

COMPOSITIONS AND METHODS FOR RAPID IDENTIFICATION AND PHENOTYPIC ANTIMICROBIAL SUSCEPTIBILITY TESTING OF BACTERIA AND FUNGI

FIELD OF THE INVENTION

The present disclosure relates to the field of molecular diagnostics, and more particularly to the identification and the determination of antimicrobial susceptibility of bacteria from biological samples.

5 BACKGROUND OF THE INVENTION

There is an urgent need for the development of rapid and convenient methods for the detection, identification and determination of antimicrobial susceptibility of bacterial pathogens in clinical samples to guide the diagnosis and treatment of infectious disease. A good example for this need is in bloodstream infections (BSI). BSI ranks among the top seven causes of death in North
10 America and Europe with an estimated 1.7 million sepsis events/ year in the US contributing to 270,000 deaths/year and \$14 billion annual US healthcare costs. A decreased time to directed antimicrobial therapy has been shown to improve morbidity and mortality in septic patients, which then results in decreased length of stay (LOS) and lower hospital costs. Faster susceptibility results will also enable more rapid antimicrobial de-escalation leading to less
15 adverse effects and decreased contribution to drug resistance. Therefore, there remains a need for the development of an assay and testing system that will provide rapid phenotypic antimicrobial susceptibility results in BSIs enabling clinicians to provide the most appropriate antimicrobial therapies more quickly leading to improved patient outcomes.

Polymicrobial bloodstream infection (BSI), defined as the presence of at least two different
20 microorganisms found from the blood cultures, has been reported increasingly, with rates ranging from 6% to 32% of all BSI episodes. The mortality rate of hospitalized patients with polymicrobial BSIs ranged from 21% to 63%, approximately twice the rate of those with monomicrobial infections.

Traditional Antimicrobial Susceptibility Testing (AST) is performed by growing a given
25 bacteria in the presence of a given antimicrobial – this can be done in both liquid culture and on solid agar media. The two most common methods of AST are: 1) Microbroth Dilution, and 2) Disk Diffusion (aka Kirby-Bauer). The microbroth dilution method provides both quantitative (Minimum Inhibitory Concentration) and qualitative (Susceptible, Intermediate, and Resistant) results. The disk diffusion method provides only qualitative results.

30 Microbroth dilution is performed by incubating a given bacteria in the presence of multiple concentrations of antimicrobial. Following incubation, growth/no growth of the bacteria is

observed at each concentration of antimicrobial. The lowest concentration at which no growth is observed is the Minimum Inhibitory Concentration (MIC), and has units of $\mu\text{g/mL}$. Established guidelines provide the “breakpoint” where experimentally determined MIC values for specific bacterial groups or species are interpreted as susceptible, intermediate, or resistant to the given antimicrobial.

Disk diffusion is performed by creating a “bacterial lawn” of a given bacteria on solid agar media, and then placing a singular antibiotic disk onto the agar media. The antibiotic in the disk diffuses into the media, and following incubation, a circular zone of inhibition around the disk is created. The diameter of the zone along with information on the bacterial species and antimicrobial is used in conjunction with reference established diameter breakpoints to determine whether the pathogen is susceptible, intermediate, or resistant to the given antimicrobial.

In general, the two most commonly used guidelines for interpreting AST results are guidelines from the: 1) Clinical Laboratory Standards Institute (CLSI), and 2) European Committee on Antimicrobial Susceptibility Testing (EUCAST). The US uses the CLSI guidelines while the European countries use the EUCAST guidelines. The current version from CLSI is M100 ED30, while the current version from EUCAST is Version 10.

SUMMARY OF THE INVENTION

The present invention relates to a polymerase chain reaction (PCR)-based rapid identification and antimicrobial susceptibility testing (ID/AST) System that supports an automated workflow for specific assay panels utilizing PCR technology for the rapid identification and/or determination of antimicrobial susceptibility of bacteria, directly from positive blood cultures for use in clinical laboratories. This system also has the capability of utilizing and analyzing samples from other sample types such as urine and respiratory infections. The PCR-based rapid ID/AST System uses the functionalities of instrumentation, consumables, reagents, and data management to provide a workflow from sample processing with reagents to result interpretation. Target identification and antimicrobial susceptibility results are outputs from the system.

The present invention also relates to a PCR-based rapid ID/AST bloodstream infection (BSI) panel that is an in vitro diagnostic test utilizing PCR technology for the rapid identification of select bacteria or fungi and performing phenotypic antimicrobial susceptibility testing (AST) on the PCR-based rapid ID/AST system. The PCR-based rapid ID/AST BSI assay can be performed directly on positive blood culture samples, on pre-positive blood cultures, or

potentially directly from patient serum. This assay is indicated as an aid in diagnosing and identifying antimicrobial susceptibility of specific pathogens that can cause bacteremia. This panel is designed to analyze the most common BSI Gram-negative and Gram-positive pathogens, with the potential for Fungi. The TaqMan 5' nuclease real-time PCR assay configuration coupled with the ID strategy should provide the capability to provide Minimum Inhibitory Concentration (MIC) and Susceptible, Intermediate and Resistant (SIR) information for polymicrobial samples, monomicrobial samples, and isolate testing as needed. These results should be used in conjunction with other clinical and laboratory findings. Standard laboratory protocols for processing positive blood cultures should be followed to ensure availability of isolates for supplemental testing as needed.

The present invention also relates to PCR-based rapid ID/AST phenotypic screening tests that are *in vitro* diagnostic tests utilizing PCR technology for the rapid identification and phenotypic susceptibility testing of a target pathogen or group of pathogens for a single drug, class of drugs, or combination of drugs. The PCR-based rapid ID/AST screening assays are performed either directly from clinical samples or from bacterial/fungal isolates. These assays will indicate the presence of problematic drug resistant pathogens to aid in patient and hospital safety. Examples of these types of tests are for Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant *Staphylococcus aureus* (VRSA), Vancomycin-resistant Enterococci (VRE), Carbapenem-Resistant Enterobacteriaceae (CRE), *Candida auris*, and MDR *Neisseria gonorrhoeae*.

Therefore, one aspect of the present invention relates to a method of using a quantitative real-time PCR assay as a reporter to simultaneously identify and determine the antimicrobial susceptibility of a group of bacteria or fungi that have similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial class. In one embodiment, the target group of bacteria or fungi are present in bloodstream infections (BSI), gastrointestinal infections, respiratory infections, urinary infections, nasal infections, rectal infections or wound infections. In one embodiment, identification of the group of bacteria or fungi is by detecting a signal that is specific to the group of bacteria or fungi. In one embodiment, the specific signal is detected by using primer and probe oligonucleotides that hybridize more selectively to a target gene that is from the group of bacteria or fungi than to the target gene that is not from the group of bacteria or fungi. In one embodiment, the target gene is selected from *rplP*, *ompA*, *tuf*, *rpoB*, *ddl*, *ddlA*, *fdnG*, *sodA*, *gyrB*, O-antigen acetylase, *ecfX*, *tusA*, CPE, *sip*, and *muc*.

In another embodiment, the group of bacteria or fungi represents a taxonomic order. In one embodiment, the taxonomic order is the order Enterobacterales. In another embodiment, the

group of bacteria or fungi comprises a taxonomic family. In one embodiment, the taxonomic family is selected from Enterobacteriaceae, Yersiniaceae, Morganellaceae, or a combination of said families. In yet another embodiment, the group of bacteria or fungi comprises a taxonomic genus. In one embodiment, the taxonomic genus is selected from *Enterococcus*, *Candida*,
5 *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, *Escherichia*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Citrobacter*, *Serratia*, *Yersinia*, *Morganella*, *Providencia*, or *Proteus*.

In another embodiment, the group of bacteria or fungi represents a taxonomic species. In one embodiment, the taxonomic species is selected from *Enterococcus faecalis* (*Efs*), *Enterococcus faecium* (*Efm*), *Escherichia coli* (*Eco*), *Klebsiella pneumoniae* (*Kpn*), *Klebsiella oxytoca* (*Kox*),
10 *Enterobacter cloacae* (*Ecl*), *Enterobacter aerogenes* (*Kae*), *Citrobacter freundii* (*Cfi*), *Citrobacter koseri* (*Cko*), *Morganella morganii* (*Mmg*), *Providencia stuartii* (*Pst*), *Proteus mirabilis* (*Pms*), *Proteus vulgaris* (*Pvs*), *Candida albicans* (*Cal*), *Candida auris* (*Cau*), *Pseudomonas aeruginosa* (*Pae*), *Acinetobacter baumannii* (*Abi*), *Acinetobacter pittii* (*Api*),
15 *Acinetobacter nosocomialis* (*Ano*), *Staphylococcus aureus* (*Sau*), *Staphylococcus epidermidis* (*Sep*), *Stenotrophomonas maltophilia* (*Sma*), *Streptococcus pneumoniae* (*Spn*), *Streptococcus agalactiae* (*Sag*), or *Streptococcus pyogenes* (*Spy*).

In yet another embodiment, the method further comprises a step selected from verifying the identification of the group of bacteria or fungi with additional primer and probe
20 oligonucleotides that hybridize more selectively to a second target gene that is from the group of target bacteria or fungi than to the second target gene that is not from the group of bacteria or fungi or determining a mechanism for an antimicrobial susceptibility phenotype or for a toxin or virulence phenotype, or both verifying and determining steps. In another embodiment, the method further comprises simultaneously identifying and determining the antimicrobial
25 susceptibility of more than one groups of bacteria or fungi wherein each group of bacteria or fungi has similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial classes.

In another aspect, the present invention relates to a method of using a quantitative real-time PCR assay as a reporter to simultaneously identify and determine the antimicrobial
30 susceptibility of bacteria of the Enterobacterales order to an antimicrobial or a class of antimicrobials by using primer and probe oligonucleotides that hybridize more selectively to a target gene that is in the Enterobacterales taxonomic order than to the target gene that is not in the Enterobacterales taxonomic order. In one embodiment, the target gene is selected from *rpIP*, *gyrB*, and *rpoB*. In one embodiment, the primer and probe oligonucleotides that hybridize more

selectively to the target gene that is in the Enterobacterales taxonomic order than to the target gene that is not in the Enterobacterales taxonomic order comprise the nucleotide sequences comprising SEQ ID NOs: 1-16. In one embodiment, the primer and probe oligonucleotides that hybridize more selectively to *gyrB* in the Enterobacterales order than to *gyrB* in the non-Enterobacterales order comprise the nucleotide sequences comprising SEQ ID NOs: 8-10.

In another aspect, the present invention relates to a method of using a multiplexed quantitative real-time PCR assay as a reporter to simultaneously identify and determine the antimicrobial susceptibility of a plurality of bacterial or fungal strains from a biological sample, i.e. from a polymicrobial biological sample. In one embodiment, the biological sample is selected from whole blood, plasma, serum, red blood cell fraction, saliva, cerebrospinal fluid, semen, stool, urine, nasal swab, wound swab, dermal swab, rectal swab, bile, lymph, sputum, lavage fluid, or a combination thereof. In one embodiment, the biological sample is whole blood, plasma, serum or a combination thereof. In one embodiment, the biological sample is cultured prior to performing the PCR assay. In another embodiment, the biological sample is a bacterial or fungal isolate. In another embodiment, the plurality of bacterial or fungal strains are grouped into at least one group of bacteria or fungi that have similar or identical clinical breakpoints for at least one antimicrobial or antimicrobial class. In one embodiment, the plurality of bacterial or fungal strains are grouped into more than one groups of bacteria or fungi wherein each group of bacteria or fungi has similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial class.

In another embodiment, the identification of the plurality of bacterial strains utilizes a plurality of strain-specific 5' nuclease (TaqMan[®]) oligonucleotide probes, each labeled with fluorescent dyes that have different emission wavelengths. In one embodiment, the identification of the plurality of bacterial strains utilizes the TAGS (Temperature Assisted Generation of Signal) technology. In another embodiment, the method further comprises a step selected from verifying the identification of the plurality of bacterial or fungal strains, or determining a mechanism for an antimicrobial susceptibility phenotype or for a toxin or virulence phenotype, or both verifying and determining steps.

In another aspect, the present invention relates to a method of using a quantitative PCR assay to simultaneously identify and determine Susceptible, Intermediate and Resistant (SIR) information for a target bacterial or fungal strain or for a target group of bacteria or fungi to an antimicrobial or a class of antimicrobials wherein the identification of the target strain or target group and the determination of SIR information are derived from one or more mathematical relationships associated with PCR data.. In one embodiment, the mathematical relationship is

selected from Threshold Cycle (Ct), Slope, Inflection of a sigmoid curve fit, Absolute Fluorescence Intensity (AFI), or Endpoint Relative Intensity (ERI). In another embodiment, the mathematical relationship is a relative expression between different antimicrobial concentrations or different antimicrobials and is selected from ΔCt , $2^{\Delta Ct}$, Δ Inflection, ΔAFI , or ΔERI . In one embodiment, the mathematical relationship is a combination of mathematical relationships selected from Threshold Cycle (Ct), Slope, Inflection of a sigmoid curve fit, Absolute Fluorescence Intensity (AFI), or Endpoint Relative Intensity (ERI). In yet another embodiment, the mathematical relationship is a combination of relative expressions between different antimicrobial concentrations or different antimicrobials and is selected from ΔCt , $2^{\Delta Ct}$, Δ Inflection, ΔAFI , or ΔERI . In another embodiment, the method further comprises a step of selected from verifying the identification of the target bacterial or fungal strains or target group of bacteria or fungi, or determining a mechanism for an antimicrobial susceptibility phenotype or for a toxin or virulence phenotype, or both verifying and determining steps. In another embodiment, the method further comprises identifying and determining the antimicrobial susceptibility of more than one target bacteria or fungi strains or more than one target groups of bacteria or fungi wherein each target strain or target group has similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial class.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. In the absence of antimicrobial (shown as Reference), bacteria have ongoing genomic DNA replication. In the presence of an antimicrobial, resistant bacteria will replicate with similar number of genome copies as the Reference, while susceptible bacteria will experience inhibition of replication resulting in fewer copies. This difference in growth provides a phenotypic readout that can be determined by qPCR.

FIG. 2. A The raw qPCR data is depicted as growth curves where fluorescence (e.g. from a TaqMan probe) is measured at each PCR cycle. For the resistant isolate, the growth curve appears similar irrespective of incubation for four hours at different antimicrobial concentrations, whereas in the susceptible isolate, a dose-dependent decrease in fluorescent intensity and increase in the number of cycles required for the signal to cross background (threshold) level, which is commonly referred as the Cycle threshold or Ct value.

FIG. 2. B The same qPCR data is represented based on the Ct value, where a resistant isolate has little or no change in Ct value as a function of antimicrobial concentration, while a susceptible isolate has a dose dependent increase in Ct value by detecting a smaller number of replicating bacteria.

FIG. 3. A Mathematical relationships such as “Slope”, “Ct”, “Inflection”, “Absolute Fluorescence Intensity (AFI)”, and “Endpoint Relative Intensity (ERI)” describe the behavior of the raw qPCR amplification curve.

FIG. 3. B These features can further be evaluated as a function of antimicrobial exposure by relating numerical values obtained in the presence of antimicrobial back to the numerical values obtained in the absence of the antimicrobial. Relative changes in these features, such as “ Δ Ct”, “ Δ Inflection” or “ Δ AFI” can then be used to determine strain MIC to a given drug allowing strain resistance and susceptibility determination utilizing approved breakpoints.

FIG. 4. Partitioning models were used to classify bacteria isolates as resistant or susceptible. Partitioning utilizes the Gini Index to determine which feature and threshold maximizes dispersion of the two classes. Only two splits are utilized to prevent overfitting and allows for graphical representation in two dimensions for easy visualization.

FIG. 5. PCR assays using primers and probe disclosed in TABLE III that target the *gyrB* gene were tested against common Gram-negative pathogens. Growth curves are observed only for the species that constitute the order *Enterobacterales* (including *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, and *P. mirabilis*). No meaningful amplification was observed for non-target organisms.

FIG. 6. PCR assays using primers and probe disclosed in TABLE II that target the *rplP* gene were tested against common Gram-negative pathogens. Growth curves are observed only for the species that constitute the order *Enterobacterales*. No meaningful amplification was observed for non-target organisms.

FIG. 7. PCR assays using primers and probe disclosed in TABLE IV that target the *rpoB* gene were tested against common Gram-negative pathogens. Growth curves were observed only for the species that constitute the order *Enterobacterales*. No meaningful amplification was observed for non-target organisms.

FIG. 8. PCR assays using primers and probe disclosed in TABLE V that target the *ompA* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Growth curves are observed only for the prevalent pathogens that are within the genus *Acinetobacter* (*A. baumannii* and *A. pittii*). No meaningful amplification was observed for non-target organisms.

FIG. 9. PCR assays using primers and probe disclosed in TABLE VI that target the *rpoB* gene were tested against common Gram-negative pathogens. Growth curves are observed only for

the prevalent pathogens that are within the genus *Acinetobacter*. No meaningful amplification was observed for non-target organisms.

FIG. 10. PCR assays using primers and probe disclosed in TABLE VII that target the *gyrB* gene were tested against common Gram-negative pathogens. Growth curves are observed only for the prevalent pathogens that are within the genus *Acinetobacter*. No meaningful amplification was observed for non-target organisms.

FIG. 11. PCR assays using primers and probe disclosed in TABLE VIII that target the *tuf* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Growth curve is observed only for pathogens within the genus *Pseudomonas* (*P. aeruginosa*). No meaningful amplification was observed for non-target organisms.

FIG. 12. PCR assays using primers and probe disclosed in TABLE IX that target the *gyrB* gene were tested against common Gram-negative pathogens. Growth curve is observed only for pathogens within the genus *Pseudomonas* (*P. aeruginosa*). No meaningful amplification was observed for non-target organisms.

FIG. 13. PCR assays using primers and probe disclosed in TABLE X that target the *rpoB* gene were tested against common Gram-negative pathogens. Growth curve is observed only for pathogens within the genus *Pseudomonas* (*P. aeruginosa*). No meaningful amplification was observed for non-target organisms.

FIG. 14. PCR assays using primers and probe disclosed in TABLE XI that target the *fdnG* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Growth curve is observed only for the species of *Stenotrophomas maltophilia*. No meaningful amplification was observed for non-target organisms.

FIG. 15. PCR assays using primers and probe disclosed in TABLE XII that target the *gyrB* gene were tested against common Gram-negative pathogens. Growth curve is observed only for the species of *Stenotrophomas maltophilia*. No meaningful amplification was observed for non-target organisms.

FIG. 16. PCR assays using primers and probe disclosed in TABLE XIII that target the *tuf* gene were tested against common Gram-negative pathogens. Growth curve is observed only for the species of *Stenotrophomas maltophilia*. No meaningful amplification was observed for non-target organisms.

FIG. 17. PCR assays using primers and probe disclosed in TABLE XV that target the *rpoB* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Growth curves are observed only for pathogens within the genus *Enterococcus* (*E. faecium* and *E. faecalis*). No meaningful amplification was observed for non-target organisms.

FIG. 18. PCR assays using primers and probes disclosed in TABLE XVI that target the *ddl* gene were tested against common Gram-positive pathogens. Growth curves are observed only for pathogens within the genus *Enterococcus* (*E. faecium* and *E. faecalis*). No meaningful amplification was observed for non-target organisms.

