The Comparative Efficacy of 0.12% Chlorhexidine and Amoxicillin to Reduce the Incidence and Magnitude of Bacteremia During Third Molar Extractions: A Prospective, Blind, Randomized Clinical Trial.

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DEDICATION

I dedicate this thesis to my wife Becca and our daughter Mia Elizabeth - and also to Lilly. You have been patient with me and have supported me during this endeavor. I love you guys.

To my parents who instilled in me a work ethic.

To God the Father, the Son, and the Holy Spirit – from whom I derive all I am and are blessed by Him.

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ABSTRACT

Objective: Although the evidence is purely circumstantial, it has been postulated that dental treatment procedures cause a transient bacteremia which may predispose patients with cardiac anomalies to IE and patients with a prosthetic joint to late prosthetic joint infections if not treated with antibiotic prophylaxis; however, there is minimal evidence-based data to support such a hypothesis. The purpose of this study is to determine the effect of a pre-procedure rinse of 0.12% chlorhexidine on the incidence and magnitude of bacteremia compared to the AHA recommended antibiotic prophylaxis guideline of 2g amoxicillin during third molar extractions. **Materials/Methods:** The research subjects were randomly assigned to one of three groups: PLAC group (receiving a placebo rinse and a placebo capsule); CHX group (receiving a 0.12% chlorhexidine rinse and a placebo capsule); and AMOX group (receiving 2g amoxicillin capsule and a placebo rinse). An IV access line was obtained for each subject and four blood draws were completed during the surgical procedure. The blood draws were processed using the Wampole™ ISOSTAT®/ISOLATOR™ Microbial System and species identification was completed using the VITEK® 2 and Biolog™ Microstation System™. **Results:** The incidence of bacteremia was analyzed using a χ^2 test with a statistical significance/α of 0.05. The PLAC group showed a 50% incidence of bacteremia, the CHX group a 60% incidence, and the AMOX group a 40% incidence (p=0.670). The magnitude of bacteremia was analyzed using the non-parametric Kruskall-Wallis test and the Friedman test with a statistical significance/α of 0.017. The PLAC group showed a

mean total bacteremia of 3.61 CFU/mL (stdev=7.09), the CHX group 2.76 CFU/mL (stdev=4.28), and the AMOX group 0.63 CFU/mL (stdev=1.33). The total bacteremia ranged from 0.0-18.20 CFU/mL in the PLAC group, 0.0-11.10 CFU/mL in the CHX group, and 0.0-4.30 CFU/mL in the AMOX group. There were 24 different bacterial species isolated in the PLAC group, 15 isolated in the CHX group, and 10 isolated in the AMOX group. Of the 33 total different bacterial species, seven (21%) were α-hemolytic and also belonged to the viridans group streptococci. In the PLAC group, five bacterial species isolated were α-hemolytic/viridans group streptococci, two isolated in the CHX group, and one isolated in the AMOX group. **Conclusion:** The results of this study may reasonably conclude an antimicrobial intervention of either a 0.12% chlorhexidine pre-procedure rinse or 2g amoxicillin antibiotic prophylaxis according to the current AHA and ADA/AAOS guidelines does not statistically reduce the incidence and magnitude of bacteremia compared to no antimicrobial intervention. However, there appeared to be three important trends: 1) a 0.12% chlorhexidine pre-procedure rinse and to a greater extent a 2g amoxicillin antibiotic prophylaxis reduced the mean magnitude and range of bacteremia in CFU/mL compared to a placebo; 2) the magnitude of bacteremia peaked within 1.5 minutes of initiating the last third molar extraction for the PLAC, CHX, and AMOX groups and decreased within 10 minutes but remained above baseline; 3) a 0.12% chlorhexidine pre-procedure rinse and to a greater extent a 2g amoxicillin antibiotic prophylaxis reduced the number of different bacterial species, including α-hemolytic/viridans group streptococci, isolated from the vasculature compared to a placebo.

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BACKGROUND AND LITERATURE REVIEW

The occurrence of a transient bacteremia originating from the oral cavity during various types of gingival sulcus manipulation is well established (Aguada et al., 1997; Aitken et al., 1995; Allison et al., 1993; Baltch et al., 1982; Baltch et al., 1988; Baltch et al., 1982; Berger et al., 1974; Bhanji et al., 2002; Brennan et al., 2007; Brown et al., 1998; Burden et al., 2004; Cannell et al., 1991; Carroll et al., 1980; Castillo et al., 2011; Cherry et al., 2007; Chung et al., 1986; Coulter et al., 1990; Crasta et al., 2009; Daly et al., 2001; Debelian et al., 1995; Diz Dios et al., 2006; Erverdi et al., 2001; Fine et al., 2010; Fine et al., 1996; Forner et al., 2006; Goker et al., 1992; Gurel et al., 2009; Hall et al., 1993; Hall et al., 1996; Hall et al., 1996; Hall et al., 1999; Head et al., 1984; Heimdahl et al., 1990; Hess et al., 1983; Hess et al., 1983; Kaneko et al., 1995; Katoh, 1992; Kinane et al., 2005; King et al., 1988; Lockhart et al., 2008; Lockhart et al., 2004; Lockhart et al., 1996; Lucas et al., 2008; Lucas et al., 2002; Lucas et al., 2000; McLaughlin et al., 1996; Morozumi et al., 2010; Nohara et al., 1995; Okabe et al., 1995; Oncag et al., 2006; Peterson et al., 1976; Pineiro et al., 2010; Rahn et al., 1995; Rajasuo et al., 2004; Roberts et al., 2006; Roberts et al., 2002; Roberts et al., 2000; Roberts et al., 1998; Roberts et al., 1997; Savarrio et al., 2005; Schlein et al., 1991; Sconyers et al., 1973; Silver et al., 1977; Sonbol et al., 2009; Takai et al., 2005; Tomas et al., 2008; Tomas et al., 2007; Tomas et al., 2007; Tomas et al., 2004; Vergis et al., 2001; Wahlmann et al., 1999; Wampole et al., 1978; Wank et al., 1976; Witzenberger et al., 1982). Such types of gingival sulcus manipulation range from the daily routine/oral hygiene activities of chewing, toothbrushing, and flossing to dental treatment procedures. Of particular

significance with regards to bacteremia originating from the oral cavity is that a thin, nonkeratinized mucosal epithelial layer separates the gingival sulcus microflora from the highly vascularized underlying tissue. Upon gingival sulcus manipulation the integrity of the epithelial layer is compromised allowing the dissemination of the oral microflora into the vasculature, which may be exacerbated by gingivitis and/or periodontal disease. The gingival sulcus, the dentition, and the other mucosal surfaces of the oral cavity are populated by a diverse, complex endogenous microflora of potentially more than 700 species of which approximately 400 species are located in the gingival sulcus/periodontal pocket (Paster et al., 2006; Wilson et al., 2007). Of the gingival sulcus/periodontal pocket microflora, 30% are streptococci primarily of the viridans group (Wilson et al., 2007). The dentition is the only nonshedding surface of the human body where the plaque bacterial concentration can exceed 10^{11} microorganisms/mg (Li et al., 2000). Consider also that a human with gingivitis and/or periodontal disease with 4-5mm probing depths equates to a surface area of approximately 10-20cm^2 and up to 30-40cm^2 in humans with 50% horizontal bone loss (Scannapieco, 2004). It therefore becomes quite apparent that the oral cavity is a common source of bacteremia that has been implicated as a cause of distant site infections (DSI).

Infective endocarditis (IE) is one type of DSI implicating oral bacteremia. Lockhart et al. (2008) state 275 bacterial species have been reported to cause IE and 170 bacterial species have been isolated from the vasculature following dental treatment procedures. In this study, Lockhart et al. (2008) isolated 98 bacterial species of

which 32 are reported to cause IE. Viridans group streptococci are reported to cause at least 50% of cases of IE (community-acquired native valve) not associated with intravenous drug use (Fowler et al., 2005). However, viridans group streptococci are endogenous microflora of the skin, oral cavity, respiratory tract, and gastrointestinal tract. It is estimated that 4-19% of IE cases are due to dental treatment procedures (Durack, 1995). Strom et al. (1998) found no evidence that dental treatment procedures were a risk factor for IE.

Although the evidence is purely circumstantial, it has been postulated that dental treatment procedures cause a transient bacteremia which may predispose patients with cardiac anomalies to IE if not treated with antibiotic prophylaxis. There is minimal evidence-based data to support such a hypothesis (Wilson et al., 2007). As a result, the American Heart Association (AHA) amended the antibiotic prophylaxis guidelines for IE in April 2007 (Wilson et al., 2007). These guidelines noted that IE is more likely to result from frequent exposure to transient bacteremias associated with daily routine/oral hygiene activities than from bacteremias induced by dental treatment procedures. It has been estimated that daily routine/oral hygiene activities may cause a bacteremia for 90 hours per month whereas a dental treatment procedure may cause a bacteremia for an average of 6 minutes (Lockhart et al., 1999). These guidelines also noted that antibiotic prophylaxis, even if 100% effective may prevent an exceedingly small number of IE cases, if any, in patients that undergo a dental treatment procedure. Since the AHA published the first guideline for the prevention of IE in 1955, there have been nine revisions that have

progressively prophylaxed less patients with fewer antibiotics. In fact, the 2007 AHA guidelines decreased the number of patients receiving IE antibiotic prophylaxis by 90% (Wilson et al., 2007). It is interesting to note that the proportion of IE cases due to viridans group streptococci has decreased during the same time period (Lockhart et al., 1996) while the overall incidence of IE has not decreased since the use of systemic antibiotic prophylaxis (Bayliss et al., 1983; Lockhart et al., 1999). Lockhart et al. (1999) also note there have been numerous documented failures of antibiotic prophylaxis. It was estimated only 6% of IE cases could be prevented by antibiotic prophylaxis in the Netherlands, which correlates to 240-480 IE cases per year in U.S. (Durack, 1995).

While the AHA has progressively prophylaxed less patients with fewer antibiotics, the American Academy of Orthopedic Surgeons (AAOS) has recommended antibiotic prophylaxis for more patients during the same time period to prevent a late prosthetic joint infection (LPJI), which is another type of DSI implicating oral bacteria. In 2003, the ADA/AAOS released a joint guideline that recommended antibiotic prophylaxis for any patient within two years of a prosthetic joint placement (ADA/AAOS, 2003). However, in 2009 the AAOS released an independent statement that contradicts the 2003 joint guideline (AAOS, 2009). The 2009 AAOS independent statement recommends antibiotic prophylaxis for any patient with a prosthetic joint, regardless of when it was placed. Similar to the circumstantial evidence noted by the AHA in regards to dental treatment procedures and IE, there

is minimal evidence-based data to conclude a transient bacteremia originating from the oral cavity predisposes a patient with a prosthetic joint to a LPJI if not treated with antibiotic prophylaxis (Aminoshariae et al., 2010; Little et al., 2010). The aforementioned change in the 2009 AAOS independent statement was based on no clinical trials, insufficiently documented case reports, and a retrospective study which found a 0.04-0.2% incidence of LPJI from circumstantial dental treatment procedures in medically-compromised patients (Aminoshariae et al., 2010; Little et al., 2010). A majority of prosthetic joint infections occur within three months of placement where the causative bacteria is usually a *staphylococcus* species, specifically *S. epidermidis* and *S. aureus* which comprise approximately 0.005% of the normal oral flora (Aminoshariae et al., 2010; Little et al., 2010). Therefore, changes to the 2003 ADA/AAOS joint guideline on antibiotic prophylaxis for prosthetic joint placement is not necessarily justified.

As previously mentioned, research has shown that daily routine/oral hygiene activities such as toothbrushing, irrigation devices, flossing, and chewing cause a transient bacteremia (Berger et al., 1974; Bhanji et al., 2002; Carroll et al., 1980; Chung et al., 1986; Crasta et al., 2009; Fine et al., 2010; Forner et al., 2006, Kinane et al., 2005; Lockhart et al., 2008; Lucas et al., 2008; Lucas et al., 2000; Schlein et al., 1991; Sconyers et al., 1973; Silver et al., 1977; Wank et al., 1976). Lockhart et al. (2009) reported a relationship between gingival disease indices and/or poor oral hygiene with an increased incidence of bacteremia following toothbrushing.

Research has also shown that dental treatment procedures such as local anesthetic administration, probing/prophylaxis/scaling/root planing, suture removal, orthodontic treatment, restorative treatment, endodontic treatment, implant placement, and dental extractions/dento-alveolar surgery cause a transient bacteremia to varying levels (Brown et al., 1998; Burden et al., 2004; Castillo et al., 2011; Coulter et al., 1990; Daly et al., 2001; Debelian et al., 1995; Gurel et al., 2009; Hall et al., 1999; Heimdahl et al., 1990; Kinane et al., 2005; King et al., 1988; Lucas et al., 2002; Lucas et al., 2000; McLaughlin et al., 1996; Okabe et al., 1995; Oncag et al., 2006; Peterson et al., 1976; Pineiro et al., 2010; Rajasuo et al., 2004; Roberts et al., 2006; Roberts et al., 2000; Roberts et al., 1998; Roberts et al., 1997; Savarrio et al., 2005; Sonbol et al., 2009; Takai et al., 2005; Tomas et al., 2008; Tomas et al., 2007 Wampole et al., 1978). Castillo et al. (2011) identified specific periodontal bacteria - primarily *P. gingivalis* and *A. actinomycetemcomitans* - in peripheral blood during scaling and root planning which correlated to the same specific periodontal bacteria subgingivally using molecular-based diagnostics.

The pre-procedure use of an antimicrobial rinse has demonstrated conflicting results in reducing the incidence of bacteremias during daily routine/oral hygiene activities and dental treatment procedures (Aguada et al., 1997; Allison et al., 1993; Cherry et al., 2007; Erverdi et al., 2001; Fine et al., 1996; Lockhart et al., 1996; Lockhart et al., 2008; Morozumi et al., 2010; Rahn et al., 1995; Tomas et al., 2007; Witzenberger et al., 1982). The different types and/or concentrations of the antimicrobial rinse along with the different methods of rinsing and/or irrigation of the gingival sulcus make

evidence-based comparisons difficult. For example, Tomas et al. (2007) reported a significant reduction in the incidence of bacteremia (control=96%, chlorhexidine=79%, p=.008) at 30 seconds following the last extraction of multiple dental extractions but Lockhart et al. (1996) did not report a significant reduction in the incidence of bacteremia (control=94%, chlorhexidine=84%, p=.27) at one minute and three minutes following the start of a single dental extraction using a 0.2% chlorhexidine pre-procedure antimicrobial rinse. Although both studies used a 0.2% chlorhexidine pre-procedure rinse, Tomas et al. (2007) filled the patient's oral cavity with the rinse for 30 seconds under general anesthesia while Lockhart et al. (1996) had the patients rinse themselves twice for 30 seconds prior to the procedure.

Antibiotic prophylaxis according to the AHA guidelines and various additional antibiotic prophylaxis regimens have demonstrated a reduced incidence, nature, magnitude, and/or duration of bacteremia during dental treatment procedures - primarily dental extractions - but are not 100% effective in preventing a bacteremia originating from the oral cavity (Aitken et al., 1995; Baltch et al., 1982; Baltch et al., 1988; Baltch et al., 1982; Brennan et al., 2007; Cannell et al., 1991; Diz Dios et al., 2006; Goker et al., 1992; Hall et al., 1993; Hall et al., 1996; Hall et al., 1996; Head et al., 1984; Hess et al., 1983; Hess et al., 1983; Kaneko et al., 1995; Katoh, 1992; Lockhart et al., 2004; Lockhart et al., 2008; Nohara et al., 1995; Roberts et al., 2002; Tomas et al., 2004; Vergis et al., 2001; Wahlmann et al., 1999). As with the pre-procedure antimicrobial rinse, the studies comparing the bacteremic efficacy of an antibiotic prophylaxis following gingival sulcus manipulation are quite variable and

again make evidence-based comparisons difficult. For example, Lockhart et al. (2004) noted a 15% ($p<.001$) incidence of bacteremia at one and a half minutes following the start of a single dental extraction (additional extractions were later completed) while Diz Dios et al. (2006) noted a 46% ($p<.001$) incidence of bacteremia at 30 seconds following the last extraction of multiple dental extractions using an antibiotic prophylaxis according to the AHA guidelines. However, Lockhart et al. (2004) studied children using 50mg/kg amoxicillin one hour preoperatively while Diz Dios et al. (2006) studied adults using 2g amoxicillin one hour preoperatively. The literature is replete with bacteremia studies originating from the oral cavity but comparisons or meta-analyses are difficult due to the protocol variability.

A review of the literature has not yielded a study that directly compares the incidence and/or magnitude of bacteremia during a dental treatment procedure using a pre-procedure antimicrobial rinse and an antibiotic prophylaxis regimen. The use of a pre-procedure antimicrobial rinse or the administration of an antibiotic according to the AHA IE and the ADA/AAOS prosthetic joint prophylaxis guidelines remains controversial with insufficient evidence-based data to support. A study directly comparing the bacteremic incidence using a 0.12% chlorhexidine pre-procedure rinse and 2g amoxicillin according to the AHA IE and the ADA/AAOS prosthetic joint prophylaxis guidelines following dental extractions would be beneficial and would provide an evidence-based reference for clinical practice. Of more significance would be to determine the magnitude/concentration of the bacteremia following

dental extractions. Although the magnitude of bacteremia required to induce IE or LPJI in susceptible patients is not known, data comparing the bacterial loads in CFU/mL of 0.12% chlorhexidine and 2g amoxicillin would provide a clearer understanding of the bacteremia dynamics caused by gingival sulcus manipulation and reduction via common antimicrobial interventions. If the use of a pre-procedure antimicrobial rinse and an antibiotic prophylaxis has a similar efficacy, then the use of an antibiotic prophylaxis during dental treatment procedures to prevent IE or LPJI in susceptible patients may become non-applicable and obsolete.

