitly aim to address the clinical impact of hVICoNS bacteremia in the NICU, but did report

prevalence data. The remaining study (68) sought to evaluate whether hV played a role in the therapeutic failure of a single infant. They reported that the infant, from whom a single CoNS isolate was sampled, died from necrotizing enterocolitis during an episode of sepsis caused by hV *S. capitis*. The authors suspected that hV and treatment with β-lactam antibiotics might have played a role in vancomycin therapy failure and subsequent death, but state that the true clinical significance of heteroresistant CoNS remains to be elucidated.

While not much is known concerning the clinical significance of hVICoNS, heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) is known to be associated with persistent bacteremia and vancomycin therapeutic failure (not limited to the NICU population)(53). For instance, Casapao and colleagues found that infections in patients with hVISA were significantly associated with higher vancomycin treatment failure and longer duration of bacteremia compared to patients with sepsis caused by vancomycin susceptible *S. aureus* (VSSA)(72). Moreover, a systematic review and meta-analysis evaluated the clinical significance of hVISA and found a 2.37 times higher glycopeptide treatment failure rate (95% CI, 1.53 to 3.67) in hVISA infections compared to VSSA infections. No significant differences, however in 30-day mortality have been found between hVISA and VSSA infections (53, 72). These clinically relevant associations found in patients with hVISA sepsis can be used as a starting point for what can be learned about hVICoNS infections, particularly in the NICU.

### *Screening methods*

Methods of screening/detection of vancomycin-intermediate heteroresistance were not uniform among the included papers. The modified E-test (BHI agar) was used in two of the included studies (6, 68). Moreover, one of these studies confirmed the positive E-test results to be true positives using the PAP-AUC method (68). PAP analysis was also used in two other studies to determine hV in the CoNS strains (7, 55). D'mello and colleagues detected hVICoNS through broth microdilution, E-test, VAN 4 screening, and PAP analysis. This study found broth microdilution to be the least sensitive method, detecting only one hV isolate. In contrast, PAP analysis detected hV in all isolates (55). Additionally, Villari and colleagues used the disk diffusion method and found all strains to be susceptible to vancomycin. The PAP approach then found that all strains displayed heterogeneous vancomycin-intermediate resistance (7). Zubair and colleagues only used the disk diffusion to detect decreased vancomycin susceptibility in their study (71), however this method is no longer considered acceptable to correctly detect hV (73). Furthermore, these authors followed the CLSI 2010 (M100-S20) guidelines, which recommended at that time that disk diffusion was not a suitable method to determine vancomycin-intermediate heteroresistance. The use of the modified E-test and/or PAP method instead may have detected far more hVICoNS among the collected isolates.

While the PAP method is the most reliable and is considered the gold standard, it is far too labour intensive for routine purposes (68, 74). Performing PAP-AUCs to detect vancomycin-intermediate heteroresistance for all CoNS strains would be ideal, however it is far from feasible in terms of resources and time. The methods of some included papers, as recommended by Walsh and colleagues (74), of performing modified E-tests and then

confirming these results with the PAP method, is a suitable alternative to performing PAP analyses for all strains (7, 55, 68). The sensitivity of the modified E-test as compared to the PAP-AUC is 82 or 96%, and the specificity is 93 or 97%, dependent on the McFarland inoculum (0.5 or 2.0 respectively)(74). The modified E-test (BHI agar) is far more reliable than standard laboratory methods with higher sensitivity and specificity when detecting heteroresistance, and has been proven to be a valid substitute to PAP (74).

***Clonality***

Clonality was evaluated in three of the five included studies. Van der Zwet and colleagues concluded that the 48 hVICoNS (of 217) strains were *S. capitis* and according to amplified-fragment length polymorphism (AFLP) with a cutoff value of 90%, all were identical. They concluded that there was a clonal spread of a single *S. capitis* strain that remained endemic in the NICU from 1998 to 2000 (68). Rasigade and colleagues determined that all of the methicillin-resistant *S. capitis* isolates that were collected from various NICUs in France belonged to the same pulsotype (typing through pulse-field gel electrophoresis, PFGE). These findings indicated a clonal spread of methicillin-resistant *S. capitis* with reduced vancomycin susceptibility in various French NICUs (6). Finally, Villari and colleagues identified four predominant clones among a total of 28, which they found to be more antibiotic resistant than the other clones. Moreover, they determined that strains from the four predominant clonal groups included the strains where growth was inhibited by the highest concentrations of glycopeptides (via PAP, 100 μg of teicoplanin per ml or 50 to 100 μg of vancomycin per ml)(7). These findings of successful hV clones in the NICU suggest that reduced glycopeptide susceptibility may play a role in their prolonged persistence. Among *S. capitis* strains in particular, it has

been suggested that vancomycin-intermediate heteroresistance is an intrinsic property of this particular species, and that increased pressure from vancomycin therapy in the NICU resulted in its selection and success (6, 55).

### *Strengths and weaknesses*

The main strength of this systematic review is that it used a comprehensive search strategy that reviews the current state of hVICoNS in the NICU. Additionally, we conducted the review according to a pre-specified protocol.

Limitations were also present in this review, the first being an insufficient number of studies to adequately interpret a funnel plot to assess the presence of publication bias. Other limitations include risk of bias in some of the included studies due to small sample size and poor methods reporting (mainly the process of selecting participants). The quality of all included studies was low as they were all observational, and the inclusion criteria restricted to either English or French studies, potentially creating bias. Moreover, the primary aim of four of the five studies was not to assess the clinical impact of hVICoNS in the NICU in terms of associations with morbidity and mortality. As addressed above, there was methodological heterogeneity, affecting the generalizability and ability to pool hVICoNS prevalence.

## Conclusion

The prevalence of hVICoNS in the NICU varied greatly in the literature, ranging from 2.3% (71) to 100% (6, 7, 55). This heterogeneity is due in part to differences in the number and species of CoNS strains investigated, clonality of strains tested, as well as the various methods of hVICoNS detection used. The data presented in this review gives an

idea of the clinical prevalence but does not help elucidate the clinical impact of hVICoNS in the NICU. Our review suggests that vancomycin-intermediate heteroresistance is much more common than previously believed. Our search, however, did not identify any studies that explicitly assessed any clinical implications of hVICoNS infections, thereby highlighting the need for research into this topic.

More detailed evaluations are needed to determine the true influence of hVICoNS on the clinical course of infection. Most of the included studies did not collect detailed clinical information from patients, and population characteristics were not separated into hVICoNS and vancomycin susceptible CoNS (VSCoNS). This must be remedied in future studies in order to better understand the clinical relevance of hVICoNS in the NICU. Ideally, this research would be completed prospectively and hVICoNS detection would follow the recommendations of Walsh et al.(74). These future studies could determine whether hVICoNS infection are associated with prolonged septicemia or higher rates of vancomycin therapeutic failure, as are hVISA, or if mortality rates differ between hVICoNS and VSCoNS.

Whether infection caused by vancomycin-intermediate heteroresistant CoNS significantly differs from those of VSCoNS may have implications as to how CoNS are currently treated in the NICU. That is, if hVICoNS are found to be associated with glycopeptide treatment failure, then other treatments should be given in replacement of vancomycin after reduced glycopeptide susceptibility is determined in the causative bacteremic agent. Furthermore, the presence of a vancomycin-intermediate resistant subpopulation in the cells may be of concern as it could lead to future vancomycin resistance. Therefore,

adherence to hospital infection control practices and surveillance of CoNS vancomycin MICs are necessary to avoid vancomycin resistance.

The findings from this systematic review underscore the need for more studies to better comprehend the clinical relevance of hVICoNS infection in the NICU. Apart from a single study where vancomycin-intermediate heteroresistance, according to the authors, may have played a role in vancomycin therapeutic failure in a single infant, there are no studies that even aim to investigate the role of hV in CoNS bacteremia. Further research is necessary to assess the true impact of hVICoNS infection and to determine its significance on patient mortality and morbidity in the NICU.

## Acknowledgements

We sincerely thank medical librarian, Ms. Genevieve Gore, who guided us through executing the initial database search for the systematic review.

## Figures and Tables

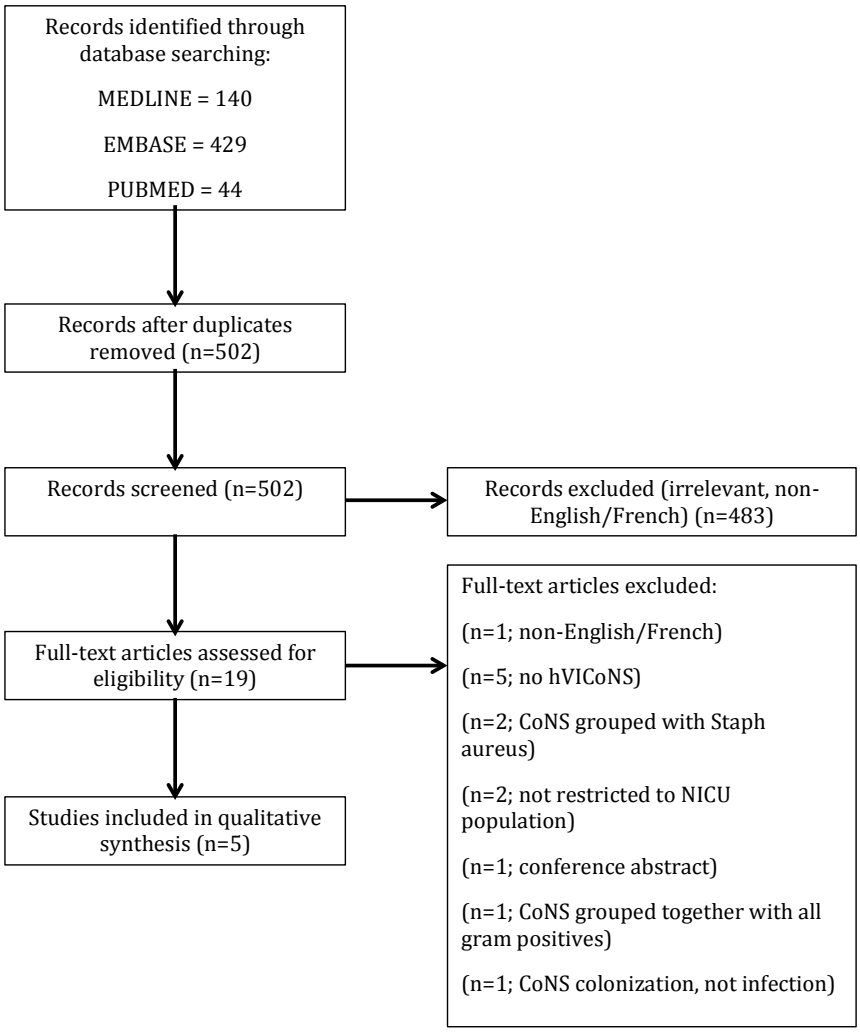


**Figure 1:** PRISMA flow diagram of identification, screening, eligibility, and inclusion of studies.

Table 1. Characteristics of included studies of hVICoNS in the NICU.

| Study | Country | Study Design | Study Population | Patients *n* | Data collection, year or year range |
|---|---|---|---|---|---|
| D'mello et al. 2007 | Australia | Retrospective laboratory based prevalence study of newborns infected with *Staphylococcus capitis*.<br>SC | Very low birth weight infants from the NICU at the Royal Women's Hospital, Melbourne | 9 *S. capitis* isolates isolated from patients | 1998-2002 |
| Rasigade et al. 2012 | France | Retrospective laboratory-based prevalence study.<br>MC | NICU infants >3 days of age from hospital centers in Caen, Limoges, Lyon, Saint-Etienne, Troyes and Versailles | 527 | January 2006 - April 2009 |
| Van der Zwet et al. 2002 | Netherlands | Retrospective observational cohort study.<br>SC | Neonates from the VU University Medical Centre NICU, located in Amsterdam | 163 | 1997-2000 |
| Villari et al. 2000 | Italy | Prospective surveillance of nosocomial infections in a NICU.<br>SC | NICU infants from the University "Frederico II" of Naples | 982 infants, 556 males (56.6%), 426 females (43.4%) | January 1996 - December 1998 |
| Zubair et al. 2011 | Pakistan | Observational cohort study, prospective.<br>SC | NICU infants attending The Children's Hospital and Institute of Child Health Lahore | 388, 252 males (65%), 136 females (35%) | 1st December 2009 - 31st December 2010 |

SC = Single center, MC = Multi center

Table 2. Published studies containing findings of isolate source, prevalence based on method of screening/detection, and clonality

| **Study** | Isolate source | No (%) CoNS isolates | Screening method | No (%) hVICoNS isolates collected | Clonality | Quality of evidence (GRADE) |
|---|---|---|---|---|---|---|
| D'mello et al. 2007 | Blood | 9 *S. capitis* isolates | Broth microdilution<br><br>E-test<br><br>VAN 4 screening<br><br>PAP analysis | 11.1% (1/9)<br><br>33.3% (3/9)<br><br>100% (9/9)<br><br>100% (9/9) | N/A | Very low |
| Rasigade et al. 2012 | Blood | 40 *S. capitis isolates* | Brain heart infusion (BHI) screen agar method to determine heteroresistance (>/1 droplet had >/ 2 colonies) | 100% (40/40) van resistant or heteroresistant (MIC>2 resistant according to EUCAST 2010 using E-test, those MIC ≤ 2 tested for heteroresistance using the BHI agar) | All methicillin-resistant *S. capitis* isolates from NICU patients in France belonged to the same pulsotype | Low |
| Van der Zwet et al. 2002 | Blood | 217 CoNS isolates | BHI agar with vancomycin with a 30-ug aztreonam disk<br><br>PAPs | 22.1% (48/217 strains)<br><br>100% (5/5 representative clinical isolates that were positive by screening) | 1 *S. capitis* strain remained endemic in the NICU since 1998 and was the causative agent for about 1/3 of CoNS bacteremia cases in the unit | Low |
| Villari et al. 2000 | Blood, tracheal/bronchial aspirate, urine, cerebrospinal fluid, purulent exudate | 81 *S. epidermidis* isolates (50.6% determined to be involved with infection) | Disk diffusion methods<br><br>PAPs | 0%<br><br>100% | Four predominant clones | Low |
| Zubair et al. 2011 | Blood | 388 CoNS isolates | Kirby-Bauer disc diffusion method | 2.3% (9/388 isolates) | N/A | Low |

Appendix 1. Search strategy

| Search: hVICoNS in the NICU<br><br>Database: Pubmed | Search: hVICoNS in the NICU<br><br>Database: EMBASE + Classic EMBASE | Search: hVICoNS in the NICU<br><br>Database: Medline |
|---|---|---|
| ((((((neonatal intensive care units[MeSH Terms]) OR ("Intensive Care, Neonatal"[Mesh] OR "Intensive Care Units, Neonatal"[Mesh])) OR nicu[Text Word])) AND ((((((Staphylococcus cohnii[title/abstract]) OR Staphylococcus caprae[title/abstract]) OR Staphylococcus capitis[title/abstract]) OR Staphylococcus warneri[title/abstract])) OR (("Staphylococcus lugdunensis"[Mesh] OR "Staphylococcus hominis"[Mesh] OR "Staphylococcus haemolyticus"[Mesh] OR "Staphylococcus epidermidis"[Mesh] OR "Staphylococcus saprophyticus"[Mesh] OR "Staphylococcus"[Mesh])))) AND (((((resistan*) OR susceptibility) OR heteroresist*) OR reduced susceptibility) OR intermediate susceptibility)) AND ((((intermediate vancomycin susceptibility) OR Vancomycin Resistance[MeSH Terms]) OR Vancomycin[MeSH Terms]) OR vancomycin[Text Word]) | 1. neonatal intensive care unit.mp.<br>2. newborn intensive care/<br>3. newborn sepsis/<br>4. nicu.mp.<br>5. newborn/<br>6. 1 or 2 or 3 or 4 or 5<br>7. Staphylococcus warneri/ or Staphylococcus capitis/ or staphylococcus.mp. or Staphylococcus cohnii/ or Staphylococcus epidermidis/ or Staphylococcus haemolyticus/ or coagulase negative Staphylococcus/ or Staphylococcus lugdunensis/ or Staphylococcus hominis/ or Staphylococcus caprae/ or Staphylococcus saprophyticus/<br>8. (epidermidis or warneri or haemolyticus or capitis or caprae or cohnii or hominis or lugdunensis or saprophyticus).mp.<br>9. Staphylococcus infection/<br>10. staphylococcal bacteremia/<br>11. 7 or 8 or 9 or 10<br>12. antibiotic resistance/<br>13. (heteroresist* or susceptibility or resistance).mp.<br>14. antibiotic sensitivity/<br>15. 12 or 13 or 14<br>16. vancomycin.mp. or vancomycin/<br>17. 6 and 11 and 15 and 16 | 1. Intensive Care Units, Neonatal/<br>2. Intensive Care, Neonatal/<br>3. nicu.mp.<br>4. (intensive care adj10 (neonat* or newborn* or new born*)).mp.<br>5. newborn.mp. or Infant, Newborn/<br>6. 1 or 2 or 3 or 4 or 5<br>7. Staphylococcal Infections/<br>8. Staphylococcus/<br>9. coagulase negative staph*.mp.<br>10. staphylococci.mp.<br>11. staphylococcus epidermidis.mp.<br>12. (epidermidis or staphylococcus warneri or warneri or staphylococcus haemolyticus or haemolyticus or staphylococcus capitis or capitis or staphylococcus caprae or caprae or staphylococcus cohnii or cohnii or staphylococcus hominis or hominis or staphylococcus lugdunesis or lugdunesis or staphylococcus saprophyticus or saprophyticus).mp.<br>13. Staphylococcus warneri/ or Staphylococcus capitis/ or Staphylococcus cohnii/ or Staphylococcus epidermidis/ or Staphylococcus haemolyticus/ or Staphylococcus lugdunensis/ or Staphylococcus caprae/ or Staphylococcus hominis/ or Staphylococcus saprophyticus/ or Staphylococcus/<br>14. 7 or 8 or 9 or 10 or 11 or 12 or 13<br>15. (heteroresist* or susceptibility or resistance).mp.<br>16. Vancomycin/<br>17. Vancomycin Resistance/<br>18. vancomycin.mp.<br>19. 16 or 17 or 18<br>20. 6 and 14 and 15 and 19 |

## Chapter 4. hVICoNS in the NICU

From my systematic review, it is apparent that the knowledge surrounding the impact hVICoNS has in the NICU is incredibly lacking. The five studies included in the review demonstrated that hVICoNS are opportunistic hospital-acquired pathogens that can remain in the hospitals for years at a time, but its clinical significance remains to be determined. There also was an incongruity amongst the published studies concerning the methods used to determine hV status. Also, while PAP-AUC is considered the gold standard for hV in *S. aureus*, this has yet to be validated in CoNS.

