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 µg/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

The present disclosure 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 disclosure 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 disclosure 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*.

In one aspect, the present disclosure refers to a method comprising performing a single quantitative real-time PCR assay as a reporter in the presence of at least one concentration of at least one antimicrobial or antimicrobial class to simultaneously identify and determine the antimicrobial susceptibility of a target group of bacteria or fungi that have similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial class, wherein the identification of the target group of bacteria or fungi is by detecting a signal that is specific to the target group of bacteria or fungi.

One example of the present disclosure 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 example, 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 example, 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 example, 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 example, the target gene is selected from *rplP, ompA, tuf, rpoB, ddl, ddlA, fdnG sodA, gyrB*, O-antigen acetylase, *ecfX, tus*A, CPE, *sip*, and *nuc*.

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

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

In another example, the group of bacteria or fungi represents a taxonomic species. In one example, the taxonomic species is selected from *Enterococcus faecalis (Efs), Enterococcus faecium (Efm), Escherichia coli (Eco), Klebsiella pneumoniae (Kpn) , Klebsiella oxytoca (Kox), 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)*, 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 example, the method further comprises a step selected from verifying the identification of the group of bacteria or fungi with additional primer and probe 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 example, the method further comprises simultaneously identifying and determining the antimicrobial 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 example, the present disclosure relates to a method of using a quantitative real-time PCR assay as a reporter to simultaneously identify and determine the antimicrobial 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 example, the target gene is selected from *rplP*, *gyrB,* and *rpoB*. In one example, 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 example, 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 disclosure refers to a method comprising performing a single multiplexed quantitative real-time PCR assay as a reporter in the presence of at least one concentration of at least one antimicrobial or antimicrobial class to simultaneously identify and determine the antimicrobial susceptibility of a plurality of bacterial or fungal strains from a biological sample.

In another example, the present disclosure 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 example, 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 example, the biological sample is whole blood, plasma, serum or a combination thereof. In one example, the biological sample is cultured prior to performing the PCR assay. In another example, the biological sample is a bacterial or fungal isolate. In another example, 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 example, 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 example, 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 example, the identification of the plurality of bacterial strains utilizes the TAGS (Temperature Assisted Generation of Signal) technology. In another example, 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 yet another aspect, the present disclosure refers to a method comprising performing a single quantitative real-time PCR assay in the presence of at least one concentration of at least one antimicrobial or class of antimicrobials 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 the antimicrobial or the 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 another example, the present disclosure 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 example, 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 example, the mathematical relationship is a relative expression between different antimicrobial concentrations or different antimicrobials and is selected from ΔCt, 2^(ΔCt), ΔInflection, ΔAFI, or ΔERI. In one example, 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 example, the mathematical relationship is a combination of relative expressions between different antimicrobial concentrations or different antimicrobials and is selected from ΔCt, 2^(ΔCt), ΔInflection, ΔAFI, or ΔERI. In another example, 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 example, 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.

CLAIMS

1. A method comprising performing a single quantitative real-time PCR assay as a reporter in the presence of at least one concentration of at least one antimicrobial or antimicrobial class to simultaneously identify and determine the antimicrobial susceptibility of a target group of bacteria or fungi that have similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial class, wherein the identification of the target group of bacteria or fungi is by detecting a signal that is specific to the target group of bacteria or fungi.

2. The method of claim 1, wherein the specific signal is detected by using primer and probe oligonucleotides that hybridize more selectively to a target gene that is from the target group of bacteria or fungi compared to the target gene that is not from the target group of bacteria or fungi.

3. The method of claim 2, wherein the target gene is selected from *rplP, ompA, tuf, rpoB, ddl, ddlA, fdnG, sodA, gyrB*, O-antigen acetylase, *ecfX, tus*A, CPE, *sip*, and *nuc*.

4. The method of any one of claims 1 to 3 further comprising a step selected from
   - verifying the identification of the target group of bacteria or fungi with additional primer and probe oligonucleotides that hybridize more selectively to a second target gene that is from the target group of target bacteria or fungi compared to the second target gene that is not from the 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.

5. The method of any one of claims 1 to 4 further comprising simultaneously identifying and determining the antimicrobial susceptibility of more than one target groups of bacteria or fungi, wherein each target group of bacteria or fungi has similar or identical clinical breakpoints for at least one antimicrobial or one antimicrobial classes.

6. The method of any one of claims 1 to 5, wherein 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.

7. A method comprising performing a single multiplexed quantitative real-time PCR assay as a reporter in the presence of at least one concentration of at least one antimicrobial or antimicrobial class to simultaneously identify and determine the antimicrobial susceptibility of a plurality of bacterial or fungal strains from a biological sample.

8. The method of claim 7, wherein the biological sample is selected from whole blood, plasma, serum, red blood cell fraction, saliva, cerebrospinal fluid, semen, urine, stool, rectal swab, nasal swab, wound swab, dermal swab, bile, lymph, sputum, lavage fluid, or a combination thereof.

9. The method of claim 8, wherein the biological sample is cultured prior to performing the PCR assay.

10. The method of any one of claims 7 to 9, wherein the biological sample is a bacterial or fungal isolate.

11. The method of any one of claims 7 to 10, wherein 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 antimicrobials or antimicrobial classes.

12. The method of claim 11, wherein 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 classes.

13. The method of any one of claims 7 to 12, wherein the identification of the plurality of bacterial or fungal strains utilizes a plurality of strain-specific 5` nuclease (TaqMan) oligonucleotide probes, each probe labeled with a fluorescent dye and each fluorescent dye having a different emission wavelength from another fluorescent dye.

14. A method comprising performing a single quantitative real-time PCR assay in the presence of at least one concentration of at least one antimicrobial or class of antimicrobials 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 the antimicrobial or the 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.

15. The method of claim 14, wherein the mathematical relationship is a relative expression between different antimicrobial concentrations or different antimicrobials and is selected from ΔCt, 2^(ΔCt), ΔInflection, ΔAFI, or ΔERI, or a combination thereof.

16. The method of any one of claims 14 and 15 wherein the mathematical relationship is a combination of Threshold Cycle (Ct), Slope, Inflection of a sigmoid curve fit, Absolute Fluorescence Intensity (AFI), or Endpoint Relative Intensity (ERI) , or a combination thereof.

17. The method of claim 15, wherein the mathematical relationship is a combination of relative expressions between different antimicrobial concentrations or different antimicrobials and is selected from ΔCt, 2^(ΔCt), ΔInflection, ΔAFI, or ΔERI, or a combination thereof.

18. The method of any one of claims 14 to 17 further comprising 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.

19. The method of any one of claims 14 to 18 further comprising 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 classes.

20. The method of any one of claims 14 to 19, wherein the PCR assay is a real-time PCR assay.