The routine use of antibiotic prophylaxis for dental treatment procedures is not without negative consequences with respect to antibiotic resistance, anaphylactic allergic responses, and cost-effectiveness. Studies have demonstrated antibiotic prophylaxis use may confer bacterial antibiotic resistance, in particular, increased oral cavity streptococci antibiotic resistance in patients with cardiac anomalies susceptible to IE and in patients with short-term doses, even after a single dose similar to the current AHA guidelines (Groppo et al., 2005; Hall et al., 2002; Harrison et al., 1985; Koh et al., 1986; Southall et al., 1983; Woodman et al., 1985). Other studies have demonstrated viridans group streptococci antibiotic resistance of normal flora (Marron et al., 2001; Seppala et al., 2003). Hall et al. (2002) report 56% of viridans group streptococci isolates were penicillin-resistant. The decreased use of short-term antibiotic prophylaxis could reduce the numbers of anaphylactic allergic responses to antibiotics (Agha et al., 2005; Bor et al., 1984; Durack et al., 1995). Idsoe et al. (1968) found 11% of patients that died from an anaphylactic allergic

response to penicillin were administered the antibiotic for surgical procedure prophylaxis. Prior to the AHA IE prophylaxis guidelines revision in 2007, it was suggested that more patients with mitral valve prolapse would die from a penicillin-induced anaphylactic allergic response than from IE if the prophylaxis were not administered (Bor et al., 1984; Idsoe et al., 1968). According to a statistical model by Agha et al. (2005), 19 cases of IE would be prevented with a net loss of 181 lives due to an anaphylaxis response per 10 million patients receiving an amoxicillin or ampicillin antibiotic prophylaxis. Overall, the incidence of a penicillin allergy ranges from 1-10% whereby an anaphylactic allergic response occurs in approximately 0.02% of patients receiving some type of penicillin (Montgomery, 1998). Approximately 300 fatal penicillin allergic reactions occur annually in the U.S. (Montgomery, 1998). Amoxicillin specifically may produce a skin rash in approximately 9% of patients (Montgomery, 1998). The decreased use of short-term antibiotic prophylaxis could also improve the cost-effectiveness of antibiotic prophylaxis (Agha et al., 2005).

The primary purpose of this study is to determine the effect of a pre-procedure rinse of 0.12% chlorhexidine on the incidence and magnitude of bacteremia compared to the AHA and the ADA/AAOS recommended antibiotic prophylaxis guideline of 2g amoxicillin during third molar extractions. A secondary purpose is to provide additional data on the incidence and magnitude of bacteremia during dental treatment procedures with or without an antimicrobial intervention.

The results of this study would primarily affect those patients with cardiac anomalies, immunosuppression, prosthetic joints, and various medical complexities who currently require antibiotic prophylaxis prior to dental treatment procedures to prevent IE, LPJI, or other DSI. In the military setting, the results of this study would have an impact primarily on the retiree and/or dependent population where issues of antibiotic resistance, antibiotic anaphylactic allergic responses, and the cost-effectiveness of antibiotic prophylaxis could be addressed. The military retiree and dependant population is growing as the life expectancy of U.S. citizens is increasing. The military retiree and dependant population is living longer with more chronic, systemic diseases. Improvements in oral hygiene and dental treatment have enabled the same population to retain more of their dentition. The relationship between systemic diseases and oral health is recognized, as in diabetes mellitus and the role of periodontal infection on systemic vasculature inflammation and cardiovascular disease. Thus, the evidence-based knowledge acquired from this study could be applied to the ever-increasing military retiree and dependant population to address antibiotic resistance, antibiotic anaphylactic allergic responses, and cost-effectiveness.

OBJECTIVE

The primary purpose of this study is to determine the effect of a pre-procedure rinse of 0.12% chlorhexidine on the incidence and magnitude of bacteremia compared to the AHA recommended antibiotic prophylaxis guideline of 2g amoxicillin during third molar extractions. A secondary purpose is to provide additional data on the incidence and magnitude of bacteremia during dental treatment procedures with or without an antimicrobial intervention.

HYPOTHESIS

Null hypothesis: There is no significant difference in the incidence and magnitude of bacteremia between the use of 0.12% chlorhexidine pre-procedure rinse and 2g amoxicillin antibiotic prophylaxis during third molar extractions. Research Question: Does the pre-procedure rinse of 0.12% chlorhexidine reduce the incidence and magnitude of bacteremia compared to 2g amoxicillin antibiotic prophylaxis during third molar extractions?

MATERIALS AND METHODS

This research study was approved by the WHMC Institutional Review Board (IRB), Federal Wide Assurance (FWA) #00001750, 59th Medical Wing Clinical Research Division - Protocol office, Lackland AFB, TX and the SGE-C office/Office of the Surgeon General, Washington, DC as a minimal risk study (FWH20110027H). Initial IRB approval was obtained January 2011, but subsequent SGE-C office review delayed the final approval until April 2011. The research protocol, informed consent document, HIPAA consent, and other required documentation were submitted according to the guidelines established by the IRB. Throughout the study, five amendments to the protocol and/or informed consent document were submitted to and approved by the IRB. An IRB progress review was completed in October 2011 with no discrepancies. An IRB audit was completed in January 2012 with no discrepancies.

Validation Procedure

The Wampole™ ISOSTAT®/ISOLATOR™ Microbial System (Inverness Medical, Princeton, NJ) is a lysis centrifugation method for quantitative blood cultures. This microbial system was used in the study to determine the incidence and magnitude of the bacteremia in CFU/mL. The microbial system was validated by inoculating 10 mL volumes of freshly drawn human blood into Isolator™ 10 tubes with known concentrations of bacteria, processing the samples, and determining the CFU/mL

recovered from the Isolator™10 tube (Attachment 1). The actual recovery was compared to the expected recovery using a paired *t* test.

Six different bacterial species were included in the validation: *Staphylococcus epidermidis* ATCC 12228; *Streptococcus anginosus* ATCC 10713; *Veillonella parvula* ATCC 10790; *Parvimonas micra* ATCC 33270; *Fusobacterium nucleatum* ATCC 25586; and *Actinomyces odontolyticus* ATCC 17929. *S. epidermidis* was chosen for the preliminary validation because of its low pathogenicity and ease of cultivation. The other organisms were chosen because of their possible association with periodontal disease. The organisms were obtained in lyophilized form from MicroBiologics® (St. Cloud, MN).

The validation of the Wampole™ ISOSTAT®/ISOLATOR™ Microbial System was completed as follows:

1. The organisms were subcultured twice to trypticase soy agar with 5% sheep blood (TSA II) (BBL™, BD, Sparks, MD). To achieve a bacterial suspension for inoculation of the blood in the Isolator™ 10 tubes, the bacterial growth was harvested from TSA II plates with a sterile, cotton tipped swab and suspended in sterile saline to a turbidity equal to a 0.5 McFarland turbidity standard (approximately 1.5 x 10^8 CFU/mL).

2. The bacterial suspensions were serially diluted with sterile saline to lower concentrations. A 100µL or 200µL aliquot of the diluted bacterial suspension (inoculum) was seeded to the 10mL of blood in the Isolator™ 10 tubes using a sterile 1 mL syringe (1mL Luer-Lok Syringe, BD, Franklin Lakes, NJ) and a sterile needle (Precision Glide™ Needle 26g x 0.5in, BD, Franklin Lakes, NJ). The stopper of the Isolator™ 10 tube was cleaned with a 70% isopropyl alcohol prep (Kendall Webcoll Alcohol Preps, Covidien, Mansfield, MA).
   Note: Different dilution schemes were used to achieve a variety of inoculum concentrations in the Isolator™10 tubes.
3. To determine the CFU of the inoculum seeded into the blood (expected recovery), aliquots (n=3) of the diluted bacterial suspensions equal to the volume of the inoculum (100 or 200 µL) were transferred to three TSA II plates. The suspension was spread evenly over the surface of the agar to achieve growth of isolated colonies that could be accurately counted. For expected counts >300 CFU additional dilutions were made and plated to achieve countable isolated colonies on the TSA II plates. After incubation (*S. epidermidis* and *S. anginosus* were incubated for 18-24 hours at 35±2°C in ambient air, *V. parvula, P. micra, F. nucleatum*, and *A. odontolyticus* were incubated for 48-72 hours at 35±2°C in anaerobic environment), the CFU on the three plates were counted and the mean was calculated. For this validation, the CFU of the inoculum (expected recovery) ranged from 1 to 600 CFU.

4. After inoculation of the blood, the Isolator™ 10 tubes were gently inverted 4-5 times then centrifuged for 35 minutes at 2700 x G in a fixed angle (30°) centrifuge (Spectrafuge 6C, Labnet International, Woodbridge, NJ) to concentrate the microorganisms. The centrifuge was allowed to stop on its own with no braking as to not disturb the bacterial concentrate.
5. After centrifugation, the Isolator™ 10 tubes were placed in the Isostat® rack located in a biological safety cabinet. Note: Steps 5- 9 were performed in the biological safety cabinet. The Isolator™ 10 tubes were handled carefully throughout as to not disturb the bacterial concentrate.
6. The tube stoppers were again cleaned with a 70% isopropyl alcohol prep. An Isostat® cap was removed from the sterile package and placed over the stopper of each Isolator™ 10 tube. The Isolator™ 10 tube with cap was placed in the Isostat® press. The hand-operated handle of the press was gently pulled down to simultaneously seat the cap and penetrate the tube stopper with a spike allowing access to the contents of the Isolator™ 10 tube.
7. For each Isolator™ 10 tube, an Isostat® supernatant pipet was used to remove and discard 9mL of the supernatant. The remaining 1mL of supernatant and pellet/bacterial concentrate was vortex mixed for a minimum of 10 seconds to disperse the pellet and obtain a homogeneous mixture of bacterial concentrate.
8. The bacterial concentrate was removed with an Isostat® concentrate pipet and distributed equally on 4 TSA II plates. The bacterial concentrate was dispensed in a straight line along the surface of the agar. To streak the

plates, the tip of the concentrate pipet was used to make about 15 - 20 passes perpendicular through the bacterial concentrate line.

9. TSA II plates with *S. epidermidis* and *S. anginosus* were incubated for 18-24 hours at 35±2°C in ambient air. TSA II plates with *V. parvula, P. micra, F. nucleatum*, and *A. odontolyticus* were incubated for 48-72 hours at 35±2°C in anaerobic environment. Plates were examined for growth after 24-72 hours for aerobes and 48-96 hours for anaerobes.
10. After incubation, the total number of CFU on all four plates was determined (actual recovery from the Isolator™ 10 tubes). The actual number of CFU recovered from the Isostat™10 tube was compared to the expected number of CFU previously determined.
11. A paired *t* test was performed to compare the mean of the CFU in the inoculum (expected recovery) to the CFU recovered using the Wampole™ ISOSTAT®/ISOLATOR™ Microbial System (actual recovery).

The paired *t* test showed a significant difference between the CFU in the inoculum and the CFU recovered from the Isolator™ 10 tube concentrate when data for all organisms was combined (p=0.0076). However, when the data was analyzed for individual organisms the only groups considered significantly different were the *F. nucleatum* groups (p=0.0230) with the actual recovery from the Isolator™ 10 tube being lower than the expected recovery in each instance. When the *F. nucleatum* data was removed from the analysis the overall difference was not significantly

different (p=0.0919). This indicates that overall the Wampole™ ISOSTAT®/ISOLATOR™ Microbial System is acceptable for performing quantitative blood cultures. However, this microbial system does not perform equally well with all organisms. It is not clear why this microbial system does not work as well with *F. nucleatum* as with the other organisms - it may be due to the colonial morphology of *F. nucleatum*. Of all the organisms tested, *F. nucleatum* is the only one with a characteristic chunky morphology making it difficult to achieve a smooth suspension in saline. Perhaps it is this characteristic that contributed to the variability seen between the expected recovery CFU and the actual recovery CFU from the Isolator™ 10ml tubes. The analysis performed well at low concentrations with a sensitivity of 1 CFU/tube or 0.1CFU/mL.

Clinical Procedure

The sample population (subjects) were patients that presented to the Wilford Hall Ambulatory Surgical Center (WHASC), Oral Surgery (OMS) Clinic, Lackland AFB, TX for third molar extractions under conscious sedation. The subjects were a convenience or presenting sample over the time of the study. The potential subjects were screened at their pre-operative (pre-op) anesthetic assessment/evaluation according to the inclusion/exclusion:

- Inclusion criteria:
  - Healthy, no systemic disease with an ASA I or II
  - Diagnosed and planned extraction #1, 16, 17, and 32 under conscious sedation
  - #17 and 32 require a mucogingival flap for extraction
  - 18 years of age or older
  - Previously received penicillin and/or amoxicillin without a hypersensitivity or allergic reaction

- Exclusion criteria:
  - Poorly controlled systemic disease with an ASA III or IV
  - Known penicillin, amoxicillin, or cephalosporin drug allergy
  - Pregnant women
  - Current immunosuppressed status
  - Active viral disease
  - Cardiac anomalies or another condition or situation requiring pre- or intra-operative use of antibiotics
  - Antibiotic use within the previous two months
  - Steroid therapy within the previous two months
  - Chlorhexidine use or other oral antimicrobial rinses within the previous two months
  - The routine use of an oral antiseptic at home
  - Gingival tissue manipulation within two hours of the procedure

Subjects that met the inclusion/exclusion criteria were asked for their voluntary participation in the study. An informed consent document and HIPAA consent were completed for each subject at their pre-op anesthetic assessment/evaluation

following an explanation of the study and answering questions in regards to the study.

The research subjects were randomized via a computer-generated model. The randomized subject log was kept confidential in an Excel® format accessed only by the primary and/or associate investigator. The subjects were assigned a study number according to the randomized subject log when they presented to their pre-op anesthetic assessment/evaluation. The Excel® format document contained the subject name, last four of the SSN, and their computer-generated randomized study number which was the only location identifying the subject with their study number. The subject was de-identified thereafter using only their study number throughout the procedure and analysis. The subjects were assigned to one of three groups based on the randomized study number:

- A control/placebo group (receiving a placebo rinse and a placebo capsule), Study number 1-10. (PLAC)
- A rinse group (receiving a 0.12% chlorhexidine rinse and a placebo capsule), Study number 11-20. (CHX)
- An antibiotic group (receiving 2g amoxicillin capsule and a placebo rinse), Study number 21-30. (AMOX)

The blood draw supplies and subject blood draw form (Attachment 2) were pre-packaged in one gallon Ziploc® bags.

Figure 1: Blood draw supplies packaged for clinical use per subject

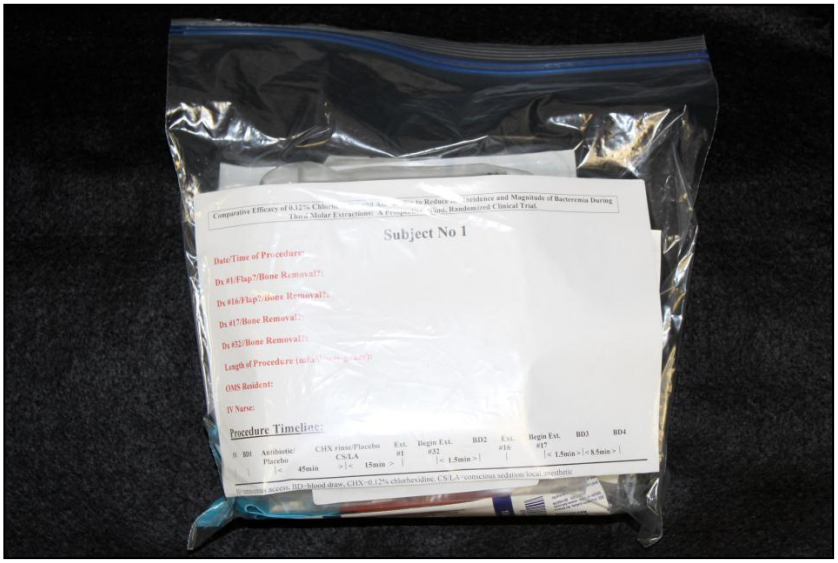
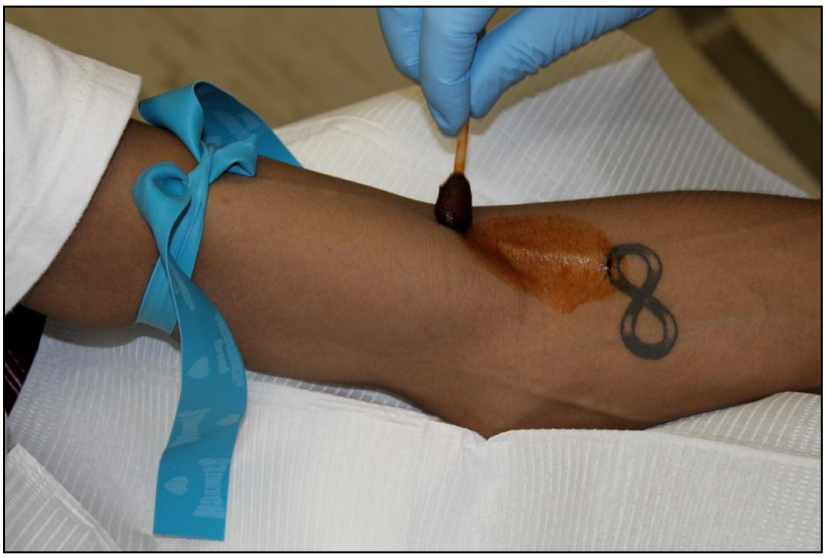


Figure 2: Isolator® 10 tubes labeled for clinical use per subject

The subjects presented to the WHASC OMS clinic approximately one hour and 15 minutes prior to the scheduled appointment. The blood draw IV access line for each subject was obtained in the following manner:

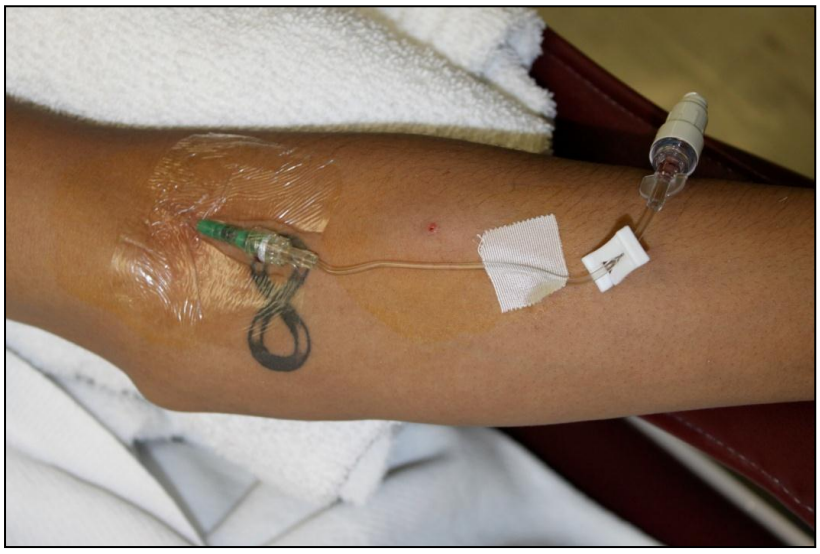
1. A one minute circular scrub in the usual manner of the IV access site with 10% povidone-iodine (Povidone-Iodine Swabstick (1's), PDI®, Inc., Orangeburg, NY).