Expanding the knowledge of hVICoNS in the NICU is necessary. Studies that specifically aim to discern the clinical importance of hVICoNS, with accurate assessments of vancomycin-intermediate heteroresistance, are needed. This was the rationale for my thesis, which consists of two further manuscripts. To address the clinical impact of hVICoNS, it is first necessary to fully understand the molecular epidemiology of hVICoNS strains. The study in the following chapter is a manuscript to be submitted for publication, which aims to give a comprehensive molecular overview of the hVICoNS CLABSI outbreak described earlier in the introduction. The cohort includes all infants with a CoNS CLABSI from either the outbreak NICU or the non-outbreak NICU. The clonality, spread, and the evolutionary relationships between the strains, as well as antibiotic susceptibilities to alternative therapies will be evaluated. I performed PFGE and MLST to molecularly type the CoNS strains from both NICUs, as well as to describe their spread and relatedness. I also performed GRD and Macro E-tests, as well as confirmation by PAP-AUC to determine hV status.

# Chapter 5. Molecular epidemiology of vancomycin-intermediate heteroresistant coagulase negative *Staphylococcus* outbreak in the neonatal intensive care unit

## Authors

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## Abstract

Coagulase negative staphylococci (CoNS) have become the leading cause of bloodstream infections (BSIs) in ICUs, particularly in premature neonates. Heterogeneous vancomycin-intermediate CoNS have been identified as sources of BSI worldwide, and its potential to emerge as a significant pathogen in the NICU remains uncertain. This study described the molecular epidemiology of an outbreak of heterogeneous vancomycin-intermediate coagulase negative staphylococci (hVICoNS) central line associated BSI (CLABSI) in a single tertiary care NICU, and compared it to a second tertiary care NICU that had not been associated with an outbreak.

Between November 2009 and April 2014, 133 CoNS CLABSIs were identified in two tertiary care NICUs in Québec. Decreased vancomycin susceptibility (hV) was identified in about 88% of all collected strains. All strains were susceptible to linezolid, and few isolates demonstrated daptomycin resistance. Great genetic diversity was observed within the collection, with thirty-one PFGE types identified. The outbreak strains were all confirmed to be vancomycin-intermediate heteroresistant and polyclonal. The study identified two major clones, PFGE types E and G (ST2), which were found in both NICUs across the five-year study period. This suggests the persistence of highly successful clones that are well adapted to the hospital environment.

hVICoNS seems more common than currently realized in the NICU, and certain hVICoNS clones can become endemic to the NICU. The reservoirs for these clones remain unknown at this time, and their identification is needed to better understand the impact of hVICoNS in the NICU and inform infection prevention strategies.

## Introduction

Coagulase-negative staphylococci (CoNS) typically reside on healthy human skin and mucus membranes, rarely cause disease, and are most frequently encountered by clinicians as contaminants of microbiological cultures. However, CoNS have increasingly become recognized as healthcare-associated pathogens, causing infections in patients with predisposing factors such as immunodeficiency and/or indwelling or implanted foreign polymer bodies (28). CoNS have also emerged as a leading cause of central line associated bloodstream infections (CLABSI) in intensive care units (ICU) with *Staphylococcus epidermidis* as the most common cause of CoNS infections (33, 61, 62), Patients in neonatal intensive care units (NICU) are particularly at risk for healthcare-associated infections (HAI), given their immature immune systems, the acuity of care needed, and the frequency of invasive procedures performed.(1, 2)

Pulse-field gel electrophoresis (PFGE) has been the most widely used technique to genetically characterize *S. epidermidis* strains. This method is used for short-term epidemiological investigations, and has been shown to be very sensitive in detecting genetic diversity among strains (37). Most recently, an improved multilocus sequence typing (MLST) scheme was developed to provide further information on the evolution, population structure, and long-term global epidemiology of *S. epidermidis* (39). With this method, Miragaia et al. have identified that the global nosocomial *S. epidermidis* population is composed of nine clonal lineages. The most predominant of these clonal lineages was clonal complex two (CC2), which itself is composed of numerous sequence types (ST); ST2 being recognized as the representative of hospital-acquired methicillin-resistant *S. epidermidis* strains (38, 75, 76).

About 90% of clinical CoNS isolates carry the *mecA* gene, which confers oxacillin (methicillin) resistance (49, 66). Therefore, vancomycin is often used as the first-line antimicrobial therapy. However, since the late 1980s, resistance to glycopeptides has been reported in CoNS. This resistance appears to be endogenous, does not seem to be associated with plasmids or transposons, and can be selected in vitro by exposure to teicoplanin or vancomycin. Although the basis for this glycopeptide resistance is still unclear, it seems to be associated with ultrastructural changes (thickened cell wall), and a reduction in the rate of autolysis (77). Reduced susceptibility to vancomycin, a phenomenon dubbed vancomycin-intermediate heteroresistance, has been well described in *Staphylococcus aureus* (hVISA) (67, 68). Vancomycin-intermediate heteroresistance (hV), where a vancomycin-intermediate resistant subpopulation of cells exists in an otherwise susceptible microbial population, has also been detected in clinical CoNS isolates (67, 69, 70).

Recently, one NICU in the province of Québec has been struggling with an outbreak of central line associated bloodstream infections (CLABSI) caused by strains of coagulase-negative *Staphylococcus* that seemed to have developed heterogeneous intermediate resistance to vancomycin (hVICoNS). In-hospital surveillance investigations so far showed that strains have demonstrated higher minimum inhibitory concentrations (MIC) to vancomycin each subsequent year following the outbreak onset.

In this study, we analyzed the molecular epidemiology of CoNS isolates that resulted in CLABSIs from two tertiary care NICUs in Québec: the first being the hospital described above with the outbreak, and the second as a hospital without a reported CoNS CLABSI outbreak. The second hospital had a stable rate of CLABSI throughout the study period,

compared to the first hospital that had a significant rise in CLABSI rates, above their baseline rate. We also studied the levels of antibiotic resistance and clinical data to compare and identify differences between CoNS isolates from the two hospitals. Further, we aimed to determine if endemic clones in the first hospital caused the outbreak, as well to determine the clonal relationships of the CoNS strains from both hospitals.

## Materials and Methods

### *Study population and setting*

The Centre Hospitalier Universitaire Ste-Justine (STJ), a level III-IV NICU, is composed of 65 beds with approximately 1100 admissions annually. STJ has an inborn patient population and also serves as a reference center for other hospitals in the province of Québec. The Montreal Children's Hospital (MCH) was a level III NICU, composed of 24 beds with approximately 450 admissions annually, prior to the move to the new hospital site in 2015. The MCH does not have in-house deliveries, but is a reference center where newborns requiring tertiary care are admitted from other hospitals centers in the province of Québec. Both NICUs participate in the mandatory provincial surveillance program for CLABSIs (SPIN-BACC) (60, 78). Additionally, both NICUs freeze and store systematically all isolated organisms from sterile body sites. The Research Ethics Board of the McGill University Health Centre approved this retrospective laboratory-based cohort study.

All patients admitted to one of the two tertiary care NICUs from November 1$^{st}$ 2009 through March 31$^{st}$ 2014 who developed a CLABSI were eligible for inclusion in the study. Patients were excluded if they had a CoNS CLABSI upon entry to the NICU.

Additionally, cases of early-onset sepsis, occurring in the first 48 hours of life, were excluded to ensure collected organisms were associated to the NICU. CoNS bloodstream isolates were identified retrospectively through clinical microbiological databases of blood cultures drawn from neonates of each hospital. Isolates were included in the study only if medical chart review and local infection control database determined that they fulfilled CDC/NHSN criteria (see definitions below). Clinical data were collected retrospectively and included: date of birth, gender, birth weight, gestational age, date of admission to and discharge from the NICU, date of sepsis-onset, and all-cause mortality.

***Definitions***

CoNS-positive blood cultures were defined using the "CDC/NHSN Surveillance Definitions for Specific Types of Infections" criteria for laboratory-confirmed primary bloodstream infections (20). According to the criteria, the patient must either i) have a recognized pathogen cultured from one or more blood cultures; the organism cultured from the blood must be unrelated to an infection at another site or ii) have at least one of the following signs/symptoms: fever (>38°C core), hypothermia (<36°C core), apnea, or bradycardia; positive culture results are unrelated to an infection at another site; and the if the organism is considered a common skin commensal (e.g., coagulase negative staphylococci), it must be cultured from two or more blood cultures drawn on the same or consecutive days, at different sites or time. Additionally, for patients with more than one CLABSI, only the initial CLABSI was considered.

***Strain identification***

Strains were identified by the VITEK-2 system (bioMérieux, Marcy-l'Etoile, France) in

both hospital laboratories, and if strain identification was not completed prior to collection for our project, sequencing of *tuf* gene was done to identify *Staphylococcus* strains to the species level (79).

### *Antimicrobial susceptibility testing*

Resistance to vancomycin was evaluated according to the Clinical & Laboratory Standards Institute (CLSI) broth microdilution reference method (susceptibility ≤4 mg/l) (56). Collected isolates were also tested for vancomycin-intermediate heteroresistance with the GRD and Macro E-tests according to CLSI and manufacturer recommendations (bioMérieux, Marcy-l'Etoile, France) (80, 81). These E-tests are more sensitive than usual automated systems to detect hV, as only a subpopulation of cells express vancomycin-intermediate heteroresistance (57). Isolates were considered hV if they were positive either by GRD E-test or Macro E-test. Furthermore, the E-test was used following manufacturer's recommendations to evaluate antibiotic susceptibility of hVICoNS strains to alternative therapies (daptomycin and linezolid). MIC interpretive criteria followed CLSI guidelines as follows: susceptible to daptomycin ≤1 mg/l and susceptible to linezolid ≤4 mg/l.

### *Population analysis profiling-area under the concentration-time curve (PAP-AUC) testing*

PAP-AUC testing was performed at the National Microbiology Laboratory in Winnipeg, Canada following two different protocols. The first protocol (PAP-AUC 1) employed an adaptation of the method described by Wootton *et al*. (82) for the detection of vancomycin-intermediate heteroresistance in *Staphylococcus aureus*. In brief, isolates of

interest were stored at -80°C until they were ready to be plated onto TSA blood agar plates. Following overnight incubation at 37°C in air, well isolated colonies were inoculated into tryptone soya broth (TSB) for overnight incubation in air. The resulting bacterial suspension turbidity was adjusted to a 2 McFarland standard using sterile 0.9% saline, and serially diluted ten-fold in sterile 0.9% saline. The undiluted, $10^3$ and $10^6$ dilutions were spiral plated using the Eddy Jet (IUL, SA; Barcelona, Spain) spiral plater onto blood-heart infusion agar plates infused with increasing concentrations of vancomycin (0, 0.5, 1, 2, 2.5, 3, 4, 6 and 8 mg/l). Plates were incubated for 48 hours in air and colonies were manually counted using the grid system provided with the Eddy Jet system. AUC ratios were generated using GraphPad Prism version 5 (LaJolla, California, USA), comparing the tested isolates against ATCC strains 29213, 700698 (Mu3), 700699 (Mu50) as well as a heteroresistant vancomycin-intermediate *Staphylococcus capitis* isolated from a Manitoba neonate. A positive PAP-AUC test was determined by an AUC ratio of ≥0.9 when the isolate was compared to Mu3 strain.

The vancomycin-intermediate heteroresistance phenotype has been shown to be unstable when isolates are serially passaged in the laboratory (83). We determined that the majority of isolates had been passaged at a minimum of five times on non-antibiotic containing media, which might have led to a loss of phenotype. To overcome this instability, a second protocol (PAP-AUC 2) was used where isolates were plated first to a TSA blood agar plate and, following overnight incubation, were then inoculated into TSB broth supplemented with 2 µg/ml of vancomycin (instead of plain TSB broth). The remainder of the protocol was unchanged. To exclude the possibility that a single passage of *Staphylococcus epidermidis* in the presence of vancomycin was able to induce hV in

previously non-hV strains, a sample of non vancomycin-intermediate heteroresistant strains of *S. epidermidis* (from MCH) were tested using this protocol.

***PFGE***

Bacterial DNAs were digested with *Sma*I and samples were run on a Chef-DR III or GenePath system (BioRad, Mississauga, On, Canada) (84). Banding patterns of *S. epidermidis* isolates were analyzed with BioNumerics software version 6.5 (Applied Maths, Austin, Texas, USA) and confirmed visually according to the criteria of Tenover et al. (85). Letters identified the different PFGE patterns. Similarity coefficients were obtained within BioNumerics by calculating Dice coefficients. Cluster analysis was done with the unweighted pair group method with arithmetic averages (UPGMA). Band position tolerances of 1% and optimization values of 0.8% were used for all analyses.

***MLST***

MLST was performed according to the protocol of Thomas et al. (39). Analysis was performed within BioNumerics using the MLST plugin related to the MLST *S. epidermidis* database (http://sepidermidis.mlst.net) for allele and sequence type (ST) assignment. The eBURST method was used to infer the evolutionary relatedness of ST (http://eburst.mlst.net/) (86).

***Statistical analysis***

Differences in gestational age, average age at sepsis onset, and average length of stay were analyzed using the Mann-Whitney test. The difference in gender, birth weight category, and 28-day mortality were using the $\chi^2$ or Fisher's exact test. *P*-values of $\leq 0.05$

were considered statistically significant. All statistical tests were performed using STATA Statistical Software, version 12 (STATA Corp, College Station, TX). The correlation between typing methods and the correlation between vancomycin-intermediate heteroresistance determination methods were evaluated using the Adjusted Rand's Index (ARI) and Wallace's coefficient (W) (87). The ARI represents the congruence between type assignments of different typing methods, taking into account chance agreement (88). Coefficients close to 0 indicate a lack of agreement among typing methods and conversely, coefficients close to 1 indicate great agreement between typing methods. Meanwhile, the Wallace coefficient estimates that isolates typed the same using one method will also be predicted as the same type given a second method. Here, a high W coefficient indicates that the type assignment given by the first method will likely be predicted the same by the second method. The ARI and W coefficients were calculated using tools available from http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home (87, 89, 90).

## Results

### *Patient and strain characteristics*

A total of 133 CoNS isolates were collected during the study period: 87 isolates from 87 patients at STJ, and 46 isolates from 35 patients at MCH. The greater number of isolates than patients at the MCH is due to three instances of polymicrobial infections, whereby two or more CoNS organisms were isolated for the same episode. Of the 133 presumed CoNS isolates collected, 1 *S. hominis*, 1 *S. capitis*, and 12 *S. warneri* were confirmed by *tuf* gene sequencing. The remaining 119 *S. epidermidis* isolates (79 from STJ and 40 from MCH) were used for further laboratory analysis and are the focus of this study.

Patients from STJ were younger than their MCH counterparts, with a mean gestational age of 27.5 weeks and a mean age at CoNS sepsis onset of 20.6 days, compared to a mean gestational age of 30.9 weeks and a mean age at CoNS sepsis onset of 72.5 days. Additionally, 41.8% of STJ patients had a birth weight less than 750g, while 32.5% of MCH patients weighed less than 750g at birth. The twenty-eight day mortality for the cohort of patients from STJ and MCH were 16 deaths (20.2%) and 1 death (2.5%), respectively. The remaining demographic characteristics of patients from STJ and MCH are summarized in Table 1.

***Antimicrobial resistance***

The antimicrobial susceptibility results are shown in Table 2. Five (6.3%) isolates from STJ were non-susceptible to daptomycin, whereas all of the isolates from MCH were susceptible. All isolates from both hospitals were susceptible to linezolid. Among isolates from MCH, 12 (30%) demonstrated reduced susceptibility to vancomycin (determined with broth microdilution, vancomycin MIC $\leq$4 mg/l), whereas all STJ isolates were susceptible. However, using the macro and GRD E-tests, all isolates (100%) from STJ demonstrated vancomycin-intermediate heteroresistance, whereas 65% of isolates from MCH were hV.

