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Comparative Evaluation of Multiplex Real-Time PCR and Conventional Culture Methods for Rapid Identification of Bloodstream Infections and Antimicrobial Resistance Markers

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ABSTRACT

Background: Bloodstream infection needs early identification of the organism and appropriate choice of antimicrobials, while culture-dependent identification and susceptibility testing need further incubation.

Objective: To compare a targeted multiplex real-time polymerase chain reaction (PCR) workflow to routine culture for pathogen identification, selected resistance markers and laboratory turnaround following blood-culture positivity.

Methods: A paired-method diagnostic study was developed around 240 consecutive, signal-positive blood-culture episodes in hospitalized adults. The comparator was subculture identification and phenotypic susceptibility, while the seven-group organism assay and reflex amplification of *mecA*, *vanA/vanB*, *blaCTX-M* and *blaNDM/blaKPC* were used as tests. The diagnostic concordance, marker-phenotype agreement, and reporting times were assessed.

Results: 210/240 episodes had an on-panel bacterial target by culture and 203/240 had an on-panel bacterial target by PCR, and 2/30 reference-negative episodes had an on-panel bacterial target by PCR. Sensitivity was 96.7%, specificity 93.3%, and overall agreement 96.3% ($\kappa=0.84$). The time from bottle positivity to species/group identification was 2.3 ± 0.7 hours for PCR and 25.8 ± 5.4 hours for culture ($p<0.001$). The detection of *mecA* was 15/16 methicillin-resistant *Staphylococcus aureus* (MRSA) and *blaCTX-M* was 34/39 phenotypically extended-spectrum beta-lactamase-positive Enterobacterales (ESBL). There was no consistent correlation between genotypic and phenotypic resistance.

Conclusion: Multiplex real-time PCR can significantly reduce the time to preliminary reporting after notification of positive blood culture, but culture and complete susceptibility testing should be performed for off-panel organisms and mechanisms.

Keywords: bloodstream infection, multiplex real-time PCR, blood culture, antimicrobial resistance, molecular diagnostics, diagnostic accuracy.

INTRODUCTION

Bloodstream infections include a variety of clinical syndromes from transient bacteremia to septic shock. The importance of their prognosis is related to the susceptibility of the host, the source of the infection, the nature of the pathogen and the time until the onset of effective treatment. The ability to communicate with the laboratory quickly is therefore an integral

part of sepsis management and not just a technical goal [1]. The problem of antimicrobial resistance also makes empiric treatment more difficult, especially when multidrug-resistant Gram-negative bacteria and methicillin-resistant staphylococci are prevalent. The current global burden of resistance is a reminder of the need for early identification of susceptible and resistant organisms [2].

Conventional blood culture is still the backbone as it allows the recovery of viable organisms, allows for assessment of any unusual agents and allows for comprehensive antimicrobial susceptibility testing. However, Gram staining, subculture, identification of the colonies and susceptibility testing follow a positive instrument alarm. The processing schedule of these sequential steps often carries over into the next day or two, and interlaboratory variations in processing schedules add further delay [3]. Therefore, treatment with broad-spectrum antimicrobials can be continued until an organism identification is available.

Specific molecular assays can be used to amplify pathogen-specific sequences directly from the positive bottle and provide a preliminary result in a few hours. A randomized study showed that integration of stewardship advice, not just the test result, is relevant to translating rapid identification into prescribing decisions [4]. Multicenter testing has already demonstrated that multiplex identification is possible from blood cultures [5] and additional genes relevant to beta-lactam, glycopeptide and carbapenem resistance have been added to the panels [6]. However, molecular panels are limited by the selection of targets, low microbial concentration, mixed cultures, and the fact that the resistance genes do not always correspond to the expressed phenotype.

While there is a growing number of high performing commercial assays described, laboratories implementing a targeted real-time PCR workflow will still need to locally validate against their isolate spectrum and workflow. Systematic evidence also suggests that measurable clinical impact is related to the use of these test results [7]. The present study was thus designed to compare the organism-level diagnostic agreement, four classes of resistance markers and reporting intervals for a multiplex real-time PCR strategy carried out as soon as a blood-culture bottle was flagged as positive.

MATERIALS AND METHODS

Design the study and determine eligibility. Plan the study and establish eligibility.

A prospective paired comparison design was planned for a clinical microbiology laboratory of a tertiary care teaching hospital for 12 months. The first episode of blood culture that was positive per adult patient (age ≥ 18 years) was included in the analytic unit. All bottles that were positive on an automated blood-culture instrument were included, regardless of Gram-stain results. Bottles that were collected or labeled incorrectly, bottles that were repeated from the same episode, and bottles that did not have enough broth remaining for both methods were excluded. Consecutive sampling was chosen to minimize discretionary specimen selection and clinical data were abstracted in a de-identified case-report format.