Figure 3: Preparation of IV access site with 10% povidone-iodine



2. IV access was obtained with an 18g angiocatheter (Acuvance® Plus Safety IV Catheter 18g x 1.25, Smiths Medical ASD, Inc-Jelco, Dublin, OH) and secured by Tegaderm™ film (Tegaderm™ Film 6cm x 7cm, 3M® HealthCare, St. Paul, MN).

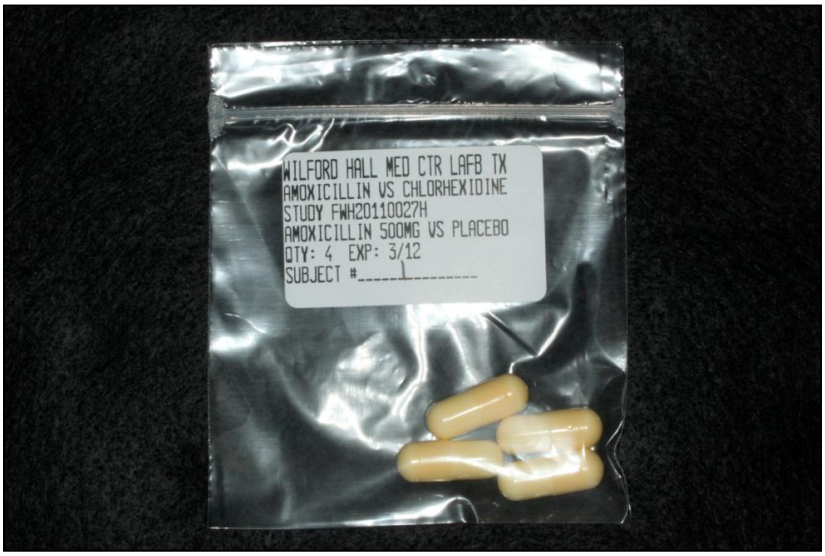
Figure 4: IV access line



3. A Maximus® 18cm minibore extension set with removable MaxPlus® Tru-Swab® connector (Medegren, Inc, Ontario, CA) was attached to the 18g angiocatheter (IV access line).

Once the IV access line was established, the first blood draw (BD) was completed as a baseline. The placebo or amoxicillin capsules were administered with a small amount of water one hour prior to the procedure. The placebo and amoxicillin capsules were packaged by and obtained from the 59th Pharmacy Squadron, Pharmacy Clinical Flight Office, Lackland AFB, TX in individually packaged plastic bags labeled with the subject number. The placebo and amoxicillin capsules were packaged as four 500mg capsules.

Figure 5: Subject placebo or amoxicillin capsules



A placebo rinse or 0.12% chlorhexidine (CHX) rinse (PerioGuard® Oral Rinse, Colgate® Oral Pharmaceuticals, New York, NY) was administered immediately prior to conscious sedation medication administration. The subject rinsed with 15mL of the placebo or CHX rinse for one minute and expectorated. The placebo and CHX

rinses were given to the subject in 30mL medicine cups (Medline Industries, Inc., Mundelein, IL). The placebo rinse was made using sterile water (1000mL Sterile Water for Irrigation, USP, Baxter Healthcare, Deerfield, IL) where blue dye (Target® aztec blue food color, Target® Brands, Inc., Minneapolis, MN) and mint extract (McCormick® pure mint extract, McCormick® and Co, Inc., Hunt Valley, MD) was added until a similar appearance, taste, and smell was obtained compared to the CHX rinse.

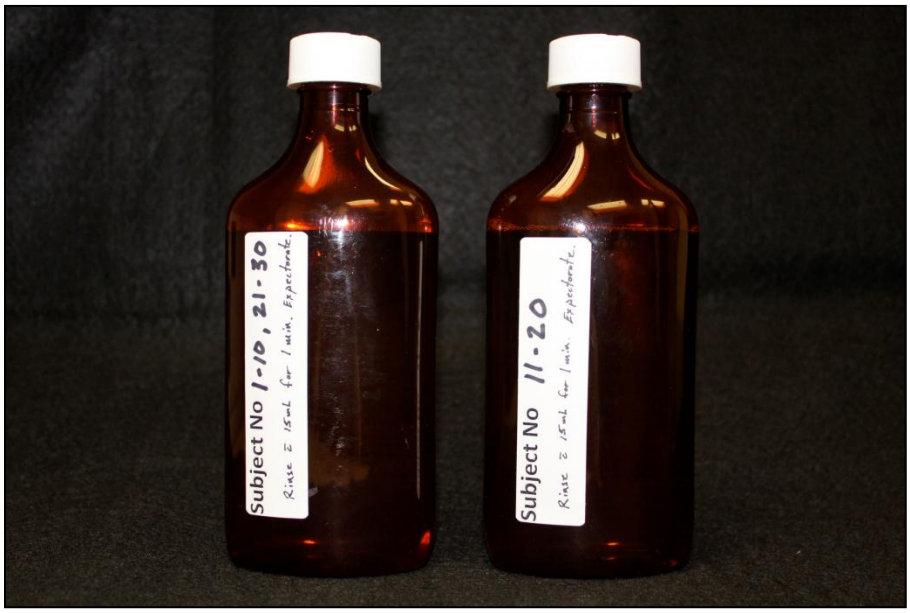
Figure 6: Ingredients for placebo rinse

Figure 7: Placebo and 0.12% CHX rinse

Figure 8: Comparison of placebo and 0.12% CHX rinse, respectively

The placebo and CHX rinses were then placed in amber-colored, light resistant bottles obtained from the 59th Pharmacy Squadron, Pharmacy Clinical Flight Office and labeled according to the study number.

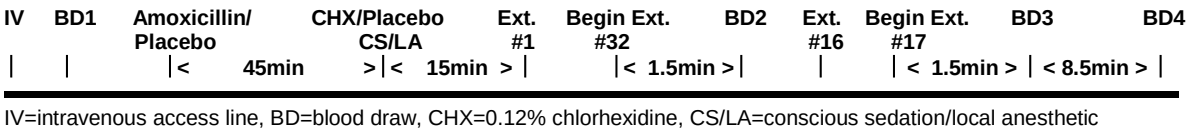
Figure 9: Subject placebo and 0.12% CHX rinses



After the blood draw IV access line was obtained, BD 1 was collected, and the placebo or 2g amoxicillin capsules were administered, a second IV access line for the conscious sedation medications was obtained in the opposite arm in a similar manner. To note, the conscious sedation medication IV access line was a standard procedure whereas the blood draw IV access line was not a standard procedure and was for research purposes only to collect the blood samples. Conscious sedation medication and local anesthetic was administered by an OMS staff, an OMS resident, or an AEGD-2 resident. Females of child-bearing potential were screened for a negative pregnancy status via a HCG lab test prior to conscious sedation medication administration. The procedure of third molar extractions was completed in the order of #1, 32, 16, and 17 by an OMS staff, an OMS resident, or an AEGD-2 resident. The second blood draw was completed 1.5 minutes following initiation of

the mucogingival flap #32. The third blood draw was completed 1.5 minutes following initiation of the mucogingival flap #17. The fourth blood draw was completed 10 minutes following initiation of the mucogingival flap #17. The four blood samples per subject were transported by a microbiology lab technician to the microbiology lab at the 59th Clinical Research Division (CRD), Lackland AFB, TX for immediate processing. All blood samples were processed within four hours of the blood draw. Refer to Figure 10 for the research protocol timeline.

Figure 10: Research protocol timeline



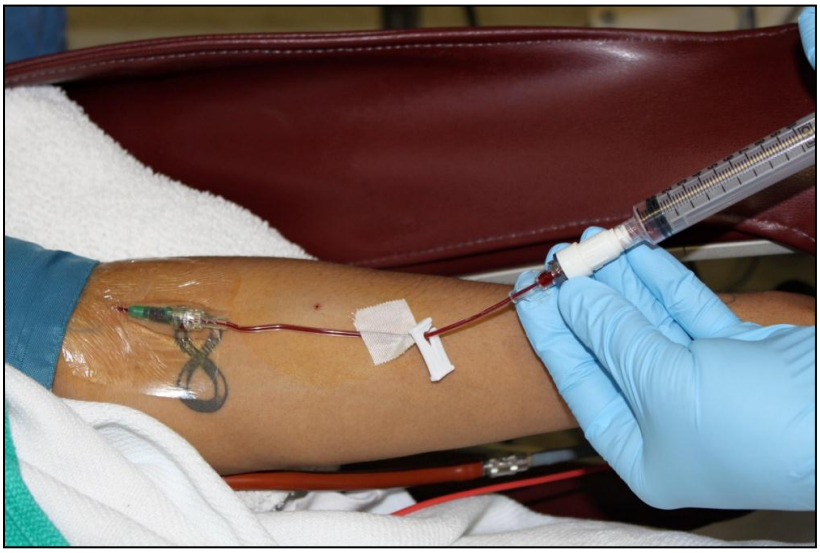
IV=intravenous access line, BD=blood draw, CHX=0.12% chlorhexidine, CS/LA=conscious sedation/local anesthetic

The four blood draws per subject were completed in the following manner:

1. Blood draw #1 was completed immediately after establishing the IV access line. The MaxPlus® Tru-Swab® connector was disinfected with a 70% isopropyl alcohol prep. Note: Steps 1-7 were completed for blood draw #1.
2. 15mL of blood was drawn from the IV access line with a sterile 20mL syringe (20mL Sterile Syringe Luer-Lok™ Tip, BD, Franklin Lakes, NJ).
3. A sterile needle (Precision Glide™ Needle 18g x 1.5in, BD, Franklin Lakes, NJ) was attached to the 20mL syringe of blood to inject the blood into the Isolator® 10 tube.

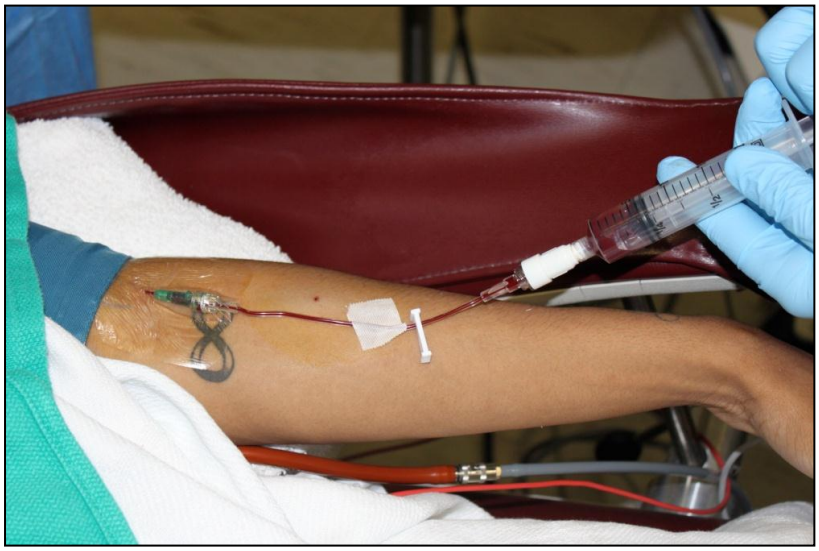
4. The stopper of the Isolator® 10 tube was disinfected with a 70% isopropyl alcohol prep.
5. 10mL of this blood was drawn into the Isolator® 10 tube discarding 4-5mL of blood. The Isolator® 10 tube was gently inverted 4-5 times. Note: The vacuum of the Isolator® 10 tube drew 10mL of blood automatically. No forceful injection was necessary.
6. The MaxPlus® Tru-Swab® connector was disinfected with a 70% isopropyl alcohol prep.
7. The IV access line was irrigated/flushed with 10mL sterile saline (10mL Sterile Saline Syringe Luer-Lok™ Tip, BD, Franklin Lakes, NJ). The IV access line was closed until blood draw #2.
8. The MaxPlus® Tru-Swab® connector was disinfected with a 70% isopropyl alcohol prep. Note: Steps 8-16 were completed for blood draws #2-4.
9. 10mL of blood and/or saline was drawn and discarded from the IV access line with a sterile 10mL syringe (10mL Sterile Syringe Luer-Lok™ Tip, BD, Franklin Lakes, NJ).

Figure 11: 10mL draw of blood and/or saline



10. The MaxPlus® Tru-Swab® connector was disinfected with a 70% isopropyl alcohol prep.
11. 15mL blood was drawn from the IV access line with a sterile 20mL syringe.

Figure 12: 15mL draw of blood sample for analysis

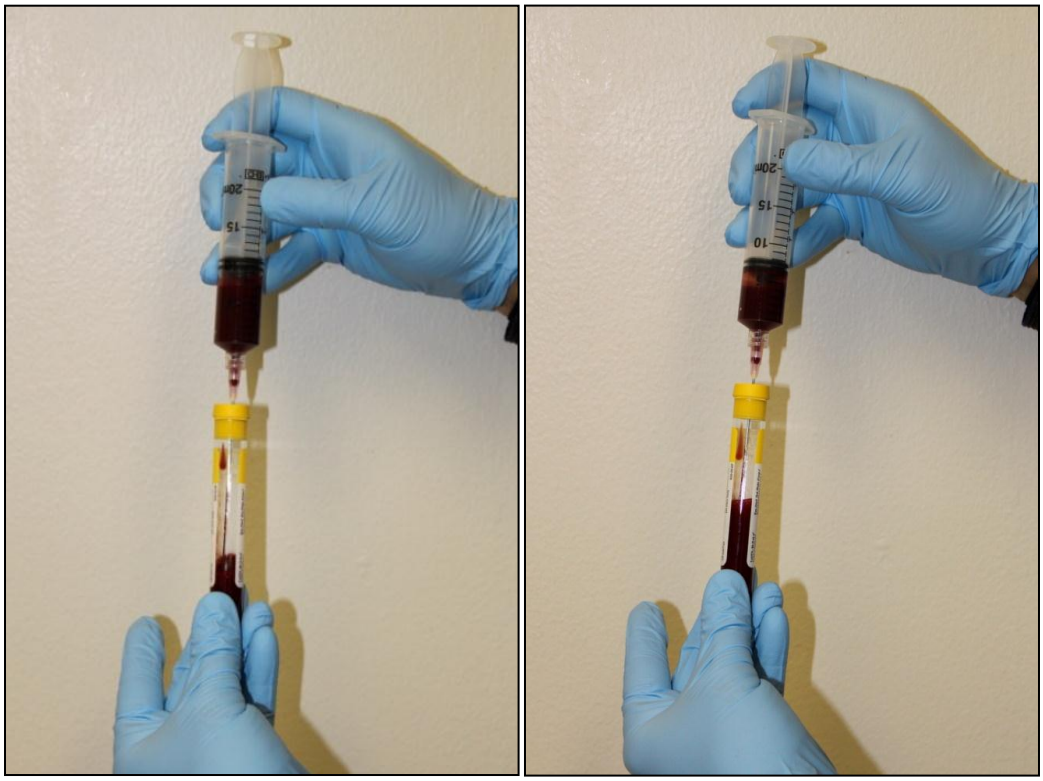


12. An 18g needle was attached to the 20mL syringe of blood to inject the blood into the Isolator® 10 tube.
13. The stopper of the Isolator® 10 tube was disinfected with a 70% isopropyl alcohol prep.

