Growth and Biofilm Formation of Bacteria Isolated from Contaminated Platelet Units

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ABSTRACT

Bacterial contamination of platelet concentrates (PCs) poses the major transfusion-associated infectious risk. Coagulase negative staphylococci (CoNS), the predominant platelet contaminants, are recognized as one of the leading causes of hospital-acquired infections due to their ability to form biofilms (surface-attached aggregates). In this study, 29 CoNS strains were characterized for their growth and biofilm formation abilities in media and PCs. Twenty-five strains were aerobic including *Staphylococcus epidermidis*, *S. capitis*, and *S. chromogenes*, while four were identified as the anaerobe *S. saccharolyticus*. Biofilm-associated *icaA* and *icaD* genes were amplified from eight strains. Interestingly, only six of those strains were biofilm-positive. Sequencing of *S. capitis icaD* revealed no mutations that could explain differences in biofilm phenotypes. Growth of CoNS in PCs varied significantly between strains. This study provides preliminary evidence that slow-growing biofilm-positive *S. epidermidis* are more likely to be missed during platelet culture, highlighting the need for improved screening methods.

To my parents,

wife, family, supervisor and friends.

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LIST OF ABBREVIATIONS

Aap	Accumulation-associated protein
Aae	Accumulation/adhesin protein
ACD	Acid Citrate Dextrose
ADP	Adenosine diphosphate
Agr	Accessory gene regulator
API	Analytical Profile Index
ATCC	American Type Culture Collection
AtlE	Autolysin E
ATR	Adverse transfusion reaction
AMPs	Antimicrobial Peptides
A492nm	Absorbance values at a 492 nm wavelength
BA	Blood Agar
BC	Buffy coat
BF	Biofilm formation
BHI	Brain heart infusion
Bhp	Bap-homologous protein
CBS	Canadian Blood Services
CFU	Colony forming units
ClfA	clumping factor A
CMV	Cytomegalovirus
CoNS	Coagulase negative staphylococci
CPD	Citrate Phosphate Dextrose
CPDA-1	Citrate Phosphate Dextrose Adenine-1
CRA	Congo red agar
D	Aspartic acid/Aspartate
d2HO	Distilled, deionized water
DNA	Deoxyribonucleic acid
Embp	Extracellular matrix binding protein
FDA	Food and Drug Administration
Fg	Fibrinogen
FnBP	Fibronictin binding proteins
G	Guanine
GP	Glycoprotein
GlcNAc	N-acetylglucosamine
GPIb	Glycoprotein Ib
h	Hour
HIV	Human immunodeficiency virus
HTLV	Human T-lymphotropic virus type
I	Isoleucine
Ica	Intercellular adhesin
Kbp	Kilo-base pair
kDa	Kilodalton
L	Leucine
LPS	Lipopolysaccharide
mM	Milimolar
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
n	Sample size
NA	Not applicable

Neg	Negative
ND	Not done, not determined
netCAD	Network Centre for Applied Development
OD	Optical density
OCS	Open Canalicular System
PAS	Platelet additive solution
PBS	Phosphate buffer saline
PCs	Platelet concentrates
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PGA	poly-gama-glutamic acid
PIA	Polysaccharide intercellular adhesin
PMN	polymorphonuclear leukocytes
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRT	Pathogen reduction technology
VC	Polyvinyl chloride
RBC	Red blood cell
rpm	Revolutions per minute
RNA	Ribonucleic acid
s	Second
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SdrG,F,H	Serine-aspartate repeat protein family
S. epi	*Staphylococcus epidermidis*
S. liq	*Serratia liquefaciens*
SDAP	Single Donor Apheresis
Spp	Species
TBS	Tris buffer saline
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSBg	Tryptic soy broth + 0.5% glucose
vWF	von Willebrand Factor
WBP	whole blood-derived platelets

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CHAPTER ONE
INTRODUCTION

1.1 TRANSFUSION MEDICINE

The science of blood transfusion was born in the first decade of the 19th century, when Dr. James Blundell performed the first successful transfusion of human blood (125). Despite Blundell's initial success, it was not until the early 20th century that the field of blood transfusion advanced dramatically. In 1901, the Austrian physician Karl Landsteiner noticed that when incompatible blood types were mixed, the red blood cells clumped, and that the receiver developed immunological reactions to the donor blood cells. This observation allowed him to discover the human blood groups, earning him the Nobel Prize in Physiology and Medicine in 1930 (4).

The first transfusions attempts had to be made directly from donor to recipient to avoid blood coagulation. In 1914, the Belgian doctor Albert Hustin performed the first blood transfusion using sodium citrate as an anticoagulant. Since then, many new biochemical additive solutions had been developed to prevent coagulation and enhance storage (95). In 1943, Loutit and Mollison introduced the Acid Citrate Dextrose (ACD) additive solution (58). In 1957, Gibson and coworkers developed the Citrate Phosphate Dextrose (CPD) and Citrate Phosphate Dextrose Adenine-1 (CPDA-1) additive solutions which are widely used to preserve blood and blood products for longer periods of time (46).

Blood was originally collected in glass bottles and transfused as whole blood until the 1960s, when the plastic bags were introduced. The sterile plastic bags facilitated the separation of whole blood into different blood components. Nowadays, one whole blood

unit can provide 2-3 therapeutic doses after separation into different blood components such as red blood cells, plasma, or platelets (21).

1.1.1 Blood Components

Blood is a highly-specialized fluid which accounts for 8% of the human body weight (2). The average of blood volume in adults is approximately five liters. Blood plays a vital role in providing the body's cells with the oxygen and nutrients required for biological processes. It also carries away unwanted waste products and carbon dioxide from the same cells. Blood also regulates the body pH and temperature (homeostasis), plays a role in coagulation, and protects from infection by circulating immune system cells.

Blood is mainly composed of plasma, erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). Plasma which circulates nutrients, such as glucose and amino acids and removes waste products such as carbon dioxide and urea, accounts for approximately 54% of whole blood volume and consists mainly of water. Red blood cells are responsible for oxygen and carbon dioxide exchange, and account for 40-50% of the total blood volume. Leukocytes are part of the body's immune system and constitute about 1% of the blood volume. Platelets make up less than 1% of the blood volume and play a role in hemostasis and preventing blood loss through the coagulation pathway (130).

1.1.2 Platelets

Platelets are two to three micrometers (μm) in diameter; they are anucleate, irregular shaped fragments and therefore not considered to be true cells. Platelets are derived from the fragmentation of giant bone marrow progenitor cells known as megakaryocytes. Each

megakaryocyte can produce between 5,000 to 10,000 platelets. The normal platelet count in healthy individuals is between 150,000 and 450,000 per μl of blood (150–450 x 10^9/L). The average lifespan of a platelet is normally between five to nine days. Old platelets are destroyed in either the spleen or the liver.

Platelet activation is initiated when a blood vessel experiences mechanical damage resulting in the exposure/release of endothelial proteins, such as von Willebrand factor (vWF). This results in the recruitment of clotting factors such as Factor VIII and collagen. Circulating platelets bind immediately to endothelial collagen with surface collagen-specific glycoprotein Ia/IIa receptors while vWF further strengthens this attachment by forming additional links between platelet glycoprotein Ib/IX/V and the collagen fibrils. These interactions activate platelets, resulting in the formation of a plug (thrombus) at the site of injury in a process known as primary hemostasis. Secondary hemostasis involves the activation of a series of coagulation factors which ultimately leads to the formation of a stable fibrin clot. Once platelets become activated, they undergo morphological changes; specifically, platelets become star-like, with a spherical body projecting many long filaments (stellate morphology). This unique shape facilitates platelet adhesion to broken blood vessels or other platelets. Moreover, platelets express many surface proteins which allow them to adhere to broken blood vessels and facilitate the formation of plugs to prevent further bleeding. In general, platelets have an important function in hemostasis by forming blood clots and preventing bleeding (104).

1.1.3 Platelet Transfusion

Platelet concentrates are prepared either by single donor apheresis (SDAP) or by pooling whole blood-derived platelets (WBP) from four to six donors(21). During SDAP

collection, blood is drained from the donor's arm and passed through a specialized centrifuge which separates platelets from the whole blood and returns the remaining blood components to the donor through the other arm. This procedure is performed by a computer-controlled closed system machine to ensure the returned blood is sterile.

WBP are prepared either by the platelet- rich-plasma (PRP) method or the buffy coat (BC) method (21). Whole blood is collected from the donor's arm into a bag containing an anticoagulant solution. PRP is prepared by spinning the whole blood at a low speed (soft spin) and extracting the PRP while the red blood cells remain in the collection container. The PRP is then spun at high speed (hard spin) to concentrate the platelets (21). During BC platelet production, whole blood is spun at high speed and the plasma and red blood cells are both extracted, leaving the buffy coat layer in the collection container. The buffy coats from four to six donations of the same ABO-Rh group are pooled together and re-suspended in one plasma unit from one of the donors. The pooled BC then undergoes a low speed spin to produce pooled platelet concentrates (19). Each SDAP unit provides 250-600ml of platelet concentrates (1-3 therapeutic doses), while 4-6 WBP units are needed to provide one therapeutic dose of approximately 300 ml.

1.1.3.1 Bacterial Contamination of Platelet Concentrates

Currently, at Canadian Blood Services, blood products are screened for bloodborne pathogens using nucleic acid, immunological or culture techniques. Blood products are screened for human immunodeficiency virus type 1 and type 2 (HIV-1, HIV-2), human T-lymphotropic virus type 1 and type 2 (HTLV-1, HTLV-2), hepatitis B, hepatitis C, West

Nile Virus, and syphilis (*Treponema pallidum*). Samples from some donors are also tested for cytomegalovirus (CMV) and Chagas disease (*Trypanosoma cruzi*) (21).

The risks associated with contamination of blood products with viral agents have dramatically decreased in recent years due to the development of very sensitive immunological and molecular techniques that detect a wide range of infectious agents following blood collection. However, bacterial contamination remains as the most prevalent transfusion-associated infectious risk (50, 66). Despite interventions such as improved donor screening, appropriate donor arm disinfection, and diversion of the initial blood aliquot, cases of adverse transfusion reactions due to bacterial contamination of blood products still occur. Between 2005 and 2010, 24 cases of transfusion-related fatalities due to bacterial contamination of blood products were in the United States alone (40). Twenty-one of these fatalities were associated with transfusion of contaminated platelet units. Overall, the number of transfusion-related fatalities remains small in comparison to the total number of transfusions, which exceeded 24 million in the United States per year (40). The United Kingdom Serious Hazard Of Transfusion (SHOT) program documented 11 confirmed adverse transfusion reactions (ATRs) due to bacterial contamination of platelet concentrates (PCs) and red blood cells (RBCs) between 2005 and 2009, while only three possible bacterial ATRs were reported in 2010 (120, 121). Between March 2004 and October 2010, Canadian Blood Services received five reports of adverse transfusion reactions, including one fatality, due to contaminated platelet concentrates (66).

Platelet concentrates are the blood product most susceptible to bacterial contamination since they are stored for 5-7 days with constant agitation at 22±2^{o}C in oxygen-permeable plastic bags. Platelet components have a physiological pH (pH ~7.4) and high concentration of glucose (approximately 500mg/dL) mainly provided by the

anticoagulant solution. Although these conditions are necessary to maintain the normal function of platelets, they make this blood product an excellent media for bacterial growth. Even a small number of bacteria present in the platelet bags can multiply to a clinically-significant level during storage and subsequently lead to post-transfusion reactions.

Gram positive bacteria, specifically coagulase negative staphylococci (CoNS) and propionibacteria, are the predominant bacterial contaminants of platelet concentrates (140). *Staphylococcus epidermidis* is the CoNS most frequently isolated from contaminated platelet concentrates. Transfusions with fatal outcomes due to contamination with this species have been reported in Canada, the United States, and Europe (28, 47, 109, 139). Other CoNS including *S. capitis, S. warneri S. saccharolyticus,* and *S. hominis* have also been isolated during bacterial screening of platelet concentrates (35, 50, 115).

1.1.3.2 Strategies used to decrease the levels of bacterial contamination in blood components

Several measures have been implemented to decrease the risk of transfusing bacterially-contaminated blood components including donor screening, skin disinfection, first aliquot diversion, pre-transfusion detection, and pathogen reduction technologies.

The majority of bacteria found in contaminated blood products are part of the normal or transient skin flora.Optimal skin disinfection of the phlebotomy site is thus essential to maximize bacterial inactivation during blood collection. Canadian Blood Services uses a one-step 2% chlorhexidine and 70% isopropyl alcohol skin cleansing kit (108).

Donor screening methods to avoid collection of potentially contaminated blood products includes body temperature determination and answering a questionnaire which includes questions related to the donor's general health and potential signs of infection or silent bacteremia(20).

Diversion of the first 30-40 ml of blood during collection has been shown to significantly reduce the level of bacterial contamination in blood components, due to the fact that most flora bacterial cells colonizing the skin are trapped in the pouch (111, 138).

Routine platelet screening for bacterial contamination has been implemented in several countries. Detection of bacteria in transfusable blood products is more difficult than viral detection since bacterial concentrations increase over time under routine blood product storage conditions. Several pre-transfusion detection methods are currently used by blood product suppliers including the Pall eBDS (eBDS; Pall Corp, East Hills, NY) and the automated BacT/ALERT® 3D culture system (bioMérieux, Marcy l'Etoile, France), which is employed by Canadian Blood Services to detect bacterial contamination in platelet concentrates (66).

The BacT/ALERT System uses liquid aerobic and anaerobic culture bottles with a colorimetric sensor at the bottom that changes color from green to yellow when pH decreases as a result of CO_2 production by growing bacteria (126).The culture bottles are inoculated with 8-10 mL of PC samples and are incubated at 36^{o}C for one to six days. This system has been validated to detect 1 to 10 CFU/mL of most common platelet contaminants (84); however, despite its high sensitivity, several reports of missed bacterial detection in platelet concentrates have been reported worldwide, resulting in severe or fatal reactions

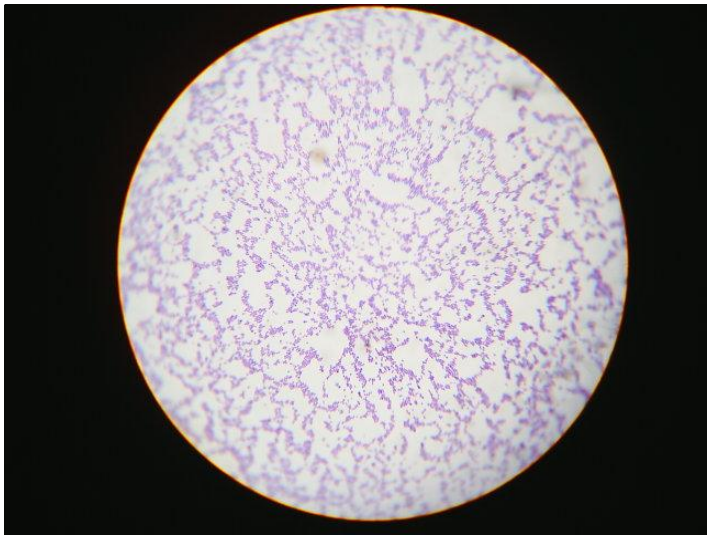
(28, 47, 66, 84, 109, 121, 139). It is likely that many false negatives that produce less severe or no reactions go unrecognised. While Canadian Blood Services performs routine testing only for aerobic bacteria, other centers such as the Dutch Sanquin Blood Bank North West use the two-bottle (aerobic and anaerobic) system which allows the detection of strict anaerobic bacteria such as *Propionibacterium acnes* and *Staphylococcus saccharolyticus.*

1.2 *STAPHYLOCOCCUS* SPP

Staphylococcus is derived from a Greek word meaning "bunch of grapes". The name was given due to the round (coccal) appearance of the cells in grape-like clusters under the light microscope. *Staphylococcus* species are characterized by their ability to retain the crystal violet dye during Gram staining, therefore they are known as Gram positive cocci (Figure1A). This species can be easily distinguished from other Gram positive cocci, such as *Streptococcus* species, which usually appear as diplococci or cocci in chains (Figure 1B). The production of the catalase enzyme, which converts hydrogen peroxide to water and oxygen resulting in bubble production, is a test used to distinguish *Streptococcus* from *Staphylococcus* species since the latter produces the catalase enzyme (Figure 2). Staphylococci are facultative anaerobes that can grow by either aerobic respiration or by fermentation (102). Although *Staphylococcus* is a non-spore-forming species, it is highly-resistant to dehydration, especially in the presence of organic substances such as blood and other tissue fluids (118).

Figure 1: Gram strain of different gram positive species. A) Gram strain of a representative *Staphylococcus* species (*S. aureus*) showing Gram positive (violet in color) cocci arranged in clusters (grape-like arrangement). B) Gram strain of a representative *Streptococcus* species (*Streptococcus pyogenes*) showing as Gram positive cocci forming long chains.

(A)



(B)

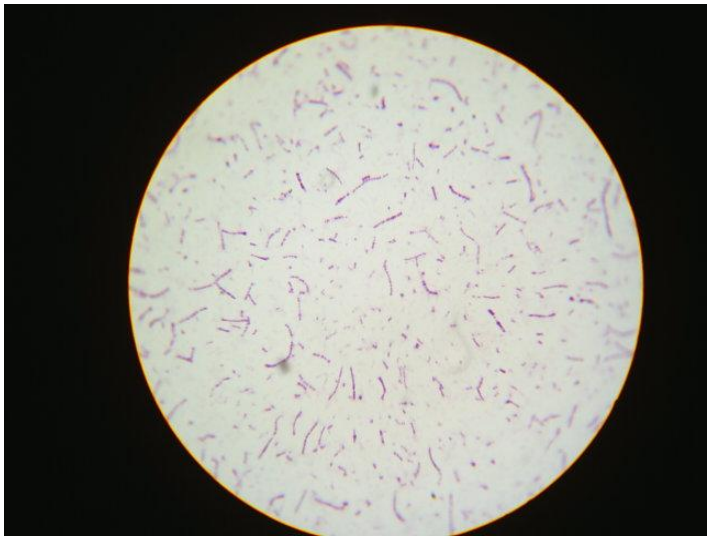
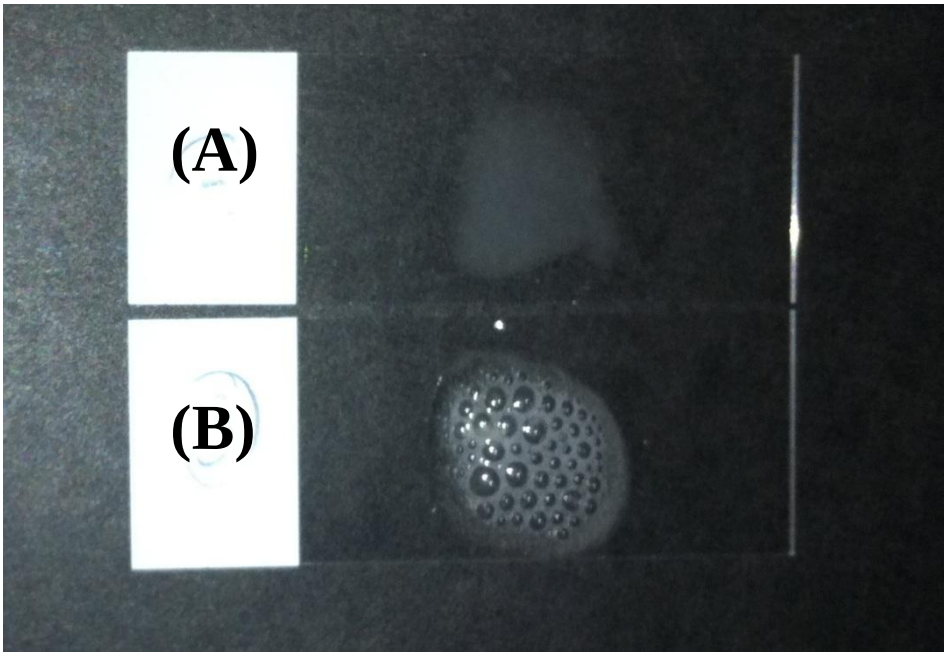


Figure 2: Catalase reaction. The catalase enzyme converts hydrogen peroxide to water and oxygen which appears as bubbles when hydrogen peroxide is added to the bacteria. A) *Streptococcus* species, (*Streptococcus aglactiae*) does not produce catalase enzyme (negative reaction), while B) *Staphylococcus* species (*S. epidermidis*) shows a positive catalase reaction.

