Genotypic false detections from blood culture bottles: Are we only seeing the tip of the iceberg?

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Rapid organism identification and detection of antimicrobial resistance genes directly from positive blood cultures has been a critically important development in the field of clinical microbiology. Testing for these is frequently accomplished using multiplexed assays that detect the presence of nucleic acids from specific microorganisms commonly found in patients with bloodstream infection. When combined with active antimicrobial stewardship interventions, such technologies have been shown to lead to improved time to initiation of appropriate antimicrobial therapy, and in turn improved patient outcomes[1].

Recently, there have been several Class 2 recalls issued by the U.S. Food and Drug Administration (FDA) because of increased risk for false-positive detections with these assays due the presence of DNA from non-viable organisms present in certain blood culture media. Such analytical performance problems put the clinical laboratories in the difficult situation of reporting potentially inaccurate results for positive blood cultures. Here, we will describe this issue in more detail, including optional strategies for the clinical laboratory to address these issues.

Multiplex nucleic-acid based assays for blood culture identification

Differences in the method of detection (nucleic acid amplification vs. probe-based detection) and panel design (multiple organisms vs. single organism) of these platforms can influence the risk of false detection from blood culture bottles. At the time of writing, various test platforms and kits are FDA-cleared for the multiplexed nucleic-acid detection of microorganisms directly from positive blood cultures.

Platforms that detect multiple organism types and utilize nucleic acid amplification include the Filmarray (Biofire Diagnostics), ePlex (Genmark Diagnostics) and iC-GPC (iCubate) platforms. The Xpert MRSA/SA BC (Cepheid) and the BD Max StaphSR (BD Molecular Diagnostics) are also direct-from-blood-culture multiplex PCR tests but specifically detect or rule out the presence of methicillin-resistant *S. aureus* (MRSA). Approaches that do not use nucleic acid amplification include the Verigene (Luminex Corporation) panels, which are probe-based, while the the Phenotest BC (Accelerate), and the PNA-FISH (OpGen) tests utilize FISH-based detection.

The FilmArray Blood Culture Identification (BCID) Panel and PhenoTest BC simultaneously target Gram-positive bacteria, Gram-negative bacteria and yeast in a single test kit. In contrast, Verigene, ePlex, iCubate and PNA-FISH systems have distinct kits that individually target Gram-positive bacteria, Gram-negative bacteria and/or yeast.

1. False-positive microorganism detection from blood culture

Beginning in 2014, several FDA-mandated device recalls have been issued due to increased risk of false-positive microorganism detection associated with certain blood culture media lots when multiplex nucleic acid-based panels are used. These recalls were categorized as "Class II," where the device could cause a temporary or reversible health problem in a patient (with slight risk of serious or deadly adverse effects) presumably due to false detection resulting in unnecessary antibiotic therapy. These recalls have included blood culture media types used for both the Bactec (Becton Dickinson) and BacT (bioMérieux) Alert Systems. To our knowledge, these recalls have not implicated blood culture media from the VersaTrek system, though this system has a smaller market share than the other two.

The first recall was caused by DNA from *Pseudomonas aeruginosa* and *Enterococcus*[2] present in BacT/Alert Standard Anaerobic (SN) blood culture bottles. There have been two separate Class 2 recalls due to increased risk for false positive *Proteus* detection by the FilmArray BCID panel. Importantly, the false detection of *Proteus* was seen in all blood culture media types manufactured by both Bactec and BacT/Alert, with a separate Class 2 recall issued in 2019 for increased false-positive *E. coli* detection in certain BacT/Alert media[3].

To date such recalls have not involved the Verigene system, which is likely due to the threshold of detection inherent with probe-based technology compared to amplification-based methods. The ePlex blood culture system obtained IVD-clearance in April 2019 and its risk for this issue is as yet unknown. However, initial data from a side-by-side platform comparison suggests that the ePlex BCID-GN Panel does not show the same false-positive *Proteus* detection as seen with the FilmArray BCID panel[4]. Whether this also applies to contaminating DNA from other organisms (e.g. *E. coli*) is currently unknown. The iCubate Gram-positive panel received IVD clearance in 2017 and the Gram-negative panel in July of 2019[5,6]. Risk for false positive detections with this platform remains unknown.