Figure 13: Disinfection of Isolator® 10 tube rubber stopper

14. 10mL of this blood was drawn into the Isolator® 10mL tube discarding 4-5mL of blood. The Isolator® 10 tube was gently inverted 4-5 times. Note: The vacuum of the Isolator® 10 tube drew 10mL of blood automatically. No forceful injection was necessary.

Figure 14: 10mL of blood sample drawn into Isolator® 10 tube



15. The MaxPlus® Tru-Swab® connector was disinfected with a 70% isopropyl alcohol prep.
16. The IV access line was irrigated/flushed with 10mL sterile saline. The IV line was closed until the next blood draw. Following blood draw #4, the IV access line was removed in the usual manner.

Figure 15: 10mL sterile saline irrigation/flush of IV access line

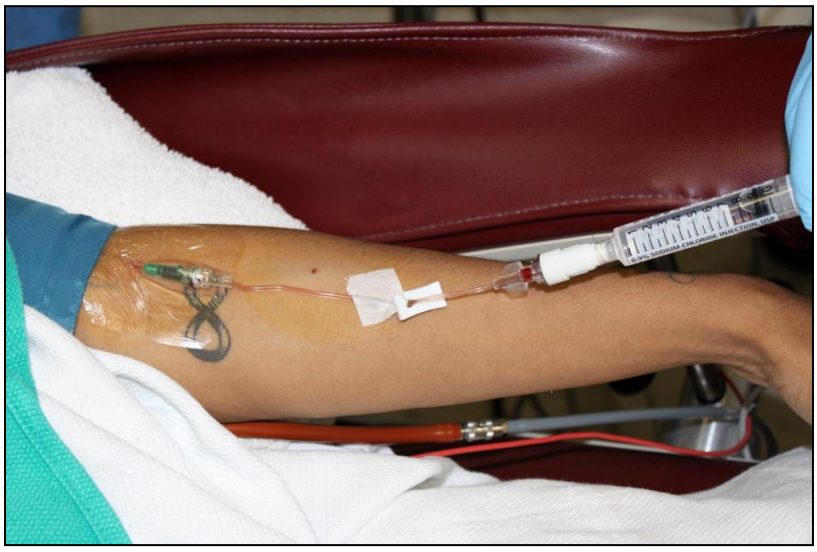


Figure 16: Four blood samples in Isolator® 10 tubes per subject

Microbiological Procedure

The 59th CRD microbiology lab processed the blood samples according to the validation procedure previously described with a few modifications:

1. The Isolator™ 10 tubes were gently inverted 4-5 times then centrifuged for 35 minutes at 2700 x G in a fixed angle (30°) centrifuge (Spectrafuge 6C, Labnet International, Woodbridge, NJ) to concentrate the microorganisms. The centrifuge was allowed to stop on its own with no braking as to not disturb the bacterial concentrate.

Figure 17: Centrifugation of Isolator™ 10 tubes

2. After centrifugation, the Isolator™ 10 tubes were placed in the Isostat® rack located in a biological safety cabinet. Note: Steps 2- 9 were performed in the

biological safety cabinet. The Isolator™ 10 tubes were handled carefully throughout as to not disturb the bacterial concentrate.

3. The tube stoppers were cleaned with a 70% isopropyl alcohol prep. An Isostat® cap was removed from the sterile package and placed over the stopper of each Isolator™ 10 tube. The Isolator™ 10 tube with cap was placed in the Isostat® press. The hand-operated handle of the press was gently pulled down to simultaneously seat the cap and penetrate the tube stopper with a spike allowing access to the contents of the Isolator™ 10 tube.

Figure 18: Biological safety cabinet

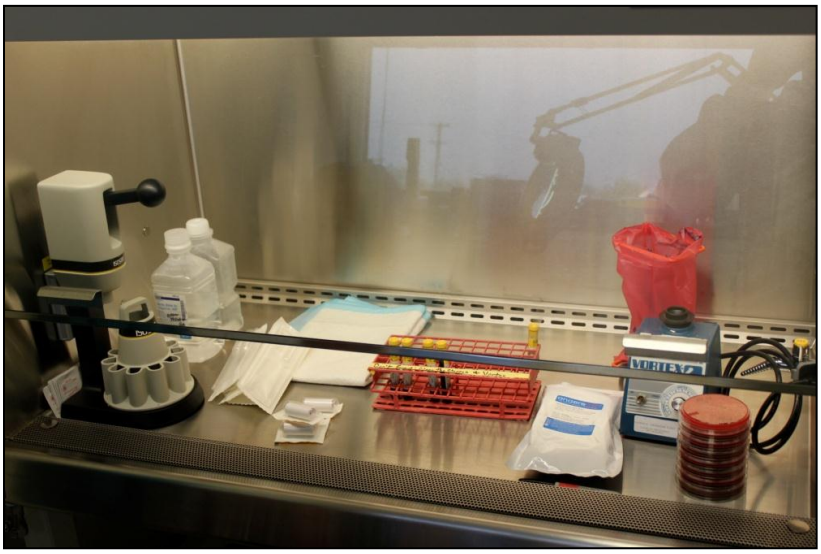
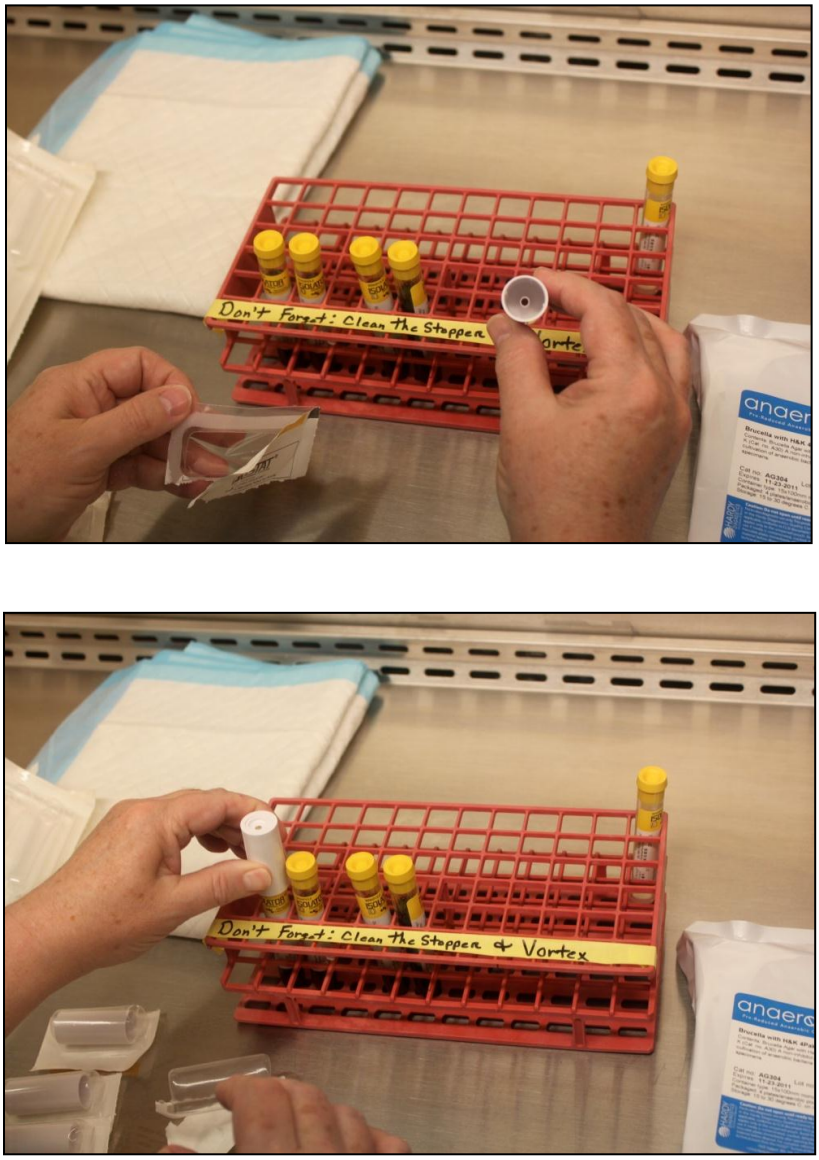
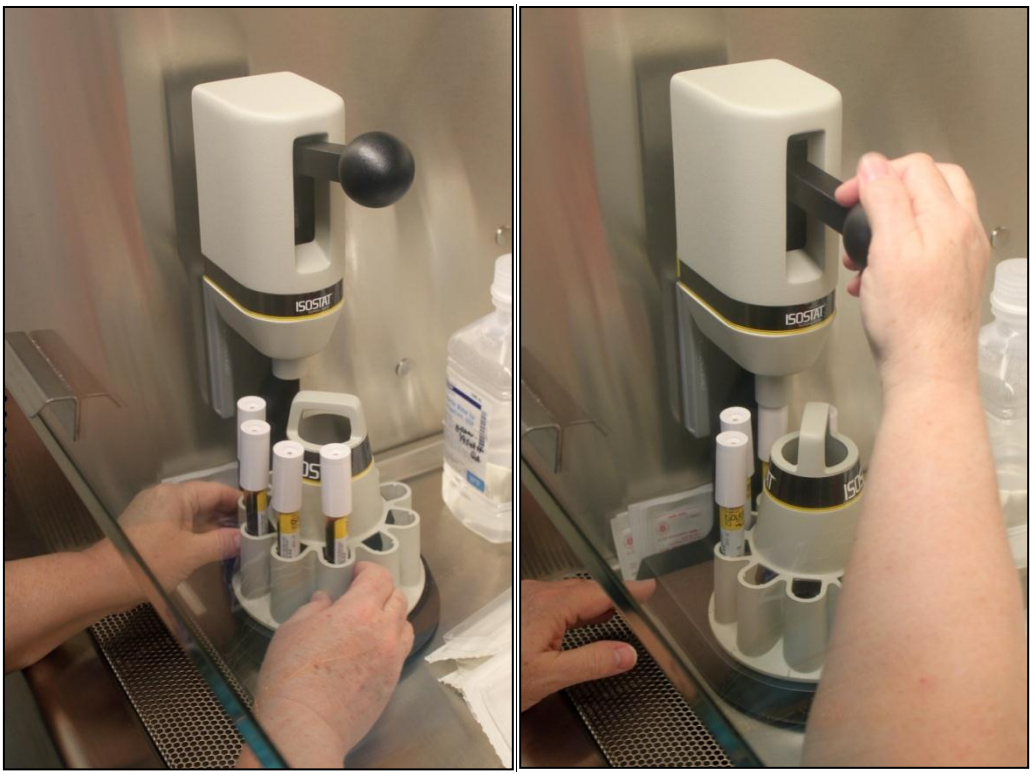


Figure 19: Placement of Isostat® cap on Isolator™ 10 tubes using Isostat® press

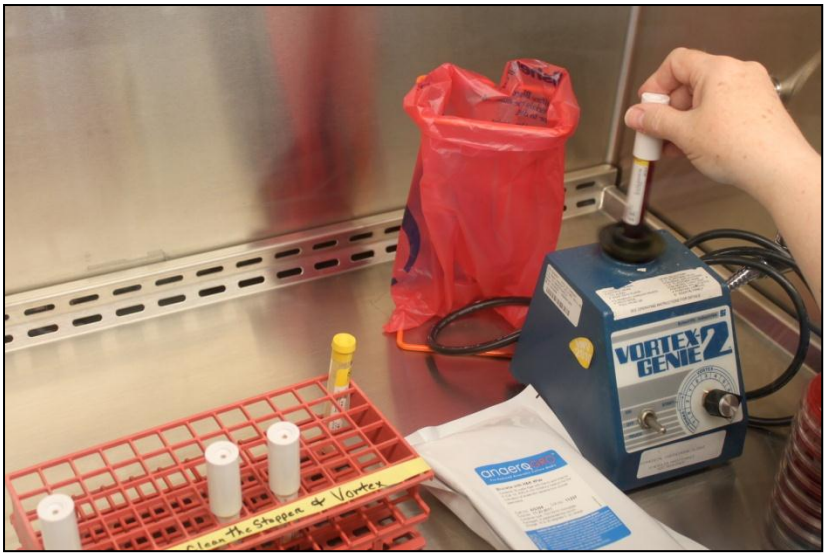




4. For each Isolator™ 10 tube, an Isostat® supernatant pipet was used to remove and discard 9mL of the supernatant. The remaining 1mL of supernatant and pellet/bacterial concentrate was vortex mixed for a minimum of 10 seconds to disperse the pellet and obtain a homogeneous mixture of bacterial concentrate.

Figure 20: 9mL supernatant removal and discard

Figure 21: 1mL remaining supernatant and pellet/bacterial concentrate vortex mix



5. The bacterial concentrate was removed with an Isostat® concentrate pipet and distributed equally on three agar plates: TSA II, chocolate agar (BBL™, BD, Sparks, MD), and Brucella blood agar with vitamin K and hemin (BHK) (Hardy Diagnostics, Santa Maria, CA). The bacterial concentrate was dispensed in a straight line along the surface of the agar. To streak the plates, the tip of the concentrate pipet was used to make about 15 - 20 passes perpendicular through the bacterial concentrate line.

Figure 22: 1mL bacterial concentrate removal

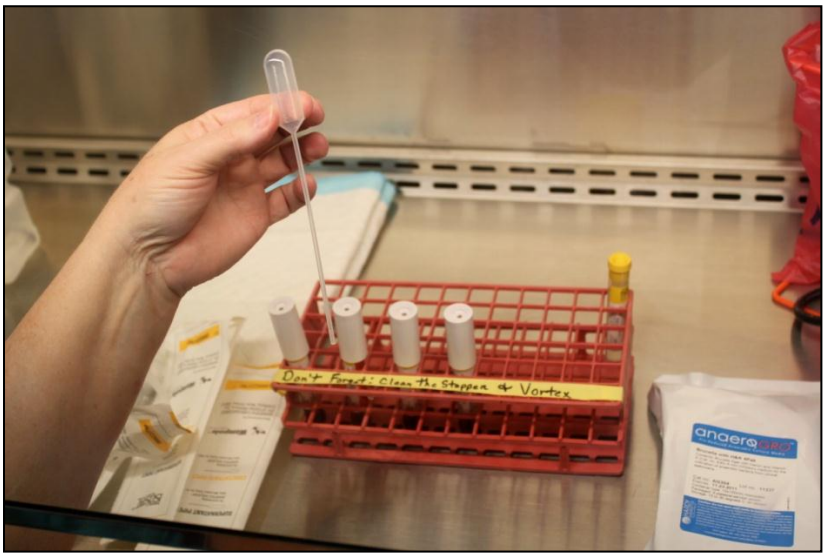
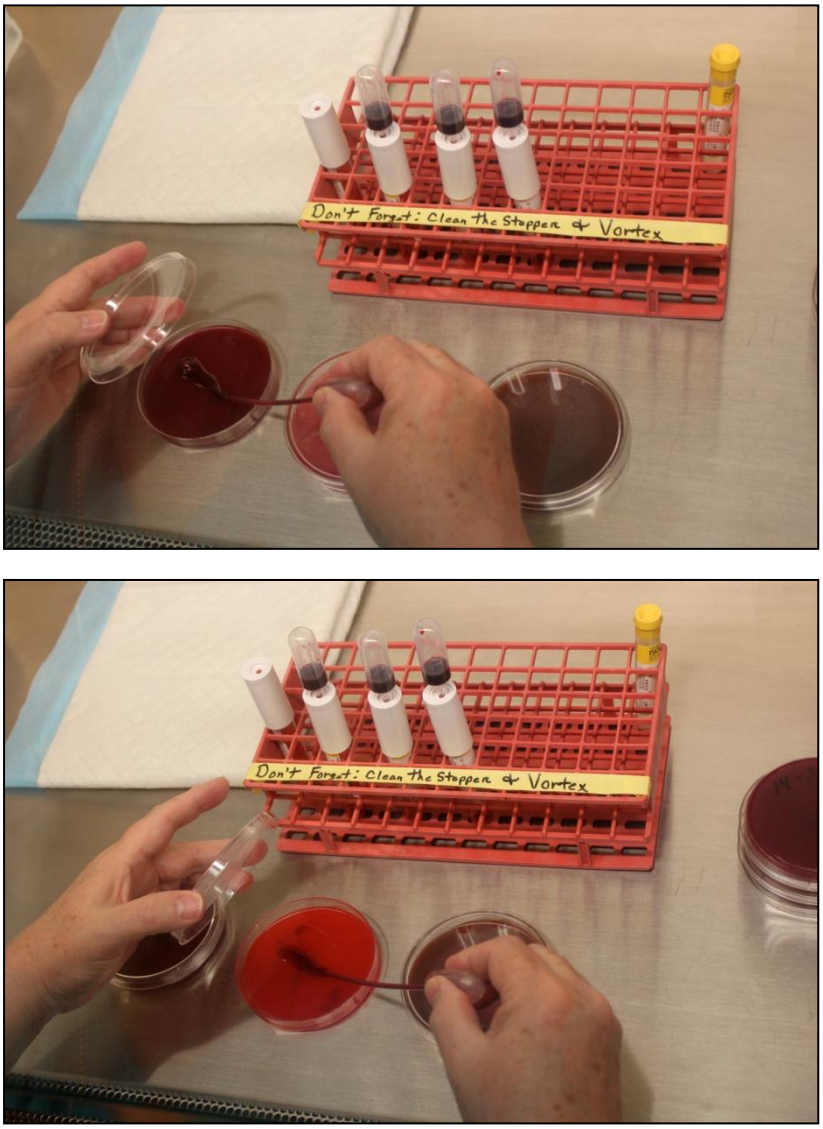
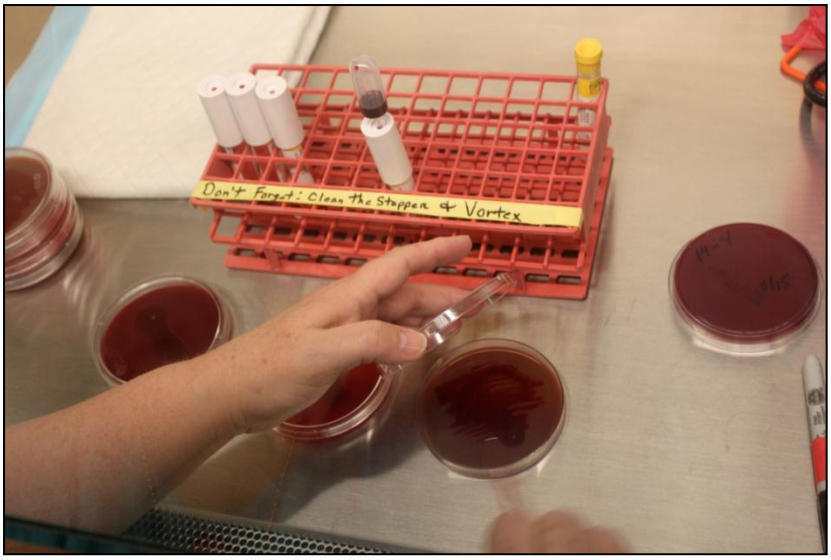