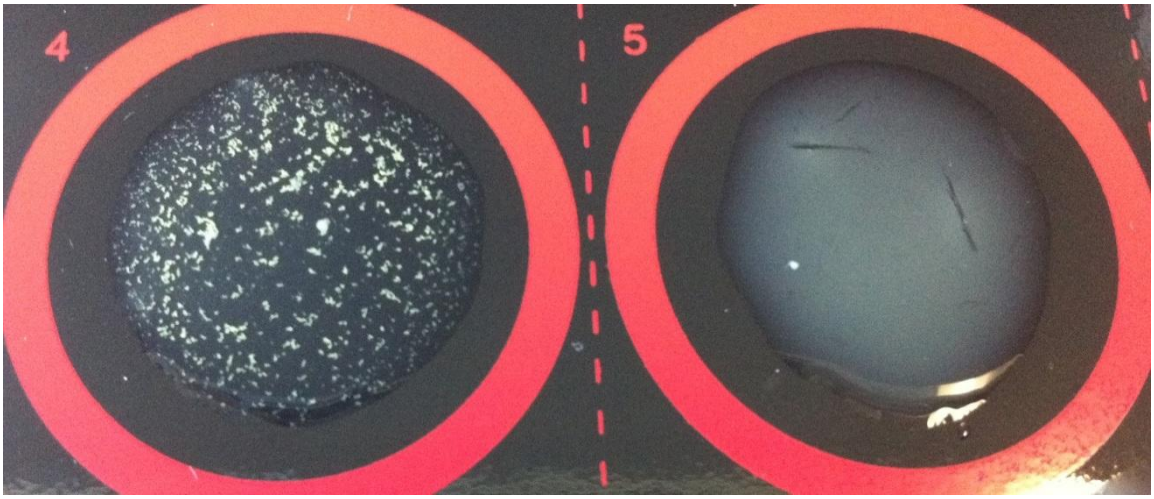

(A)
(B)

There are at least 40 species belonging to the *Staphylococcus* genus. Of these, one has three subspecies, nine have two subspecies, while the rest do not have any subspecies (57). Staphylococci are divided into two main groups: coagulase-positive *Staphylococcus* and coagulase-negative *Staphylococcus* (CoNS). Coagulase-positive staphylococci produce the enzyme coagulase, which converts fibrinogen to fibrin and causes blood clot formation (Figure 3) (136). Coagulase-positive staphylococci include six species: *S. aureus, S. intermedius, S. hyicus, S. lutrae, S. pseudointermedius,* and *S. schleiferi* subsp.*coagulans* (42).

CoNS are part of the normal flora of different human tissues like the skin, upper respiratory tract, vagina, and mucous membranes. CoNS were previously considered to be harmless microorganisms; however, in recent years, many accounts of bacteremia associated with CoNS have been reported, especially in immunocompromised patients, intravenous drug users, and neonates (90, 136). There are approximately 41 species/subspecies belonging to the CoNS group (77)**.** Of these, 21 species are associated with human disease including: *S. epidermidis, S. capitis, S. saprophyticus, S. saccharolyticus, S. warneri,* and *S. hominis* (77).

Figure 3: Coagulase reaction. Coagulase enzyme converts fibrinogen to fibrin and causes blood clot formation. A) Positive coagulase test (*S. aureus*), and B) negative coagulase test *(S. epidermidis).*

(A) **(B)**



1.2.1 *Staphylococcus epidermidis*

In 1884, Friedrich Rosenbach, a German physician and microbiologist, first distinguished *S. aureus* from *S. albus* by the appearance of yellow versus white colonies on blood agar. *S. albus* is now known as *S. epidermidis* (116).

S. epidermidis is the most widespread CoNS. This bacterium is mostly isolated from human epithelia and considered to be the predominant colonizer of the skin, axillae (armpits), head, and nose (112). Based on epidemiological studies, it has been shown that healthy individuals can carry up to 24 different strains of *S. epidermidis* at a time (148). The genetic complexity of this species allows it to survive and grow in different harsh environments. *S. epidermidis* is currently recognized as one of the leading pathogens in nosocomial (hospital-acquired) infections. *S. epidermidis* has the ability to colonize catheters and other biomedical devices, particularly affecting immunocompromised and neonate patients (99, 100).

Infections produced by *S. epidermidis* include: endocarditis (native and prosthetic heart valves), osteomyelitis, meningitis, otitis media, cerebrospinal fluid (CSF) shunt infection, urinary tract infection, peritoneal dialysis catheter infection, infections in prosthetic joints, and adverse transfusion reactions caused by contaminated blood products (18, 22, 25, 34, 38, 53, 62)**.**

Different virulence factors have been demonstrated in *S. epidermidis* such as the SepA protease involved in resistance to antimicrobial peptides (AMPs), the Aps system which regulates AMP resistance mechanisms and other exoenzymes, such as SepA and GluSE, which are involved in lipase maturation and degradation of fibrinogen and complement factor C5, respectively(37, 75, 76, 124, 135). *S. epidermidis* also produces toxins such as the pro-inflammatory cytolysin (PSMs) (89, 133, 144), and the haemolytic

N-formylated alpha-helical peptide δ-toxin that can cause lysis of red blood cells (44, 97, 136). In addition, the ability to synthesize biofilms significantly increases *S. epidermidis* virulence.

1.3 BACTERIAL BIOFILM FORMATION

A biofilm is a surface-associated multicellular aggregation of microbial cells which are embedded in an extracellular polymeric matrix. The matrix protects the biofilm cells and facilitates intercellular communication (5). It has been shown that microbial cells growing as a biofilm are both physiologically and genetically distinct from their counterpart planktonic cells (free-floating bacteria)(97).

1.3.1 *S. epidermidis* Biofilm Formation

Certain strains of *S. epidermidis* are able to produce biofilms, which facilitates their attachment and aggregation to specific surfaces, as well as their protection from antimicrobial agents and clearance by the immune system.

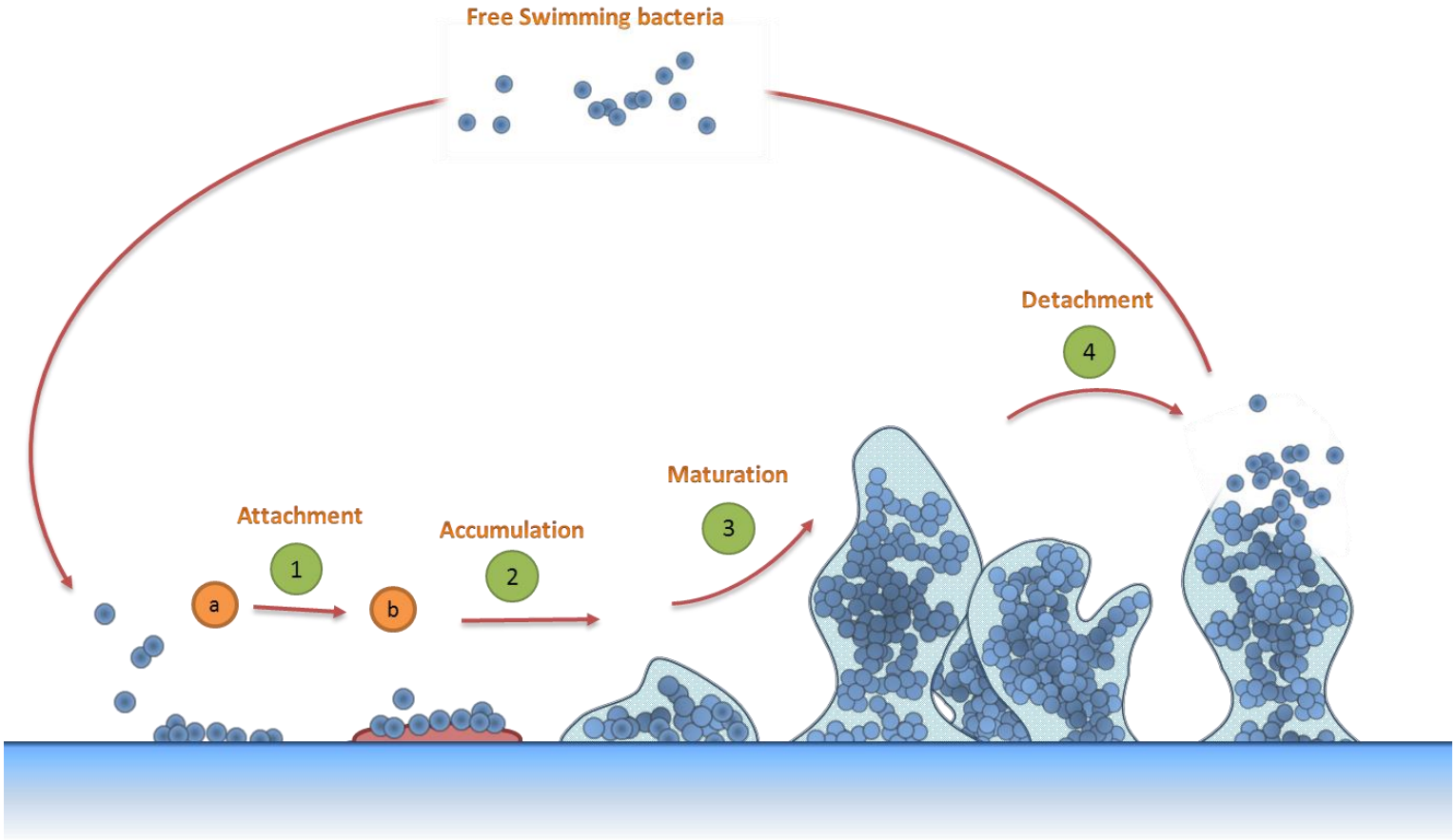
The exact mechanism by which *S. epidermidis* is able to produce a functional, mature biofilm is not clearly understood. However, based on what is known, *S. epidermidis* biofilm formation can be divided into a four-phase process including: initial adhesion of bacterial cells to a surface, accumulation and aggregation into a multicellular structure, formation of mature stable biofilms, and detachment and dispersal of bacterial cells from the biofilm (Figure 4).

Initial adhesion of bacterial cells to biomaterial surfaces is a fundamental step in the formation of a mature biofilm. *S. epidermidis* develops adhesive forces by releasing specific proteins to create a hydrophobic environment on the bacterial cell surface and this

subsequently facilitates the colonization of biomaterial surfaces. Polarity changes and non-specific physicochemical factors might also be involved in this step (68). The bi-functional adhesion and autolysin surface protein AtlE and the intercellular adhesion protein Bap (also known as Bhp in *S. epidermidis*) are contributing factors to the increased hydrophobicity of the staphylococcal cell surface (59, 128).

Bacteria can adhere directly to polymeric biomaterial surfaces or bind to host proteins covering biomaterial surfaces including collagen, fibrinogen, fibrinectin, vitronectin, and elastin. *S. epidermidis* carries a wide range of surface proteins that facilitate the primary attachment to host matrix proteins. These proteins are called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which bind irreversibly to surface matrix proteins. SdrG, SdrF, and SdrH, which belong to the serine-aspartate repeats family, are examples of MSCRAMMs (87). SdrG (also known as Fbe), the most extensively studied MSCRAMM, binds to fibrinogen. Moreover, Bowden *et al.* [62] showed that *S. epidermidis* SdrG is necessary and sufficient for the attachment of this pathogen to fibrinogen-coated materials. SdrF is responsible for mediating staphylococcal binding to type 1 collagen (6) while the specific ligand of SdrH has not been identified (10, 87).

Figure 4: Steps of biofilm formation. Biofilm formation is divided into four steps**:** 1) initial adhesion of bacterial cells to surfaces, a) attachment to biomaterial surfaces, b) attachment to host matrix proteins coated the surfaces, 2) aggregation into multicellular structures, 3) formation of mature stable biofilms, and 4) dispersal of bacterial cells from the biofilms. Figure modified from: Otto *et al.* 2009 (96).


Free Swimming bacteria
Detachment
4
Maturation
3
Attachment
1
Accumulation
2
a
b

Other *S. epidermidis* proteins able to bind to host proteins include the extracellular lipase GehD which adheres to different collagen types including I, II, and IV (11), and the fibronectin binding protein Embp (63, 142). Autolysins AtlE and Aae also have low affinities for binding various matrix proteins such as fibrinogen, fibronectin, and vitronectin (61). Table 1 summarizes the most important *S. epidermidis* surface-associated proteins as well as the important virulence factors that mediate biofilm formation in *S. epidermidis.*

After the initial adhesion, cell-cell interaction prevents the dissemination of the initial colonizing bacteria and facilities the formation of mature biofilms. *S. epidermidis* releases various surface macromolecules and proteins that facilitate the intercellular aggregation. Polysaccharide intercellular adhesin (PIA) is involved in the cell-to-cell adhesion during *S. epidermidis* biofilm formation (81, 82). PIA is a linear homoglycan composed of β-1,6-linked N-acetylglucosamine residues and carries up to 15% de-acetylated amino groups. The positive and negative charges can be simultaneously introduced to the polysaccharide by substitution with ester-linked succinate and phosphate residues; the ionic interaction caused by these positive and negative charges within the polysaccharide could explain its function in linking different cells within the biofilm (79, 80)

PIA biosynthesis is directed by enzymes encoded by the *icaADBC* gene operon. IcaA and IcaD are transmembrane proteins involved in the synthesis of N-acetylglucosamine (GlcNAc) polymers. The elongation and export of the resultant polymer is believed to be controlled by the IcaC membrane protein. After export, the IcaB de-acetylase enzyme is responsible for de-acetylation of some of the GlcNAc residues needed

to provide the polymer with the cationic character that is essential for surface attachment (Figure 5)(113).

Despite the fact that PIA is considered to be essential for *S. epidermidis* biofilm formation, some strains lacking the *ica* genes have been isolated from biofilm-associated infections (128). Recently, it has been demonstrated that certain surface proteins mediate biofilm formation in *ica*-negative *S. epidermidis* strains. The surface proteins Bap and Aap have been shown to mediate and participate in PIA-independent biofilm formation (Table 1), (9, 64). This finding supports the fact that *S. epidermidis* can use different pathways to form mature biofilms (96).

Figure 5: Biosynthesis of the exopolysacharide poly-N-acetyleglucosamine (PIA). IcaA and IcaD are trans-membrane proteins involved in the synthesis of N-acetylglucosamine (GlcNAc) polymers (1). The IcaC membrane protein controls the elongation and export of the polymer (2). The IcaB protein is responsible for de-acetylation of GlcNAc residues (3). Figure modified from: Otto *et al.* 2009 (96).

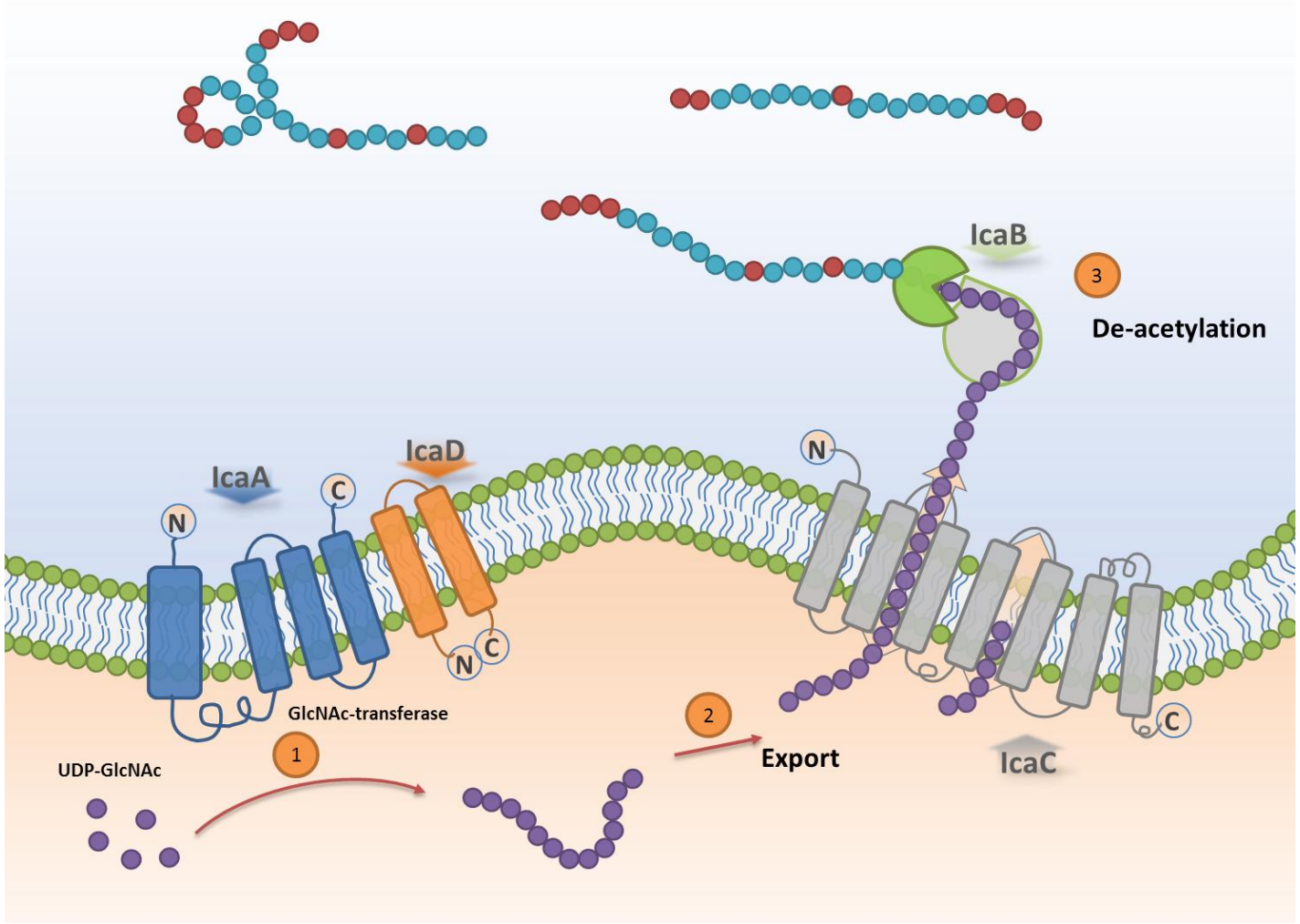

IcaB
3
De-acetylation
IcaD
IcaA
C
N
N
C
N
C
GlcNAc-transferase
2
Export
1
UDP-GlcNAc
C
IcaC

Table 1: *S. epidermidis* virulence factors

Factor	Function	Reference
Attachment to abiotic surfaces		
AtlE	Adhesion and autolysin surface protein which mediates attachment to polystyrene and binds to vitronectin	(59)
Biofilm-associated protein Bap (Bhp in *S.epidermidis)*	Intercellular adhesion protein	(128)
Aae	A bi-functional autolysin and adhesin with a bacteriolytic activity	(61)
SSP-1 and SSP-2	Attachment to polystyrene	
Attachment to host matrix proteins		
SdrG	Binds to fibrinogen	(55)
SdrF	Binds to collagen type I	(6, 7)
GehD	Bind to collagen types I, II, and IV	(11)
Aae	Bind to various matrix proteins such as fibrinogen, fibronectin, and vitronectin	(61) 16
Embp	Binds to fibrinectin	(63, 142)
Intercellular aggregation		
PIA	Intercellular polysaccharide adhesin	(60, 79)
Biofilm-associated protein Bap (Bhp in *S.epidermidis)*	Intercellular adhesion protein	(60, 128)
Accumulation-associated protein Aap	Intercellular adhesion protein	(60, 64)
Protection from immune clearance		
PIA	Protects from IgG, AMPs, phagocytosis and complement	(74, 137)
PGA	Protects from AMPs and phagocytosis	(70)
SepA protease and Aps system	Involved in AMP resistance	(75, 76)
Exoenzymes		
Metalloprotease Sep-1	Iinvolved in lipase maturation, possibly tissue damage, AMP resistance	(37, 75, 135)
Serine protease GluSE	Degradation of fibrinogen and complement factor C5	(37, 93)
Toxins		
PSMs	Pro-inflammatory cytolysins	(89, 133, 144)

The basic structure of a biofilm can be portrayed as a community of bacterial cells embedded in a matrix which is separated by channels. This matrix functions as a physical/chemical barrier against antimicrobial compounds; biofilm-associated cells have been shown to exhibit a 1000-fold greater resistance to antibiotics as compared to their planktonic (free-floating) counterparts (24) and demonstrate reduced clearance by the innate immune system(49, 50). The water channels facilitate the exchange of nutrients and waste (36). Mature biofilms are heterogeneous micro-environments composed of a wide range of biomaterials including polysaccharides, proteins, nucleic acids, and phosphates(30). Water is the major component of the biofilm matrix (147), while bacteria account for less than 50% of the total volume of the biofilm (30).

The 140 kDa accumulation-associated protein (Aap) is shown to play an important role in biofilm accumulation and maturation in *S. epidermidis* (64). The protein Bhp in *S. epidermidis* is a homologues of the biofilm-associated protein Bap in *S. aureus* and has also been proposed to promote biofilm maturation by increasing intercellular adhesion (33).

The PIA produced by *S. epidermidis* plays a crucial role in protection from immunoglobulin G (IgG), AMPs, phagocytosis, and complement in addition to its essential role in biofilm formation (Table 1). Furthermore, *S. epidermidis* produces poly-gama-glutamic acid (PGA) exopolymers encoded by the *cap* locus. PGA plays an important role in biofilm protection from neutrophil phagocytosis, complement action, antibodies, and anti-microbial peptides (Table 1) (23, 56, 105, 134) Environmental factors such as glucose, salt, and ethanol can trigger *S. epidermidis* biofilm production and maturation. (65, 69).

In addition to intercellular aggregation, biofilms also undergo a detachment process. Mechanical disruption of the biofilm as well as biofilm-induced dispersal plays an important role in detachment of biofilm cells. Due to the exposure to strong disruptive

forces, bacterial cells may disperse from the biofilm and can then colonize another surface to form new biofilm communities (72, 106). Enzymatic degradation of biofilm exopolymers, as well as disruption of non-covalent interactions by detergent-like molecules, such as sigma-toxins, are known to facilitate biofilm detachment (83).

Despite microbial biofilms sharing a lot of similarities with regard to function and structure, differences exist which make each biofilm a unique community(127). Surface properties, nutrient availability, the composition of the microbial community and external stressors are all considered factors that play a role in the uniqueness of each biofilm (127).