***PFGE***

Of the 119 *S. epidermidis* isolates, six were untypeable by the PFGE method. The remaining 113 isolates were grouped into 31 distinct PFGE patterns (Appendix 1 and Table 3). Twenty-one PFGE patterns were STJ-specific and of these, 11 PFGE patterns were represented by a single isolate (singletons). Five PFGE patterns were MCH-specific,

of which 4 PFGE patterns were singletons. Finally, 4 PFGE types (AB, D, E, and G) harbored isolates from both hospitals. The PFGE pattern including the highest number of strains was PFGE pattern G (29 isolates (25.4%), 22 from STJ and 7 from MCH), followed by PFGE patterns E (28 isolates (24.6%), 8 from STJ and 20 from MCH) and Q (7 isolates (6.1%), all STJ).

***Time distribution of PFGE patterns***

PFGE pattern G was the most predominant at STJ. The first pattern G clone from STJ appeared in Q1 2010, and remained an etiology for CLABSI until the end of the study period (Figure 1). PFGE pattern E from STJ was also a major clonal group, first appearing later in Q3 2011, and spanned the study period until Q4 2013. Clones belonging to PFGE pattern Q were limited to STJ (the third predominant clone) and also appeared later in Q3 2011. These strains became more frequent from 2013-2014. Conversely, PFGE pattern E from MCH was the predominant clone, and was found from the start of the study period until Q3 2013. The other major clone at MCH, PFGE pattern G, was found from Q2 2010 until the end of the study period.

***MLST sequencing***

Thirty-nine isolates were typed by MLST (at least one selected per PFGE pattern and all isolates that were untypeable by PFGE). In total, 14 different STs were identified, 10 (71.4%) of which were represented by a single isolate (Table 3 and Appendix 1). ST2 was the most common ST, compromising 53.8% (21 of 39) of the typed isolates, including all untypeable isolates. There were 8 STs unique to STJ, 4 STs unique to MCH, and 2 STs that were common to both NICUs. All of the 14 STs have previously been

recorded in the MLST database (as of January 2016). The eBURST algorithm clustered these STs into one major clonal complex (clonal complex 2, CC2) (Figure 2). It was previously suggested that CC2 could be separated into clusters and subgroups (37). However, with the MLST database populating, new subgroup founders were identified by the eBURST algorithm. Our isolates were classified into 11 different subgroups amongst the CC2.

***Relationship of PFGE types to MLST***

To measure the concordance between PFGE and MLST (Table 3 and Figure S1), ARI and Wallace coefficients were calculated. The ARI was 0.054, indicating a very low congruence between PFGE and MLST. The concordance of type assignments between PFGE and MLST were also low ($W_{PFGE \rightarrow MLST}$ = 0.567 [0.345-0.789], $W_{MLST \rightarrow PFGE}$ = 0.028 [0.000 – 0.101]). For instance, PFGE pattern E consists of isolates typed ST2 and ST54. Conversely, strains typed ST2 consists of isolates belonging to 14 PFGE patterns, including E and G. Therefore here, ST is not predictive of PFGE pattern, and vice-versa.

***Relationship of PFGE patterns to antimicrobial resistance***

At the MCH, 15 of 20 (75%) PFGE pattern E isolates were vancomycin-intermediate heteroresistant, as well as 2 of 8 (25%) PFGE pattern G isolates. While all the MCH isolates were daptomycin-susceptible, there was a correlation between vancomycin-intermediate heteroresistance and increased daptomycin MICs. For instance, pattern G hV strains had higher daptomycin MICs than the vancomycin susceptible strains (0.38 mg/l vs. 0.19 mg/l, $p$=0.036). This correlation of higher levels of vancomycin-intermediate resistance and daptomycin resistance was also found among the isolates

from STJ. All isolates from STJ were hV and the daptomycin MIC50 and 90 for STJ were higher than those for MCH (Table 2). Further, from STJ, five of the six daptomycin non-susceptible strains were from PFGE pattern E.

### *PAP-AUC*

Thirty-six isolates were tested by PAP-AUC (at least one selected per PFGE pattern and all isolates that were untypeable by PFGE amongst the hV isolates). In addition, 10 isolates from MCH that were not hV by E-tests were tested (see Materials and Methods section). Following the first protocol (PAP-AUC 1), none of the 36 isolates demonstrated vancomycin-intermediate heteroresistance. However, with the second protocol (PAP-AUC 2), 32 isolates (88.9%) showed vancomycin-intermediate heteroresistance. The 10 isolates that were hV negative by E-tests were not shown to be hV with either PAP-AUC protocol (Appendix 1).

## Discussion

In this study, we demonstrated the clonal dissemination and endemic persistence of two major clonal groups within two NICUs in Québec over a 5-year period. From the data, *S. epidermidis* was the most frequently isolated organism in CLABSI cases. Using results from the GRD and Macro E-tests, all strains from the outbreak hospital (STJ) were hV, as were about 65% of strains from MCH. Moreover, isolates with hV of the same PFGE pattern and ST were isolated from patients in the two NICUs. This indicates that hVICoNS can persist within hospitals and may be more common than previously expected. However, the clinical significance of hVICoNS remains unknown. In *S. aureus*, vancomycin resistance is associated with prolonged bacteremia, high-inoculum infections,

and vancomycin therapeutic failure (53). To date, the clinical impact of hVICoNS in the NICU has yet to be addressed.

Vancomycin-intermediate heteroresistance determination among isolated strains using the broth microdilution (BMD) and E-test methods were discordant (ARI and W < 0.11), although this is not uncommon. E-tests are far more reliable than routine laboratory methods in detecting vancomycin-intermediate heteroresistance, with higher sensitivity and specificity (55, 57). Compared to BMD methods, E-tests use higher volumes of inoculum, and plating allows for visibility of micro-colonies in the zones of inhibition (91). Here, we showed that MIC determination using the routine laboratory method is not foretelling of hV status, and that surveillance of vancomycin MICs alone may be inappropriate to monitor hV.

Persistence of hVICoNS in the NICU has been reported in other studies, with prevalence ranging from 22.1 to 100% of collected CoNS isolates (6-8, 55). Additionally, the clonality of hVICoNS in the NICU has been previously described. For instance, Rasigade et al. reported the clonal spread of a *S. capitis* strain with reduced vancomycin susceptibility to various NICUs throughout France (6). Moreover, Villari et al. found four predominant *S. epidermidis* clones over a three-year period in Italy (7). They emphasized the importance of cross-transmission and selective pressure from glycopeptide use in the high prevalence of hVICoNS in the NICU. These findings of successful hVICoNS clones suggest that reduced glycopeptide susceptibility may play a role in their prolonged persistence.

Due to the rise in vancomycin-intermediate heteroresistance, we evaluated the strains

antimicrobial susceptibilities to known alternative therapies. All of the strains were susceptible to linezolid, even though over the final two years of our study, 29 STJ patients were treated with linezolid. Resistance could have been expected after administration of linezolid due to therapeutic pressure and selection of linezolid-resistance sub-types, but this was not seen. The reported prevalence of linezolid resistance is rare, with about 2% of clinical CoNS isolates exhibiting resistance globally (46). However, it has been discovered that CoNS species acquire resistance after linezolid treatment much more rapidly than *S. aureus* (47). An outbreak of linezolid-resistant CoNS has recently been reported in a tertiary referral university hospital in Ireland. The outbreak was quickly contained through infection control practices and cessation of linezolid use (92). Although linezolid is an alternative to vancomycin, the potential for rapid acquisition of linezolid resistance in CoNS following treatment and trends of increasing rates of resistance is of concern (47, 93, 94).

About 6.3% of strains from STJ were non-susceptible to daptomycin. Interestingly, daptomycin was never used as a treatment in either hospital throughout the study period. Daptomycin resistance without prior treatment is not unusual. Several studies have highlighted the relationship between daptomycin resistance and vancomycin resistance/ vancomycin-intermediate heteroresistance despite a lack of exposure to daptomycin in clinical isolates of *Staphylococcus aureus* (95-97). These studies concluded that a thickened cell wall, which is a common feature and mechanism of resistance in clinical vancomycin resistant/hV *S. aureus*, could inhibit daptomycin's activity on the cell membrane. Daptomycin is a bactericidal agent which acts by targeting and permeating the cell membrane, resulting in leakage of ions and ultimately cell death (98). A

thickened cell wall could therefore make it difficult for daptomycin molecules to pass through the cell wall and reach its target. Nunes et al. used transmission electron microscopy to determine if the thickness of cell walls changed with varying levels of vancomycin susceptibility (54). Using derivative CoNS strains that grew on high concentrations of vancomycin or teicoplanin, they compared cell wall thickness and found that all derivative strains had thicker cell wall than the parental strains. Another mechanism for daptomycin resistance, which has been identified in *S. aureus*, is mutations in the *mprF* gene. This mutation results in the increase of positive charges along the cell surface, thereby reducing daptomycin binding by repulsing the cationic daptomycin molecules (98, 99). However, mutations in the *mprF* gene have not been found in a majority of clinical VISA/hVISA isolates (96, 98). Our study did not look into either of these mechanisms of daptomycin resistance, and confirming whether or not mutations in the *mprF* gene are also found in CoNS, or determining cell-wall thickness in hV strains would be important. Future studies are needed to fully understand daptoymcin resistance among CoNS, and would greatly contribute to the expanding knowledge of hVICoNS.

Great genetic diversity was observed within the collection of strains from the two NICUs. In particular, STJ had a higher degree of genetic diversity than MCH, with 21 versus 5 PFGE patterns specific to each hospital. The diversity found in each hospital is in line with previous studies that investigated the molecular epidemiology of clinical *S. epidermidis* isolates (93, 100, 101). Further, molecular typing of the collected isolates using MSLT demonstrated that ST2 was the predominant sequence type in this study, comprising more than 50% of all PFGE patterns, followed by ST5 (10.3%). All of the

identified STs in our study belonged to the CC2, the primary clonal complex found worldwide. Our findings are comparable to other studies of *S. epidermidis* in US hospitals, which found ST2 and ST5 to be the most common sequence type in their collections (93, 102). PFGE patterns and ST were not correlated in our study; in particular ST2 consisted of 14 different PFGE patterns. In the study of Cherifi et al., who aimed to describe the molecular epidemiology of *S. epidermidis* in Belgian hospitals as well as the community, they found that ST2 was made of only 2 PFGE patterns (100). The higher number in our study is somewhat surprising; nonetheless, ST2 has been identified in the large majority of clinical methicillin-resistant *S. epidermidis* strains (38, 75, 100).

Recently, two studies attempted to elucidate the population structure of *S. epidermidis*, using different clustering approaches than the BURST algorithm used in our study. The first study utilized a Bayesian model-based clustering approach with MLST data, and the second study utilized a whole genome sequencing approach (103, 104). Thomas et al (103). grouped the *S. epidermidis* population into 6 genetic clusters (GC) and Méric *et al. (104)* grouped *S. epidermidis* into 3 groups. In both cases, clustering of *S. epidermidis* isolates was consistent with previously used methods. According to the Thomas *et al*. scheme (103), our isolates clustered into GC1, GC5 and GC6. While GC 1 and 6 appear to have a more generalist lifestyle, GC5 appears to be suited to a more nosocomial lifestyle, which represents the majority of the MLST typed isolates in our study. Additionally, according to the Méric *et al*. scheme (104), all of our isolates clustered into group A, within occurs a high number of recombination events. This could in part explain the great PFGE diversity observed in our study.

PFGE patterns E and G were found in both NICUs, suggesting that the same *S. epidermidis* clones could have successfully colonized both hospitals. The commonality of these clones to both NICUs is not due to transfers, as between-NICU transfers did not occur during the study period. Additionally, PFGE pattern E did not appear at STJ until August 2011, while pattern E strains were found at the MCH since the start of the study period in November 2009. Additionally, looking at antimicrobial resistance, the levels of daptomycin resistance from these strains at MCH around August 2011 were 0.25 mg/l, while the first pattern E clones at STJ were resistant (1.5 mg/l). This suggests that the PFGE pattern E clones may not have the same reservoir/origin in both institutions. Finally, PFGE patterns E, G, and Q belong to the most prevalent and successful strain type found worldwide (ST2) (38, 105). Therefore, it is not surprising to find PFGE patterns E and G ubiquitous in either of the NICUs in our study.

PFGE has been shown to be highly discriminatory, and is useful for determining clonal relationships and short-term, local epidemiology (37, 38). MLST is also discriminatory, and is used for long-term global epidemiology (38). We used PFGE to infer clonality in the collection of strains and used MLST to give evolutionary context of the strains in terms of population structure within the hospitals and globally. Low ARI and W coefficients were calculated when correlating PFGE and MLST in our study, indicating that these typing methods were not congruent with one another. Miragaia et al. (37) found similar results, with ARI and W for PFGE and MLST <0.75. They suggested that high rates of recombination among *S. epidermidis* was the reason for the lack of congruence (37, 38). Therefore, the use of both methods was needed to infer the extent and evolution of clonality within and between the two hospitals.

Controversy remains regarding the vancomycin-intermediate heteroresistance phenomenon in *S. epidermidis*, particularly in view of the methods for detection and the stability of the hV phenotype (83). PAP-AUC is generally recognized as the gold standard for hV determination (57, 82), and has been used in other studies to confirm hVICoNS (7, 8, 55, 106). However, this method is laborious and not available in routine diagnostic microbiology laboratories. In addition, the interpretation criteria for the PAP-AUC were developed and validated for *S. aureus*, not for CoNS (57, 82). We used two different protocols for PAP-AUC, taking into account the possible phenotype's instability. Our results show that the strains either harbored an unstable vancomycin-intermediate heteroresistance phenotype, or that hV in CoNS may be inducible in the presence of vancomycin. Both of these hypotheses must be investigated further. The clinical impact of vancomycin-intermediate heteroresistance and its association with treatment failure could not be addressed in this study. Finally, specific testing protocol and interpretation criteria should be developed to accurately determine vancomycin-intermediate heteroresistance in CoNS, since using criterion developed for *S. aureus* may not be entirely suitable to confirm hV in CoNS strains.

Limitations were present in this study. The study was completed retrospectively and thus had to rely on information recorded in medical charts. Moreover, only the first positive blood culture from each patient was used to determine hV. Doing so may have disregarded the manifestation of hV in subsequent CLABSI cultures from the MCH. While we used the CDC/NHSN definition to ensure our CoNS isolated from CLABSI cultures were true pathogens, it is possible that we included contaminated blood cultures. These false-positives would overestimate the CLABSI prevalence in our study.

Furthermore, the detection of two major clonal groups in our study could be due to a lack of discriminatory power of PFGE. However, we detected a large number of PFGE patterns in both NICUs, which makes this unlikely.

This study was the first to investigate a CLABSI outbreak caused by hVICoNS in the NICU. It aimed to describe the molecular epidemiology of the outbreak in a single tertiary care NICU in Québec, and compare it to a second tertiary care NICU in the same province that had not seen an increase in their CLABSI rate. The outbreak strains were all confirmed to be heteroresistant vancomycin-intermediate, based on routinely available methods and PAP-AUC, and were polyclonal. The presence of vancomycin-intermediate resistant subpopulations in the collection of isolates may be of concern as it could lead to future vancomycin resistance. Consistent surveillance of vancomycin MICs is therefore crucial to avoid further resistance; however MICs alone may be inappropriate to monitor hV. Finally, the study identified two major clones, PGFE patterns E and G (ST2), which were found in both NICUs. This suggests the persistence of highly successful clones that are well adapted to the hospital environment. The reservoirs for these clones remain unknown at this time, and identification of the reservoirs is needed to better understand the impact of hVICoNS in the NICU and inform infection prevention strategies.