Figure 23: Plating and streaking of bacterial concentrate onto TSA II, chocolate, and BHK agar plates



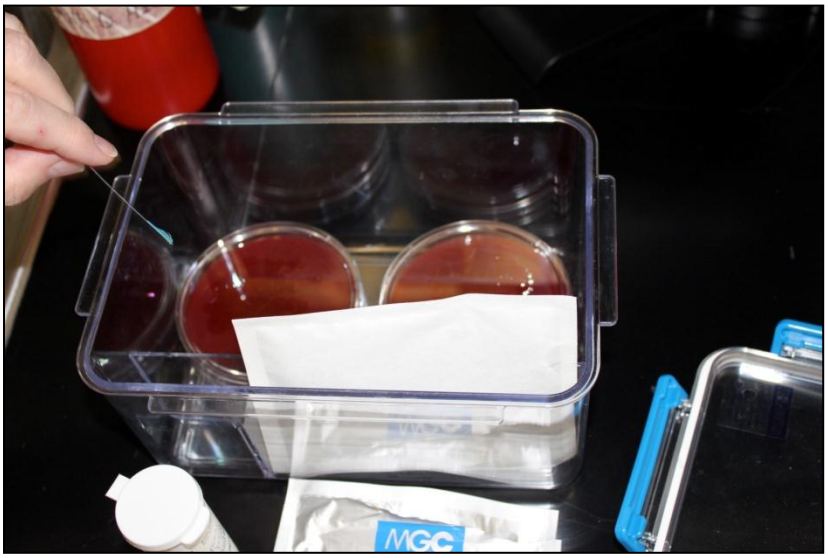


6. The TSA II and chocolate agar plates were incubated aerobically in 5-10% CO_2 at 35 ± 2°C. Plates were examined for growth after two days of incubation.

Figure 24: Aerobic incubation

7. The BHK plate was incubated anaerobically at 35 ± 2°C. Plates were examined for growth after four days of incubation.

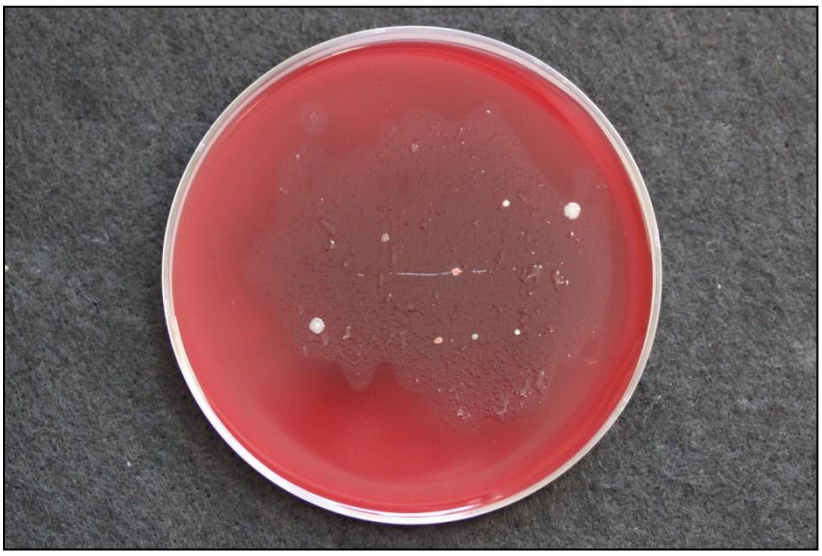
Figure 25: Anaerobic incubation



8. Colonies were counted and grouped by colonial morphology. Hemolytic reaction was recorded for colony types growing on TSA II plates. For each colony type the concentration/magnitude of the bacteria in the blood, stated as CFU/mL, was calculated using the following formula:

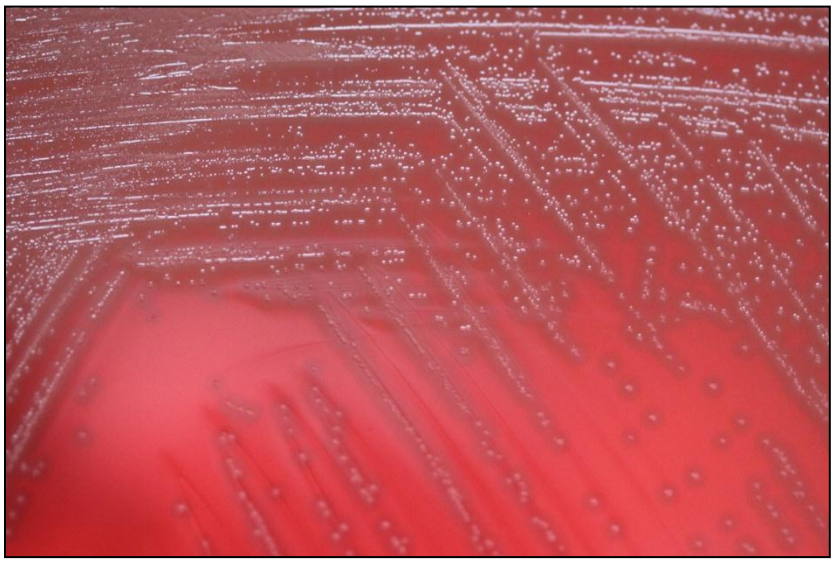
$$\text{CFU/mL} = \frac{\text{Total number of CFU all plates}}{\text{Number of plates on which the organism would be expected to grow}} \times \frac{\text{Number of plates inoculated}}{\text{Blood volume}}$$

Figure 26: Bacterial colony growth on TSA II, chocolate, and BHK agar plates



9. Following primary isolation, each colony type was subcultured to TSA II or BHK plates to obtain a pure culture and verify the required environmental growth conditions (aerobic or anaerobic). A Gram stain was performed on each pure culture and the isolate was transferred to trypticase soy broth with 20% glycerol and frozen at -70°C.

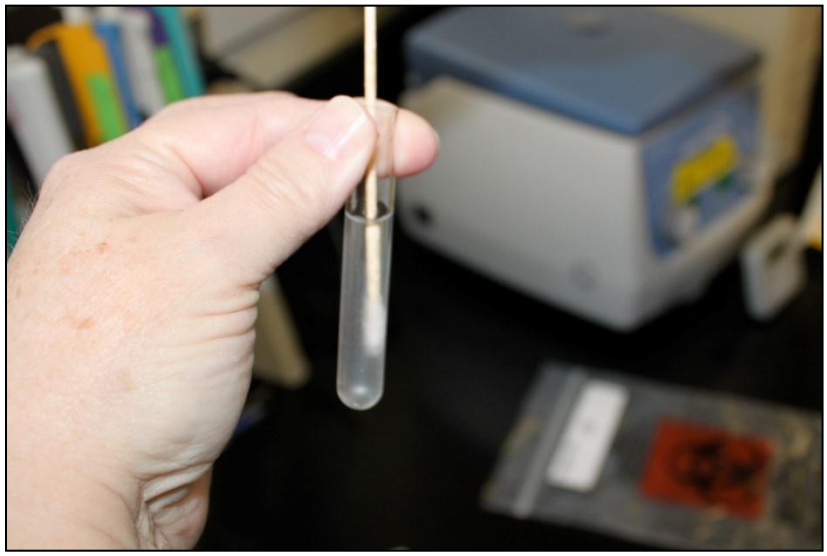
Figure 27: Hemolytic strep pure culture

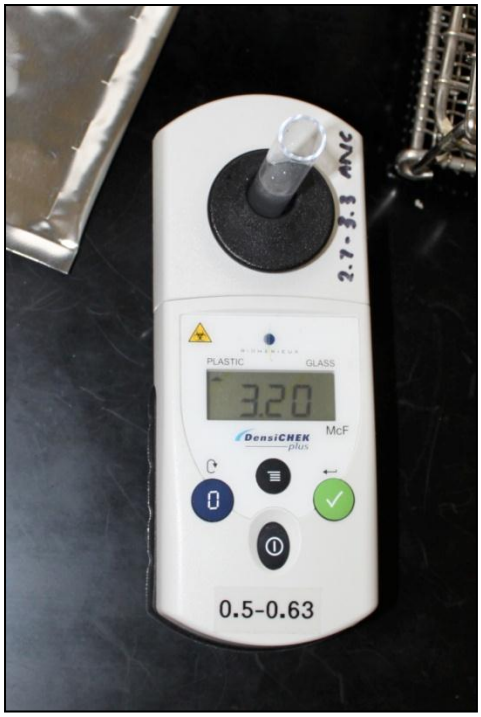


10. Identification of the bacterial isolates was attempted on the VITEK® 2 Compact bacterial identification system (bioMerieux, Inc, Durham, NC). The VITEK® 2 is an automated instrument for identification and susceptibility testing of aerobic Gram positive and Gram negative bacteria and identification testing of anaerobic Gram positive and Gram negative bacteria. The database includes approximately 350 clinically relevant organisms. The VITEK® 2 test cards are made up of 30 to 47 microwells containing identification substrates. The cards are inoculated with a bacterial suspension and placed in the incubator/reader module. Growth and activity within each test well is monitored by optical reading of the cards. Reading is performed once every 15 minutes with a multichannel fluorometer and photometer to record fluorescence, turbidity, and colorimetric signals. Information is sent to a workstation equipped with software to manage test

results, determine isolate identification, and offer reliable result validation via the Advanced Expert™ System.

Figure 28: Preparation and identification of bacterial pure culture using VITEK® 2 Compact




2.7-3.3 ANC
PLASTIC
GLASS
3.20
McF
DensiCHEK plus
0.5-0.63


ANC
24421131010854813 3
2

11. Bacterial isolates not identified immediately by the VITEK® 2 were stored at -70°C until identification on the Biolog™ Microstation System™ (BioLOG, Hayward, CA) was completed. The MicroStation System™ is another instrument for identifying and characterizing microorganisms with a database of 526 Gram negative aerobes, 339 Gram positive aerobes, and 361 anaerobes. The Microstation System™ technology uses each microorganism's ability to use particular carbon sources or chemical sensitivity assays to produce a unique pattern or "phenotypic fingerprint" for that microorganism. A microorganism respires as it begins to use the carbon sources in the wells of the MicroPlate™. Among bacteria, this respiration process reduces a tetrazolium redox dye where the wells change to a purple color. The end result is a pattern of colored wells on the MicroPlate™ that is characteristic for that microorganism. The pattern is read by a fiber optic reading instrument termed the MicroStation Reader. The fingerprint data was fed into the software to search the database for a species identification.

STATISTICAL ANALYSIS

Calculation of the required sample size was determined using a power of 80% to determine an effect size of 0.6 or approximately 1.2 standard deviations difference among the means. Based on this calculation, it was determined 10 subjects per group was sufficient to detect a statistically significant difference in the magnitude of bacteremia in CFU/mL for three groups and four measures.

RESULTS

Over the course of six months, 37 subjects completed an ICD and HIPAA document to participate in the study. However, the blood draws on seven subjects were not completed due to technical or logistical issues: an IV line sufficient for blood draws was not established in three subjects; only two of the four blood draws could be completed due to a collapsed/negative pressure of the vein in three subjects; and no microbiology lab support in one subject.

Demographic Data

The study population consisted of 30 subjects total with 10 per PLAC, CHX, or AMOX groups. The mean subject age was 21.8 years with a range of 18-29 years. A one-way ANOVA found no significant difference in the mean age among the three groups ($p=0.473$). There were 23 male and 7 female subjects. A χ^2 test found no difference in gender among the three groups ($p=0.475$). The mean surgical procedure length was 42.0 minutes with a range of 11-78 minutes. A one-way ANOVA found no significant difference in the mean procedure length among the three groups ($p=0.632$) (Table 1 - Appendix).

Descriptive Data

There was no statistically significant difference in the subjects' third molar extraction diagnosis, number of surgical flaps, or number of bone removal procedures among the PLAC, CHX, or AMOX groups according to χ^2 tests (Tables 2-5 - Appendix). However, for tooth #32, the PLAC group appeared to have a larger number of

subjects (9/10) requiring bone removal compared the CHX group (5/10) and the AMOX group (6/10).

There was no statistically significant difference in the surgeon performing the surgical procedure (p=0.234) and the investigator initiating the IV line (p=0.642) among the PLAC, CHX, or AMOX groups according to χ^2 tests.

Incidence of Bacteremia

There was no statistically significant difference in the incidence of bacteremia among the three groups. The incidence of bacteremia is defined as at least one positive culture of the four blood draws per subject and reported as a percentage. The incidence of bacteremia was analyzed using a χ^2 test with a statistical significance/α of 0.05. The PLAC group showed a 50% incidence of bacteremia, the CHX group a 60% incidence, and the AMOX group a 40% incidence (p=0.670) (Table 6 - Appendix).

Magnitude of Bacteremia

There was no statistically significant difference in the magnitude of bacteremia among the PLAC, CHX, or AMOX groups. The magnitude of bacteremia is defined as CFU/mL. The data was not normally distributed and was therefore analyzed using the non-parametric Kruskall-Wallis test and the Friedman test. A Bonferroni correction was applied because multiple comparisons were done between groups with a statistical significance/α of 0.017. The mean total bacteremia includes all four

blood draws. The PLAC group showed a mean total bacteremia of 3.61 CFU/mL (stdev=7.09), the CHX group 2.76 CFU/mL (stdev=4.28), and the AMOX group 0.63 CFU/mL (stdev=1.33). The total bacteremia ranged from 0.0-18.20 CFU/mL in the PLAC group, 0.0-11.10 CFU/mL in the CHX group, and 0.0-4.30 CFU/mL in the AMOX group (Table 7 - Appendix).

The Kruskal-Wallis test found no significant difference between the PLAC, CHX, or AMOX groups for a particular blood draw. For blood draw (BD) 1, the mean bacteremia was 0.03 CFU/mL (stdev=0.11, p=0.595), for BD 2 was 0.49 CFU/mL (stdev=2.13, p=0.172), for BD 3 was 1.52 CFU/mL (stdev=3.89, p=0.463), and for BD 4 was 0.29 CFU/mL (stdev=0.60, p=0.499) (Table 8 - Appendix). The Friedman test found no significant difference between the four blood draws within the PLAC, CHX, or AMOX groups. In the PLAC group, the mean bacteremia was 0.00, 1.26, 1.90, and 0.45 CFU/mL for BD 1-4 (stdev=0.00, 3.67, 5.36, 0.83, p=0.031), respectively. In the CHX group, the mean bacteremia was 0.04, 0.18, 2.37, and 0.17 CFU/mL for BD 1-4 (stdev=0.13, 0.29, 4.11, 0.24, p=0.062), respectively. In the AMOX group, the mean bacteremia was 0.05, 0.02, 0.30, and 0.26 CFU/mL for BD 1-4 (stdev=0.16, 0.06, 0.73, 0.60, p=0.310), respectively (Table 8 - Appendix).

Nature of Bacteremia/Bacterial Species Identification

A total of 33 different bacterial species were isolated among the PLAC, CHX, and AMOX groups (Tables 9-11 - Appendix). Of these 33 bacterial species, two bacterial species were not identified but were categorized as gram positive rods – one bacterial isolate was an aerobe and the other was an anaerobe. Also, a

fusobacterium isolate was not identified in the PLAC group. There were 24 different bacterial species isolated in the PLAC group, 15 isolated in the CHX group, and 10 isolated in the AMOX group. Of the 33 different bacterial species, seven (21%) were α-hemolytic and also belonged to the viridans group streptococci. In the PLAC group, five bacterial species isolated were α-hemolytic/viridans group streptococci, two isolated in the CHX group, and one isolated in the AMOX group.