FIG. 19. PCR assays using primers and probe disclosed in TABLE XVII targeting the *gyrB* gene were tested against common Gram-positive pathogens. Growth curves are observed only for pathogens within the genus *Enterococcus* (*E. faecium* and *E. faecalis*). No meaningful amplification was observed for non-target organisms.

FIG. 20. PCR assays using primers and probe disclosed in TABLE XVIII that target the *CPE* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Growth curve is observed only for pathogens within the species *Staphylococcus aureus*. No meaningful amplification was observed for non-target organisms.

FIG. 21. PCR assays using primers and probe disclosed in TABLE XIX that target the *gyrB* gene were tested against common Gram-positive pathogens. Growth curve is observed only for pathogens within the species *Staphylococcus aureus*. No meaningful amplification was observed for non-target organisms.

FIG. 22. PCR assays using primers and probe disclosed in TABLE XX that target the *ddlA* gene were tested against common Gram-positive pathogens. Growth curve is observed only for pathogens within the species *Staphylococcus aureus*. No meaningful amplification was observed for non-target organisms.

FIG. 23. PCR assays using primers and probe disclosed in TABLE XXII that target the *gyrB* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Growth curve is observed only for pathogens within the species *Streptococcus agalactiae*. No meaningful amplification was observed for non-target organisms.

FIG. 24. PCR assays using primers and probe disclosed in TABLE XXIII that target the *sip* gene were tested against common Gram-positive pathogens. Growth curve is observed only for

pathogens within the species *Streptococcus agalactiae*. No meaningful amplification was observed for non-target organisms.

FIG. 25. PCR assays using primers and probe disclosed in TABLE XXIV that target the *ddlA* gene were tested against common Gram-positive pathogens. Growth curve is observed only for pathogens within the species *Streptococcus agalactiae*. No meaningful amplification was observed for non-target organisms.

FIG. 26. PCR assays using primers and probe disclosed in TABLE XXVI that target the *RDN18* (*18s rRNA*) gene were tested against common fungal pathogens: *C. albicans* and *C. auris*. Growth curves are observed only for pathogens within the genus *Candida*. Gram-negative and positive organisms were also tested and showed no meaningful amplification (data not shown).

FIG. 27. PCR assays using primers and probe disclosed in TABLE XXVII that target the *RDN58* (*5.8srRNA*) gene were tested against common fungal pathogens: *C. albicans* and *C. auris*. Growth curves are observed only for pathogens within the genus *Candida*. Gram-negative and positive organisms were also tested and showed no meaningful amplification (data not shown).

FIG. 28. PCR assays using primers and probe disclosed in TABLE XXVIII that target the *16s* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Growth curves are observed for all Gram-negative pathogens.

FIG. 29. PCR assays using primers and probe disclosed in TABLE XXVIII that target the *16s* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Growth curves are observed for all Gram-positive pathogens.

FIG. 30-1 and 30-2. Established Minimum Inhibitory Concentration (MIC) breakpoints for Gram-negative bacterial organisms as determined by the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30. Breakpoints are used to interpret MIC results from Antimicrobial Susceptibility Testing, and to classify “groupings” of organisms as either Susceptible, Intermediate, or Resistant to a given antimicrobial. Groupings of organisms can be at differing levels, including, but not limited to, species, genus, order, or a specific biochemical property.

FIG. 31. Established Minimum Inhibitory Concentration (MIC) breakpoints for Gram-positive bacterial organisms as determined by the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30. Breakpoints are used to interpret MIC results from Antimicrobial Susceptibility Testing, and to classify “groupings” of organisms as either Susceptible,

Intermediate, or Resistant to a given antimicrobial. Groupings of organisms can be at differing levels, including, but not limited to, species, genus, order, or a specific biochemical property.

FIG. 32. Established Minimum Inhibitory Concentration (MIC) breakpoints for fungal organisms (yeast) as determined by the Clinical and Laboratory Standards Institute (CLSI) document M60 ED 1. Breakpoints are used to interpret MIC results from Antifungal Susceptibility Testing (AFST), and to classify “groupings” of organisms as either Susceptible, Intermediate, or Resistant to a given antifungal. Groupings of organisms can be at differing levels, including, but not limited to, species, genus, order, or a specific biochemical property. Of note is that while AFST is recommended for *Candida auris*, neither CLSI or CDC currently have established breakpoints for the species; instead, AFST results from closely related *Candida* spp. and expert opinion are used to determine the susceptibility of *C. auris* isolates to a given antifungal.

FIG. 33. Rapid Identification and phenotypic antimicrobial susceptibility testing of *Enterobacteriales* utilizing three distinct target genes (*gyrB*, *rplP*, and *rpoB*) and three classes of antibacterial agents: fluoroquinolone (CIP), aminoglycoside (GEN), and carbapenem (MEM).

FIG. 34. Rapid Identification and phenotypic antimicrobial susceptibility testing of *P. aeruginosa* utilizing three distinct target genes (*tuf*, *gyrB*, *rpoB*) and three classes of antibacterial agents: fluoroquinolone (CIP), aminoglycoside (GEN), and carbapenem (MEM).

FIG. 35. Rapid Identification and phenotypic antimicrobial susceptibility testing of *Acinetobacter* utilizing three distinct target genes (*ompA*, *rpoB*, and *gyrB*) and three classes of antibacterial agents: fluoroquinolone (CIP), aminoglycoside (GEN), and carbapenem (MEM).

FIG. 36. Rapid Identification and phenotypic antimicrobial susceptibility testing of *S. aureus* utilizing three distinct target genes (*gyrB*, *ddlA*, *tuf*) and one class of antibacterial agent: cephalosporin (FOX).

FIG. 37. Rapid Identification and phenotypic antimicrobial susceptibility testing of *Enterococcus faecium* utilizing three distinct target genes (*rpoB*, *ddl*, and *gyrB*) and two classes of antibacterial agents: beta-lactam (AMP) and glycopeptide (VAN).

FIG. 38. Primers and Probes that target the *RDN18* and *RDN58* genes to be used for rapid identification and phenotypic antimicrobial susceptibility testing of *Candida*.

FIG. 39. Primers and Probes that target the *16s* gene to be used for rapid identification and phenotypic antimicrobial susceptibility testing of any given Gram-negative or Gram-positive bacteria.

FIG. 40A. Inclusivity and exclusivity performance of a Gram-negative pathogen PCR multiplex

master mix. FIG. 40B. Breakpoint groups, channels and dye wavelengths and names of the multiplex PCR assay.

FIG. 41A. Inclusivity and exclusivity performance of a Gram-positive pathogen PCR multiplex master mix. FIG. 41B. Breakpoint groups, channels and dye wavelengths and names of the multiplex PCR assay.

FIG. 42. Thresholds to distinguish between susceptible and resistant can be unique to each primer/probe set and are determined using statistical separation of populations as outlined in FIG 4. The thresholds associated with ciprofloxacin susceptibility are shown for A) *Acinetobacter baumannii* (Abi), B) Enterobacteriaceae (Entero), and C) *Pseudomonas aeruginosa* (Pae), and are based on changes in Ct value, Relative Fluorescence Intensity (RFI), and Slope prior to the Ct fluorescence value at three different antibiotic concentrations.

FIG. 43. A) The distribution of resistant and susceptible isolates is shown for *A. baumannii* (Abi), *E. cloacae* (Ecl), *E. coli* (Eco), *K. aerogenes* (Kae), *K. pneumoniae* (Kpn), and *P. aeruginosa* (Pae). B) Sensitivity, specificity, and categorical agreement for ciprofloxacin across species using the thresholds in FIG 42, where sensitivity and specificity are as defined in EXAMPLE 21.

FIG. 44. Thresholds to distinguish between susceptible and resistant can be unique to each primer/probe set and are determined using statistical separation of populations as outlined in FIG 4. The thresholds associated with gentamicin susceptibility are shown for A) *Acinetobacter baumannii* (Abi), B) Enterobacteriaceae (Entero), and C) *Pseudomonas aeruginosa* (Pae), and are based on Inflection cycle, changes in Absolute Fluorescence Intensity (AFI), and Goodness of Fit for the curve fit to the raw fluorescence data.

FIG. 45. A) The distribution of resistant and susceptible isolates is shown for *A. baumannii* (Abi), *E. cloacae* (Ecl), *E. coli* (Eco), *K. aerogenes* (Kae), *K. pneumoniae* (Kpn), and *P. aeruginosa* (Pae). B) Sensitivity, specificity, and categorical agreement for gentamicin across species using the thresholds in FIG 44, where sensitivity and specificity are as defined in EXAMPLE 21.

FIG. 46. Thresholds to distinguish between susceptible and resistant can be unique to each primer/probe set and are determined using statistical separation of populations as outlined in FIG 4. The thresholds associated with meropenem susceptibility are shown for A) *Acinetobacter baumannii* (Abi), B) Enterobacteriaceae (Entero), and C) *Pseudomonas aeruginosa* (Pae), and are based on changes in Ct value relative to no antibiotic and the lowest antibiotic concentration, the absolute Ct value, and the Absolute Fluorescence Intensity (AFI).

FIG. 47. A) The distribution of resistant and susceptible isolates is shown for *A. baumannii* (Abi), *E. cloacae* (Ecl), *E. coli* (Eco), *K. aerogenes* (Kae), *K. pneumoniae* (Kpn), and *P. aeruginosa*

(Pae). B) Sensitivity, specificity, and categorical agreement for meropenem across species using the thresholds in FIG 46, where sensitivity and specificity are as defined in EXAMPLE 21.

FIG. 48. A) Workflow for testing isolates directly from positive blood culture samples, which were created by spiking bacteria into whole blood, separating red blood cells, inoculating plasma containing bacteria into a commercial blood culture bottle, incubating overnight, and then following standard assay workflow. B) Change in Ct values as a function of antibiotic (Gentamicin) for various resistant and susceptible isolates, showing that phenotypic results can be obtained on bacteria directly from positive blood culture.

FIG 49. Polymicrobial AST where a 1:1 ratio of two Gram-negative organisms (Kpn and Abi) with different susceptibility combinations were co-incubated together in the absence or presence of three different antibiotics at varying concentrations. Each species displayed the appropriate phenotype in the corresponding detection channel as indicated by a Delta CT threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results for a variety of polymicrobial scenarios.

FIG 50. Polymicrobial AST where a 1:1 ratio of one Gram-negative organism (Kpn) and one Gram-positive organism (Sar) with different susceptibility combinations were co-incubated together in the absence or presence of three different antibiotics at varying concentrations. Each species displayed the appropriate phenotype in the corresponding detection channel as indicated by a Delta CT threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results for a variety of polymicrobial scenarios. N/A indicates that there is no clinically relevant interpretation for the corresponding bacteria-drug combination.

FIG 51. Polymicrobial AST where a 1:1 ratio of one Gram-negative organism (Kpn) and one fungal organism (Cal) with different susceptibility combinations were co-incubated together in the absence or presence of one antibiotic at varying concentrations. The Gram-negative species displayed the appropriate phenotype in the corresponding detection channel as indicated by a Delta CT threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results for a variety of polymicrobial scenarios. N/A indicates that there is no clinically relevant interpretation for the corresponding organism-drug combination. Cal susceptibility for fluconazole is indicated.

FIG 52. Polymicrobial AST where a 1:1 ratio of two Gram-positive organisms (Efs and Sar) with different susceptibility combinations were co-incubated together in the absence or presence of one antibiotic at varying concentrations. Both species displayed the appropriate phenotype in the corresponding detection channel as indicated by a delta-Ct threshold that

separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results for a variety of polymicrobial scenarios.

FIG. 53. Polymicrobial AST where a 1:1 ratio of one Gram-positive organism (Sar) and one fungal organism (Cal) with different susceptibility combinations were co-incubated together in the absence or presence of one antibiotic at varying concentrations. The Gram-positive species displayed the appropriate phenotype in the corresponding detection channel as indicated by a delta-Ct threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results for a variety of polymicrobial scenarios. N/A indicates that there is no clinically relevant interpretation for the corresponding organism-drug combination. Cal susceptibility for fluconazole is indicated.

FIG. 54. PCR assays using primers and probes disclosed in TABLE XXXIX that target the blaKPC, blaVIM, blaNDM, and blaOXA-48 genes were tested against Gram-negative pathogens with known mechanisms of carbapenem resistance. Positive (Pos) signals were observed only for the targeted resistance mechanism.

FIG. 55. PCR assays using primers and probes disclosed in TABLE XL that target the *citC* gene of *P. Stuartii* and *invA* gene of *Salmonella* were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *C. freundii*, *P. stuartii*, *P. rettgeri*, *S. enterica*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Only species-specific growth curves were observed. These results represent non-limiting examples of species-specific detection sets that allow improved breakpoint based AST calling for some specific species within the order Enterobacterales.

FIG. 56. PCR assays using primers and probes disclosed in TABLE XLI that target *gyrB* gene of *S. agalactiae*, *ddlA* gene of *S. agalactiae*, *tuf* gene of *S. pneumoniae*, and *speB* gene of *S. pyogenes* were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Only species-specific growth curves were observed. These results represent non-limiting examples of species-specific detection sets that allow improved breakpoint based AST calling for some specific species within the *Staphylococcus* and *Streptococcus* genera.

FIG. 57. The left panel shows the thresholds to distinguish between susceptible and resistant strains in an ID-AST PCR assay that utilized non-species-specific primer/probe sets (e.g. sets that hybridize to a target gene in the Enterobacterales order) may be confounded by species-specific differences in phenotype manifestation. The right panel shows the use of species-specific primer/probe sets that provide species identification would enable separate

interpretation for each individual species, leading to improved Categorical Agreement to CLSI standards.

FIG. 58. Data interpretation strategy wherein a sigmoidal function is fit to the raw PCR curve data followed by calculation of curve parameters and features. Features are then compared
5 between the presence of various antibiotic concentrations and the no-antibiotic reference to derive relative feature changes. Relative change features are then input into separate machine learning algorithms along with the ground truth MIC in order to train predictive models. Trained models then participate as a voting ensemble to return the final predicted MIC.

FIG. 59. Diagram indicating how Species ID, Antimicrobial Susceptibility Testing, Resistance
10 Mechanism detection, and Universal *16s* rRNA phenotypic information is combined to return a result, wherein Species ID is used to select the appropriate algorithm for MIC prediction which is then compared to the appropriate breakpoints from regulatory bodies to determine susceptibility information.

DETAILED DESCRIPTION OF THE INVENTION

15 Definitions

The disclosed methods may include performing at least one cycling step that includes amplifying one or more portions of the nucleic acid molecule gene target from a sample using one or more pairs of primers. A “sample” or “biological sample” as used herein refers to a sample that includes but is not limited to whole blood, plasma, serum, red blood cell fraction,
20 saliva, cerebrospinal fluid, semen, stool, urine, , rectal swab, bile, lymph, sputum, lavage fluid, or a combination thereof. “Primer(s)” as used herein refer to oligonucleotide primers that specifically anneal to the target bacterial gene, and initiate DNA synthesis therefrom under appropriate conditions producing the respective amplification products. Each of the discussed primers anneals to a target within or adjacent to the respective target nucleic acid molecule such that at least a portion of each amplification product contains nucleic acid sequence
25 corresponding to the target. The one or more amplification products are produced provided that one or more of the target bacterial gene nucleic acid is present in the sample, thus the presence of the one or more of target bacterial gene amplification products is indicative of the presence of that bacterial strain in the sample. The amplification product should contain the nucleic acid sequences that are complementary to one or more detectable probes for target bacterial gene.
30 “Probe(s)” as used herein refer to oligonucleotide probes that specifically anneal to nucleic acid sequence encoding the target bacterial gene. Each cycling step includes an amplification step,

a hybridization step, and a detection step, in which the sample is contacted with the one or more detectable probes for detection of the presence or absence of the bacterial strain in the sample.

As used herein, the term “amplifying” refers to the process of synthesizing nucleic acid molecules that are complementary to one or both strands of a template nucleic acid molecule.

- 5 Amplifying a nucleic acid molecule typically includes denaturing the template nucleic acid, annealing primers to the template nucleic acid at a temperature that is below the melting temperatures of the primers, and enzymatically elongating from the primers to generate an amplification product. Amplification typically requires the presence of deoxyribonucleoside triphosphates, a DNA polymerase enzyme (e.g., Platinum[®] Taq) and an appropriate buffer
10 and/or co-factors for optimal activity of the polymerase enzyme (e.g., MgCl₂ and/or KCl).

The term “primer” as used herein is known to those skilled in the art and refers to oligomeric compounds, primarily to oligonucleotides but also to modified oligonucleotides that are able to “prime” DNA synthesis by a template-dependent DNA polymerase, i.e., the 3’-end of the, e.g., oligonucleotide provides a free 3’-OH group whereto further “nucleotides” may be attached by
15 a template-dependent DNA polymerase establishing 3’ to 5’ phosphodiester linkage whereby deoxynucleoside triphosphates are used and whereby pyrophosphate is released. Therefore, there is – except possibly for the intended function – no fundamental difference between a “primer”, an “oligonucleotide”, or a “probe”.

The term “hybridizing” refers to the annealing of one or more probes to an amplification product.

- 20 Hybridization conditions typically include a temperature that is below the melting temperature of the probes but that avoids non-specific hybridization of the probes.

The term “5’ to 3’ nuclease activity” refers to an activity of a nucleic acid polymerase, typically associated with the nucleic acid strand synthesis, whereby nucleotides are removed from the 5’ end of nucleic acid strand.

- 25 The term “thermostable polymerase” refers to a polymerase enzyme that is heat stable, i.e., the enzyme catalyzes the formation of primer extension products complementary to a template and does not irreversibly denature when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded template nucleic acids. Generally, the synthesis is initiated at the 3’ end of each primer and proceeds in the 5’ to 3’ direction along
30 the template strand. Thermostable polymerases have been isolated from *Thermus flavus*, *T. ruber*, *T. thermophilus*, *T. aquaticus*, *T. lacteus*, *T. rubens*, *Bacillus stearothermophilus*, and *Methanothermus fervidus*. Nonetheless, polymerases that are not thermostable also can be employed in PCR assays provided the enzyme is replenished.

The term “complement thereof” refers to nucleic acid that is both the same length as, and exactly complementary to, a given nucleic acid.

The term “extension” or “elongation” when used with respect to nucleic acids refers to when additional nucleotides (or other analogous molecules) are incorporated into the nucleic acids.

5 For example, a nucleic acid is optionally extended by a nucleotide incorporating biocatalyst, such as a polymerase that typically adds nucleotides at the 3' terminal end of a nucleic acid.

The terms “identical” or percent “identity” in the context of two or more nucleic acid sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same, when compared and aligned for maximum correspondence, 10 e.g., as measured using one of the sequence comparison algorithms available to persons of skill or by visual inspection. Exemplary algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST programs, which are described in, e.g., Altschul et al. (1990) “Basic local alignment search tool” *J. Mol. Biol.* 215:403-410, Gish et al. (1993) “Identification of protein coding regions by database similarity search” *Nature* 15 *Genet.* 3:266-272, Madden et al. (1996) “Applications of network BLAST server” *Meth. Enzymol.* 266:131-141, Altschul et al. (1997) “Gapped BLAST and PSI-BLAST: a new generation of protein database search programs” *Nucleic Acids Res.* 25:3389-3402, and Zhang et al. (1997) “PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation” *Genome Res.* 7:649-656.