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## Acknowledgements

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## Figures and Tables

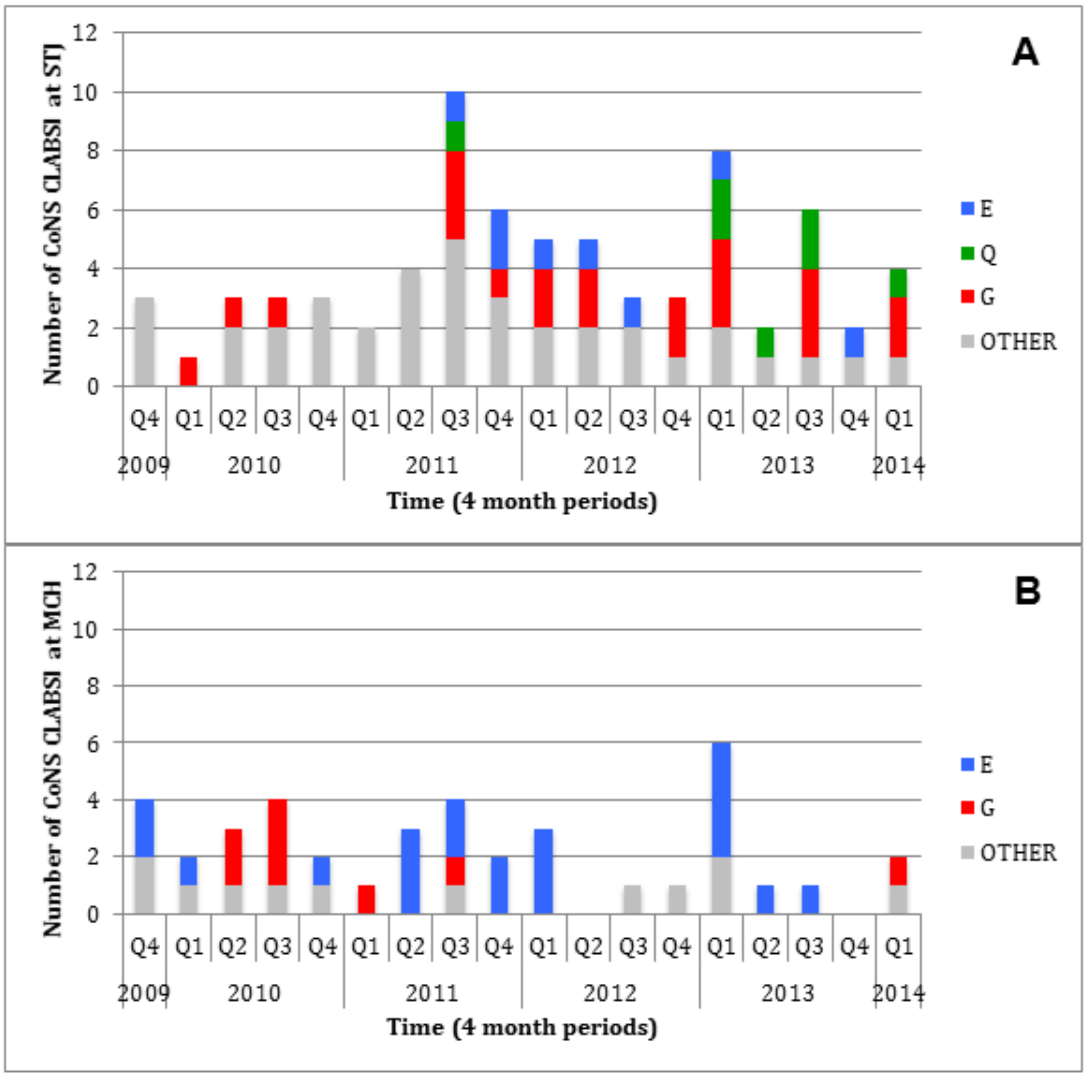



Figure 1. PFGE pattern distribution of hVICoNS infection from November 2009 until March 2014. Q1-Q4 represents four month-periods (quarter) within a year. Panel A shows cases from STJ and panel B shows cases from MC

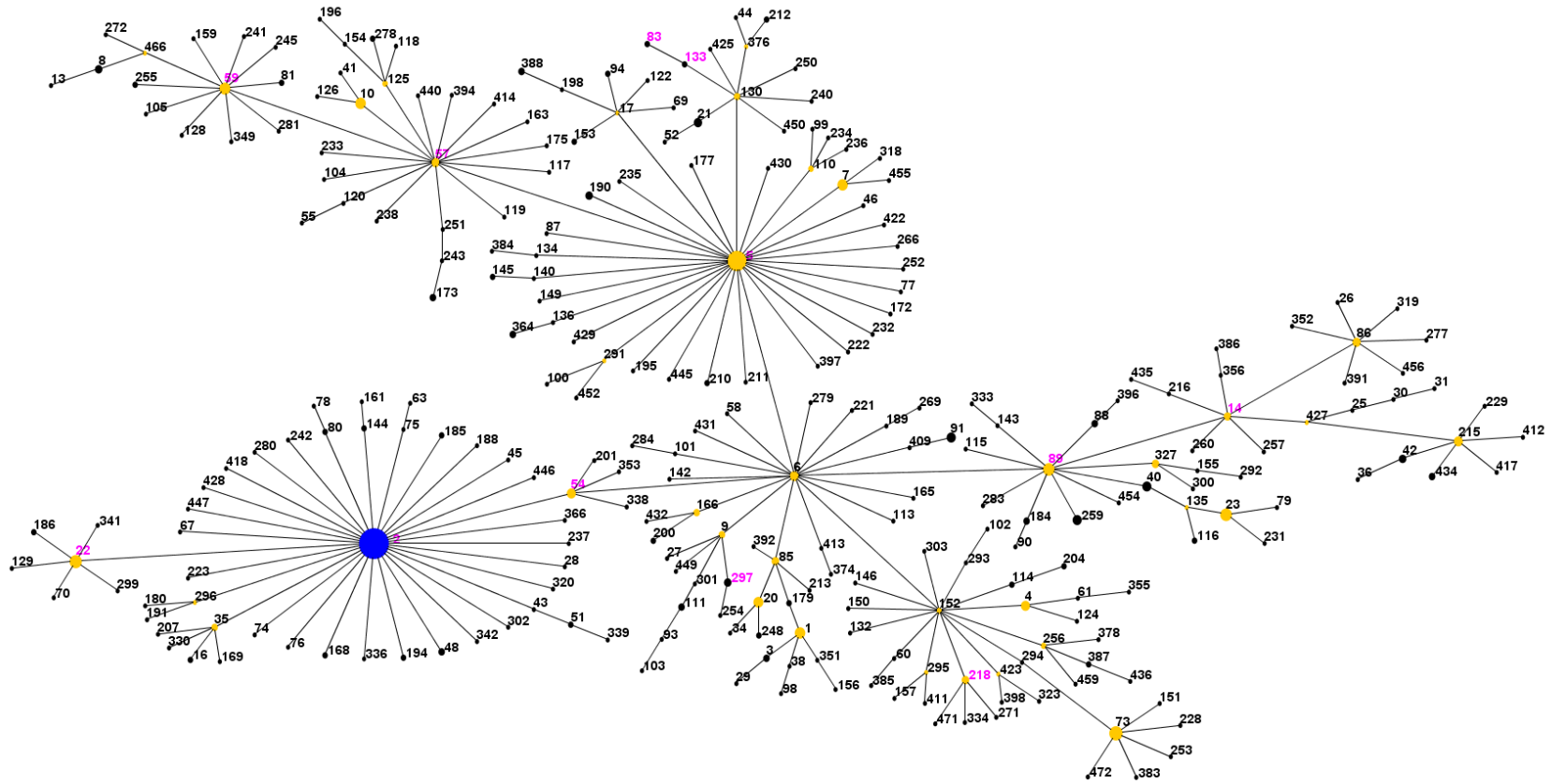


Figure 2. eBURST analysis of *S. epidermidis* CC2 using all 448 strain types (STs) available in the MLST database as of January 2016. Each ST is represented by a dot, and extending lines connect single-locus variants. The size of the dot is proportional to the number of isolates for each ST. The blue dot (ST2) represents the putative founder of CC2. The yellow dots represent putative subgroup founders. The pink STs represent those in our study.

Table 1. Patient characteristics of *Staphylococcus epidermidis* isolates according to their hospital of origin.

| Patient characteristics | STJ (*n*=79) | MCH (*n*=40) | *P*-value |
|---|---|---|---|
| Female, *n* (%) | 38 (48.1%) | 15 (35.4%) | 0.27 |
| Gestational Age, mean (range in weeks) | 27.5 (23.1-39.4) | 30.9 (23.9-39.4) | 0.0014 |
| Average age at sepsis onset, mean (range in days) | 20.6 (2-97) | 72.5 (4-217) | < 0.0001 |
| Birth weight category | | | 0.007 |
| ≤ 750 g | 33 (41.8%) | 13 (32.5%) | |
| 751-1000 g | 25 (31.6%) | 6 (15%) | |
| 1001-1500 g | 12 (15.2%) | 5 (12.5%) | |
| 1501-2500 g | 5 (6.3%) | 8 (20%) | |
| > 2500 g | 4 (5.1%) | 8 (20%) | |
| Average length of stay, mean (range in days) | 87.5 (0-257) | 79.6 (8-386) | 0.27 |
| 28-day mortality, *n* (%) | 16 (20.2%) | 1 (2.5%) | 0.011 |

Table 2. Antimicrobial susceptibility patterns of *S. epidermidis* isolates according to their hospital of origin.

| | STJ (*n*=79) | | | MCH (*n*=40) | | |
|---|---|---|---|---|---|---|
| Antimicrobial Agent | $MIC_{50}$ (mg/l) | $MIC_{90}$ (mg/l) | *n* (% resistant) | $MIC_{50}$ (mg/l) | $MIC_{90}$ (mg/l) | *n* (% resistant) |
| Daptomycin | 0.75 | 1 | 5 (6.3%) | 0.38 | 0.5 | 0 (0%) |
| Linezolid | 1 | 1.5 | 0 (0%) | 1 | 1.5 | 0 (0%) |
| Antimicrobial Agent | $MIC_{50}$ (mg/l) | $MIC_{90}$ (mg/l) | *n* (% reduced susceptibility)[a] | $MIC_{50}$ (mg/l) | $MIC_{90}$ (mg/l) | *n* (% reduced susceptibility) |
| Vancomycin: | | | | | | |
| Broth microdilution | 2 | 2 | 0 (0%) | 2 | 4 | 12 (30%) |
| Antimicrobial Agent | | | *n* (% intermediate heteroresistant) | | | *n* (% intermediate heteroresistant) |
| Vancomycin: | | | | | | |
| GRD Etest | | | 79 (100%) | | | 24 (60%) |
| Macro Etest | | | 42 (53.2%) | | | 13 (32.5%) |
| Total[b] | | | 79 (100%) | | | 26 (65%) |

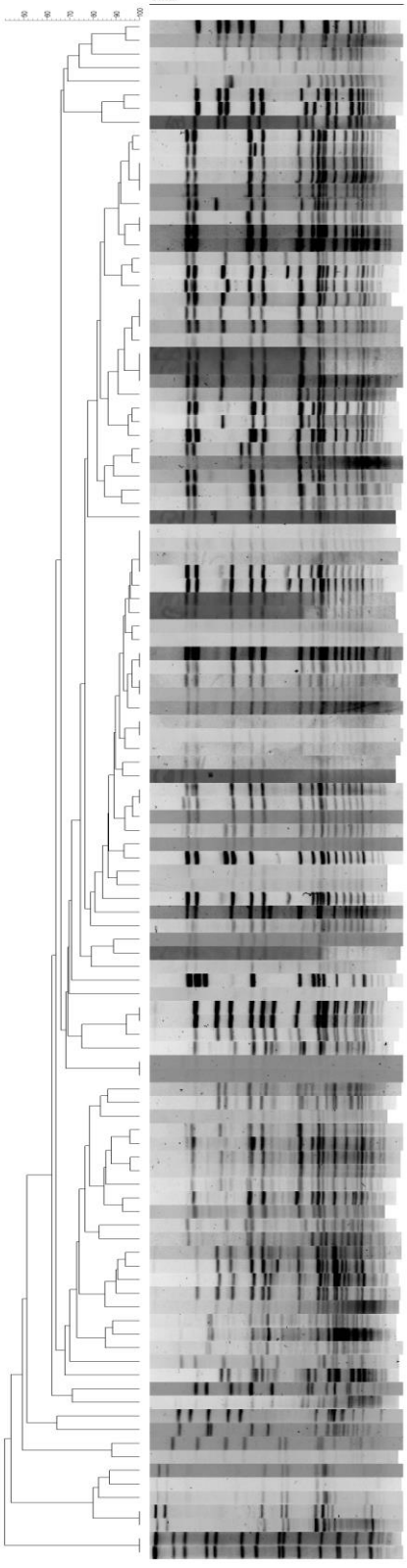
[a] Reduced vancomycin susceptibility, MIC ≥4 mg/l

[b] Isolates were considered vancomycin-intermediate heteroresistant if they were positive either by GRD E-test or Macro E-test.

Table 3. Distribution of *S. epidermidis* PFGE and MLST types.

| | Group 1<br>Common to both hospitals | Group 2<br>Unique to STJ | Group 3<br>Unique to MCH |
|---|---|---|---|
| Number of isolates | 65 | 47 | 7 |
| PFGE types (Number of isolates) | AB (5), D (3), E (28), G (29) | A (2), AC (2), AD (1), B (1), C (1), F (1), H (1), I (2), J (1), L (1), N (1), O (2), P (3), Q (7), R (2), S (5), T (3), U (1), V (1), Y (2), Z (1), untypeable (6) | AA (1), K (1), M (3), W (1), X (1) |
| MLST type | ST2, ST5 | ST22, ST57, ST59, ST83, ST89, ST133, ST225, ST457 | ST14, ST54, ST218, ST297 |

**Appendix 1**. Dendrogram of the PFGE pattern for the 119 *S. epidermidis* isolates causing CLABSIs (*n*=31). Information about MLST type and heterogeneous vancomycin-intermediate status was also included. Abbreviations: Y= yes, N= No. (Image on following page)




