"Answers in Hours" A Randomized Controlled Trial Using Microbiome Metagenomics for Bile Duct Cultures

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LIST OF ABBREVIATIONS

AE	Adverse Event/Adverse Experience
CFR	Code of Federal Regulations
CRF	Case Report Form
DSMB	Data and Safety Monitoring Board
FDA	Food and Drug Administration
GCP	Good Clinical Practice
HIPAA	Health Insurance Portability and Accountability Act
ICU	Intensive Care Unit
IDE	Investigational Device Exemption
IRB	Institutional Review Board
NS	Nanopore Sequencing
PHI	Protected Health Information
PI	Principal Investigator
SAE	Serious Adverse Event/Serious Adverse Experience
SOP	Standard Operating Procedure
SSI	Surgical Site Infection
TRAG	Tissue Request Acquisition Group
UADE	Unanticipated Adverse Device Effect

Study Summary

Title	"Answers in Hours" A Randomized Controlled Trial Using Microbiome Metagenomics for Bile Duct Cultures
Running Title	"Answers in Hours"
IRB Protocol Number	21-004234
Phase	Pilot
Methodology	Randomized 1:1 Controlled Trial
Overall Study Duration	3 years
Subject Participation Duration	90 days
Objectives	Reduce the rate of bacterobilia driven surgical site infection (SSI) in patients undergoing pancreatic head resection by providing surgical team with Oxford Nanopore (ONT) microbial sequencing data in the post-operative setting. Reduce cost of care through reduction in SSI and improved antibiotic stewardship.
Number of Subjects	140
Diagnosis and Main Inclusion Criteria	Any adult, male or female, over age 18, undergoing pancreaticoduodenectomy or total pancreatectomy for benign or malignant indication.
Study Device	GridION Sequencing Instrument
Reference therapy	Standard culture techniques and prolonged peri-operative antibiotics
Statistical Methodology	For continuous variables, t-test will be performed and for binary variables chi-square or fisher's exact testing will be performed as appropriate. Universal and multivariable linear regression adjustment will be performed if any baseline imbalances in demographic or clinical variables are detected

1 Introduction

This document is a protocol for a human research study. This study will be carried out in accordance with the procedures described in this protocol, applicable United States government regulations and Mayo Clinic policies and procedures.

1.1 Background

Postoperative infections, specifically surgical-site infections (SSI), are a significant source of morbidity in patients undergoing pancreatic head resection and have been reported in over 25% of patients [1,2]. Infectious complications significantly increase the risk of delayed gastric emptying, pancreatic fistula, biliary fistula, abdominal collection, sepsis, acute respiratory failure, pulmonary complications, cardiovascular complications, and mortality [3–5]. Patients who develop SSI are also less likely to receive adjuvant chemotherapy, more likely to experience delays in adjuvant therapy, and have reduced overall survival [6–9]. Overall, complications arising from SSIs have been shown to have a direct effect on mortality, varying from 33% to 77% increased risk of mortality [10], and 75% of SSI-associated deaths are directly attributable to the SSI [11–13].

The increased rates of morbidity and mortality associated with SSI have led to the current surgical perioperative antibiotic prophylaxis guidelines developed jointly by the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, the Surgical Infection Society, and the Society for Healthcare Epidemiology of America which recommend the administration of cefazolin, cefoxitin, cefotetan, ceftriaxone, ampicillin-sulbactam, clindamycin/ vancomycin + aminoglycoside or aztreonam or fluoroquinolone, or metronidazole + aminoglycoside or fluoroquinolone 60 – 120 minutes prior to surgical incision to reduce the risk of SSI during pancreatic/ biliary tract surgeries[14]. This has led to extensive 'blind' use of broad-spectrum antibiotics which are associated with their own risks. Excessive usage of broad-spectrum antibiotics contributes towards the emergence of antimicrobial resistance (AMR), and their usage has been associated with acute adverse events[15], and increased risk of developing sepsis [16], cancer [17–20] ,and heart disease[21–24], as well as other antibiotic associated complicated such as *Clostridium difficile* infections [25–28].

Many patients who undergo pancreatic head resection have obstructive jaundice prior to surgery due to compression or occlusion of the common bile duct. To alleviate these symptoms, patients often undergo preoperative biliary drainage, most commonly by insertion of a biliary stent. This creates a conduit between the biliary tree and the intestinal lumen, resulting in translocation of intestinal microbes into the normally sterile bile duct systems. Numerous studies have demonstrated and confirmed an association between preoperative biliary stenting and the occurrence of SSI [3,6–8,29], and as such, some institutions have opted to administer postoperative antibiotics[30–32,32–35]. In some cases, postoperative administration of broad-spectrum prophylactic antibiotics is performed without any assessment of the microbes present or sensitivities, largely due to the lack of available microbial testing infrastructure, large-volume, and nearby testing labs. In others, detection and characterization of biliary microbial contamination is currently achieved by performing standard bile cultures and

antimicrobial susceptibility testing (AST) on diverse polymicrobial community. Standard bile cultures, however, can take days to weeks to yield actionable results [36,37] and are typically received over the course of 2 – 4 days. Bile cultures are often polymicrobial, a mixture of anaerobic and aerobic species, carry multiple antimicrobial resistant (AMR) phenotypes, and contain fungal species [38–41]. This contributes towards delays in timely results as aerobic cultures (up to 5 days to complete), anaerobic cultures (up to 14 days to complete), and fungal cultures (up to 24 days to complete) must be performed, and additional testing must be carried out to determine the different microbial species present in the sample before AST can be carried out. Results are often generated over the course of several days, and this temporal separation between the initial broad-spectrum treatment and the acquisition of all the diagnostic information often results in uncertainty and the administration of multiple empiric antimicrobials [42].

At our institute the current standardized approach is administer a perioperative prophylaxis course of ceftriaxone plus metronidazole 60 minutes prior to surgery (Figure 1). Antibiotic treatment is continued for 5-days postoperatively for patients undergoing pancreatic head resection, and the selection and duration of this specific broad-spectrum prophylactic regiment is based on recommended surgical guidelines and our own historical SSI culture data [43]. During surgery, bile swab and aspirate samples are collected and transferred to the Pathology lab to undergo standard bile cultures and AST. If the bile cultures are eventually negative for microbial growth, the patient will be taken off the broad-spectrum antibiotics following completion of the 5-day prophylactic regimen (Figure 1). If the bile cultures are positive, then antibiotic treatment will be extended for 10 days, and therapy is tailored based on organisms and resistances identified in the bile cultures (Figure 1). Introduction of this approach in 2014 resulted in significant decrease in hospital stay, reduced the need for interventional radiology procedures, and decreased the risk of postoperative pancreatic fistula grade [43]. Within the first year of introduction, our approach significantly decreased inpatient cost of care, decreased the total 30-day costs, and saved a total of $1.1 million , in addition to improving patient outcome [43].

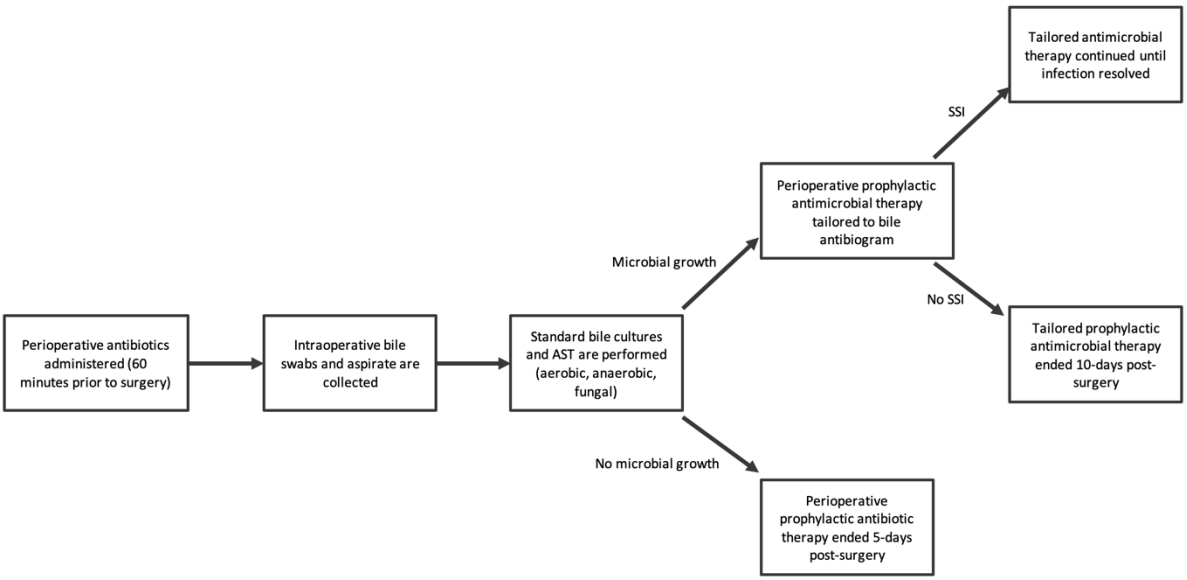

Figure 1: Schematic diagram of the current standardized perioperative prophylactic antibiotic treatment plan for patients undergoing total pancreatectomy and pancreaticoduodenectomy at our institution.

Since the introduction of our standardized approach, we have improved antibiotic tailoring by developing methods of increasing detection of anaerobic species through our use of bile aspirate in addition to collection of bile swabs [38]. However, similar to other institutions, it takes an average of 4 days for final bacterial culture results to be generated [44], and the majority of cultured microbial species have been found to exhibit multi-resistance phenotypes. This means that for the first 4-days post-surgery many of our patients at risk of developing SSI are receiving suboptimal antimicrobial therapy. This provides potential pathogens a window of opportunity to grow and propagate prior to introduction of tailored antimicrobial therapy. Rapid methods of detecting microbial species and antimicrobial resistance phenotypes are required to shorten this window of opportunity. Our data shows that in patients with positive bile cultures it took an average of 7 days to complete microbial identification and AST for bacterial and fungal species (range = 3 – 24 days), and it took 24 days to confirm the absence of biliary microbes (5 days for aerobic cultures, 14 days for anaerobic cultures, 24 days for fungal cultures). In particular, delays in detecting *Candida* species, was of concern due to the increased risk of SSI in patients with biliary candidiasis [45]. In our own patient cohort, 17% of patients were positive for biliary candidiasis, and the microbe was the presumptive cause of 22% of SSIs. In patients with biliary candidiasis, it took an average length of 4.6 days for fungal identification, and an additional 6 days for anti-fungal susceptibility testing to be complete. As anti-fungals are not part of the recommended perioperative prophylactic antimicrobial treatment course, it meant that that during the first 2 weeks post-surgery these patients were at increased risk of SSI due to untreated biliary candidiasis.

A single diagnostic test that would enable simultaneous identification of bacterial species (aerobic, anaerobic, fastidious), fungal species, and antimicrobial resistance in an expeditated

fashion would be highly advantageous. It would allow for all the microbial information to be generated in a single report, enabling the administration of optimal tailored antibiotics in the early postoperative window without the need for continuous changes to the treatment regimen as new information becomes available. It would also enable early cessation of antimicrobial prophylaxis if no microbes are detected, potentially reducing the complications associated with use of broad-spectrum antibiotics.

We hypothesized that use of metagenomic Oxford Nanopore (ONT) sequencing, a real-time method of sequencing that has been demonstrated to detect microbial species in as little as 6 hours [42,46,47], could be developed as a tool for rapid detection and characterization of biliary microbes. We have since developed methods of detecting intraoperative bile microbes, and have recently published the promising results of a prospective clinical trial that was performed to determine the feasibility and utility of ONT sequencing in detection of microbial species and antibiotic resistance genes using intra-operative bile from patients undergoing pancreatic head resection [44,48]. Our preliminary data would suggest that not only is this technique feasible, but our ONT sequencing results highly correlate with traditional culture microbial identification and does so with a median time of 14 hours compared to 98 hours for traditional culture, a turnaround time improvement of 92%. Furthermore, the results of our study found that when standard cultures (SC) were negative (i.e. no further antibiotic therapy needed) the ONT perfectly correlated. This suggested that ONT could be used to identify patients with negative biliary microbes, enabling a more rapid cessation of unnecessary antimicrobial therapy in patients. This would improve antibiotic stewardship, a continual goal in surgical patient care.

Given these promising preliminary results, we aim to reduce the rate of SSI while minimizing overutilization of antibiotic therapy in patients undergoing pancreatic head resection by providing supplemental ONT data in addition to standard clinical cultures. This will provide not only an important potential diagnostic, but will also elucidate microbial communities within contaminated bile, potentially leading to improved targeted approaches in the future.

1.2 Investigational Device

The investigation device being utilized for this research study is the Oxford Nanopore Technologies (ONT) R9.4.1 flow cells and the ONT GridION MK1 Sequencing Instrument (Device number GXB03025). ONT sequencing will be performed using MinKNOW (version 21.11.7), MinKNOW Core (4.5.4), Bream (6.3.5) and Guppy (5.1.13) software. Microbial species identification will be achieved using Porechop (0.2.4), BBMap (35.85) and Centrifuge (1.0.4) software, and detection of antimicrobial resistance genes will be achieved using ResFinder 4.1 software[49,50]. The ResFinder 4.1 tool, developed and maintained by the Center for Genomic Epidemiology (CGE), was selected following research that demonstrated it to be as reliable as standard antimicrobial susceptibility testing (broth microdilution, disc diffusion) for detecting clinically relevant AMR phenotypes [51]. Additionally, the tool utilizes a database of horizontally acquired chromosomal gene mutations that confer AMR, and thus use of ResFinder 4.1 will enable us to link predicted AMR phenotypes to specific microbial species.

To ensure that no potential resistance phenotypes are missed we will also use the PlasmidFinder (2.0.1)[52] and CARD (CARD 2020)[53] tools to check for any plasmid-acquired AMR phenotypes.

If there are new releases of the software prior to the trial, the software will be updated to the latest versions and all samples in the trial will be sequenced and analyzed using the same software versions.

1.3 Intended use of Investigational Device

The ONT GridION MK1 Sequencing Instrument (Device number GXB03025) will be used in conjunction with an optimized phenol chloroform DNA extraction to perform metagenomic sequencing on DNA extracted from intraoperative bile aspirate samples taken from patients undergoing total pancreatectomy and pancreaticoduodenectomy. The technology will utilize ONT sequencing and the data output will be basecalled read sequences in the fastq file format. Sequenced reads will be analyzed for microbial identification and AMR prediction.

1.4 Clinical Data to Date

This study will use data that has been collected under the following research study: "Comparative Analysis of Bile Microorganisms in Patients Undergoing Major Hepatobiliary Operations", IRB# 19-011419 and published in two peer-reviewed journals [44,48]. In brief, these studies involved analysis of intraoperative bile aspirate samples collected from 42 patients undergoing pancreatic head resection (total pancreatectomy, n = 9; pancreaticoduodenectomy, n = 33) at Mayo Clinic, Rochester (see Table 1 for patient characteristics). Metagenomic ONT sequencing was performed on bile aspirates to identify microbial species (bacterial, fungal) and antibiotic resistance genes. Sequencing results were then compared to bile cultures performed as part of Mayo Clinic's standard of care clinical practice to determine how ONT sequencing compared to current 'Gold Standard' culture practices [44,48].

To determine the optimum ONT protocol for detection of microbial species and antibiotic resistance genes present in the bile aspirates, different methods of DNA extraction, use of host DNA depletion, and use of different AMR gene databases were evaluated [48]. In total, intraoperative biliary microbes were detected in 54.7% (23/42) of patients undergoing total pancreatectomy or pancreaticoduodenectomy using SC techniques. This included 35.7% (15/42) of patients with positive bacterial cultures, 2.4% (1/42) of patients with positive fungal cultures, and 16.7% of patients with positive fungal and bacterial cultures. The male sex, use of biliary instrumentation, and performance of vascular reconstruction during surgery was significantly correlated with the detection of biliary microbes.