DISCUSSION

This study was a randomized, blind, placebo-controlled prospective clinical trial to evaluate the comparative incidence and magnitude of bacteremia between a pre-procedure 0.12% chlorhexidine rinse and 2g amoxicillin antibiotic prophylaxis during third molar extractions. A review of the literature did not yield a study that directly compared the incidence and/or magnitude of bacteremia during a dental treatment procedure using a pre-procedure antimicrobial rinse and an antibiotic prophylaxis regimen. The use of a 0.12% chlorhexidine pre-procedure rinse is not a standard of care, but is commonly used prior to dental extractions. The use of 2g amoxicillin antibiotic prophylaxis is not a standard of care in healthy patients with no cardiac anomalies or prosthetic joints and is not otherwise indicated for such use. The primary purpose of this study was to determine the effect of a pre-procedure rinse of 0.12% chlorhexidine on the incidence and magnitude of bacteremia compared to the AHA and the ADA/AAOS recommended antibiotic prophylaxis guideline of 2g amoxicillin during third molar extractions. A secondary purpose was to provide additional data on the incidence and magnitude of bacteremia during dental treatment procedures with or without an antimicrobial intervention.

The PLAC, CHX, and AMOX groups were similar with no statistically significant differences in regards to subjects' age and gender, surgical procedure length, third molar extraction diagnosis, number of surgical flaps, number of bone removal procedures, surgeon completing the procedure, and investigator initiating the IV line.

Thus, bacteremia comparisons among the PLAC, CHX, and AMOX groups were justified.

Although there was no statistically significant difference among the PLAC, CHX, and AMOX groups in regards to the incidence and magnitude of bacteremia, there appeared to be a few important trends. One trend was it appeared as though the PLAC group (3.61 CFU/mL) resulted in the highest mean magnitude of bacteremia followed by the CHX group (2.76 CFU/mL) and then the AMOX group (0.63 CFU/mL) (Figure 29 - Appendix). Also, the PLAC group (0.0-18.20 CFU/mL) displayed a larger range of magnitude values followed by the CHX group (0.0-11.10 CFU/mL) and then the AMOX group (and 0.0-4.30 CFU/mL) (Figure 30 - Appendix). These findings are consistent with previous studies (Hall et al., 1996; Heimdahl et al., 1990; Lockhart et al., 2008; Roberts et al., 2006) that evaluated the magnitude of bacteremia during dental extractions. Heimdahl et al. (1990) found a mean bacteremia of 1.34 CFU/mL with a range of 0.0-9.88 CFU/mL during the extraction of one mandibular third molar that was either a complete bony impaction (no communication with the oral cavity) or a partial bony impaction (communication with the oral cavity). Hall et al. (1996) found a median bacteremia of 2.05 CFU/mL and 0.72 CFU/mL in patients administered an erythromycin or clindamycin antibiotic prophylaxis, respectively, during a single tooth/non-third molar extraction. Ten minutes following the extraction, Hall et al. (1996) found a decreased median bacteremia of 0.60 CFU/mL and 0.30 CFU/mL in the same patients, respectively. Roberts et al. (2006) found a median bacteremia of 2.73 CFU/mL at one minute post

extractions and 0.32 CFU/mL at 15 minutes post extractions in children. Lockhart et al. (2008) failed to detect a bacteremia below their methodology study threshold of 10^4 CFU/mL.

The magnitude of bacteremia data was not normally distributed and therefore a RM-ANOVA could not be used to analyze the data. Instead, a Kruskal-Wallis test and a Friedman test for non-parametric data had to be used for the statistical analysis. Because multiple comparisons were used in the statistical analysis, a more stringent p-value of 0.017 was used which made it more difficult to detect differences among the groups. There are two possible reasons no significant difference was observed among the PLAC, CHX, or AMOX groups: 1) an incorrect power analysis and/or insufficient subjects to detect a difference; or 2) there actually was no difference among the groups. The power analysis was accomplished prior to submission of the protocol to the IRB and determined 10 subjects per group was sufficient to detect a statistically significant difference in the magnitude of bacteremia in CFU/mL for three groups and four measures. However, the power analysis assumed a RM-ANOVA/parametric test would be used for the statistical analysis.

A second trend was it appeared as though the magnitude of bacteremia peaked at BD 3 for the PLAC, CHX, and AMOX groups with a bacteremia remaining above baseline at BD 4 (Figure 31). These findings are consistent with other similar studies (Diz Dios et al., 2006; Heimdahl et al., 1990; Lockhart et al., 2008; Lockhart et al., 2004; Rajasuo et al., 2004; Roberts et al., 2006; Tomas et al., 2007) whereby

a transient bacteremia peaks within five minutes of an extraction and decreases significantly at 10-15 minutes post extraction. The PLAC group displayed a more gradual increase of bacteremia to a peak at BD 3 whereas the CHX group displayed a spike at BD 3. The AMOX group displayed a lower, consistent level of bacteremia throughout the BDs.

A third trend was the reduction in different bacterial species isolated in the CHX and AMOX groups compared to the PLAC group. There were 24 different bacterial species isolated in the PLAC group, 15 isolated in the CHX group, and 10 isolated in the AMOX group for a total of 33 different bacterial species (Tables 9-11, Figure 32 - Appendix). Of these 33 bacterial species, two bacterial species were not identified but were categorized as gram positive rods – one bacterial isolate was an aerobe and the other was an anaerobe. The aerobic isolate had a colonial composition that was 'chunky' which did not allow a satisfactory suspension for identification using the VITEK® 2 or Biolog™ Microstation System™. The anaerobic isolate was lost upon subculture and not available for identification. A fusobacterium isolate was not identified in the PLAC group and may represent *fusobacterium nucleatum*. Of the 33 different bacterial species, seven (21%) were α-hemolytic and also belonged to the viridans group streptococci (Figure 32 - Appendix). In the PLAC group, five bacterial species isolated were α-hemolytic/viridans group streptococci, two isolated in the CHX group, and one isolated in the AMOX group. Two α-hemolytic/viridans group streptococci - *streptococcus australis* and *streptococcus parasanguinis* - were isolated in this study not previously reported by other similar studies (Hall et al.,

1996; Heimdahl et al., 1990; Lockhart et al., 2008; Lockhart et al., 2004; Lockhart et al., 1996; Roberts et al., 2006; Tomas et al., 2007).

In two subjects bacterial isolates were recovered at BD 1/baseline. *Staphylococcus hominis* was isolated in one subject of the AMOX group only at BD 1. In the other subject of the CHX group, *staphylococcus epidermidis* was isolated at BD 1 and 2. It is interesting to note that in this same subject of the CHX group, *streptococcus mitis/oralis* (viridans group streptococci) was isolated only in BD 1 – the microorganism was not later recovered in BD 2-4. It appears as though this subject presented with a baseline transient bacteremia of potential oral origin prior to a dental treatment procedure. This could be one example of the antimicrobial effectiveness of a 0.12% chlorhexidine pre-procedure rinse. *Staphylococcus hominis* and *staphylococcus epidermidis* are commensal microorganisms of the skin and likely represent contamination during initiation of the IV line.

The results of this study found a 50% incidence of bacteremia in the PLAC group, 60% for CHX, and 40% for AMOX where incidence is defined as at least one positive culture of the four blood draws per subject. A more detailed evaluation of the data reveals 14/40 (35%) of PLAC BDs had a positive culture, 15/40 (38%) for CHX, and 7/40 (18%) for AMOX. The incidence of bacteremia found in this study appears lower than other similar studies (Diz Dios et al., 2006; Hall et al., 1996; Heimdahl et al., 1990; Lockhart et al., 2008; Lockhart et al., 2004; Lockhart et al., 1996; Roberts et al., 2006; Tomas et al., 2007). However, differences among the

patients and dental extractions may account for this discrepancy. Other similar studies included medically-compromised and/or developmental disabled patients and erupted teeth which required extraction due to caries and/or periodontal disease. For example, Hall et al. (1996) found a 79% and 84% incidence of bacteremia in patients administered an erythromycin or clindamycin antibiotic prophylaxis, respectively, during a single tooth/non-third molar extraction due to caries and/or periodontal disease. In another study, Heimdahl et al. (1990) found a 55% incidence of bacteremia in patients during the extraction of one mandibular third molar that was either a complete bony impaction (no communication with the oral cavity) or a partial bony impaction (communication with the oral cavity).

As previously discussed, there is a more diverse microflora associated with the gingival sulcus/periodontal pocket. As the present study included third molar extractions - in which there may have been no oral cavity communication - and a mean subject age of 21.8 years, the development of a more complex microflora may not have been established. Okabe et al. (1995) found a higher incidence of bacteremia in older patients (86.8%) compared to younger patients less than 20 years old (42.9%).

The results of this study are based upon a healthier patient sample than the target patient population – those patients with cardiac anomalies, prosthetic joints, immunosuppression, and various medical complexities for whom antibiotic prophylaxis is currently recommended for the prevention of IE, LPJI, or other DSI.

However, a reasonable and logical extrapolation of the results based on the sampled population can be applied to the target patient population.

After a review of the literature, it is believed this study was the first to evaluate the incidence and/or magnitude of bacteremia during a dental treatment procedure using a pre-procedure antimicrobial rinse and an antibiotic prophylaxis regimen. A study by Morozumi et al. (2010) evaluated the incidence and magnitude of bacteremia during scaling/root planing using an essential oil-containing antiseptic and azithromycin antibiotic. However, the antiseptic oral rinse was used as a subgingival irrigation one week prior to the scaling/root planing procedure while the azithromycin antibiotic was administered for three days prior to the scaling/root planing procedure. In contrast, this study evaluated a common clinical practice of using a 0.12% chlorhexidine pre-procedure rinse and the recommended antibiotic prophylaxis regimen according to the current AHA and ADA/AAOS guidelines during a dental treatment procedure.

A future research study including the extraction of erupted teeth with oral cavity communication and a gingival sulcus/periodontal pocket using the current protocol and design would be beneficial. Such a study could evaluate patients with periodontal disease who may harbor the more virulent red-complex bacterial species - *porphyromonas gingivalis*, *treponema denticola*, and *tannerella forsythia* - and classify their oral health based on gingival and/or plaque indices. As previously mentioned, the relationship between systemic diseases and oral health is

recognized, as in diabetes mellitus and the role of periodontal infection on systemic vasculature inflammation and cardiovascular disease. As noted by the 2007 AHA IE guidelines, optimal oral health may reduce bacteremia and the risk of IE more than antibiotic prophylaxis (Wilson et al., 2007). Also, Lockhart et al. (2009) found bacteremia following toothbrushing was associated with poor oral hygiene and gingival bleeding.

CONCLUSION

The null hypothesis was there is no significant difference in the incidence and magnitude of bacteremia between the use of 0.12% chlorhexidine pre-procedure rinse and 2g amoxicillin antibiotic prophylaxis during third molar extractions. According to the results and statistical analysis of this study, the null hypothesis is not rejected and there is no difference between a 0.12% chlorhexidine pre-procedure rinse and 2g amoxicillin antibiotic prophylaxis antimicrobial intervention in regards to the incidence and magnitude of bacteremia in CFU/mL. Assuming a correct power analysis and sufficient subjects to detect a difference, the results of this study may reasonably conclude an antimicrobial intervention of either a 0.12% chlorhexidine pre-procedure rinse or 2g amoxicillin antibiotic prophylaxis according to the current AHA and ADA/AAOS guidelines does not statistically reduce the incidence and magnitude of bacteremia compared to no antimicrobial intervention.

However, there appeared to be three important trends:

1. A 0.12% chlorhexidine pre-procedure rinse and to a greater extent a 2g amoxicillin antibiotic prophylaxis reduced the mean magnitude and range of bacteremia in CFU/mL compared to a placebo.
2. The magnitude of bacteremia peaked within 1.5 minutes of initiating the last third molar extraction for the PLAC, CHX, and AMOX groups and decreased within 10 minutes but remained above baseline.
3. A 0.12% chlorhexidine pre-procedure rinse and to a greater extent a 2g amoxicillin antibiotic prophylaxis reduced the number of different bacterial species, including α-hemolytic/viridans group streptococci, isolated from the vasculature compared to a placebo.

The results of this study once again demonstrate an antibiotic prophylaxis does not prevent the occurrence of bacteremia during a dental treatment procedure. The results of this study also demonstrate an antimicrobial oral rinse may decrease the level of bacteremia during a dental treatment procedure, but not to a level comparable to the systemic administration of an antibiotic. The results of this study should be interpreted in the context of other studies.

APPENDIX

Attachment 1: Validation Procedure Document

MV 40M-008
30 Nov 10

METHOD VALIDATION

ISOSTAT ISOLATOR 10

1. PRINCIPLE

The WAMPOLE ISOSTAT Microbial system uses lysis centrifugation technology to concentrate microorganisms in a blood culture sample resulting in faster isolation and increased recovery of organisms from the blood. The ISOSTAT System is capable of providing quantitative information to assist in determining the magnitude of bacteremia at the time of specimen collection.

This report describes the procedures used to validate the ISOSTAT ISOLATOR 10 system for use in the 59th CRD laboratory.

2. MEDIA AND REAGENTS

2.1 Trypticase soy agar with 5% sheep blood (TSA II), Becton Dickinson Microbiology Systems, Cockeysville, MD, #21239. Stored at 2-8°C

2.2 0.9% Sodium chloride. Baxter Healthcare Corporation, Deerfield, IL, #2F7124. Stored at room temperature.

3. EQUIPMENT AND UNIQUE SUPPLIES

3.1 Ambient air incubator, 35±2°C. Incubator ECN 92625 was used for this validation.

3.2 ISOLATOR 10 microbial tubes. Evacuated tube specifically designed for collection and concentration of microorganisms from blood. Wampole Laboratories, Cranbury, New Jersey, #50C7. Stored at 2 to 40°C.

3.3 ISOSTAT Press, rack, and press head. Small hand-operated press used to apply the cap. The press head accommodates the ISOLATOR 10 tube. The rack supports up to ten ISOLATOR 10 microbial tubes during processing. Wampole Laboratories, Cranbury, New Jersey, #50C2.

3.4 ISOSTAT Consumables. Wampole Laboratories, Cranbury, New Jersey, #50C9.

3.4.1 ISOSTAT Cap. Plastic cap which penetrates top stopper of ISOLATOR 10 tube and permits access to tube's contents through a membrane-covered port.

3.4.2 ISOSTAT Supernatant pipet. Plastic pipet used for removing supernatant fluid from ISOLATOR 10 microbial tubes.

3.4.3 ISOSTAT Concentrate pipet. Plastic pipet for removing concentrate from ISOLATOR 10 microbial tubes to agar plates.

3.5 Mitsubishi Pack-Rectangular Jar, 2.5 L. Mitsubishi Gas Chemical Company, Inc. New York, NY, #50-25.

3.6 Mitsubishi Pack-Anaero anaerobic gas generating system. Mitsubishi Gas Chemical Company, Inc. New York, NY, #10-01. Stored at 2-25°C.

3.7 Dry Anaerobic Indicator Strips. BD BBL, Sparks, MD., #271051. Stored at 15-25°C.

3.8 Spectrafuge 6C centrifuge, ECN 114911.

3.9 One mL Syringe, Luer-Lok tip, with .01 mL volume markings. BD, Franklin Lakes, NJ, #309628.

3.10 Precision Glide needles, 26G½. BD, Franklin Lakes, NJ, #305111.

4. SPECIMEN

4.1 Ten ml of fresh human blood drawn into an ISOLATOR tube and spiked with known microorganisms. Tubes containing short draws were not used in this validation.

4.2 The organisms used in this validation were obtained in lyophilized form from MicroBiologics, St. Cloud, MN, and were subcultured twice before being used in testing. The organisms, except for *Staphylococcus epidermidis*, were chosen because of their known association with periodontal disease. The ISOSTAT system is being validated at this time primarily to support dental research. Preliminary work was done with *S. epidermidis* because of its low pathogenicity and ease of cultivation.

4.2.1 *Staphylococcus epidermidis* ATCC 12228.
4.2.2 *Streptococcus anginosus* ATCC 10713.
4.2.3 *Veillonella parvula* ATCC 10790.
4.2.4 *Parvimonas micra* ATCC 33270, formerly known as *Micromonas micros* and *Peptostreptococcus micros.*
4.2.5 *Fusobacterium nucleatum* ATCC 25586.
4.2.6 *Actinomyces odontolyticus* ATCC 17929.

4.3 All ISOLATOR tubes were processed within 2 hours of being inoculated with organisms.

5. PROCEDURE

5.1 Each test organism was subcultured to TSA II and incubated for 18-24 hours at 35±2°C in ambient air (*S. epidermidis* and *S. anginosus*) or 48-72 hours at 35±2°C in anaerobic environment (*V. parvula, P. micra, F. nucleatum*, and *A. odontolyticus*)

5.2 A suspension of the test organism equal in turbidity to a 0.5 McFarland turbidity standard was prepared in sterile saline.

5.3 The bacterial suspensions were serially diluted in sterile saline. Note: Different dilution schemes were used to achieve a variety of concentrations of bacteria in the ISOLATOR 10 tubes.

5.4 The stoppers of the ISOLATOR tubes were cleaned with an alcohol pad and then an aliquot (100 or 200 µL) of the diluted bacterial suspension (Inoculum) was added to the 10 mL of blood in the ISOLATOR tube using a sterile syringe and needle. The sample was mixed by gently inverting the tube several times.

5.5 To determine the number of colony forming units (CFU) in the Inoculum, aliquots (n=3) of the diluted bacterial suspension, equal to the volume of the Inoculum (100 or 200 µL), were transferred to TSA II plates. The suspension was spread evenly over the surface of the agar to achieve growth of isolated colonies that could be accurately counted.