20 A “modified nucleotide” in the context of an oligonucleotide refers to an alteration in which at least one nucleotide of the oligonucleotide sequence is replaced by a different nucleotide that provides a desired property to the oligonucleotide. Exemplary modified nucleotides that can be substituted in the oligonucleotides described herein include, e.g., a C5-methyl-dC, a C5-ethyl-dC, a C5-methyl-dU, a C5-ethyl-dU, a 2,6-diaminopurine, a C5-propynyl-dC, a C5-propynyl- 25 dU, a C7-propynyl-dA, a C7-propynyl-dG, a C5-propargylamino-dC, a C5-propargylamino-dU, a C7-propargylamino-dA, a C7-propargylamino-dG, a 7-deaza-2-deoxyxanthosine, a pyrazolopyrimidine analog, a pseudo-dU, a nitro pyrrole, a nitro indole, 2'-O-methyl Ribo-U, 2'-O-methyl Ribo-C, an N4-ethyl-dC, an N6-methyl-dA, and the like. Many other modified nucleotides that can be substituted in the oligonucleotides are referred to herein or are otherwise 30 known in the art. In certain embodiments, modified nucleotide substitutions modify melting temperatures (T_m) of the oligonucleotides relative to the melting temperatures of corresponding unmodified oligonucleotides. To further illustrate, certain modified nucleotide substitutions can reduce non-specific nucleic acid amplification (e.g., minimize primer dimer formation or the like), increase the yield of an intended target amplicon, and/or the like in some embodiments.

Examples of these types of nucleic acid modifications are described in, e.g., U.S. Pat. No. 6,001,611.

The term “TAGS” or “Temperature Assisted Generation of Signal” (disclosed in U.S Patent Publication No. 2018/0073064) is a multiplexing technology that enables the measurement of multiple individual targets in each fluorescence channel by collecting fluorescence data at different temperatures during thermal cycling. Consequently, TAGS multiplexing with two or three temperature channels can double or triple the number of resolvable targets per optical channel. In principle, this technology can be deployed on any quantitative PCR (qPCR) instrument capable of collecting more than one fluorescence read per PCR cycle.

The term “antimicrobial” refers to an agent or drug used to treat a microbial infection, usually by killing the microorganism or by inhibiting its growth. Antimicrobials may include antibiotics for treating bacterial infection, antifungals for treating fungi infection, antiprotozoals for treating protozoan infection and antivirals for treating viral infection. Antimicrobials are classified in several different manners (and referred as “antimicrobial class”) and include classification by mechanism of action (e.g. inhibition of cell wall synthesis, inhibition of protein or nucleic acid synthesis, disruption of cell membrane), by source (e.g. from natural sources or synthetic), and by chemical structure (e.g. β -lactams, aminoglycosides, macrolides, quinolones etc.).

Examples of antimicrobial classes include but are not limited to: Allylamines, Amidinopenicillins, Aminocyclitols, Aminoglycosides, Amphenicols, Ansamycins, B-3-Glucan synthase inhibitors, Carbapenems, Cephalosporins, Glycylcyclines, Cyclic polypeptides, Glycopeptides, Imidazoles, Lincosamides, Lipopeptides, Macrolides and ketolides, Monobactams, Nitrofurantoin, Nitroimidazoles, Oxazolidinones, Penicillins, Phosphonic acid derivatives, Pleuromutilins, Polyenes, Polymyxins, Pseudomonic acids, Quinolones, Riminofenazines, Steroid antibacterials, Streptogramins, Sulfonamides, dihydrofolate reductase inhibitors and combinations, Sulfones, Tetracyclines, and Triazoles.

Examples of antimicrobial drugs or agents include but are not limited to: naftifine, mecillinam, spectinomycin, chloramphenicol, rifampicin, caspofungin, meropenem, ceftriaxone, cefepime, ceftaroline, tigecycline, bacitracin, vancomycin, miconazole, clindamycin, daptomycin, erythromycin, telithromycin, aztreonam, nitrofurantoin, metronidazole, linezolid, ampicillin, fosfomycin, retapamulin, amphotericin-B, colistin, mupirocin, ciprofloxacin, clofazimine, fusidic acid, quinupristin/dalfopristin, sulfamethoxazole, trimethoprim, dapson, chlortetracycline, and fluconazole.

The term “Minimum Inhibitory Concentration” or “MIC” refers to the lowest concentration of an antimicrobial required to inhibit the growth of an organism. In classical culture-based tests, the MIC is determined when the bacteria are added to wells containing growth media and varying concentrations of the antimicrobial. The concentration of antimicrobial is doubled in each successive well and the MIC is found by identifying the well with the lowest antimicrobial concentration in which there is no visible growth after an incubation period. The term “breakpoint” refers to a chosen concentration of an antimicrobial that defines whether a species of bacteria is susceptible or resistant to the antimicrobial. If the MIC is less than or equal to the susceptibility breakpoint the bacteria is considered susceptible to the antimicrobial. If the MIC is greater than this value the bacteria is considered intermediate or resistant to the antimicrobial. Breakpoints are an integral part of modern microbiology laboratory practice and are used to define susceptibility and resistance to antibacterials. Depending on the testing method, they are expressed as either a concentration (in mg/liter or µg/ml) or a zone diameter (in mm). In general, all susceptibility testing methods require breakpoints, also known as interpretive criteria, so that the results of the tests can be interpreted as susceptible, intermediate, or resistant and reported as such to a broad range of clinicians. “Clinical breakpoints” which refer to those concentrations (MICs) that separate strains where there is a high likelihood of treatment success from those bacteria where treatment is more likely to fail. In their simplest form, these breakpoints are derived from prospective human clinical studies comparing outcomes with the MICs of the infecting pathogen.

Detection and Identification of Infectious Pathogens

The present disclosure provides methods to detect infectious pathogens, for example, bacteria strains that cause bloodstream infections. The methods comprise the steps of amplifying portions of target genes by PCR using strain-specific, species-specific, genus-specific, family-specific, or order-specific primer sequences and detecting the amplification products using strain-specific, species-specific, genus-specific, family-specific, or order-specific probe nucleic acid sequences. Target gene selection was the result of an *in silico* search of the public sequence database, as well as a literature search for nucleic acid sequences that are specific to a species (e.g. *E. coli*) or to an order (e.g. Enterobacterales) and discriminate against other strains and families. As a result of the search, the following target genes were identified:

Enterobacterales order: *rplP*, *ompA*, *tuf*, *gyrB*, *rpoB*

Enterobacteriaceae family: *rplP*, *ompA*, *tuf*, *gyrB*, *rpoB*

Enterococcus genus: *tuf*, *rpoB*, *sodA*, *ddl*, *gyrB*

Pseudomonas genus: *gyrB*, O-antigen acetylase, *rpoB*, *ecfX*, *tuf*

Acinetobacter genus: *ompA*, *tusA*, *rpoB*, *gyrB*

Strenotrophomonas maltophilia: *fdnG*, *gyrB*, *tuf*

Staphylococcus aureus: CPE, *gyrB*, *mic*, *rpoB*, *tuf*, *ddlA*

5 *Staphylococcus epidermidis*: *altE*, *femA*

Coagulase-negative *Staphylococci*: *rpoB*, *tuf*, *sodA*

Streptococcus genus: *tuf*, *gyrB*, *sip*, *ddlA*

Streptococcus pneumoniae: *lytA*, *SP2020*, *piaB*

Proteus mirabilis: *UreR*, *UreC*

10 *Candida albicans*: *ACT*, *RPB-1*, 5.8s ribosomal RNA, 18s ribosomal RNA

For detection of bacteria belonging to the Enterobacterales order or to the Enterobacteriaceae family, primers and probes to amplify the *rplP* gene encoding for the ribosomal L16 protein are provided (SEQ ID NO: 1-3, TABLE I). Addition of a second probe (SEQ ID NO: 4, TABLE I) further extends inclusivity to include other prevalent pathogens in the order Enterobacterales, such as the strains *Serratia marcescens* and *Proteus mirabilis*. Nucleic acids other than those exemplified herein can also be used to detect highly specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 1-4, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 1-4, or a complement of SEQ ID NOs: 1-4 and the variant.

TABLE I: Oligonucleotides for detecting Enterobacteriaceae family or Enterobacterales order

Primers and Probes that hybridize to <i>rplP</i> gene in Enterobacteriaceae/Enterobacterales strains				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	RM_ENTF	1	TAATCGGCAGTTTCGCTG<t _B B dC>	t _{BB} _dC= t-butylbenzyl-dC
Reverse primer	RM_ENTRP	2	GTTCCCGGACAAACCGATC<t _{BB} dA>	t _{BB} _dA= t-butylbenzyl-dA
Probe 1	RM_ETP02	3	<Cy5.5>TTCACGGGCCA<BHQ_2>GCTCTTCCGGAACACCGTC CAT<Phos>	<Cy5.5>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate

Probe 2	RM_ETP02B	4	<Cy5.5>CTGGATCAGGG<BHQ_2>CAACCCAATACTCCACGTTACC<Phos>	<Cy5.5>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate
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The detection of bacteria belonging to the Enterobacterales order can also comprise of other primers and probes for amplifying the *rplP* gene (SEQ ID NOs: 5-7, TABLE II), as well as primers and probes for amplifying the *gyrB* gene that encodes the DNA gyrase subunit B protein (SEQ ID NOs: 8-10, TABLE III) and primers and probes for amplifying the *rpoB* gene that encodes the DNA-dependent RNA polymerase (SEQ ID NOs: 11-16, TABLE IV). Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 5-7, 8-10, 11-16, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 5-7, 8-10, 11-16, or a complement of SEQ ID NOs: 5-7, 8-10, 11-16 and the variant.

TABLE II: Oligonucleotides for detecting Enterobacterales order

Primers and Probes that hybridize to <i>rplP</i> gene in Enterobacteriaceae/Enterobacterales strains				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2891	5	AATTCGTAATAATGCACAAAGGCC	
Reverse primer	SEGP2892	6	GCTTAAGTGCACGGGTCATAGCA	
Probe	SEGP2893	7	<FAM>TGGTCGTCT<ZEN>GAC TGCACGTCAGATCGAAGC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE III: Oligonucleotides for detecting Enterobacterales order

Primers and Probes that hybridize to <i>gyrB</i> gene in Enterobacteriaceae/Enterobacterales strains				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1899	8	TGTCGAATTCTTATGACTCCTCAGTA	
Reverse primer	SEGP1901	9	CGCGAGCGCTTCGTCGA	

Probe	SEGP2016	10	<HEX>CCGGTCTGC<ZEN>ACC ACATGGTATTTCGAGGTGG<3IA BkFQ>	<HEX>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker
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TABLE IV: Oligonucleotides for detecting Enterobacterales order

Primers and Probes that hybridize to <i>rpoB</i> gene in Enterobacteriaceae/Enterobacterales strains				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2799	11	TGGTAAACGTCCACAAGTTCT GGA	
Reverse primers	SEGP2800 SEGP2802 SEGP2821	12 13 14	CATACTGCCCTTCAGGATCTTG C GTAGCTGACATATTGCAGTTC AGCA CCGTTCTGACCGTCCGGATC	
Probes	SEGP2804 SEGP2822	15 16	<FAM>TATCTCCTT<ZEN>TCTA TCCAGCTTGACTCGTTTC AGA AGTTTATCGA<3IABkFQ> <FAM>TC TCC TTTC<ZEN>T ATCCAGCTGGATTCATTC CAG AAATTCATTGAAC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

For detection of bacteria belonging to the species *Acinetobacter baumannii* (Abi), primers and probes for amplifying the *ompA* gene encoding for the outer membrane protein A are provided (see TABLE V). Nucleic acids other than those exemplified herein can also be used to detect *Acinetobacter* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 17-19, 20-22, 29-31 a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 17-19, 20-22, 29-31 or a complement of SEQ ID NOs: 17-19, 20-22, 29-31 and the variant.

TABLE V: Oligonucleotides for detecting *Acinetobacter baumannii*

Primers and Probes that hybridize to <i>ompA</i> gene in <i>A. baumannii</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	RM_AFP01	17	TTGGTGGTCACTTGAAG<t_BB_dC>	t_BB_dC= t-butylbenzyl-dC
Reverse primer	RM_ARP02	18	TTTCTGGCTTGTATTGGT<t_BB_dC>	t_BB_dC= t-butylbenzyl-dC
Probe	RM_P02	19	<HEX_Thr>ACTCCAG<BHQ_2> TTGCTCCACAACCACAAGAG<Phos>	<HEX_Thr>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate
Forward primer	SEGP2603	20	TTATCTTTAGCTCGTGCTAACT CTGTTAAA	
Reverse primer	SEGP2606	21	GCACGACCTTCTTTAGTTTGT TGTCA	
Probe	SEGP2769	22	<ATTO>TCTACTCA<BHQ_2>A GGTTCGCTTGGGATCAACCG ATTGCT<Phos>	<ATTO>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate
Forward primer	SEGP1813	29	ACCCTAACGCTACTGCACGT	
Reverse primer	SEGP1815	30	GGTTGATCCCAAGCGAAACCT	
Probe	SEGP1951	31	<ATTO>TCGAAGGT<BHQ_2>C ACACAGATAACACT<Phos>	<ATTO>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate

The detection of *Acinetobacter baumannii* can also comprise of primers and probes for amplifying the *rpoB* gene (SEQ ID NOs: 23-25, TABLE VI) and for amplifying the *gyrB* gene (SEQ ID NOs: 26-28, TABLE VII). Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 23-25, 26-28, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 23-25, 26-28, or a complement of SEQ ID NOs: 23-25, 26-28, and the variant.

TABLE VI: Oligonucleotides for detecting *Acinetobacter baumannii*

Primers and Probes that hybridize to <i>rpoB</i> gene in <i>Acinetobacter baumannii</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2590	23	CATACTCATATACCGAAAAG AAACGG	

Reverse primer	SEGP2593	24	CTATACTCAACAAATTCTAA AGCAGC	
Probe	SEGP2594	25	<FAM>CGCGAAGAT<ZEN>AT CGGTCTCC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE VII: Oligonucleotides for detecting *Acinetobacter baumannii*

Primers and Probes that hybridize to <i>gyrB</i> gene in <i>Acinetobacter baumannii</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2626	26	ACAGAACAAACCAATGAAA AGGCTTATGATTC	
Reverse primer	SEGP2628	27	ACCATATGGTGTAAACCGGT ACC	
Probe	SEGP2629	28	<FAM>AAAGTATTA<ZEN>C GTGATTAGATGCAGTTCGTA AACGTCCGGGT<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

For detection of bacteria belonging to the species *Pseudomonas aeruginosa* (Pae), primers and probes for amplifying the *tuf* gene encoding elongation factor (SEQ ID NOs: 32-34, TABLE VIII), the *gyrB* gene (SEQ ID NOs: 35-37, TABLE IX) and the *rpoB* gene (SEQ ID NOs: 38-40, TABLE X) are provided. Nucleic acids other than those exemplified herein can also be used to detect *Pseudomonas* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 32-34, 35-37, 38-40, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 32-34, 35-37, 38-40, or a complement of SEQ ID NOs: 32-34, 35-37, 38-40 and the variant.

TABLE VIII: Oligonucleotides for detecting *Pseudomonas aeruginosa*

Primers and Probes that hybridize to <i>tuf</i> gene in <i>Pseudomonas aeruginosa</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2341	32	CCGTGCAGAAGCTGGTAG	
Reverse primer	SEGP2342	33	GAGATCGAGAACACGTCTTCG	
Probe	SEGP2343	34	<FAM>TTCCGGAGC<ZEN>CGG TTCGTGCCATCG<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE IX: Oligonucleotides for detecting *Pseudomonas aeruginosa*

Primers and Probes that hybridize to <i>gyrB</i> gene in <i>Pseudomonas aeruginosa</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2630	35	GGAGTACAACATCGACAAGC TGC	
Reverse primer	SEGP2631	36	CGCTCGATCAGCTCGGGC	
Probe	SEGP2632	37	<FAM>CACAACATC<ZEN>AT CATCATGACCGATGCTGACGT CGAC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

5 TABLE X: Oligonucleotides for detecting *Pseudomonas aeruginosa*

Primers and Probes that hybridize to <i>rpoB</i> gene in <i>Pseudomonas aeruginosa</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2634	38	GGCGTGCTGAAGATCGTCAA	
Reverse primer	SEGP2637	39	ACCGGCATGATCACCGAGA	
Probe	SEGP2640	40	<FAM>CGCATCCAG<ZEN>CC GGGCGACAAGATGG<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

For detection of bacteria belonging to the species *Stenotrophomonas maltophilia* (*S. maltophilia*), primers and probes for amplifying the *fdnG* gene (SEQ ID NOs: 41-43, TABLE XI), the *gyrB* gene (SEQ ID NOs: 44-46, TABLE XII) and the *tuf* gene (SEQ ID NOs: 47-49, TABLE XIII) are provided. Nucleic acids other than those exemplified herein can also be used to detect *Stenotrophomonas* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 41-43, 44-46, 47-49, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 41-43, 44-46, 47-49, or a complement of SEQ ID NOs: 41-43, 44-46, 47-49, and the variant.

TABLE XI: Oligonucleotides for detecting *Stenotrophomonas maltophilia*

Primers and Probes that hybridize to <i>fdnG</i> gene in <i>Stenotrophomonas maltophilia</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2532	41	CCAAGCTCGACAAGCCGTAC	
Reverse primer	SEGP2538	42	GCTTGGAGAACGCGCTGATC	
Probe	SEGP2544	43	<FAM>TGCAGGCGT<ZEN>ACGAGCTGATGAACGAAGGC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XII: Oligonucleotides for detecting *Stenotrophomonas maltophilia*

Primers and Probes that hybridize to <i>gyrB</i> gene in <i>Stenotrophomonas maltophilia</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2578	44	TGCAGTGGACCGACTCCTA	
Reverse primer	SEGP2579	45	CTGCTTGGCGATGCCGTTCT	
Probe	SEGP2580	46	<FAM>GACGATGTA<ZEN>CTGCTTCACCAAC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XIII: Oligonucleotides for detecting *Strenotrophomonas maltophilia*

Primers and Probes that hybridize to <i>tuf</i> gene in <i>Strenotrophomonas maltophilia</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2572	47	GCACCAAGCCGCACGTCA	
Reverse primer	SEGP2573	48	GTGCGCGGTCGAGATCGT	
Probe	SEGP2574	49	<FAM>CAAGACCAC<ZEN>GCTGACCGC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

For detection of bacteria belonging to the *Enterococcus* genus, primers and probes for amplifying the *tuf* gene (SEQ ID NOs: 50-52, TABLE XIV), the *rpoB* gene (SEQ ID NOs: 53-55, TABLE XV), the *ddl* gene encoding xxxx (SEQ ID NOs: 56-61, TABLE XVI), and the *gyrB* gene (SEQ ID NOs: 62-66, TABLE XVII) are provided. Nucleic acids other than those exemplified herein can also be used to detect *Enterococcus* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 50-52, 53-55, 56-61, 62-66, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 50-52, 53-55, 56-61, 62-66, or a complement of SEQ ID NOs: 50-52, 53-55, 56-61, 62-66 and the variant.