Table 1: Clinical characteristics of patients included in our preliminary study

Clinical feature	Total	Biliary microbes $^{+}$	Biliary microbes $^{-}$	*P value*
Age, mean (IQR)	61 (22 - 71)	66.3 (46.3 – 81.3)	58.0 (18 – 82.6)	0.0858
BMI, mean (IQR)	26.4 (19 – 40.4)	26.3 (19 – 40.4)	26.4 (19.7 – 36.2)	0.9295
Sex, % male (*n*)	54.8 (23)	38.1 (16)	16.7 (7)	0.0375
Surgery indication, % (*n*)				0.892
Pancreatic ductal adenocarcinoma	45 (29)	50 (21)	19.0 (8)	
Intraductal Papillary Mucinous Neoplasm	7.1 (3)	0 (0)	7.1 (3)	
Neuroendocrine tumors	11.9 (5)	2.4 (1)	9.5 (4)	
Other	11.9 (5)	2.4 (1)	9.5 (4)	
ASM Score, mean (IQR)	2.6 (1 – 4)	2.7 (2 – 4)	2.5 (1 – 3)	0.1874
Type of surgery				0.9716
Total pancreatectomy, % (*n*)	21 (9)	11.9 (5)	9.5 (4)	
Pancreaticoduodenectomy, % (*n*)	79 (33)	42.9 (18)	35.7 (15)	
Biliary instrumentation, % (*n*)	52 (22)	52.4 (22)	0 (0)	<0.0001
Vascular reconstruction, % (*n*)	38 (16)	31.0 (13)	7.1 (3)	0.0079

BMI, body mass index; *IQR*, interquartile range

Comparison of our optimized ONT sequencing protocol SC results revealed that our ONT protocol had identified 100% of biliary microbial contamination cases, detected 75% of cultured bacterial species and 76% of cultured fungal species, and predicted 81% of antibiotic resistance phenotypes observed using SC results [44,48]. With regards to detection of common biliary pathogens [3,54–57], our ONT protocol improved detection of biliary candidiasis, detected 100% of cases of biliary *Escherichia coli* and biliary *Klebsiella*, and detected 83% of biliary *Enterobacter*, 76.5% of biliary *Enterococcus*, 93.8% of biliary *Streptococcus*. However, ONT did not detect one case of *Pseudomonas* infection and one case of *Staphylococcus aureus*, indicating as proposed in this study that SC techniques should be run alongside ONT protocols to ensure that no microbial species are missed. As for detection of pathogens of high clinical concern (i.e. ESBL, CRE species), ONT similar detection rates compared to SC, and potentially improved detection of *E. coli, K. oxytoca, K. pneumoniae,* and *Citrobacter freundii*. Overall, ONT improved detection of microbial species (particularly with regards to anaerobic species and fungal species) and predicted a significantly higher number of antibiotic resistance phenotypes than were identified using SC. Significantly, the results turnaround for ONT sequencing was just 14 hours compared to an average turnaround time of 98 hours for SC. On average, it took 4.8 days (range = 1 – 15 days) to complete microbial identification and an additional 2 days (range = 0 – 9 days) to complete AST for the culture positive samples. Delays greater than 5 days in completing microbial identification occurred in samples with multiple aerobic species of the same genus (*Enterococcus* spp., *Klebsiella* spp., *Streptococcus* spp.), anaerobic species (*Schaalia (Actinomyces) turicensis, Finegoldia magna, Veillonella sp., Actinomyces johnsonii, Parvimonas micra*), and/ or fungal species Saccharomyces cerevisiae, *Candida* spp.), and on average, optimal antimicrobial therapy was delayed for 7 days. Use of a test that would enable microbial identification and prediction of AMR phenotypes within 24hrs post-surgery, would, therefore, have significantly shortened the delay in patients receiving optimized tailored antimicrobial therapy post-surgery.

Table 2: Detection of common biliary pathogens and pathogens of high clinical concern using standard culture (SC) and our Oxford Nanopore sequencing (ONT) protocol

Detection of common biliary microbes			
Microbial species	**Patients with positive SC result, *n* (%)**	**Patients with positive ONT sequencing result, *n* (%)**	**Patients with positive SC & ONT sequencing result, *n* (%)**
Enterobacter spp.	6 (14.3)	6 (14.3)	5 (11.9)
Enterococcus spp.	17 (40.5)	13 (31.0)	13 (31.0)
Escherichia coli	3 (7.1)	5 (9.5)	3 (7.1)
Klebsiella spp.	11 (26.2)	14 ()	11 (26.2)
Pseudomonas spp.	1 (2.4)	0 (0)	0 (0)
Staphylococcus aureus	1 (2.4)	0 (0)	0 (0)
Streptococcus spp.	16 (38.1)	19()	15 (35.7)
Candida spp.	5 (11.9)	7 (16.7)	5 (11.9)
Detection of pathogens of high clinical concern			
Enterobacter aerogenes	0 (0)	0 (0)	0 (0)
Enterobacter cloacae	5 (11.9)	6 (14.3)	4 (9.5)
Escherichia coli	3 (7.1)	5 (11.9)	3 (7.1)
Haemophilus influenzae	0 (0)	0 (0)	0 (0)
Klebsiella oxytoca	7 (16.7)	10 (23.8)	7 (16.7)
Klebsiella pneumoniae	4 (9.5)	7 (16.7)	4 (9.5)
Kluyvera species	0 (0)	0 (0)	0 (0)
Neisseria gonorrhoeae	0 (0)	0 (0)	0 (0)
Proteus mirabilis	0 (0)	0 (0)	0 (0)
Pseudomonas aeruginosa	1 (2.4)	0 (0)	0 (0)
Salmonella enterica	0 (0)	0 (0)	0 (0)
Citrobacter freundii	4 (9.5)	6 (14.3)	3 (7.1)
Serratia marcescens	3 (7.1)	1 (2.4)	1 (2.4)

SSI occurred in 21.4% (9/42) patients within 30 days post-surgery, and an additional two patients developed SSI 34 days post-surgery and 42 days post-surgery, respectively. In total, 44% (4/9) of these patients had positive intraoperative SC and ONT results, and 22% (2/9) of patients had positive post-operative abdominal fluid cultures. In the cases where post-operative organisms were detected, *Candida albicans* was the most commonly detected (2/4 patients) followed by *Enterococcus faecalis* (1/4 patients), highlighting the importance of improved

detection of *Candida* species in these patients. Comparison of SC and ONT predictive power found no difference with regards to identifying patients at risk of SSI.

1.5 Study Rationale and Risk Analysis (Risks to Benefits Ratio)

1.5.1 Study Rationale

SSIs are one of the most common and costly of hospital-acquired infections within the US, accounting for 20% of all hospital-acquired infections[13]. A single SSI increases hospital stay by an average of 9.7 days and accounts for a 2- to 11-fold increase in the risk of mortality [13]. Patients with pancreatic cancer are clinically vulnerable and have a particularly high risk of SSI following pancreatic head resection, with global incidence rates ranging from 25 – 45% of patients undergoing the procedure [1,2]. The occurrence of a SSI increases the rate of mortality and the need for additional invasive procedures, and it can result in delay or failure to complete adjuvant chemotherapy [9,58].

Rapid etiologic diagnosis of biliary microbial contamination can facilitate timely and rational post-operative antimicrobial therapy, reducing the risk of a SSI developing. SCs, however, can take many days to return actionable results[36,37] and are typically received over the course of 4-7 days. Bile cultures are often polymicrobial, a mixture of anaerobic and aerobic species, carry multiple antibiotic resistant phenotypes, and contain fungal species [38–41]. This contributes towards delays in timely results, and the temporal separation between the initial broad-spectrum treatment and the acquisition of all the diagnostic information often results in uncertainty and the administration of multiple empiric antimicrobials[42].

Next-generation sequencing platforms, such as Illumina and ION Torrent, have been widely used in the identification and characterization of microbial species, including previous characterization of the bile microbiome in healthy and diseased states [59,60]. However, in practice, these technologies require at least 16 hours for the sequencing run alone and analysis of the sequencing data can only be performed once the sequence run has concluded[47,61].

ONT sequencing has been demonstrated to successfully characterize microbial populations present in a range of clinical samples and situations in as little as 6 hours [42,46,47,62–77]. Moreover, we have previously demonstrated that ONT sequencing provides a rapid, comprehensive, and accurate profile of microbial pathogens and antibiotic resistance phenotypes present in intraoperative bile aspirates matching SC techniques [44,48]. Collectively, this suggests that the incidence rate of SSIs occurring in patients undergoing pancreatic head resection may potentially be reduced by the use of ONT sequencing to rapidly characterize intraoperative biliary microbial contamination and guide early targeted antimicrobial therapy post-surgery, more rapidly than SCs Additionally, use of ONT sequencing would improve antibiotic stewardship by reducing the use of broad-spectrum antibiotics, and patients would benefit either by receiving a more targeted antimicrobial approach or by being taken off unnecessary broad-spectrum antibiotics earlier which currently is not possible, subsequently reducing the

risk of clinically relevant antibiotic-associated adverse events. Clinical risk to the patient is minimal.

1.5.2 Anticipated Risks

ONT sequencing will be utilized alongside current SCs. We will use SC to ensure that the care patients receive will be no worse than current practice. In other words, culture will ensure that ONT only improves patient care and outcome. In the event that ONT results and culture assessment contradict, the care plan will prioritize the results of standard culture in the event of treatment failure (Table 3). The risks associated with participation in this study are no greater than for those patients not on the study also undergoing identical operations. The only therapeutic interventions this trial includes is alteration or cessation of postoperative antimicrobial therapy based on either ONT or SCs. In order to minimize any theoretical risks in the ONT arm, such as missed microbial species and/or antibiotic resistance phenotype or ineffective antibiotic regimen, SC data will be utilized in both arms and antibiotic therapy will be adjusted if SCs suggest broader coverage (Table 3). There will be no alteration in the standard clinical postoperative care of patients in this study.

Table 3: Incorporation of ONT results in clinical management decisions

ONT Result	Clinical management decision
A negative ONT result	Discontinue antibiotics after 24 hours of peri-operative coverage
A positive ONT result for a single pathogen typical of biliary or gastrointestinal flora	Adjust and/or narrow antibiotic therapy to cover single pathogen. If single pathogen deemed clinically insignificant (ie. lactobacillus), discontinue antibiotics after 24 hours of peri-operative coverage.
A positive ONT result for multiple pathogens	Adjust and/or narrow antibiotic therapy to cover all pathogens, excluding those deemed clinically insignificant. If all pathogens are considered clinically insignificant, discontinue antibiotics after 24 hours of peri-operative coverage.
A positive ONT result positive for rare or unexpected flora	Adjust and/or narrow antibiotic therapy to cover rare or unexpected flora, excluding those deemed clinically insignificant.
ONT results different from SC	Adjust and/or narrow antibiotic therapy to cover pathogens found in ONT results, excluding those deemed clinically insignificant. SC results to only be considered in the event of treatment failure with antibiotic therapy targeted towards ONT results.

1.5.3 Potential Benefits

Potential benefits associated for patients who participate in this study include:

- Earlier diagnosis of biliary microbial contamination
- Earlier targeted antimicrobial therapy post-surgery

  - Reduces window of opportunity for pathogenic species to propagate and cause infection
  - Reduced risk of SSIs
  - Potential candidate for earlier chemotherapy
- Earlier cessation of unnecessary antimicrobial therapy following negative result of biliary aspirates
  - Cost saving from stoppage of IV antibiotic administration
  - Potential candidate for earlier chemotherapy
  - Reduced gut microbiome disruption and decreased risk of adverse events following antibiotics
- Wider impact – reduction of broad-spectrum antibiotic usage, subsequently improving antibiotic stewardship and avoid antibiotic associated complications

The technology will improve antibiotic stewardship by rapidly identifying which patients require antimicrobial therapy, reducing time spent on broad-range antimicrobial therapy, and decreasing the risk of emerging resistance by ensuring that patients who do require antimicrobial therapy are on an optimal treatment regimen. Patients will directly benefit through early diagnosis of biliary microbial contamination. This will enable patients who require antimicrobial therapy to receive early targeted antimicrobial therapy post-surgery, potentially reducing the risk of infection by decreasing the window of opportunity for pathogenic species to propagate. Patients who do not require antimicrobial therapy will benefit by being taken off therapy earlier, reducing patient exposure to broad-spectrum antibiotics and the risk of adverse events and long-term health impacts associated with unnecessary antimicrobial therapy.

The data from this study will provide an important scientific characterization of the biliary flora in patients undergoing pancreatic head resection. Given the high risk of infection, better characterization of these microbes using untargeted approaches will provide a more complete picture of potential pathogen risks on a population level. The scientific knowledge gained in this study may influence future studies and therapeutic possibilities, especially with regard to underassessed species such as fungi and obligate anaerobes.

1.6 Anticipated Duration of the Clinical Investigation

The anticipated duration of this study is 2 years. Direct participation for participants lasts until surgical specimens have been obtained. Additional follow-up data will be collected from the medical record for up to 90 days following the procedure.

2 Study Objectives

2.1 Hypothesis:

The use of ONT sequencing, a novel metagenomic technique, will result in faster identification of organisms from intra-operatively collected bile samples and allow for early treatment of biliary microbial contamination. Supplementing SC with ONT sequencing data will allow patients to be treated earlier in the post-operative setting with tailored antibiotics, which will reduce the rate of biliary microbial contamination related SSIs in patients undergoing pancreatic head resection as compared to SC alone.

2.2 Primary Objective

To improve antibiotic stewardship (reducing duration of peri-operative prophylactic antibiotic regimen, reducing administration of broad-spectrum antibiotic) by providing surgical team with rapid ONT sequencing data in the early post-operative setting.

Success Criteria: Significantly reduced antibiotic regimen duration and use of broad-spectrum antibiotics, corrected for key co-variates ($p<0.1$).

2.2 Secondary Objective

To reduce the cost of care through reduction in SSI and improved antibiotic stewardship.

Success Criteria: Significantly reduced rates of SSI and reduced total hospital costs, corrected for key co-variates ($p<0.1$).

2.1 General Design

This will be a prospective 1:1 randomized controlled trial with the following study arms:

Arm 1*: Patient Group 1*

Standard Care Only. Patients will undergo intra-operative bile aspiration (as is routinely performed) with SC of the aspirate. All patients will receive standard prophylactic antibiotics according to practice standards and will be continued for up to 5 days as is routine. If bile cultures are positive, antimicrobials will be continued for a complete 10-day course of antibiotics (Figure 2).

Arm 2*: Patient Group 2*

Standard Care + ONT. Patients will undergo intra-operative bile aspiration (as is routinely performed) with standard cultures AND ONT sequencing of the aspirate. All patients will receive standard prophylactic antibiotics according to practice standards. ONT sequencing results will be made available to providers through electronic medical record (EMR) and team electronic notifications. Patients with negative ONT results will be taken off the standard prophylactic antibiotics and patients with positive ONT results will have antimicrobial therapy adjusted according to ONT sequencing results. However, if SCs are positive or suggest alternative/broader coverage then these results

will guide therapy and not ONT results. If bile cultures are positive, antimicrobials will be continued for a complete 10-day course of antibiotics (Figure 2).

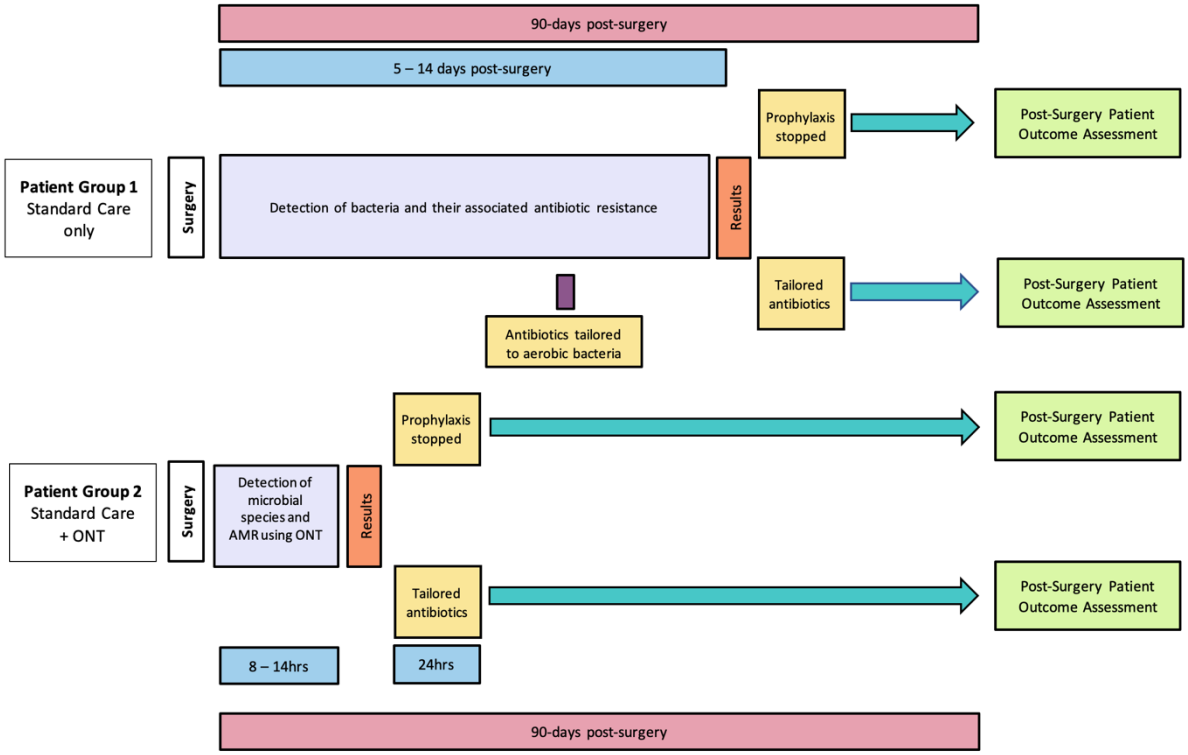

Figure 2: A schematic diagram of our proposed study to investigate the use of Oxford nanopore sequencing to detect and characterize biliary microbial contamination

The study will include patients undergoing elective pancreaticoduodenectomy or total pancreatectomy for any clinical indication. Patients will be excluded from the study if they are < 18 years of age, enrolled in any similar clinical trials involving use of peri-operative antibiotics, are undergoing distal pancreatectomy, or undergoing emergency total pancreatectomy or emergency pancreaticoduodenectomy (Table 4). Following recruitment to the study, patients will be randomly assigned intraoperatively/post-operative to either the control group (Arm 1) or the interventional group (Arm 2). We will use stratified randomization with age (below 65 years vs 65 years and above), sex, operation type (pancreaticoduodenectomy vs total pancreatectomy), biliary stenting (yes vs no), and patient ASA score as stratification factors.