<u>What is responsible for this issue?</u>
All IVD-cleared blood culture media have an intended use that is limited to the cultivation of bacteria and fungi present in blood. Blood culture media have multiple ingredients from diverse sources including animal, plant and yeast extracts to name a few. It is critical to note, that the intended use of blood culture media has thus far only required sterility on the part of the manufacturer. No current requirement for blood culture media to be free of microorganism nucleic acid exists. Thus, while the final product is sterile, the ingredients themselves may on occasion contain microbial DNA from non-viable organisms.

The increased risk of false-positive *Proteus* detections with the FilmArray BCID assay has been thoroughly investigated by the device manufacturer. The presence of *Proteus* DNA was demonstrated in various lots of both Bactec and BacT/Alert media. Importantly, this is not a universal problem, with only certain media lots affected by this issue. It has been determined that the quantity of non-viable microorganism DNA leading to false-positive detections is orders of magnitude lower than the concentration of organism that triggers detection of the positive growth by blood culture systems. In the case of *Proteus*, contaminating DNA causing false-positive detections was present at an equivalent

concentration of $1x10^4$-$1x10^5$ CFU/ml compared with concentrations of $1x10^9$ CFU/mL for true positive *Proteus* detections[7]. Importantly, detection of nucleic acid from nonviable *Proteus* in blood culture may be the tip of the proverbial iceberg, with false-positive detections noted for several distinct gastrointestinal molecular panels due to contaminating DNA in Cary-Blair transport media.

Potential laboratory strategies for dealing with false positive microorganism detection in blood cultures

Critically, reporting of false-positive detections can result in patients being prescribed inappropriate antimicrobial therapy. Furthermore, this can erode clinician confidence in laboratory testing. Thus, it is imperative that clinical laboratories be aware of these issues and take steps to reduce the risk of inadvertent reporting of false-positive detections.

It is expected that labs using amplification technology and broad panels will be the most affected by this issue: false detection with the BioFire BCID panel was responsible for the blood culture media recalls discussed above. Probe-based detected is expected to be superior at avoiding false detection of contaminating DNA. Of course contamination may not be limited to *Proteus* DNA, but logically a Gram-positive only panel, as with the Verigene and ePlex systems, will not inadvertently detect a false-positive Gram-negative like *Proteus*.

Positive blood cultures should always receive a Gram stain and it is imperative that laboratories always correlate direct identification assay results with the Gram stain result. Recent CAP checklist updates mandate "spot checks" to verify that culture results match direct detection results. This correlation between Gram stain and culture would not, for example, be possible to rule out false-positive organism detection with gastrointestinal panels performed on stool specimens.

Difficulties in the correlation between blood culture direct detection and stain results can arise when a Gram-negative organism is observed by Gram-stain but the only organism detection is *Proteus*. This has the potential for laboratories to incorrectly report a blood culture bottle positive for a Gram-negative bacterium not detected by the BCID panel (e.g. *Bacteroides*, *Stenotrophomonas maltophilia*, etc.) as positive for *Proteus*. The laboratory should carefully examine the Gram stain morphology of the organism prior to molecular assay result release if contamination is suspected.

One of our laboratories (SBW) has taken a conservative approach to this issue, choosing to never report a positive *Proteus* detected by the BCID panel (see Table 1). While the aforementioned reporting strategy may work when an issue is already known, it may not be effective when initially encountering a new false positivity issue. Laboratories should therefore always be suspicious for the potential of false positive results anytime multiple detections are present and exercise caution when reporting the presence of organisms beyond what is observed by Gram stain.

Laboratories may mitigate the risk of reporting inaccurate results from molecular blood culture tests by:

1) Ensuring the Gram stain matches the results from the molecular test
2) Always performing a sub-culture of the positive blood culture
3) Confirming that organism morphology matches the molecular test results, the next day when growth is visible on solid growth media
4) Review of past cultures from the patient (if present) to ensure consistency
5) Being aware of this issue, and reporting any suspected false-positive results to their manufacturer for investigations
6) All these elements should be a part of the quality system implemented by the laboratory as a part of the molecular test.