Little is known about *S. epidermidis* mechanisms of biofilm dispersal. The IcaR is a negative regulator of PIA synthesis which could promote bacterial detachment (27). In addition, the *agr* (accessory gene regulator) locus, a quorum-sensing system that acts in response to cell density, has been shown to act at the biofilm detachment stage (134). The *luxS* quorum sensing system is also involved in *S. epidermidis* biofilm detachment. It has been shown that these two quorum-sensing systems repress *S. epidermidis* biofilm formation and increase bacterial dispersal (143, 145). Besides these two main quorum-sensing systems, other factors that mediate biofilm formation have been identified including the global stress response alternative sigma factor B (103) and the global gene regulator SarA which positively influence biofilm formation in *S. epidermidis* (129).

1.3.2 *S. epidermidis* Biofilm Formation in Platelet Concentrates

Studies conducted in Dr. Ramirez's laboratory have demonstrated that *S. epidermidis* is capable of forming biofilms under platelets storage conditions. This species has the ability to adhere to the plastic surface of platelet storage bags and to platelet aggregates (49). Assessment of biofilm-forming ability of 13 CoNS isolated from contaminated platelet

preparations in Canada revealed that a high proportion of these strains possess biofilm-associated genes and were able to form biofilms (50).

1.3.3 Biofilm Formation by Other CoNS

Other CoNS are also able to form biofilms and cause serious nosocomial infections (94). Although not well studied as in *S. epidermidis*, several biofilm proteins have been identified in other CoNS. For example, the autolysin Aas and the intercellular adhesin Ssp from *S. saprophyticus* participate in biofilm formation on uroepithelial cell surfaces of young women resulting in severe urinary infections (77). *S. lugdunensis*, which causes more serious infections such as endocarditis, sepsis, and brain abscesses, produces an extracellular mucus known as Slush (*S. lugdunensis synergistic haemolysin*). The presence of the biofilm-associated *icaADBC* genes and production of biofilms have been demonstrated in strains of *S. capitis, S. haemolyticus*, *S. homini*, *S. warneri*, and *S. auricularis* isolated from patients of a neonatal intensive care unit(35).

1.4 BACTERIA-HOST INTERACTION

Despite the fact that platelets are known to play an essential role in hemostasis, their abilities to function as antimicrobial agents have also been demonstrated. Platelets are an important part of the host defence system because of their ability to produce antimicrobial peptides as well as secrete chemokines and cytokines which are stored in the platelet's alpha-granules (119).

Bacterial-platelet interactions can occur either directly through bacterial surface proteins (67) or indirectly through plasma bridging molecules that link the bacterial and platelet surface receptors (92). Bacterial binding to platelets results in platelet activation

and thrombus formation, which can result in the formation of a septic embolism or in the consumption of activated platelets and thrombocytopenia.

S. aureus-platelet interactions are probably the best characterized between bacteria and platelets. *S. aureus* carries different surface proteins that can bind to platelets and stimulate platelet activation. The clumping factor A (ClfA) contains a ~500 residue fibrinogen binding domain and the fibronectin binding proteins FnBPA/B seem to be the dominant surface proteins mediating platelet aggregation and activation (41). The same platelet-binding mechanism has been demonstrated for *S. epidermidis* with the homologous protein SdrG (16). *S. aureus* utilizes vWF as a bridging molecule to bind to platelet glycoprotein Ib (GPIb) via its surface protein A (31). There is evidence that the engulfment of *S. aureus* by platelets is enhanced after platelet activation has occurred (101, 146).

White (141) demonstrated that engulfment of *S. aureus* by platelets is very different than ingestion of the same organism by polymorphonuclear leukocytes. He demonstrated that platelets do not act as phagocytes; rather, they act as "coverocytes". When platelets were challenged with bacteria, they responded in a unique way to cover as much bacterial surface as possible which might play a role in immune evasion to the covered bacteria.

1.5 HYPOTHESES AND OBJECTIVES

1.5.1 Hypothesis

Bacteria isolated from platelet units implicated in adverse transfusion reactions are more likely to exhibit slow growth and to form biofilms under platelet storage conditions.

1.5.2 Objectives

1. To identify CoNS isolated from contaminated platelets units using biochemical and molecular techniques.
2. To compare growth rates of CoNS in growth media (trypticase soy broth, TSB) and platelet concentrates.
3. To study the ability of CoNS strains to form biofilms in TSB and in platelet concentrates.
4. To investigate the relation between ability for biofilm formation and missed bacterial detection during platelet screening by an automated culture system.

CHAPTER TWO
MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

A total of 29 CoNS strains isolated from tainted platelet units were characterized during this study (Table 2). Eleven and 17 CoNS were kindly provided by our collaborators, Drs. Michael Jacobs and Roslyn Yomtovian (Case Western Reserve University, US), and Dr. Dirk de Korte (Sanquin Blood Bank North West, The Netherlands), respectively. While the American strains were isolated from contaminated PCs that had been transfused, the Dutch strains were isolated from contaminated units that were captured during platelet testing and therefore were not transfused. The bacterial concentrations of the American strains present in the platelet units at the time of transfusion are described in Table 2. The unique Canadian strain used during this study was isolated during re-testing of outdated platelet concentrates which had yielded negative results during initial screening. *S. epidermidis* strains ATCC 12228 and ATCC 35984, *Staphylococcus aureus* ATCC 27664, *Streptococcus agalactiae* ATCC 12927 and *Micrococcus luteus* ATCC 4698 were purchased from The American Type Culture Collection (Manassas,VA) and used as control strains for the bacteria identification experiments conducted in this study. To prepare frozen stocks, all CoNS strains were cultured on trypticase soy agar (TSA, BD, Sparks, MD,USA). After aerobic overnight incubation, a well-separated single colony was isolated and sub-cultured onto a TSA plate to ensure purity of the strain. On the following day, colonies were collected and suspended in 8 ml of brain-heart infusion broth (BHI, Biosciences, Franklin Lakes, NJ) containing 15% glycerol. One-ml aliquots of the broth suspension were stored at -80°C.

Table 2: CoNS strains used in the present study

Origin	Strain ID	Bacterial concentration (CFU/ml) at the time of transfusion	Transfusion reaction (Yes/No)
American	11-92	1.0×10^4	Yes
	02-96	1.0×10^2	Yes
	03-96	6.0×10^2	No
	04-96	3.0×10^3	No
	07-98-1	9.5×10^2	No
	07-98-2	2.5×10^2	No
	07-98-3	8.0×10^1	No
	07-98-4	1.4×10^3	No
	10-99	1.0×10^8	Yes
	07-04	4.6×10^5	Yes
	09-04	3.0×10^2	No
Canadian	02-09	N/Ap	N/Ap
Dutch	1025504	N/Ap	N/Ap
	1025512	N/Ap	N/Ap
	1025517	N/Ap	N/Ap
	1025521	N/Ap	N/Ap
	1025522	N/Ap	N/Ap
	1025524	N/Ap	N/Ap
	1025525	N/Ap	N/Ap
	1025527	N/Ap	N/Ap
	1025667	N/Ap	N/Ap
	1025679	N/Ap	N/Ap
	1025548	N/Ap	N/Ap
	1025549	N/Ap	N/Ap
	1025550	N/Ap	N/Ap
	1025673	N/Ap	N/Ap
	930892-4	N/Ap	N/Ap
	21073-5	N/Ap	N/Ap
	22334-6	N/Ap	N/Ap

N/Ap, Not applicable

2.2 BACTERIAL IDENTIFICATION

A set of biochemical and molecular tests were chosen for the speciation of the CoNS strains used in this study.

2.2.1 Catalase Test

Catalase is an enzyme that breaks down hydrogen peroxide into oxygen and water. This test is mainly used to differentiate *Staphylococcus* species (catalase-positive) from *Streptococcus* species (catalase-negative).

The following strains were used as controls:

Staphylococcus aureus ATCC 27664 (positive control)

Streptococcus agalactiae ATCC 12927 (negative control)

2.2.2 Coagulase Test

Coagulase is a protein which possesses a prothrombin-like activity. This test is used to differentiate CoNS from coagulase-positive staphylococci such as *S. aureus.* The same previous control strains were used.

2.2.3 Lysostaphin Resistance Test

This test is used to differentiate *Staphylococcus* species, which are sensitive to lysostaphin produced by *Micrococcus* species, which displays resistance to this enzyme. The reaction is based on the activity of the endopeptidase lysostaphin which breaks down the interpeptidic pentaglycin bridges of the peptidoglycan of the staphylococcal cell wall. A bacterial cell

suspension was prepared in 200 µl of sterile saline from a pure 18-24 hours old culture, followed by addition of 200 µl of lysostaphin reagent. The suspension was then incubated aerobically at 37°C for two hours and examined for solution clearance (Figure 6A).

The following strains were used as controls:

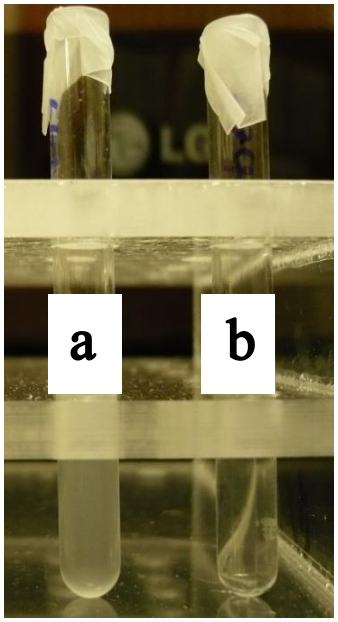
Micrococcus luteus ATCC 4698	Positive (turbid) resistance to the reagent
Staphylococcus aureus ATCC 27664	Negative (Clear) strong sensitivity to the reagent
Staphylococcus epidermidis ATCC 12228	Negative (Clear) weak sensitivity to the reagent

2.2.4 Analytical Profile Index

The identification of the CoNS strains was carried out using the Analytical Profile Index (API) Staph Kit (BioMérieux, Lyon, France). Strains were allowed to grow aerobically on P agar (10g Peptone, 0.7g $MgCl_2$, 5g K_2SO_4, 7.5g agar and 5ml glycerol in 495ml dH_2O) for 18-24 hours at 37°C. A few colonies were taken to make a homogeneous bacterial suspension prepared in API Staph medium with a turbidity equivalent to a 0.5 McFarland Standard. API microtubes were inoculated with the bacterial suspension and incubated aerobically for 18-24 h at 37°C. Catalase, coagulase, and lysostaphin resistance tests were done in parallel for each strain according to the manufacturers' procedures. During incubation, bacterial metabolic activity is visualized by color changes in the strips; the profile of metabolic tests is then used to identify the unknown organism using an algorithm established by the manufacturer (Figure 6B).

Figure 6: Biochemical tests for staphylococcal identification. A) Lysostaphin resistance test showing positive (a) and negative (b) reactions. B) Twenty biochemical tests are included in the API Staph strip, which provide a unique profile code for the identification of *Staphylococcus* spp.

(A)


a
b

(B)


api STAPH
0 GLU FRU MNE MAL LAC TRE MAN XLT MEL
NIT PAL VP RAF XYL SAC MDG NAG ADH URE
api STAPH
0 GLU FRU MNE MAL LAC TRE MAN XLT MEL
NIT PAL VP RAF XYL SAC MDG NAG ADH URE
api STAPH
0 GLU FRU MNE MAL LAC TRE MAN XLT MEL
NIT PAL VP RAF XYL SAC MDG NAG ADH URE

2.2.5 Molecular Confirmation of *S. epidermidis* Strains

S. epidermidis identification was confirmed was PCR amplification of the *divIVA* gene which is implicated in cell division of other Gram-positive bacteria (39, 110) and has been shown to provide a clear distinction between *S. epidermidis* from other CoNS (85). A primer pair previously designed in Dr. S. Ramirez' lab that amplifies *S.epidermidis divIVA* was used to amplify this gene by PCR. The primer pair includes the forward primer Staphdiv-FW (5'-GCG CGT CGA CAT GCC TTT TAC ACC AAG TG-3') and reverse primer and Sep*div*-REV (5'-GCG CGG ATC CTT AAT TAT TTG ATG TTG ATTG-3').

A master mix containing 75.5 ul of sterile ddH_2O, 10 µl 10x PCR buffer containing 15 mmol/ L $MgCl_2$ (Qiagen Canada, Inc., Mississauga, Ontario, Canada), 2 µl dNTP mix (0.2 mmol/L of each dNTP; New England Biolabs, Ipswich, MA), 1 µl of each primer (0.2 µg/µl), 0.5 µl HotStar Taq Plus DNA polymerase (2.5 U/reaction; (Qiagen Canada, Inc., Mississauga, Ontario, Canada) and 10 µl of bacterial cell suspension, which provided the chromosomal template DNA. Bacterial cell suspensions were adjusted to a 0.5 McFarland turbidity standard (Hardy Diagnostics, Santa Maria, CA), by inoculating 2-3 colonies into nuclease-free, sterile ddH_2O. PCR amplification was performed in a thermal cycler PCR system (Mastercycler ep, Eppendorf, Hamburg, Germany) using the following program: an initial denaturation of 15 minutes at 95°C, 35 cycle of denaturation for 30 s at 94°C, annealing for 30 s at 46°C, and extension for 60 s at 72°C, final extension for 10 min at 72°C, and maintained at 4°C. Agarose gel electrophoresis (2% agarose in Tris-acetate-EDTA) was used to resolve the PCR products. A molecular weight marker (1kb Plus DNA Ladder, New England Biolabs) ddH_2O, and positive *S.epidermidis* ATCC35984 and negative controls (water; no DNA template) were included with each experiment.

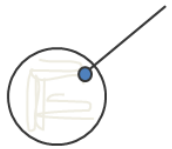
2.3 GROWTH STUDIES

2.3.1 Growth Curves of CoNS in Laboratory Media (TSB) Under Ideal Growth Conditions

CoNS strain were streaked onto TSA plates and incubated aerobically for 24 hours at 37°C. Distinct colonies were selected and inoculated into 15 ml of TSB. The cell suspensions were then incubated aerobically for 24 hours at 37°C with agitation at 260 rpm. A culture flask containing 100 ml TSB was inoculated with the bacterial cell suspension to an OD_{550} = 0.05 which corresponds to approximately $1X10^7$ colony-forming units per mL (CFU/mL). The flask was incubated aerobically at 37°C with agitation at 260 rpm. One-hundred µl samples were taken every two hours for a period of 10 hours. The samples were serially-diluted up to 7-fold where required, and the last 3 dilutions were plated on TSA plates in duplicate. The next day, colonies were counted on plates containing between 30 and 300 colonies and bacterial concentration was determined (Figure 7). Each growth curve was repeated once in duplicate, unless otherwise stated.

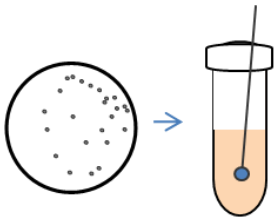
Figure 7: Experimental design for growth rate studies in TSB. The experimental strain was streaked onto TSA plate followed by inoculation into TSB media. The bacterial suspensions were then adjusted to OD_{550} = 0.05 in TSB in 100ml cultures and incubated aerobically. Optical density was measured every two hours and bacterial samples were recovered, serially-diluted, and plated for enumeration.

Streaking



Subculture sample on TSA
At 37°C/overnight

Inoculation



2-3 colonies with 15ml TSB
At 37°C/overnight
with agitation ~260 rpm

Adjusting

adjust the sample
OD550 =0.05 (~10^7 CFU/ml).

Incubation

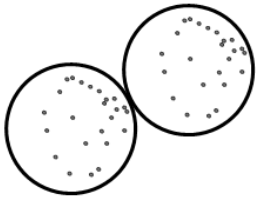
In TSB at 37°C
with agitation ~260 rpm for 10h

Measuring

Measure OD_{550}
every 2 hours + culture plates

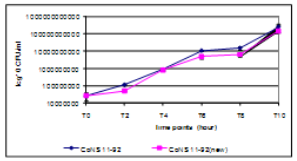
Inoculate plates with serial dilution

Counting



Count colonies

Analysis



Use appropriate statistical
program/ draw graph

2.3.2 Growth Curves of CoNS in Laboratory Media (TSB) Under Platelet Storage Conditions

Six CoNS strains were randomly selected and streaked onto TSA plates which were incubated aerobically for 24 hours at 37°C. Well-separated colonies were selected and inoculated into 15 ml of TSB. The cell suspensions were then incubated aerobically for 24 hours at 37°C with agitation at 260 rpm. Next, the bacterial cultures were adjusted to OD_{550nm} = 0.05 and serially-diluted to a final concentration of approximately 10^2 CFU/ml (Figure 8). One ml of the 10^2 CFU/mL suspension was used to inoculate 99 mL TSB which was then divided into two tissue culture flasks, each containing 50 ml of culture, and incubated under platelet storage conditions (room temperature with constant agitation at 60 rpm for 5 days). One-hundred μl samples were taken every day, serially-diluted if needed, for the 5-day incubation period and cultured on TSA. Following overnight incubation at 37°C, colonies were counted for plates containing between 30 and 300 colonies and bacterial concentration was determined (Figure 9A). Each growth curve was repeated once in duplicate, unless stated otherwise.

2.3.3 Growth Curves of CoNS in Platelet Concentrates under Platelet Storage Conditions

The experiments were conducted in the same manner as described above with the exception of the culture media; instead of TSB media, bacteria were grown in outdated platelet concentrates. One-hundred ul samples were drawn every day, serially-diluted and incubated for 5 days, and cultured on TSA, followed by colony counting (Figure 9B).

Figure 8: Serial dilution procedure to achieve a final concentration of ~10^2CFU/ml.

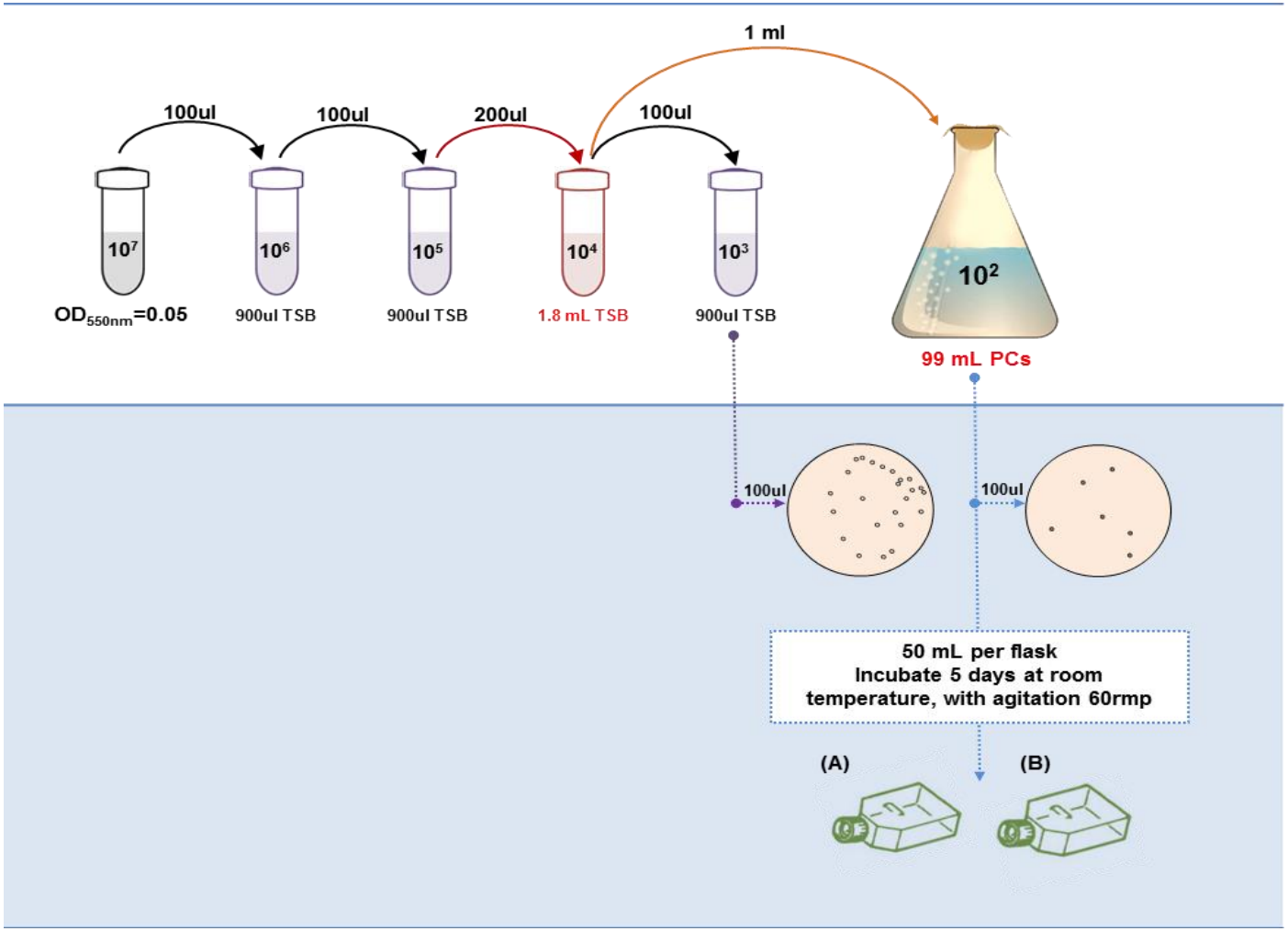
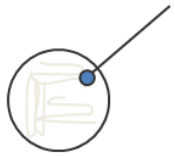

1 ml
100ul
100ul
200ul
100ul
10^7
10^6
10^5
10^4
10^3
10^2
OD_{550nm}=0.05
900ul TSB
900ul TSB
1.8 mL TSB
900ul TSB
99 mL PCs
100ul
100ul
50 mL per flask
Incubate 5 days at room temperature, with agitation 60rmp
(A)
(B)

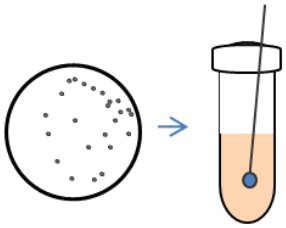
Figure 9: Experimental design for growth rate studies in TSB and platelet concentrates.