| Strain number | PFGE pattern | MLST type | Hospital | Date of collection | hVICoNS (E-test) | hVICoNS (PAP-AUC 1) | hVICoNS (PAP-AUC 2) |
|---|---|---|---|---|---|---|---|
| ID113379 | A | 2 | STJ | 2011-06-14 | Y | N | Y |
| ID133618 | A | 133 | STJ | 2010-07-25 | Y | N | Y |
| ID133614 | AD | 22 | STJ | 2012-04-05 | Y | N | Y |
| ID133576 | B | 59 | STJ | 2012-01-13 | Y | N | Y |
| ID115145 | C | 22 | STJ | 2011-10-05 | Y | N | Y |
| ID136439 | D | 5 | MCH | 2008-10-17 | Y | N | Y |
| ID136448 | D | | MCH | 2010-03-24 | Y | | |
| ID133563 | D | 5 | STJ | 2012-09-16 | Y | N | Y |
| ID136393 | E | | MCH | 2007-04-16 | Y | | |
| ID136444 | E | | MCH | 2009-04-19 | Y | | |
| ID136436 | E | | MCH | 2008-03-04 | Y | | |
| ID136440 | E | | MCH | 2009-01-20 | Y | | |
| ID136611 | E | | MCH | 2007-04-16 | Y | | |
| ID136396 | E | | MCH | 2007-08-17 | N | N | N |
| ID136368 | E | | MCH | 2005-11-01 | Y | | |
| ID136397 | E | | MCH | 2007-08-27 | Y | | |
| ID136375 | E | 54 | MCH | 2006-02-18 | Y | N | Y |
| ID136432 | E | | MCH | 2007-10-08 | Y | | |
| ID136433 | E | | MCH | 2007-12-25 | Y | | |
| ID136446 | E | | MCH | 2009-08-26 | Y | | |
| ID133580 | E | | STJ | 2011-11-20 | Y | | |
| ID133581 | E | | STJ | 2011-12-24 | Y | | |
| ID133607 | E | | STJ | 2012-05-27 | Y | | |
| ID133615 | E | | STJ | 2012-03-29 | Y | | |
| ID133560 | E | | STJ | 2012-08-11 | Y | | |
| ID133593 | E | | STJ | 2013-01-10 | Y | | |
| ID136614 | E | | MCH | 2009-02-08 | Y | | |
| ID133577 | E | 2 | STJ | 2011-07-28 | Y | N | Y |
| ID136386 | E | | MCH | 2006-10-15 | N | | |
| ID136437 | E | | MCH | 2008-03-26 | Y | | |
| ID136441 | E | | MCH | 2009-02-08 | Y | | |
| ID136370 | E | | MCH | 2005-11-23 | N | N | N |
| ID136392 | E | | MCH | 2007-04-30 | Y | N | Y |
| ID133648 | E | | STJ | 2013-10-14 | Y | | |
| ID136435 | E | | MCH | 2008-01-23 | N | | |
| ID136442 | E | | MCH | 2009-03-21 | N | N | N |
| ID133565 | F | 2 | STJ | 2012-09-19 | Y | N | Y |
| ID115116 | G | | STJ | 2010-08-31 | Y | | |
| ID133578 | G | | STJ | 2011-08-02 | Y | | |
| ID133606 | G | | STJ | 2012-05-29 | Y | | |
| ID136391 | G | | MCH | 2007-01-12 | Y | | |
| ID136447 | G | | MCH | 2010-03-02 | N | N | N |
| ID133611 | G | | STJ | 2013-01-27 | Y | | |
| ID133583 | G | | STJ | 2013-01-22 | Y | | |
| ID133584 | G | | STJ | 2013-08-26 | Y | | |
| ID133592 | G | | STJ | 2012-12-16 | Y | | |
| ID136383 | G | | MCH | 2006-09-09 | N | | |
| ID136395 | G | | MCH | 2007-07-18 | N | N | N |
| ID133579 | G | | STJ | 2011-11-26 | Y | | |
| ID133587 | G | | STJ | 2013-02-10 | Y | | |
| ID136610 | G | | MCH | 2006-07-20 | N | N | N |
| ID113377 | G | | STJ | 2011-07-14 | Y | | |
| ID133595 | G | | STJ | 2014-03-05 | Y | | |
| ID116086 | G | | STJ | 2011-09-29 | Y | | |
| ID133562 | G | | STJ | 2012-05-31 | Y | | |
| ID133594 | G | | STJ | 2012-10-24 | Y | | |
| ID116322 | G | | STJ | 2012-02-06 | Y | | |
| ID127475 | G | | STJ | 2013-08-13 | Y | | |
| ID133572 | G | 2 | STJ | 2010-02-11 | Y | N | Y |
| ID133646 | G | | STJ | 2013-09-22 | Y | | |
| ID115137 | G | | STJ | 2010-06-18 | Y | | |
| ID136609 | G | | MCH | 2006-04-15 | N | N | N |
| ID133598 | G | | STJ | 2014-03-03 | Y | | |
| ID133616 | G | | STJ | 2012-02-19 | Y | | |
| ID136382 | G | 2 | MCH | 2006-08-20 | Y | N | Y |
| ID136379 | G | | MCH | 2006-04-05 | N | N | N |
| ID133609 | H | | STJ | 2012-04-03 | Y | | |
| ID115139 | I | 2 | STJ | 2011-03-18 | Y | N | Y |
| ID133591 | I | | STJ | 2012-12-13 | Y | | |
| ID113383 | J | 89 | STJ | 2011-07-10 | Y | N | Y |
| ID136438 | K | 218 | MCH | 2008-09-29 | N | | |
| ID133602 | L | 2 | STJ | 2013-12-25 | Y | N | Y |
| ID136374 | M | 2 | MCH | 2005-11-18 | Y | N | Y |
| ID136443 | M | | MCH | 2009-03-21 | Y | | |
| ID136369 | M | | MCH | 2005-11-14 | Y | | |
| ID133574 | N | 2 | STJ | 2009-11-27 | Y | N | Y |
| ID115125 | O | | STJ | 2010-10-25 | Y | | |
| ID115147 | O | 2 | STJ | 2010-10-15 | Y | N | Y |
| ID113381 | P | | STJ | 2011-07-04 | Y | | |
| ID115117 | P | 5 | STJ | 2010-09-06 | Y | | |
| ID133597 | P | 2 | STJ | 2014-02-19 | Y | N | Y |
| ID133604 | Q | | STJ | 2014-01-10 | Y | | |
| ID133612 | Q | 2 | STJ | 2013-05-30 | Y | N | Y |
| ID133613 | Q | | STJ | 2013-01-26 | Y | | |
| ID133649 | Q | | STJ | 2013-09-30 | Y | | |
| ID127483 | Q | | STJ | 2013-08-18 | Y | | |
| ID113388 | Q | | STJ | 2011-07-13 | Y | | |
| ID133590 | Q | | STJ | 2013-03-08 | Y | | |
| ID115122 | R | 225 | STJ | 2011-12-05 | Y | N | N |
| ID115126 | R | 2 | STJ | 2011-01-09 | Y | N | Y |
| ID113382 | S | | STJ | 2011-07-04 | Y | | |
| ID115151 | S | | STJ | 2010-11-12 | Y | | |
| ID133573 | S | 2 | STJ | 2009-11-12 | Y | N | Y |
| ID134586 | S | | STJ | 2009-12-28 | Y | | |
| ID113376 | S | | STJ | 2011-07-24 | Y | | |
| ID115138 | T | | STJ | 2011-12-04 | Y | | |
| ID115158 | T | | STJ | 2010-05-11 | Y | N | N |
| ID133619 | T | | STJ | 2013-06-29 | Y | | |
| ID115148 | U | 59 | STJ | 2011-08-05 | Y | N | N |
| ID115114 | V | 5 | STJ | 2010-04-21 | Y | N | N |
| ID136377 | W | 14 | MCH | 2006-02-23 | N | | |
| ID136394 | X | 297 | MCH | 2007-07-15 | N | N | N |
| ID133617 | Z | 457 | STJ | 2013-02-24 | Y | N | Y |
| ID136384 | AA | | MCH | 2006-09-09 | Y | | |
| ID113373 | Y | | STJ | 2011-05-01 | Y | | |
| ID133585 | Y | 57 | STJ | 2013-02-12 | Y | N | Y |
| ID133575 | AB | 2 | STJ | 2011-04-01 | Y | N | Y |
| ID136387 | AB | | MCH | 2006-11-20 | N | N | N |
| ID133586 | AB | | STJ | 2013-08-15 | Y | | |
| ID136608 | AB | | MCH | 2006-04-15 | Y | | |
| ID136613 | AB | | MCH | 2009-03-21 | Y | | |
| ID113375 | AC | 83 | STJ | 2011-06-17 | Y | | |
| ID133582 | AC | | STJ | 2012-01-07 | Y | | |
| ID113372 | Untypeable | 2 | STJ | 2011-07-20 | Y | N | Y |
| ID113374 | Untypeable | 2 | STJ | 2011-06-14 | Y | N | Y |
| ID115129 | Untypeable | 2 | STJ | 2010-11-24 | Y | N | Y |
| ID115135 | Untypeable | 2 | STJ | 2010-10-13 | Y | N | Y |
| ID133561 | Untypeable | 2 | STJ | 2012-09-02 | Y | N | Y |
| ID134587 | Untypeable | 2 | STJ | 2011-12-27 | Y | N | Y |

## Chapter 6. The Clinical Impact of hVICoNS

The second manuscript provided an overview of the molecular epidemiology of the hVICoNS outbreak in a single NICU in Québec and compared it to a second NICU without an outbreak. The study demonstrated a high prevalence of vancomycin-intermediate heteroresistance amongst the strains, as well as uncovered two major clonal groups shared between the 2 NICUs. This study was consistent with the 5 studies included in the systematic review (manuscript 1) that certain hVICoNS strains may persist and become endemic in the NICU. Further, the manuscript highlighted that the heterogeneous vancomycin-intermediate phenotype may either be unstable in CoNS or inducible in the presence of vancomycin. These hypotheses should be investigated further, and methods to determine hV must be adapted specifically for CoNS. Further, when comparing our results from the E-tests to the modified PAP-AUC, there were no missed hV strains, and little more than 10% of the tested strains were misclassified as hV. As E-tests are significantly less laborious and can be done in-house, the clinical relevance of using E-tests versus PAP-AUC should be assessed to conclude the true value of using PAP-AUC to determine hV status.

As the clinical impact of hVICoNS has yet to be addressed, the following manuscript, which is to be submitted for publication, seeks to fill in this knowledge gap. Further, the systematic review highlighted that the majority of included studies did not collect detailed clinical information from patients, and population characteristics were not separated into hVICoNS and vancomycin susceptible CoNS (VSCoNS). This was remedied in the following manuscript, where clinical data was extracted retrospectively

from patient's charts and analyzed. In particular, heteroresistant vancomycin-intermediate *S. aureus* have been shown to be associated with prolonged bacteremia. As well, clinicians working in the outbreak NICU suspected that hVICoNS might be associated with prolonged bacteremia and thrombocytopenia. Therefore, to verify these associations in CoNS, I compared the duration of bacteremia and the risk of thrombocytopenia in neonates with an hVICoNS CLABSI to neonates with a vancomycin-susceptible CoNS CLABSI, adjusting for potential confounders. If there are no significant differences in the duration of bacteremia or risk of thrombocytopenia, it suggests that vancomycin-intermediate heteroresistance may not impact the morbidity of CoNS infection in the NICU. Moreover, using hV results from tests that are routinely available (GRD and macro E-test) and from PAP-AUC that are much more labor-intensive but considered as the gold standard, I will assess if the associations (or lack of) identified remain. Taking all these results together may suggest that expensive and time-consuming techniques to determine hV status (PAP-AUC) may not be necessary for routine purposes.

# Chapter 7. Central line associated bloodstream infections in the NICU: Does vancomycin-intermediate heteroresistance of coagulase negative staphylococci matter?

## Authors

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## Abstract

Objective: To determine whether the duration of bacteremia, as well as risk of thrombocytopenia, differed between patients in the NICU with a central line associated bloodstream infection (CLABSI) due to a vancomycin-intermediate heteroresistant *Staphylococcus epidermidis* (hVISE) compared a vancomycin susceptible *S. epidermidis*.

Design: A retrospective cohort study from November 2009, through April 2014.

Patients and Settings: A total of 114 patients with coagulase negative staphylococci (CoNS) CLABSI from two tertiary-care NICUs in Québec.

Results: 111 patients were included in the final analysis; 98 had an hVISE infection. The median duration of bacteremia was 4 days (range; 0-33) for patients with hVISE and 4 days (range; 2-8 days) for patients without hVISE. The duration of bacteremia was not significantly different between those with and without hVISE infection (*B*: -0.56; 95% CI: -2.76 to 1.65). Further, the risk of thrombocytopenia for patients with and without hVISE was not significantly different (OR: 0.42, 95% CI: 0.076 to 2.72).

Conclusions: hVISE was not shown to be associated with a longer duration of bacteremia, nor greater risk of thrombocytopenia. Heterogeneous vancomycin intermediate resistance in *S. epidermidis* may not have a significant clinical role in infants with CLABSI. However, due to lack of power, larger studies are needed before concluding on the need for hV identification in a clinical laboratory.

## Introduction

Coagulase negative staphylococci (CoNS) are among the leading causes of bloodstream infections (BSIs) in intensive care units (ICU). Premature neonates are particularly at risk, given their immature immune system, frequent use of indwelling catheters, and prolonged hospital stays (13).

The great majority of clinical CoNS isolates are methicillin resistant, with *mecA* – the gene conferring resistance – found in about 90% of isolates (43, 107). Vancomycin is therefore often used as the antimicrobial therapy of choice. Reduced susceptibility to glycopeptides, also known as vancomycin-intermediate heteroresistance (hV), has been well described in clinical *Staphylococcus aureus* isolates, and is defined as the detection of a vancomycin-intermediate resistant subpopulation of cells in an otherwise vancomycin susceptible microbial population. hV has been reported in clinical CoNS isolates, but unlike with *S. aureus,* CoNS vancomycin-intermediate heteroresistance is not yet a widely accepted concept.

While hVISA has been shown to have an impact on patient morbidity, vancomycin-intermediate heteroresistant coagulase negative staphylococci (hVICoNS) infection and its significance on patient mortality and morbidity in the NICU remains unknown. In the context of a recent outbreak of hVICoNS central line associated bloodstream infections (CLABSI) in a tertiary-care NICU in Québec, clinicians reported that infants with an hVICoNS CLABSI seemed to have more prolonged bacteremia and thrombocytopenia than expected. Therefore, the first aim of this study was to determine whether the duration of CLABSI in patients with an hVICoNS was different compared to patients

with a vancomycin susceptible (VSCoNS). The second aim was to determine if the risk of thrombocytopenia was different in patients with an hVICoNS CLABSI compared to patients with a VSCoNS CLABSI.

## Methods

### *Study design and patient population*

A retrospective cohort study was conducted in two NICUs of the Greater Montreal Area: the Centre Hospitalier Universitaire Sainte-Justine (a level III-IV NICU) and the Montreal Children's Hospital (a level III NICU). Both NICUs are tertiary care units in the province of Québec. Patients admitted to these two NICUs from November 1st 2009 through March 31st 2014 and developed a CLABSI due to a *S. epidermidis* were included in the analyses. We used CLABSI cases adjudication as identified by the local infection control services and reported to the Québec Nosocomial Infections Surveillance Program (SPIN) (17, 108). Patient information such as demographics, treatment, and outcomes were collected only if medical chart review determined that they fulfilled CDC/NHSN criteria for CoNS CLABSI (20). Patients were excluded if they had a CoNS CLABSI upon entry to the NICU, or if the infection was polymicrobial, i.e., where 2 or more organisms were isolated in their first positive blood culture. Further, only patients with a *S. epidermidis* CLABSI were used for further analysis and are the focus of this study (hVISE and VSSE). The Research Ethics Board of the McGill University Health Centre approved the study.

### *Heteroresistance*

Vancomycin-intermediate heteroresistance (hV) was determined using GRD and Macro E-tests, and confirmed with population analysis profile – area under the curve (PAP-AUC) as described by Chong et al. (109). A CoNS was considered hV if it was positive by E-test. hV status, as determined by PAP-AUC, was used in sensitivity analyses.

### *Variable Definitions*

The first outcome of interest was duration of bacteremia, defined as the number of days between the first positive blood culture for *S. epidermidis* and the first negative blood culture. We assessed whether hVISE CLABSIs lasted longer compared to vancomycin susceptible (VS) *S. epidermidis* CLABSIs. The second outcome of interest was thrombocytopenia. We assessed whether patients with an hVISE CLABSI had a greater risk of thrombocytopenia compared to patients with a VS *S. epidermidis* CLABSI. Thrombocytopenia was defined as a platelet count of $<50 \times 10^9$/L within 7 days prior to the first positive blood culture (110).

Cholestasis was defined as direct bilirubin (DBIL) concentration > 176.8 μmol/L (1) at the time of the first positive blood culture (111). Necrotizing enterocolitis was defined according to the modified Bell's staging criteria: NEC of stage ≥ IIA within one week (7 days) prior to the first positive blood culture (112). Catheter dwell time was calculated as the number of days between catheter insertion and removal. Finally, we recorded which antimicrobial therapy (vancomycin or linezolid) was used to treat the patient's CLABSI.

### *Statistical Analysis*

Categorical variables were analyzed using the $X^2$ or the Fisher exact test, and continuous variables were analyzed using the Student *t*-test or the Mann-Whitney test. For the first outcome, simple linear regression was used to evaluate the association between independent variables and duration of bacteremia. We also used multiple linear regression to evaluate the association between hVISE CLABSI and duration of bacteremia, while adjusting for identified confounding variables. For the second objective, univariate logistic regression was used to evaluate the association between independent variables and thrombocytopenia. Multivariable logistic regression was then used to analyze the association between hVISE CLABSI and the risk of thrombocytopenia, adjusting for confounders. Sensitivity analyses were performed using a second definition of vancomycin-intermediate heteroresistance (modified PAP-AUC) for both outcomes. Statistical analyses were performed using STATA Statistical Software, version 12 (STATA Corp, College Station, TX).

## Results

### *General comparisons*

Of the 114 patients who had a CLABSI caused by *S. epidermidis* during the study period, three patients were excluded due to polymicrobial infections. Ninety-eight of the remaining 111 study patients had a vancomycin-intermediate heteroresistant *S.epidermidis* (hVISE) CLABSI.

***hVISE***

Table 1 compares patients with hVISE infection to patients with vancomycin-susceptible *S. epidermidis* (VSSE) infections. Patients with hVISE infections weighed less, were more likely to be mechanically ventilated, and were more likely treated with linezolid when compared to patients with VSSE infections. The median duration of bacteremia (from the first positive blood culture to the first negative blood culture) was 4 days (range: 0 days [patient died within the day of the first positive blood culture] - 33 days) for patients with hVISE, and 4 days (range: 2-8 days) for patients without hVISE. 54.1% of patients with an hVISE CLABSI had thrombocytopenia, compared to 53.8% of patients with a VSSE CLABSI.

***Duration of Bacteremia: Simple Linear Regression***

Table 2 gives the simple linear regression analyses for each independent predictor variable for duration of bacteremia. hV status was not a significant predictor for duration of bacteremia (*B*: -2.89, 95% CI: -2.34 to 1.75). Thrombocytopenia was positively associated with duration of bacteremia; patients with thrombocytopenia had a greater duration of bacteremia by 1.74 days compared to patients without thrombocytopenia.

***Duration of Bacteremia: Multiple Linear Regression***

Table 2 also gives the final multiple linear regression model that assessed the association between hVISE infection and duration of bacteremia. After adjusting for covariates known to confound this association (a priori and from simple linear regression), the duration of bacteremia was not different between infants with hVISE infection compared to those with VSSE infection. Cholestasis was positively associated with duration of

bacteremia (*B*: 2.38, 95% CI: 0.49 to 4.27), as was thrombocytopenia (B: 1.66, 95% CI: 0.24 to 3.08). The model could not significantly predict the duration of bacteremia amongst neonates with a *S. epidermidis* CLABSI (F 12.98 = 1.60, p = 0.104), and the final model only accounted for 16.4% of the explained variability in the duration of bacteremia.

***Thrombocytopenia: Univariate Logistic Regression***

Table 3 gives the univariate logistic regression for each independent predictor variable of thrombocytopenia. hV status was not a significant predictor of thrombocytopenia (OR: 1.14, 95% CI: 0.36 to 3.65). Neonates with a birth-weight of either 1001-1500 g, 1501-2500 g, or >2500 g had a lower risk (OR: 0.27, 0.24, 0.13, respectively) of thrombocytopenia than those with a birth weight ≤ 750 g. Duration of bacteremia was associated with thrombocytopenia, with a 1.24 times greater risk of thrombocytopenia for a single day increase in bacteremia duration.

***Thrombocytopenia: Multivariable Logistic Regression***

Table 3 also shows the results of a multivariable logistic regression of the association between hVISE and thrombocytopenia. After adjusting for confounders (a priori and from univariate logistic regression), hV status was not significantly associated with thrombocytopenia (OR: 0.42, 95% CI: 0.076 to 2.72). Neonates with a higher birth weight (between 1501-2500 g or >2500 g) had a lower risk of thrombocytopenia (OR: 0.18 and 0.071, respectively) than neonates with a birth-weight ≤750 g. Duration of bacteremia was associated with thrombocytopenia, with a 1.22 times (95% CI: 1.01 to 1.46) greater risk of thrombocytopenia per a one day increase in bacteremia duration.