Long-term solution to false-positive organism detection

It is likely that assay manufacturers will take a multi-pronged approach to this issue. Firstly, future assay versions can be modified to increase the lower limit of detection for organisms that are known to be more frequent contaminants of blood culture media. This approach has been shown to be effective with the next generation of the FilmArray BCID Panel (BCID 2 Panel), with no false-positive detections noted with this panel in preliminary studies[7]. Secondly, assurance of nucleic acid-free blood culture media would be an obvious strategy to prevent this problem. Despite these measures, continued vigilance on the part of clinical microbiology laboratories will remain necessary to mitigate the risk of reporting of false-positive results.

2. False-positive and false-negative detection of antimicrobial resistance genes from positive blood culture bottles

Some blood culture identification assays include detection of antimicrobial resistance genes for both Gram-positive and Gram-negative organisms. Reports of false-negative results for the Cepheid Xpert MRSA/SA test for detecting methicillin-resistant *Staphylococcus aureus* from blood culture led the FDA in 2010 to issue a Class 1 device recall, a category designation reserved for situations most likely to result in negative health consequences or deaths in patients[8]. As described above, false-positive organism detections have been connected to blood culture media itself being contaminated during production with trace amounts of nucleic acid. In contrast, clinical and biological factors can result in false-positive or false-negative detection of antimicrobial resistance genes. For this reason it is essential that laboratories be vigilant in correlating detection of resistance markers with phenotypic susceptibility testing results. To date, the extent of false detection of antimicrobial resistance appears to be low. Reported and published cases most often involve one of the most commonly isolated antibiotic resistant bacteria: methicillin-resistant *Staphylococcus aureus* (MRSA).

Arguably the most common scenario of false-positive resistance detection comes from a mixed blood culture bottle positive for both methicillin-susceptible *S. aureus* (MSSA) and *mecA*-containing coagulase negative *Staphylococcus* spp. (CoNS), commonly *mecA*-positive

Staphylococcus epidermidis. To our knowledge, there are currently no published data on the frequency of this occurrence in blood cultures. In nares MRSA screening, co-colonization of MSSA and methicillin-resistant CoNS was shown to result in false positive MRSA screening reports on earlier testing platforms[9]. Helpfully, more current versions of the direct-from-blood-culture Cepheid Xpert MRSA/SA and the BD Max Staph SR tests, and the forthcoming FilmArray BCID2 panel include an additional target for detection of *mec* (*SSCmec-orfX*) right-extremity junction (MREJ). When positive, this indicates insertion of the staphylococcal cassette chromosome (which contains *mecA*) into the *S. aureus* genome. Panels that include specific CoNS targets, including *S. epidermidis*, could help clarify mixed MSSA and *mecA*-containing CoNS cultures[10].

Recently, a troubling phenomenon has been reported, so-called "stealth[13]MRSA." These are strains of *S. aureus* that harbor the *mecA* gene but that test susceptible to beta-lactams *in vitro*. Methicillin resistance in these strains arises only in the presence of subclinical concentrations of antibiotics. In these "dormant" MRSA or "oxacillin-susceptible MRSA" isolates, *mecA* is present, but mutations cause sequence instability leading to variable expression[13]. Thus, PBP2a may not be expressed in detectable amounts, and isolates will test as susceptible to oxacillin and cefoxitin. Recent work shows that induction of resistance can occur following exposure to antibiotics results from secondary mutations causing reversion and functional expression of *mecA*[11]. These isolates present a challenge to clinical laboratories because of the discordance between *mecA* detection and phenotypic susceptibility to beta-lactam antibiotics but more importantly, represent a scenario where the patient may fail beta-lactam therapy if the phenotypic test results are used for therapy selection.