Streaking



Subculture sample on TSA
At 37°C/overnight

Inoculation

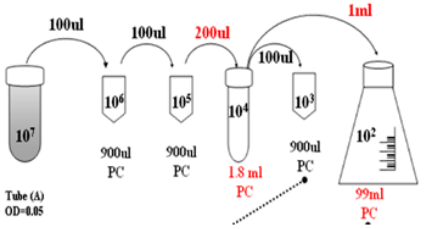


2-3 colonies with 15ml TSB
At 37°C/overnight
with agitation ~260 rpm

Adjusting

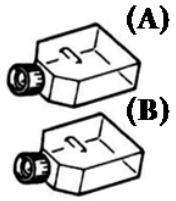
adjust the sample
OD550 =0.05 (~10^7 CFU/ml).

Dilution



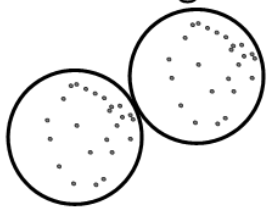
Serial dilution
A)In TSB
B) In PCs

Incubation



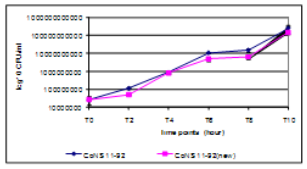
Divide the last dilution into two TC flask
Incubate at 22±2°C with agitation ~60 rpm, for 5days
culture plates every day

Counting



Count colonies

Analysis



Use appropriate statistical program/ draw graph

2.3.4 Growth Curves in Platelet Concentrates versus Platelet-Poor-Plasma

Platelet-Poor-Plasma (PPP) was created by centrifugation of platelet concentrates for 15 min at 3000 rpm followed by filtration though sterile 0.45 um size filters (Sartorius Corp., Edgewood, NY). Filtered platelet concentrates should have an approximate 1000-fold reduction in platelet content as reported previously in Dr. Ramirez' lab (49). A strain that was fast-growing (*S. epidermidis* 07-04) and a strain that was slow growing (*S. epidermidis* 02-96) in PCs were selected for these experiments.

2.3.5 Growth Curves of Anaerobic CoNS Strains in Platelet Concentrates

Platelet concentrates were inoculated with strict anaerobic CoNS strains 1025548, 1025549, 1025550, and 1025673 (Table 2) at initial target concentrations of ~10 to ~10^2 CFU/ml (low) and ~10^3 to ~10^4 CFU/ml (high). Platelet samples were taken daily for five days to determine bacterial concentrations by plating serial dilutions in duplicate on blood agar plates. Plates were incubated at 37^{o}C under anaerobic conditions in HP0011A anaerobic jars (Oxoid, Nepean, ON, Canada) for up to 72 hours before colony counting and determination of bacterial concentrations. All growth curves were repeated twice in duplicate, in different platelet preparations.

2.4 BIOFILM FORMATION STUDIES

The ability of CoNS strains to form biofilms was determined using three different approaches: screening for slime formation, molecular amplification of the biofilm-associated genes *icaA* and *icaD*, and crystal violet staining assay.

2.4.1 Determination of Slime Formation by a Congo Red Agar Assay

All strains were studied for their ability to produce slime by streaking them on Congo Red Agar (CRA) plates followed by aerobic incubation for 24 hours at 37°C. As described by Freeman *et al* (43), CRA was prepared by dissolving 18.5g brain-heart infusion (BHI) and 7.5g agar into 400ml dH2O.The mixture was then autoclaved, and left to cool down to 60°C in a water bath. Meanwhile, 18g saccharose (Sigma-Aldrich Canada Ltd) and 0.4g Congo Red Dye (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) were dissolved in 100mL dH_2O, and filter-sterilized using less or equal to 0.2u size filters. The filtered mixture was added to the autoclaved media. CRA plates were prepared with 20ml media each plate, storage at 4°C. Rough, dull, black colonies indicate a slime production due to the binding of Congo red dye to biofilm polysaccharide; the non-slime producing phenotype is characterized by the presence of smooth, red/ pink colonies.

2.4.2 PCR Amplification of Biofilm-Associated *icaA* and *icaD* Genes from *S. epidermidis* and *S. capitis*

S. epidermidis RP62 (also known as ATCC 35984) GeneBank sequence NC_002976.3 was used to design specific primers for *S. epidermidis icaA* and *icaD* using the Primer Designer program (Version 2.0.1, C 1990-91, Scientific and Educational Software). For detection of *S. epidermidis icaA,* primers Sepi_icaA-FW (5'-GCG CCT GGA TAG TAG GAT CGA TTT AC -3') and Sepi_icaA-REV (5'- GCG CTT ACC GTT GGA TAT TGC CTC T -3')which anneal to nucleotides 51 to 72 and 1218 to 1239, respectively, were synthesized. For the amplification of *S. epidermidis icaD,* primer pair SepicaD-FW (5'-AAG CCC AGA CAG AGG CAA TAT CCA -3') and SepicaD-REV (5'-AGT ACA AAC AAA CTC ATC

CAT CCG A-3) which anneal to nucleotides 7 to 30 and 215 to 239, respectively, were synthesized.

S. capitis GeneBank sequence AY146582.1 was used to design specific primers for *S. capitis icaA* and *icaD* using the Primer Designer program. For detection of *S. capitis icaA*, primers ScapicaA-FW (5'-CAA TAA TCT TAT TCT TCA AT-3') and ScapicaA-REV (5'- ATG TCC ACC TGG AGC CCA TC -3') which anneal to nucleotides 1 to 20 and 980 to 1000, respectively, were synthesized. For the amplification of *S. capitis icaD*, the primers ScapicaD-FW2 (5'-TTC GTA TTA ATT ATG ACC CA -3') and ScapicaD-REV (5'-ACG ACC TTT CTT AAT TTT TTG G-3') which anneal at positions 900 to 919 and 1661 to 1682, respectively, were used. All primers were synthesized by Integrated DNA Technologies Inc.(Coralville, IA, USA).

A master mix tube was prepared containing 1 µl of each of two selected primers at a concentration of 0.2 µg/µl with the following reagents: 75.5 µl of sterile ddH_2O, 10 µl 10x PCR buffer containing 15 mmol/ L $MgCl_2$ (Qiagen), 2 µl dNTP mix (0.2 mmol/L of each dNTP; New England Biolabs), 0.5 µl HotStar Taq Plus DNA polymerase (2.5 U/reaction; Qiagen), and 10 µl of bacterial cell suspension, which provided the chromosomal template DNA. Bacterial cell suspensions were adjusted to a 0.5 McFarland turbidity standard (Hardy Diagnostics), by inoculating 2-3 colonies into nuclease-free, sterile ddH_2O. The final reaction used for volume PCR amplification was 100 µl. PCR amplification of S. epidermidis icaA and icaD genes was performed in a thermal cycler PCR system (Mastercycler ep, Eppendorf): initial denaturation for 15 minutes at 95°C, followed by 50 cycles of denaturation for 60 seconds at 94°C, annealing for 60 seconds at 58°C, and extension for 30 seconds at 72°C. For amplification of *icaA and icaD* genes of *S. capitis* strains the following PCR conditions were used; annealing for 60 seconds at 46°C for *icaA*,

and 53.°C for *icaD*, and extension for 80 seconds at 72°C; 10 minutes at 72°C; and hold at 4°C. Agarose gel electrophoresis (2% agarose in Tris-acetate-EDTA) was used to analyzing the PCR mixture. A molecular weight marker (1kb Plus DNA ladder, New England Biolabs) ddH_2O, and positive icaA and icaD genes (*S. epidermidis* ATCC 35984) and negative (*S. epidermidis* ATCC 12228) controls were included with all experiments.

2.4.2.1 Sequencing of *S. capitis icaD*

PCR products corresponding to the *icaD* gene from *S. capitis* were resolved by agarose gel electrophoresis, cleaned with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocols, and submitted for sequencing to StemCore-DNA Sequencing Facility (Ottawa, Ontario).

Nucleotide sequences were then translated and protein alignments were performed using the MultiAlin program, designed by Florence Corpet (29).

2.4.3 Biofilm Formation in Regular Media

Cultures of CoNS grown in TSB supplemented with 0.5% glucose (TSBg) were incubated aerobically overnight at 37°C with agitation (260 rpm). The cultures were diluted to OD_{600}=0.1 in 10 ml of TSBg. Three-ml aliquots were transferred into each well of a 6-well tissue culture plate (Corning Inc., Corning, NY). The tissue culture plates were incubated aerobically overnight at 37°C without agitation. The next day, supernatants of each well were removed using an aspiration/filtration vacuum system (Model 2515, Welch, Rietschel Thomas, Sheboygan, WI) followed by gentle rinsing with sterile phosphate-buffered saline (PBS), pH 7.4 (three times, 3 ml per well). A Gram crystal violet dye solution (0.3%, BD Biosciences, Sparks, MD) was used to stain the wells for 30 min at room temperature with

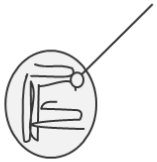
agitation at 100 rpm (2 ml of dye per well). The dye was then removed and the wells were rinsed three more times with PBS (3 ml per well) as above. A mixture composed of 80% ethanol: 20% acetone (vol/vol;Fisher Scientific, Fair Lawn, NJ) was added (3 ml per well) to elute the stain from the wells; the elution solution was incubated at room temperature for 15 min with constant agitation at 100 rpm. The eluted solution from each well was then transferred into 6 wells of a 96-well microtitter plate (Corning; 200 ul per well). A microplate reader (Expert Plus, ASYS Hitech GmbH, city, Austria) was used to measure the absorbance at a 492-nm wavelength (A492nm;Figure 10). Extra wells containing sterile TSBg were also measured as negative controls. The blank values were subtracted from values obtained from the experimental samples determine the value of stained biofilm on the wells. *S. epidermidis* ATCC 12228 (non-biofilm-forming) and ATCC 35984 (biofilm-forming) were used as negative and positive controls, respectively. All assays were repeated in triplicate at least two independent times**.**

2.4.4 Biofilm Formation in Platelet Concentrates

Biofilm formation assays were performed in fresh (2-3 day-old) platelet concentrates with selected CoNS strains 02-96, 07-04, 02-09, and 09-04 (Table 2). Ten-ml platelet samples were inoculated with overnight cultures corresponding to OD_{600} =0.1 Aliquots of 3 ml were transferred into three wells of 6-well tissue culture plates (Corning Inc.) which were then incubated under platelet storage conditions (room temperature with agitation for five days). Biofilm formation was determined by a crystal violet semi-quantitative assay as described above for biofilms grown in TSBg (Figure 10).

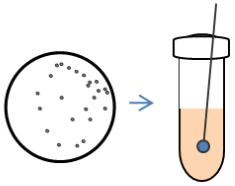
Figure 10: The crystal violate assay performed in either media (TSBg) or in platelet concentrates (PCs).

Streaking



Subculture sample on TSA
At 37°C/overnight

Inoculation

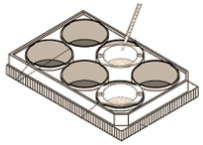


2-3 colonies with 15ml TSBg
At 37°C/overnight
with agitation ~260 rpm

Adjusting

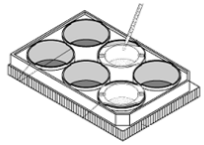
Adjust the sample
OD600 =0.1

Transferring



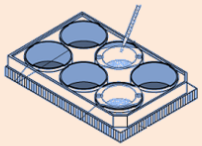
6-well tissue culture plate
3 mL/well
A) In TSBg at 37°C/overnight)
B) In PCs at 22±2 °C/5 days

Washing



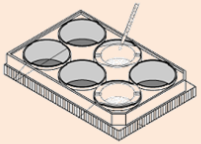
wash with PBS (pH 7.4)

Staining



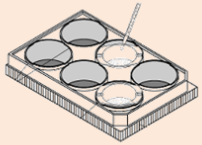
stain with Crystal Violet
(0.3%)

Washing



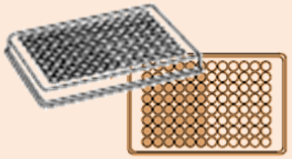
wash with PBS (pH 7.4)

De-staining



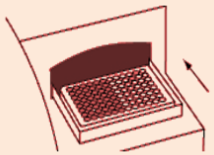
De-stain with
Ethanol: Acetone (80:20)

Transferring



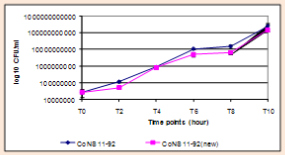
96-well tissue
culture microtitre plate
200 µL/well

Measuring



Measure OD492
with microplate reader

Analysing



Use appropriate statistical
program/ draw graph

2.5 MISSED BACTERIAL DETECTION EXPERIMENTS

These experiments were designed to determine if biofilm formation during platelet storage contributes to missed detection of CoNS during platelet screening with the BacT/ALERT®3D automated culture system. The strains *S. epidermidis* 02-96 and *S. epidermidis* 07-04 were used in these experiments.

2.5.1 Testing of Platelet Bag Coupons

One-cm^2 platelet bag coupons were excised from platelet bags and suspended in two flasks containing 200 ml of TSBg. Flasks were inoculated with *S. epidermidis* 02-96 and *S. epidermidis* 07-04 to a final concentration corresponding to approximately 0.3 CFU/ml. After 24 hours of static incubation at 37°C, the coupons were removed and washed three times with PBS for 60 seconds. Following washing, each coupon was transferred into a culture flask containing 100 ml of platelet concentrates. After incubation for 24h under platelet storage conditions (room temperature with agitation), aliquots of 8 to 10 ml were transferred to 10 blood products aerobic (BPA) BacT/ALERT culture bottles which were incubated in the BacT/ALERT®3D system until bottles became positive or for up to 6 days (Figure 11).

2.5.2 Testing Using a Sampling Site Coupler

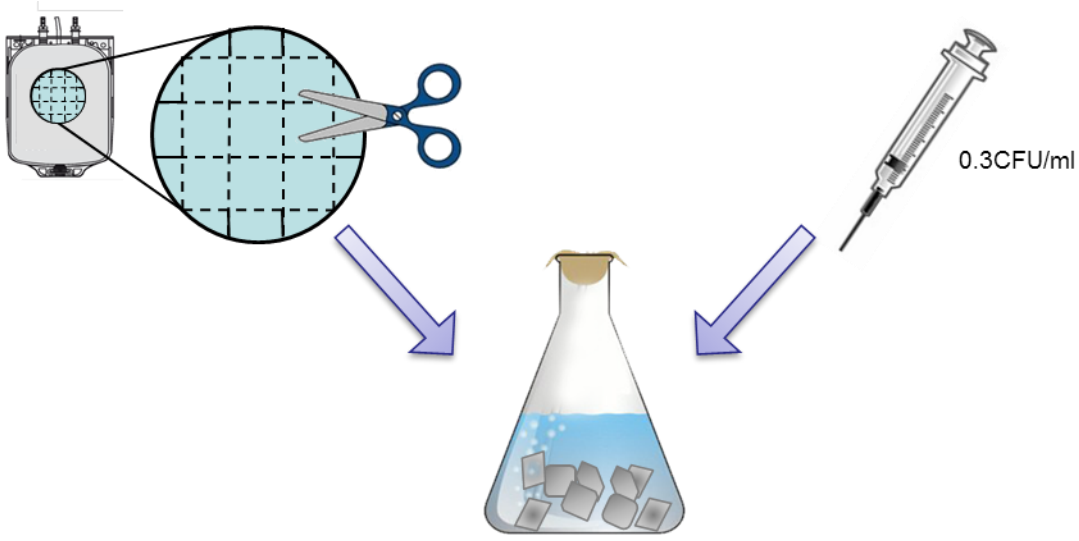
The sampling site coupler found on the exterior of platelet storage bags was used as a surface on which to grow bacterial biofilms since this site represents a potential source of contamination during platelet recovery. The top part of sampling site couple was immersed in a flask containing TSBg media which had been previously inoculated with

~0.3 CFU/mL of either *S. epidermidis* 02-96 or *S. epidermidis* 07-04. The flasks were then sealed with parafilm and incubated at 37°C for 24h. The next day, the sampling site coupler was washed with PBS for 60 sec with agitation (100 rpm). A needle was then inserted through the contaminated site couple to mimic the action of blood collection/venipuncture. Samples of 10 ml were collected and transferred to tissue culture flasks and further incubated for 24 h under platelet storage conditions (room temperature with agitation). Eight to 10 ml samples were transferred into BPA culture bottles followed by incubation in the BactT/ALERT®3D system until flagged positive or for up to 6 days (Figure 12).

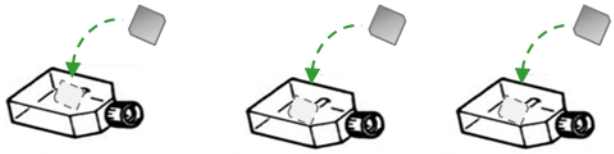
2.5.3 Inoculation of TSBg in Pre-conditioned Platelet Containers with Low Bacterial Concentrations

Four platelet units were drained and the platelet bags containing residual platelet residue on the inner walls (pre-conditioned) were used for these experiments. Each bag was filled with 200 ml of TSBg, and inoculated with *S. epidermidis* 02-96 and *S. epidermidis* 07-04, (Table 2) at final concentrations of approximately 0.1 CFU/ml (20 CFU/bag) and 0.3 CFU/ml (60 CFU/bag). The platelet bags were then incubated under platelet storage conditions (room temperature with agitation). After two hours, 8 to 10 ml samples were then transferred to 10 BPA culture bottles which were incubated in the BacT/ALERT® 3D system until positive or for up to 6 days. The experiment was repeated 5 and 3 times for the 0.1 and 0.3 CFU/ml inocula, respectively.

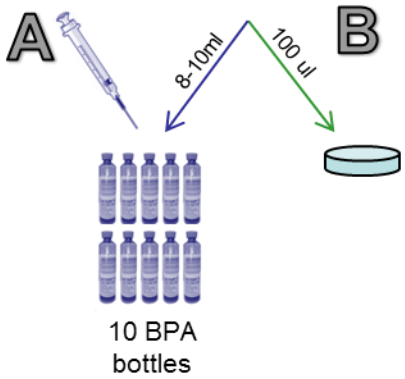
Figure 11: Procedure for testing platelet bag coupons for bacterial growth



Incubate for 24h at 37°C, static



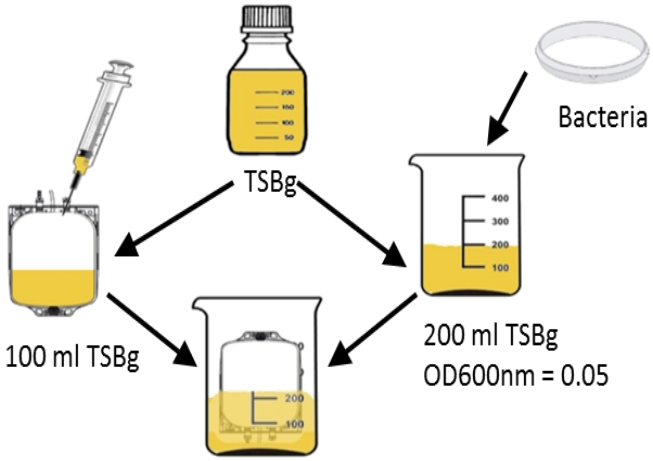
Incubate for 24-30h at Platelet Storage Conditions

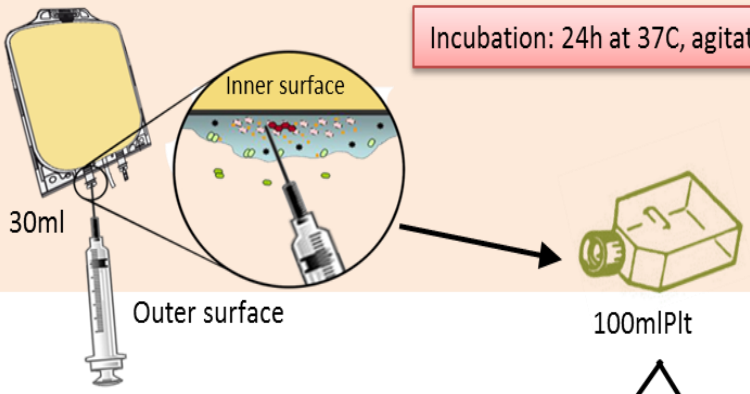


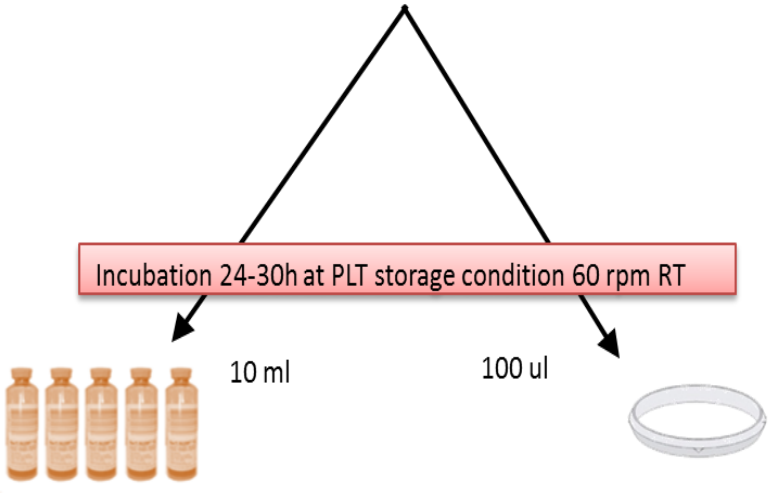
Incubate until flagged +ve or for Max 6 days.