Table 4: Patient inclusion and exclusion criteria for the clinical trial

Inclusion Criteria	• > 18 years of age • Scheduled for elective total pancreatectomy or pancreaticoduodenectomy
Exclusion Criteria	• < 18 years of age

	• Emergency total pancreatectomy or pancreaticoduodenectomy • Patients scheduled for distal pancreatectomy • Women who are pregnant • Patients without the cognitive capacity to consent • Patients enrolled in similar clinical trials involving use of perioperative antibiotics

Bile will be collected via bile duct aspiration. Bile aspiration will be performed with a 16-gauge needle and 3 cc syringe prior to bile duct division (Figure 3), and aspirated material will be aseptically transferred into sterile aerobic and anaerobic transport vials. Samples will be concurrently transported to the Pathology lab and the microbiome lab to undergo SC techniques and ONT sequencing, respectively. Standardization of bile collection and surgical procedure will be assured as all surgeries will be performed by the same lead surgeon, Dr. Mark Truty. Surgical variables that have been associated with increased SSI risk will be recorded. This will include patient sex, age, BMI, ASA score, the volume of blood loss, whether the patient had a biliary stent inserted prior to surgery, closure technique, and whether vascular reconstruction was performed during surgery.

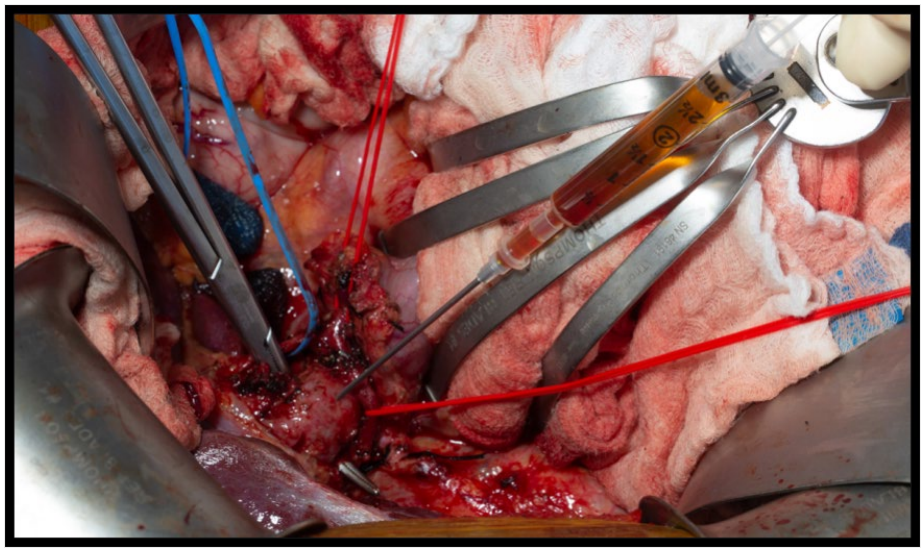

Figure 3: Collection of intraoperative bile aspirate during pancreatic head resection (total pancreatectomy, pancreaticoduodenectomy)

SCs obtained from intraoperative bile aspiration for both study arms will be performed in compliance with clinical standards. This is routinely performed by the Pathology lab and no changes to these protocols are necessary (see Supplementary Materials S1 – S6) for culture methods and AST protocols). Results will be reported in the EMR which will be accessible by the clinical team, as is the current clinical practice. ONT sequencing for those assigned to Study Arm 2 will be performed on a 1cc liquid bile sample collected fresh from the operating room after specimen release from the Pathology lab. Approval from our institutional TRAG

(Tissue Request Acquisition Group), will be requested for managing of intraoperative samples and will notify the study team of sample receipt. After collection, microbial DNA will be extracted and sequenced in preparation for reading per protocols [44,48]. In brief, Porechop and BBMap will be used to remove adaptor sequences and low complexity reads. The filtered reads will then be aligned using Centrifuge in order to determine species identification. Bile aspirates will be declared microbial positive when 500 or more microbial reads are detected. Additionally, 50 reads per million (RPM) ratio (RPM-r), defined as RPM-r = RPM_{sample} / $RPM_{negative\ control}$, will be used as a minimum threshold to reduces biases caused by different sequencing depth and mitigate concerns regarding potential microbial contamination, as described in previous studies [47,73,78,79]. Relative abundance of the microbial species detected will then be determined and high abundant species (species with a relative abundance greater than 1%) will then be reported to the care team. The sequenced reads will also be used to identify acquired chromosomal AMR genes and putative AMR phenotypes using ResFinder 4.1[51]. A minimum coverage of 95% and a minimum sequence identity of 95% will be used, and predicted phenotype and source organism of the identified AMR gene will be reported to the care team. To ensure that no potential resistance phenotypes are missed we will also use the PlasmidFinder and CARD tools with the default settings to check for any plasmid-acquired AMR phenotypes. This will be a critical step as CRE and ESBL genes are located on plasmids. However, due to these genes being located on plasmids, use of PlasmidFinder and CARD will not be used to declare presence of CRE- and/or ESBL-organisms but instead inform the Surgical Clinical Pharmacy and the Pathology laboratory about their potential presence in the patient sample

After sequencing data is gathered, samples will be destroyed. The data results from the ONT sequencing testing will be entered into EMR as a research clinic note for documentation and results will additionally be emailed to the medical providers on the care team. Decisions on antibiotic regimen will be made with the assistance of Surgical Clinical Pharmacy with changes in regimens as deemed appropriate based on standard cultures and ONT results, as described in Table 5. A negative ONT result will result in discontinuation of perioperative antibiotics after 24 hours while a positive ONT result will be used to guide early targeted antibiotic therapy (Table 5). With regards to early discontinuation of antibiotics, we are confident that this will not adversely impact patient care as our preliminary data demonstrated that ONT resulted in no false negative calls. However, as this is a new technology, SC will be performed alongside ONT, and if microbial growth is detected in the cultures, the clinician will have the option of reverting patient care to current standard of practice. This option will also be available in the event of treatment failure in patients with positive ONT results (Table 5).

Given that currently clinically available tests will be available to the care team with the additional ONT sequencing as supplemental data there will be minimal risk to subjects participating in this study.

Table 5 Incorporation of ONT results in clinical management decisions

ONT Result	Clinical management decision
A negative ONT result	Discontinue antibiotics after 24 hours of peri-operative coverage

A positive ONT result for a single pathogen typical of biliary or gastrointestinal flora	Adjust and/or narrow antibiotic therapy to cover single pathogen. If single pathogen deemed clinically insignificant (ie. lactobacillus), discontinue antibiotics after 24 hours of peri-operative coverage.
A positive ONT result for multiple pathogens	Adjust and/or narrow antibiotic therapy to cover all pathogens, excluding those deemed clinically insignificant. If all pathogens are considered clinically insignificant, discontinue antibiotics after 24 hours of peri-operative coverage.
A positive ONT result positive for rare or unexpected flora	Adjust and/or narrow antibiotic therapy to cover rare or unexpected flora, excluding those deemed clinically insignificant.
ONT results different from SC	Adjust and/or narrow antibiotic therapy to cover pathogens found in ONT results, excluding those deemed clinically insignificant. SC results to only be considered in the event of treatment failure with antibiotic therapy targeted towards ONT results.

Databases Used

For the analysis of our endpoints, we are relying on sequence homology for two main tasks, mainly, (1) the identification of species and (2) identification of AMR phenotypes. For species identification, we use the refseq genome database (downloaded on 12/18/2020) with all data from the following higher order taxa groups included: human, protozoa, viral, bacterial, and fungal. For the identification of AMR phenotypes, we use the ResFinder database (downloaded on 06/04/2021, version 4.1) which contains the acquired chromosomal resistance genes, ensuring we know which species is carrying a given resistance gene. For plasmid AMR gene identification, we will use the PlasmidFinder-specific database (downloaded on 7.14.2021/version 2.0.1) and the CARD data (downloaded on 07/07/21, version CARD 2020). These databases undergo regular updates since they were first developed. To ensure the analysis remains consistent throughout the study, databases will not be updated for the duration of the study. In the unlikely event that we require a software/ database update in order to run these tools (compatibility issues with future institutionally required OS update, inability to restore databases from archives after computational disaster), we will validate any newer versions of the database and/or algorithms using the data and culture gold standards from our published study and recently collected data and will only proceed if we have comparable performance in accuracy for species and AMR identification.

Patient Follow-up and Safety Monitoring

Direct study participation will last until surgical samples have been collected. Follow-up data will be collected from the medical record for 90 days following the surgical procedure in order to satisfy the study objectives. Event monitoring will be daily while inpatient and weekly following patient discharge (Figure 3). Adverse events will be assessed daily, including occurrence of SSI, type of SSI, and severity of SSI (Table 6). Serious Adverse events will be reported to the IRB following Mayo Clinic policies and FDA guidances. In the event of a SSI, we will treat and monitor appropriately using standard care procedures which include additional culture testing, drainage, and imaging.

Table 6: Classes of surgical site infections that will be assessed and recorded

Surgical Site Infection Class	**Surgical Site Infection definition**
Superficial incisional	Only skin and subcutaneous tissue involvement
Deep incisional	Facia or muscular layers
Organ space	Involvement of any part of the body that was opened or manipulated during the surgical procedure

The overall outcome of the study will be statistically assessed every 2 weeks and any statistically significant differences between the study arms will be reported to the data safety board (p-value < 0.05 for the primary outcome or SSI rate). Criteria for stopping the study will include significantly worse patient outcomes ($p<0.05$) in Arm 2 of the clinical trial (Table 7), significant deviations from the protocol as determined by IRB, or any other safety issues identified by the data safety monitoring board.

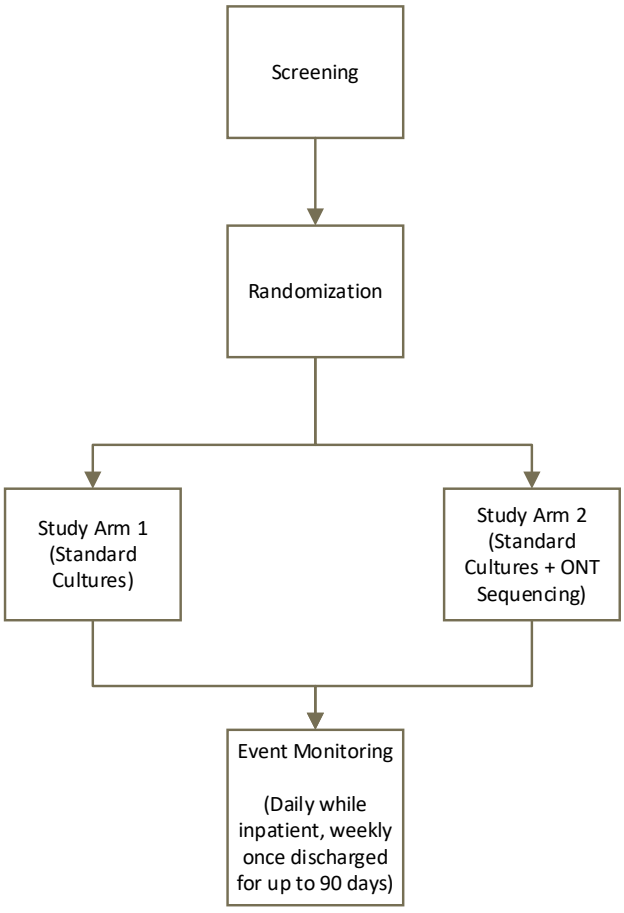

Figure 3: A schematic figure of the clinical trial protocol

Table 7: Stopping Rules-Clinical criteria for stopping the clinical trial

Patient Outcome	Criteria for ending the clinical trial
Mortality	Rates of mortality significantly higher in ARM 2 of the clinical trial ($P < 0.05$)
Morbidity	Rates of morbidity significantly high in Arm 2 of the clinical trial ($P < 0.05$)
SSI	Rates of SSI significantly higher in ARM 2 of the clinical trial
Antibiotic adverse events	Number of antibiotic adverse events significantly higher in ARM 2 of the clinical trial ($P < 0.05$)
Pancreatic fistula	Rates of pancreatic fistula significantly higher in ARM 2 of the clinical trial ($P < 0.05$)
Biliary fistula	Rates of biliary fistula significantly higher in ARM 2 of the clinical trial ($P < 0.05$)
Intra-abdominal abscess	Rates of intra-abdominal abscess significantly higher in ARM 2 of the clinical trial ($P < 0.05$)
Pulmonary complications	Rates of pulmonary complications significantly higher in ARM 2 of the clinical trial ($P < 0.05$)
Cardiovascular complications	Rates of cardiovascular complications significantly higher in ARM 2 of the clinical trial ($P < 0.05$)

Data Safety Monitoring Board

The Department of Surgery Data Safety Monitoring Board will be responsible for analysis of the results on a bi-yearly basis (every 6 months) in order to evaluate outcomes as the is enrolling.

2.2 Primary Study Endpoints

- Reduced administration of antibiotics with a non-significant ($p>0.05$) effect on SSI rate

2.3 Secondary Study Endpoints

- Cost effectiveness (reduced total hospital costs)
- Timeliness of sample analysis
- Reduction in SSI

3 Subject Selection, Enrollment and Withdrawal

Any adult, male or female, over age 18, undergoing pancreaticoduodenectomy or total pancreatectomy for benign or malignant indication.

Target accrual: 140 patients.

3.1 Inclusion Criteria

- ≥18 yr M or F
- Undergoing pancreaticoduodenectomy or total pancreatectomy for any benign or malignant indication with informed consent

3.2 Exclusion Criteria

- Women who are pregnant
- Patients who are institutionalized or incarcerated
- Patients without the cognitive capacity to consent
- Patients undergoing emergency pancreaticoduodenectomy or total pancreatectomy
- Patients enrolled in similar clinical trials involving use of perioperative antibiotics

3.3 Subject Recruitment, Enrollment and Screening

Patients scheduled for pancreaticoduodenectomy or total pancreatectomy for benign or malignant indication at Mayo Clinic Rochester will be approached by the study team prior to their surgery if they meet the enrollment criteria. Recruitment methods may include electronic invitations to the study through the patient portal or email. After receipt of the initial study invitation, a study coordinator will contact the patient by phone to review the study and answer any questions. If the patient is interested in participating, the study coordinator will consent the patient via paper or electronic consenting methods.

3.3.1 When and How to Withdraw Subjects

Participants will be withdrawn from the study if they no longer meet enrollment requirements or wish to withdrawal consent. If a participant chooses to withdraw their consent, no additional testing or data collection will be performed.

3.3.2 Data Collection and Follow-up for Withdrawn Subjects

Additional data collection during the follow-up period will not occur for subjects who have been withdrawn from the research study.

4 Study Device

4.1 Description

Please refer to the manufacturing information/instructions for use provided by Oxford Nanopore Technologies for full device description. Briefly, the GridION MK1 is a ONT sequencing device that will be used to examine microbial DNA in bile cultures from subjects undergoing pancreatic head resection. The same GridION instrument (device ID GXB03025)

will be used to test each sample. Serial number/lot number information for the sterile, single time use flow cells/ reagents will be recorded on a device accountability log. MinKnow, Guppy, Porechop, BBMap, Centrifuge, and ResFinder 4.1 software will be used for data analysis. The GridION device will be stored in a secure laboratory and only be accessible to trained study staff. In the event the GridION needs replacement, we will contact the manufacturer for a replacement and validate performance using residual DNA from our published studies. A GridION instrument will be considered validated if microbial species are detected in the expected proportions. We will then record the new GridION serial number and date put into service for this study.

ONT sequencing and analysis will only be performed by our Postdoctoral Fellow trained in the technology, and there are no medical or surgical procedures involved in the use of the device

4.2 Method for Assigning Subjects to Treatment Groups

Two potential confounding variables exist in this patient population: operation type (pancreaticoduodenectomy vs total pancreatectomy) and pre-operative biliary stenting. Therefore, to eliminate potential bias between arms, patients will be stratified according to operation type (pancreaticoduodenectomy/total pancreatectomy) and biliary stenting (Y/N). Stratified patients will be assigned to arms by the study team using Medidata Rave with pre-determined assignments.

Given the prospective nature of this study, it will include subjects previously diagnosed with a specific disease and add newly diagnosed subjects in the future as patients are accrued.

4.3 Masking/Blinding of Study

This is an open, randomized trial. Subjects and study team members will not be blinded.

4.4 Storage

The GridION sequencing instrument will be located in Dr.Walther-Antonio 's lab. The device will only be accessible by study staff.

4.5 Cleaning/Sterilization Procedures (optional)

DNA extraction and sequencing library preparation will be performed in a UV biosafety cabinet category 2 hood to ensure sterile conditions throughout the library preparation steps. Prior to sequencing Flush Buffer will be loaded into the ONT R9.4.1 flow cells in order to remove the storage solution prior to use. To ensure that no cross-contamination occurs, a new, sterile flow cell will be used for each patient.