TABLE XIV: Oligonucleotides for detecting *Enterococcus* genus

Primers and Probes that hybridize to <i>tuf</i> gene in <i>Enterococcus</i> genus				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1632	50	GACAAACCATTTCATGATGCCAG	
Reverse primer	SEGP1631	51	AACTTCGTCACCAACGCGAAC	
Probe	SEGP1633	52	<HEX>CTGGACGTG<ZEN>GTACTGTTGCTAC<3IABkFQ>	<HEX>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XV: Oligonucleotides for detecting *Enterococcus* genus

Primers and Probes that hybridize to <i>rpoB</i> gene in <i>Enterococcus</i> genus				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2522	53	CCGTCCAGTGGTAGCAAGTATCA	
Reverse primer	SEGP2525	54	ACCATAGTGAGAGTAGTGAACTCA	
Probe	SEGP2770	55	<HEX>TTGACTCGT<ZEN>GACCGTGCCGGTTATGAAGTTCG<3IABkFQ>	<HEX>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XVI: Oligonucleotides for detecting *Enterococcus* genus

Primers and Probes that hybridize to <i>ddl</i> gene in <i>Enterococcus</i> genus				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1624 SEGP1627	56 57	CGTAGCATTCTATGATTATGAAGC C GACAGGAAAGAACTAGGAGGAC	
Reverse primer	SEGP1625 SEGP1628	58 59	CATCGTGTAAGCTAACTTCG AAACAGACACATCGTGCT	
Probe	SEGP1626 SEGP1629	60 61	<HEX>CAGATTCCA<ZEN>GCCGA AGTGCC<3IABkFQ> <HEX>CACTTCTGC<ZEN>CGCCAT ACAACAA<3IABkFQ>	<HEX>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

5 TABLE XVII: Oligonucleotides for detecting *Enterococcus* genus

Primers and Probes that hybridize to <i>gyrB</i> gene in <i>Enterococcus</i> genus				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2882 SEGP2884	62 63	AGCAACGATCCTGAAAAATGCGA ATTGTTTCATC AGTAAAGATCCGGAAAAATGCGA ATTATTTATC	
Reverse primer	SEGP2885 SEGP2886	64 65	AAAGACCGGATCTCTTCATTTGCC AATGACCGAATTTCTTCATTGGCT	

Probe	SEGP2888	66	<FAM>CCAATTCGT<ZEN>GGGAA AATCTTGAATGTTGAGAAAGCAAG CA<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker
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For detection of bacteria belonging to the species *Staphylococcus aureus* (*S. aureus*), primers and probes for amplifying the *CPE* gene encoding a protein involved in capsular formation (SEQ ID NOs: 67-69, 72 TABLE XVIII), the *gyrB* gene (SEQ ID NOs: 73-75, TABLE XIX), the *ddlA* gene (SEQ ID NOs: 76-78, TABLE XX). For detection of bacteria belonging to the *Staphylococcus* genus, primers and probes for amplifying the *tuf* gene (SEQ ID NOs: 79-81, TABLE XXII) are provided. Nucleic acids other than those exemplified herein can also be used to detect *Staphylococcus* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 67-69, 72, 73-75, 76-78, 79-81, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 67-69, 72, 73-75, 76-78, 79-81, or a complement of SEQ ID NOs: 67-69, 72, 73-75, 76-78, 79-81 and the variant.

TABLE XVIII: Oligonucleotides for detecting *Staphylococcus aureus*

Primers and Probes that hybridize to <i>CPE</i> gene in <i>Staphylococcus aureus</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1490	67	AAGATAAGCTTATTGAACAAGGA CATC	
Reverse primer	SEGP1491	68	CTTGAGGTGAATTGTTGTGAACC	
Probe	SEGP1492	72	<FAM>TTAGGAATC<ZEN>AATTA TGGAA GTGACCTCGT<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker
Probe	SEGP1493	69	<HEX>TTAGGAATC<ZEN>AATTA TGGAAAGTCGACCTCGT<3IABkFQ>	<HEX>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XIX: Oligonucleotides for detecting *Staphylococcus aureus*

Primers and Probes that hybridize to <i>gyrB</i> gene in <i>Staphylococcus aureus</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2792	73	CACAAGTCGCACGTACAGTG	
Reverse primer	SEGP2793	74	ATTCTTCAGGACTTTTACTAGA GCAATCG	
Probe	SEGP2794	75	<FAM>TAAATCAGC<ZEN>GTTA GATGTAGCAAGTC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XX: Oligonucleotides for detecting *Staphylococcus aureus*

Primers and Probes that hybridize to <i>ddlA</i> gene in <i>Staphylococcus aureus</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2932	76	ATCTACTGATGAGCTTCATT AGAAAATGGA	
Reverse primer	SEGP2933	77	TCAAAAAGTCCTTGAATCGTG CCA	
Probe	SEGP2935	78	<FAM>CGCTTGAGA<ZEN>TT TCACAGCTATTGAAAGAAAG TAGTTCAGGACAA<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

5 TABLE XXI: Oligonucleotides for detecting *Staphylococcus* genus

Primers and Probes that hybridize to <i>tuf</i> gene in <i>Staphylococcus</i> genus				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1835	79	CCGTGTTGAACGTGGTCAAA TCAAA	
Reverse primer	SEGP1836	80	AGCAGCTAATACTTGACCAC GTTGTA	
Probe	SEGP1838	81	<FAM>AGACTACGC<ZEN>T GAAGCTGGTGAC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

For detection of bacteria belonging to the species *Streptococcus agalactiae* (*S. agalactiae*), primers and probe for amplifying the *gyrB* gene (SEQ ID NOs: 121-123, TABLE XXII), the *sip* gene encoding the surface immunogenic protein (SEQ ID NOs: 82-84, TABLE XXIII), and the *ddlA* gene (SEQ ID NOs: 85-87, TABLE XXIV) are provided. For detection of bacteria

5 belonging to the *Streptococcus* genus, primers and probes for amplifying the *tuf* gene (SEQ ID NOs: 100-102, TABLE XXV) are provided. Nucleic acids other than those exemplified herein can also be used to detect *Streptococcus* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include,

10 e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a sequence selected from SEQ ID NOs: 121-123, 82-84, 85-87, 100-102 a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 121-123, 82-84, 85-87, 100-102 or a complement of

15 SEQ ID NOs: 121-123, 82-84, 85-87, 100-102 and the variant.

TABLE XXII: Oligonucleotides for detecting *Streptococcus agalactiae*

Primers and Probes that hybridize to <i>gyrB</i> gene in <i>Streptococcus agalactiae</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2921	121	ACCACTGTATTTGATTTGAT AAATTAGCCAAA	
Reverse primer	SEGP2922	122	TTCTCATTGATAAACTCAACG TATGAACCTA	
Probe	SEGP2923	123	<FAM>ACTAAGAAT<ZEN>CT CCATTTTCAGACAAGCGAGAA GGTCAAGAAGTTG<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XXIII: Oligonucleotides for detecting *Streptococcus agalactiae*

Primers and Probes that hybridize to <i>sip</i> gene in <i>Streptococcus agalactiae</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2204	82	ATCCTGAGACAACACTGACA	
Reverse primer	SEGP2205	83	TTGCTGGTGTTTCTATTTTCA	

Probe	SEGP2206	84	<CY5.5>ATCAGAAGAGT<BHQ_2>CATACTGCCACTTC<Phos>	<CY5.5>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate
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TABLE XXIV: Oligonucleotides for detecting *Streptococcus agalactiae*

Primers and Probes that hybridize to <i>ddlA</i> gene in <i>Streptococcus agalactiae</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2947	85	CACAAGAATTTGATGAAATGC CATCTTCA	
Reverse primer	SEGP2949	86	ACAATTGCATTATCATCATAG ATATCACTTGA	
Probe	SEGP2951	87	<FAM>TAATGACAA<ZEN>AC CAAAC TGTTGATTTAGACAAAATGGT TCGTCCA<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

TABLE XXV: Oligonucleotides for detecting *Streptococcus* genus

Primers and Probes that hybridize to <i>tuf</i> gene in <i>Streptococcus</i> genus				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1705	100	GTACAGTTGCTTCAGGACGTATC	
Reverse primer	SEGP1706	101	ACGTTTCGATTTTCATCACGTTG	
Probe	SEGP1709.1	102	<ATTO>TTCCGTA<BHQ_2>AACA ACTTGACGAAGGTCTTG<Phos>	<ATTO>: Fluorophore <ZEN>: Quencher <Phos>: Phosphate

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For detection of the common fungal pathogens: *Candida albicans* and *Candida auris*, primers and probe for amplifying the *18s* ribosomal RNA (*18s* rRNA) gene (SEQ ID NOs: 88-90, TABLE XXVI), and the *5.8s* ribosomal RNA (*5.8s* rRNA) gene (SEQ ID NOs: 91-93, TABLE XXVII) are provided. Nucleic acids other than those exemplified herein can also be used to detect *Candida* genus-specific pathogen grouping target genes in a sample. For example, functional variants can be evaluated for specificity and/or sensitivity by those of skill in the art using routine methods. Representative functional variants can include, e.g., one or more deletions, insertions, and/or substitutions in the target gene primers and probes disclosed herein. More specifically, embodiments of the oligonucleotides each include a nucleic acid with a

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sequence selected from SEQ ID NOs: 88-90, 91-93, a substantially identical variant thereof in which the variant has at least, e.g., 80%, 90%, or 95% sequence identity to one of SEQ ID NOs: 88-90, 91-93, or a complement of SEQ ID NOs: 88-90, 91-93 and the variant.

TABLE XXVI: Oligonucleotides for detecting *Candida*

Primers and Probes that hybridize to 18s rRNA gene in <i>Candida</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1712	88	CGTTTTTCATTAATCAAGAA CGAAAGTTA	
Reverse primer	SEGP1713	89	ACCGATCCCTAGTCGGCAT A	
Probe	SEGP1716	90	<FAM>AGACTACGA<ZEN> CGGTATCTGATCATCTTCG ATCCC<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

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TABLE XXVII: Oligonucleotides for detecting *Candida*

Primers and Probes that hybridize to 5.8s rRNA gene in <i>Candida</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1718	91	ACAACGGATCTCTTGTT CTC	
Reverse primer	SEGP1719	92	GCAATGTGCGTTCAAAGA TTCGA	
Probe	SEGP1722.1	93	<FAM_Thr>TCGATGAAG<B HQ_2>AACGCAGCGAAAT GCGATACG<Phos>	<FAM_Thr>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate

For detection of all types of bacteria, a primer and probe combination for amplifying a conserved region in the 16s ribosomal RNA (16s rRNA) gene (SEQ ID NOs: 94-96, TABLE XXVIII) is provided.

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TABLE XXVIII: Oligonucleotides for detecting general bacteria

Primers and Probes that hybridize to 16S rRNA in bacteria (Gram-positive and Gram-negative)				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP1830	94	TCCTACGGGAGGCAGCA GT	
Reverse primer	SEGP1831	95	GGACTACCAGGGTATCT AATCCTGTT	
Probe	SEGP1895.1	96	<CFR_635>CGTATTACCG CGGCT<BHQ_2>GCTGGC AC<Phos>	<CFR_635>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate

In one embodiment, the above-described sets of primers and probes are used not only for the detection and for identification of infectious bacteria strains but also in the performance of an antimicrobial susceptibility testing (AST) assay. Therefore, the present invention discloses methods and compositions for performing quantitative real-time PCR reactions whereby identification (ID) of bacteria and testing of their antimicrobial susceptibility (AST) are determined simultaneously at a single assay setting.

A functionally active variant of any of the primers and/or probes disclosed herein may be identified by using the primers and/or probes in the disclosed methods. A functionally active variant of a primer and/or probe described herein pertains to a primer and/or probe that provide a similar or higher specificity and sensitivity in the described method or kit as compared to the respective sequence of the primer and/or probe described herein.

The variant may, e.g., vary from the sequence of the primers and probes described herein by one or more nucleotide additions, deletions or substitutions such as one or more nucleotide additions, deletions or substitutions at the 5' end and/or the 3' end of the respective sequence of the primer and/or probe described herein. As detailed above, a primer (and/or probe) may be chemically modified, i.e., a primer and/or probe may comprise a modified nucleotide or a non-nucleotide compound. A probe (or a primer) is then a modified oligonucleotide. "Modified nucleotides" (or "nucleotide analogs") differ from a natural "nucleotide" by some modification but still consist of a base or base-like compound, a pentofuranosyl sugar or a pentofuranosyl sugar-like compound, a phosphate portion or phosphate-like portion, or combinations thereof. For example, a "label" may be attached to the base portion of a "nucleotide" whereby a "modified nucleotide" is obtained. A natural base in a "nucleotide" may also be replaced by, e.g., a 7-deazapurine whereby a "modified nucleotide" is obtained as well. The terms "modified

nucleotide” or “nucleotide analog” are used interchangeably in the present application. A “modified nucleoside” (or “nucleoside analog”) differs from a natural nucleoside by some modification in the manner as outlined above for a “modified nucleotide” (or a “nucleotide analog”).

- 5 Oligonucleotides including modified oligonucleotides and oligonucleotide analogs that amplify a nucleic acid molecule encoding any of the target genes can be designed using, for example, a computer program such as OLIGO (Molecular Biology Insights Inc., Cascade, Colo.). Important features when designing oligonucleotides to be used as amplification primers include, but are not limited to, an appropriate size amplification product to facilitate detection (e.g., by
10 electrophoresis), similar melting temperatures for the members of a pair of primers, and the length of each primer (i.e., the primers need to be long enough to anneal with sequence-specificity and to initiate synthesis but not so long that fidelity is reduced during oligonucleotide synthesis). Typically, oligonucleotide primers are 8 to 50 nucleotides in length (e.g., 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, or 50 nucleotides in length).
15 In some embodiments oligonucleotide primers are 40 or fewer nucleotides in length.

- In addition to a set of primers, the methods may use one or more probes in order to detect the presence or absence of target genes. The term “probe” refers to synthetically or biologically produced nucleic acids (DNA or RNA), which by design or selection, contain specific nucleotide sequences that allow them to hybridize under defined predetermined stringencies
20 specifically (i.e., preferentially) to “target nucleic acids”, in the present case to a target gene nucleic acid. A “probe” can be referred to as a “detection probe” meaning that it detects the target nucleic acid.

- In some embodiments, the described target gene probes can be labeled with at least one fluorescent label. In one embodiment, the target gene probes can be labeled with a donor
25 fluorescent moiety, e.g., a fluorescent dye, and a corresponding acceptor moiety, e.g., a quencher. In one embodiment, the probe comprises or consists of a fluorescent moiety and the nucleic acid sequences comprise or consist of the probe sequences disclosed herein.

- Designing oligonucleotides to be used as probes can be performed in a manner similar to the design of primers. Embodiments may use a single probe or a pair of probes for detection of the
30 amplification product. Depending on the embodiment, the probe(s) use may comprise at least one label and/or at least one quencher moiety. As with the primers, the probes usually have similar melting temperatures, and the length of each probe must be sufficient for sequence-specific hybridization to occur but not so long that fidelity is reduced during synthesis.

Oligonucleotide probes are generally 15 to 40 (e.g., 16, 18, 20, 21, 22, 23, 24, or 25) nucleotides in length.

Polymerase Chain Reaction (PCR)

U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159, and 4,965,188 disclose conventional PCR techniques. PCR typically employs two oligonucleotide primers that bind to a selected nucleic acid template (e.g., DNA or RNA). Primers useful in some embodiments include oligonucleotides capable of acting as points of initiation of nucleic acid synthesis within the described target gene nucleic acid sequences. A primer can be purified from a restriction digest by conventional methods, or it can be produced synthetically. The primer is preferably single-stranded for maximum efficiency in amplification, but the primer can be double-stranded. Double-stranded primers are first denatured, i.e., treated to separate the strands. One method of denaturing double stranded nucleic acids is by heating.

If the template nucleic acid is double-stranded, it is necessary to separate the two strands before it can be used as a template in PCR. Strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One method of separating the nucleic acid strands involves heating the nucleic acid until it is predominately denatured (e.g., greater than 50%, 60%, 70%, 80%, 90% or 95% denatured). The heating conditions necessary for denaturing template nucleic acid will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 90°C to about 105°C for a time depending on features of the reaction such as temperature and the nucleic acid length. Denaturation is typically performed for about 30 sec to 4 min (e.g., 1 min to 2 min 30 sec, or 1.5 min).

If the double-stranded template nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature that promotes annealing of each primer to its target sequence on the described target gene nucleic acid molecules. The temperature for annealing is usually from about 35°C to about 65°C (e.g., about 40°C to about 60°C; about 45°C to about 50°C). Annealing times can be from about 10 sec to about 1 min (e.g., about 20 sec to about 50 sec; about 30 sec to about 40 sec). The reaction mixture is then adjusted to a temperature at which the activity of the polymerase is promoted or optimized, i.e., a temperature sufficient for extension to occur from the annealed primer to generate products complementary to the template nucleic acid. The temperature should be sufficient to synthesize an extension product from each primer that is annealed to a nucleic acid template, but should not be so high as to denature an extension product from its complementary template (e.g., the temperature for

extension generally ranges from about 40°C to about 80°C (e.g., about 50°C to about 70°C; about 60°C). Extension times can be from about 10 sec to about 5 min (e.g., about 30 sec to about 4 min; about 1 min to about 3 min; about 1 min 30 sec to about 2 min).

5 PCR assays can employ nucleic acid such as RNA or DNA (cDNA). The template nucleic acid need not be purified; it may be a minor fraction of a complex mixture, such as nucleic acid contained in human cells. Nucleic acid molecules may be extracted from a biological sample by routine techniques such as those described in *Diagnostic Molecular Microbiology: Principles and Applications* (Persing et al. (eds), 1993, American Society for Microbiology, Washington D.C.). Nucleic acids can be obtained from any number of sources, such as plasmids, 10 or natural sources including bacteria, yeast, protozoa viruses, organelles, or higher organisms such as plants or animals.

The oligonucleotide primers are combined with PCR reagents under reaction conditions that induce primer extension. For example, chain extension reactions generally include 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 15 mM MgCl₂, 0.001% (w/v) gelatin, 0.5-1.0 µgprotodenatured 15 template DNA, 50 pmoles of each oligonucleotide primer, 2.5 U of Taq polymerase, and 10% DMSO). The reactions usually contain 150 to 320 µM each of dATP, dCTP, dTTP, dGTP, or one or more analogs thereof.

The newly synthesized strands form a double-stranded molecule that can be used in the succeeding steps of the reaction. The steps of strand separation, annealing, and elongation can 20 be repeated as often as needed to produce the desired quantity of amplification products corresponding to the target nucleic acid molecules. The limiting factors in the reaction are the amounts of primers, thermostable enzyme, and nucleoside triphosphates present in the reaction. The cycling steps (i.e., denaturation, annealing, and extension) are preferably repeated at least once. For use in detection, the number of cycling steps will depend, e.g., on the nature of the 25 sample. If the sample is a complex mixture of nucleic acids, more cycling steps will be required to amplify the target sequence sufficient for detection. Generally, the cycling steps are repeated at least about 20 times, but may be repeated as many as 40, 60, or even 100 times.