If +ve: Gram stain and identification.

Figure12: Procedure for testing the sampling site coupler for bacterial contamination


Bacteria
TSBg
100 ml TSBg
200 ml TSBg
OD600nm = 0.05


Incubation: 24h at 37C, agitation
Inner surface
30ml
Outer surface
100mlPlt


Incubation 24-30h at PLT storage condition 60 rpm RT
10 ml
100 ul

2.6 STATISTICAL ANALYSES

Mean and standard deviation of the growth rate and biofilm formation graphs were calculated using Microsoft Excel 2003 (Microsoft Canada, Mississauga, ON). To compare biofilm formation between slow- and fast-growing strains in platelet concentrates, mixed models with random effects were fitted to perform comparisons between the biofilm-negative strain *S. epidermidis* ATCC 12228 and each of the other strains. Dunnett adjustment was applied to correct the larger false positive error due to multiple comparisons. Differences in growth during the missed detection experiments were calculated by pairwise comparison using the statistical analysis system SAS 9.1.3 software (SAS Institute Inc., Cary, NC). A value of $p<0.05$ was interpreted as statistically-significant.

CHAPTER THREE
RESULTS

3.1 CHARACTERIZATION OF COAGULASE-NEGATIVE *STAPHYLOCOCCUS* STRAINS

3.1.1 Bacterial Identification and Confirmation

A panel of tests was selected to identify the 29 strains examined in this thesis including macro- and microscopic morphology, Gram staining, production of catalase and coagulase, resistance to lysostaphin, and biochemical identification using the API system. All CoNS strains produced round, white colonies on TSA plates and formed grape-shape clusters which stained Gram positive, and yielded a positive catalase reaction. All aerobic strains were also coagulase negative and sensitive to lysostaphin, confirming their identity as *Staphylococcus* species. However, API 20A was unsuccessful in identifying the anaerobic strains.

Twenty-five aerobic strains were identified including 20 *S. epidermidis*, four *S. capitis* and one *S. chromogenes* (Table 3). Four anaerobic strains were also studied but they could not be identified using the API 20A system. These strains were subsequently identified as *S. saccharolyticus* by our collaborator in the Netherlands using MALDI-TOF and 16S ribosomal RNA methods (115)(Table 3).

A molecular approach, determination of the presence of the cell division *divIVA* gene by PCR, was used to distinguish *S. epidermidis* from other CoNS strains (85). A primer pair previously designed in Dr. S. Ramirez' lab that amplifies *S. epidermidis divIVA* was used. All 20 *S. epidermidis* strains were positive for the presence of the expected ~777

bp band corresponding to the *divIVA* gene while *divIVA* was not amplified from the other CoNS (Table 3, Figure 13).

Table 3: Biochemical identification and molecular confirmation of CoNS

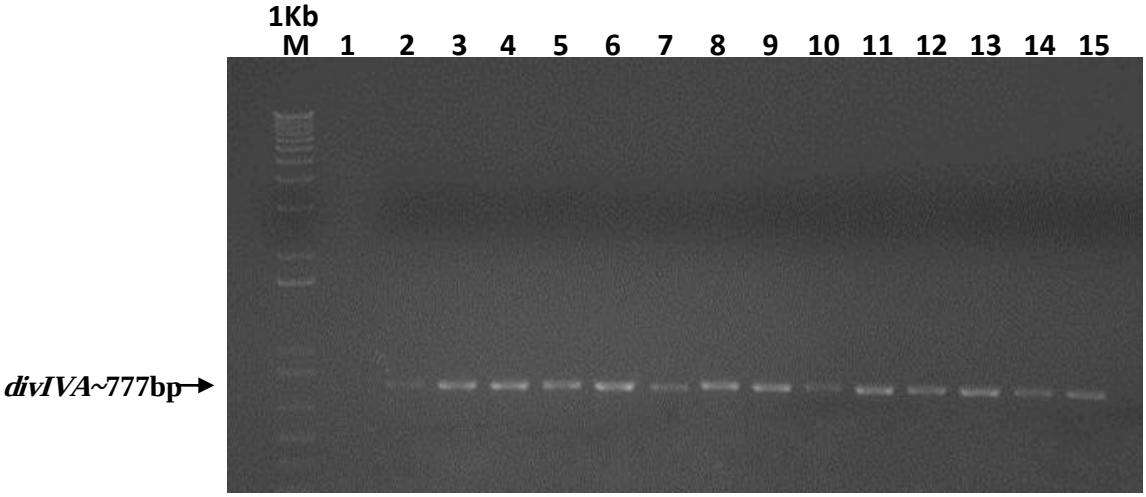
Origin	Strain	API Staph Identification (%)*	Presence of *S. epidermidis divIVA*
American	11-92	*S. epidermidis* (98.1)	Pos
	02-96	*S. epidermidis* (98.8)	Pos
	03-96	*S. epidermidis* (97.8)	Pos
	04-96	*S. epidermidis* (97.8)	Pos
	07-98-1	*S. epidermidis* (97.4)	Pos
	07-98-2	*S. epidermidis* (97.8)	Pos
	07-98-3	*S. epidermidis* (97.8)	Pos
	07-98-4	*S. epidermidis* (79.4)	Pos
	10-99	*S. epidermidis* (90.8)	Pos
	07-04	*S. epidermidis* (97.8)	Pos
	09-04	*S. epidermidis* (97.8)	Pos
Canadian	02-09	*S. epidermidis* (90.4)	Pos
Dutch	1025504	*S. epidermidis* (90.4)	Pos
	1025512	*S. capitis* (96.60)	Neg
	1025517	*S. capitis* (99.7)	Neg
	1025521	*S. capitis* (99.0)	Neg
	1025522	*S. epidermidis* (98.1)	Pos
	1025524	*S. epidermidis* (99.4)	Pos
	1025525	*S. capitis* (99.8)	Neg
	1025527	*S. epidermidis* (97.8)	Pos
	1025667	*S. chromogenes* (98.7)	Neg
	1025679	*S. epidermidis* (98.1)	Pos
	1025548	*S. saccharolyticus*¶	Neg
	1025549	*S. saccharolyticus*¶	Neg
	1025550	*S. saccharolyticus*¶	Neg
	1025673	*S. saccharolyticus*¶	Neg
	930892-4	*S. epidermidis* (97.9)	Pos
	21073-5	*S. epidermidis* (98.1)	Pos
	22334-6	*S. epidermidis* (97.3)	Pos

* (%) identification accuracy provided by API algorithm, cut off > 85%.

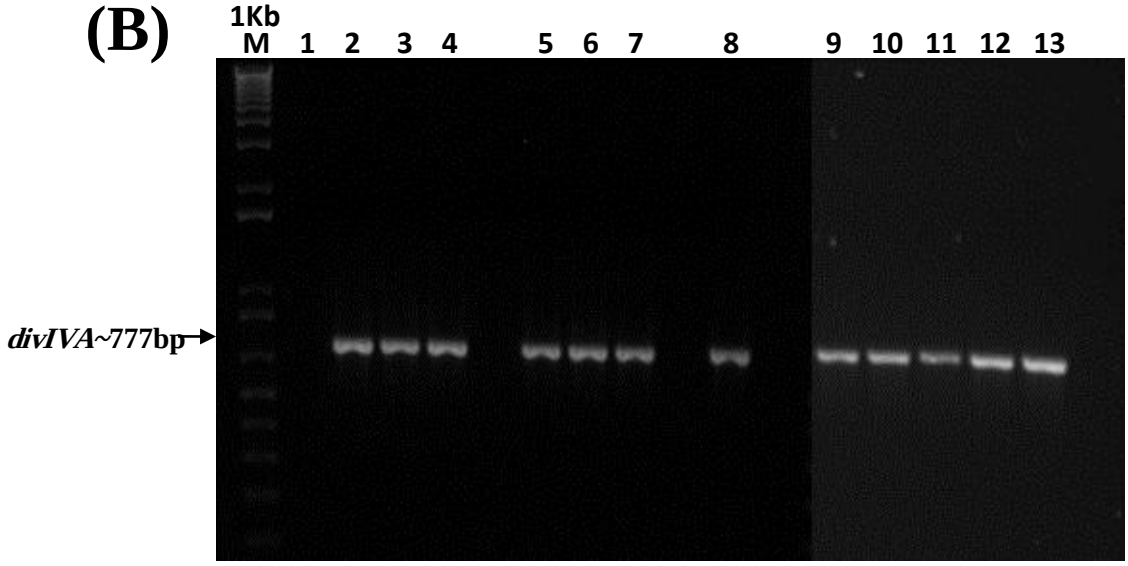
¶ Anaerobic strains identified using MALDI-TOF and 16S ribosomal RNA methods (115).

Figure 13: PCR Amplification of *S. epidermidis divIVA* gene. A) American and Canadian strains. (M), molecular weight marker; (1) negative control (ddH_2O); (2) strain 11-92; (3) strain 02-96; (4) strain 03-96; (5) strain 04-96; (6) strain 07-98-1; (7) strain 07-98-2; (8) strain 07-98-3; (9) strain 07-98-4 ; (10) strain 10-99; (11) strain 07-04; (12) strain 09-04; (13) strain 02-09; (14) ATCC strain 12228 (positive control); (15) ATCC strain 35984 (positive control). **B) Dutch strains**. **(**M), molecular weight marker; (1) negative control (ddH_2O); (2 and 13) ATCC strain 35984 (positive control); (3 and 12) ATCC strain 12228 (positive control); (4) strain 1025679; (5) strain 1025527; (6) strain 1025524; (7) strain 1025522; (8) strain 1025504; (9) strain 930892-4; (10) strain 21073-5; (11) strain 22334-6. The expected *divIVA* fragment is ~777bp.

(A)


1Kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
divIVA~777bp

(B)


1Kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13
divIVA~777bp

3.2 GROWTH RATE STUDIES OF CoNS IN MEDIA AND PLATELET CONCENTRATES

3.2.1 Growth Curves of CoNS strains in Media (TSB)

The growth dynamics of the CoNS strains were studied in three different environments: 1) in trypticase soy broth (TSB) under optimal growth conditions [37^{o}C and constant agitation, on horizontal shaker (~60 rpm)]; 2) in TSB under platelet storage conditions (22±2^{o}C, with constant agitation for 5 days); and 3) in outdated platelet concentrates under platelet storage conditions. All aerobic strains showed continuous growth in TSB under optimal growth conditions (37^{o}C with constant agitation) (Figure 14). The initial concentrations varied from ~10^{6} to ~10^{7} CFU/ml, while the final bacterial concentrations after 10 hours of incubation ranged from between ~ 10^{10} to ~10^{12} CFU/ml.

Six strains were randomly selected for studying the growth rates in TSB under platelet storage conditions. These strains include: *S. epidermidis* 11-92, *S. epidermidis* 02-96, *S. epidermidis* 03-96, *S. epidermidis* 04-96, *S. epidermidis* 10-99, and *S. epidermidis* 02-09. All strains either maintained their initial concentrations or demonstrated decreased viability over the five-day incubation period (Figure 15). Based on these results, the remaining strains were not grown under these conditions.

Figure 14: Growth curves of aerobic CoNS strains in TSB media. Bacterial suspensions in TSB were incubated for 10 hours with a constant agitation at ~260 rpm. Mean ± standard deviation of OD values of two independent experiments performed in duplicate, are shown.

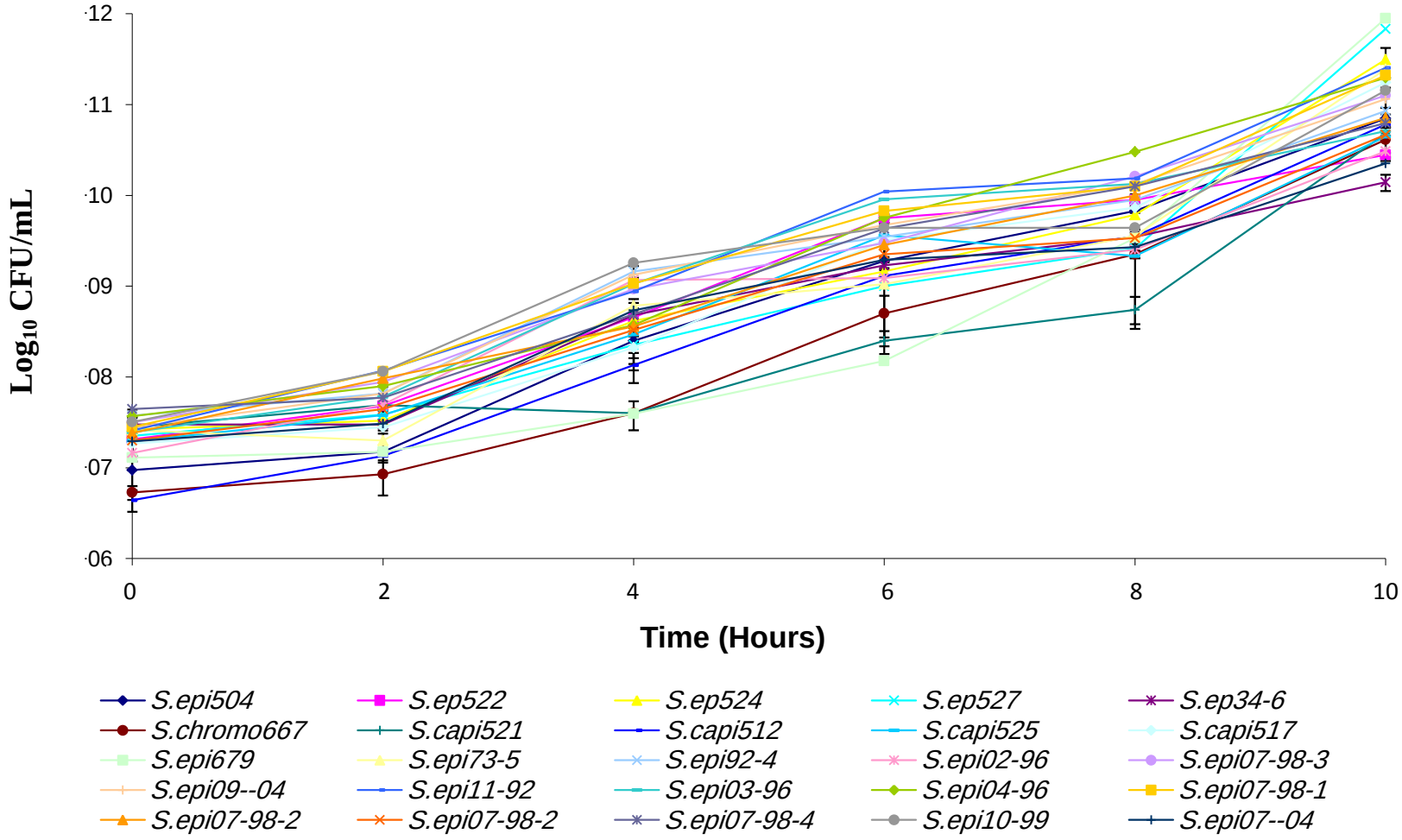
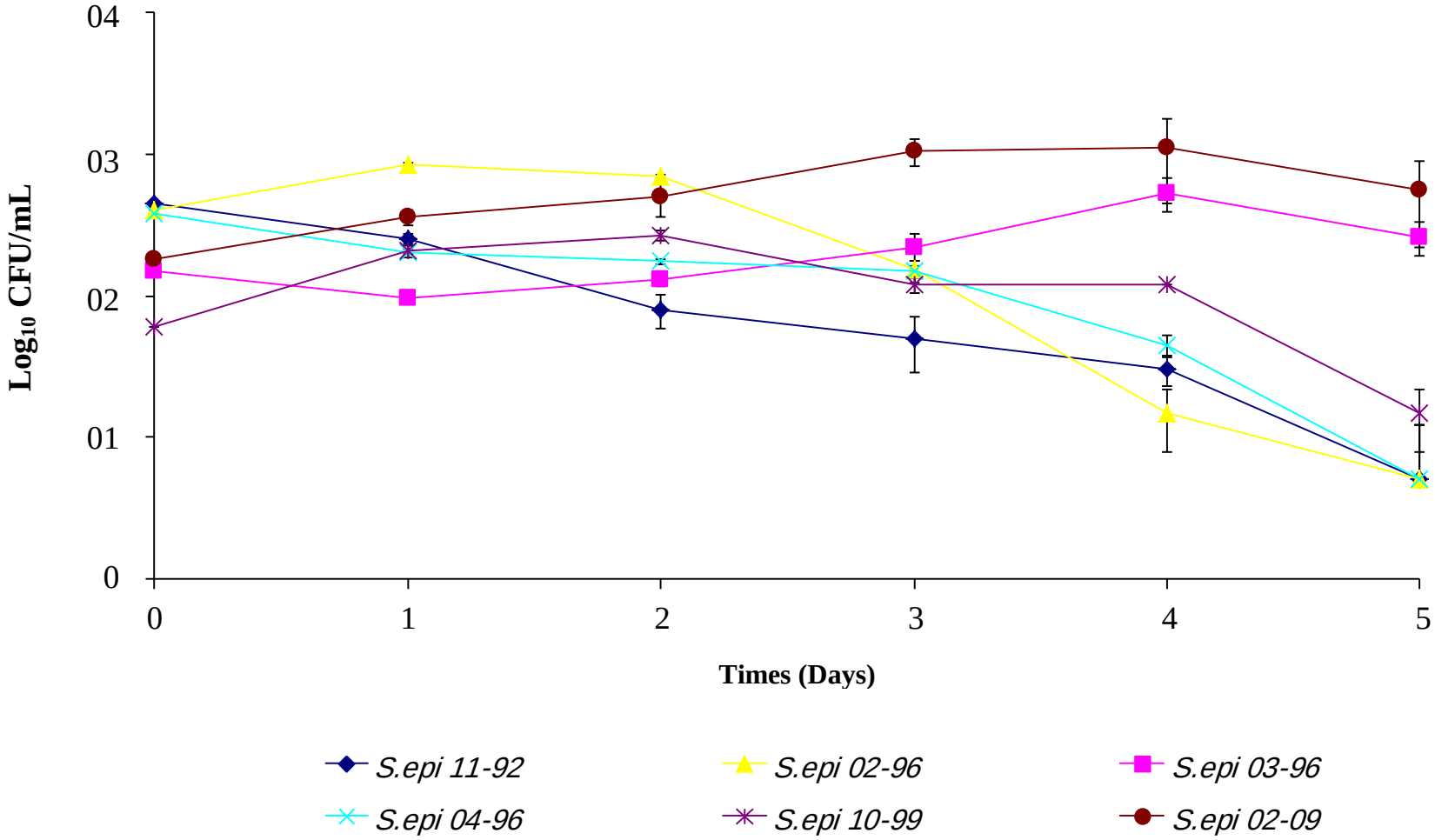

Log10 CFU/mL
12
11
10
09
08
07
06
0
2
4
6
8
10
Time (Hours)
S.epi504
S.ep522
S.ep524
S.ep527
S.ep34-6
S.chromo667
S.capi521
S.capi512
S.capi525
S.capi517
S.epi679
S.epi73-5
S.epi92-4
S.epi02-96
S.epi07-98-3
S.epi09--04
S.epi11-92
S.epi03-96
S.epi04-96
S.epi07-98-1
S.epi07-98-2
S.epi07-98-2
S.epi07-98-4
S.epi10-99
S.epi07--04

Figure 15: Growth curves of CoNS in TSB under platelet storage conditions (22±2^{o}C for 5 days with agitation at ~60rpm). Mean of ± standard deviation of OD values of two independent experiments performed in duplicate are shown.


04
03
02
01
0
Log10 CFU/mL
0
1
2
3
4
5
Times (Days)
S.epi 11-92
S.epi 02-96
S.epi 03-96
S.epi 04-96
S.epi 10-99
S.epi 02-09

3.2.2 Growth Curves of CoNS strains in Platelet Concentrates

Strains were grown under platelet storage conditions in platelet concentrates to gain insight into the effect of platelets and plasma factors on the growth patterns of CoNS strains. Due to the limited access to fresh platelets, it was decided to use recently-outdated platelet units (6 to 10 days old) to conduct these experiments. All platelet units had been pre-tested for sterility. Interestingly, the growth rates of CoNS strains cultured in platelet concentrates differed considerably in comparison to the growth rates in TSB. The initial concentration of all strains was approximately 10^2 CFU/ml and two growth patterns were observed in platelet concentrates among CoNS strains based on the lag phase as well as the final concentration attained after 5 days of incubation. A slow grower group, for strains reached final concentrations ranging from 10^3 to 10^6 CFU/ml, with a lag phase of 2 to 3 days, and a fast grower group, which reached final concentrations $\geq 10^9$ CFU/ml with a lag phase of 1 to 2 days (Figure 16).