5 Study Procedures

5.1 Enrollment Visit

At the time of enrollment, the informed consent will be reviewed with the participant and informed consent will be obtained.

Consented patients will be de-identified in compliance with the Health Insurance Portability and Accountability Act of 1996 (HIPAA) and patient identifiers will be stored electronically with access limited to members of the research team on a need-to-know basis. Patient demographics and baseline collection information will be gathered from the participant's medical record and documented in the Medidata Rave database. Demographics and baseline collection data collection will include age, sex, indication for surgery, ASA, major-comorbidities, pre-operative biliary stenting/manipulation, and pre-surgical cholangitis, etc.

5.2 Sample Collection Visit

Following enrollment procedures, 2 cc of residual bile collected during surgery will be aliquoted into cryovial and placed on ice (not frozen). It will be picked up by the study team at the time of collection for ONT sequencing. Tissue Request Acquisition Group (TRAG) approval will be requested for managing of samples. The sample will be aliquoted from the clinical collection and so collection will not add additional time to the surgery procedure. Participants will be randomized in Medidata Rave to a study arm based on operation type and pre-operative biliary stenting using a dynamic stratification. Participants will be randomized in a 1:1 ratio.

5.3 Follow up Data Collection

Outcomes will be abstracted from the medical record for 90-days post-operatively and will include outcomes of primary endpoints that include: duration of perioperative antibiotic regimen, total number of antibiotics administered, and administration of broad-spectrum antibiotics vs narrow-spectrum antibiotics. Secondary endpoints will include SSI rate, SSI severity (superficial, deep, organ/space, bloodstream, or urine), and total hospital costs. Standard post-pancreatectomy surgical outcomes will also be collected including: total hospital length of stay (days), ICU length of stay (days), need for and type or interventional procedures, pancreas-specific complications (pancreatic leak/grade, delayed gastric emptying, and post pancreatectomy hemorrhage) hospital readmission, and death. In addition, all culture results and ONT sequencing results will be captured and compared. Additional clinical data will be gathered if deemed appropriate for outcome analysis. Patients will be assigned a study identification number at the beginning of the study to aide in securing identifiable patient information. All patient information will be stored in a secure Medidata Rave database that

will be accessed by designated study members only. Statistical analysis will be performed to compare outcomes between study arms as described in analysis section of this application.

6 Statistical Plan

6.1 Sample Size Determination

Data for power analysis based on pilot study data (IRB# 19-011419). Power analysis is based on improvement in antibiotic stewardship with the following assumptions:

- Treatment groups are random and independent
- Microbial detection positivity rate using standard cultures = 54%
- Microbial detection positivity rate using nanopore sequencing = 77%

Analytical method:

- Sample size per group = 66 (total N = 132), Target size=70 per arm, total N=140
- Power = 80%, alpha (type I error) = 0.05, beta (type II error) = 0.2

6.2 Statistical Methods

Descriptive Statistics

The primary analysis of this study will be a modified intention to treat analysis (ITT), performed to determine how use of ONT changed patient treatment, i.e. did use of ONT improve antibiotic stewardship. Proportions of patients with detection of microbes of interest will be estimated along with exact 95% binomial confidence intervals. Comparisons of the proportions between the two study arms will be compared using Fisher's exact test or Chi-square test as appropriate. We will calculate the time from randomization to first microbial detection within a patient. We will report the mean difference in time to first microbial detection within 96 hours post randomization in both treatment arms, together with a 95% confidence interval. Comparison of time to first microbial detection within 96 hours post randomization, will be performed using Wilcoxon rank sum test or two sample t-test as appropriate. If there are any baseline imbalances in demographic or clinical variables between the two groups, we will perform the analysis using univariable and multivariable linear regression adjusting for these variables with imbalances as possible confounders. Duration of antibiotics, total number of different antibiotics, and broad-spectrum antibiotics vs narrow-spectrum antibiotics administered in the two arms will be compared to determine if use of ONT improved antibiotic stewardship by significantly reducing the duration of perioperative antibiotics and use of broad-spectrum antibiotics

Secondary analysis will evaluate SSI's will be evaluated and classified using ACS NSQIP definitions and data collected will include mortality rate, SSI incidence rate, ICU length of stay, total length of hospital stay, need for interventional procedures, and hospital readmission. Statistical analysis will be performed by the study team to determine if use of ONT significantly improved patient outcome compared to patients who received standard of care

only. The secondary endpoint of the study is a binary endpoint i.e. whether patients develop SSI (yes, no). Association between the secondary outcome and predictor variables will be assessed using univariable and multivariable logistic regression.

In both primary and secondary analysis, the following variables will be assessed for inclusion in the regression models

- Age at time of surgery DOB
- BMI at time of surgery RAVE calculate
- Sex
- Diabetes mellitus co-morbidity at time of surgery (clarifications needed for data collection)
- Cancer diagnosis
- ASA score at time of surgery
- Administration of chemotherapy prior to surgery (from what timepoint or date of last chemo?)
- Presence of biliary stent at time of surgery
- Blood loss during surgery
- Length of surgery (skin to skin, open to close?)
- Vascular reconstruction (arterial, venous) during surgery
- Closure technique
- Pancreatic leak risk score (when done? In record?)
- Surgical drains (what is needed)
- Post-op TPN

Variables with p-values less than 0.15 will be considered as potential candidates for a multivariable model. We will report odds ratios and 95% confidence interval. The area under the receiver operating characteristic (ROC) curve will be estimated as a measure of the ability of the model to discriminate between patients who had SSI within 90 days versus those who did not. An area under the ROC estimate of 0·7–0·8 will be regarded as acceptable, 0·8–0·9 will be regarded as excellent, and more than 0·9 will be regarded as outstanding.

We will also assess cost effectiveness of using ONT compared to SC. To achieve this, we will compare the cost to perform SC testing and ONT testing against costs associated with post-operative patient care. This will include the total cost to administer perioperative antibiotics, costs associated with length of hospital stay, and the costs associated with the need of additional procedures and tests associated with SSI (i.e. post-operative SC and antibiotic susceptibility testing, additional antibiotics, wound debridement, abdominal abscess drainage, CT scan, US scan).

For continuous variables, t-test will be performed and for binary variables chi-square or fisher's exact testing will be performed as appropriate. Additional analysis will be performed as necessary to determine differences in study arms. Should additional analysis beyond the scope of our abilities is necessary then a statistician will be employed.

Inpatient related costs associated with each intervention arm will be analyzed and compared with the assistance of the Robert D. and Patricia E. Kern Center for Science of Health Care in order to understand the economic impact of each intervention arm.

Handling of Missing Data

Missing data could arise from lost samples or from a catastrophic failure of computer storage and backup systems. If any of these were to occur before an intervention could be determined using ONT sequencing, we will note these events and revert the patient to Standard of Care group.

Primary Hypothesis:

The use of ONT sequencing, a novel metagenomic technique, will result in more rapid identification of organisms in intra-operatively collected bile samples and allow for earlier treatment of biliary microbial contamination. Supplementing SC with ONT sequencing data will allow patients to be treated earlier in the post-operative setting with tailored antibiotics, which will reduce the rate of biliary microbial contamination related SSIs in patients undergoing pancreatic head resection as compared to standard cultures alone and will improve antimicrobial stewardship by avoiding unnecessary use of antibiotics.

7 Safety and Adverse Events

7.1 Definitions

Unanticipated Adverse Device Effect (UADE)
A UADE is any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with, a device if that effect, problem or death was not previously identified in nature, severity, or degree of incidence in the investigational plan or IDE application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects.

Adverse Effect (Event)
Any untoward medical occurrence in a subject involved in clinical study of an investigational device; regardless of the causal relationship of the problem with the device or, if applicable, other study related treatment(s).

Associated with the investigational device: There is a reasonable possibility that the adverse effect may have been caused by the investigational device.

Life-threatening adverse effect: Any adverse effect that places the subject, in the view of either the investigator or the sponsor, at immediate risk of death from the effect **as it occurred**.

It does not include a reaction that, had it occurred in a more severe form, might have caused death.

Serious adverse effect: An adverse effect is considered "serious" if, in the view of either the investigator or the sponsor, it results in any of the following outcomes:

- death
- a life-threatening AE
- inpatient hospitalization or prolongation of existing hospitalization
- a persistent or significant disability/incapacity
- a congenital anomaly/birth defect.

Unanticipated adverse effect: Any adverse effect, the nature, specificity, severity, or frequency of which is not consistent with the risk information in the clinical study protocol or elsewhere in the current IDE application.

General Physical Examination Findings
At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

Hospitalization, Prolonged Hospitalization or Surgery
Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as an unanticipated adverse device effect unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for an adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should **not** be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

Post-study Adverse Event
All unresolved adverse events should be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the local investigator should instruct each subject to report, to the local investigator, any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study. The local investigator

should notify the study regulatory sponsor of any death or adverse event occurring at any time after a subject has discontinued or terminated study participation that may reasonably be related to this study. The sponsor should also be notified if the local investigator should become aware of the development of problems, cancer or of a congenital anomaly in a subsequently conceived offspring of a subject that has participated in this study.

Preexisting Condition
A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

Unanticipated Problems Involving Risk to Subjects or Others (UPIRTSO)
Any unanticipated problem or adverse event that meets all of the following three criteria:

- Serious: Serious problems or events that results in significant harm, (which may be physical, psychological, financial, social, economic, or legal) or increased risk for the subject or others (including individuals who are not research subjects). These include: (1) death; (2) life threatening adverse experience; (3) hospitalization - inpatient, new, or prolonged; (4) disability/incapacity - persistent or significant; (5) birth defect/anomaly; (6) breach of confidentiality and (7) other problems, events, or new information (i.e. publications, DSMB reports, interim findings, product labeling change) that in the opinion of the local investigator may adversely affect the rights, safety, or welfare of the subjects or others, or substantially compromise the research data, **AND**
- Unanticipated: (i.e. unexpected) problems or events are those that are not already described as potential risks in the protocol, consent document, not listed in the Investigator's Brochure, or not part of an underlying disease. A problem or event is "unanticipated" when it was unforeseeable at the time of its occurrence. A problem or event is "unanticipated" when it occurs at an increased frequency or at an increased severity than expected, **AND**
- Related: A problem or event is "related" if it is possibly related to the research procedures.

7.2 Recording of Adverse Events

All adverse events occurring during the study period must be recorded. All observed or volunteered adverse effects (serious or non-serious) and abnormal test findings, regardless of the treatment group if applicable or suspected causal relationship to the investigational device or if applicable other study treatment or diagnostic product(s) will be recorded in the subjects' case history. For all adverse effects sufficient information will be pursued and or obtained as to permit; an adequate determination of the outcome, an assessment of the casual relationship between the adverse effect and the investigational device or, if applicable other study treatment or diagnostic product. The clinical course of each event should be followed until resolution, stabilization, or until it has been ultimately determined that the study treatment or participation

is not the probable cause. Serious adverse events that are still ongoing at the end of the study period must be followed up, to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be at least possibly related to the study treatment or study participation should be recorded and reported immediately.

Causality and severity assessment
The sponsor-investigator will promptly review documented adverse effects and abnormal test findings to determine 1) if the abnormal test finding should be classified as an adverse effect; 2) if there is a reasonable possibility that the adverse effect was caused by the investigational device or other study treatments; and 3) if the adverse effect meets the criteria for a serious adverse effect.

If the sponsor-investigator's final determination of causality is "unknown and of questionable relationship to the investigational device or other study treatments," the adverse effect will be classified as associated with the use of the investigational device or other study treatments for reporting purposes. If the sponsor-investigator's final determination of causality is "unknown but not related to the investigational device or other study treatments," this determination and the rationale for the determination will be documented in the respective subject's case history.

7.1 Sponsor-Investigator Reporting of Unanticipated Adverse Device Effects and Unanticipated Problems

When an adverse event has been identified, the study team will take appropriate action necessary to protect the study participant and then complete the Study Adverse Event Worksheet and log. The sponsor-investigator will evaluate the event and determine the necessary follow-up and reporting required.

The sponsor-investigator will promptly review documented Unanticipated Adverse Device Effects and as necessary shall report the results of such evaluation to FDA within 10 working days and Mayo IRB within 5 working days of initial notice of the effect. Thereafter the sponsor-investigator will submit such additional reports concerning the effect as requested.

7.1.1

7.1.2 Sponsor-Investigator Reporting, Notifying Mayo IRB

The sponsor-investigator will report to the Mayo IRB any UPIRTSOs and Non-UPIRTSOs according to the Mayo IRB Policy and Procedures.

7.1.3 Sponsor-Investigator Reporting: Notifying the FDA

The sponsor-investigator will report to the FDA all unanticipated adverse device effects according to the required reporting timelines, formats and regulations.

The sponsor-investigator will submit a completed FDA Form 3500A to the FDA's Center for Devices and Radiological Health for any observed or reported adverse effect that is determined to be an unanticipated adverse device effect. A copy of this completed form will be provided to the DSMB and all participating sub-investigators.

The completed FDA Form 3500A will be submitted to the FDA as soon as possible and, in no event, later than 10 working days after the sponsor-investigator first receives notice of the adverse effect.

If the results of the sponsor-investigator's follow-up evaluation shows that an adverse effect that was initially determined to not constitute an unanticipated adverse device effect does, in fact, meet the requirements for reporting; the sponsor-investigator will submit a completed FDA Form 3500A as soon as possible, but in no event later than 10 working days, after the determination was made.

For each submitted FDA Form 3500A, the sponsor-investigator will identify all previously submitted reports that that addressed a similar adverse effect experience and will provide an analysis of the significance of newly reported adverse effect in light of any previous, similar report(s).

Subsequent to the initial submission of a completed FDA Form 3500A, the sponsor-investigator will submit additional information concerning the reported adverse effect as requested by the FDA.

Reporting Process
Unanticipated Adverse Device Effect reports will be submitted on FDA Form 3500A.
The contact information for submitting reports is:

Food and Drug Administration
Center for Devices and Radiological Health
Document Mail Center - WO66-G609
10903 New Hampshire Avenue
Silver Spring, Maryland 20993-0002

Deviations from the investigational plan.

The sponsor-investigator shall notify Mayo IRB (see 21 CFR 56.108(a) (3) and (4)) of any deviation from the investigational plan to protect the life or physical well-being of a subject in an emergency. Such notice shall be given as soon as possible, but in no event later than 5

working days after the emergency occurred. Except in such an emergency, prior approval by the sponsor-investigator is required for changes in or deviations from a plan, and if these changes or deviations may affect the scientific soundness of the plan or the rights, safety, or welfare of human subjects, FDA and IRB notification in accordance with 21 CFR 812.35(a) also is required.

7.2 Medical Monitoring and Data Safety Monitoring Board

The Mayo Clinic Department of Surgery Data Safety Monitoring Board will serve as the monitoring committee for this study. The function of a DSMB is to review data and endpoints on a timeline set forth by the Data Safety Monitoring Plan (DSMP) in the IRB approved protocol with members independent of the investigators and direct study team.

It is the responsibility of the sponsor-investigator to oversee the safety of the study. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above, as well as the construction and implementation of a site data and safety-monitoring plan (see Section 10 Auditing, Monitoring and Inspecting). Medical monitoring will include a regular assessment of the number and type of serious adverse events.

Patient's will be monitored 24/7 by a team composed of the consultant surgeon, general surgery residents, advanced nurse practitioners, and physician assistants throughout their hospital stay. Patients will also be monitored and cared for by the primary care team composed of nurses, nurse assistants, pharmacists, and other consulting services in order to best take care of the patient. Diagnostics tests to evaluate for SSI will include physical examination, laboratory testing, and imaging such as computed tomography (CT) and ultrasound (US). Patients with complicated hospital courses will return for a clinical visit after approximately 2 weeks with cross sectional CT imaging and laboratory testing with further follow up visits as clinically indicated. In the instance of an uneventful hospital course, patient's will return at 4 weeks from their date of discharge for a clinical visit with CT imaging and laboratory testing. After this first routine follow-up appointment with surgery, patients will continue to follow with medical oncology and with the surgical clinic on an as needed basis.

8 Data Handling and Record Keeping

8.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (long term survival status that the subject is alive) at the end of their scheduled study period.

8.2 Source Documents

ONT sequenced reads will be stored as fastq and fast5 files. Detection of microbial species will be stored as original ckraken files,in excel spreadsheets, and in Medidata Rave. Information of antimicrobial resistance genes detected in the ONT sequencing data will be stored as fsa, txt, excel files, and Medidata Rave. Original records of patient outcome will be stored in clinical charts (hospital records), and extrapolated patient clinical data will be stored in excel spreadsheets and Medidata Rave.

Source data comprise all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial. When applicable, information recorded on the CRF shall match the Source Data recorded on the Source Documents.

8.3 Case Report Forms

A Case Report Form (CRF) will be completed for each subject enrolled into the clinical study. The investigator-sponsor will review, approve and sign/date each completed CRF; the investigator-sponsor's signature serving as attestation of the investigator-sponsor's responsibility for ensuring that all clinical and laboratory data entered on the CRF are complete, accurate and authentic.