***Power***

We did not detect a significant difference in the duration of bacteremia between patients with hVISE and VSSE sepsis. The power of our study to detect a 1-day difference in the duration of bacteremia between those with hVISE versus VSSE was 34%, not giving us enough power to detect a significant association between duration of bacteremia and hV status. Additionally, the resulting confidence intervals for hV status were wide (95% CI: -1.19 to 2.46).

***Sensitivity Analysis***

Table 2 and 3 summarize the results of the sensitivity analyses performed to explore whether the duration of bacteremia varied if we used the hV status obtained from clinical laboratory E-tests or a modified, gold-standard, PAP-AUC (Table 4). The sensitivity and specificity of the E-tests compared to the PAP-AUC in our dataset are 100% and 44.8%, respectively. For the first objective, using simple linear regression, the duration of bacteremia between those with and those without an hVISE infection remained non-significant (*B*: 0.50, 95% CI: 0.99 to 1.99). Using multiple linear regression analysis, hV remained a non-significant risk factor for duration of bacteremia (*B*: 0.63, 95% CI: -1.19 to 2.46). Furthermore, cholestasis, thrombocytopenia, and birth-weight ≤750 g remained positively associated with duration of bacteremia. Similarly for thrombocytopenia, hV status remained not associated with thrombocytopenia on univariate (OR: 1.09, 95% CI: 0.47 to 2.56) and multivariable analysis (OR: 0.58, 95% CI: 0.16 to 2.10). A greater risk of thrombocytopenia remained for a 1-day increase in duration of bacteremia, as did a lower risk of thrombocytopenia in neonates with a birth-weight >2500 g.

## Discussion

This study examined the clinical impact of hVISE CLABSI isolates from two tertiary care NICUs in Québec. We did not find a significant association between vancomycin-intermediate heteroresistant *S. epidermidis* infection and duration of bacteremia or risk of thrombocytopenia. Our study was however, underpowered as hV was much more prevalent in our cohort than previously anticipated. The number of patients without an hVISE strain represented less than 15% of the total cohort of CLABSI patients when using the E-tests available in clinical laboratories, and 26% when using the PAP-AUC method. Future studies assessing the clinical impact of hV status should be wary of the potential for high hV prevalence in the NICU.

Previous reports detecting hVISE in the NICU have not identified, nor sought to identify, its impact on patient morbidity and mortality (6-8, 55). However, studies have demonstrated the clinical impact of vancomycin-intermediate heteroresistance in *Staphylococcus aureus* (hVISA). While not limited to the NICU population, hVISA has been shown to be associated with persistent bacteremia and vancomycin failure (53, 72). A systematic review of the significance of hVISA determined that vancomycin therapeutic failure (also defined as persistent bacteremia) was commonly associated with hVISA. Moreover, the systematic review found that the odds of vancomycin therapeutic failure were 2.37 times higher (95% CI, 1.53 to 3.67) in hVISA infections compared to vancomycin susceptible *Staphylococcus aureus* (VSSA) infections (53). In contrast, our findings do not suggest that decreased vancomycin susceptibility increases the morbidity of coagulase negative *Staphylococcus*, as it does in *S. aureus*.

Our study consistently found a positive association between duration of bacteremia and thrombocytopenia, as detected in the 7 days before the first positive blood culture (Table 2, 3). Previously, a new syndrome of CoNS sepsis characterized by prolonged bacteremia and thrombocytopenia had been identified in the NICU (113). The authors attributed the syndrome to changes in the features of CoNS, highlighting that patient care practice and patient characteristics between those with and those without prolonged bacteremia were similar. Given these findings, we wondered if hV status could have explained this syndrome, as clinicians also thought that there was an increase in the incidence of thrombocytopenia and prolonged duration of CLABSI in our study population. It was posited that vancomycin-intermediate heteroresistance modified the clinical manifestation of CoNS CLABSI and thereby increased the risk of thrombocytopenia and duration of bacteremia in neonates. However, our multivariable analyses did not demonstrate an association between duration of bacteremia and hV or between thrombocytopenia and hV. Thrombocytopenia in the 7 days prior to CLABSI onset may be a marker of more severe infection that could manifest as a prolonged bacteremia.

A protective association between neonates with a larger birth weight (between 1501-2500 g or >2500 g) and risk of thrombocytopenia was identified through our study. Previous studies evaluating sepsis-induced thrombocytopenia in neonates have also found a greater incidence of thrombocytopenia in low/very low-birth weight infants (114-116). Christensen et al. reported that the rate of thrombocytopenia in neonates <1000 g was two-fold that of the general NICU population (115).

The study also demonstrated a positive association between duration of bacteremia and cholestasis. Although the incidence of sepsis-associated cholestasis in neonates ranges

from 20-60% (117), the presence of cholestasis upon the first positive blood culture, like preceding thrombocytopenia, could be a marker of severe CoNS bloodstream infections and should be investigated further.

For *S. aureus* bloodstream infections, expensive and time-consuming techniques, such as population analysis profile (PAP-AUC), are used to identify vancomycin-intermediate heteroresistant strains; as usual laboratory techniques fail to detect the subpopulation of hV cells (57, 82). Our study does not show an overwhelming increase in duration of bloodstream infection associated with hV status. However, due to our lack of power, we cannot conclude that additional tests to detect hV are not needed. Our results show that the E-tests available in routine use (Macro E-test and GRD) when compared to PAP-AUC have a good sensitivity (100%) but a poor specificity (45%). In the multiple linear regression, there was no difference in the regression analyses when we used the E-tests or the PAP-AUC results. Awaiting more evidence to support the increase in morbidity or mortality associated with hV status, more laborious techniques, such as PAP-AUC may not be needed and could be replaced by GRD and Macro E-tests. Further, since our study did not provide evidence that hV status in CoNS impacts patient morbidity, routine determination of hV status in clinical laboratories may even be unnecessary. Although CoNS bloodstream infections are not as severe as infections with other pathogens, they are associated with a higher relative risk of bronchopulmonary dysplasia and adverse neurodevelopmental impairments, including cerebral palsy (21-24). Consequently, CoNS are an important nosocomial pathogen and care should be taken to reduce CoNS infections.

Vancomycin-intermediate heteroresistance has previously been suggested to be an intrinsic property of *S. capitis* (55). Moreover, the genome of *S. epidermidis* has been shown to be highly flexible (33). It is therefore possible that vancomycin-intermediate heteroresistance may play a role in helping CoNS species survive and persist in hospitals, while not changing its morbidity. Furthermore, the presence of a vancomycin-intermediate resistant subpopulation in the cells may be of concern as heteroresistance is considered a precursor that may or may not lead to complete resistance (118). As vancomycin was administered in nearly 75% of the CLABSI cases in our cohort, the possibility of vancomycin resistance is obviously of great concern. Therefore, the institution and adherence to infection control practices, antimicrobial stewardship, and surveillance of CoNS vancomycin MICs are necessary to avoid vancomycin resistance.

Our study had several strengths. First, our cohort included all infants with a *S. epidermidis* CLABSI from the two hospitals. Second, the sensitivity analysis, which used different inclusion criteria for hV, did not find a difference in the duration of bacteremia nor the risk of thrombocytopenia. This suggests that our utilization of the hV status obtained from E-tests is justified. Further, it indicates that using PAP-AUC to determine hV, which is far more laborious than modified E-tests, may be unnecessary (57). There were limitations with our study, including it being retrospective in nature. We also excluded patients with polymicrobial infections, potentially losing information on the clinical impact of hV. Finally, our study was underpowered as hV status was far more prevalent than previously hypothesized.

In summary, our findings suggest that patients with vancomycin-intermediate heteroresistant CoNS do not significantly differ from those with vancomycin susceptible

CoNS in terms of duration of bacteremia and risk of thrombocytopenia. The lack of an increase in morbidity suggests that time-consuming techniques to assess vancomycin-intermediate heteroresistance in CoNS, such as PAP-AUC, may by unnecessary. Additionally, the absence of an association between vancomycin-intermediate heteroresistance and severity of CLABSI could be the result of CoNS adaptation to the clinical setting, and hV being an intrinsic property in certain CoNS species.

# Tables

Table 1. Baseline characteristics between patients who had an hV *S. epidermidis* CLABSI compared to those who did not have an hV *S. epidermidis* CLABSI.

| Variable | Patients with hV (n=98) | Patients without hV (n=13) | *P* |
|---|---|---|---|
| Patient characteristics | | | |
| Gender | | | 0.33 |
| Female | 44 (44.9%) | 4 (30.8%) | |
| Method of delivery | | | 0.77 |
| C-section | 57 (58.2%) | 7 (53.8%) | |
| Birth-weight category | | | 0.010 |
| ≤ 750 g | 36 (36.7%) | 4 (30%) | |
| 751-1000 g | 28 (28.6%) | 1 (7.7%) | |
| 1001-1500 g | 16 (16.3%) | 1 (7.7%) | |
| 1501-2500 g | 11 (11.2%) | 2 (8.7%) | |
| > 2500 g | 7 (7.1%) | 5 (3.8%) | |
| Ventilation | | | 0.002 |
| None | 15 (15.3%) | 7 (53.8%) | |
| Non-invasive | 23 (23.4%) | 0 | |
| Mechanical | 60 (61.2%) | 6 (46%) | |
| Necrotizing enterocolitis | 30 (30.6%) | 1 (7.7%) | 0.083 |
| Cholestasis | 24 (24.5%) | 1 (7.7%) | 0.17 |
| Thrombocytopenia | 53 (54.1%) | 7 (53.8%) | 0.99 |
| Infection characteristics | | | |
| Catheter days, median (range) | 14 days (0-97) | 12 days (0-26) | 0.21 |
| Catheter removed <48 hrs. prior to sepsis | 86 (87.7%) | 11 (84.6%) | 0.75 |
| Catheter inserted for >10 days prior to sepsis | 51 (52%) | 6 (54.5%) | |
| Duration of bacteremia, median (range) | 4 days (0-33) | 4 days (2-8) | 0.24 |
| Age at sepsis onset, median (range) | 19 days (2-177) | 23 days (4-210) | 0.11 |
| Treatment, Vancomycin | 69 (70.4%) | 13 (100%) | 0.022 |
| Vancomycin treatment > 7 days | 57 (58.2%) | 11 (84.6%) | |
| MIC Vancomycin (ug/mL), median (range)* | 2 (0.5 to 8) | 2 (1 to 4) | |

* MIC = Minimum inhibitory concentration for each strain

Table 2. Simple and multiple linear regression models predicting duration of bacteremia for 111 patients with *S. epidermidis* CLABSI.

| Independent Variable | Simple Linear Regression | | Multiple Linear Regression (MLR) | | Sensitivity Analysis (MLR) | |
|---|---|---|---|---|---|---|
| | *B*-coefficient* | 95% CI | *B*-coefficient | 95% CI | *B*-coefficient | 95% CI |
| hVICoNS | -2.98 | -2.34 to 1.75 | -0.56 | -2.76 to 1.65 | 0.63 | -1.19 to 2.46 |
| Gender: Male | -0.13 | -1.46 to 1.20 | … | … | … | … |
| Method of delivery: C-section | -0.99 | -1.43 to 1.23 | … | … | … | … |
| Birth-weight (BW) category | | | | | | |
| ≤ 750 g | Ref | Ref | Ref | Ref | Ref | Ref |
| 751-1000 g | -1.05 | -2.74 to 0.63 | -0.86 | -2.57 to 0.85 | -0.89 | -2.60 to 0.82 |
| 1001-1500 g | -1.07 | -3.07 to 0.93 | -0.57 | -2.66 to 1.52 | -0.74 | -2.89 to 1.40 |
| 1501-2500 g | 0.15 | -2.06 to 2.36 | 0.28 | -2.19 to 2.73 | 0.34 | -2.10 to 2.79 |
| > 2500 g | -1.44 | -3.72 to 5.87 | -0.22 | -2.73 to 2.29 | 0.013 | -2.44 to 2.46 |
| Ventilation | | | | | | |
| None | Ref | Ref | … | … | … | … |
| Non-invasive | -1.87 | -3.92 to 0.17 | … | … | … | … |
| Mechanical | -0.77 | -2.46 to 0.92 | … | … | … | … |
| Necrotizing enterocolitis | -1.08 | -2.53 to 0.37 | -1.26 | -2.79 to 0.28 | -1.40 | -2.95 to 0.15 |
| **Cholestasis** | 1.29 | -0.26 to 2.85 | **2.38** | **0.49 to 4.27** | **2.26** | **0.39 to 4.13** |
| **Thrombocytopenia** | **1.74** | **0.46 to 3.03** | **1.66** | **0.24 to 3.08** | **1.73** | **0.31 to 3.13** |
| Catheter days, median (range) | -0.016 | -0.062 to 0.029 | … | … | … | … |
| Catheter removed <48 hrs. prior to sepsis | -0.24 | -1.99 to1.51 | … | … | … | … |
| Catheter inserted for >10 days prior to sepsis | -0.34 | -1.65 to 0.98 | -0.42 | -1.84 to 0.99 | -0.41 | -1.83 to 1.01 |
| Age at sepsis onset, median (range) | -0.0084 | -0.026 to 00091 | -0.011 | -0.032-0.010 | -0.0068 | -1.03 to 0.015 |
| Treatment, Vancomycin | 0.12 | -1.49 to 1.51 | … | … | … | … |
| Vancomycin treatment > 7 days | 0.096 | -1.25 to 1.45 | 0.22 | -1.16 to 1.61 | 0.38 | -1.03 to 1.79 |
| MIC Vancomycin (ug/mL) | -0.15 | -0.73 to 0.43 | -0.56 | -2.76 to 1.65 | -0.29 | -0.91 to 0.33 |

*B = regression coefficient, estimates the increase in the duration of bacteremia per unit increase of continuous predictors, or in the yes versus no group for binary/categorical predictors; 95% CI = 95% confidence interval.

Table 3. Univariate and multivariable logistic regression of independent variables for prediction of the risk thrombocytopenia in 111 patients with *S. epidermidis* CLABSI.

| Independent Variable | Univariate Logistic Regression | | Multivariable Logistic Regression (MLR) | | Sensitivity Analysis (MLR) | |
|---|---|---|---|---|---|---|
| | OR* | 95% CI | OR | 95% CI | OR | 95% CI |
| hVICoNS | 1.14 | 0.36 to 3.65 | 0.42 | 0.076 to 2.72 | 0.58 | 0.16 to 2.10 |
| Gender: Male | 1.04 | 0.49 to 2.21 | … | … | … | … |
| Method of delivery: C-section | 2.36 | 1.09 to 5.12 | 2.29 | 0.87 to 6.00 | 2.34 | 0.89-6.15 |
| Birth-weight category | | | | | | |
| ≤ 750 g | Ref | Ref | Ref | Ref | Ref | Ref |
| 751-1000 g | 0.72 | 0.26 to 2.03 | 0.59 | 0.18 to 1.93 | 0.60 | 0.18 to 1.95 |
| **1001-1500 g** | **0.27** | **0.081 to 0.87** | 0.26 | 0.066 to 1.02 | 0.29 | 0.072 to 1.17 |
| **1501-2500 g** | **0.24** | **0.064 to 0.88** | **0.18** | **0.032 to 0.99** | **0.21** | **0.040 to 1.10** |
| **> 2500 g** | **0.13** | **0.029 to 0.56** | **0.071** | **0.0099 to 0.51** | **0.092** | **0.016 to 0.54** |
| Ventilation | | | | | | |
| None | Ref | Ref | … | … | … | … |
| Non-invasive | 0.45 | 0.13 to 1.47 | … | … | … | … |
| Mechanical | 1.14 | 0.42 to 3.04 | … | … | … | … |
| Necrotizing enterocolitis | 1.29 | 0.56 to 3.02 | … | … | … | … |
| Cholestasis | 1.19 | 0.48 to 2.94 | … | … | … | … |
| **Duration of bacteremia** | **1.24** | **1.05 to 1.47** | **1.22** | **1.01 to 1.46** | **1.23** | **1.02 to 1.47** |
| Total catheter days | 0.98 | 0.96 to 1.01 | … | … | … | … |
| Catheter removed <48 hrs. prior to sepsis | 1.06 | 0.39 to 2.87 | … | … | | |
| Catheter inserted for >10 days prior to sepsis | 0.95 | 0.45 to 2.01 | 2.17 | 0.76 to 6.16 | 1.87 | 0.69 to 5.06 |
| Age at sepsis onset, median (range) | 0.98 | 0.97 to 0.99 | 0.99 | 0.97 to 1.00 | 0.99 | 0.97 to 1.00 |
| Treatment, Vancomycin | 1.35 | 0.57 to 3.20 | 1.22 | 0.42 to 3.54 | 1.18 | 0.41-3.41 |
| Vancomycin treatment > 7 days | 1.06 | 0.99 to 1.13 | … | … | … | … |
| MIC Vancomycin (ug/mL) | 1.15 | 0.80 to 1.63 | 1.64 | 0.98 to 2.72 | 1.57 | 0.94 to 2.63 |

* OR = odds ratio; 95% CI = 95% confidence interval

Table 4. Vancomycin heteroresistance status of CoNS obtained from GRD/Macro E-tests or modified PAP-AUC.

| | Modified PAP-AUC | | |
|---|---|---|---|
| GRD/Macro E-test | hV | Not hV | Total |
| hV* | 82 | 16 | 98 |
| Not hV | 0 | 13 | 13 |
| Total | 82 | 29 | 111 |

*hV: Heterogeneous resistance to vancomycin

## Chapter 8. Discussion and Conclusion

Vancomycin-intermediate heteroresistance in *S. aureus* is known to have a negative impact on patients, being associated with persistent bacteremia and vancomycin treatment failure (53, 72). However, the hV phenomenon has yet to be accepted in CoNS, and its clinical relevance remains unknown. Therefore, the goal of this thesis was to elucidate the clinical burden and significance of hVICoNS, particularly in the NICU.