Based on these results, it was decided to investigate the contribution of platelet concentration to the growth behaviour of CoNS. A fast-growing strain, *S. epidermidis* 07-04 and a slow-growing strain, *S. epidermidis* 02-96, were selected for these experiments. The strains were grown in either regular platelet concentrates or filtered platelet concentrates known as platelet-poor-plasma (PPP). No difference was observed in the growth rate of these strains upon platelet depletion; both strains maintained the same growth pattern in either PCs or PPP (Figure 17).

Figure 16: Growth curves in platelet concentrates. Growth curves of aerobic CoNS in platelet concentrates under platelet storage conditions (22±2^{o}C for 5 days with agitation at ~60rpm). Mean of ± standard deviation of OD values of two independent experiments, each performed in duplicate, are shown.

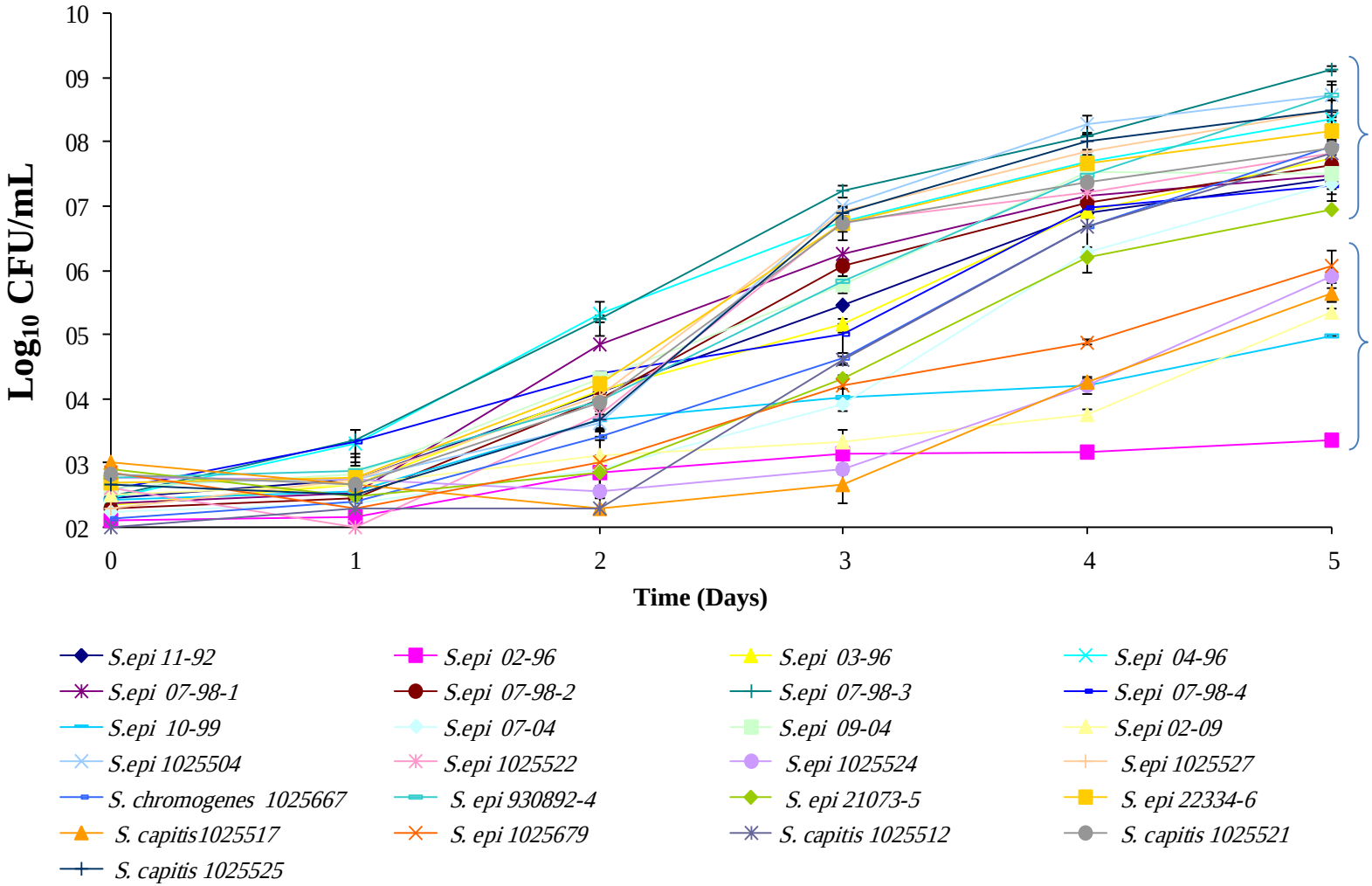
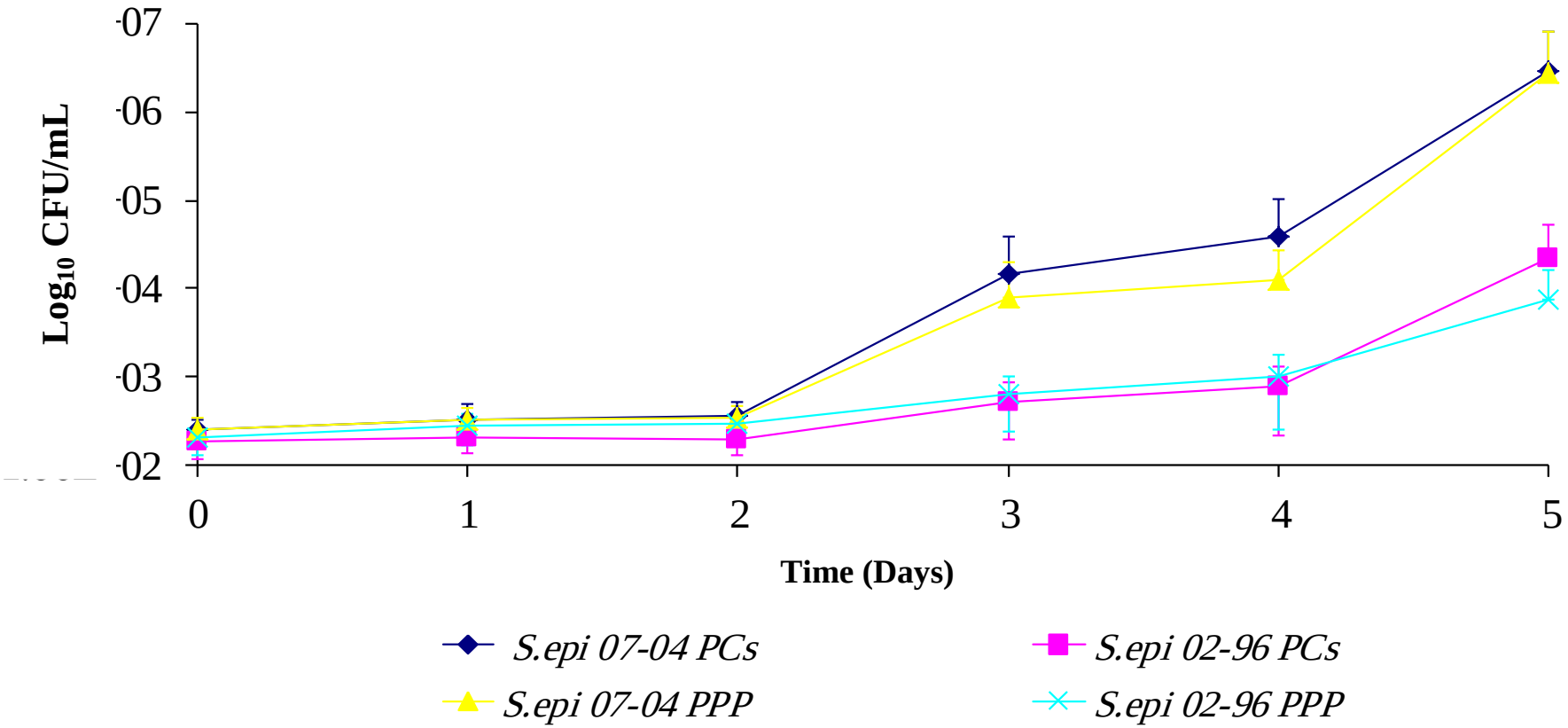

Log10 CFU/mL
10
09
08
07
06
05
04
03
02
0
1
2
3
4
5
Time (Days)
S.epi 11-92
S.epi 02-96
S.epi 03-96
S.epi 04-96
S.epi 07-98-1
S.epi 07-98-2
S.epi 07-98-3
S.epi 07-98-4
S.epi 10-99
S.epi 07-04
S.epi 09-04
S.epi 02-09
S.epi 1025504
S.epi 1025522
S.epi 1025524
S.epi 1025527
S. chromogenes 1025667
S. epi 930892-4
S. epi 21073-5
S. epi 22334-6
S. capitis1025517
S. epi 1025679
S. capitis 1025512
S. capitis 1025521
S. capitis 1025525

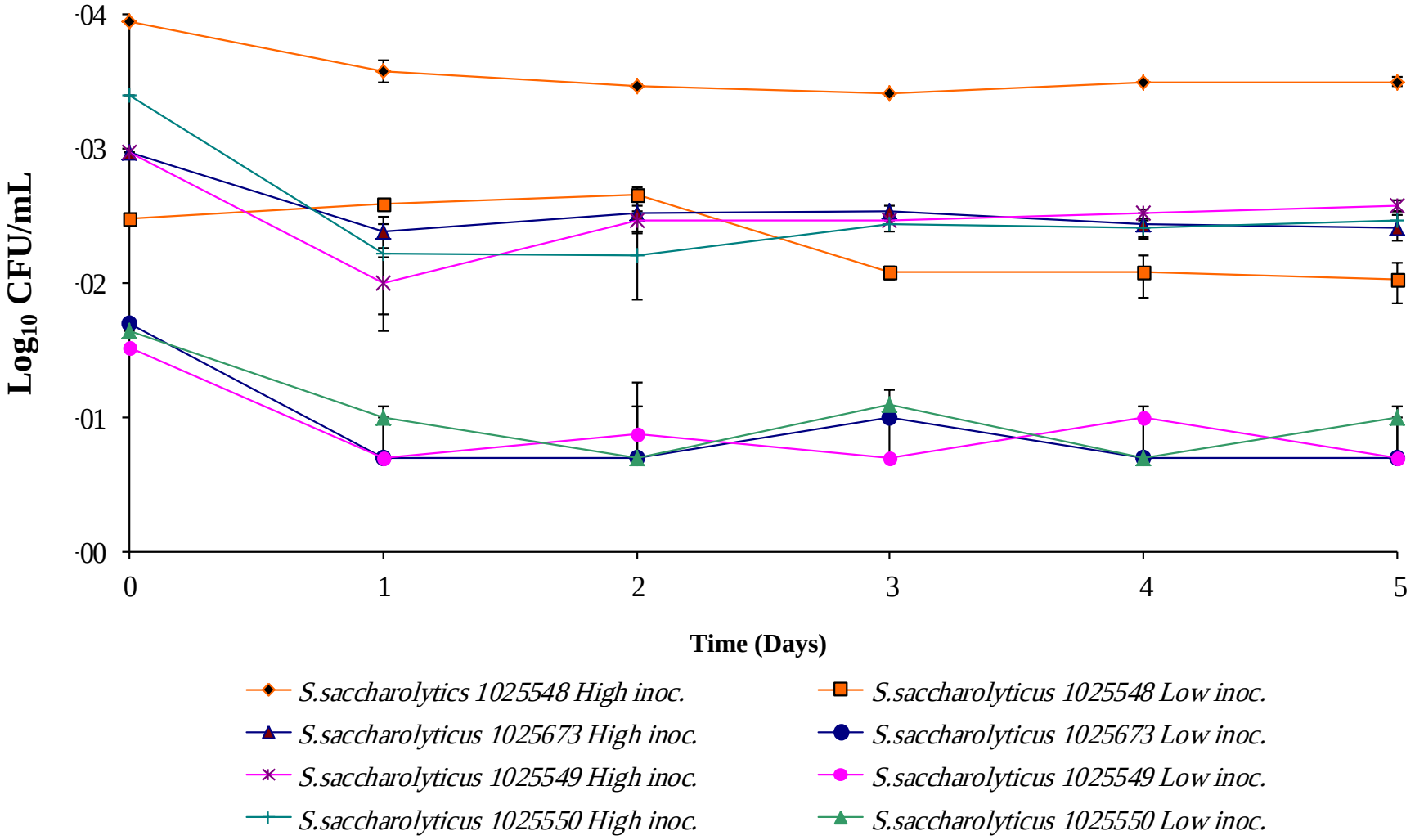
Figure 17: Growth curves in in platelet concentrates and platelet poor plasma. Growth of fast-growing strain *S. epidermidis* 07-04 and slow-growing strain *S. epidermidis* 02-96 in platelet concentrates (PCs) and platelet poor plasma (PPP) under platelet storage conditions (22±2^{o}C for 5 days with agitation ~60rpm). Mean of ± standard deviation of OD values of two independent experiments, each performed in duplicate, are shown.


Log10 CFU/mL
07
06
05
04
03
02
0
1
2
3
4
5
Time (Days)
S.epi 07-04 PCs
S.epi 02-96 PCs
S.epi 07-04 PPP
S.epi 02-96 PPP

3.2.3 Growth Curves of Anaerobic *S. saccharolyticus* Strains in Platelet Concentrates

Four anaerobic *S. saccharolyticus* strains (1025548, 1025549, 1025550, and 1025673; Table 3) were evaluated for their ability to grow in the aerobic platelet storage environment. As observed in Figure 18, all strains maintained their viability, but did not increase their concentrations during the 5 days of incubation. A summary of these results has been recently published in the peer-reviewed journal *Transfusion*(3, **Appendix 1**).

Figure 18: Growth curves of the anaerobic *S. saccharolyticus* strains under aerobic platelet storage conditions. Platelet concentrates were inoculated with low (~10^2) and high (~10^4) CFU/m. Mean of ± standard deviation of OD values of duplicate experiments is shown.


·04
·03
·02
·01
·00
Log10 CFU/mL
0
1
2
3
4
5
Time (Days)
S.saccharolytics 1025548 High inoc.
S.saccharolyticus 1025548 Low inoc.
S.saccharolyticus 1025673 High inoc.
S.saccharolyticus 1025673 Low inoc.
S.saccharolyticus 1025549 High inoc.
S.saccharolyticus 1025549 Low inoc.
S.saccharolyticus 1025550 High inoc.
S.saccharolyticus 1025550 Low inoc.

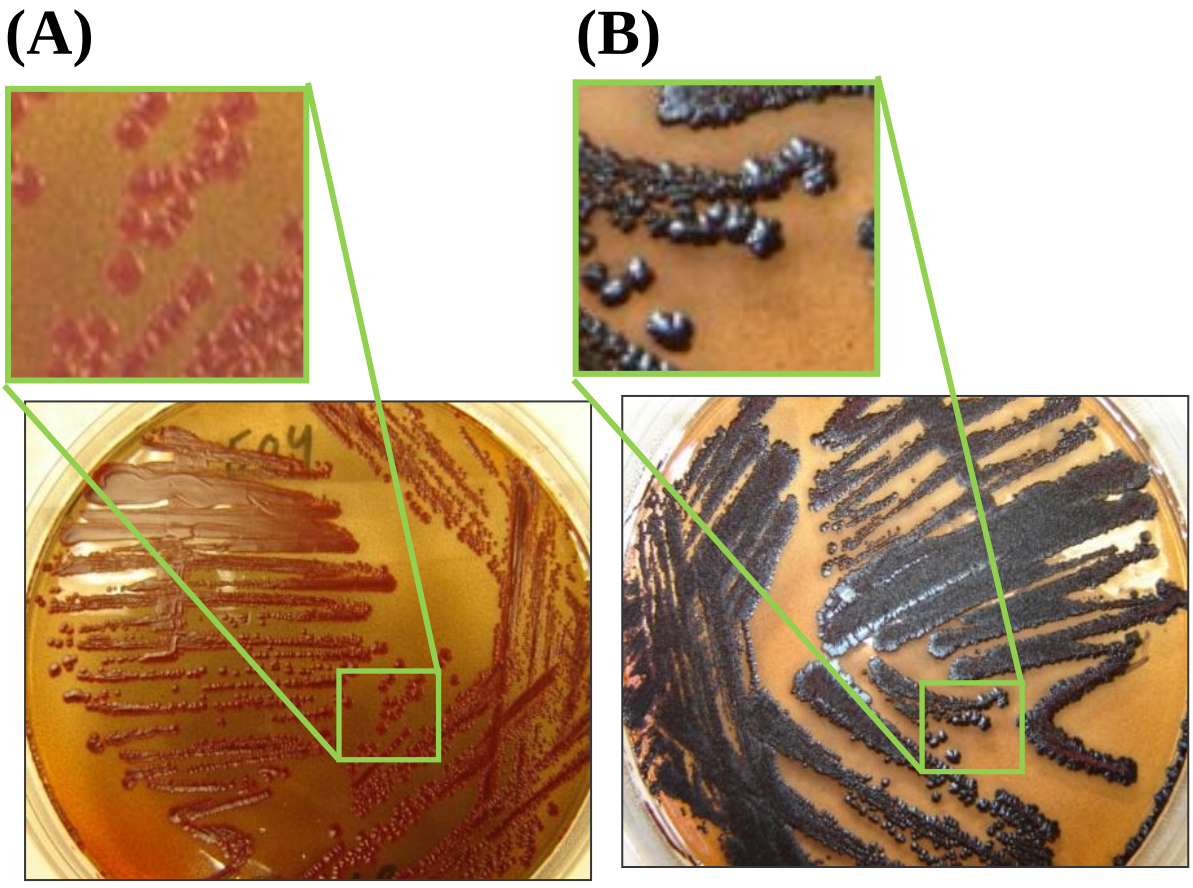
3.3 BIOFILM FORMATION BY CoNS

The biofilm-forming ability of the CoNS strains used in this study was characterized using three criteria: 1) their ability to produce slime on Congo Red Agar (CRA); 2) the presence of biofilm-associated genes *icaA* and *icaD*; and 3) biofilm formation in either TSB media supplemented with 0.5% glucose (TSBg) or in platelet concentrates measured by crystal violet staining. *S. epidermidis* strains ATCC 12228 and ATCC 35984 were used as negative and positive controls, respectively, for all assays (49).

3.3.1 Determination of Slime Production

All strains were grown on CRA plates incubated for 24 h at 37^{o}C. Visual examination of bacterial colonies demonstrated that six out of the 29 strains were able to produce rough, dull, black colonies on CRA representing slime production while the others showed red to pink colonies representing the non-slime producing phenotype (Figure 19). The slime-producing strains included four American isolates (*S. epidermidis* 02-96, *S. epidermidis* 07-98-2, *S. epidermidis* 07-98-4, and *S. epidermidis* 07-04) and two Dutch strains (*S. capitis* 1025517 and *S. epidermidis* 21973-5; Table 4).

Figure 19: Assessment of biofilm-forming ability by congo red agar. ATCC strains cultured on Congo Red Agar A) ATCC strain 12228, a non-slime-producing strain, appears as smooth red-to- pink colonies, B) ATCC strain 35984, a slime-producing strain, appears as rough, dull black colonies.

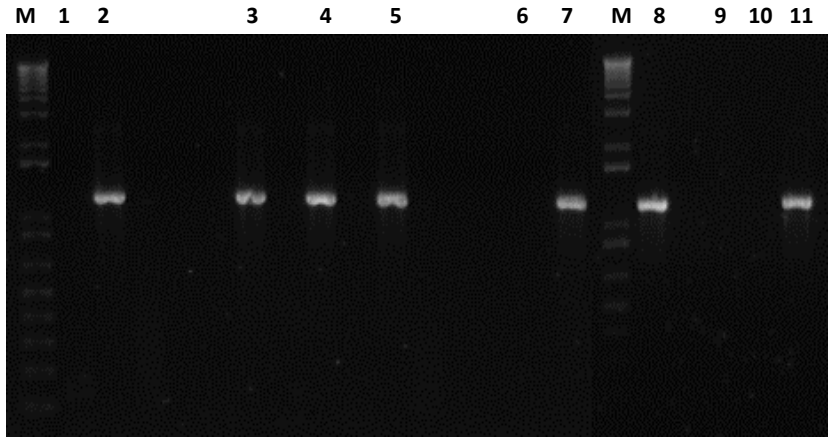

(A)
(B)

3.3.2 PCR Amplification of Biofilm-Associated Genes

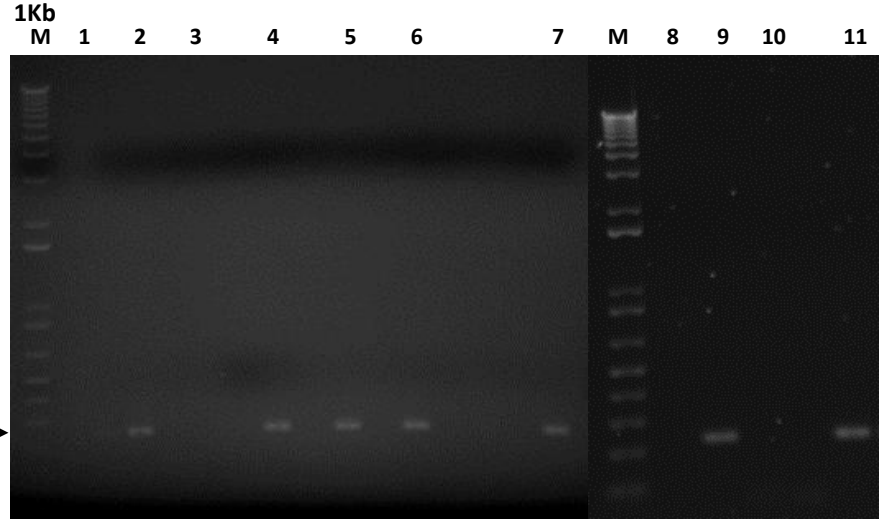
The *icaA* and *icaD* genes, which encode two of the enzymes responsible of production of polysaccharide intercelluler adhesin (PIA), were PCR-amplified from *S. epidermidis* and *S. capitis* isolates. The four slime-producing American strains (*S. epidermidis* 02-96, *S. epidermidis* 07-98-2, *S. epidermidis* 07-98-4, and *S. epidermidis* 07-04) were positive for the presence of *icaA* and *icaD*. Four Dutch strains were *icaA* and *icaD* positive: *S. capitis* 1025517, *S. capitis* 1025521, *S. capitis* 1025525, and *S. epidermidis* 21073-5 (Figure 20).