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. If a space on the CRF is left blank because the procedure was not done or the question was not asked, "N/D" will be entered in RAVE. If the item is not applicable to the individual case, write "N/A". All entries should be printed legibly in ink on the Source Document Worksheet.

Data Handling and Record Keeping

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- What protected health information (PHI) will be collected from subjects in this study
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(This information is contained within the Mayo IRB Informed Consent Template Section 14) In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts will be made to obtain permission to collect at least vital status (long term survival status that the subject is alive) at the end of their scheduled study period.

Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

Case Report Forms

Data will be captured at each participating site by qualified study staff who will perform primary data collection from source-document reviews to electronic case report forms (eCRF) via Medidata Rave, the information technology endorsed by Mayo Clinic's Clinical Trial Management System (CTMS) as described in Appendix/Attachment (please fill in Appendix/Attachment location info within protocol). Data reported in the eCRF derived from source documents should be consistent with the source documents or the discrepancies should be explained. Data will be entered for this study utilizing one or a combination of the following methods:

1. Data may be captured electronically, without use of paper.
2. Data may be transcribed from the Electronic Medical Record (EMR-an electronic source that must be available for review) into an EDC system, without use of paper.
3. Data may be captured on paper (considered source documentation) and transcribed into the EDC system, BUT paper documentation must be retained and available for review.

- **Data Management**

Study sites will transcribe subject source data into eCRFs using Medidata Rave. The Medidata
Rave system is compliant with 21 CFR (Code of Federal Regulations) Part 11 FDA (Food and Drug Administration) requirements. Edit checks, electronic queries, and audit trails are built into the system to ensure accurate and complete data collection and security. Data will be transmitted via the internet from investigational sites to a central hosting site, utilizing state-of-the-art encryption mechanisms to ensure security and confidentiality.

- **Data Processing**

All data is entered into electronic case report forms (eCRF's) through the Medidata Rave system. Case report forms are automatically rolled out based on a predetermined, visit based schedule to improve study staff workflow and data quality. Data is exported nightly to a secure FTP for analysis and reporting.

- **Data Security and Confidentiality**

The Medidata Rave database access model is role based and fully auditable at the study, form, and field levels. Data is de-identified whenever possible and the ability to update data is limited to necessary staff. **Access is managed by the Mayo Clinic Research Service Center, under a controlled and monitored access request system.** Metadata's platform specifically supports Electronic Record and Electronic Signature (ER/ES) requirements, including US 21 CFR part 11.

- **Data Quality Assurance/Data Clarification Process**

Each eCRF contains edit checks and custom functions to ensure the highest possible data quality. Only necessary eCRF's are available for data entry to reduce the possibility of erroneous entry.

- **Data Clarification Process**

The edit checks and custom functions on the eCRF's trigger queries requesting the attention of appropriate study staff. The fields are marked in pink to allow study staff to quickly identify the data fields that require attention or actions. Additionally, secure email notifications are sent for adverse event tracking and monitoring.

Clinical Trial Management Systems (CTMS)

CTMS is the Mayo Clinic Research Committee-endorsed institutional resource for clinical data management. CTMS is a robust institutional effort initiated in 2010 to address emerging changes within the data and statistical coordinating centers affiliated with NCI-funded cooperative groups. In 2010, NCI selected Medidata Rave® (http://www.mdsol.com/) as the required data collection tool for all cooperative studies. To capitalize on Mayo Clinic and the NCI's investment in Medidata Rave®, Mayo Clinic formalized a three-tier data management infrastructure with the Medidata Rave® product as the premier system.

Medidata Rave® is a product for multi-center clinical trials conducted under 21 CFR Part 11 requirements. This web-based system provides ease of use coupled with an integrated randomization module (Medidata Balance™), custom reporting, robust data validation routines, and straightforward integration with SAS.

- *Electronic Data Capture:* Medidata Rave® allows for data collection in multisite studies. During the course of the data entry into Medidata Rave®, the system provides real-time within-case report form (CRF) and inter-CRF data consistency verification. Medidata Rave® is flexible in nature so that all data can be entered even if "required" fields and or other consistency checks requirements are not satisfied. The system uses an internal "flagging" or "query" system to distinguish the valid from the invalid data thereby ensuring compliance with the FDA guidance document "Computerized Systems Used in Clinical Trials." All data discrepancy issues are tracked and audited by the system to ensure the highest quality data is available for analysis and study reporting.

  Contained within the CTMS initiative at Mayo Clinic is a diverse set of administrative and technical personnel to support the development and implementation of clinical trials in Medidata Rave®. While the time necessary to program Rave's electronic case report forms (eCRFs) has been directly budgeted, the CTMS initiative supports protocol independent activities such as software/server maintenance, data standards, institutional system integrations, SAS data, and training of study personnel through institutional resources.

  The dedicated VPN connection between Mayo Clinic and Medidata provides the conduit for data connectivity. Clinical trial data hosted in Medidata is accessible when needed for SAS using the SAS On Demand Connection, in combination with Mayo Clinic's SAS Pipeline program, which creates a common and direct combination of the metadata (labels, formats, etc.) and data (raw values) into SAS datasets on a scheduled (nightly) basis. This process removes the need to separately label and format the entire clinical trial database separately in SAS.

- *Medidata Balance™:* Randomization encounters challenges in complex multisite clinical studies in which random assignment to study drug must be completed prior

Data Processing

Data processing will be performed by the study investigator, study coordinators and finally by a qualified statistician. Given that this is a single center study, there will not be any transfer of data. All data pertinent to the study will be stored in a central location accessible to only study personnel.

8.4 Records Retention

The sponsor-investigator will maintain records and essential documents related to the conduct of the study. These will include subject case histories and regulatory documents.

The sponsor-investigator will retain the specified records and reports during the study and for the longer of the following;

1. As outlined in the Mayo Clinic Research Policy Manual –"Retention of and Access to Research Data Policy" http://mayocontent.mayo.edu/research-policy/MSS_669717,

9 Study Monitoring, Auditing, and Inspecting

9.1 Study Monitoring Plan

The investigator will allocate adequate time for such monitoring activities. The Investigator will also ensure that the monitor or other compliance or quality assurance reviewer is given access to all the study-related documents and study related facilities (e.g. pharmacy, diagnostic laboratory, etc.), and has adequate space to conduct the monitoring visit.

9.2 Auditing and Inspecting

The sponsor-investigator will permit study-related monitoring, audits, and inspections by the IRB, the monitor, and government regulatory agencies, of all study related documents (e.g., source documents, regulatory documents, data collection instruments, study data etc.). The sponsor-investigator will ensure the capability for inspections of applicable study-related facilities (e.g., pharmacy, diagnostic laboratory, etc.).

Participation as a sponsor-investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable compliance offices.

10 Ethical Considerations

This study is to be conducted according to United States government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted local Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study. The decision of the IRB concerning the conduct of the study will be made in writing to the sponsor-investigator before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in

this study. This consent form will be submitted with the protocol for review and approval by the IRB for the study. The formal consent of a subject, using the Approved IRB consent form, must be obtained before that subject undergoes any study procedure. The consent form must be signed and dated by the subject or the subject's legally authorized representative, and the individual obtaining the informed consent.

11 Study Finances

11.1 Funding Source

The research study is supported by institutional funds provided in the form of a Transform the Practice award from the Center for Individualized Medicine, Mayo Clinic.

12 Publication Plan

Link to protocol registration site for ClinicalTrials.gov: https://register.clinicaltrials.gov/ .

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14 Supplementary Materials

S1. Standard cultures performed on the bile samples

Swab samples were inoculated into thioglycolate broth (thio), chocolate blood agar (CBA), tryptic soy agar with 5% sheep blood (BAP), eosin-methylene blue agar and Columbia CNA agar with 5% sheep blood, colistin and nalidixic acid (CNA). Bile fluid aspirate samples were aseptically removed from the transport vial and one aliquot of fluid placed onto each of chocolate blood agar, BAP, eosin-methylene blue agar, and CNA culture media for aerobic culture. Aerobic culture plates were incubated at 35°C in 5% CO_2 and examined daily for growth for up to 5 days. Anaerobic culture media were inoculated in parallel and included CDC anaerobe 5% sheep blood, phenylethyl alcohol blood agar, laked *Brucella* blood agar with kanamycin and vancomycin and a BAP used as an aerotolerance screen.

S2: Protocol for the identification of aerobic cultures

PROCEDURE:

1. Culture reading schedule:
    a. Based on the time the culture was set up, read cultures in two batches beginning at 8:00 a.m. and 12:30 p.m. to allow for a minimum incubation time of 16 hours. At 8:00 a.m. move plates cultured by the midnight tech (found on the bottom shelf of the incubator) to the appropriate a.m. shelf to complete their incubation. Culture set up Initial examination 8:00 a.m. - 4:00 p.m. 4:00 p.m. – Midnight, Midnight- 8:00 a.m. 8:00 a.m., the next day 12:30 p.m., the next day 8:00 a.m., the next day
    b. The schedule for reading urine cultures is found in Urine Culture [011327].
    c. The schedule for reading stool cultures is found in Stool Culture [011342].
2. Initial examination of GEN and GENS cultures: Open the DGEN1 work list. Sort the work list by last name to separate bench I and bench II orders.
    a. Separate all positive cultures from the negative cultures.
    b. Review the requested test order(s), patient demographics, medical record number, ordered tests and special instructions from any paperwork received, and match with the plate identification on day 1. File all negative culture paperwork on day one at the bench.
    c. Mark all negative orders as the plates and thioglycollates are placed in a rack for further incubation.
        - If the order only has GC-Lect plates, RLABL the culture and reincubate the plate with the bench subcultures.
        - Body fluids incubated in Bactec bottles will not have paper work or media at the bench. Open the order from the worklist to check that the media setup is bottles or check the FX instruments to be sure there are bottles incubating before you mark an order as negative. d. Add the "No growth to date" test

comment to all of the selected negative orders. The orders will be removed from the work list when the work list is refreshed.
- Choose "Define TC". Select appropriate test (e.g., GEN). Select the NGTD test comment from the keypad. Choose preliminary status, and add results.
- If the culture is positive at a later date the "No growth to date" comment should be Hidden by placing a (?) before the comment.

3. Initial examination of SPUT/SPUTS, CGAS, CGBS, GC and GEN/GENS cultures from a cornea donor source (held 7 days).
    a. Add the RLABL media to the order and use the sticker to label a culture divider. Aerobic Culture Examination and Documentation Guidelines [PROC 011336.021]
    b. All orders that have had the RLABL media added will be removed from the DGEN1 work list when you refresh.
    c. Negative cultures are reincubated in the bench subculture reincubate racks and looked at daily.
4. Negative culture re-examination of GEN/GENS cultures (Days 2-5):
    a. Perform work in the BSC wearing safety glasses and gloves.
    b. Examine reincubated plates daily and discard negative plates at 5 days.
    c. Examine sub plates from body fluids subcultured from Bactec bottles daily.
        - Reincubate RCBA plates subcultured from body fluid bottles that flag as a false positive (NOS) for a total of 48 hours before tossing as negative.
        - Result the RLABL media to move the culture back to the worklist. The order will be finalized on day 5 from the DGEN2 work list.
    d. Examine thioglycollate broths daily and discard negative broths at 5 days.
        - Return negative brain heart infusion broths containing foreign body specimens (e.g., bullets, glass shards, metal fragments (not surgical hardware) to Initial Processing. Specimens will be saved in the Initial Processing area for one year for medico-legal purposes.
        - Return heart valves to be saved to Initial Processing area for final disposition (some valves may be forwarded to the Pathology Lab) after culture workups are complete. Refer to Initial Processing procedures for details.
    e. For positive plates and thioglycollates: Verify by Gram stain that the organism is not a select agent (e.g. Brucella sp., Francisella sp., etc.) before removing plates from the BSC for workup. See Biosafety Policy for the Bacteriology Lab [036885].
        - Initiate a Pathogen Alert Worksheet [051193] if a select agent or highly infectious organism is suspected.
    f. RLABL and workup any positive cultures. If a highly infectious or select agent is suspected keep the plates in the BSC.

g. Bench I personnel will open the DGEN2 work list after bench I and II have finished the reincubate process.
    1) Mark all orders that are 2-5 days old. NOTE: For day 5 cultures, if the received date is later than the collected date, wait a full 5 days after the received date before you mark the order.
    2) Autoresult the orders:
        - The "No growth to date" test comment will be updated in the final report for 2-4 day old cultures with a preliminary status. Orders will stay on the worklist.
        - In the electronic workcard a test comment of "?This order was updated and reported after X days" will document that the culture media was reviewed daily. X= day reviewed.
    3) The "No growth after 5 days of incubation" test comment will be added to the 5 day old cultures with a final status. These orders will be removed from the worklist when the worklist is refreshed.

5. Workup of GEN/GENS cultures on Day 1:
    a. Enter the order using Result Entry.
    b. Add the RLABL media to the order and use the sticker to label a culture divider. All orders that have RLABL media added will be removed from the DGEN1 work list when you refresh.
    c. Recheck the DGEN1 work list. The work list should be empty. Troubleshoot any remaining orders.
    d. Keep all positive paperwork with the pending culture and file at the bench when the culture is finalized.
    e. Review the requested test order(s), patient demographics, medical record number, ordered tests and special instructions from any paperwork received, and match with the plate identification each day the culture is worked on.
        - Notate sterile collection circumstances in the Common Media Comments. "OR collected specimen" should be noted by IP in the Order Comments; however Ultrasound (US) and computed tomography (CT) guided collections will need to be manually identified and notated
    f. Check the Order Comments and Culture Comments: This is where information communicated from Initial Processing (i.e. "OR collected") will be found. Copy the comments into the culture work card as a Common Media Comment.
    g. Compare the computer-generated order number to the media labels used to identify the solid/liquid media inoculated for the culture each time the culture is reviewed or there is work performed on the culture.
    h. Review direct Gram stain result, if applicable. If the direct Gram stain does not correlate with the growth on the culture, the direct Gram stain should be reviewed.
        - Refer to Gram Stain [011334] for instructions on resolving Gram stain delta checks.

i. The core benches will check the DGSD and DGSDN resulting worklists daily to resolve any orders that qualify.
j. Document work in the electronic workcard. The date is entered by the system. Continue to record colonial morphology, quantitation, order additional tests and record results. Most of these choices will be provided by using the keyboards set up in the system. Additional information on LIS entry can be found in SCC SoftMic Functions – Bacteriology Laboratory [046139].
k. Reincubate all plates on positive cultures to provide a total of 2 days of incubation.
l. Hold final report on orders that have been referred to a specialty area e.g. RMYC, RSPL, RANA until the referral media has been resulted.
m. Identify all aerobic organisms according to Organism Identification [011332].
n. Perform susceptibility testing once every 7 days per organism per source unless specifically requested by phone to perform more often.
o. Refer to the appropriate source specific procedure for further workup instructions.
p. Charge for testing performed by adding the bill only charges under the RBILL media.

6. Workup of Multiple Positive Cultures
   a. Determine that a patient has multiple cultures from the same anatomical source. A "left" and a "right" site cannot be considered the same.
   b. When multiple cultures from a patient yield isolates with identical colonial morphology, it is only necessary to identify the isolate from one culture. The identity of morphologically similar isolates from subsequent cultures may, therefore, be presumed. This applies only to cultures from the same, consecutive, or every other day if there are fresh plates for comparison of morphologies. Exceptions: Isolates of gram positive-cocci (except S. aureus) or gram positive-bacilli may utilize the same by morphology comparison only after 2 orders are verified to be the same identification by Bruker testing.
   c. Document in the workcard that the isolate is "same by morphology as" and record the order number of the culture/isolate that was identified. "Same by morph" is approved as shorthand for the above phrase.
   d. Do not bill for identification of isolates that are identified by morphology comparison.
7. Thioglycollate workup: NOTE: If growth in thioglycollate is questionable, do not stain until day 5 to minimize the chance of contaminating the broth.
   a. Thioglycollate workup when primary media is positive: Gram stain any positive thioglycollate broth. Subculture to appropriate aerobic media if stain suggests growth of an organism not already recovered on plated media. Consider additional subculture media (i.e., CBA, BCYE) or special incubation conditions. "***CT" = CT guided "***US" = ultrasound Aerobic Culture
   b. Thioglycollate workup when primary media is negative for organism seen in thio Gram stain: If only thioglycollate broth is positive anaerobic organisms could be present, Gram stain the broth and subculture both aerobically and anaerobically (RBAPS and RCBAS, RSBS). Take the RSBS to initial

processing and place in the CO2 holding jar (lid up). Set the timer for two hours and confirm that the CO2 is flowing.

1) Review aerobic subculture after 1 day of incubation, work up aerobic growth if present. Reincubate negative plates for 1 additional day.
2) Anaerobe lab will return the RSBS to the bench after 2 days of incubation. If anaerobic growth is present proceed with identification according to Organism Identification [011332].