Fluorescence Resonance Energy Transfer (FRET)

FRET technology (see, for example, U.S. Pat. Nos. 4,996,143, 5,565,322, 5,849,489, and 30 6,162,603) is based on a concept that when a donor fluorescent moiety and a corresponding acceptor fluorescent moiety are positioned within a certain distance of each other, energy transfer takes place between the two fluorescent moieties that can be visualized or otherwise detected and/or quantitated. The donor typically transfers the energy to the acceptor when the

donor is excited by light radiation with a suitable wavelength. The acceptor typically re-emits the transferred energy in the form of light radiation with a different wavelength. In certain systems, non-fluorescent energy can be transferred between donor and acceptor moieties, by way of biomolecules that include substantially non-fluorescent donor moieties (see, for example, US Pat. No. 7,741,467).

In one example, an oligonucleotide probe can contain a donor fluorescent moiety and a corresponding quencher, which may or not be fluorescent, and which dissipates the transferred energy in a form other than light. When the probe is intact, energy transfer typically occurs between the donor and acceptor moieties such that fluorescent emission from the donor fluorescent moiety is quenched the acceptor moiety. During an extension step of a polymerase chain reaction, a probe bound to an amplification product is cleaved by the 5' to 3' nuclease activity of, e.g., a Taq Polymerase such that the fluorescent emission of the donor fluorescent moiety is no longer quenched. Exemplary probes for this purpose are described in, e.g., U.S. Pat. Nos. 5,210,015, 5,994,056, and 6,171,785. Commonly used donor-acceptor pairs include the FAM-TAMRA pair. Commonly used quenchers are DABCYL and TAMRA. Commonly used dark quenchers include BlackHole Quenchers™ (BHQ), (Biosearch Technologies, Inc., Novato, Cal.), Iowa Black™, (Integrated DNA Tech., Inc., Coralville, Iowa), BlackBerry™ Quencher 650 (BBQ-650), (Berry & Assoc., Dexter, Mich.).

In another example, two oligonucleotide probes, each containing a fluorescent moiety, can hybridize to an amplification product at particular positions determined by the complementarity of the oligonucleotide probes to the target nucleic acid sequence. Upon hybridization of the oligonucleotide probes to the amplification product nucleic acid at the appropriate positions, a FRET signal is generated. Hybridization temperatures can range from about 35° C to about 65° C for about 10 sec to about 1 min.

Fluorescent analysis can be carried out using, for example, a photon counting epifluorescent microscope system (containing the appropriate dichroic mirror and filters for monitoring fluorescent emission at the particular range), a photon counting photomultiplier system, or a fluorimeter. Excitation to initiate energy transfer, or to allow direct detection of a fluorophore, can be carried out with an argon ion laser, a high intensity mercury (Hg) arc lamp, a fiber optic light source, or other high intensity light source appropriately filtered for excitation in the desired range.

As used herein with respect to donor and corresponding acceptor moieties "corresponding" refers to an acceptor fluorescent moiety or a dark quencher having an absorbance spectrum that overlaps the emission spectrum of the donor fluorescent moiety. The wavelength maximum of

the emission spectrum of the acceptor fluorescent moiety should be at least 100 nm greater than the wavelength maximum of the excitation spectrum of the donor fluorescent moiety. Accordingly, efficient non-radiative energy transfer can be produced there between.

- Fluorescent donor and corresponding acceptor moieties are generally chosen for (a) high efficiency Forster energy transfer; (b) a large final Stokes shift (>100 nm); (c) shift of the emission as far as possible into the red portion of the visible spectrum (>600 nm); and (d) shift of the emission to a higher wavelength than the Raman water fluorescent emission produced by excitation at the donor excitation wavelength. For example, a donor fluorescent moiety can be chosen that has its excitation maximum near a laser line (for example, Helium-Cadmium 442 nm or Argon 488 nm), a high extinction coefficient, a high quantum yield, and a good overlap of its fluorescent emission with the excitation spectrum of the corresponding acceptor fluorescent moiety. A corresponding acceptor fluorescent moiety can be chosen that has a high extinction coefficient, a high quantum yield, a good overlap of its excitation with the emission of the donor fluorescent moiety, and emission in the red part of the visible spectrum (>600 nm).
- Representative donor fluorescent moieties that can be used with various acceptor fluorescent moieties in FRET technology include fluorescein, Lucifer Yellow, B-phycoerythrin, 9-acridineisothiocyanate, Lucifer Yellow VS, 4-acetamido-4'-isothio-cyanatostilbene-2,2'-disulfonic acid, 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin, succinimidy 1-pyrenebutyrate, and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid derivatives.
- Representative acceptor fluorescent moieties, depending upon the donor fluorescent moiety used, include LC Red 640, LC Red 705, Cy5, Cy5.5, Lissamine rhodamine B sulfonyl chloride, tetramethyl rhodamine isothiocyanate, rhodamine x isothiocyanate, erythrosine isothiocyanate, fluorescein, diethylenetriamine pentaacetate, or other chelates of Lanthanide ions (e.g., Europium, or Terbium). Donor and acceptor fluorescent moieties can be obtained, for example, from Molecular Probes (Junction City, Oreg.) or Sigma Chemical Co. (St. Louis, Mo.).

The donor and acceptor fluorescent moieties can be attached to the appropriate probe oligonucleotide via a linker arm. The length of each linker arm is important, as the linker arms will affect the distance between the donor and acceptor fluorescent moieties. The length of a linker arm can be the distance in Angstroms (Å) from the nucleotide base to the fluorescent moiety. In general, a linker arm is from about 10 Å to about 25 Å. The linker arm may be of the kind described in WO 84/03285. WO 84/03285 also discloses methods for attaching linker arms to a particular nucleotide base, and also for attaching fluorescent moieties to a linker arm. An acceptor fluorescent moiety, such as an LC Red 640, can be combined with an oligonucleotide which contains an amino linker (e.g., C6-amino phosphoramidites available

from ABI (Foster City, Calif.) or Glen Research (Sterling, VA)) to produce, for example, LC Red 640-labeled oligonucleotide. Frequently used linkers to couple a donor fluorescent moiety such as fluorescein to an oligonucleotide include thiourea linkers (FITC-derived, for example, fluorescein-CPG's from Glen Research or ChemGene (Ashland, Mass.)), amide-linkers
5 (fluorescein-NHS-ester-derived, such as CX-fluorescein-CPG from BioGenex (San Ramon, Calif.)), or 3'-amino-CPGs that require coupling of a fluorescein-NHS-ester after oligonucleotide synthesis.

As described herein, amplification products can be detected using labeled hybridization probes that take advantage of FRET technology. One FRET format utilizes TaqMan[®] technology to
10 detect the presence or absence of an amplification product, and hence, the presence or absence of the target gene. TaqMan[®] technology utilizes one single-stranded hybridization probe labeled with, e.g., one fluorescent dye and one quencher, which may or may not be fluorescent. When a first fluorescent moiety is excited with light of a suitable wavelength, the absorbed energy is transferred to a second fluorescent moiety or a dark quencher according to the
15 principles of FRET. The second moiety is generally a quencher molecule. During the annealing step of the PCR reaction, the labeled hybridization probe binds to the target DNA (i.e., the amplification product) and is degraded by the 5' to 3' nuclease activity of, e.g., the Taq Polymerase during the subsequent elongation phase. As a result, the fluorescent moiety and the quencher moiety become spatially separated from one another. As a consequence, upon
20 excitation of the first fluorescent moiety in the absence of the quencher, the fluorescence emission from the first fluorescent moiety can be detected. By way of example, an ABI PRISM[®] 7700 Sequence Detection System (Applied Biosystems) uses TaqMan[®] technology, and is suitable for performing the methods described herein for detecting the presence or absence of the target gene in the sample.

25 Molecular beacons in conjunction with FRET can also be used to detect the presence of an amplification product using the real-time PCR methods. Molecular beacon technology uses a hybridization probe labeled with a first fluorescent moiety and a second fluorescent moiety. The second fluorescent moiety is generally a quencher, and the fluorescent labels are typically located at each end of the probe. Molecular beacon technology uses a probe oligonucleotide
30 having sequences that permit secondary structure formation (e.g., a hairpin). As a result of secondary structure formation within the probe, both fluorescent moieties are in spatial proximity when the probe is in solution. After hybridization to the target nucleic acids (i.e., amplification products), the secondary structure of the probe is disrupted and the fluorescent

moieties become separated from one another such that after excitation with light of a suitable wavelength, the emission of the first fluorescent moiety can be detected.

Another common format of FRET technology utilizes two hybridization probes. Each probe can be labeled with a different fluorescent moiety and are generally designed to hybridize in close proximity to each other in a target DNA molecule (e.g., an amplification product). A donor fluorescent moiety, for example, fluorescein, is excited at 470 nm by the light source of the LightCycler® Instrument. During FRET, the fluorescein transfers its energy to an acceptor fluorescent moiety such as LightCycler®-Red 640 (LC Red 640) or LightCycler®-Red 705 (LC Red 705). The acceptor fluorescent moiety then emits light of a longer wavelength, which is detected by the optical detection system of the LightCycler® instrument. Efficient FRET can only take place when the fluorescent moieties are in direct local proximity and when the emission spectrum of the donor fluorescent moiety overlaps with the absorption spectrum of the acceptor fluorescent moiety. The intensity of the emitted signal can be correlated with the number of original target DNA molecules (e.g., the number of target strain/family genomes). If amplification of target nucleic acid occurs and an amplification product is produced, the step of hybridizing results in a detectable signal based upon FRET between the members of the pair of probes.

Generally, the presence of FRET indicates the presence of the target gene in the sample, and the absence of FRET indicates the absence of the target gene in the sample. Inadequate specimen collection, transportation delays, inappropriate transportation conditions, or use of certain collection swabs (calcium alginate or aluminum shaft) are all conditions that can affect the success and/or accuracy of a test result, however. Using the methods disclosed herein, detection of FRET within, e.g., 45 cycling steps is indicative of the presence of the target strain/family of interest.

Representative biological samples that can be used in practicing the methods include, but are not limited to respiratory specimens, fecal specimens, blood specimens, dermal swabs, nasal swabs, wound swabs, blood cultures, skin, and soft tissue infections. Collection and storage methods of biological samples are known to those of skill in the art. Biological samples can be processed (e.g., by nucleic acid extraction methods and/or kits known in the art) to release target gene nucleic acid or in some cases, the biological sample can be contacted directly with the PCR reaction components and the appropriate oligonucleotides.

Melting curve analysis is an additional step that can be included in a cycling profile. Melting curve analysis is based on the fact that DNA melts at a characteristic temperature called the melting temperature (T_m), which is defined as the temperature at which half of the DNA

duplexes have separated into single strands. The melting temperature of a DNA depends primarily upon its nucleotide composition. Thus, DNA molecules rich in G and C nucleotides have a higher T_m than those having an abundance of A and T nucleotides. By detecting the temperature at which signal is lost, the melting temperature of probes can be determined.

- 5 Similarly, by detecting the temperature at which signal is generated, the annealing temperature of probes can be determined. The melting temperature(s) of the probes from the amplification products can confirm the presence or absence of the target strain/family of interest in the sample. Within each thermocycler run, control samples can be cycled as well. Positive control samples can amplify target nucleic acid control template (other than described amplification products of target genes) using, for example, control primers and control probes. Positive control samples can also amplify, for example, a plasmid construct containing the target nucleic acid molecules. Such a plasmid control can be amplified internally (e.g., within the sample) or in a separate sample run side-by-side with the patients' samples using the same primers and probe as used for detection of the intended target. Such controls are indicators of the success or failure of the amplification, hybridization, and/or FRET reaction. Each thermocycler run can also include a negative control that, for example, lacks target template DNA. Negative control can measure contamination. This ensures that the system and reagents would not give rise to a false positive signal. Therefore, control reactions can readily determine, for example, the ability of primers to anneal with sequence-specificity and to initiate elongation, as well as the ability of probes to hybridize with sequence-specificity and for FRET to occur.

In an embodiment, the methods include steps to avoid contamination. For example, an enzymatic method utilizing uracil-DNA glycosylase is described in U.S. Pat. Nos. 5,035,996, 5,683,896 and 5,945,313 to reduce or eliminate contamination between one thermocycler run and the next.

- 25 Conventional PCR methods in conjunction with FRET technology can be used to practice the methods. In one embodiment, a LightCycler[®] instrument is used. The following patent applications describe real-time PCR as used in the LightCycler[®] technology: WO 97/46707, WO 97/46714, and WO 97/46712.

- The LightCycler[®] can be operated using a PC workstation and can utilize a Windows NT operating system. Signals from the samples are obtained as the machine positions the capillaries sequentially over the optical unit. The software can display the fluorescence signals in real-time immediately after each measurement. Fluorescent acquisition time is 10-100 milliseconds (msec). After each cycling step, a quantitative display of fluorescence vs. cycle number can be continually updated for all samples. The data generated can be stored for further analysis.

As an alternative to FRET, an amplification product can be detected using a double-stranded DNA binding dye such as a fluorescent DNA binding dye (e.g., SYBR[®] Green or SYBR[®] Gold (Molecular Probes)). Upon interaction with the double-stranded nucleic acid, such fluorescent DNA binding dyes emit a fluorescence signal after excitation with light at a suitable wavelength.

5 A double-stranded DNA binding dye such as a nucleic acid intercalating dye also can be used. When double-stranded DNA binding dyes are used, a melting curve analysis is usually performed for confirmation of the presence of the amplification product.

It is understood that the embodiments of the present disclosure are not limited by the configuration of one or more commercially available instruments.

10 **Real-time PCR for Phenotypic Antimicrobial Susceptibility Testing (AST)**

Although quantitative real-time PCR (qPCR or qRT-PCR) can identify and quantify bacteria in samples with high specificity and sensitivity, its reliability in performing phenotypic based AST using growth of bacteria in the presence of antimicrobials has not been demonstrated with consistency. The present invention utilizes mathematical relationships derived from PCR growth curves to make the determination of whether a tested bacteria strain is susceptible, intermediate or resistant (SIR) to a given antimicrobial. The principle behind a phenotypic AST test using PCR is depicted in FIG. 1. In the absence of antimicrobial (shown as Reference), bacteria have ongoing genomic DNA replication. In the presence of an antimicrobial, resistant bacteria will replicate with similar number of genome copies as the Reference, while susceptible bacteria will experience inhibition of replication resulting in fewer copies. This difference in growth provides a phenotypic readout that can be determined by qPCR.

Raw data from a hypothetical qPCR experiment in which either a resistant or a susceptible bacteria strain is incubated for four hours with various concentrations of an antimicrobial shown in FIG. 2. In FIG. 2A, the raw qPCR data is depicted as growth curves where fluorescence (e.g. from a TaqMan[®] probe) is measured at each PCR cycle. For the resistant isolate, the growth curve appears similar irrespective of incubation for four hours at different antimicrobial concentrations, whereas in the susceptible isolate, a dose-dependent decrease in fluorescent intensity and increase in the number of cycles required for the signal to cross background (threshold) level, which is commonly referred as the Cycle threshold or Ct value. In FIG. 2B the same qPCR data is represented based on the Ct value, where a resistant isolate has little or no change in Ct value as a function of antimicrobial concentration, while a susceptible isolate has a dose dependent increase in Ct value by detecting a smaller number of replicating bacteria.

qPCR data can be used to determine whether a strain is susceptible, intermediate or resistant to a given antimicrobial by exploring a variety of mathematical relationships that are shown on FIG. 3. FIG 3A describes mathematical relationships such as “Slope”, “Ct”, “Inflection”, “Absolute Fluorescence Intensity (AFI)”, and “Endpoint Relative Intensity (ERI)” describe the behavior of the raw qPCR amplification curve. As shown in FIG. 3B, these features can further be evaluated as a function of antimicrobial exposure by relating numerical values obtained in the presence of antimicrobial back to the numerical values obtained in the absence of the antimicrobial. Relative changes in these features, such as ΔCt or ΔAFI can then be used to determine strain MIC to a given drug allowing strain resistance and susceptibility determination utilizing approved breakpoints.

From the calculated differences in the mathematical features, simplified partitioning models such as that depicted in FIG. 4 were used to classify bacteria isolates as resistant or susceptible. Partitioning utilizes the Gini Index to determine which feature and threshold maximizes dispersion of the two classes. Only two splits are utilized to prevent overfitting and allows for graphical representation in two dimensions for easy visualization.

EXAMPLES

The following examples, tables and figures are provided to aid the understanding of the subject matter, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

EXAMPLE 1 PCR conditions

Real-time PCR detection of target genes were performed using the **cobas**[®] 6800/8800 systems platforms (Roche Molecular Systems, Inc., Pleasanton, CA). The final concentrations of the amplification reagents are shown below:

TABLE XXIX: PCR Amplification Reagents

<i>Master Mix Component</i>	<i>Final Conc (45.0uL)</i>	
DMSO	5.4	%
NaN3	0.027	%
Potassium acetate	120.0	mM
Glycerol	3.0	%
Tween 20	0.015	%
NaN3	0.027	%
Tricine	60.0	mM
NTQ21-46AAptamer	0.222	μ M

UNG Enzyme	10.0	U
Z05D-DNA Polymerase	45.0	U
dATP	400.0	μ M
dCTP	400.0	μ M
dGTP	400.0	μ M
dUTP	800.0	μ M
Forward primer oligonucleotides	0.50	μ M
Reverse primer oligonucleotides	0.50	μ M
Probe oligonucleotides	0.15	μ M
Manganese Acetate	3.30	mM
Trizma Base	12	μ M
Methylparaben	0.08	%

The following table shows the typical thermoprofile used for PCR amplification reaction:

TABLE XXX: PCR Thermoprofile

Program Name	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C / s)	Cycles	Analysis Mode
Pre-PCR	50	None	00:02:00	4.4	1	None
	94	None	00:00:05	4.4		
	55	None	00:02:00	2.2		
	60	None	00:06:00	4.4		
	65	None	00:04:00	4.4		
1st Measurement	95	None	00:00:05	4.4	5	Quantification
	55	Single	00:00:30	2.2		
2nd Measurement	91	None	00:00:05	4.4	45	Quantification
	58	Single	00:00:25	2.2		
Cooling	40	None	00:02:00	2.2	1	None

- 5 The Pre-PCR program comprised initial denaturing and incubation at 55°C, 60°C and 65°C for reverse transcription of RNA templates. Incubating at three temperatures combines the advantageous effects that at lower temperatures slightly mismatched target sequences (such as genetic variants of an organism) are also transcribed, while at higher temperatures the formation of RNA secondary structures is suppressed, thus leading to a more efficient transcription. PCR
- 10 cycling was divided into two measurements, wherein both measurements apply a one-step setup (combining annealing and extension). The first 5 cycles at 55°C allow for an increased inclusivity by pre-amplifying slightly mismatched target sequences, whereas the 45 cycles of the second measurement provide for an increased specificity by using an annealing/extension temperature of 58°C.