Figure 20: PCR amplification of *S. epidermidis icaA* (A), *S. epidermidis icaD* (B), *S. capitis icaA* (C), and *S. capitis icaD* (D) genes. (M), molecular weight marker; (1 and 9); negative control (ddH_2O); (2) strain 02-96; (3) strain 07-98-2; (4) strain 07-98-4; (5) strain 07-04; (6 and 10) ATCC strain 12228, (*icaAD*-negative); (7 and 11) ATCC strain 35984, (*icaAD*-positive); (8) strain 21073-5; (12) *S. capitis* strain 1025517; (13) strain *S. capitis* 1025521, (14) *S. capitis* strain 1025525.

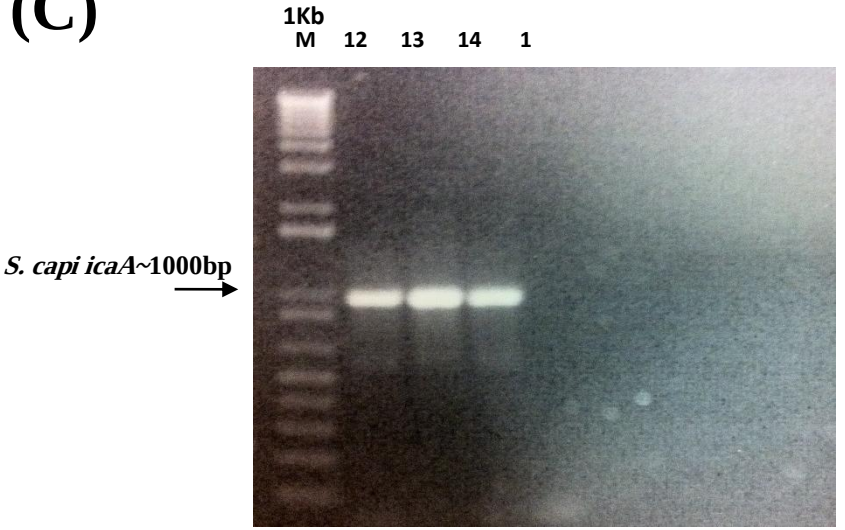
(A)


M 1 2 3 4 5 6 7 M 8 9 10 11
icaA~1189bp

(B)


1Kb
M 1 2 3 4 5 6 7 M 8 9 10 11
icaD~239bp

(C)


1Kb
M 12 13 14 1
S. capi icaA~1000bp

(D)

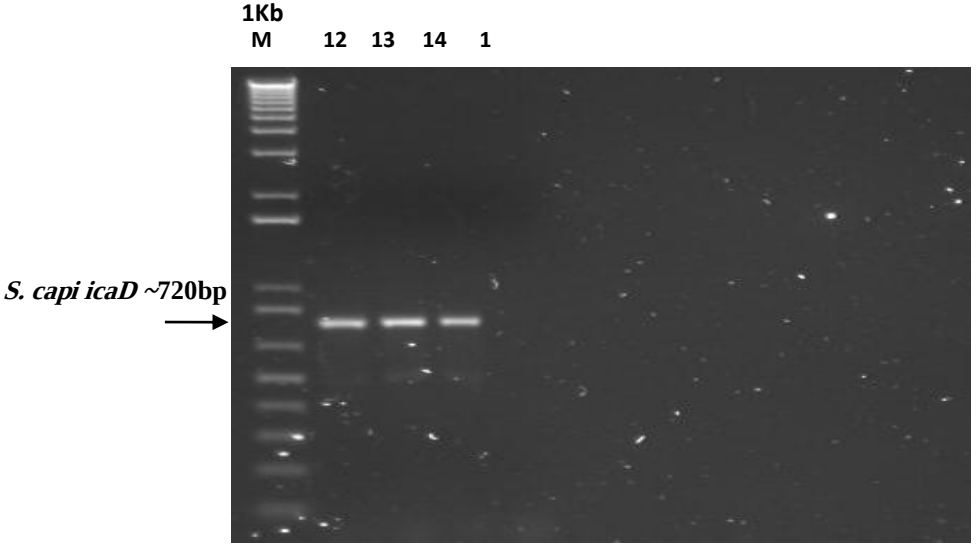

1Kb
M 12 13 14 1
S. capi icaD ~720bp

Table 4: Slime production, presence of biofilm-associate genes, and biofilm formation by CoNS strains

Strain	*icaA*	*icaD*	Slime	Biofilm	
				TSBg	PCs
S. epidermidis 02-96	Pos	Pos	Pos	Pos	Pos
S. epidermidis 07-04	Pos	Pos	Pos	Pos	Pos
S. epidermidis 09-04	Neg	Neg	Neg	Neg	Pos
S. epidermidis 02-09	Neg	Neg	Neg	Neg	Pos
S. epidermidis 07-98-2	Pos	Pos	Pos	Pos	ND
S. epidermidis 21073-5	Pos	Pos	Pos	Pos	ND
S. epidermidis 07-98-4	Pos	Pos	Pos	Neg	ND
S. epidermidis 11-92	Neg	Neg	Neg	Neg	ND
S. epidermidis 03-96	Neg	Neg	Neg	Neg	ND
S. epidermidis 04-96	Neg	Neg	Neg	Neg	ND
S. epidermidis 07-98-1	Neg	Neg	Neg	Neg	ND
S. epidermidis 07-98-3	Neg	Neg	Neg	Neg	ND
S. epidermidis 10-99	Neg	Neg	Neg	Neg	ND
S. epidermidis 930892-4	Neg	Neg	Neg	Neg	ND
S. epidermidis 1025504	Neg	Neg	Neg	Neg	ND
S. epidermidis 22334-6	Neg	Neg	Neg	Neg	ND
S. epidermidis 1025522	Neg	Neg	Neg	Neg	ND
S. epidermidis 1025524	Neg	Neg	Neg	Neg	ND
S. epidermidis 1025527	Neg	Neg	Neg	Neg	ND
S. epidermidis 1025679	Neg	Neg	Neg	Neg	ND
S. capitis 1025512	Neg	Neg	Neg	Neg	Pos
S. capitis 1025517	Pos	Pos	Pos	Pos	Pos
S. capitis 1025521	Pos	Pos	Neg	Pos	Pos
S. capitis 1025525	Pos	Pos	Neg	Neg	Pos
S.saccharolyticus 1025548	Neg	Neg	Neg	ND	ND
S.saccharolyticus 1025549	Neg	Neg	Neg	ND	ND
S.saccharolyticus 1025550	Neg	Neg	Neg	ND	ND
S.saccharolyticus 1025673	Neg	Neg	Neg	ND	ND
S. chromogenes 1025667	Neg	Neg	Neg	Neg	ND

Neg: Negative; Pos: Positive; ND: Not done

3.3.3 Biofilm Formation in Media and Platelet Concentrates

Primary screening for biofilm formation ability was performed using both slime production and molecular amplification of the *icaAD* genes; however, these assays do not confirm the ability of the strains to form an actual biofilm in a specific environment. To test for biofilm formation, the CoNS strains were cultured in TSBg media (TSB plus 0.5% glucose) and in platelet concentrates using six-well tissue culture plates following protocols previously established in the Ramirez laboratory (49).

Inoculated TSBg was incubated at 37^{o}C for 24 hours at static motion, before proceeding with crystal violet staining. Five of the six slime-forming strains (*S. epidermidis* 02-96, *S. epidermidis* 07-98-2, *S. epidermidis* 07-04, *S. capitis* 1025517, and *S. epidermidis* 21073-5) formed biofilms in TSBg, while *S. epidermidis* 07-98-4 failed to form biofilms in TSBg despite the presence of the *icaA* and *icaD* genes as well as its ability to produce slime (Table 4, Figure 21).The cut-off level was chosen based on the optical density of the negative control *S. epidermidis* ATCC 12228 plus the standard deviation.

Greco *et al*, (50) have previously demonstrated the importance of using fresh platelet concentrates to perform accurate biofilm formation assays. However, due to the fact that fresh platelet concentrates are important for transfusions, only eight out of the 29 strains were selected to study biofilm formation in fresh platelet concentrates. These strains included *S. epidermidis* 02-96 (slow-growing in platelet concentrates and biofilm-forming in TSBg), *S. epidermidis* 07-04 (fast-growing in platelet concentrates and biofilm-forming in TSBg), *S. epidermidis* 02-09 (slow-growing in platelet concentrates and non-biofilm-forming in TSBg), *S. epidermidis* 09-04 (fast-growing in platelet concentrates and non-biofilm-forming in *TSBg), S. capitis* 1025512, *S. capitis* 1025517, *S. capitis* 1025521 *and S.*

capitis 1025525. The assay was repeated four and three independent times, each time in triplicate, for *S.epidermidis* and *S. capitis*, respectively (Figures 22 and 23). All tested strains were able to form biofilms in platelet concentrates, although a high variability in the amount of biofilm formed was observed between assays. Statistical analysis showed a significant difference in biofilm formation among the two fast-growing strains *S. epidermidis* 07-04 and *S. epidermidis* 09-04 ($p=0.0333$) with more biofilm formed by S. epidermidis 09-04. While biofilm formation between the two slow-growing strains (*S. epidermidis* 02-96 and *S. epidermidis* 02-09) was not significantly different ($p=0.4747$). However, the biofilm formation between the slow-growers (*S. epidermidis* 02-09 and *S. epidermidis* 02-96) and fast growers (*S. epidermidis* 07-04 and *S. epidermidis* 09-04) was significantly different ($p <0.0001$) with more biofilm formed by the slow grower strains. The negative control *S. epidermidis* ATCC 12228 was also able to produce biofilms in platelet concentrates, confirming previous observations in the Ramirez laboratory (49).

Figure 21: Biofilm formation in TSBg. Biofilm formation of CoNS strains in TSB supplemented with 0.5% glucose as determined by crystal violet straining and OD determination at 492 nm. ATCC strain 12228, a biofilm-negative control strain and ATCC strain 35984, a biofilm-positive control strain, are included. Mean of ± standard deviation of OD values of two independent experiments each performed in triplicate, are shown.

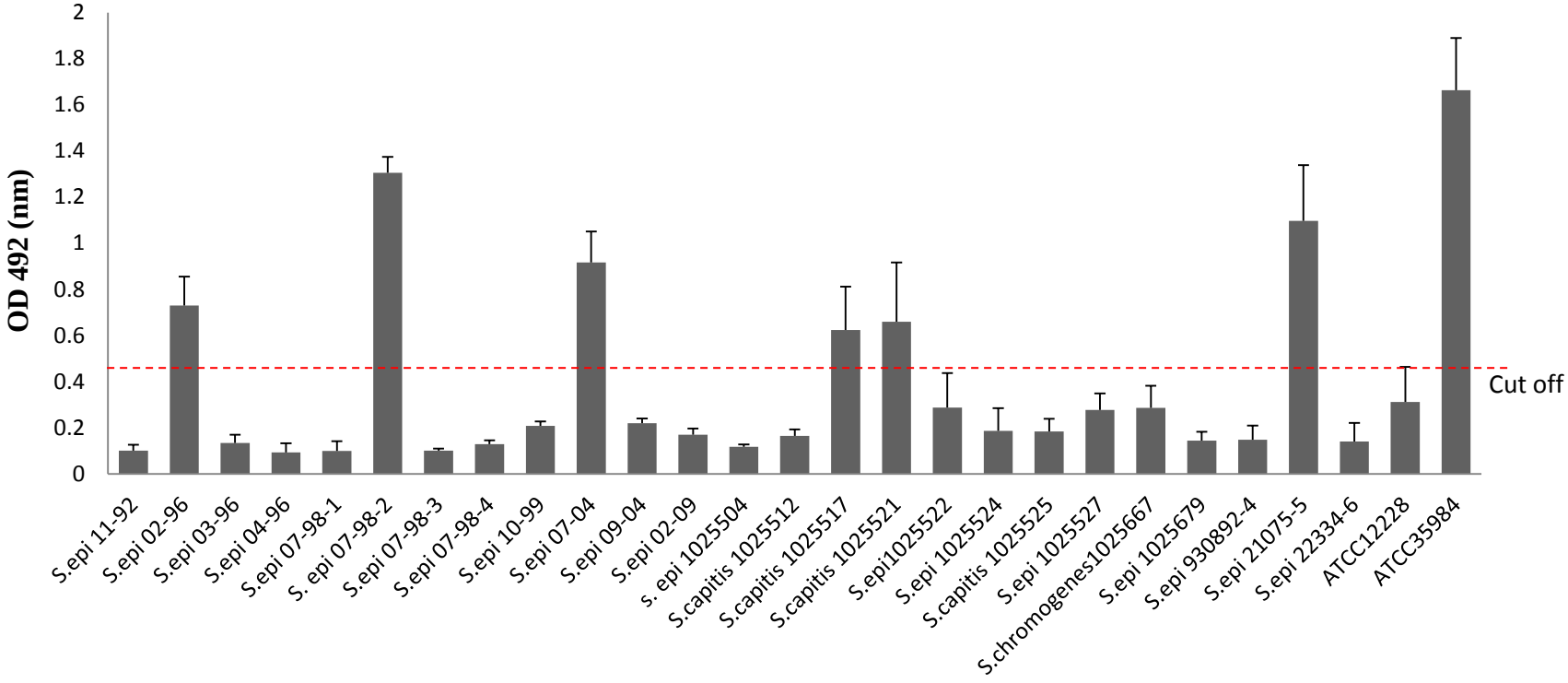

OD 492 (nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cut off
S.epi 11-92
S.epi 02-96
S.epi 03-96
S.epi 04-96
S.epi 07-98-1
S. epi 07-98-2
S.epi 07-98-3
S.epi 07-98-4
S.epi 10-99
S.epi 07-04
S.epi 09-04
S.epi 02-09
s. epi 1025504
S.capitis 1025512
S.capitis 1025517
S.capitis 1025521
S.epi1025522
S.epi 1025524
S.capitis 1025525
S.epi 1025527
S.chromogenes1025667
S.epi 1025679
S.epi 930892-4
S.epi 21075-5
S.epi 22334-6
ATCC12228
ATCC35984

Figure 22: Biofilm formation of *S. epidermidis* strains in fresh platelet concentrates. Biofilm formation of *S.epidermidis* strains (*S. epidermidis* 02-96, *S. epidermidis* 07-04, *S. epidermidis* 02-09, and *S. epidermidis* 09-04) in platelet concentrates as determined by crystal violet straining. ATCC strain 12228, a biofilm-negative control strain and ATCC strain 35984, a biofilm-positive control strain, are included. Mean of ± standard deviation of OD values of four independent experiments, each performed in triplicate, are shown.

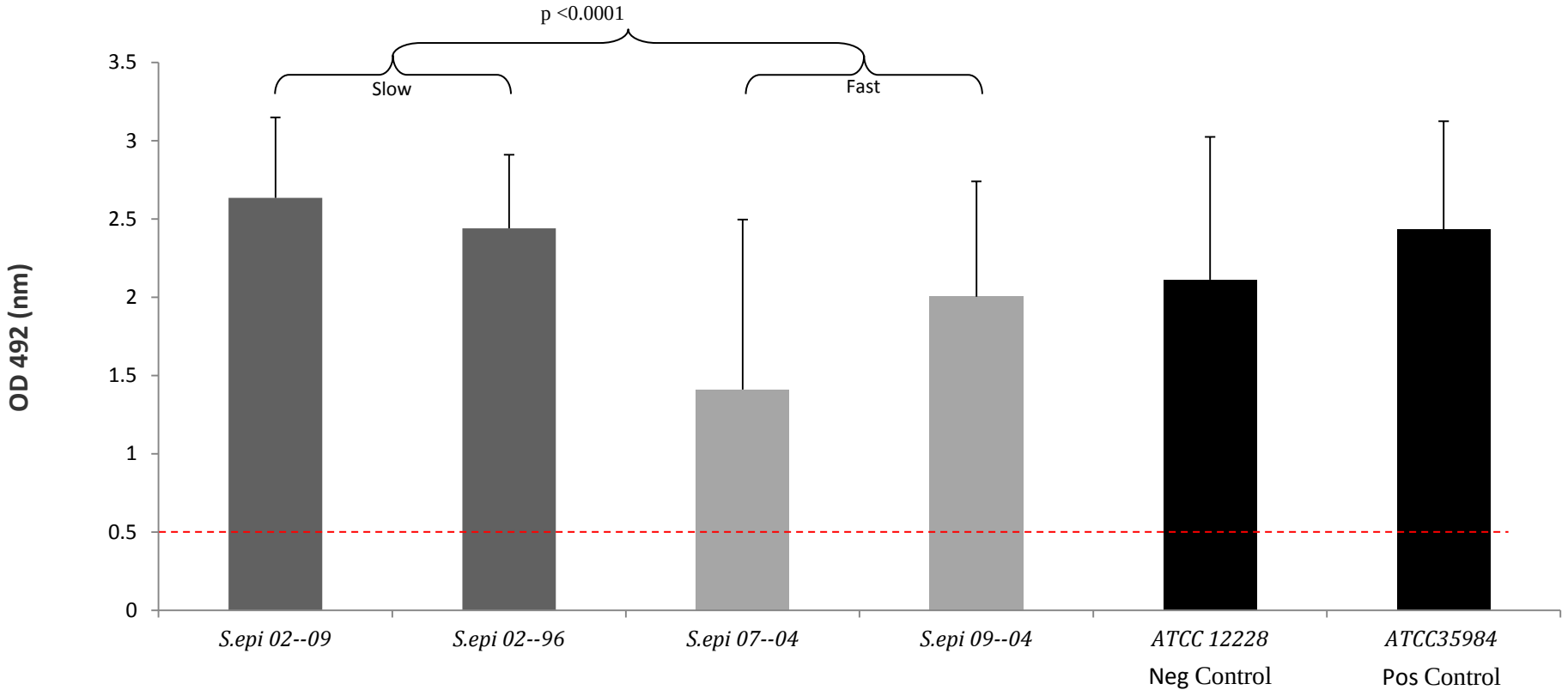
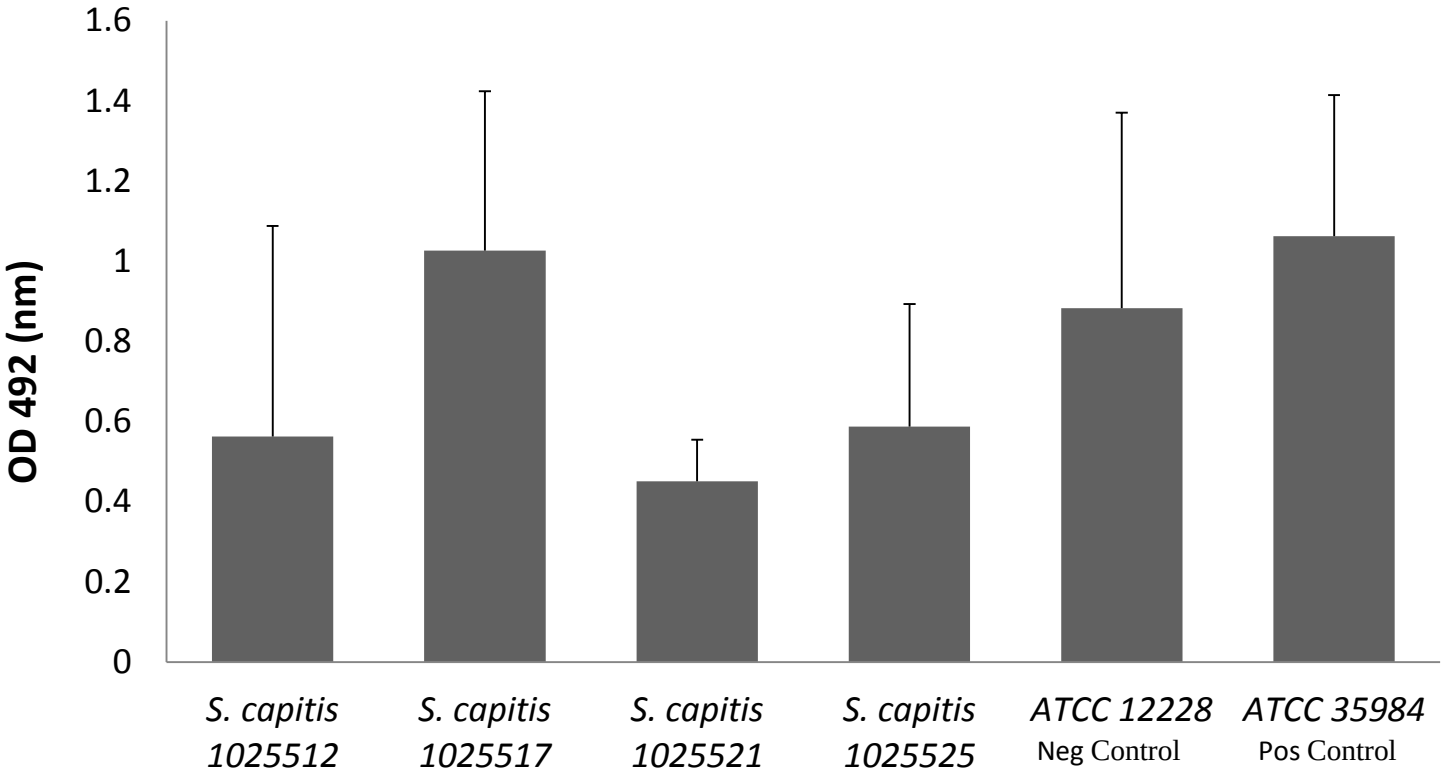

p <0.0001
Slow
Fast
OD 492 (nm)
3.5
3
2.5
2
1.5
1
0.5
0
S.epi 02--09
S.epi 02--96
S.epi 07--04
S.epi 09--04
ATCC 12228
Neg Control
ATCC35984
Pos Control

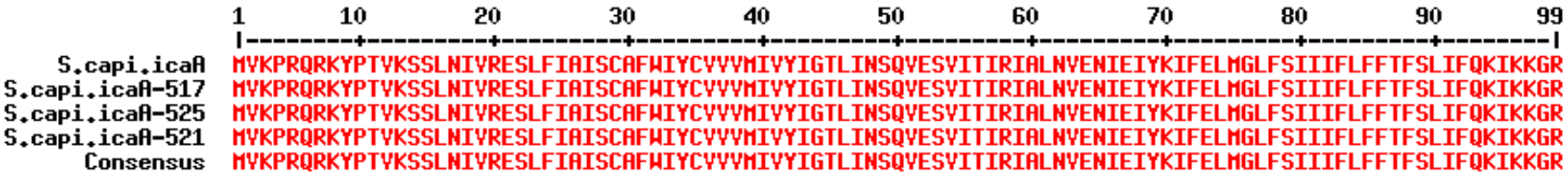
Figure 23: Biofilm formation of *S. capitis* strains in fresh platelet concentrates. Biofilm formation of *S. capitis* strains (*S. capitis* 1025517, *S. capitis* 1025521, *and S. capitis* 1025525) in platelet concentrates as determined by crystal violet straining. ATCC strain 12228, a biofilm-negative control strain and ATCC strain 35984, a biofilm-positive control strain, are included. Mean of ± standard deviation of OD values of three independent experiments, each performed in triplicate, are shown.