5.6 The ISOLATOR tubes were centrifuged at 5400 RPM (2800 x G) for 30 minutes. The centrifuge was allowed to stop on its own, with no braking, so that the concentrate would not be disturbed. Note: Centrifuging in the Spectrafuge 6C centrifuge at > 5400 RPM resulted in broken tubes.

5.7 After centrifugation, each ISOLATOR tube was carefully placed in the ISOSTAT rack. Tubes were handled carefully so as not to disturb the microbial concentrate. Note: Steps 5.7 – 5.16 were performed in the biological safety cabinet.

5.8 The tube stoppers were cleaned with alcohol pads.

5.9 An ISOSTAT cap was removed from the sterile package and placed over the stopper of each ISOLATOR tube.

5.10 Each tube, in turn, was positioned with its cap under the press head. The handle was gently pulled down as far as possible to allow the spike to penetrate the stopper and seat the cap firmly.

5.11 For each tube, an ISOSTAT supernatant pipet was removed from the sterile package and the bulb on the pipet was squeezed to collapse it completely. Maintaining pressure on the bulb the stem of the pipet was inserted as far as possible into the tube through the cap. When the pipet was in as far as it would go the pressure on the bulb was slowly released to allow the supernatant to be drawn into the pipet. Complete withdrawal was indicated by the appearance of bubbles in the pipet bulb and air in the stem.

5.12 When all supernatant fluid had been withdrawn, the pipets were discarded in the biohazard waste container.

5.13 Each tube was vortex mixed vigorously for at least 10 seconds to achieve a homogenous mixture of concentrate.

5.14 For each tube, an ISOSTAT concentrate pipet was removed from the sterile package and the bulb was squeezed to collapse it completely. Maintaining pressure on the bulb the stem of the pipet was inserted into the tube through the cap so that the pipet tip reached the bottom of the tube. The pressure on the bulb was released to allow the concentrate to be drawn into the pipet. Care was taken to remove all of the concentrate.

5.15 The pipets were removed from the ISOLATOR tubes and the concentrate was distributed equally on 4 TSA II plates. The concentrate was dispensed in a straight line along surface of agar.

5.16 To streak the plates the tip of the concentrate pipet was used to make about 15 - 20 passes perpendicular to original inoculum line. The streak lines were kept away from the edges of the plates.

5.17 The plates were incubated immediately at 35 ± 2°C in ambient air or anaerobic environment depending on the requirements of the test organism. The plates were incubated agar side down to maintain contact of inoculum with agar and to prevent the concentrate from dripping onto lid.

5.18 Plates were examined for growth after 24-72 hours for aerobes and 48-96 hours for anaerobes.

5.19 To establish the approximate number of colony forming units (CFUs) in the Inoculum the number of CFUs on the three plates inoculated directly from the diluted bacterial suspension were counted and the mean was calculated.

5.20 To determine the total number of CFU recovered from the ISOLATOR the number of CFU on the four plates inoculated with ISOLATOR concentrate were counted and added together.

5.21 Paired *t* test was performed to compare the mean of the CFU in the Inoculum to the CFU recovered from the ISOLATOR.

6. RESULTS

6.1 See Attachment 1, Table 1 for raw data and calculation of mean from Inoculum.

6.2 The plating method used to determine the CFU in the Inoculum was previously validated and shown to have a variability of ±20%. That work was accomplished using plate counts of 30 to 300 CFU, the industry recommended range to obtain reliable data. This validation included counts < 30 CFU. See Attachment 2, Table 2. The RSD for counts > 30 CFU ranged from 1.33 to 23.56, comparable with previous work. The RSD for counts ranging from 10 to 30 CFU ranged from 2.84 to 27.09, still comparable with previous work. For counts < 10 CFU the RSDs were high ranging from 21.53 to 173.21. The difficulty of working with such low concentration bacterial suspensions is apparent. A difference of 1 CFU can change the RSD dramatically. Careful examination of the raw data and other statistical data (see sections 6.3 and 6.4) indicates that this method is acceptable for use in this validation.

6.3 Paired *t* test showed a significant difference between the CFU in the Inoculum and the CFU recovered from the ISOLATOR concentrate when data for all organisms was combined. See Attachment 3, Table 3. However, when the data was analyzed for individual organisms the only groups considered significantly different were the *F. nucleatum* groups. See Attachment 3, Table 4, top table. When the *F. nucleatum* data was removed from the analysis the overall difference was not significantly different. See Attachment 3, Table 4, bottom table. This indicates that overall the test is acceptable for performing quantitative blood cultures, however, it does not perform equally well with all organisms. It is not clear why the test does not work as well with the *F. nucleatum* as with the other organisms. It may have something to do with the colonial morphology of the *F. nucleatum*. Of all the organisms tested the *F. nucleatum* is the only one which has a characteristic chunky morphology making it difficult to achieve a smooth suspension in saline. Perhaps it is this characteristic that contributed to the variability seen between the Inoculum CFU and the CFU recovered from the ISOLATOR.

6.4 The test performed well at low concentrations with a sensitivity of 1 CFU/tube. See Attachment 4, Table 5. However, it must be noted that none of the *F. nucleatum* data fell within the 0-5 CFU range.

7. SAFETY

Universal precautions were observed at all times.

8. ROBUSTNESS

8.1 This test is sensitive to minor changes in technique. Proper centrifugation speed and time are critical to ensure the optimum recovery of organisms and to avoid tube breakage. Care must be taken at all times not to disturb the concentrate.

8.2 The test does not perform equally well with all organisms.

9. PROCEDURE NOTES

9.1 Proper centrifugation speed is critical to ensure the optimum recovery of organisms and to avoid tube breakage.

9.2 The use of a biological safety hood is recommended to prevent contamination of the culture plates.

9.3 Allow centrifuge to stop on its own. DO NOT USE THE BRAKE as this can disturb the concentrate and decrease the recovery of organisms.

9.4 Do not squeeze the bulb on the supernatant pipet after insertion into the ISOLATOR tube. Bubbles will disturb the concentrate.

10. REFERENCES

The Wampole ISOSTAT System Resource Guide, Wampole ISOSTAT System, Wampole Laboratories, Cranbury, New Jersey, IN-050C1-03. Issued: April 2000.

11. ATTACHMENTS

11.1 Table 1: Raw data. Calculation of mean CFU from Inoculum.

11.2 Table 2: Variability of plating method used to determine the number of CFU inoculated into the ISOLATOR.

11.3 Table 3: *t* test data sorted by organism and Table 4: *t* test data sorted by Inoculum concentration.

11.4 Table 5: Sensitivity data.

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ISOSTAT ISOLATOR 10 Method Validation

Attachment 1

Table 1: Raw data. Calculation of mean CFU from Inoculum

Organism	CFU from inoculum	Mean CFU from Inoculum	CFU from Isolator 10
A. odontolyticus	5,6,13	8	10
A. odontolyticus	31,47,33	37	42
A. odontolyticus	443,442,418	434	402
F. nucleatum	18,0,10	9	3
F. nucleatum	6,15,8	10	2
F. nucleatum	21,20,20	20	9
F. nucleatum	31, 32, 30	31	10
F. nucleatum	31, 32, 30	31	14
F. nucleatum	31, 32, 30	31	18
F. nucleatum	254, 269, 219	247	194
P. micra	7,5,11	8	7
P. micra	10,11,10	10	11
P. micra	605	605	594
S. anginosus	0, 0, 0	0	1
S. anginosus	0, 0, 0	0	0
S. anginosus	0, 0, 1	1	0
S. anginosus	1, 0, 0	1	1
S. anginosus	2,3,4	3	2
S. anginosus	3, 3, 2	3	2
S. anginosus	2,3,4	3	3
S. anginosus	2,3,4	3	3
S. anginosus	5, 0, 3	3	4
S. anginosus	10, 16, 14	13	10
S. anginosus	40,34,28	34	22
S. anginosus	40,34,28	34	30
S. anginosus	63,74,50	62	69
S. anginosus	112 ,90, 117	106	96
S. anginosus	152,150,148	150	164
S. anginosus	152,150,148	150	168
S. anginosus	152,150,148	150	171
S. epi	0,1,4	2	1
S. epi	0,1,4	2	1
S. epi	0,1,4	2	3
S. epi	12,8,9	10	9
S. epi	12,8,9	10	13
S. epi	23,21,25	23	16
S. epi	105,141,131	126	93
S. epi	105,141,131	126	121
S. epi	105,141,131	126	121
V. parvula	15,20,26	20	10
V. parvula	38,32,42	37	12
V. parvula	82,85,94	87	41

Table 2: Variability of plating method used to determine the number of CFU inoculated into the ISOLATOR

CFU from Inoculum					
TSAII 1	TSAII 2	TSAII 3	Mean	StDev	RSD
0	0	1	0.33	0.58	173.21
1	0	0	0.33	0.58	173.21
0	1	4	1.67	2.08	124.90
3	3	2	2.67	0.58	21.65
5	0	3	2.67	2.52	94.37
2	3	4	3.00	1.00	33.33
2	3	4	3.00	1.00	33.33
7	5	11	7.67	3.06	39.85
5	6	13	8.00	4.36	54.49
18	0	10	9.33	9.02	96.63
6	15	8	9.67	4.73	48.89
12	8	9	9.67	2.08	21.53
12	8	9	9.67	2.08	21.53
10	11	10	10.33	0.58	5.59
10	16	14	13.33	3.06	22.91
15	20	26	20.33	5.51	27.09
21	20	20	20.33	0.58	2.84
23	21	25	23.00	2.00	8.70
32	32	30	31.33	1.15	3.69
40	34	28	34.00	6.00	17.65
31	47	33	37.00	8.72	23.56
38	32	42	37.33	5.03	13.48
63	74	50	62.33	12.01	19.27
82	85	94	87.00	6.24	7.18
112	90	117	106.33	14.36	13.51
105	141	131	125.67	18.58	14.79
152	150	148	150.00	2.00	1.33
254	269	219	247.33	25.66	10.37
443	442	418	434.33	14.15	3.26

Table 3: *t* test data sorted by organism

	Paired *t* test	
Organism	P value	*n*
All organisms	0.0076	42
S. epi	0.1691	9
S. anginosus	0.4190	17
A. odontolyticus	0.5552	3
F. nucleatum	0.0230	7
P. micra	0.4274	3
V. parvula	0.1226	3

Table 4: *t* test data sorted by Inoculum concentration

Including *Fusobacterium nucleatum*

	Paired *t* test	
CFU	P value	*n*
All concentrations	0.0076	42
0-10	0.2933	19
0-30	0.0287	23
>30	0.8104	19

Without *Fusobacterium nucleatum*

	Paired *t* test	
CFU	P value	*n*
All concentrations	0.0919	35
0-10	not done	
0-30	0.1890	20
>30	not done	

Table 5: Sensitivity data

Organism	Mean CFU Inoculum	CFU from Isolator 10
S. anginosus	0	1
S. anginosus	0	0
S. anginosus	1	0
S. anginosus	1	1
S. epi	2	1
S. epi	2	1
S. epi	2	3
S. anginosus	3	2
S. anginosus	3	3
S. anginosus	3	3
S. anginosus	3	2
S. anginosus	3	4
Paired t test	P value	0.5035

Attachment 2: Subject blood draw form

Comparative Efficacy of 0.12% Chlorhexidine and Amoxicillin to Reduce the Incidence and Magnitude of Bacteremia During Third Molar Extractions: A Prospective, Blind, Randomized Clinical Trial.

Subject No 1

Date/Time of Procedure:

Dx #1/Flap?/Bone Removal?:

Dx #16/Flap?/Bone Removal?:

Dx #17/Bone Removal?:

Dx #32//Bone Removal?:

Length of Procedure (min/drugs-gauze):

Surgeon:

IV Start:

Procedure Timeline:

IV	BD1	Antibiotic/ Placebo	CHX rinse/Placebo CS/LA	Ext. #1	Begin Ext. #32	BD2	Ext. #16	Begin Ext. #17	BD3	BD4
|	|	| < 45min	>| < 15min > |		| < 1.5min >|		|	| < 1.5min >|	< 8.5min > |

IV=intravenous access, BD=blood draw, CHX=0.12% chlorhexidine, CS/LA=conscious sedation/local anesthetic

Clinic Procedure:

1. Establish IV access for blood draws as follows:
   a. 1 min circular scrub in usual manner of IV access site with 10% povidone-iodine (PDI® Povidone-Iodine Swabstick)
   b. IV access with an Acuvance® 18g angiocatheter secured by 3M® Tegaderm™ film
   c. Attachment of a Maximus® 18cm minibore extension set with removable MaxPlus® Tru-Swab® connector by Medegren, Inc.
2. Obtain blood draw #1 as follows:
   a. Disinfect MaxPlus® Tru-Swab® connector with a 70% isopropyl alcohol prep then draw and discard 10mL of blood and/or saline from the IV access line with a BD® 10mL syringe with luer-lok™ tip
   b. Disinfect MaxPlus® Tru-Swab® connector again with a 70% isopropyl alcohol prep and draw 15mL blood from the IV access with a BD® 20mL syringe with luer-lok™ tip
   c. Attach a BD® 18g 1.5 PrecisionGlide®needle to the 20mL syringe of blood to inject the blood into the Isolator® tube
   d. Disinfect end of Isolator® tube with a 70% isopropyl alcohol prep and inject 10mL of this blood into the Isolator® tube discarding 4-5mL of blood
   e. Disinfect MaxPlus® Tru-Swab® connector with a 70% isopropyl alcohol prep and irrigate/rinse the IV access line with 10mL sterile saline closing the IV access line until the next blood draw
3. Administer the 2g Amoxicillin or Placebo capsule
4. Establish IV access for conscious sedation meds using the same prep procedure as previously described
5. Administer the 15mL of 0.12% CHX rinse or Placebo rinse– subject to rinse for 1 min and expectorate
6. Obtain blood draws #2, 3, 4 as previously described according to the timeline
7. Place Isolator® tubes of blood in the labeled biohazard bag for the microbiology lab to pick-up and transfer

Table 1: Demographic Data

	Age (mean/stdev)*	Gender (M:F)	Proc Length (mean/stdev)**
PLAC	21.2/1.6	9:1	45.8/18.8
CHX	21.6/2.7	7:3	37.8/23.1
AMOX	22.6/3.1	7:3	42.4/12.0
p-value	**p=0.473**	**p= 0.475**	**p=0.632**

*****Years
******Minutes

Table 2: Descriptive Data Tooth #1

	Diagnosis*			Surgical Flap		Bone Removal	
	Mal	**ST Imp**	**PB Imp**	**Yes**	**No**	**Yes**	**No**
PLAC	4	4	2	6	4	2	8
CHX	4	5	1	6	4	2	8
AMOX	4	5	1	6	4	1	9
p-value	**p=0.958**			**p=1.000**		**p=0.787**	

***Mal:** malposed, **ST Imp:** soft tissue impaction, **PB Imp:** partial bony impaction

Table 3: Descriptive Data Tooth #16

	Diagnosis*			Surgical Flap		Bone Removal	
	Mal	**ST Imp**	**PB Imp**	**Yes**	**No**	**Yes**	**No**
PLAC	4	4	2	6	4	2	8
CHX	6	4	0	4	6	1	9
AMOX	6	1	3	5	5	3	7
p-value	**p=0.258**			**p=0.670**		**p=0.535**	

***Mal:** malposed, **ST Imp:** soft tissue impaction, **PB Imp:** partial bony impaction

Table 4: Descriptive Data Tooth #17

	Diagnosis*			Surgical Flap		Bone Removal	
	ST Imp	**PB Imp**	**CB Imp**	**Yes**	**No**	**Yes**	**No**
PLAC	3	6	1	10	0	7	3
CHX	4	6	0	10	0	7	3
AMOX	4	4	2	10	0	6	4
p-value	**p=0.612**			**p=N/A****		**p=0.861**	

***ST Imp:** soft tissue impaction, **PB Imp:** partial bony impaction, **CB Imp:** complete bony impaction

**No statistics computed because surgical flap was an inclusion criteria and constant

Table 5: Descriptive Data Tooth #32

	Diagnosis*			Surgical Flap		Bone Removal	
	ST Imp	PB Imp	CB Imp	Yes	No	Yes	No
PLAC	2	6	2	10	0	9	1
CHX	4	6	0	10	0	5	5
AMOX	4	5	1	10	0	6	4
p-value	**p=0.572**			**p=N/A****		**p=0.142**	

***ST Imp:** soft tissue impaction, **PB Imp:** partial bony impaction, **CB Imp:** complete bony impaction

**No statistics computed because surgical flap was an inclusion criteria and constant

Table 6: Incidence of Bacteremia

	Incidence	
	Positive	Negative
PLAC	5	5
CHX	6	4
AMOX	4	6
p-value	**p=0.670**	

Table 7: Total Mean Magnitude of Bacteremia and Range

	Total bacteremia* (mean/stdev)	Total bacteremia range*
PLAC	3.61/7.09	0.0 - 18.20
CHX	2.76/4.28	0.0 - 11.10
AMOX	0.63/1.33	0.0 - 4.30

***CFU/mL

Table 8: Mean Magnitude of Bacteremia per Blood Draw

	BD 1* (mean/stdev)	BD 2* (mean/stdev)	BD 3* (mean/stdev)	BD 4* (mean/stdev)	p-value
PLAC	0.00/0.00	1.26/3.67	1.90/5.36	0.45/0.83	**p=0.031**
CHX	0.04/0.13	0.18/0.29	2.37/4.11	0.17/0.24	**p=0.062**
AMOX	0.05/0.16	0.02/0.06	0.30/0.73	0.26/0.60	**p=0.310**
Total	0.03/0.11	0.49/2.13	1.52/3.89	0.29/0.60	
p-value	**p=0.595**	**p= 0.172**	**p=0.463**	**p=0.499**	