The first step in exploring the clinical relevance of hVICoNS in the NICU was to conduct a systematic review, as reported in Chapter 3. Five cohort, surveillance, and laboratory-based studies, published between 1980-2014, were identified. The prevalence of hVICoNS ranged from 2.3% to 100%, the wide-range owing to inconsistent definitions and methods of hV determination among the included studies. Further, the evaluation of hVICoNS prevalence in some studies was limited as their methods of determining vancomycin-intermediate heteroresistance were inadequate. The review also highlighted that certain hVICoNS strains might persist and even become endemic in the NICU. However, none of these studies addressed the clinical impact of hVICoNS, thereby highlighting a knowledge gap of the possible clinical significance of vancomycin-intermediate heteroresistance in CoNS.

Following this systematic review, an original analysis was performed to describe the molecular epidemiology of an outbreak of hVICoNS CLABSI in a single NICU in Québec, which was compared to a second tertiary care NICU in Québec that had not been associated with an outbreak. This manuscript can be found in Chapter 5. Vancomycin-intermediate heteroresistance was identified in about 88% of all collected strains, and all

strains from the outbreak NICU were confirmed to be hV. The collection of strains was genetically diverse, with thirty-one PFGE types identified. Particularly, two major clones were found in both NICUs spanning across the five-year study period, suggesting that successful, well-adapted clones may persist in a clinical setting. This finding of persistent clones in the NICU was consistent with the systematic review. Moreover, it is necessary to determine what makes these persistent strains well adapted in comparison to intermittent strains to fully comprehend CoNS's success in the clinical environment.

This original analysis also used MLST to molecularly type the collection of strains. The two major clones (PFGE types E and G) belonged to the same clinically successful strain type (ST2), consistent with previous studies evaluating the molecular epidemiology of hospital-acquired *S. epidermidis*. In terms of a clinical context, using both PFGE and MLST typing methods are needed to provide a comprehensive overview of the spread and evolution of *S. epidermidis* for both short-term and long-term epidemiology.

To address the clinical relevance of hVICoNS, a second original analysis was performed on the same cohort. This manuscript was presented in Chapter 7. This analysis found that patients with vancomycin-intermediate heteroresistant CoNS do not significantly differ from those with vancomycin susceptible CoNS in terms of duration of bacteremia and risk of thrombocytopenia. However, the study was significantly underpowered, as hV was much more prevalent than previously hypothesized.

A previous report from Vancouver showed an increase in CoNS bloodstream infection duration and increased incidence of thrombocytopenia in their NICU, without a clear explanation. We thought that hV, given our clinicians' hunch of prolonged bloodstream

infections and thrombocytopenia, could have explained the situation. However, the absence of association between hV and more severe features of infection (prolonged bacteremia duration and thrombocytopenia), does not allow us to conclude that hV is actually a virulence factor. hV may be the result of selective pressure from vancomycin usage in hospitals or a laboratory artifact. Further, vancomycin-intermediate heteroresistance has been suggested to be an intrinsic property in *S. capitis* (55). This hypothesis could be applied to other CoNS species, such as *S. epidermidis*, and should be evaluated in the future. In line with D'mello et al. (55), older clinical *S. epidermidis* strains going back decades, before routine vancomycin use, could be evaluated for hV. If hV were found in these strains, it would suggest that hV is an inherent property of *S. epidermidis* and that hV status may not be indicative of further CoNS virulence.

Alternatively, vancomycin-intermediate heteroresistance in CoNS may be a laboratory artefact associated with the use of more sensitive methods to determine antimicrobial susceptibility. The second manuscript highlighted that when using PAP-AUC, said to be the gold standard for the detection of hV status in *S. aureus*, to determine hV status, the hV phenotype was unstable and needed to be induced by the presence of vancomycin. These laboratory findings should be investigated to determine the relevance of hV testing, using time-consuming research methods. In addition, testing protocols and interpretation criteria developed specifically for *S. aureus* are currently being used. These may not be suitable to confirm hV in CoNS strains, and procedures specific to CoNS should be developed.

The original studies had the advantages of using an entire cohort of patients with a CoNS CLABSI, as well as performing sensitivity analyses to confirm results. However, several

limitations were present, such as the potential of including contaminated blood cultures, only using the first positive blood culture, excluding polymicrobial infections, and the studies being retrospective in nature. In addition, vancomycin-intermediate heteroresistance was much more prevalent in the cohort than previously believed, which resulted in the third manuscript being significantly underpowered.

The lack of increase in morbidity calls into question the true clinical relevance of vancomycin-intermediate heteroresistance in CoNS. While the study was underpowered, hV status does not appear to modify the clinical manifestation of CoNS CLABSI. This suggests that laborious techniques to assess hV status in CoNS, such as PAP-AUC, may by unnecessary. However, routine determination of hV status in clinical laboratories needs to be further studied to determine their necessity. Nevertheless, before concluding that hV may have no clinical relevance, larger, better-powered studies should be undertaken to provide further evidence on the true impact of hVICoNS on patient morbidity and mortality. Pending this future research, it is difficult to conclude with confidence whether or not hV has a clinical impact on premature infants in the NICU. Despite these limitations, this study was the first of its kind to evaluate the clinical relevance of vancomycin-intermediate heteroresistance in CoNS. This study aimed to fill the knowledge gap surrounding hVICoNS, and provides a better understanding of the clinical impact of hV in NICU patients. It also serves to highlight future directions for prospective, larger scale studies.

## References


1. **BRODIE SB, SANDS KE, GRAY JE, PARKER RA, GOLDMANN DA, DAVIS RB, RICHARDSON DK.** 2000. Occurrence of nosocomial bloodstream infections in six neonatal intensive care units. The Pediatric infectious disease journal **19:**56-65.
2. **Schulman J, Stricof R, Stevens TP, Horgan M, Gase K, Holzman IR, Koppel RI, Nafday S, Gibbs K, Angert R.** 2011. Statewide NICU central-line-associated bloodstream infection rates decline after bundles and checklists. Pediatrics **127:**436-444.
3. **Otto M.** 2009. Staphylococcus epidermidis—the'accidental'pathogen. Nature Reviews Microbiology **7:**555-567.
4. **Sieradzki K, Villari P, Tomasz A.** 1998. Decreased susceptibilities to teicoplanin and vancomycin among coagulase-negative methicillin-resistant clinical isolates of staphylococci. Antimicrobial agents and chemotherapy **42:**100-107.
5. **Center KJ, Reboli AC, Hubler R, Rodgers GL, Long SS.** 2003. Decreased vancomycin susceptibility of coagulase-negative staphylococci in a neonatal intensive care unit: evidence of spread of Staphylococcus warneri. Journal of Clinical Microbiology **41:**4660-4665.
6. **Rasigade JP, Raulin O, Picaud JC, Tellini C, Bes M, Grando J, Ben Said M, Claris O, Etienne J, Tigaud S, Laurent F.** 2012. Methicillin-resistant Staphylococcus capitis with reduced vancomycin susceptibility causes late-onset sepsis in intensive care neonates. PLoS ONE [Electronic Resource] **7:**e31548.
7. **Villari P, Sarnataro C, Iacuzio L.** 2000. Molecular epidemiology of Staphylococcus epidermidis in a neonatal intensive care unit over a three-year period. Journal of Clinical Microbiology **38:**1740-1746.
8. **Van Der Zwet WC, Debets-Ossenkopp YJ, Reinders E, Kapi M, Savelkoul PH, Van Elburg RM, Hiramatsu K, Vandenbroucke-Grauls CM.** 2002. Nosocomial spread of a Staphylococcus capitis strain with heteroresistance to vancomycin in a neonatal intensive care unit. Journal of Clinical Microbiology **40:**2520-2525.
9. **Becker K, Heilmann C, Peters G.** 2014. Coagulase-negative staphylococci. Clinical microbiology reviews **27:**870-926.
10. **Kloos WE, Musselwhite MS.** 1975. Distribution and persistence of Staphylococcus and Micrococcus species and other aerobic bacteria on human skin. Applied microbiology **30:**381-395.
11. **Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, Bouffard GG, Blakesley RW, Murray PR, Green ED.** 2009. Topographical and temporal diversity of the human skin microbiome. science **324:**1190-1192.
12. **Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R.** 2009. Bacterial community variation in human body habitats across space and time. Science **326:**1694-1697.

13. **Nash C, Chu A, Bhatti M, Alexander K, Schreiber M, Hageman JR.** 2013. Coagulase Negative Staphylococci in the Neonatal Intensive Care Unit: Are We Any Smarter? NeoReviews **14:**e284-e293.
14. **Pfaller MA, Herwaldt LA.** 1988. Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. Clinical Microbiology Reviews **1:**281-299.
15. **Duguid I, Evans E, Brown M, Gilbert P.** 1992. Growth-rate-independent killing by ciprofloxacin of biofilm-derived Staphylococcus epidermidis evidence for cell-cycle dependency. Journal of antimicrobial chemotherapy **30:**791-802.
16. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK.** 2008. Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infection Control **29:**996-1011.
17. **Fontela PS, Platt RW, Rocher I, Frenette C, Moore D, Fortin É, Buckeridge D, Pai M, Quach C.** 2012. Epidemiology of central line–associated bloodstream infections in Quebec intensive care units: A 6-year review. American journal of infection control **40:**221-226.
18. **Institut National de Santé Publique Québec.** 2015. Bactériémies nosocomiales panhospitalières: Résultats de surveillance 2014-2015. https://www.inspq.qc.ca/infectionsnosocomiales/spin-bactot/surveillance-2014-2015. Accessed January 27, 2016.
19. **von Eiff C, Peters G, Heilmann C.** 2002. Pathogenesis of infections due to coagulasenegative staphylococci. The Lancet infectious diseases **2:**677-685.
20. **Horan TC, Andrus M, Dudeck MA.** 2008. CDC/NHSN surveillance definition of health care–associated infection and criteria for specific types of infections in the acute care setting. American journal of infection control **36:**309-332.
21. **Jean-Baptiste N, Benjamin DK, Cohen-Wolkowiez M, Fowler VG, Laughon M, Clark RH, Smith PB.** 2011. Coagulase-negative staphylococcal infections in the neonatal intensive care unit. Infection Control **32:**679-686.
22. **Liljedahl M, Bodin L, Schollin J.** 2004. Coagulase-negative staphylococcal sepsis as a predictor of bronchopulmonary dysplasia. Acta Paediatrica **93:**211-215.
23. **Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, Higgins RD, Health NIoC, Network HDNR.** 2004. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. Jama **292:**2357-2365.
24. **Mittendorf R, Roizen N, Moawad A, Khoshnood B, Lee K-S.** 1999. Association between cerebral palsy and coagulase-negative staphylococci. The Lancet **354:**1875-1876.
25. **Krediet TG, Jones ME, Gerards LJ, Fleer A.** 1999. Clinical outcome of cephalothin versus vancomycin therapy in the treatment of coagulase-negative staphylococcal septicemia in neonates: relation to methicillin resistance and mec A gene carriage of blood isolates. Pediatrics **103:**E29.
26. **Healy CM, Palazzi DL, Edwards MS, Campbell JR, Baker CJ.** 2004. Features of invasive staphylococcal disease in neonates. Pediatrics **114:**953-961.

27. **Healy C, Baker C, Palazzi D, Campbell J, Edwards M.** 2013. Distinguishing true coagulase-negative Staphylococcus infections from contaminants in the neonatal intensive care unit. Journal of Perinatology **33:**52-58.
28. **Rogers KL, Fey PD, Rupp ME.** 2009. Coagulase-negative staphylococcal infections. Infectious disease clinics of North America **23:**73-98.
29. **de Allori MCG, Jure MÁ, Romero C, de Castillo MEC.** 2006. Antimicrobial resistance and production of biofilms in clinical isolates of coagulase-negative Staphylococcus strains. Biological and Pharmaceutical Bulletin **29:**1592-1596.
30. **Götz F, Perconti S, Popella P, Werner R, Schlag M.** 2014. Epidermin and gallidermin: staphylococcal lantibiotics. International Journal of Medical Microbiology **304:**63-71.
31. **Ogston A.** 1881. Report upon micro-organisms in surgical diseases. British medical journal **1:**369. b362.
32. **Urwin R, Maiden MC.** 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends in microbiology **11:**479-487.
33. **Widerström M, Wiström J, Sjöstedt A, Monsen T.** 2012. Coagulase-negative staphylococci: update on the molecular epidemiology and clinical presentation, with a focus on Staphylococcus epidermidis and Staphylococcus saprophyticus. European journal of clinical microbiology & infectious diseases **31:**7-20.
34. **Sloos J, Dijkshoorn L, Vogel L, Van Boven C.** 2000. Performance of Phenotypic and Genotypic Methods To Determine the Clinical Relevance of Serial Blood Isolates ofStaphylococcus epidermidis in Patients with Septicemia. Journal of clinical microbiology **38:**2488-2493.
35. **Becker K, Harmsen D, Mellmann A, Meier C, Schumann P, Peters G, Von Eiff C.** 2004. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of Staphylococcus species. Journal of clinical microbiology **42:**4988-4995.
36. **Argemi X, Riegel P, Lavigne T, Lefebvre N, Grandpré N, Hansmann Y, Jaulhac B, Prévost G, Schramm F.** 2015. Implementation of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry in Routine Clinical Laboratories Improves Identification of Coagulase-Negative Staphylococci and Reveals the Pathogenic Role of Staphylococcus lugdunensis. Journal of clinical microbiology **53:**2030-2036.
37. **Miragaia M, Carrico J, Thomas J, Couto I, Enright M, De Lencastre H.** 2008. Comparison of molecular typing methods for characterization of Staphylococcus epidermidis: proposal for clone definition. Journal of clinical microbiology **46:**118-129.
38. **Miragaia M, Thomas J, Couto I, Enright M, De Lencastre H.** 2007. Inferring a population structure for Staphylococcus epidermidis from multilocus sequence typing data. Journal of bacteriology **189:**2540-2552.
39. **Thomas JC, Vargas MR, Miragaia M, Peacock SJ, Archer GL, Enright MC.** 2007. Improved multilocus sequence typing scheme for Staphylococcus epidermidis. Journal of clinical microbiology **45:**616-619.
40. **Milisavljevic V, Wu F, Cimmotti J, Haas J, Della-Latta P, Larson E, Saiman L.** 2005. Genetic relatedness of Staphylococcus epidermidis from infected infants

and staff in the neonatal intensive care unit. American journal of infection control **33:**341-347.

41. **Huebner J, Pier GB, Maslow JN, Muller E, Shiro H, Parent M, Kropec A, Arbeit RD, Goldmann DA.** 1994. Endemic Nosocomial Transmission of Staphylococcus epidermidis Bacteremia Isolates in a Neonatal Intensive Care Unit over 1O Years. Journal of Infectious Diseases **169:**526-531.
42. **Low DE, Schmidt BK, Kirpalani HM, Moodie R, Ford-Jones EL, Kreiswirth B, Matlow A.** 1992. An endemic strain of Staphylococcus haemolyticus colonizing and causing bacteremia in neonatal intensive care unit patients. Pediatrics **89:**696-700.
43. **Venkatesh MP, Placencia F, Weisman LE.** 2006. Coagulase-negative staphylococcal infections in the neonate and child: an update. Seminars in Pediatric Infectious Diseases **17:**120-127.
44. **Duah M.** 2010. Daptomycin for methicillin-resistant Staphylococcus epidermidis native-valve endocarditis: a case report. Annals of clinical microbiology and antimicrobials **9:**9.
45. **Jones RN, Ross JE, Castanheira M, Mendes RE.** 2008. United States resistance surveillance results for linezolid (LEADER Program for 2007). Diagnostic microbiology and infectious disease **62:**416-426.
46. **Gu B, Kelesidis T, Tsiodras S, Hindler J, Humphries RM.** 2013. The emerging problem of linezolid-resistant Staphylococcus. Journal of Antimicrobial Chemotherapy **68:**4-11.
47. **Tewhey R, Gu B, Kelesidis T, Charlton C, Bobenchik A, Hindler J, Schork NJ, Humphries RM.** 2014. Mechanisms of linezolid resistance among coagulase-negative staphylococci determined by whole-genome sequencing. MBio **5:**e00894-00814.
48. **Mermel LA, Allon M, Bouza E, Craven DE, Flynn P, O'Grady NP, Raad II, Rijnders BJ, Sherertz RJ, Warren DK.** 2009. Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America. Clinical infectious diseases **49:**1-45.
49. **Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I.** 1997. Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. The Lancet **350:**1670-1673.
50. **Howden BP, Davies JK, Johnson PD, Stinear TP, Grayson ML.** 2010. Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. Clinical microbiology reviews **23:**99-139.
51. **Maor Y, Hagin M, Belausov N, Keller N, Ben-David D, Rahav G.** 2009. Clinical features of heteroresistant vancomycin-intermediate Staphylococcus aureus bacteremia versus those of methicillin-resistant S. aureus bacteremia. Journal of Infectious Diseases **199:**619-624.