OD 492 (nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
S. capitis 1025512
S. capitis 1025517
S. capitis 1025521
S. capitis 1025525
ATCC 12228 Neg Control
ATCC 35984 Pos Control

3.3.4 Alignment of *S. capitis* IcaD Proteins

As shown in Table 4, three out of the four *S. capitis* strains were *icaAD*-positive (*S. capitis* 1025517, *S. capitis* 1025521, *and S. capitis* 1025525). However, only *S. capitis* 1025517 was able to produce slime on CRA and form biofilms in TSBg and PCs. To investigate whether the differences in the ability to form slime and biofilms were due to mutations in *icaD*, the gene was amplified from each strain and the PCR amplicons were sent for sequencing; the translated proteins were aligned. No differences were found in the IcaD proteins of the four strains.

Figure 24: *S. capitis* IcaD protein alignment.


1 10 20 30 40 50 60 70 80 90 99
S.capi.icaA MVKPRQRKYPTVKSSLNIVRESLFIAISCAFWIYCVVVMIVYIGTLINSQVESVITIRIALNVENIEIYKIFELMGLFSIIIFLFFTFSLIFQKIKKGR
S.capi.icaA-517 MVKPRQRKYPTVKSSLNIVRESLFIAISCAFWIYCVVVMIVYIGTLINSQVESVITIRIALNVENIEIYKIFELMGLFSIIIFLFFTFSLIFQKIKKGR
S.capi.icaA-525 MVKPRQRKYPTVKSSLNIVRESLFIAISCAFWIYCVVVMIVYIGTLINSQVESVITIRIALNVENIEIYKIFELMGLFSIIIFLFFTFSLIFQKIKKGR
S.capi.icaA-521 MVKPRQRKYPTVKSSLNIVRESLFIAISCAFWIYCVVVMIVYIGTLINSQVESVITIRIALNVENIEIYKIFELMGLFSIIIFLFFTFSLIFQKIKKGR
Consensus MVKPRQRKYPTVKSSLNIVRESLFIAISCAFWIYCVVVMIVYIGTLINSQVESVITIRIALNVENIEIYKIFELMGLFSIIIFLFFTFSLIFQKIKKGR

3.4 RELATION BETWEEN ABILITY FOR BIOFILM FORMATION AND MISSED DETECTION DURING PLATELET SCREENING

Three experiments were designed to provide preliminary evidence for a relationship between the ability of CoNS strains to form biofilms during platelet storage could and missed detection during routine platelet screening with the automated culture BacT/ALERT®3D system. The strains used for these experiments were *S. epidermidis* 02-96 (slow-grower in platelet concentrates) and *S. epidermidis* 07-04 (fast-grower in platelet concentrates); both presenting a biofilm-positive phenotype on platelet concentrates. Based on the results of these studies (Figure 21) and previous research in the Ramirez laboratory (49, 50), biofilm formation by *S. epidermidis* has been shown to occur in platelet concentrates even by strains traditionally known to be "biofilm-negative", and therefore no negative controls are available that can be used for these experiments.

3.4.1 Test 1: Platelet Bag Coupons

Bacterial biofilms were grown on 1 cm^2 platelet bag coupons which were used to inoculate platelet cultures. Upon 24 h incubation under platelet storage conditions, simulating the routine practice of platelet quarantine, 8 to 10 ml were inoculated into each of 10 BacT/ALERT®3D BPA bottles and incubated in the BacT/ALERT®3D system. All bottles became positive within 8 hours of inoculation.

3.4.2 Test 2: Sampling Site Coupler

The sampling site coupler is a site found on the exterior of platelet storage bags and serves as the site from which samples are extracted/introduced through a sterile, single-use needle. The sample site coupler was used as a surface on which bacterial biofilms were grown. A

needle was then inserted through the contaminated sample site coupler to mimic the action of blood collection/venipuncture. Aliquots of 8 to 10ml were then transferred to BPA culture bottles and incubated in the BacT/ALERT system. All inoculated bottles were positive within 19 hours of inoculation.

3.4.3 Test 3: Inoculation of Pre-conditioned Platelet Bags

The previous two approaches used high initial bacterial inocula which do not represent the levels of contamination found on the skin of blood donors. Therefore, a third assay was designed. Cultures of the slow grower *S. epidermidis* 02-96 and the fast grower *S. epidermidis* 07-04, which both display biofilm-positive phenotypes in regular media and platelet concentrates, respectively, were introduced into pre-conditioned platelet bags containing 200 ml of TSBg. Pre-conditioned platelet bags were prepared by gently removing the platelet concentrates out, but leaving host proteins adhered to the inner surface of the containers. The cultures were inoculated at two initial concentrations (~0.1 and ~0.3 CFU/ml), which represent real-life levels of contamination in platelet concentrates. The experiment was repeated five and three independent times for each concentration, respectively.

Using this approach, it was observed that the slow grower *S. epidermidis* 02-96 was detected in 60% (30/50) and in 97.7% (29/30) of the bottles inoculated with ~0.1 and ~0.3 CFU/ml, respectively, while the fast grower *S .epidermidis* 07-04 was detected in 74% (37/50) and 93.3% (28/30) of the bottles inoculated with ~0.1 and ~0.3 CFU/ml, respectively. Pairwise comparison showed a significant difference between the two inoculated concentrations (~0.1 and ~0.3 CFU/ml) for *S. epidermidis* 02-96 and *S. epidermidis* 07-04 ($p=0.0005$ and $p = 0.0008$, respectively). Interestingly, there was a

significant difference in the number of positive bottles detected between the two strains at the lower concentration of ~0.1 CFU/ml ($p = 0.0036$). The number of bottles detected with initial inocula of with ~0.3 CFU/ml was not significantly different for the two strains ($p=0.4439$).

CHAPTER FOUR
DISCUSSION

4.1 PREVALENCE OF CoNS AS PLATELET CONTAMINANTS

Bacterial contamination of blood products continues to be the most prevalent infectious risk in transfusion medicine (8, 107). Among the different blood products, platelet preparations are considered to be the most susceptible blood product to contamination with bacteria (66). Unlike other blood components, that are mainly stored statically in low temperature environments, platelet concentrates are stored with constant agitation at 22±2^{o}C for up to 5 days, providing a suitable growth environment for many bacterial species. Platelet concentrates are characterized by a physiological pH (pH ~7.4) and high glucose content (approximately 500mg/dL) mainly provided by anticoagulant solutions present in the storage bags. Storage bags are designed to maintain the normal function of platelets by permitting oxygen permeability. Even a small number of bacteria present in the platelet bags can multiply to a clinically-significant level during storage and subsequently lead to post-transfusion reactions (8, 86, 98, 109, 117).

Gram positive and Gram negative bacteria alike have been isolated during screening of platelet concentrates (98, 109). According to reported studies, Gram positive skin and environmental contaminants are the predominant identified bacterial species isolated from platelet concentrates (66). Among the skin flora, coagulase-negative staphylococci and *Propionibacterium acnes* are the major contaminants of platelet concentrates; the latter is mainly detected when anaerobic bottles are used (123, 139). Within the coagulase-negative staphylococci, *S. epidermidis* is the predominant platelet contaminant. This bacterium resides on the skin of healthy donors and therefore is introduced into the collection bag at

the time of phlebotomy. According to recent studies, other coagulase-negative staphylococci including *S. capitis*, *S. warneri*, *S. saccharolyticus* and *S. hominis* have also been found to contaminate platelet concentrates (115).

In this thesis, *S. epidermidis* was the predominant species identified. This finding supports previous studies, which demonstrated that *S. epidermidis* is the most commonly skin flora microorganism isolated during platelet screening (12, 47, 66, 78, 109). Other aerobic coagulase negative staphylococci were also identified in this study, including *S. chromogenes* and *S. capitis*. The former has been reported to contaminate whole blood (17), while *S. capitis* been documented as one of the important platelet contaminants (115). Interestingly, results from this thesis also showed that the strict anaerobe *S. saccharolyticus* is able to survive, but not to multiply, under platelet storage conditions likely due to the requirement of this species for a strict anaerobic environment, which is missing in the aerated platelet containers (3). Similar studies have been conducted with the common anaerobic platelet contaminant *Propionibacterium acnes*, which is unable to proliferate under platelet storage conditions and does not reach clinically-significant concentrations (123). Therefore, the clinical relevance in transfusion medicine of *P. acnes* is still unclear and under debate (115). According to Störmer *et al* (123), patients who received platelet concentrates contaminated with *P. acnes* neither showed symptoms of febrile transfusion complications nor there was evidence of any inflammatory event associated with transfusion. Incidences of transfusion reactions due to contaminated platelet concentrates with *S. saccharolyticus* have not been reported in the literature; however, both *P. acnes* and *S. saccharolyticus* have been involved in serious infections such as prosthetic valve endocarditis (54, 73). In addition, other strict anaerobes such as *Clostridium perfringens* and *Eubacterium limosum*, have been implicated in fatal transfusion reactions (40,

88).Therefore, the potential of anaerobic species to cause serious transfusion reactions cannot be underestimated. Noticeably, Canada only screens platelet concentrates with aerobic culture bottles while other countries such as The Netherlands use the two-bottle culture system which allows the detection of a great diversity of both aerobic and anaerobic bacteria, in addition to improved detection of some facultative anaerobes (13, 115).

4.2 BACTERIAL GROWTH BY CoNS IN PLATELET CONCENTRATES

Growth studies performed in this thesis showed that all strains proliferated at a similar rate in regular laboratory media (TSB) under their optimal growth conditions (37^{o}C/ agitation); however, they were not able to grow in the same media under platelet storage conditions (room temperature/ agitation), implying that the different temperature might be the main factor affecting the growth dynamics of CoNS. Similarly, Akly *et al* (1) reported that *S. epidermidis* was not able to grow at room temperature. However, if the temperature were the limiting factor, *S. epidermidis* would not be able to growth in platelet concentrates which are stored at 22-24^{o}C. According to Störmer *et al* [23], lactic acid production by metabolizing platelets promotes *S. epidermidis* proliferation due to pH decrease in platelet concentrates. Further studies should confirm that the pH is the limiting factor by adjusting pH in regular media and confirm growth promotion of *S. epidermidis* under acidic conditions.

In the present study, staphylococcal growth in platelet concentrates varied within strains independently of the platelet preparation. These results are in contrast to the study reported by Störmer *et al* (122), which reported that bacterial proliferation in platelet concentrates was not strain-specific. At this time it is unknown which factors would drive differential growth in platelet concentrates by various strains of *S. epidermidis*.

Interestingly, in this study there were no reductions in the growth rates of two *S. epidermidis* strains upon platelet depletion by ~1,000 times, when compared to platelet rich plasma. In contrast, results obtained by Greco *et al* (51), have shown that reduction in platelet concentration resulted in a significantly decreased growth of *S. epidermidis*. Flow cytometry assays to determine platelet counts were not performed in this thesis therefore, post-filtration reduction in platelet content was not confirmed, which could explain the discrepancy in growth between the two studies. Another explanation for the differences observed may be due to the initial bacterial concentration used in each study. While of the initial inoculum used in this thesis was from 10 to 100 CFU/ ml, Greco *et al* (51), inoculated platelet concentrates with 0.2 to 2 CFU/ml. Furthermore, differences in strain-specific factors might have contributed to the discrepancy in results.

4.3 BIOFILM FORMATION BY CoNS IN PLATELET CONCENTRATES

Greco *et al* (49) have shown that the platelet storage environment promotes *S. epidermidis* biofilm formation with cells adhering to the inner surface of the platelet containers and to platelets. Physiological pH and glucose provided by the anticoagulant solutions in the platelet units support survival, growth, and biofilm formation of *S. epidermidis* (50, 122). Biofilm-positive CoNS might utilize the presence of glucose to form biofilms as it is required to synthesize N-acetyl glucosamine, the main constituent of the exopolysaccharide (biofilm matrix) (45, 82).

A previous study in the Ramirez' laboratory showed that a surprisingly high proportion of *S. epidermidis* isolated from healthy donors carried *ica* genes and therefore had the potential for biofilm formation (50). Similarly, in this study, eight (27.6%) of strains captured during platelet screening carried the *ica* genes demonstrating once more

that potentially-virulent strains are distributed in the community. Missed detection of these strains during platelet screening may lead to severe consequences. However, the clinical impact of transfusion of contaminated platelet units on recipient depends on many factors including the virulence of the bacterial species, concentration, and health status of the recipient.

Biofilm formation by *S. epidermidis* is usually related to the presence of proteins encoded by the *icaADBC* gene operon (60, 79). In the current study, the ability of biofilm formation by CoNS isolated from contaminated platelet units was investigated using several approaches. PCR amplification of *icaA* and *icaD* genes responsible for PIA production revealed eight positive CoNS isolates. Of those, five were *S. epidermidis* and three *S. capitis*. The slime production test on Congo Red Agar showed that six out of the eight *ica*-positive strains were able to produce black colonies indicating a slime producing ability. However, neither the presence of *ica* genes nor slime production promise the ability for biofilm formation. Slime-negative *S. capitis* 1025521 was able to form biofilms in TSBg while *S. epidermidis* 07-98-4, which was *ica*-positive and slime-positive, was not able to form biofilms (Table 4). The failure to produce biofilms by *ica*-positive strains has been previously reported in the literature for *S. epidermidis* and other staphylococcal species (32, 132). Cramton *et al* (32) observed that *S. aureus* may display biofilm negative phenotypes *in vitro* despite having the *ica* locus and speculated that the biofilm formation is highly sensitive to the growth conditions. Regulation or specific mutations in *ica* genes the might be some of the factors for negative biofilm phenomena.

In the current work, biofilm formation experiments in platelet concentrates were done with four *S. epidermidis* strains including slow and fast growers in platelet concentrates which were both biofilm-positive in regular media (TSBg). Results from these

experiments demonstrated that the slow growers formed significantly more biofilms than the fast growers. Therefore, it is possible that the slow growth observed is due to the biofilm formation which leads to less bacteria available for sampling and not actual reduction in the growth dynamics of these strains. In any case, slow growth and biofilm formation during platelet storage contribute to missed detection during platelet screening posing a very important transfusion infectious risk.

S. epidermidis and *S. capitis* strains studied in this thesis considered to be "biofilm-negative" in regular media, formed biofilms in platelet concentrates. It has been demonstrated that some *S. epidermidis* can utilize different pathways to form biofilms other than PIA production, which requires the presence of the *icaADBC* operon (26, 71). Evidence of *ica*-independent biofilm mechanisms has been reported elsewhere. Tormo *et al*. (128) demonstrated that strains producing the surface protein Bap, which were *ica*-negative, were able to form biofilms. The accumulation-associated protein Aap, characterized by Hussain *et al* (64), has also been implicated in biofilm accumulation of *ica*-negative *S. epidermidis* strains (114). Other surface factors such as AtlE and teichoic acids and their role in biofilm development have been well documented in literature (52, 59). Therefore, it is possible that *S. epidermidis* biofilms in platelet concentrates have either a polysaccharide (ie, PIA) or protein (*ica*-independent) matrix. Further studies are needed to confirm the nature of the biofilms formed in platelet concentrates. Digestion using protease would be used to determine the nature of the biofilm matrix.

4.4 RELATIONSHIP BETWEEN BIOFILM FORMATION IN PLATELET CONCENTRATES AND MISSED BACTERIAL DETECTION BY THE AUTOMATED CULTURE BacT/ALERT® 3D SYSTEM

Stewart *et al*(48) showed that *Serratia marcescens* isolates implicated in adverse transfusion reactions formed biofilms in platelet concentrates which correlated with reduced detection by the BacT/ALERT System. Therefore, the relation between biofilm formation of *S. epidermidis* and missed detection has been further investigated in the present thesis. Testing platelet bag's coupons colonized with *S. epidermidis* biofilms as well as simulating the action of blood collection by using sampling site couplers did not prove to be good models for this study likely due to bacterial overgrowth obtained on both surfaces. In contrast, inoculation of pre-conditioned platelet concentrate bags with low bacterial concentrations of biofilm-positive *S. epidermidis* strains demonstrated that that a slow-growing isolate was more likely to be missed during screening with the BacT/ALERT system than the faster-growing strain. Although a slow growth behaviour by *S. epidermidis* has been associated with incidences of missed detection during platelet screening (14, 15, 91), this study is the first to report the relation between slow growth and increased biofilm formation in platelet concentrates. It is possible that the "slow growth" is attributed to decreased recovery due to biofilm formation and not to a real reduction in the growth rate of strong biofilm formers (122).

A drawback of the missed detection experiments was the small number of strains tested, and especially, that only biofilm positive strains were used. Therefore, it would be important to include biofilm negative controls to fully investigate the influence of biofilm phenomena on missed detection of this species during routine laboratory screening. Using platelet concentrates instead of the pre-conditioned TSBg media, would provide additional

results to support the preliminary findings reported herein. However, there are no negative *S. epidermidis* controls for biofilm formation in platelet concentrates, since all strains tested so far are able to form biofilms in this medium.

In this thesis, it was hypothesized that slow growth and biofilm formation was predominant in CoNS strains implicated in adverse transfusion reactions (ATRs). Out of the four strains involved in ATRs (11-92, 02-96, 10-99 and 07-04, Table 2), only two were able to form biofilms in regular media and platelet concentrates (02-96 and 07-04). While 02-96 was a slow grower, strain 07-04 reached high concentrations in platelet concentrates. Therefore a correlation between growth, biofilm formation and clinical outcome in platelet recipients was not observed, probably due to the small number of strains studied. Overall, this thesis provided novel evidence that links biofilm formation during platelet storage with slow growth and missed detection. These results could be exploited to develop better bacterial detection methods with the long term goal of improving the safety of the platelet supply for Canadians.

4.5 FUTURE STUDIES

This study has generated a series of questions that can be resolved in future studies:

1. What is the nature of the biofilms formed by *ica*-negative strains in platelet concentrates?
2. Which bacteria and platelet factors are involved in biofilm formation during platelet storage by strains otherwise considered to be biofilm-negative?
3. Which regulatory factors affect biofilm formation during platelet storage?
4. Do anaerobic CoNS form biofilms in platelet concentrates?
5. Which factors can be targeted to develop mechanisms to prevent/reduce staphylococcal biofilm formation during platelet storage?
6. Can an animal model be developed to test the link between biofilm formation during platelet storage and the clinical outcome of platelet recipients?

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- Dr. Samra Uzicanin from Canadian Blood Services performed the biochemical identification and growth curves of the US CoNS strains.

- Drs. Michael Jacobs, Roslyn Yomtovian (Case Western Reserve University), and Dirk de Korte (Sanquin Blood Bank North West) provided CoNS strains for this thesis.

- Dr. Qi-Long Yi from Canadian Blood Services performed the statistical analyses of this thesis.