* CFU/mL

Table 9: PLAC Group Bacterial Identification

	Bacterial Species Identification	Gram stain	Type	BDs Isolated*
1	***Actinomyces meyeri***	G Pos.	Rod, Anaerobe	3
2	***Actinomyces naeslundii***	G Pos.	Rod, Anaerobe	7
3	***Clostridium cadaveris***	G Pos.	Rod, Anerobe	3
4	***Corynebacterium mimutissimum***	G Pos.	Rod, Aerobe	1
5	***Finegoldia magna***	G Pos.	Cocci, Anaerobe	1
6	***Fusobacterium species***	G Neg.	Rod, Anaerobe	2
7	***Gemella species***	G Pos.	Cocci, Aerobe	2
8	***Kocuria species***	G Pos.	Cocci, Aerobe	1
9	***Lactobacillus acidophilus***	G Pos.	Rod, Anaerobe	1
10	***Neisseria subflava***	G Neg.	Cocci, Aerobe	1
11	***Parvimonas micra***	G Pos.	Cocci, Anaerobe	1
12	***Peptoniphilus asaccharolyticus***	G Pos.	Cocci, Aerobe	3
13	***Prevotella oralis***	G Neg.	Rod, Anaerobe	1
14	***Propionibacterium acnes***	G Pos.	Rod, Anaerobe	1
15	***Staphylococcus capitis***	G Pos.	Cocci, Aerobe	1
16	***Staphylococcus hominis***	G Pos.	Cocci, Aerobe	1
17	***Streptococcus australis***	G Pos.	Cocci, α-Hemolytic	1
18	***Streptococcus constellatus***	G Pos.	Cocci, α-Hemolytic	1
19	***Streptococcus gordonii***	G Pos.	Cocci, α-Hemolytic	2
20	***Streptococcus mitis/oralis***	G Pos.	Cocci, α-Hemolytic	2
21	***Streptococcus parasanguinis***	G Pos.	Cocci, α-Hemolytic	1
22	***Streptococcus porcinus***	G Pos.	Cocci, β-Hemolytic	1
23	***Unidentified species***	G Pos.	Rod, Aerobe	1
24	***Unidentified species***	G Pos.	Rod, Anaerobe	1

*****Number of times bacterial species was isolated in different BD

Table 10: CHX Group Bacterial Identification

	Bacterial Species Identification	Gram stain	Type	BDs Isolated*
1	***Actinomyces meyeri***	G Pos.	Rod, Anaerobe	2
2	***Actinomyces naeslundii***	G Pos.	Rod, Anaerobe	10
3	***Clostridium bifermentans***	G Pos.	Rod, Anaerobe	1
4	***Clostridium cadaveris***	G Pos.	Rod, Anerobe	1
5	***Clostridium subterminale***	G Pos.	Rod, Anaerobe	1
6	***Fusobacterium nucleatum***	G Neg.	Rod, Anaerobe	1
7	***Lactobacillus gasseri***	G Pos.	Rod, Anaerobe	2
8	***Leuconostoc mesenteroides***	G Pos.	Cocci, Aerobe	1
9	***Parvimonas micra***	G Pos.	Cocci, Anaerobe	1
10	***Peptoniphilus asaccharolyticus***	G Pos.	Cocci, Aerobe	1
11	***Prevotella melaninogenica***	G Neg.	Rod, Anaerobe	2
12	***Propionibacterium acnes***	G Pos.	Rod, Anaerobe	3
13	***Streptococcus anginosus***	G Pos.	Cocci, α-Hemolytic	1
14	***Streptococcus mitis/oralis***	G Pos.	Cocci, α-Hemolytic	1
15	***Staphylococcus epidermidis***	G Pos.	Cocci, Aerobe	2

*****Number of times bacterial species was isolated in different BD

Table 11: AMOX Group Bacterial Identification

	Bacterial Species Identification	Gram stain	Type	BDs Isolated*
1	***Actinomyces israelii***	G Pos.	Rod, Anaerobe	2
2	***Actinomyces meyeri***	G Pos.	Rod, Anaerobe	3
3	***Actinomyces naeslundii***	G Pos.	Rod, Anaerobe	1
4	***Finegoldia magna***	G Pos.	Cocci, Anaerobe	1
5	***Peptoniphilus asaccharolyticus***	G Pos.	Cocci, Aerobe	1
6	***Prevotella melaninogenica***	G Neg.	Rod, Anaerobe	2
7	***Propionibacterium acnes***	G Pos.	Rod, Anaerobe	1
8	***Staphylococcus epidermidis***	G Pos.	Cocci, Aerobe	1
9	***Staphylococcus hominis***	G Pos.	Cocci, Aerobe	1
10	***Streptococcus sanguinis***	G Pos.	Cocci, α-Hemolytic	1

*****Number of times bacterial species was isolated in different BD

Table 12: Bacterial Identification Comparison

	Total Different Bacterial Isolates*	α-Hemolytic/Viridans Group Streptococci Isolates*
PLAC	24	5
CHX	15	2
AMOX	10	1
Total	33	7

***Number of different bacterial isolates/species

Figure 29: Total Mean Magnitude of Bacteremia

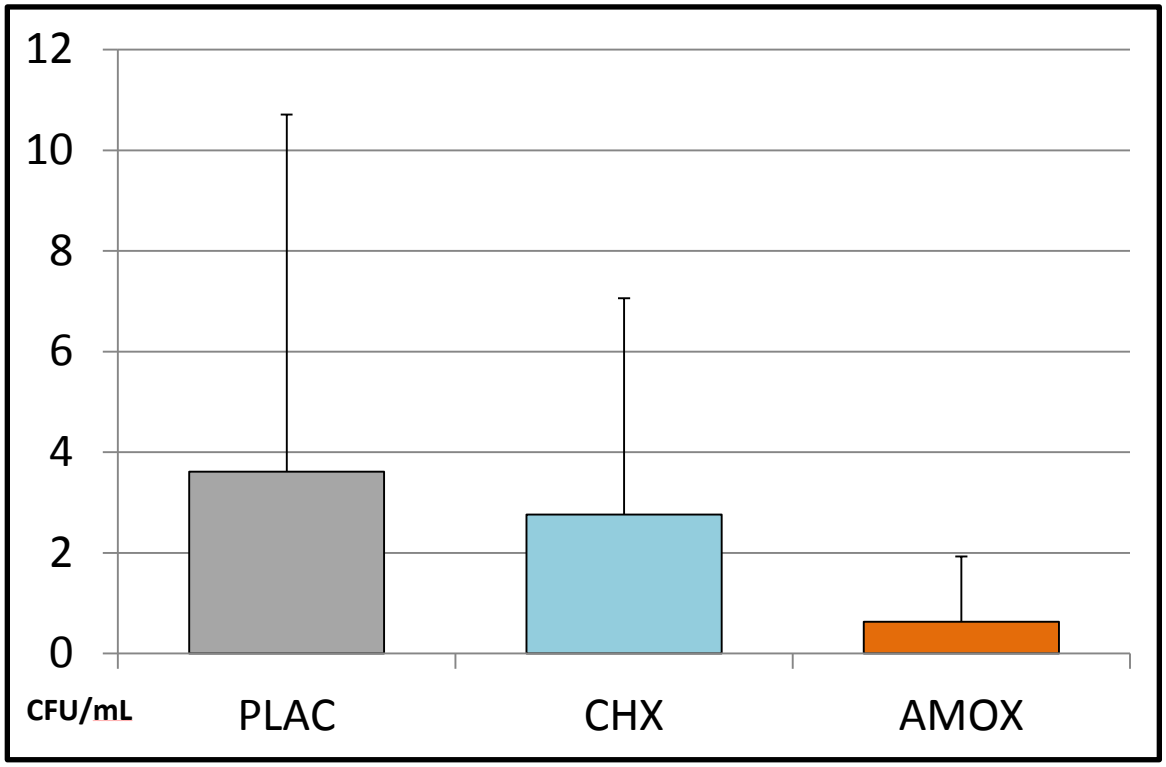


Figure 30: Mean Magnitude of Bacteremia Range

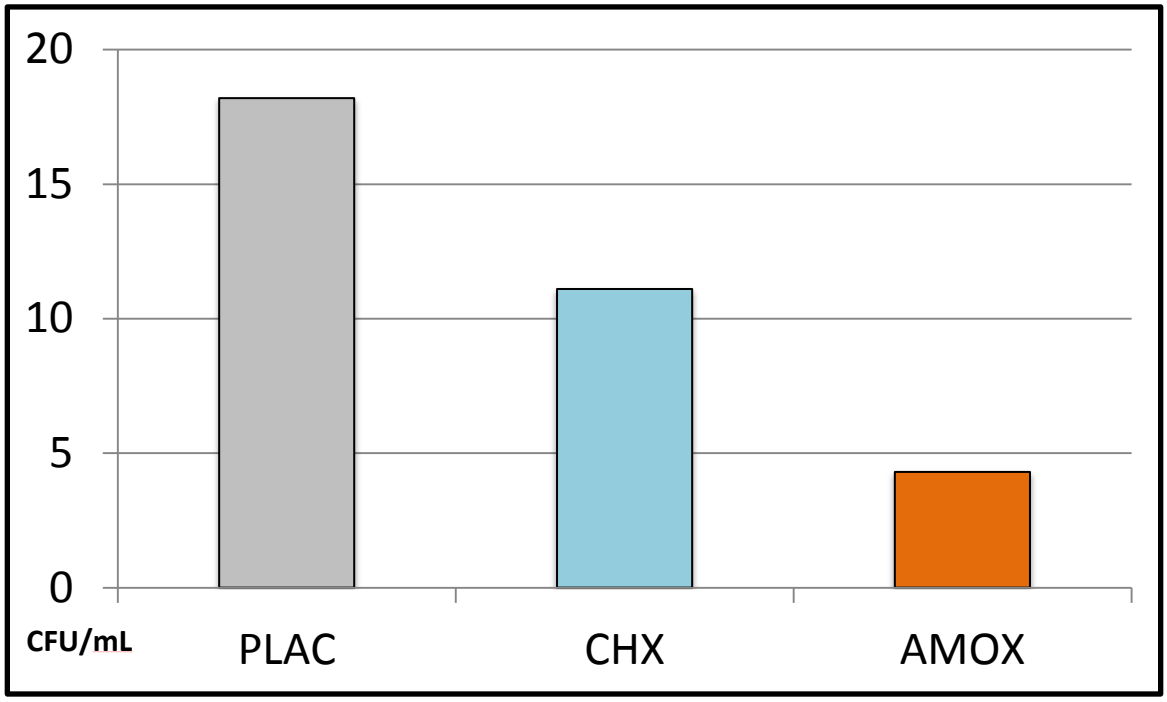


Figure 31: Blood Draws 1-4 Mean Magnitude of Bacteremia

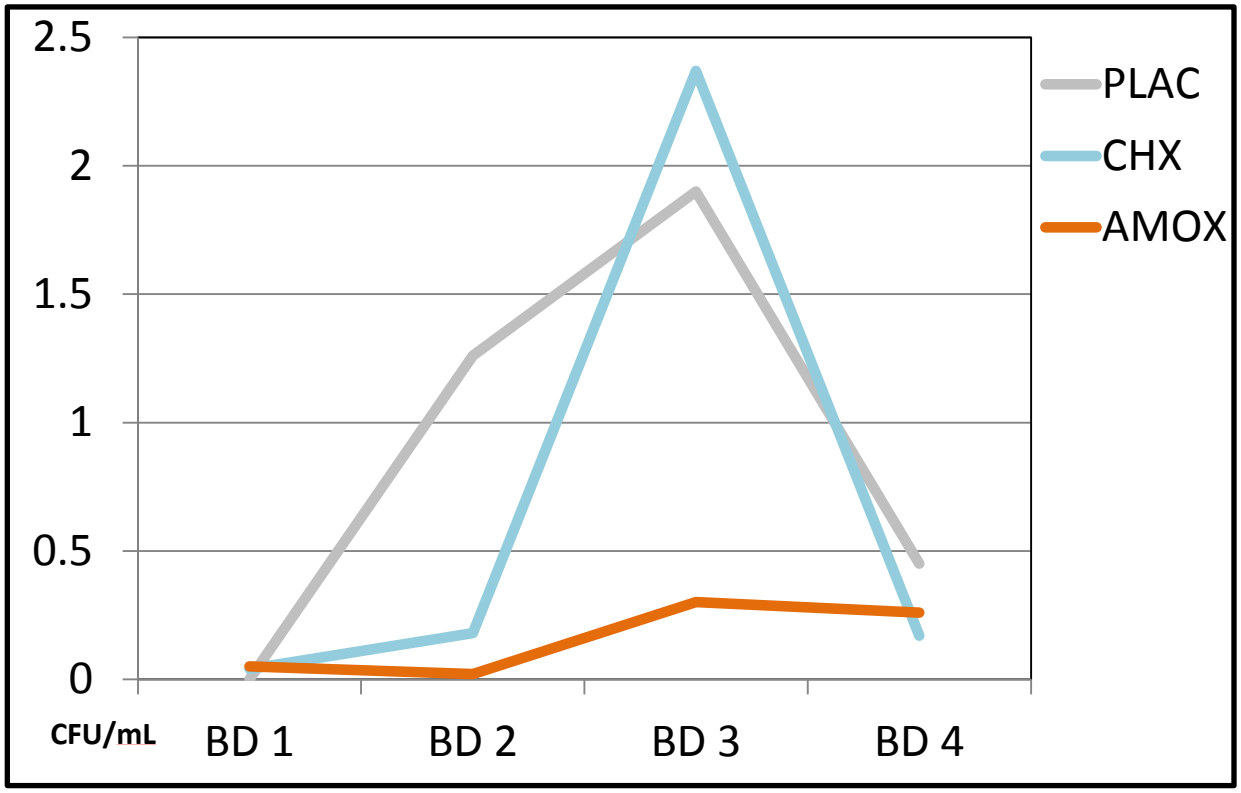
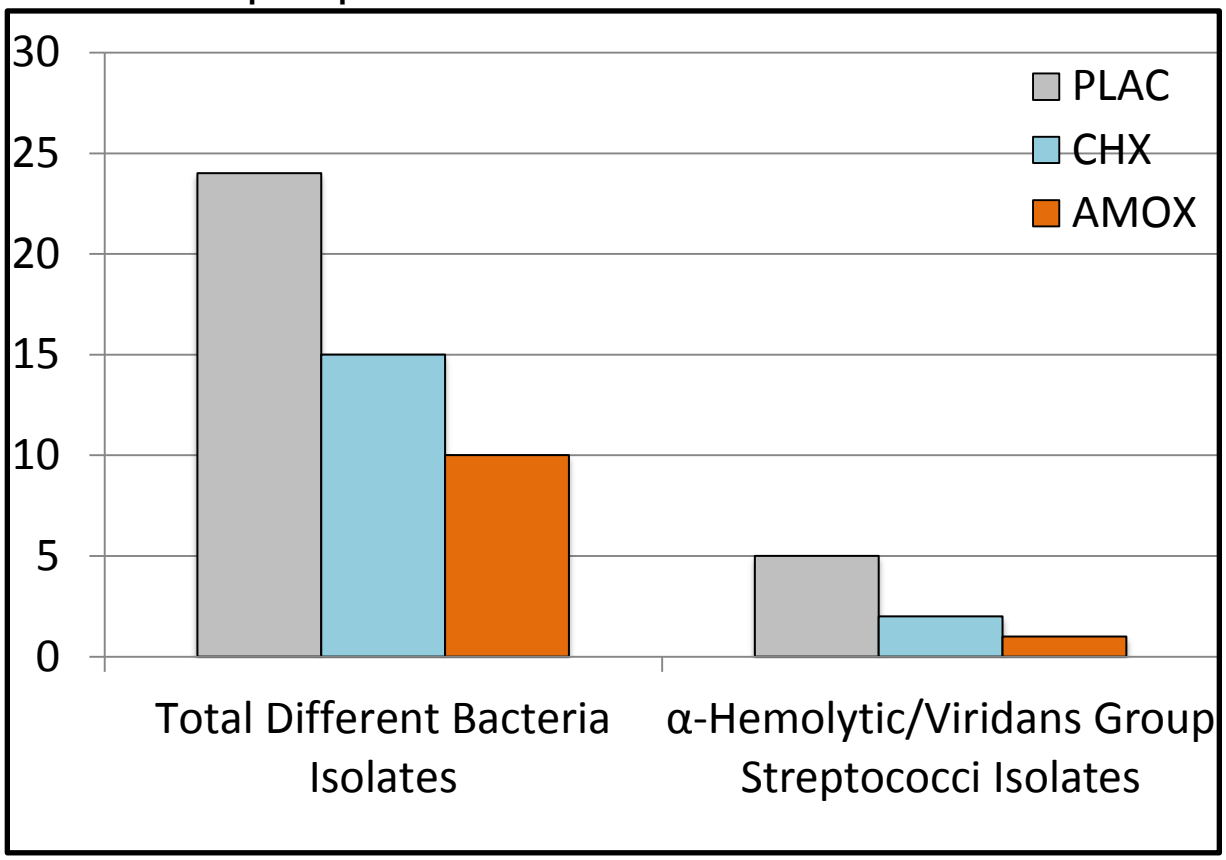


Figure 32: Total Different Bacterial Isolates and α-Hemolytic/Viridans Group Streptococci Isolates



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