8. Workup of plates referred to the bench from the Anaerobe lab:
    a. Anaerobe lab will order a ^RXANA media under the general culture and place the plate in the "XANAE LAB" general bench rack.
    b. General bench staff will compare the morphology to their culture plates.
        - If the isolate is the same as the aerobic culture record a "same as" statement under the RXANA plate.
        - If the isolate is different from the aerobic culture order a RBWU# under the RXANA plate and proceed with workup based on the categories in 1 and 2 above. Anaerobe lab will provide the isolate quantity. If the quantity is 1-4+ consult with management on what to report.
    c. The RXANA media must be resulted before the General culture can be finalized.
    d. Aerobic isolates reported after the general culture is finalized will be flagged as an additional report by the LIS.
9. Completion of specimens referred to anaerobe Lab from the bench.
    a. ^RANA is ordered on all specimens submitted to the anaerobe lab for workup. Submit the ^RANA sticker to anaerobe lab when submitting an isolate from the general bench.
    b. If no ANAE(S) is ordered, the anaerobe staff will enter a Common Media Comment under the aerobic culture "Wait for anaerobes" to indicate that processing is in progress. The aerobic culture should be held on the bench referral wall until anaerobe processing is complete.
        - When complete, the anaerobe finalizer will review the work, result the RANA media, add a common media comment of "Anaerobe work-ups complete", write "ANAE complete" on the culture divider and return it to the bench. The culture can then be finalized.
    c. If an ANAE(S) culture is ordered, then the referral will be processed under the ANAE(S) and the general bench can finalize their culture.
10. Media labeling guidelines:

a. Label subculture plates with the media label and note the WORKUP number and date.
b. Label biochemical tubes with the media label and note the WORKUP number and date. For biochemical sets (multiple tubes inoculated at the same time, placed in rows in a rack), label the first tube in the set and place subsequent tubes in a row; labeling is not required.

PROCEDURE: Use the following table(s) to identify possible pathogenic bacteria from patient specimens. The primary testing scheme should be utilized whenever feasible. The secondary testing should be utilized when the primary testing scheme is inconclusive or unavailable. Refer to Bruker MALDI-TOF MS Reporting-Scheme-Bacteriology [048215] for MALDI-TOF acceptance ranges and resulting guidelines.

Organisms suspected of being a select agent Identification of Francisella spp., Brucella spp., Burkholderia mallei/pseudomallei, Bacillus anthracis, Yersinia pestis, and N. meningitidis can be performed by MALDI-TOF when plate preparation steps are performed in the BSC. Refer isolate to Special Procedures Laboratory if any of these organisms are suspected. • Isolates received with a request to rule out B. cereus Biovar anthracis must be submitted to MDH in order for the biovar to be determined. • Alert a member of the management team when a select agent or N. meningitidis is suspected.

S2: Table 1

Gram-negative bacilli				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing [H]**	**Comments**
***Acinetobacter* spp.**		MALDI-TOF	Blue centers on EMB: Strip oxidase (-) TSI (K/K) **Report *Acinetobacter* spp.**	
***Brucella* spp.** ***Francisella* spp.**	Filmy growth at 24 hours **See comments**	**Perform all in BSC**: stain tiny-cb Tape all plates MALDI-TOF Refer entire culture/paperwork to SPL		**Perform all plate manipulation in BSC, see Fastidious Fermenting Gram-negative Bacilli / Gram-negative Cocci Identification [034753] or LRN flowcharts (see link above). Notify QC**

				and Supervisor of all isolates reported.
Gram-negative bacillus, resembling *Capnocytophaga* spp.	Spreading "oil slick" yellow colonies on CBA or BA	MALDI-TOF	Stain: long thin gnb with pointed ends, Drop oxidase (-) Catalase (-) **Prelim Gram-negative bacillus, resembling *Capnocytophaga* spp.**	
***Citrobacter* spp.**	Darkened agar on BA	MALDI-TOF	TSI (A/A, H2S) Tube indole (-) **Report *C. freundii* complex** TSI (K/A) Tube indole (+) **Report *C. koseri***	Refer to SPL if secondary testing is inconclusive. Foul odor is characteristic
Eikenella corrodens	Pitting agar	MALDI-TOF	Stain: small gnb Drop oxidase (+) Catalase (-) **Prelim Gram-negative bacillus**	Bleach-like odor is characteristic

Gram-negative bacilli				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing H**	**Comments**
Escherichia coli	Green Metallic Sheen on EMB Non-mucoid	Quick indole (+) **OR** MALDI-TOF if the BA is discolored indicating a different identification e.g., *Citrobacter* spp., *Morganella morganii*	MALDI-TOF	**MALDI-TOF does not differentiate between *E. coli* and *Shigella* spp.** Indole odor is characteristic Document the listed morphology in the LIS if using biochemical testing
	Beta-hemolytic on BA (Mucoid or Non-mucoid)	Quick indole (+) Strip oxidase (-) **OR** MALDI-TOF if the BA is discolored indicating a different identification e.g., *Citrobacter* spp., *Morganella morganii*	MALDI-TOF	

	Lactose positive on EMB and non-hemolytic on BA	MALDI-TOF	Quick indole (+) Strip oxidase (-) PYR (-)	
	Lactose negative on EMB and non-hemolytic on BA	MALDI-TOF	**Urine sources:** Quick indole (+) Strip oxidase (-) MUG (+)	
***Haemophilus* spp.**	Tan on CBA No growth on BA	MALDI-TOF	Stain: small gnb, Drop oxidase (+) **Prelim Gram-negative bacillus**	AST needs both genus and species to send susceptibilities
***Proteus* spp.**	Swarming (+/-)	MALDI-TOF	Ornithine (+) Tube indole (-) **Report *Proteus mirabilis*** Ornithine (-) Tube indole (-) **Report *Proteus penneri*** Ornithine (-) Tube indole (+) **Report *Proteus hauseri/vulgaris***	Preliminary report: **Swarming**: "Gram-negative bacillus resembling *Proteus"* (RPRO) **Non-swarming:** "Gram-negative bacillus"
Pseudomonas aeruginosa	"Fringy" and beta-hemolytic **Plus a third characteristic:** Silver sheen, obvious grape/corn chip odor or pigment production	Strip oxidase (+)	MALDI-TOF	*Pseudomonas otitidis* resembles *P. aeruginosa* in morphology. Use caution when using odor as a definitive characteristic; *P. otitidis* is more stinky. Document the listed morphology in the LIS if using primary testing

Gram-negative bacilli				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing H**	**Comments**
	"Watery" and beta-hemolytic **Plus a third characteristic:** Silver sheen, obvious grape/corn chip odor, or pigment production	Strip oxidase (+)	MALDI-TOF	If watery isolate has no odor or non-hemolytic and MALDI-TOF is inconclusive, refer to SPL for 16S rRNA gene sequencing to rule out possible ***Inquilinus limosus***.

				Document the listed morphology in the LIS if using primary testing
Stenotrophomonas maltophilia	Gun-metal gray colonies on BA	MALDI-TOF	Strip oxidase (-), TSI (K/K), Lysine with oil overlay (+) **Report *Stenotrophomonas maltophilia***	AST needs both genus and species to send susceptibilities.
Gram-negative bacilli, not listed above		MALDI-TOF		
[H] When no secondary testing is listed and MALDI-TOF does not provide a satisfactory identification, refer to SPL if full identification is required per the source specific SOP.				

S2: Table 2

Staphylococci [A,C]				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing [H]**	**Comments**
***Micrococcus* spp**.	Yellow pigmented	MALDI-TOF	**Sterile sources:** Stain: gpc in tetrads/clusters, **Prelim Gram-positive cocci**	
Rothia mucilaginosa	Sticky	MALDI-TOF	**Sterile sources:** Stain: gpc in tetrads/clusters, **Prelim Gram-positive cocci**	
Staphylococcus aureus	**Typical**: Large White/Creamy, Usually beta-hemolytic	Stain: gpc in tetrads/clusters Staphaurex (+) **OR** MALDI-TOF		
	Atypical: Clear grayish (cell wall deficient)	MALDI-TOF	Stain: gpc in tetrads/clusters, Staphaurex (+) **Report *Staphylococcus aureus***	
***Staphylococcus*, coagulase negative**	White, Usually non-hemolytic	MALDI-TOF [G]	Stain: gpc in tetrads/clusters,	

			Staphaurex (-) **Report *Staphylococcus*, coagulase negative**	
Staphylococcus lugdunensis	White/Creamy, Non-hemolytic or slightly beta-hemolytic	MALDI-TOF	Stain: gpc in tetrads/clusters, Staphaurex (- or +) PYR (+) Tube ORN w/oil (+) **Report *Staphylococcus lugdunensis***	
Staphylococcus saprophyticus	White, Non-hemolytic Grows on EMB	MALDI-TOF	Stain: gpc in tetrads/clusters, Staphaurex (- or +) **Prelim Gram-positive cocci, resembling *Staphylococcus***	
***Aerococcus* spp.**	Alpha-hemolytic, Whitish	MALDI-TOF	**Sterile source:** Stain: gpc resembling Staph **Prelim Gram-positive cocci** **Urine:** Stain from plate gpc resembling staph Stain from THIO broth resembles Staph **Prelim Gram-positive cocci**	*Aerococcus* spp. may be PYR (+) and resemble *Enterococcus* spp. but AST will usually be Pen (S).
Gram-positive cocci, not listed above		MALDI-TOF		

[A] Multiple positive cultures of GPC (except *S. aureus*) on all sources: if morphology is identical on multiple orders, use MALDI-TOF to identify the first two orders. If the same genus and species is confirmed on both orders you may use the "same by morph" rule to report additional orders. If different identifications are found do not "same by morph" any orders.
[C] Perform MALDI-TOF or refer all vancomycin resistant Gram-positive cocci (or coccobacillus) as a possible *Leuconostoc* (notification by AST reporter).
[G] Species level identification of isolates by MALDI-TOF is optional when susceptibilities are not being performed.
[H] When no secondary testing is listed and MALDI-TOF does not provide a satisfactory identification, refer to SPL if full identification is required per the source specific SOP.

S2: Table 3

Streptococci [A, B, C]

Suspected Organism Identification	Morphology	Primary Testing	Secondary Testing [H]	Comments
***Abiotrophia/ Granulicatella* spp.**	Alpha-hemolytic Growth on CBA No growth on BA	MALDI-TOF	**Sterile sources:** Stain: gpc in pairs/chains or pleomorphic, Catalase (-) **Prelim Gram-positive cocci**	
***Enterococcus* spp**.	Alpha-hemolytic Non-hemolytic Occasionally Beta-hemolytic	MALDI-TOF	Stain: gpc in pairs/chains, Catalase (-) PYR (+) [If Beta: StrepPro (D+)] **Report *Enterococcus* spp**.	If PYR (-) consider testing by MALDI-TOF if you suspect *S. bovis* group
Streptococcus pneumoniae	Alpha-hemolytic Sunken center Dimpled Dime/checker shaped	Stain: gpc lancet shaped in pairs, or short chains Catalase (-) Deoxycholate (+) Perform Optochin/Taxo P disk (Susceptible, zone >14 mm) if deoxycholate is questionable **OR** MALDI-TOF		
***Streptococcus bovis* group (SBOVI)**	Alpha-hemolytic Non-hemolytic	MALDI-TOF		***S. bovis*** group resembles *Enterococcus* spp. morphologically but is PYR (-)
***Streptococcus viridans* group**	Alpha hemolytic Non-hemolytic	MALDI-TOF	Stain: GPC in pairs/chains and Catalase (-), PYR(-) Perform MUG test when isolated in multiple cultures from bloods and any CSF culture. If MUG(+), refer to	Perform deoxycholate if morphology questionable for *S. pneumoniae* from bloods, CSF, respiratory and isolates

			SPL for 16S rRNA gene sequencing to rule out ***S. suis***. **Report *Streptococcus viridans* group**	from the neck and above.
Streptococcus suis	Alpha hemolytic, Non-hemolytic, May be mucoid	MALDI-TOF		
***Streptococcus*, beta hemolytic, non-groupable (SBHNG)**	Beta hemolytic	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-) StrepPro: (A), (B), (C), (D), (F), (G) all (=) **Report SBHNG**	
***Streptococcus pyogenes* (*Streptococcus*, group A)** [E]	Beta-hemolytic, Alpha-prime (mixture of beta and non-beta colonies)	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-), StrepPro (A+) and one other group (-) PYR (+) **Report *Streptococcus pyogenes***	Report as *Streptococcus pyogenes* regardless of testing method.
***Streptococcus agalactiae* (*Streptococcus*, group B)**	Beta-hemolytic	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-), StrepPro (B+) and one other group (-) **Report *Streptococcus agalactiae***	Report as *Streptococcus agalactiae* regardless of testing method.
	Non-hemolytic	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-) StrepPro (B+) and one other group (-) **Prelim Gram-positive cocci, resembling *Streptococcus***	
***Streptococcus dysgalactiae* (*Streptococcus*, group C)**	Beta-hemolytic	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-)	

			StrepPro (C+) and one other Group (-) **Report *Streptococcus*, group C**
***Streptococcus anginosus* group (*Streptococcus*, group F)**	Beta-hemolytic May have buttery odor [D]	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-) StrepPro (F+) and one other group (-) **Report *Streptococcus*, group F**
***Streptococcus dysgalactiae* (*Streptococcus*, group G)**	Beta-hemolytic	MALDI-TOF	Stain: gpc in pairs and chains, Catalase (-) StrepPro (G+) and one other group (-) **Report *Streptococcus*, group G**
Gram-positive cocci, not listed above		MALDI-TOF	

[A] Multiple positive cultures of GPC (except *S. aureus*) on all sources: if morphology is identical on multiple orders, use the MALDI-TOF to identify the first two orders. If the same genus and species is confirmed on both orders you may use the "same by morph" rule to report additional orders. If different identifications are found do not "same by morph" any orders.
[B] Perform MALDI-TOF or refer to SPL all vancomycin resistant Gram-positive cocci (or coccobacillus) as a possible *Leuconostoc* (notification by AST reporter).
[C] Perform MALDI-TOF or refer all vancomycin resistant Gram-positive cocci (or coccobacillus) as a possible *Leuconostoc* (notification by AST reporter).
[D] Buttery odor is valid only from a blood agar plate, not a chocolate plate.
[E] *Streptococcus anginosus* is usual microbiota in respiratory and genital/rectal sources. It can be beta-hemolytic (usually small colonies with "butterscotch" odor) and Strep Pro A(+). *S. anginosus* will be PYR (-) but *S. pyogenes* will be PYR (+).
[H] When no secondary testing is listed and MALDI-TOF does not provide a satisfactory identification, refer to SPL if full identification is required per the source specific SOP.

S2: Table 4

Gram-positive bacillus [F]				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing** [H]	**Comments**
***Bacillus* spp.**	Beta-hemolytic (morph rules out *B. anthracis*)	MALDI-TOF	Stain : Large gpb/gvb, with spores +/- Catalase (+) **See comments**	**Refer to SPL for Species Identification if:** • _CSF culture is positive and direct Gram stain is positive with *Bacillus* spp. • _Other sterile sources have **multiple** cultures positive with *Bacillus* spp.
Large spore forming aerobic Gram-positive bacillus, not *Bacillus cereus* or *Bacillus anthracis* (SAG NCA)	Non-hemolytic	**Perform in BSC** MALDI-TOF	**Perfom in BSC** until *B. anthracis* is ruled out with MOT (+) Catalase (+), MOT (+), EYA (-/NG), Esculin slant for spores (+) Reincubate esculin slant and stain for spores, from both the esculin slant and the oldest BA plate, for up to 5 days. MALDI-TOF: *B. anthracis* ruled out and MALDI does not provide a genus level **Report Soft code SAG NCA**	
Large Gram-positive bacillus resembling *B. anthracis*	**Non-hemolytic and Sticky, ground glass morphology**	**Tape plates and Refer to SPL to rule out possible *B. anthracis***		**Perform in BSC** MOT (-), EYA (+ Lecithinase)
Arcanobacterium haemolyticum	Beta-hemolytic	MALDI-TOF	Stain: small Gram-positive bacillus, Catalase (-), **Prelim Small Gram-positive bacillus**	
***Corynebacterium* spp. / Coryneform, this category might include:** *Trueperella* spp., *Brevibacterium casei,* *Rothia* spp.,	Non-hemolytic, usually small white, can be dry or yellow	**Sterile source**: MALDI-TOF	Stain: small Gram-positive bacillus, Catalase (+), Motility (-) **Prelim Small Gram-positive bacillus,**	Motility performed to rule out *Listeria* spp.