Arguably most worrisome from a clinical standpoint is the possibility of false-negative reports of resistance. This can occur when tests are unable to detect known targets of resistance, for example *mecC,* known to be the resistance gene in some strains of MRSA[12]. False negatives can also arise due to alterations in targeted genetic elements. Recently this issue was seen with *S. aureus* isolates with genetic alterations tested using the Cepheid Xpert MRSA/SA BC test. Isolates with deletions or insertions in the *S. aureus* target (*spa*) or MREJ were not detected via this platform and isolates were reported as MSSA[11]. As a correction to this rare issue, reporting rules were updated: previously detection of *mecA, spa* and MREJ were all required to trigger a report of MRSA. With the updated reporting algorithm, *mecA* along with *spa* or the MREJ target, instead of all three, results in a report of MRSA[11]. Surveillance and investigation of discordant genotypic and phenotypic resistance results will be necessary to identify sequence variants not detected by current assays. Manufacturers will hopefully continue to update panels to detect such variants.

<u>Potential laboratory strategies for dealing with false-positive *mecA* detection</u>

The obvious risk to patients if a laboratory reports a resistant organism as susceptible certainly motivates a "better safe than sorry" approach in the detection of resistance determinants. Thus, the more rapidly the lab can confirm the phenotypic susceptibility results, the better.

One strategy could be to automatically drop a cefoxitin disk onto the agar subculture of every blood culture bottle with MRSA or *mecA/C* detected. Note that this approach is not considered a valid cefoxitin susceptibility test. Rather, it could be a screen to indicate a possible mixture of MSSA and methicillin-resistant CoNS present in the same culture. Additionally, in the case of stealth MRSA, some isolates show cefoxitin-induced expression of functional *mecA* and PBP2a. Thus, growth from the cefoxitin zone margin could be used for testing PBP2a, direct *mecA* PCR and additional susceptibility testing[14]. The performance characteristics of this approach for the detection of stealth MRSA from blood culture subcultures is currently not known but may be helpful in some instances.

When faced with apparent discrepancy in *mecA*-expression in oxacillin/cefoxitin-susceptible *S. aureus*, laboratories should confirm *S. aureus* identity, repeat susceptibility testing, and (if available) *mecA* specific PCR testing. For laboratories that refer susceptibility test results, when turn-around-times will already be prolonged, resolving discrepancies associated with a mixed infection at the reference laboratory will add increased time and cost. The sending laboratory could consider performing a PBP2a test on *S. aureus* growing from a culture set with MRSA detected by nucleic acid testing for blood culture sets that would not undergo susceptibility testing, since the bottles were not the first set positive.

Conclusions

False detections from blood cultures represent a substantial challenge for clinical laboratories. With respect to false detection of DNA from non-viable organisms, the ultimate and definitive solution would be screening of blood culture media components for the presence of contaminating microorganism DNA prior to media manufacture. It is currently unclear whether this will occur, and if it does occur, whether it will be associated with increased media cost to the consumer.

Detection of resistance determinants from other organism's DNA present within the same blood culture bottle, or missing detections due to genetic alterations, is problematic. Hopefully, these problems will be mitigated in the future with upgraded panels that detect more sequence variants.

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Table 1: Potential approach to BCID *Proteus* reporting

Gram Stain	BCID Detection	Result Reporting
Gram-negative Rod	*Enterobacteriaceae* and *Proteus,* KPC Negative	***"Indeterminate BCID results"***
Gram-negative Rod	*Enterobacteriaceae and Proteus, KPC* Positive	**Do NOT report. Consult for guidance.**
Gram-negative Rod	*Enterobacteriaceae and Proteus* plus one of the organisms below with or without KPC detected *E. coli* *Enterobacter cloacae* complex *Klebsiella oxytoca* *Klebsiella pneumoniae* *Serratia marcescens* *Acinetobacter baumannii* *Haemophilus influenza* *Pseudomonas aeruginosa*	Report the other organism (e.g. *E.coli*) and/or KPC marker result. **Do NOT report *Proteus* result.**
Gram-positive Cocci (Clusters, Pairs, etc.); ***Yeast;*** ***Gram-negative Diplococci;*** ***Gram-positive rods***	*Enterobacteriaceae* and *Proteus* detected plus target that is consistent with Gram Stain result	Report the target that is consistent with Gram Stain result **Do NOT report *Proteus* result.**
For any other scenario, or if unsure, consult with Lab Director.		