52. **Charles PG, Ward PB, Johnson PD, Howden BP, Grayson ML.** 2004. Clinical features associated with bacteremia due to heterogeneous vancomycin-intermediate Staphylococcus aureus. Clinical infectious diseases **38:**448-451.
53. **van Hal SJ, Paterson DL.** 2011. Systematic review and meta-analysis of the significance of heterogeneous vancomycin-intermediate Staphylococcus aureus isolates. Antimicrobial agents and chemotherapy **55:**405-410.
54. **Nunes APF, Teixeira LM, Iorio NLP, Bastos CCR, de Sousa Fonseca L, Souto-Padrón T, Dos Santos KRN.** 2006. Heterogeneous resistance to vancomycin in Staphylococcus epidermidis, Staphylococcus haemolyticus and Staphylococcus warneri clinical strains: characterisation of glycopeptide susceptibility profiles and cell wall thickening. International journal of antimicrobial agents **27:**307-315.
55. **D'Mello D, Daley AJ, Rahman MS, Qu Y, Garland S, Pearce C, Deighton MA.** 2008. Vancomycin heteroresistance in bloodstream isolates of Staphylococcus capitis. Journal of Clinical Microbiology **46:**3124-3126.
56. **Patel J, Cockerill F, Alder J, Bradford P, Eliopoulos G, Hardy D.** 2014. Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. CLSI standards for antimicrobial susceptibility testing **34:**1-226.
57. **Walsh TR, Bolmström A, Qwärnström A, Ho P, Wootton M, Howe RA, MacGowan AP, Diekema D.** 2001. Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. Journal of clinical microbiology **39:**2439-2444.
58. **Wootton M, Howe R, Hillman R, Walsh T, Bennett P, MacGowan A.** 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. Journal of Antimicrobial Chemotherapy **47:**399-403.
59. **Wootton M, MacGowan AP, Walsh TR, Howe RA.** 2007. A multicenter study evaluating the current strategies for isolating Staphylococcus aureus strains with reduced susceptibility to glycopeptides. Journal of clinical microbiology **45:**329-332.
60. **Fontela PS, Platt RW, Rocher I, Frenette C, Moore D, Fortin É, Buckeridge D, Pai M, Quach C.** 2011. Surveillance Provinciale des Infections Nosocomiales (SPIN) Program: implementation of a mandatory surveillance program for central line-associated bloodstream infections. American journal of infection control **39:**329-335.
61. **Gaynes RP, Edwards JR, Jarvis WR, Culver DH, Tolson JS, Martone WJ.** 1996. Nosocomial infections among neonates in high-risk nurseries in the United States. National Nosocomial Infections Surveillance System. Pediatrics **98:**357-361.
62. **Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK.** 2002. Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics **110:**285-291.

63. **Jean-Baptiste N, Benjamin DK, Jr., Cohen-Wolkowiez M, Fowler VG, Jr., Laughon M, Clark RH, Smith PB.** 2011. Coagulase-negative staphylococcal infections in the neonatal intensive care unit. Infect Control Hosp Epidemiol **32:**679-686.
64. **Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, Higgins RD, National Institute of Child H, Human Development Neonatal Research N.** 2004. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. JAMA **292:**2357-2365.
65. **Krediet TG, Jones ME, Janssen K, Gerards LJ, Fleer A.** 2001. Prevalence of Molecular Types and mecA Gene Carriage of Coagulase-Negative Staphylococci in a Neonatal Intensive Care Unit: Relation to Nosocomial Septicemia. Journal of Clinical Microbiology **39:**3376-3378.
66. **Venkatesh MP, Placencia F, Weisman LE.** 2006. Coagulase-negative staphylococcal infections in the neonate and child: an update. Semin Pediatr Infect Dis **17:**120-127.
67. **Garrett DO, Jochimsen E, Murfitt K, Hill B, McAllister S, Nelson P, Spera RV, Sall RK, Tenover FC, Johnston J, Zimmer B, Jarvis WR.** 1999. The emergence of decreased susceptibility to vancomycin in Staphylococcus epidermidis. Infection Control & Hospital Epidemiology **20:**167-170.
68. **Van Der Zwet WC, Debets-Ossenkopp YJ, Reinders E, Kapi M, Savelkoul PHM, Van Elburg RM, Hiramatsu K, Vandenbroucke-Grauls CMJE.** 2002. Nosocomial spread of a Staphylococcus capitis strain with heteroresistance to vancomycin in a neonatal intensive care unit. Journal of Clinical Microbiology **40:**2520-2525.
69. **Sieradzki K, Villari P, Tomasz A.** 1998. Decreased susceptibilities to teicoplanin and vancomycin among coagulase-negative methicillin-resistant clinical isolates of staphylococci. Antimicrobial Agents & Chemotherapy **42:**100-107.
70. **Duah M.** 2010. Daptomycin for methicillin-resistant Staphylococcus epidermidis native-valve endocarditis: a case report. Ann Clin Microbiol Antimicrob **9:**9.
71. **Zubair M, Zafar A, Ejaz H, Hafeez S, Javaid H, Javed A.** 2011. Incidence of coagulase negative staphylococci in neonatal sepsis. Pakistan Journal of Medical and Health Sciences **5:**716-719.
72. **Casapao AM, Leonard SN, Davis SL, Lodise TP, Patel N, Goff DA, LaPlante KL, Potoski BA, Rybak MJ.** 2013. Clinical Outcomes in Patients with Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Bloodstream Infection. Antimicrobial agents and chemotherapy **57:**4252-4259.
73. **Jones RN.** 2006. Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict clinical outcomes or detect resistant strains. Clinical Infectious Diseases **42 Suppl 1:**S13-24.
74. **Walsh TR, Bolmstrom A, Qwarnstrom A, Ho P, Wootton M, Howe RA, MacGowan AP, Diekema D.** 2001. Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. J Clin Microbiol **39:**2439-2444.

75. **Gordon RJ, Miragaia M, Weinberg AD, Lee CJ, Rolo J, Giacalone JC, Slaughter MS, Pappas P, Naka Y, Tector AJ.** 2012. Staphylococcus epidermidis colonization is highly clonal across US cardiac centers. Journal of Infectious Diseases **205:**1391-1398.
76. **Widerström M, McCullough CA, Coombs GW, Monsen T, Christiansen KJ.** 2012. A multidrug-resistant Staphylococcus epidermidis clone (ST2) is an ongoing cause of hospital acquired infection in a Western Australian hospital. Journal of clinical microbiology**:**JCM. 06456-06411.
77. **Biavasco F, Vignaroli C, Varaldo P.** 2000. Glycopeptide resistance in coagulase-negative staphylococci. European Journal of Clinical Microbiology and Infectious Diseases **19:**403-417.
78. **Blanchard AC, Fortin E, Rocher I, Moore DL, Frenette C, Tremblay C, Quach C.** 2013. Central line–associated bloodstream infection in neonatal intensive care units. Infection Control **34:**1167-1173.
79. **Lévesque S LY, Domingo MC, Massé C, Bernatchez H, Gaudreau C, Tremblay C.** In press. Enteroccocus pallens as a potential novel human pathogen: three cases of spontaneous bacterial peritonitis. JMM Case Reports.
80. **Yusof A, Engelhardt A, Karlsson Å, Bylund L, Vidh P, Mills K, Wootton M, Walsh TR.** 2008. Evaluation of a new Etest vancomycin-teicoplanin strip for detection of glycopeptide-intermediate Staphylococcus aureus (GISA), in particular, heterogeneous GISA. Journal of clinical microbiology **46:**3042-3047.
81. **Turner J, Howe RA, Wootton M, Bowker KE, Holt HA, Salisbury V, Bennett PM, Walsh TR, MacGowan AP.** 2001. The activity of vancomycin against heterogeneous vancomycin-intermediate methicillin-resistant Staphylococcus aureus explored using an in vitro pharmacokinetic model. Journal of Antimicrobial Chemotherapy **48:**727-730.
82. **Wootton M, Howe R, Hillman R, Walsh T, Bennett P, MacGowan A.** 2001. A modified population analysis profile method to detect Staphylococcus aureus with decreased susceptibilities to vancomycin in a UK hospital. J Antimicrob Chemother **47:**399-404.
83. **Plipat N, Livni G, Bertram H, Thomson RB.** 2005. Unstable vancomycin heteroresistance is common among clinical isolates of methiciliin-resistant Staphylococcus aureus. Journal of clinical microbiology **43:**2494-2496.
84. **Mulvey M, Chui L, Ismail J, Louie L, Murphy C, Chang N, Alfa M.** 2001. Development of a Canadian standardized protocol for subtyping methicillin-resistant Staphylococcus aureus using pulsed-field gel electrophoresis. Journal of clinical microbiology **39:**3481-3485.
85. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. Journal of clinical microbiology **33:**2233.
86. **Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. Journal of bacteriology **186:**1518-1530.

87. **Carrico J, Silva-Costa C, Melo-Cristino J, Pinto F, De Lencastre H, Almeida J, Ramirez M.** 2006. Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant Streptococcus pyogenes. Journal of Clinical Microbiology **44:**2524-2532.
88. **Hubert L, Arabie P.** 1985. Comparing partitions. Journal of classification **2:**193-218.
89. **Pinto FR, Melo-Cristino J, Ramirez M.** 2008. A confidence interval for the Wallace coefficient of concordance and its application to microbial typing methods. PLoS One **3:**e3696.
90. **Severiano A, Pinto FR, Ramirez M, Carriço JA.** 2011. Adjusted Wallace coefficient as a measure of congruence between typing methods. Journal of clinical microbiology **49:**3997-4000.
91. **El-Halfawy OM, Valvano MA.** 2015. Antimicrobial Heteroresistance: an Emerging Field in Need of Clarity. Clinical microbiology reviews **28:**191-207.
92. **O'Connor C, Powell J, Finnegan C, O'Gorman A, Barrett S, Hopkins K, Pichon B, Hill R, Power L, Woodford N.** 2015. Incidence, management and outcomes of the first cfr-mediated linezolid-resistant Staphylococcus epidermidis outbreak in a tertiary referral centre in the Republic of Ireland. Journal of Hospital Infection.
93. **Mendes RE, Deshpande LM, Costello A, Farrell DJ.** 2012. Analysis of the molecular epidemiology of Staphylococcus epidermidis clinical isolates from USA hospitals. Antimicrobial agents and chemotherapy**:**AAC. 00279-00212.
94. **Farrell DJ, Mendes RE, Ross JE, Sader HS, Jones RN.** 2011. LEADER Program results for 2009: an activity and spectrum analysis of linezolid using 6,414 clinical isolates from the United States (56 medical centers). Antimicrobial agents and chemotherapy**:**AAC. 01729-01710.
95. **Cui L, Tominaga E, Neoh H-m, Hiramatsu K.** 2006. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate Staphylococcus aureus. Antimicrobial agents and chemotherapy **50:**1079-1082.
96. **Pillai SK, Gold HS, Sakoulas G, Wennersten C, Moellering RC, Eliopoulos GM.** 2007. Daptomycin nonsusceptibility in Staphylococcus aureus with reduced vancomycin susceptibility is independent of alterations in MprF. Antimicrobial agents and chemotherapy **51:**2223-2225.
97. **Sakoulas G, Alder J, Thauvin-Eliopoulos C, Moellering RC, Eliopoulos GM.** 2006. Induction of daptomycin heterogeneous susceptibility in Staphylococcus aureus by exposure to vancomycin. Antimicrobial Agents and Chemotherapy **50:**1581-1585.
98. **Bayer AS, Schneider T, Sahl HG.** 2013. Mechanisms of daptomycin resistance in Staphylococcus aureus: role of the cell membrane and cell wall. Annals of the New York Academy of Sciences **1277:**139-158.
99. **Jones T, Yeaman MR, Sakoulas G, Yang S-J, Proctor RA, Sahl H-G, Schrenzel J, Xiong YQ, Bayer AS.** 2008. Failures in clinical treatment of Staphylococcus aureus infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrobial agents and chemotherapy **52:**269-278.

100. **Cherifi S, Byl B, Deplano A, Nonhoff C, Denis O, Hallin M.** 2013. Comparative epidemiology of Staphylococcus epidermidis isolates from patients with catheter-related bacteremia and from healthy volunteers. Journal of clinical microbiology **51:**1541-1547.
101. **Bogado I, Limansky A, Sutich E, Marchiaro P, Marzi M, Putero J, Viale A.** 2002. Molecular characterization of methicillin-resistant coagulase-negative staphylococci from a neonatal intensive care unit. Infection Control **23:**447-451.
102. **Wong A, Reddy SP, Smyth DS, Aguero-Rosenfeld ME, Sakoulas G, Robinson DA.** 2010. Polyphyletic emergence of linezolid-resistant staphylococci in the United States. Antimicrobial agents and chemotherapy **54:**742-748.
103. **Thomas JC, Zhang L, Robinson DA.** 2014. Differing lifestyles of Staphylococcus epidermidis as revealed through Bayesian clustering of multilocus sequence types. Infection, Genetics and Evolution **22:**257-264.
104. **Méric G, Miragaia M, De Been M, Yahara K, Pascoe B, Mageiros L, Mikhail J, Harris LG, Wilkinson TS, Rolo J.** 2015. Ecological overlap and horizontal gene transfer in Staphylococcus aureus and Staphylococcus epidermidis. Genome biology and evolution **7:**1313-1328.
105. **Schoenfelder SM, Lange C, Eckart M, Hennig S, Kozytska S, Ziebuhr W.** 2010. Success through diversity–how Staphylococcus epidermidis establishes as a nosocomial pathogen. International Journal of medical microbiology **300:**380-386.
106. **Satola SW, Lessa FC, Ray SM, Bulens SN, Lynfield R, Schaffner W, Dumyati G, Nadle J, Patel JB, Active Bacterial Core surveillance MI.** 2011. Clinical and laboratory characteristics of invasive infections due to methicillin-resistant Staphylococcus aureus isolates demonstrating a vancomycin MIC of 2 micrograms per milliliter: lack of effect of heteroresistant vancomycin-intermediate S. aureus phenotype. Journal of Clinical Microbiology **49:**1583-1587.
107. **Diekema D, Pfaller M, Schmitz F, Smayevsky J, Bell J, Jones R, Beach M.** 2001. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clinical Infectious Diseases **32:**S114-S132.
108. **Quach C, Milstone AM, Perpête C, Bonenfant M, Moore DL, Perreault T.** 2014. Chlorhexidine Bathing in a Tertiary Care Neonatal Intensive Care Unit: Impact on Central Line–Associated Bloodstream Infections. Infection Control **35:**158-163.
109. **Jasmine Chong CQ, Ana C. Blanchardb, Philippe Guillaume Poliquind, George Golding, Céline Laferrière, Simon Lévesque.** 2016. Molecular epidemiology of vancomycin heteroresistant-intermediate coagulase negative Staphylococcus outbreak in the neonatal intensive care unit. In preparation
110. **Stephan F, de Montblanc J, Cheffi A, Bonnet F.** 1999. Thrombocytopenia in critically ill surgical patients: a case-control study evaluating attributable mortality and transfusion requirements. Critical Care **3:**151.
111. **Costa S, Maggio L, Sindico P, Cota F, De Carolis MP, Romagnoli C.** 2010. Preterm small for gestational age infants are not at higher risk for parenteral nutrition–associated cholestasis. The Journal of pediatrics **156:**575-579.

112. **Kliegman R, Walsh M.** 1987. Neonatal necrotizing enterocolitis: pathogenesis, classification, and spectrum of illness. Current problems in pediatrics **17:**219-288.
113. **Khashu M, Osiovich H, Henry D, Al Khotani A, Solimano A, Speert DP.** 2006. Persistent bacteremia and severe thrombocytopenia caused by coagulase-negative Staphylococcus in a neonatal intensive care unit. Pediatrics **117:**340-348.
114. **Charoo BA, Iqbal J, Iqbal Q, Mushtaq S, Bhat AW, Nawaz I.** 2009. Nosocomial sepsis-induced late onset thrombocytopenia in a neonatal tertiary care unit: a prospective study. Hematology/oncology and stem cell therapy **2:**349-353.
115. **Christensen R, Henry E, Wiedmeier S, Stoddard R, Sola-Visner M, Lambert D, Kiehn T, Ainsworth S.** 2006. Thrombocytopenia among extremely low birth weight neonates: data from a multihospital healthcare system. Journal of Perinatology **26:**348-353.
116. **Guida JD, Kunig AM, Leef KH, McKenzie SE, Paul DA.** 2003. Platelet count and sepsis in very low birth weight neonates: is there an organism-specific response? Pediatrics **111:**1411-1415.
117. **Fuchs M, Sanyal AJ.** 2008. Sepsis and cholestasis. Clinics in liver disease **12:**151-172.
118. **Falagas M, Makris G, Dimopoulos G, Matthaiou D.** 2008. Heteroresistance: a concern of increasing clinical significance? Clinical Microbiology and Infection **14:**101-104.