Dermabacter hominis, *Corynebacterium otitidis*, *Curtobacterium* spp., *Microbacterium* spp., Other small Gram-positive bacillus			**resembling *Corynebacterium or* small Gram-positive bacillus as appropriate**	
		Lower respiratory sources when predominant organism: MALDI-TOF to rule out possible *C. pseudodiphtheriticum*	Stain: coryneform, Catalase (+), Urea (+), Refer to SPL to rule out possible *C. pseudodiphtheriticum*	
		Urine sources when predominant or >105: MALDI-TOF to rule out possible *Corynebacterium* group F1, *C. urealyticum*, or *C. regelii*	Stain: coryneform, Catalase (+), Urea (=) **Report Urogenital microbiota** Urea (+) **Report Small Gram-positive bacillus, resembling *Corynebacterium,* urease producer**	
		Other, non-sterile sources: Stain: gpb or coryneform, Catalase (+) **OR** MALDI-TOF		Refer to source specific SOPs for reporting guidance.
Erysipelothrix rhusiopathiae/tonsillarum	Alpha-hemolytic	MALDI-TOF	Stain: small, slender gpb, **Prelim Small Gram-positive bacillus**	
Gardnerella vaginalis	Small white non-hemolytic on CNA	MALDI-TOF	Stain: small Gram +/- bacillus, **Prelim Gram-negative bacillus**	
Gardnerella vaginalis	Small white non-hemolytic on CNA	MALDI-TOF	Stain: small Gram +/- bacillus, **Prelim Gram-negative bacillus**	

[F] Multiple positive cultures on all sources: if morphology is identical on multiple orders, use the MALDI-TOF to identify the first two orders. If the same genus and species is confirmed on both orders you may use the "same by morph" rule to report additional orders. If different identifications are found do not "same by morph" any orders.
[H] When no secondary testing is listed and MALDI-TOF does not provide a satisfactory identification, refer to SPL if full identification is required per the source specific SOP.

S2: Table 5

Miscellaneous				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing [H]**	**Comments**
Moraxella catarrhalis	Sticky "hockey puck", Grey/white opaque coloring Growth on BA	**Perform in BSC:** Stain: gnc (if performed) MALDI-TOF		
Neisseria gonorrhoeae	Clear, tan colony No growth on BA	**Perform in BSC:** Stain: gnc (if performed) MALDI-TOF **See Comment:**		Refer to *Neisseria gonorrhoeae* Culture [011279] If patient is ≤ 15 years old Refer to SPL for further testing
Neisseria meningitidis	Large, clear, tan colony Grows on BA	**Perform in BSC:** Stain: gnc (if performed) MALDI-TOF		**Perform all work, including susceptibilities, in a BSC with suspect *N. meningitidis.* Notify QC and Supervisor of all isolates reported.**
Yeast	Domed, Waxy Feelers	Gram stain, unless "feelers" present	Refer to Mycology if source is sterile, blood, catheter tips. (RMYC) per Referral of Fungal/Yeast Isolates to the Mycology Lab [011338]	**Yeast**
Filamentous Fungi	Fuzzy		Refer to Mycology from all sources except respiratory and stool. (RMYC) per Referral of Fungal/Yeast Isolates to the Mycology Lab [011338]	**Filamentous Fungi**
***Mycobacterium*, *Nocardia*, or other aerobic actinomycete** (e.g., *Gordonia, Rhodococcus, Streptomyces, Tsukamurella*)	Sticky Chalky/crusty	Stain: beaded and/or branching Gram-positive bacillus or beaded filamentous Gram-positive bacillus	Refer to Mycology/TB (RTBR)	**Rapid growing *Mycobacterium, Nocardia*, or other aerobic actinomycete** Musty odor is characteristic

[H] When no secondary testing is listed and MALDI-TOF does not provide a satisfactory identification, refer to SPL if full identification is required per the source specific SOP.

S2: Table 6

Anaerobic Organisms				
Suspected Organism Identification	**Morphology**	**Primary Testing**	**Secondary Testing**	**Comments**
Aerotolerant non-spore forming anaerobes e.g., *Actinomyces* spp., *Cutibacterium* spp.	Alpha-hemolytic, Non-hemolytic Small white	Stain: small gpb may be Coryneform or branchy Catalase (+/-), aerobic growth is (-)/(wk+)		**Refer to anaerobe lab (RANA) if:** • _ANAE(S)/ ACT is pending • Non-swab specimen from blood, bone, joint or central nervous system (CNS) **Report as "non-spore forming Gram-positive bacillus not further identified" (NGPB-NFI)** • Sterile source, ANAE/ ACT not ordered • Nonsterile source, isolated <48 or when pure/predominant and reporting is preferred **Report with usual microbiota if:** • Isolate > 48 hours from nonsterile source

Obligate anaerobes	Any isolate(s) from blood, bone, joint or central nervous system (CNS) culture in pure or mixed culture or Pure cultures from sterile sources acceptable for anaerobic culture	Appropriate aerobic and anaerobic subcultures: Aerobic growth (-), Anaerobic growth (+) Refer to Anaerobe lab (RANA) for species level identification and reporting	See specific source SOP for further guidelines. Anaerobes are reported under the general culture if a separate anaerobe culture is not ordered. *C. tertium* and *Letptotrichia* sp. should be treated as obligate anaerobes regardless of growth.
	Thio with pure culture of anaerobe from a source unacceptable for anaerobic culture (includes swabs)	Appropriate aerobic subcultures: Aerobic growth on CBA (-) and BA (-), Report anaerobe as: Possible anaerobic organism isolated in broth only, not further identified (PANFI)	
	Thio with mixed culture (more than one anaerobe, or mixture of aerobes and anaerobes) from source other than bone, joint, or CNS	Appropriate aerobic subcultures: Aerobic growth (-), Report anaerobe(s) as: Organism in mixed culture from broth only, possible anaerobe present-not further identified (OPMC)	

Back-up Identification of Enteric Gram Negative Bacilli

Order a work-up, quantitate, and describe the organism as usual. Order the long set media ^RTSI, ^RLYS, ^RCIT, ^RUREA, ^RORN, ^RMOT, ^RIND, ^RMALN. If Klebsiella sp.is suspected add ^RMR and ^RVP. If Morganella sp or Providencia sp is suspected add ^RLIA. Inoculate media, labeling the first tube in the rack with the date and a small, RLABL sticker. Incubate the media set in room air at 35oC. Read tubes after 16 to 24 hours of incubation.

Interpret tube reactions using Table 7 of this SOP. Any organisms falling outside the descriptions in Table 1 must be referred to the Special Procedures Laboratory for further identification. Add the RSPL test to the workcard.

S2: Table 7

Organism	TSI				Lysine	Citrate	Urea	Ornithine	Motility[B]	Indole	Malonate	Comments
	slant	butt	gas	H2S								
***Shigella* sp.**	K	A	=	=	=	=	=	+/=	=	=	=	**Must be referred for serological confirmation**

Edwardsiella tarda	K	A	+	+	+	=	=	+	+	+	=	
Escherichia coli	A/K	A	+/=	=	+/=	=	=	+	+	+	=	
***Salmonella* sp.**	K	A	+/=	+	+	+/=	=	+/=	+	=	=	**Must be referred for serological confirmation**
Citrobacter freundii	K/A	A	+	+	=	+	+/=	+/=	+	=	+/=	
Citrobacter (diversus) koseri	A	A	+	=	=	+	+/=	+	+	+	+	
***Klebsiella* sp.** Add MR/VP	A/K	A	+	=	+/=	+	+	=	=	+/=	+	Do strip oxidase on LYS= to rule out *Aeromonas* sp.
Enterobacter cloacae	A/K	A	+	=	=	+	+/=	+	+	=	+	Do strip oxidase to rule out *Aeromonas* sp. Refer if organism has GMS
Klebsiella (Enterobacter) aerogenes	A	A	+	=	+	+	=	+	+	=	+/=	Do DNase if malonate = to rule out *Yersinia sp*.
Yersinia sp.	K/A	A	+/=	=	+/=	+	+/=	+	+	=	=	**Must be referred for confirmation**
Morganella morganii Add LIA	K	A	+	=	+/=	=	+	+	+	+	=	LIA R/A
***Providentia* sp.** Add LIA	K/A	A	+	=	=	+	+/=	=	+	+	=	LIA R/A **Must be referred for confirmation**
[A] Charts in Gram-Negative Bacilli Identification [008515] may be consulted as necessary to attempt to distinguish between the species within a genus. [B] Non-motile variants occur in all motile species.												

S3: Aerobic Antimicrobial Susceptibility testing

PROCEDURE:

A. Initial Set Up of ZMMLS, SUSC2, CARNP Samples
    1. Remove samples to be processed from all packaging material.
        a. Discard all packaging material in a red biohazard bag.
        b. Match up client paperwork, if available, to the respective sample.
    2. In Result Entry, scan the bar code for the sample.
        a. Verify patient information on the sample labels and available paperwork match.
        b. Review the following areas for any information pertaining to organism identification and specific testing requested.
            1) Client provided paperwork if available.
                - Free text information relevant to testing in the Common media comment field. Include "Per paperwork" to indicate the origin of the information.
            2) Labels on sample. o Free text information relevant to testing in the Comment media comment field. Include "Per slant" to indicate the origin of the information.
            3) Site field.
            4) All comment fields including Order comments, Culture comments and Micro Order Entry comments.
                - Copy and paste any relevant information from Order comment, Culture comments or Micro Order Entry comments into the Common Media comment field.
        c. Add media RLABL to the order.
            - This can be done either using the Add Media button or from the RBILL keypad in the workcard.
        d. In workcard view under the original media RMMLS, add the media code that corresponds to the type of media received from the client.

        e. Using the ASTWU keypad under the media received, add the appropriate subculture media and also add a bacterial workup. The subculture media ordered will depend upon the identification provided by the client.

S3: Table 1

Identification provided by client	Subculture media to be used	Additional comments
Gram-negative bacilli (*Enterobacterales*, Nonfermenters, *Pasteurella* spp.)	BA, EMB	Add CBA as needed

Haemophilus spp. and HACEK organisms (*Haemophilus* spp., *Aggregatibacter actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens* and *Kingella kingae*)	CBA, BA, EMB	
N. meningitidis/Gram-negative cocci	CBA, BA	Tape all plates
N. gonorrhoeae	CBA	
Gram-positive bacteria	BA	Add CBA as needed
Campylobacter spp.	CBA	Incubated in Campy jar using campy atmosphere.
Helicobacter pylori	BA	Incubated in Campy jar with moistened towel using campy atmosphere. H. pylori media (HP media) can also be added.

f. Under the work up media, add appropriate susceptibility testing. (RSENS, RDAPT, RTIG, RCANP, etc).
   - Indicate any specific antimicrobials requested or information that should be written on the BAMS tube at the time that susceptibilities are picked.

g. Enter appropriate information into the isolate tab. Do not verify at this time. NOTE: This step does not have to be performed at the time of ordering work in the workcard. However, it does need to be completed before susceptibility results can be sent from BAMS to the MIC tab.
   - Entering from the isolate tab:
     a) Organism number.
     b) Appropriate panel (MIC, KB and/or BP tab).
     c) Organism identification,
     d) Isolate comment–all MCL isolates will have comment, "Organism identified by client." added.
     e) Media ID–enter appropriate bacterial workup.
   - Entering from the workcard, under bacterial workup
     a) Using the DBWU1 keypad, select >DAST
     b) Using the DAST keypad, add ^;RSENS;&OIBC under the work up media
     c) Add organism identification code after the "^", but before the first ";" (e.g ^PSEAER;RSENS;&OIBC)
     d) Select "Ok"
     e) Select "Yes" for the Micro Results Entry- "Do you want to add the isolate?"

h. Save order and print labels.

3. Prepare culture divider.
   a. Sticker the culture divider using the large RLABL sticker.
   b. Write organism identification on the divider.

c. Write any specific antimicrobials requested on the divider

4. Label client sample with the small sticker of the media received. (i,e., RSLNT)
5. Label subculture plates and susceptibility tube with remaining stickers.
6. All client samples are subcultured in the Biological Safety Cabinet (BSC) using appropriate personal protective equipment. (Lab coat, gloves pulled over the cuff of the lab coat and safety eyewear.) Refer to the Biosafety Policy for the Bacteriology Lab [036885] for additional guidance. (e.g., International clients).
7. Incubate subculture plates at appropriate atmosphere and temperature.
8. Any sample submitted without an organism identification, should not be subcultured until the isolate identification is provided by the client.
    a. Submit a case ticket to MCL.
    b. Set the sample and labeled divider aside while waiting for a case resolution.
9. Client samples are filed in racks on the shelves at the end of the AST bench.

B. Initial Set Up of Samples with a Combination of ZMMLS and Molecular Lab Testing Related to Antimicrobial Resistance (KPNRP, OXVRP, GNRG) Aerobic Antimicrobial Susceptibility Testing Initial Setup and Workflow Information [PROC 061663.004]
    1. Isolates with Molecular Lab testing will only be delivered to AST when there is also a ZMMLS test ordered. Samples that only have Molecular testing ordered will be delivered to Molecular Lab directly.
    2. Remove samples to be processed from all packaging material. a. Discard all packaging material in a red biohazard bag. b. Match up client paperwork, if available, to the respective sample.
    3. In Order Entry, scan the bar code for the sample.
        a. Verify patient information on the sample labels and available paperwork match.
        b. Review the order comment field for organism identification information.
        c. Print 3 copies of the standard label. Stickers are used for the following.
            1) Culture divider.
            2) BA (also write organism identification on plate).
            3) EMB.
        d. Save order.
        e. Any sample submitted without an organism identification, should not be subcultured until the isolate identification is provided by the client.
            1) Submit a case ticket to MCL.
            2) Set the sample and labeled divider in the remedy box while waiting for a case resolution.

C. ZMMLS/SUSC2/CARNP: Day 2
    1. Gather all subculture plates and culture dividers set up the previous day.

2. Examine all subculture plates in the BSC. Plates will not be removed from BSC until potential highly infectious organisms can be ruled out. Refer to the Biosafety Policy for the Bacteriology Lab [036885] for criteria that must be met before plates can be removed from BSC.
3. For cultures that have been deemed safe to remove from the BSC, open the workcard view of Result entry.
    a. Answer the subculture media with "Growth" or "No Growth". If growth is weak it may be necessary to tape and reincubate plates. This should also be indicated in the workcard.
    b. Under the Bacterial workup add the following.
        1) Colony morphology
            i. Use descriptors consistent with Organism Identification [011332].
        2) Additional sub culture plates.
        3) Additional AST testing requested/appropriate due to organism identification.
    c. Date stamp RSENS media with the date susceptibility testing will be performed.
    d. Date stamp any additional testing and media ordered in the workcard.
    e. Save culture.
        i. Isolate will remain unverified and no status is applied to the order until the susceptibility results are ready to be reported.
    f. Culture dividers are filed on the Reporting side for resulting the next day.
    g. Sub plates will be saved at the end of the bench for 1 week.

D. ZMMLS in Combination with Molecular Testing Related to Antimicrobial Resistance (KPNRP, OXVRP, GNRG)-Day 2
    1. Follow the steps outlined above in ZMMLS/SUSC2/CARNP:Day
    2. BA plates are delivered to the Molecular bench for testing.

S4: Anaerobic cultures

PROCEDURE:

Processing and Incubation

1. Initial Processing staff will incubate inoculated THIO and TSA plate in CO015577$_2$ at 35°C.
2. All other anaerobe media will be placed into a CO2 holding jar, lid up, for 2 hours or until the jar is full.

3. Jars will be incubated in the Anaerobe Laboratory incubator at 35°C until the morning of day 3.

24 hour Pre-examination of cultures for aerobic growth

1. The afternoon of the day after inoculation, remove the THIOs and TSA plates from the incubator and separate the positive THIOs and TSA plates from those with no growth.
2. If a THIO appears positive and the aerobic plate is negative, order RLABL and an aerotolerance subculture in SoftLabMic. Inoculate subculture mediaand incubate the TSA in ambient atmosphere, the CBA in CO2atmosphere and the CDC plate in anaerobic atmosphere.
3. If a primary TSA plate has growth, and there are no organisms being processed under the GEN(S)culture, subculture the primary TSA morphology(ies) to TSA plate(s)and incubate in CO2atmosphere.

48 hour Primary Examination

1. Unpack thetwo-dayold GasPak® jars. Check anaerobic indicators on remaining jars, and documenting TotalQC.
   O Used jars will need to be bleached before being returned to circulation.
2. Separate out ANAE(S)/ACT, GEN(S), GENB, IDENT, B, and CFRC(S) CBA plates.
   O Return IDENT and CFRC(S) plates to SPL.

O Return GENS thio and isolate subs to their respective benches.

   O Subculture plates from Bactec bottles (xBTAN) will be evaluated by ANAE lab regardless of source; GENS body fluids, GENB tissues, and B/BBLD blood products.
3. Separate positive anaerobe cultures from negative.
   O Note that if any one of the primary media(THIO, SBS, RB,PEA, LVK, or TSA) has growth the culture is considered positive.
   O Segregate duodenal cultures (source: SMBA) from other cultures. These will be processed separately.
4. ANAE(S)/ACT cultures(other than SMBA's)that are "No growth" are marked and auto-resulted from the DANAR resulting worklist by scanning a representative anaerobe plate from each culture and selecting the 'Auto-result' function.
5. Refresh the DANAR worklist to eliminate all no growth cultures.
6. Place negative plates into metal canisters and incubate on the heated side of the anaerobic chamber until plates are 7 days old. These will be checked Monday, Wednesday, and Friday's for growth.
7. Incubate negative THIOs in the non-CO235ºC incubator for 14 days and check daily for growth.
8. SIBO cultures:
   O Process according to Quantitative Culture for Small Intestine Bacterial Overgrowth (SIBO)[012095].
9. Scan the remaining positive specimens in order to add an RLABL to the order.

a. Select Define MC from the right-hand menu.
b. Click OK in the select tests window to define the media comment under test ANAE(S)/ACT.
c. Type in RBILL in the select media window followed by OK.
d. In the DBILL window select RLABL from the keypad and then OK.
e. Select Add Results from the right-hand menu.
f. In the upper tool bar select Refresh. This will print a card label foreach positive specimen.