EXAMPLE 2 PCR using primers/probes for detecting Enterobacterales order

Using the PCR conditions described in Example 1, PCR assays using forward primer RM_ENTF (SEQ ID NO: 1), reverse primer RM_ENTRP (SEQ ID NO: 2) and both probes RM_ETP02 (SEQ ID NO: 3) and RM_ETP02B (SEQ ID NO: 4) that target the *rplP* gene were tested against five bacteria strains from the Enterobacterales order: *E. Coli*, *K. pneumonia*, *E. cloacae*, *S. marcescens*, *P. mirabilis*, and two bacteria strains from non-Enterobacterales order : *P. aeruginosa*, and *A. baumannii*. The concentration of the starting material that used ranged between 1e8 and 5e8 CFU/ml for the culture fluid for all the strains (overnight cultures previously stored in glycerol) except for *S. marcescens* in which DNA (~ 1e7 copies/μl) was used. No sample preparation was performed for the culture fluids. The results of this experiment showed that growth curves are observed only for the five strains of the Enterobacterales order family but not for the two non-Enterobacterales strains, thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting Enterobacterales. Similar experiments were performed testing the *P. aeruginosa*-specific primers and probe (SEQ ID NOs: 5-8) and the *A. baumannii*-specific primers and probe (SEQ ID NOs: 9-11) which also showed good specificity and exclusivity (data not shown).

PCR assays using forward primer SEGP1899 (SEQ ID NO: 8), reverse primer SEGP1901 (SEQ ID NO: 9) and probe SEGP2016 (SEQ ID NO: 10) that target the *gyrB* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Gram-positive organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 5, growth curves were observed only for the species that constitute the order Enterobacterales (including *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, and *P. mirabilis*). No meaningful amplification was observed for non-target organisms (*S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting Enterobacterales.

PCR assays were performed for other primers and probe combinations designed to target the *rplP* gene in Enterobacterales. PCR assays using forward primer SEGP2891 (SEQ ID NO: 5), reverse primer SEGP2892 (SEQ ID NO: 6) and probe SEGP2893 (SEQ ID NO: 7) that target the *rplP* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Gram-positive organisms were also tested and showed no

meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control which was 0 ng/μL. As shown in FIG. 6, growth curves were observed only for the species that constitute the order Enterobacterales (including *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, and *P. mirabilis*). No meaningful amplification was observed for non-target organisms (*S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting Enterobacterales.

Similar results were obtained in PCR assays using forward primer SEGP2799 (SEQ ID NO: 11), reverse primers SEGP2800 (SEQ ID NO: 12), SEGP2802 (SEQ ID NO: 13), and SEGP2821 (SEQ ID NO: 14), in combination with probes SEGP2804 (SEQ ID NO: 15) and SEGP2822 (SEQ ID NO: 16) that target the *rpoB* gene. As shown in FIG. 7, growth curves were observed only for the species that constitute the order Enterobacterales (including *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, and *P. mirabilis*). No meaningful amplification was observed for non-target organisms (*S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting Enterobacterales.

EXAMPLE 3 PCR using primers/probes for detecting *Acinetobacter* genus

PCR assays using forward primer SEGP2603 (SEQ ID NO: 20), reverse primer SEGP2606 (SEQ ID NO: 21), and probe SEGP2769 (SEQ ID NO: 22) that target the *ompA* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Gram-positive organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 8, growth curves were observed only for the prevalent pathogens that are within the genus *Acinetobacter* (*A. baumannii* and *A. pittii*). No meaningful amplification was observed for non-target organisms (*E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, and *P. aeruginosa*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *Acinetobacter*.

Similar results were obtained in PCR assays using forward primer SEGP2590 (SEQ ID NO: 23), reverse primer SEGP2593 (SEQ ID NO: 24), and probe SEGP2594 (SEQ ID NO: 25) that

target the *rpoB* gene (as shown in FIG. 9), and in PCR assays using forward primer SEGP2626 (SEQ ID NO: 26), reverse primer SEGP2628 (SEQ ID NO: 27) and probe SEGP2629 (SEQ ID NO: 28) that target the *gyrB* gene (as shown in FIG. 10). These experiments demonstrate good inclusivity and exclusivity profiles for both combinations of primers and probes for detecting
5 *Acinetobacter*.

EXAMPLE 4 PCR using primers/probes for detecting *Pseudomonas aeruginosa*

PCR assays using forward primer SEGP2341 (SEQ ID NO: 32), reverse primer SEGP2342 (SEQ ID NO: 33) and probe SEGP2343 (SEQ ID NO: 34) that target the *tuf* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Gram-positive organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 11, growth curves were observed only for pathogens within the genus *Pseudomonas* (*P. aeruginosa*). No meaningful
10 amplification was observed for non-target organisms (*E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *A. baumannii*, and *A. pittii*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *P. aeruginosa*.

Similar results were obtained in PCR assays using forward primer SEGP2630 (SEQ ID NO: 35), reverse primer SEGP2631 (SEQ ID NO: 36), and probe SEGP2632 (SEQ ID NO: 37) that target the *gyrB* gene (as shown in FIG. 12), and in PCR assays using forward primer SEGP2634 (SEQ ID NO: 38), reverse primer SEGP2637 (SEQ ID NO: 39) and probe SEGP2640 (SEQ ID NO: 40) that target the *rpoB* gene (as shown in FIG. 13). These experiments demonstrate good inclusivity and exclusivity profiles for both combinations of primers and probes for detecting
20 *P. aeruginosa*.

EXAMPLE 5 PCR using primers/probes for detecting *Stenotrophomonas maltophilia*

PCR assays using forward primer SEGP2532 (SEQ ID NO: 41), reverse primer SEGP2538 (SEQ ID NO: 42) and probe SEGP2544 (SEQ ID NO: 43) that target the *fdnG* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Gram-positive organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 14, growth curves were
30

observed only for the species of *Stenotrophomonas maltophilia* (*S. maltophilia*). No meaningful amplification was observed for non-target organisms (*E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *S. maltophilia*.

Similar results were obtained in PCR assays using forward primer SEGP2578 (SEQ ID NO: 44), reverse primer SEGP2579 (SEQ ID NO: 45), and probe SEGP2580 (SEQ ID NO: 46) that target the *gyrB* gene (as shown in FIG. 15), and in PCR assays using forward primer SEGP2572 (SEQ ID NO: 47), reverse primer SEGP2573 (SEQ ID NO: 48) and probe SEGP2574 (SEQ ID NO: 49) that target the *tuf* gene (as shown in FIG. 16). These experiments demonstrate good inclusivity and exclusivity profiles for both combinations of primers and probes for detecting *S. maltophilia*.

EXAMPLE 6 PCR using primers/probes for detecting *Enterococcus* genus

PCR assays using forward primer SEGP2522 (SEQ ID NO: 53), reverse primer SEGP2525 (SEQ ID NO: 54), and probe SEGP2770 (SEQ ID NO: 55) that target the *rpoB* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Gram-negative organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 17, amplification curves are observed only for pathogens within the genus *Enterococcus* (*E. faecium* and *E. faecalis*). No meaningful amplification was observed for non-target organisms (*S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *S. aureus*, and *S. epidermidis*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *Enterococcus*.

Similar results were obtained in PCR assays using forward primers SEGP1624 (SEQ ID NO: 56) and SEGP1627 (SEQ ID NO: 57), reverse primers SEGP1625 (SEQ ID NO: 58) and SEGP1628 (SEQ ID NO: 59), and probes SEGP1626 (SEQ ID NO: 60) and SEGP1629 (SEQ ID NO: 61) that target the *ddl* gene (as shown in FIG. 18). Good specificity was also observed in PCR assays using forward primers SEGP2882 (SEQ ID NO: 62) and SEGP2884 (SEQ ID NO: 63), reverse primers SEGP2885 (SEQ ID NO: 64) and SEGP2886 (SEQ ID NO: 65) and probe SEGP2888 (SEQ ID NO: 66) that target the *gyrB* gene (as shown in FIG. 19). These experiments demonstrate good inclusivity and exclusivity profiles for both combinations of primers and probes for detecting *Enterococcus*.

EXAMPLE 7 PCR using primers/probes for detecting *Staphylococcus aureus*

PCR assays using forward primer SEGP1490 (SEQ ID NO: 67), reverse primer SEGP1491 (SEQ ID NO: 68) and probe SEGP1492 (SEQ ID NO: 72) that target the *CPE* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Gram-negative organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 20, meaningful growth curves were observed only for pathogens within the species *Staphylococcus aureus*. No meaningful amplification was observed for non-target organisms (*S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, and *S. epidermidis*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *S. aureus*.

Similar results were obtained in PCR assays using forward primer SEGP2792 (SEQ ID NO: 73), reverse primer SEGP2793 (SEQ ID NO: 74), and probe SEGP2794 (SEQ ID NO: 75) that target the *gyrB* gene (as shown in FIG. 21), and in PCR assays using forward primer SEGP2932 (SEQ ID NO: 76), reverse primer SEGP2933 (SEQ ID NO: 77) and probe SEGP2935 (SEQ ID NO: 78) that target the *ddlA* gene (as shown in FIG. 22). These experiments demonstrate good inclusivity and exclusivity profiles for both combinations of primers and probes for detecting *S. aureus*.

EXAMPLE 8 PCR using primers/probes for detecting *Streptococcus agalactiae*

PCR assays using forward primer SEGP2921 (SEQ ID NO: 121), reverse primer SEGP2922 (SEQ ID NO: 122) and probe SEGP2923 (SEQ ID NO: 123) that target the *gyrB* gene were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Gram-negative organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown on FIG. 23, meaningful growth curves were observed only for pathogens within the species *Streptococcus agalactiae*. No meaningful amplification was observed for non-target organisms (*S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*), thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *S. agalactiae*.

Similar results were obtained in PCR assays using forward primer SEGP2204 (SEQ ID NO: 82), reverse primer SEGP2205 (SEQ ID NO: 83), and probe SEGP2206 (SEQ ID NO: 84) that

target the *sip* gene (as shown in FIG. 24), and in PCR assays using forward primer SEGP2947 (SEQ ID NO: 85), reverse primer SEGP2949 (SEQ ID NO: 86) and probe SEGP2951 (SEQ ID NO: 87) that target the *ddlA* gene (as shown in FIG. 25). These experiments demonstrate good inclusivity and exclusivity profiles for both combinations of primers and probes for detecting
5 *S. agalactiae*.

EXAMPLE 9 PCR using primers/probes for detecting *Candida* genus

PCR assays using forward primer SEGP1712 (SEQ ID NO: 88), reverse primer SEGP1713 (SEQ ID NO: 89), and probe SEGP1716 (SEQ ID NO: 90) that target the *RDN18* (*18s rRNA*) were tested against common fungal pathogens: *Candida albicans* and *Candida auris*. Gram-
10 negative and positive organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 26, meaningful amplification curves were observed only for pathogens within the genus *Candida*. No meaningful amplification was observed for non-target organisms, thereby demonstrating good
15 inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting *Candida*.

PCR assays using forward primer SEGP1718 (SEQ ID NO: 91), reverse primer SEGP1719 (SEQ ID NO: 92), and probe SEGP1722.1 (SEQ ID NO: 93) that target the *RDN58* (*5.8s rRNA*) gene were tested against *C. albicans* and *C. auris*. Gram-negative and positive organisms were
20 also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control that was 0 ng/μL. As shown in FIG. 27, meaningful amplification curves were observed only for pathogens within the genus *Candida*. No meaningful amplification was observed for non-target organisms, thereby demonstrating good inclusivity and exclusivity profiles for this particular
25 combination of primers and probes for detecting *Candida*.

EXAMPLE 10 PCR for detecting common Gram-negative and Gram-positive pathogens

PCR assays using forward primer SEGP1830 (SEQ ID NO: 94), reverse primer SEGP1831 (SEQ ID NO: 95) and probe SEGP1895.1 (SEQ ID NO: 96) that target the *16s* gene were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides
30 the no template control that was 0 ng/μL. As shown on FIG. 28, amplification curves were

observed for all Gram-negative pathogens, thereby demonstrating this particular combination of primers and probes for the detection of common Gram-negative pathogens.

This same combination of primers and probe that target the *16s* gene was also tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis* under identical concentrations. As shown on FIG. 29, amplification curves were observed for all Gram-positive pathogens, thereby demonstrating this particular combination of primers and probes for the detection of common Gram-positive pathogens.

EXAMPLE 11 Interpretation of AST Results

In general, the two most commonly used guidelines for interpreting Antimicrobial Susceptibility Testing (AST) results are guidelines from the: 1) Clinical Laboratory Standards Institute (CLSI), and 2) European Committee on Antimicrobial Susceptibility Testing (EUCAST). The US uses the CLSI guidelines while the European countries use the EUCAST guidelines. The current version from CLSI is M100 ED30, "Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition", and available via URL: clsi.org/standards/products/microbiology/documents/m100. The current version from EUCAST is Version 10, "The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020" and available via URL: www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf.

In FIG. 30-1 and 30-2, the established Minimum Inhibitory Concentration (MIC) breakpoints indicated as µg/mL for a number of Gram-negative bacterial organisms (Enterobacterales order, *P. aeruginosa*, *Acinetobacter* genus, *S. maltophilia*) as determined by the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30. Breakpoints are used to interpret MIC results from Antimicrobial Susceptibility Testing, and to classify "groupings" of organisms as either Susceptible, Intermediate, or Resistant to a given antimicrobial. Groupings of organisms can be at differing levels, including, but not limited to, species, genus, order, or a specific biochemical property.

In FIG. 31, the established Minimum Inhibitory Concentration (MIC) breakpoints indicated as µg/mL for a number of Gram-positive bacterial organisms (*S. aureus* and *S. lugdunensis*, *S. epidermidis*, *Enterococcus* genus, *S. pneumonia*, Streptococcus/β-hemolytic, Viridans Streptococcus) as determined by the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30. Breakpoints are used to interpret MIC results from Antimicrobial

Susceptibility Testing, and to classify “groupings” of organisms as either Susceptible, Intermediate, or Resistant to a given antimicrobial. Groupings of organisms can be at differing levels, including, but not limited to, species, genus, order, or a specific biochemical property. In FIG. 32, the established Minimum Inhibitory Concentration (MIC) breakpoints indicated as $\mu\text{g/mL}$ for fungal organisms (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida auris*) as determined by the Clinical and Laboratory Standards Institute (CLSI) document M60 ED 1. Breakpoints are used to interpret MIC results from Antifungal Susceptibility Testing (AFST), and to classify “groupings” of organisms as either Susceptible, Intermediate, or Resistant to a given antifungal. Groupings of organisms can be at differing levels, including, but not limited to, species, genus, order, or a specific biochemical property. Of note is that while AFST is recommended for *Candida auris*, neither CLSI or CDC currently have established breakpoints for the species; instead, AFST results from closely related *Candida* spp. and expert opinion are used to determine the susceptibility of *C. auris* isolates to a given antifungal.

15 **EXAMPLE 12 PCR ID/AST Assay Protocol**

Methods and Materials:

- a. Prepare antimicrobial (Abx) Plate ahead of time and store at -80°C until needed
 - i. Diluent – Cation Adjusted Mueller Hinton Broth (CAMHB)
 - ii. Final Vol – 50 μL /well
 - 20 iii. Remove from -80°C and let thaw at 30min/Room Temp prior to use

TABLE XXXI: Antimicrobial (Abx) Plate Layout

Antibiotic (Abx) Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
B	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
C	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
D	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
E	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
F	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
G	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx
H	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx	0 Abx	1/4 Abx	1/2 Abx	1 Abx	2 Abx	4 Abx

- b. Overnight cultures in CAMHB – 37°C/16-18hrs/500rpm
 - i. 10 μL glycerol stock + 490 μL CAMHB in 2mL 96-well Deep Well Plate
- 25 c. Normalize cultures to 1.00E+06 CFU/mL by Optical Density (OD)
- d. Prepare Test Plate by adding 50 μL normalized test isolate to appropriate wells of Abx Plate as outlined

TABLE XXXII Test Isolate Layout on Abx Plate**Test Isolate Layout on Antibiotic (Abx) Plate**

	1	2	3	4	5	6	7	8	9	10	11	12
A				Isolate_1							Isolate_9	
B				Isolate_2							Isolate_10	
C				Isolate_3							Isolate_11	
D				Isolate_4							Isolate_12	
E				Isolate_5							Isolate_13	
F				Isolate_6							Isolate_14	
G				Isolate_7							Isolate_15	
H				Isolate_8							Isolate_16	

- e. Incubate Test Plate at 37°C/4hrs/no shaking
- f. Prepare PCR Reagents according to TABLE IV
- 5 g. Add 45µl Master Mix to each well of a PCR Assay Plate
- h. Following incubation, stamp 5µl/well of Test Plate to PCR Assay Plate
- i. Final Vol – 50µl/well
- i. For Test Plate, continue incubating at 37°C/12-16hrs/no shaking to determine reference method Minimum Inhibitory Concentration (MIC)
- 10 j. Load PCR Assay Plate to **cobas®** z 480 instrument and run under conditions of TABLE IV.
- k. Following incubation read Test Plate for MIC and determine phenotypic Susceptible/Intermediate/Resistant interpretation.

EXAMPLE 13 Real-Time PCR ID and AST Assay of Enterobacterales order

- 15 Rapid identification and phenotypic antimicrobial susceptibility testing of *Enterobacterales* utilizing three distinct target genes, *gyrB*, *rplP*, and *rpoB*, and three classes of antibacterial agents ciprofloxacin (fluoroquinolone), gentamicin (aminoglycoside), and meropenem (carbapenem) was performed. The primer/probe sets used were as follows. For *gyrB*, SEQ ID NO: 8 (forward primer), SEQ ID NO: 9 (reverse primer), SEQ ID NO: 10 (probe); for *rplP*,
- 20 SEQ ID NO: 5 (forward primer), SEQ ID NO: 6 (reverse primer), SEQ ID NO: 7 (probe); for *rpoB*, SEQ ID NO: 11 (forward primer), SEQ ID NOs: 12-14 (reverse primers), SEQ ID NOs: 15-16 (probe). The antimicrobial susceptibility of *K. pneumoniae* strains 0143 (antimicrobial resistant strain) and 16565 (antimicrobial sensitive strain) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30 to determine their
- 25 resistance and susceptibility to the given antimicrobials, respectively. Each strain was inoculated at 5e5 CFU/mL into wells containing various concentrations of the indicated antimicrobials, and after 4h of incubation were subjected to PCR-based rapid ID/AST testing using the protocol of Example 12.

The results are shown on FIG. 33. The percentages on the Y-axis depicted as “Fold Change Abx level 1” were determined using the calculation $2^{-(\text{Abx_Level_1_Ct} - \text{Reference_Ct})}$ or $2^{-\Delta\text{Ct}}$. The Abx Level indication does not relate to an actual concentration that was used but is rather an indication of which 2-fold dilution is being referred to where Abx Level 1 is the lowest concentration and each Level up is 2-fold higher concentration (see TABLE XXXI). To give an example of how Fold Change is calculated, if the Reference_Ct value (i.e. the Ct value with no antimicrobial added) is 20 and the Abx_Level_1_Ct value is 22, then the Fold Change = $2^{-(22-20)} = 2^{-2} = \frac{1}{2} \times \frac{1}{2} = 25\%$. Based on these calculations, strains resistant to the antimicrobial which have lower ΔCt values will have higher “Fold Change” values than strains that are sensitive to the antimicrobial which have higher ΔCt values, and in FIG. 33, Abx Level 1 was able to produce the best separation between the resistant strain Kpn 0143 and the sensitive strain Kpn 16565. These data further indicate that all three gene targets (*gyrB*, *rplB*, *rpoB*) can be utilized to obtain correct susceptibility results for both Kpn strains, in determining sensitivity or resistance to ciprofloxacin, gentamicin, and meropenem. However, there may be instances in which any given target gene may perform better or worse for determining sensitivity or resistance to a given antimicrobial. Taken together, these results indicate that different target genes and alleles can be utilized for rapid PCR-based ID/AST of the *Enterobacterales* order as long as the primers and probes can exhibit correct inclusivity and exclusivity criteria for *Enterobacterales* as was shown in Example 2, FIGS 5-7.