10. Investigate any specimens remaining on the DANAR work list and address accordingly.
    O Common causes of 'missing' cultures include.
        1) Ordered but not acceptable (e.g. swab) with test not yet credited.
        2) Set up close to midnight and closed in the wrong day's jar.
        3) Placed into bottles with test not yet credited.
        4) Ordered but accidentally not set up.
    •When this occurs an evaluation of specimen quality and viability needs to be assessed prior to determining whether the culture can be set-up.

S5: Anaerobic Antimicrobial Susceptibility testing

PROCEDURE:

Preparation of Agar Dilution Plates

1. Remove eight 200mL, eight 150mL bottles, and six 100mL bottles and bottles of Brucella agar from the walk-in refrigerator on Friday afternoon. Loosen bottle caps and set on counter in AST lab for Sunday evening lab personnel to melt the following morning. For non-routine antibiotic plates, technologists may melt agar at any time using the microwave oven in Antimicrobial Susceptibility Lab.
2. Cool freshly melted agar in a 50°C water bath (at least 30 minutes).
3. Remove seventeen 10mL aliquots of laked sheep blood from the -20°C freezer on Monday morning and allow to thaw at room temperature.
4. Prepare dilutions according to the dilution schemes found in Attachment 1 and Attachment 2.

Table 2: Antibiotics and Test Concentrations

Drug	Abbr	Concentrations (mcg/mL)
Amoxicillin-Clavulanate	ANAMC	4/2, 8/4
Ampicillin-Sulbactam	ANSAM	0.5/0.25, 1/0.5, 2/1, 4/2, 8/4, 16/8, 32/16
Cefotaxime	ANCTX	16, 32
Ceftriaxone	ANCRO	16, 32
Ciprofloxacin	ANCIP	1,2
Clindamycin	ANCC	2, 4
Ertapenem	ANETP	4, 8

Imipenem	ANIPM	0.03, 0.06, 0.12, 0.25, 0.5, 4, 8, 16
Meropenem	ANMEM	4, 8
Metronidazole	ANMET	2, 4, 8, 16
Minocycline	ANMI	4, 8
Moxifloxacin	ANMX	2, 4
Penicillin	ANP	0.5,1
Piperacillin-Tazobactam	ANTZP	16/4, 64/4
Rifampin	ANRIF	0.03, 0.06, 0.12, 0.25
Vancomycin	ANVA	0.5,1,2,4

1. Add 5 mL of laked sheep blood per 100 mL of Brucella agar.
2. Prepare each dilution plate by adding the indicated volume of antimicrobial. After thorough mixing, pour agar contents into 6 petri dishes which have been labeled with the antimicrobial abbreviation code, the concentration and date on the lowest concentration plate of any given drug. If bubbles form on the surface of the media, immediately flame the surface with a Bunsen burner. Place the lid ajar and allow media to solidify.
3. Rinse out the 100 mL media bottles, ensure there are no traces of blood in the bottle or cap, and return to Media Lab black bins for processing.
4. Store agar dilution Brucella blood agar plates in stacks of dilution sets at 2-8°C (anaerobe refrigerator) for up to 7 days.
    - Exceptions;
        a) Amoxicillin-Clavulanate plates must beused on the day of preparation
        b) Imipene plates must be used within 3 days of preparation

Table 3: Routine Antimicrobial Testing Performed Based on Organism Group The antimicrobials listed (except vancomycin) are included on the routine panel. Those marked with 'X' are routinely reported. Specimen source and site limitations are noted in column headings.	**Clindamycin** (not urine or CSF)	**Ertapenem** (not CSF)	**Metronidazole**	**Moxifloxacin**	**Penicillin**	**Piperacillin-Tazobactam**	**MinocyclineA** (not CSF)	**VancomycinA**
• β-lactamase positive anaerobic gram-negative bacilli • *Bacteroides* spp[B] • *Parabacteroides* spp[B] • *Phocaeicola* spp[B]	X	X	X			X		
• β-lactamase negative anaerobic gram-negative bacilli • Anaerobic cocci • Non-spore forming gram positive bacilli (other than those listed below)	X		X		X[F]			
• Large spore-forming GPB (such as *Clostridium, Lachnoanaerobaculum, Paraclostridium, Enterocloster, etc)*	X	X	X		X	X		
• *Cutibacterium, Propionimicrobium, Arachnia,* and *Propionibacterium* species[C]				X	X[F]		X	
• *Actinomyces, Schaalia, Gleimia, Pauljensenia, Buchananella, Bowdeniella, Winkia* species[C,D]	X				XF			
• Staphylococcus saccharolyticus[A,E]	X		X		X			

• *Clostridioides difficile* recovered from feces/intestinal isolates			X					X

A – There will be no interpretive categories reported
B - β-lactamase testing not performed on *Bacteroides, Parabacteroides,* or *Phocaeicola* spp
C – Metronidazole has little or no activity against these organisms
D – Add isolate comment of 'This organism routinely demonstrates resistance to metronidazole'
E – Add isolate comment of 'Susceptibility testing performed anaerobically'
F – Ertapenem and piperacillin-tazobactam will be released on isolates that test as penicillin resistant

Patient Testing

1. Assess subculture plates at 24-48 hours of incubation for sufficient growth and purity. Confirm that appropriate QC organisms and a sufficient number of Schaedler's broth tubes are available. Broth must spend a minimum of 4 hours reducing in chamber prior to use.
2. Order ^RANS in the workcard for each patient isolate that will be set up. Ensure that the organism is entered into the isolate tab with associated RANAD panel.
3. Setup/Modify Worksheet In BAMS, create a board utilizing the appropriate panel(s) and name it ANA (if more than one board will be set up, number the boards sequentially; e.g. ANA1, ANA2).
    a. See Reporting Anaerobic Antimicrobial Susceptibilities with the BAMS System [058400] for information on utilizing the BAMS system.
    b. See Attachment 4 for panel descriptions.
    c. In the associated worksheet, assign the isolates to positions corresponding to the order on the Seed Plate Worksheet. Print a copy to retain.
4. Inoculation of plates

NOTE: Inoculated antimicrobial plates must be closed into anaerobic jars within 30 minutes from the time the Schaedler's broth is removed from the anaerobic chamber.

a. Remove antimicrobial plates as needed for the day's susceptibilities from the refrigerator and allow to equilibrate to room temperature. See Table 3 for routine susceptibilities performed per organism group. **NOTE:** If testing a swarming *Clostridium* sp, an additional SB plate per anaerobe jar will be required.
b. Working in the unheated side of the chamber, suspend organisms in Schaedler's broth using the Grant bio Densitometer to adjust the broth to a turbidity of 0.5-0.6 McFarland.
c. Attach the replicator pin holder to the support arm.
d. Pipet India ink into well #1 of the seed plate to serve as an orientation marker. Fill well approximately halfway.
e. Pipet an aliquot of each control and patient sample into its assigned well using a plastic transfer pipet. 1) Fill wells approximately half full. Overfilling may cause mixing due to splashing.
    i. 2) Do not fill wells with spreading *Clostridium* sp at this time.
f. Inoculate plates in the following order: 1) SB control plate(s) (omit spreading *Clostridium*, if applicable).
    i. Special drugs, if requested but not to be reported on the swarming *Clostridium*
    ii. Fill well(s) with spreading *Clostridium* sp isolate(s).
    iii. Stamp second SB control plate.
    iv. Remaining susceptibility plates.
g. Inspect each plate to verify that each position has been inoculated. If no inoculation has occurred, a 10 mcL calibrated loop may be used to inoculate that spot or the organism must be retested. Do not re-stamp plates.
h. Allow inoculated media to stand (without inverting) to permit the moisture to absorb into the agar.
i. Perform a purity check by streaking 1 mcL from each well to half of a CDC blood agar plate.
j. Place plates in a GasPak jar with indicator and Anaero-Pak without tipping or inverting the plates. Incubate at 33-37°C for 42-48 hours.
k. Remove the India ink from the seed block with a transfer pipet followed by a swab. Place block and pins into the ethanol soaking pans on the cart in AST. Safety eyewear is required while handling alcohol. Soak the seed plate and replicator pin holder in 70% ethyl alcohol for a minimum of 30 minutes. Evening shift will scrub seed plate and replicator pin holder with a brush and detergent, rinsing

REPORTING/INTERPRETING RESULTS:

Reading the Plates

See Reporting Anaerobic Antimicrobial Susceptibilities with the BAMS System [058400].

1. Read plates
    a. Retrieve the worksheet from the "to read" shelf and:
        i. Update the identifications on genus-level and Gram stain only listings as possible.
        ii. Update any anatomic source listed as SIT or OTHER- AST results may not be released without a confirmed source.
    b. Examine purity plates to verify pure isolates were tested. Mixes are invalid and must be retested.
    c. Examine the control plate(s) for growth.
        i. If multiple jars were utilized, compare the control plates to one another. If each of the control plates appears to be growing at the same strength the whole board may be compared to a single plate. If the plates appear to have different levels of growth, then the antibiotics plates must be compared to the control plate associated with that jar.
        ii. Failure of an organism to grow on the control plate is termed a Growth Control Failure (GCF). No antibiotics will be reported for that isolate. Re-testing may ensue depending on organism identification. See procedural notes for exceptions.
    d. **Read the endpoint as that concentration where a marked change occurs in the appearance of growth as compared to control plate inoculum.** The marked change in growth might be a change from confluent growth to a haze, <10 tiny colonies, or one to three normal-sized colonies. See Attachment 3 for pictorial reference.
2. Confirm that all QC organisms are within range.
3. Assess isolate antibiotic patterns to ensure that confirmatory testing is not required. See Table 4.
    a. Organisms which are resistant to antibiotics where the 'Expected Result' is susceptible should be evaluated prior to reporting.
    b. If it is determined that an isolate demonstrates an unusual resistance pattern, additional testing should be conducted to ensure that the result is clinically accurate. Testing may include, but is not limited to, confirmation of ID and/or confirmation of AST result by repeat testing.
    c. Consultation of the Mayo Antibiogram App can assist in determining if an organism is demonstrating an unusual level of resistance. http://mayoweb.mayo.edu/antibiogram/antibiogram.html
4. Assess β-lactamase results in conjunction with the penicillin result.
5. In rare cases an organism may be β-lactamase negative but penicillin resistant. In these cases, repeat the β-lactamase testing to confirm a negative result and report ertapenem and piperacillin-tazobactam in addition to the routine antimicrobials.

6. *Bacteroides*, *Parabacteroides,* and *Phocaeicola* spp. are expected to be β-lactamase positive, however rare species may not possess β-lactamase enzymes. Organisms in these genera that present as penicillin susceptible should be investigated. Perform a β-lactamase test, and if the result is negative, report the β-lactamase result and penicillin result instead of ertapenem and piperacillin-tazobactam.
7. Send results.

Reporting Results

1. Refer to Table 3 for specifics on which antimicrobials to report and Table 4 for their interpretations. The MIC and interpretation will be displayed in the final report. These tables also list expected results. Further antibiogram information can be obtained from:
   o Mayo Clinic Antimicrobial Therapy Quick Guide
   o http://mayoweb.mayo.edu/antibiogram/antibiogram.html
2. β-lactamase negative *Porphyromonas* spp. are routinely reported as "Additional susceptibility testing not performed per laboratory criteria - this organism is predictable susceptible to penicillin" in conjuction with the β-lactamase result. Susceptibility testing may be attempted if phoned request is received.
3. Organisms that have been repeated for confirmatory purposes, but which fail to grow for additional testing should be reported as "Growth not adequate for additional susceptibilities." (&BASN). If AST pattern is suspect, consultation with management, the Microbiology Fellows, or the Lab Director should be considered.
4. Organisms that do not grow on the BAMS control plates should be repeated once more before adding an isolate comment of "Unable to perform susceptibility testing. Organism did not grow on test medium." (&BUPS)
   o Do not repeat susceptibility testing on Porphyromonas spp. or slow growing pigmented anaerobic gram negative bacilli that fail to grow on the control media on the first attempt. These organisms generally do not grow rapidly enough to achieve readable results. Add "Unable to perform susceptibility testing. Organism did not grow on control media" (&BUPS) isolate comment.

Table 4: Reporting Guidelines for Anaerobic Bacteriaa						
Antibiotic (Soft code)	Additional Testing / Notes	Report on:	Interpretation (µg/mL)			Expected Results
			S	I	R	
Clindamycin (ANCC)	• Do not report on CSF sources • Do not report on URINE sources	• Morphologies other than small Gram-positive bacilli resembling *Cutibacterium*	≤2	4	>4	
Metronidazole (ANMET)	• Verify purity and/or aerotolerance on organisms that are metronidazole resistant • Confirm resistance on anaerobic organisms other than small non-sporeforming Gram-positive bacilli before releasing result	• Morphologies other than small Gram-positive bacilli resembling *Cutibacterium* sp, *Actinomyces* sp, and related genera	≤8	16	>16	• Most obligate anaerobic organisms should be metronidazole susceptible • *Staph. saccharolyticus* and aerotolerant GPB such as *Actinomyces* sp, should be resistant
Penicillin (ANP)		•Morphologies other than β-lactamase positive gram negative bacilli • Do not release on organisms that are β-lactamase positive[c]	≤0.5	1	>1	• Susceptible • *Clostridium* and *Eggerthella* sp may be resistant • Anaerobic GNC are commonly intermediate or resistant
Ertapenem (ANETP)	• Do not report on CSF sources • Repeat testing to confirm resistance before reporting • Freeze organism if resistant	• β-lactamase positive anaerobic gram negative bacilli[c] • Penicillin resistant organisms	≤4	8	>8	Susceptible
Piperacillin-Tazobactam		• β-lactamase positive anaerobic gram negative bacilli[c]	≤16/4	64/4	>64/4	Susceptible

(ANTZP)		• Penicillin resistant organisms				
Moxifloxacin (ANMX)	• Freeze organism if resistant	• Cutibacterium sp • *Propionimicrobium sp* • *Propionibacterium* sp • Arachnia sp	≤2	4	>4	Susceptible
Minocycline (ANMI)	• Do not report on CSF sources • Freeze organism if resistant	• Cutibacterium sp • *Propionimicrobium* sp • *Propionibacterium* sp • Arachnia sp	No interpretive categories exist, report MIC only[b]			≤4

Interpretation Codes
S: Susceptible
I: Intermediate
R: Resistant
N: Non susceptible
D: Susceptible dose dependent
No interpretation: Add the test comment }MYST "There are no established interpretive guidelines for agents reported without interpretations."

Table 4: Reporting Guidelines for Anaerobic Bacteria[a]

Antibiotic (Soft code)	Additional Testing / Notes	Report	Interpretation (µg/mL)			Expected Results
			S	I	R	
Amoxicillin-Clavulanate (ANAMC)		By special request only	≤4/2	8/4	>8/4	
Ampicillin-Sulbactam (AMSAM)		By special request only	≤4/2	16/8	≥32/16	
Cefotaxime (ANCTX)		By special request only	≤16	32	>32	
Ceftriaxone		By special request only	≤16	32	>32	

(ANCRO)	• Organisms which are Penicillin susceptible and Ceftriaxone resistant should be verified before reporting					
Ciprofloxacin (ANCIP)	• Do not report on CSF sources	By special request only	No interpretive categories exist, report MIC only[b]			
Imipenem (ANIPM)	• Do not report on CSF sources	By special request only	≤4	8	>8	
Meropenem (ANMEM)	• Repeat testing to confirm resistance before reporting • Freeze organism if resistant	By special request only	≤4	8	>8	
Rifampin (ANRIF)	• *C. acnes* only	By special request only	No interpretive categories exist, report MIC only[b f]			≤0.03
Vancomycin (ANVA)	• Organisms other than *C. acnes* & *Clostridioides difficile*	By special request only	No interpretive categories exist, report MIC only[b]			
			ECV[d]	ECV[e]		
	• *Cutibacterium acnes* & *Clostridioides difficile*	By special request only	<2	≥4		

[a] *Staphylococcus saccharolyticus* should have MIC's reported without interpretive criteria.
[b] Add comment "There are no interpretive guidelines for agents reported without interpretations." Into report.
[c] The criterion "β-lactamase positive" refers to organisms that have tested positive for β-lactamase, and to organisms that are presumed to be β-lactamase positive (eg. *Bacteroides* and *Parabacteroides* spp).
[d] "This MIC is consistent with the Epidemiological Cutoff Value (ECV) observed in isolates WITHOUT acquired resistance; however, correlation with treatment outcome is unknown. Infectious Disease consult is suggested." Otherwise there is no interpretation.
[e] "This MIC is consistent with the Epidemiological Cutoff Value (ECV) observed in isolates WITH acquired resistance; however, correlation with treatment outcome is unknown. Infectious Disease consult is suggested." Otherwise there is no interpretation.
[f] "The range of MIC values of *C. acnes* isolates presumed to lack resistance mechanisms is <= 0.03 mcg/mL."