20 **EXAMPLE 14 Real-Time PCR ID and AST Assay of *Pseudomonas aeruginosa***

Rapid Identification and phenotypic antimicrobial susceptibility testing of *Pseudomonas aeruginosa* utilizing three distinct target genes, *tuf*, *gyrB*, *rpoB*, and three classes of antibacterial agents, ciprofloxacin, gentamicin, and meropenem, was performed. The primer/probe sets used were as follows. For *tuf*, SEQ ID NO: 32 (forward primer), SEQ ID NO: 33 (reverse primer), SEQ ID NO: 34 (probe); for *gyrB*, SEQ ID NO: 35 (forward primer), SEQ ID NO: 36 (reverse primer), SEQ ID NO: 37 (probe); for *rpoB*, SEQ ID NO: 38 (forward primer), SEQ ID NO: 39 (reverse primer), SEQ ID NO: 40 (probe). The antimicrobial susceptibility of *P. aeruginosa* strains 16657 (resistant) and 17816 (sensitive) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30 to determine their resistance and susceptibility to the given antimicrobials, respectively. Each strain was inoculated at 5×10^5 CFU/mL into wells containing various concentrations of the indicated antimicrobials, and after 4h of incubation were subjected to PCR-based rapid ID/AST testing using the protocol of Example 12. The results, as shown in FIG. 34, indicate that all three gene targets can be utilized

to obtain correct susceptibility results for both *P. aeruginosa* strains, though some target alleles do seem to perform better for some antimicrobials. Taken together, these results indicate that different target genes and alleles can be utilized for rapid PCR-based ID/AST given they provide the correct inclusivity and exclusivity criteria for *P. aeruginosa* (see FIGs. 11-13).

5 **EXAMPLE 15 Real-Time PCR ID and AST Assay of *Acinetobacter baumannii***

Rapid Identification and phenotypic antimicrobial susceptibility testing of *Acinetobacter baumannii* utilizing three distinct target genes, *ompA*, *rpoB*, *gyrB*, and three classes of antibacterial agents, ciprofloxacin, gentamicin, and meropenem, was performed. The primer/probe sets used were as follows. For *ompA*, SEQ ID NO: 20 (forward primer), SEQ ID
10 NO: 21 (reverse primer), SEQ ID NO: 22 (probe); for *rpoB*, SEQ ID NO: 23 (forward primer), SEQ ID NO: 24 (reverse primer), SEQ ID NO: 25 (probe); for *gyrB*, SEQ ID NO: 26 (forward primer), SEQ ID NO: 27 (reverse primer), SEQ ID NO: 28 (probe). The antimicrobial susceptibility of *A. baumannii* strains 17694 (resistant) and 16421 (sensitive) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30 to
15 determine their resistance and susceptibility to the given antimicrobials, respectively. Each strain was inoculated at 5e5 CFU/mL into wells containing various concentrations of the indicated antimicrobials, and after 4h of incubation were subjected to PCR-based rapid ID/AST testing using the protocol of Example 12. The results, as shown in FIG. 35, indicate that all three gene targets can be utilized to obtain correct susceptibility results for both Abi strains,
20 though some target alleles do seem to perform better for some antimicrobials. Taken together, these results indicate that different target genes and alleles can be utilized for rapid PCR-based ID/AST given they provide the correct inclusivity and exclusivity criteria for *A. baumannii* (see FIGs. 8-10).

EXAMPLE 16 Real-Time PCR ID and AST Assay of *Staphylococcus aureus*

25 Rapid Identification and phenotypic antimicrobial susceptibility testing of *Staphylococcus aureus* utilizing three distinct target genes (*gyrB*, *ddlA*, *tuf*) and one class of antibacterial agent, cefoxitin (cephalosporin) was performed. The primer/probe sets used were as follows. For *gyrB*, SEQ ID NO: 73 (forward primer), SEQ ID NO: 74 (reverse primer), SEQ ID NO: 75 (probe); for *ddlA*, SEQ ID NO: 76 (forward primer), SEQ ID NO: 77 (reverse primer), SEQ ID NO: 78
30 (probe); for *tuf*, SEQ ID NO: 79 (forward primer), SEQ ID NO: 80 (reverse primer), SEQ ID NO: 81 (probe). The antimicrobial susceptibility of *S. aureus* strains 15509 (resistant) and 16405 (sensitive) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30 to determine their resistance and susceptibility to the given

antimicrobials, respectively. Each strain was inoculated at 5e5 CFU/mL into wells containing various concentrations of the indicated antimicrobial, and after 4h of incubation were subjected to PCR-based rapid ID/AST testing using the protocol of Example 12. The results, as shown in FIG. 36, indicate that all three gene targets can be utilized to obtain correct susceptibility results for both *S. aureus* strains, though some target alleles do seem to perform better. Taken together, these results indicate that different target genes and alleles can be utilized for rapid PCR-based ID/AST given they provide the correct inclusivity and exclusivity criteria for *S. aureus* (see FIGs. 21-22).

EXAMPLE 17 Real-Time PCR ID and AST Assay of *Enterococcus faecium*

Rapid Identification and phenotypic antimicrobial susceptibility testing of *Enterococcus faecium* utilizing three distinct target genes (*rpoB*, *ddl*, *gyrB*) and two classes of antibacterial agents, ampicillin (beta-lactam) and vancomycin (glycopeptide) was performed. The primer/probe sets used were as follows. For *rpoB*, SEQ ID NO: 53 (forward primer), SEQ ID NO: 54 (reverse primer), SEQ ID NO: 55 (probe); for *ddl*, SEQ ID NOs: 56-57 (forward primers), SEQ ID NOs: 58-59 (reverse primers), SEQ ID NOs: 60-61 (probes); for *gyrB*, SEQ ID NOs: 62-63 (forward primers), SEQ ID NOs: 64-65 (reverse primers), SEQ ID NO: 66 (probe). The antimicrobial susceptibility of *E. faecium* strains 18483 (resistant) and 18446 (sensitive) were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) document M100 ED 30 to determine their resistance and susceptibility to the given antimicrobials, respectively. Each strain was inoculated at 5e5 CFU/mL into wells containing various concentrations of the indicated antimicrobial, and after 4h of incubation were subjected to PCR-based rapid ID/AST testing using the protocol of Example 12. The results, as shown in FIG. 37, indicate that all three gene targets can be utilized to obtain correct susceptibility results for both *E. faecium* strains, though some target alleles do seem to perform better. Taken together, these results indicate that different target genes and alleles can be utilized for rapid PCR-based ID/AST given they provide the correct inclusivity and exclusivity criteria for *E. faecium* (see FIGs. 17-19).

EXAMPLE 18 Real-Time PCR ID and AST Assay of *Candida* genus

Rapid Identification and phenotypic antimicrobial susceptibility testing of *Candida* can be performed with the target genes *RDN18* (18s ribosomal RNA) and *RDN58* (5.8s ribosomal RNA) using the primers and probes as shown in FIG. 38 which are for *RDN18*: SEQ ID NO: 88 (forward primer), SEQ ID NO: 89 (reverse primer), SEQ ID NO: 90 (probe); and for *RDN58*:

SEQ ID NO: 91 (forward primer), SEQ ID NO: 92 (reverse primer), SEQ ID NO: 93 (probe).
The correct inclusivity and exclusivity criteria for *Candida* are shown in FIGs. 26-27.

EXAMPLE 19 Generic Real-Time PCR ID and AST Assay

Rapid Identification and phenotypic antimicrobial susceptibility testing of any given Gram-negative or Gram-positive bacteria can be performed with the widely conserved *16s* ribosomal RNA gene as target and using the primers and probe as shown in FIG. 39, which are SEQ ID NO: 94 (forward primer), SEQ ID NO: 95 (reverse primer), and SEQ ID NO: 96 (probe).

EXAMPLE 20 Multiplex PCR ID Assay with Breakpoint Groups

FIG. 40A shows the inclusivity and exclusivity performance of a Gram-negative pathogen PCR multiplex master mix. The *Acinetobacter* PCR detection set utilized forward primer SEGP2603 (SEQ ID NO: 20), reverse primer SEGP2606 (SEQ ID NO: 21) and probe SEGP2769 (SEQ ID NO: 22) that targets the *ompA* gene (see TABLE V) and assay results are reported in channel 1. The *Pseudomonas aeruginosa* PCR detection set utilized forward primer SEGP2341 (SEQ ID NO: 32), reverse primer SEGP2342 (SEQ ID NO: 33) and probe SEGP2343 (SEQ ID NO: 34) that targets the *tuf* gene (see TABLE VIII) and assay results are reported in channel 2. The *Enterobacteriales* PCR detection set utilized forward primer SEGP1899 (SEQ ID NO: 8), reverse primer SEGP1901 (SEQ ID NO: 9) and probe SEGP2016 (SEQ ID NO: 10) and targets the *gyrB* gene (see TABLE III) and assay results are reported in channel 3. General bacterial PCR detection set utilized forward primer SEGP1830 (SEQ ID NO: 94), reverse primer SEGP1831 (SEQ ID NO: 95) and probe SEGP1895.1 (SEQ ID NO: 96) targets the *16s* rRNA gene (see TABLE XXVIII) and assay results are reported in channel 4. Lastly, a generic internal control PCR detection set utilized forward primer SEGP1952 (ACAACCGCGCCATACATGTCAAGA<t_BB_dC>; SEQ ID NO: 97), reverse primer SEGP1953 (GTCGGGCCGCTTATACAGT ACCA<t_BB_dC>; SEQ ID NO: 98) and probe SEGP1954 (<CY5.5>TGCGCGTCCCG<BHQ_2>TTTTGATACTTCGTAACGGTGC<Phos>; SEQ ID NO: 99) and assay results are reported in channel 5. The breakpoint groups, channels, and dye wavelengths are summarized in FIG. 40B. The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples. The multiplex reaction was tested against genomic DNA isolated from common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. Additionally, no meaningful amplification was observed with genomic DNA isolated from common Gram-positive pathogens (data not shown). As designed, amplification

curves are observed in the correct channel for desired pathogens, indicating the multiplex reaction has very strong inclusivity and exclusivity.

FIG. 41A shows the inclusivity and exclusivity performance of a Gram-positive pathogen PCR multiplex master mix. The *Streptococcus* PCR detection set utilized forward primer SEGP1705 (SEQ ID NO: 100), reverse primer SEGP1706 (SEQ ID NO: 101) and probe SEGP1709.1 (SEQ ID NO: 102) that targets the *tuf* gene (see TABLE XXV) and assay results are reported in channel 1. The *Staphylococcus* PCR detection set utilized forward primer SEGP1835 (SEQ ID NO: 79), reverse primer SEGP1836 (SEQ ID NO: 80) and probe SEGP1838 (SEQ ID NO: 81) that targets the *tuf* gene (see TABLE XXI) and assay results are reported in channel 2. The *Enterococcus* PCR detection set utilized forward primer SEGP2522 (SEQ ID NO: 53), reverse primer SEGP2525 (SEQ ID NO: 54) and probe SEGP2770 (SEQ ID NO: 55) that targets the *rpoB* gene (see TABLE XV) and assay results are reported in channel 3. General bacterial PCR detection set utilized forward primer SEGP1830 (SEQ ID NO: 94), reverse primer SEGP1831 (SEQ ID NO: 95) and probe SEGP1895.1 (SEQ ID NO: 96) that targets the *16s* rRNA gene (see TABLE XXVIII) and assay results are reported in channel 4. Lastly, a generic internal control PCR detection set utilized forward primer SEGP1952 (SEQ ID NO: 97), reverse primer SEGP1953 (SEQ ID NO: 98) and probe SEGP1954 (SEQ ID NO: 99) and assay results are reported in channel 5. The breakpoint groups, channels, and dye wavelengths are summarized in FIG. 41B. The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples. The multiplex reaction was tested against purified genomic DNA from common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. Additionally, no meaningful amplification was observed with genomic DNA isolated from common Gram-negative pathogens (data not shown). As designed, amplification curves are observed in the correct channel for desired pathogens, indicating the multiplex reaction has very strong inclusivity and exclusivity.

EXAMPLE 21 Analysis of PCR-AST Assay

FIG. 42 contains graphs from a series of PCR-AST assays on diverse Gram-negative strains showing different thresholds that can be used to distinguish between susceptible and resistant isolates of multiple pathogen groups separated into different channels and interpreted using statistical separation of populations as outlined in FIG. 4. The thresholds associated with ciprofloxacin susceptibility are shown for A) *Acinetobacter baumannii* (Abi) using primers/probe of SEQ ID NOs: 17-19 that target the *ompA* gene, B) Enterobacteriaceae (Entero) using primers/probe of SEQ ID NOs: 1-3 that target the *rplP* gene, and C) *Pseudomonas*

aeruginosa (Pae) using primers/probe as shown in TABLE XXXIII and target the O-antigen acetylase gene, and are based on change in Ct value at 2 µg/mL ciprofloxacin relative to no ciprofloxacin control (Δ Ct), Relative Fluorescence Intensity (RFI) at 0.5 µg/mL ciprofloxacin and Δ Ct at 1 µg/mL ciprofloxacin, and Slope prior to the Ct fluorescence value at 0.5 µg/mL ciprofloxacin (Slope) and Δ Ct at 1 µg/mL ciprofloxacin.

TABLE XXXIII Oligonucleotides for detecting *Pseudomonas aeruginosa*

Primers and Probes that hybridize to O-antigen acetylase gene in <i>P. aeruginosa</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	RM_PFP01	130	ACGTTTTCCTTCGCTG<t_BB_dA>	t_BB_dA= t-butylbenzyl-dA
Reverse primer 1	RM_PRP02	131	GTACAGTGACCAGCCAT<t_BB_dC>	t_BB_dC= t-butylbenzyl-dC
Reverse primer 2	RM_PRP04	132	GCGAAACAATCCAGGCCAT<t_BB_dC>	t_BB_dC= t-butylbenzyl-dC
Probe	RM_P04	133	<FAM_Thr>CCTACG<BHQ_2>TGAATGCGCTGTTCGATGCGTTGGC<Phos>	<FAM_Thr>: Fluorophore <BHQ_2>: Quencher <Phos>: Phosphate

FIG. 43A) shows the distribution of the resistant and susceptible isolates that were tested for Abi, Pae, and for the Enterobacteriaceae family subdivided into the strains *E. cloacae* (Ecl), *E. Coli* (Eco), *K. aerogenes* (Kae), and *K. pneumonia* (Kpn). The sensitivity, specificity, and categorical agreement for ciprofloxacin across species using the thresholds in FIG. 42 are shown in FIG. 43B). Sensitivity is defined as Total Positives / (Total Positives + False Negatives); Specificity is defined as Total Negatives / (Total Negatives + False Positives); Categorical Agreement is defined as (Total Positives + Total Negatives) / (Total Positives + False Negatives + Total Negatives + False Positives).

FIG. 44 contains graphs from a second PCR-AST assay where thresholds associated with gentamicin susceptibility are shown for A) Abi, B) Entero, and C) Pae, using the respective primers/probe sets, and are based on Inflection cycle at 1 µg/mL gentamicin, changes in Absolute Fluorescence Intensity (Δ AFI) at 1 µg/mL gentamicin and 8 µg/mL gentamicin, and Goodness of Fit for the curve fit to the raw fluorescence data at 16 µg/mL gentamicin and Δ AFI at 4 µg/mL gentamicin. FIG. 45A) shows the distribution of the resistant and susceptible isolates that were tested for Abi, Pae, and for the Enterobacteriaceae strains *Enterobacter cloacae* (Ecl), *Escherichia coli* (Eco), *Klebsiella aerogenes* (Kae) and *Klebsiella pneumonia*

(Kpn). The sensitivity, specificity and categorical agreement for gentamicin across species using the thresholds in FIG. 44 are seen in FIG 45B).

FIG. 46 contains graphs from a third PCR-AST assay where thresholds associated with meropenem susceptibility are shown for A) Abi, B) Entero, and C) Pae, using the respective
 5 primers/probe sets, and are based on change in Ct value at 4 µg/mL meropenem relative to no meropenem control (Δ Ct), change in Ct value at 4 µg/mL meropenem relative to the lowest meropenem concentration at 0.25 µg/mL (Δ Abx-Ct) and the absolute Ct value (Ct) at 0.25 µg/mL meropenem, and the Absolute Fluorescence Intensity (AFI) at 1 µg/mL meropenem and Δ Ct at 4 µg/mL meropenem. FIG. 47A) shows the distribution of the resistant and susceptible
 10 isolates that were tested for Abi, Pae, and for the Enterobacteriaceae strains Ecl, Eco, Kae and Kpn. The sensitivity, specificity and categorical agreement for gentamicin across species using the thresholds in FIG. 46 are seen in FIG 47B).

FIG. 48A) describes the workflow for testing bacteria isolates directly from positive blood culture samples, which were created by spiking a fixed concentration of bacteria into whole
 15 blood, separating red blood cells, inoculating plasma containing bacteria into a commercial blood culture bottle, incubating overnight, and then following the PCR-AST assay protocol as described in EXAMPLE 12 for testing isolates known for being resistant or susceptible to gentamicin. FIG. 48B) shows the results of this experiment where the change in Ct value (Δ Ct) are used to distinguish between resistant and susceptible isolates, showing that phenotypic
 20 results can be obtained on bacteria directly from positive blood culture.

EXAMPLE 22 Multiplex ID-AST PCR Assay of Polymicrobial Samples

A. Kpn/Abi Multiplex PCR ID-AST assay was performed in a polymicrobial sample where a 1:1 ratio of two Gram-negative organisms, *Klebsiella pneumonia* (Kpn) and *Acinetobacter baumannii* (Abi) with different susceptibility combinations were co-incubated together in the
 25 absence or presence of three different antibiotics, ciproflaxacin, gentamicin and meropenem, at varying concentrations. Detection of the Kpn signal was from an ATTO-labeled probe and detection of the Abi signal was from a HEX-labeled probe. Primers and probes used in this assay are shown in TABLE XXXIV and the results are shown on FIG. 49. Each species displayed the appropriate phenotype in the corresponding detection channel as indicated by a
 30 delta-Ct threshold that separates susceptible (sensitive) and resistant strains, thereby providing accurate antimicrobial susceptibility results for this polymicrobial situation.

TABLE XXXIV Oligonucleotides Used in Kpn and Abi ID-AST Assay

Primers and Probes used in polymicrobial ID-AST assay with <i>Klebsiella pneumoniae</i> and <i>Acinetobacter baumannii</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primers	SEGP1899 SEGP1813	8 29	TGTCGAATTCTTATGACTCCTCCA GTAACCCTAACGCTACTGCACGT	
Reverse primers	SEGP1901 SEGP1815	9 30	CGCGAGCGCTTCGTCGA GGTTGATCCCAAGCGAAACCT	
Probes	SEGP2016 SEGP1951	10 31	<HEX>CCGGTCTGC<ZEN>ACCAC ATGGTATTCGAGGTGG<3IABkFQ> <ATTO>TCGAAGGT<BHQ_2>CAC ACAGATAACACT<Phos>	<HEX>: Fluorophore <ATTO>: Fluorophore <ZEN>: Quencher <BHQ_2>: Quencher <3IABkFQ>: 3' Blocker <Phos>: 3' Blocker

B. Kpn/Sar Multiplex PCR ID-AST assay was performed in a polymicrobial sample where a 1:1 ratio of one Gram-negative organism *Klebsiella pneumonia* (Kpn) and one Gram-positive organism *Staphylococcus aureus* (Sar) with different susceptibility combinations were co-incubated together in the absence or presence of three different antibiotics, ciprofloxacin, cefoxitin and meropenem, at varying concentrations. Detection of the Kpn signal was from a HEX-labeled probe and detection of the Sar signal was from a FAM-labeled probe. Primers and probes used in this assay are shown in TABLE XXXV and the results are shown on FIG. 50.

N/A indicates that there is no clinically relevant interpretation for the corresponding bacteria-drug combination. Each species displayed the appropriate phenotype in the corresponding detection channel as indicated by a delta-Ct threshold that separates susceptible (sensitive) and resistant strains, thereby providing accurate antimicrobial susceptibility results for this polymicrobial situation.

TABLE XXXV Oligonucleotides Used in Kpn and Sar ID-AST Assay

Primers and Probes used in polymicrobial ID-AST assay with <i>Klebsiella pneumoniae</i> and <i>Staphylococcus aureus</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primers	SEGP1899 SEGP1835	8 79	TGTCGAATTCTTATGACTCCTCCAGTA CCGTGTTGAACGTGGTCAAATCAAA	
Reverse primers	SEGP1901 SEGP1836	9 80	CGCGAGCGCTTCGTCGA AGCAGCTAATACTTGACCACGTTGTA	
Probes	SEGP2016 SEGP1838	10 81	<HEX>CCGGTCTGC<ZEN>ACCACATG GTATTCGAGGTGG<3IABkFQ> <FAM>AGACTACGC<ZEN>TGAAGCTG GTGAC<3IABkFQ>	<HEX>: Fluorophore <FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

C. Kpn/Cal Multiplex PCR ID-AST assay was performed in a polymicrobial sample where a 1:1 ratio of one Gram-negative organism, *Klebsiella pneumoniae* (Kpn), and one fungal organism, *Candida albicans* (Cal) with different susceptibility combinations were co-incubated together in the absence or presence of two different antibiotics, ciprofloxacin and meropenem, at varying concentrations. Detection of the Kpn signal was from a HEX-labeled probe and detection of the Cal signal was from a FAM-labeled probe. Primers and probes used in this assay are shown in TABLE XXXVI and the results are shown on FIG. 51. N/A indicates that there is no clinically relevant interpretation for the corresponding organism-drug combination. Cal susceptibility for fluconazole is indicated. The Kpn strains displayed the appropriate phenotype in the corresponding detection channel as indicated by a delta-Ct threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results.

TABLE XXXVI Oligonucleotides Used in Kpn and Cal ID-AST Assay

Primers and Probes used in polymicrobial ID-AST assay with <i>Klebsiella pneumoniae</i> and <i>Candida albicans</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primers	SEGP1899 SEGP1712	8 88	TGTCGAATTCTTATGACTCCTCCAGTA CGTTTTCATTAATCAAGAACGAAAGTTA	

Reverse primers	SEGP1901 SEGP1713	9 89	CGCGAGCGCTTCGTCGA ACCGATCCCTAGTCGGCATA	
Probes	SEGP2016 SEGP1716	10 90	<HEX>CCGGTCTGC<ZEN>ACCACATGGT ATTCGAGGTGG<3IABkFQ> <FAM>AGACTACGA<ZEN>CGGTATCTGA TCATCTTCGATCCC<3IABkFQ>	<HEX>: Fluorophore <FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

D. Efs/Sar Multiplex PCR ID-AST assay was performed in a polymicrobial sample where a 1:1 ratio of two Gram-positive organisms, *Enterococcus faecalis* (Efs) and *Staphylococcus aureus* (Sar), with different susceptibility combinations were co-incubated together in the absence or presence of vancomycin at varying concentrations. Detection of the Efs signal was from a HEX-labeled probe and detection of the Sar signal was from a FAM-labeled probe. Primers and probes used in this assay are shown in TABLE XXXVII and the results are shown on FIG. 52. Both species displayed the appropriate phenotype in the corresponding detection channel as indicated by a delta-Ct threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results for this polymicrobial situation.

TABLE XXXVII Oligonucleotides Used in Efs and Sar ID-AST Assay

Primers and Probes used in polymicrobial ID-AST assay with <i>Enterococcus faecalis</i> and <i>Staphylococcus aureus</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primers	SEGP1624 SEGP1835	56 79	CGTAGCATTCTATGATTATGAAGCC CCGTGTTGAACGTGGTCAAATCAAA	
Reverse primers	SEGP1625 SEGP1836	58 80	CATCGTGTAAGCTAACTTCG AGCAGCTAATACTTGACCACGTTGTA	
Probes	SEGP1626 SEGP1838	60 81	<HEX>CAGATTCCA<ZEN>GCCGAAGTG CC<3IABkFQ> <FAM>AGACTACGC<ZEN>TGAAGCTGG TGAC<3IABkFQ>	<HEX>: Fluorophore <FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

E. Sar/Cal Multiplex PCR ID-AST assay was performed in a polymicrobial sample where a 1:1 ratio of one Gram-positive organism, *Staphylococcus aureus* (Sar) and one fungal organism *Candida albicans* (Cal), with different susceptibility combinations were co-incubated

together in the absence or presence of cefoxitin at varying concentrations. Detection of the Sar signal was from a FAM-labeled probe and detection of the Cal signal was from a HEX-labeled probe. Primers and probes used in this assay are shown in TABLE XXXVIII and the results are shown on FIG. 53. N/A indicates that there is no clinically relevant interpretation for the corresponding organism-drug combination. Cal susceptibility for fluconazole is indicated. The Sar strains displayed the appropriate phenotype in the corresponding detection channel as indicated by a delta-Ct threshold that separates susceptible and resistant isolates, providing accurate antimicrobial susceptibility results.

TABLE XXXVIII Oligonucleotides Used in Sar and Cal ID-AST Assay

Primers and Probes used in polymicrobial ID-AST assay with <i>Staphylococcus aureus</i> and <i>Candida albicans</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primers	SEGP1835 SEGP1712	79 88	CCGTGTTGAACGTGGTCAAATCAAA CGTTTTCATTAATCAAGAACGAAAGTTA	
Reverse primers	SEGP1836 SEGP1713	80 89	AGCAGCTAATACTTGACCACGTTGTA ACCGATCCCTAGTCGGCATA	
Probes	SEGP1838 SEGP1717	81 134	<FAM>AGACTACGC<ZEN>TGAAGCTGGT GAC<3IABkFQ> <HEX>AGACTACGA<ZEN>CGGTATCTGA TCATCTTCGATCCC<3IABkFQ>	<HEX>: Fluorophore <FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

EXAMPLE 23 PCR Assays for Determining Mechanism of Carbapenem Resistance

PCR assays that target the blaKPC, blaVIM, blaNDM, and blaOXA-48 genes were tested against Gram-negative pathogens with known mechanisms of carbapenem resistance: *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*, *E. coli* and *K. aerogenes*. The primers and probes used in this assay are listed in TABLE XXXIX. The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples other than the no template control. The results of the experiment are shown on FIG. 54. Growth curves, depicted as Positive (Pos) in the figure were observed only for the targeted resistance mechanism. No meaningful amplification was observed for non-target resistance mechanisms, thereby demonstrating good inclusivity and exclusivity profiles for this particular combination of primers and probes for detecting common mechanisms of carbapenem resistance.

TABLE XXXIX Oligonucleotides Used in Carbapenem Resistance PCR Assay

Gene Target	Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
blaKPC	Forward Primer	SEGP2124	103	GCGATACCACGTTCCGTCTG	
blaVIM		SEGP2135	104	CCGAGTGGTGAGTATCCGAC	
blaNDM		SEGP2127	105	TTTGGCGATCTGGTTTTCCG	
blaOXA-48		SEGP2133	106	GGCACGTATGAGCAAGATGC	
blaKPC	Reverse Primer	SEGP2125	107	CGGTCGTGTTCCCTTTAGC	
blaVIM		SEGP2136	108	GAATGCGTGGAATCTCGTTC	
blaNDM		SEGP2128	109	ATCAAACCGTTGGAAGCGAC	
blaOXA-48		SEGP2134	110	GTTTGACAATACGCTGGCTGC	
blaKPC	Probe	SEGP2126	111	<CFR_635>AGCGGCAGCAGTTT<BHQ_2>GTTGATTG<Phos>	<CFR_635>: Fluorophore <BHQ_2>: Quencher <Phos>: 3' Blocker
blaVIM		SEGP2137	112	<CFR_635>CGCTGTATCAATCA A<BHQ_2>AAGCAACTCATCA<Phos>	
blaNDM		SEGP2129	113	<CFR_635>AGACATTCCGTGCGA<BHQ_2>GCTGGC<Phos>	
blaOXA-48		SEGP2346	114	<CFR_635>TCGGGCAATGT<BHQ_2>AGACAGTTTCTGGCTCGA CG<Phos>	

EXAMPLE 24 species-specific PCR ID-AST assays

PCR assays using forward primer SEGP2164 (SEQ ID NO: 115), reverse primer SEGP2166 (SEQ ID NO: 116) and probe SEGP2167 (SEQ ID NO: 117) that target the *citC* gene of *P. stuartii*, and also forward primer SEGP2119 (SEQ ID NO: 118), reverse primer SEGP2121 (SEQ ID NO: 119) and probe SEGP2120 (SEQ ID NO: 120) that target the *invA* gene of *Salmonella* were tested against common Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *K. aerogenes*, *S. marcescens*, *P. mirabilis*, *C. freundii*, *P. stuartii*, *P. rettgeri*, *S. enterica*, *S. maltophilia*, *P. aeruginosa*, *A. baumannii*, and *A. pittii*. The sequences are shown in TABLE XL. Gram-positive organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control which was 0 ng/μL. As shown in FIG. 55, meaningful amplification curves were species-specific. No meaningful amplification was observed for non-target organisms, thereby demonstrating good inclusivity and exclusivity profiles for the four combinations of primers and probes for detecting the respective Gram-

negative target species. These results represent non-limiting examples of species specific detection sets that allow improved breakpoint based AST calling for some specific species within the order *Enterobacterales*. CLSI guidelines indicate that some *Enterobacterales* species such as those shown here are resistant to specific aminoglycoside drugs, while the majority of *Enterobacterales* species are not.

TABLE XL Oligonucleotides for detecting *P. stuartii* and *Salmonella*

Primers and Probes targeting <i>citC</i> gene of <i>P. stuartii</i> and <i>invA</i> gene of <i>Salmonella</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2164 SEGP2119	115 118	GCCATCGTGATGAATGCCAATCC TGACCATTTC AATGGGA ACTCTGC	
Reverse primer	SEGP2166 SEGP2121	116 119	TCAGAGCCTTTGTGGATAGTGAGA AGATCGCCAATCAGTCCTAACGA	
Probe	SEGP2167 SEGP2120	117 120	<FAM>TTGGCTACA<ZEN>TTTATT TGTCGTCAAAGAAGATACCTCACG CTTCC<3IABkFQ> <FAM>CAAAGGCGA<ZEN>GCAGC CGCTCAGTATTGAGGA<3IABkFQ>	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker

PCR assays using forward primer SEGP2921 (SEQ ID NO: 121), reverse primer SEGP2922 (SEQ ID NO: 122) and probe SEGP2923 (SEQ ID NO: 123) that target the *gyrB* gene of *S. agalactiae*, forward primer SEGP2947 (SEQ ID NO: 85), reverse primer SEGP2949 (SEQ ID NO: 86) and probe SEGP2951 (SEQ ID NO: 87) that target the *ddlA* gene of *S. agalactiae*, forward primer SEGP2777 (SEQ ID NO: 124), reverse primer SEGP2778 (SEQ ID NO: 125) and probe SEGP2779 (SEQ ID NO: 126) that target the *tuf* gene of *S. pneumoniae* and forward primer SEGP2113 (SEQ ID NO: 127), reverse primer SEGP2114 (SEQ ID NO: 128) and probe SEGP2115 (SEQ ID NO: 129) that target the *speB* gene of *S. pyogenes* were tested against common Gram-positive pathogens: *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis*. The sequences are shown in TABLE XLI. Gram-negative organisms were also tested and showed no meaningful amplification (data not shown). The concentration of the genomic DNA was roughly 2-10 ng/μL for all samples, besides the no template control which was 0 ng/μL. As shown in FIG. 56, meaningful amplification curves were species-specific. No meaningful amplification was observed for non-target organisms, thereby demonstrating good inclusivity and exclusivity profiles for the four combinations of primers and probes for detecting the respective Gram-positive target species.

These results represent non-limiting examples of species-specific detection sets that allow improved breakpoint based AST calling for some specific species within the *Staphylococcus* and *Streptococcus* genera (see CLSI breakpoint tables in FIG. 31), by having these ID-wells a more general ID/AST detection set such as those shown in FIG. 41 can be utilized.

5 **TABLE XLI Oligonucleotides for detecting *S. agalactiae*, *S. pneumoniae* and *S. pyogenes***

Primers and Probes targeting <i>gyrB</i> gene of <i>S. agalactiae</i> , <i>ddlA</i> gene of <i>S. agalactiae</i> , <i>tuf</i> gene of <i>S. pneumoniae</i> , <i>speB</i> gene of <i>S. pyogenes</i>				
Oligonucleotide Type	Oligonucleotide Name	SEQ ID NO:	Sequence	Modifications
Forward primer	SEGP2921	121	ACCACTGTATTGATTTTGATAAATTAGCCAAA	
	SEGP2947	85	CACAAGAATTTGATGAAATGCCATCTTCA	
	SEGP2777	124	GTGACTCTAAATACGAAGACATCGTT	
	SEGP2113	127	CGGAAGAAGCCGTCAGAGAC	
Reverse primer	SEGP2922	122	TTCTCATTGATAAACTCAACGTATGAACCTA	
	SEGP2949	86	ACAATTGCATTATCATCATAGATATCACTTGA	
	SEGP2778	125	GCAATGGTTTGTGTCAGTGTACG	
	SEGP2114	128	ATGGTGCTGACGGACGTAAC	
Probe	SEGP2923	123	<FAM>ACTAAGAAT<ZEN>CTCCATTTCAGACA	<FAM>: Fluorophore <ZEN>: Quencher <3IABkFQ>: 3' Blocker
	SEGP2951	87	AGCGAGAAGGTCAAGAAGTTG<3IABkFQ>	
	SEGP2779	126	<FAM>TAATGACAA<ZEN>ACCAAAGTGTGAT	
	SEGP2115	129	TTAGACAAAATGGTTCGTCCA<3IABkFQ>	

The impact of using species-specific primer/probe sets in making accurate calls in PCR ID-AST assays can be seen in FIG. 57. In the left panel, use of non-species-specific primers and probes may result in the inability to discriminate between susceptible and resistant strains. In contrast, in the right panel, the use of species-specific primer/probe sets that provide identification enables separate interpretation for each individual species, leading to improved Categorical Agreement to the CLSI breakpoint guidelines.

EXAMPLE 25 Interpretation of PCR ID-AST assay data

FIG. 58 depicts a workflow of a PCR ID-AST assay data interpretation strategy wherein a sigmoidal function is fit to the raw PCR curve data followed by calculation of curve parameters and features. Features are then compared between the presence of various antibiotic concentrations and the no-antibiotic reference to derive relative feature changes. Regression modeling of feature values and changes across antibiotic levels are also used to generate additional features that correspond to the feature dose-response relationship, and may include the use of Ordinary Least Squares, Ridge Regression, Lasso, Elastic Net, Bayesian Regression,

or Logistic Regression models. Features are then assembled into a dataframe and are input into separate machine learning algorithms along with the ground truth MIC, or ground truth S/I/R, in order to train predictive models, which may include Neural Networks, Tree-Based Models, Support Vector Machine, or Nearest-Neighbor classifiers. Training consists of splitting the data into a training set and a hold-out test set, followed by splitting the training set into k-folds with crossvalidation to search the appropriate hyperparameter space for each type of classifier. Models are then selected based on average crossvalidation scores as well as performance on the held-out test set. Trained models then participate as an ensemble to return the final predicted MIC, which can be based on unweighted voting, weighted voting, average probabilities, weighted probabilities, or interpreted by a downstream classifier of the aforementioned classifier types.

FIG. 59 shows a diagram indicating how species ID, Antimicrobial Susceptibility Testing, Resistance Mechanism detection, and Universal 16s rRNA phenotypic information is combined to return a result, wherein species ID is used to select the appropriate algorithm for MIC prediction which is then compared to the appropriate breakpoints from regulatory bodies to determine susceptibility information. Detection of a resistance mechanism associated with the antibiotic that was tested can then influence the susceptibility result that is returned depending on whether its presence is consistent with the predicted MIC. In the absence of a species ID, the 16s rRNA phenotypic information can be used to return a generic MIC with no susceptibility result, which can be used in conjunction with a species ID that is determined in an alternative fashion, such as mass spectrometry.

There are specific examples of using algorithmic elements to improve breakpoint based AST calling. One example is the use of resistance mechanism detection to adjust phenotype result calling. Each resistance mechanism can have one or more antibiotic substrates associated with its activity which are known a priori. These mechanisms can also have different time-frames in which their activity can be detected. Some of these resistance mechanisms do not have robust activity within 4 hours and can only be detected phenotypically after much longer incubation times (12-24 hours). For these resistance mechanisms an organism may be identified as susceptible to a given drug simply because the resistance mechanism has not manifested sufficiently within a 4 hour time frame. Detection of these types of resistance mechanisms via separate PCR wells allows for the correction of discordant phenotypic results. A specific example is *Serratia marsecens* that sometimes encodes a SME carbapenemase resistance mechanism which is inducible, but not within a 4 hour time frame. Thus these resistant *S. marsecens* strains will appear to be phenotypically susceptible to meropenem, but detection of

the SME gene will allow the correct phenotypic prediction which is meropenem resistance. Another example is the use of phenotypic susceptibility from one or more antibiotics to predict susceptibility for other antibiotics. Because resistance mechanisms often have overlapping substrate specificity this means that susceptibility to some antibiotics is directly correlated with
5 susceptibility to other antibiotics. Likewise, resistance to some antibiotics is directly correlated with resistance to other antibiotics. This is similar to the Expert Rules system that many AST product manufacturers employ whereas data collected from the PCR ID-AST assays of the present invention would be employed as an adjunct to other methods of phenotypic result interpretation. A specific example would be a strain that is susceptible to the antibiotic
10 ertapenem will always be susceptible to the antibiotic meropenem due to the nature of carbapenemases and their substrate specificity which is always higher for degradation of ertapenem. Similarly, any strain that is resistant to meropenem will also be resistant to ertapenem for the same reason.

While the foregoing invention has been described in some detail for purposes of clarity and
15 understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made. For example, all the techniques and apparatus described above can be used in various combinations.