Uncommon on-panel organisms and off-panel samples were represented by the target of 240 evaluable episodes. Assuming on-panel sensitivity of about 95%, the number of reference-positive specimens would be at least 150, which would give an approximate half-width of the confidence interval of 3.5 percentage points. This was the minimum that was planned for, but estimates for rare species and resistance genes were still likely to be imprecise.

Conventional laboratory workflow

A direct Gram stain was done immediately after a positive instrument alert, and subculture onto blood agar, chocolate agar and MacConkey agar as appropriate. The material suspected of being yeast was also cultured on fungal medium. Routine biochemical/automated identification and corroborative tests were used to characterize pure colonies, as needed. Susceptibility was determined by a validated automated MIC method with confirmatory disk diffusion or gradient diffusion, as appropriate; interpretations were based on the current CLSI standards adopted by the laboratory. Results and confirmatory tests for methicillin resistance, glycopeptide resistance, extended-spectrum beta-lactamase phenotype and carbapenem nonsusceptibility were examined. Culture adjudication was completed without the use of the index PCR result.

The workflow for multiplex real-time PCR.

200 μ L of mixed bottle broth was processed for nucleic-acid extraction within 1 hour of positivity alert with an internal amplification control and extraction-negative controls. Validated multiplex reactions for *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, coagulase-negative staphylococci (as a group), *Enterococcus* spp., *Pseudomonas aeruginosa* and the *Acinetobacter baumannii* complex were performed using fluorescent hydrolysis probes. Equivocal signals were followed by repeat testing and prespecified species/group targets and analytical cutoffs (cycle threshold ≤ 35 with acceptable control amplification) were applied consistently. A multiplex reaction was used to detect *mecA*, *vanA/vanB*, *blaCTX-M*, *blaNDM* and *blaKPC*. The marker targets were used to identify the susceptibility of a plausible corresponding cultured pathogen and not to claim the susceptibility of an unidentified pathogen.

The PCR technologist was not aware of the results of the culture other than the initial direct gram stain. The discordant results were reviewed for extraction controls, replicate amplification, organism purity and identification records. If the signal was for two pathogens in a specimen that was otherwise monomicrobial, it was not confirmed until other microbiological results were obtained. Reporting time was defined as the period between instrument positivity and validated organism or resistance-marker result, not between venepuncture and instrument positivity, to ensure that the comparison was of the post-positivity diagnostic interval.

Statistical analysis

Numbers were summarized as n (%), continuous measures were summarized as mean $\pm$ standard deviation, and diagnostic performance was summarized as two-sided 95% binomial confidence intervals. Operational comparators for target-level sensitivity, specificity, positive predictive value, and negative predictive value were culture recovery of an organism included in the assay. Cohen's κ measured binary agreement. Within-pair differences were examined and paired identification times compared by a paired-samples t-test or a Wilcoxon signed-rank test was prespecified. Only taxonomically appropriate isolates with a defined phenotypic comparator were used in the calculation of marker performance. The criteria for statistical significance was $p < 0.05$ and the analyses were considered exploratory and not a test of patient outcome.

RESULTS

A total of 240 distinct positive-alert episodes were eligible for comparison. Patients had a mean age of 57.4 ± 16.2 years and 142 (59.2%) were male. Intensive-care patients contributed 96 episodes (40.0%), reflecting an enriched population at risk of complicated infection. Culture established 210 organisms within the seven targeted categories; 20 specimens yielded organisms outside the PCR panel and 10 produced no confirmed viable isolate on subculture (Table 1).

Table 1. Clinical and microbiological characteristics of the paired blood-culture cohort (N=240)

Characteristic	Value
Mean age, years	57.4 $\pm$ 16.2
Male sex	142 (59.2%)
Intensive care / general ward / emergency	96 (40.0%) / 104 (43.3%) / 40 (16.7%)
Central vascular catheter present	94 (39.2%)
Antibiotics received before culture collection	121 (50.4%)
On-panel Gram-negative bacteria	120 (50.0%)
On-panel Gram-positive bacteria	90 (37.5%)
Off-panel culture isolates	20 (8.3%)
Positive instrument signal without confirmed growth	10 (4.2%)
Total episodes assessed	240 (100.0%)

Percentages are based on all 240 positive-alert episodes; the last four microbiological categories are mutually exclusive.

PCR correctly identified 203 of 210 on-panel isolates, giving a target-level sensitivity of 96.7% (203/210). Two apparent organism detections occurred among the 30 off-panel/no-growth specimens, yielding a specificity of 93.3% (28/30), positive predictive value of 99.0% (203/205), and negative predictive value of 80.0% (28/35). Binary agreement was 231/240 (96.3%; $\kappa = 0.84$). The seven false-negative results occurred across different groups without a dominant organism-specific failure pattern (Table 2).

Table 2. Conventional culture versus multiplex real-time PCR for on-panel organism identification

Culture-defined organism	Culture, n	PCR detected, n	Detection rate
<i>Escherichia coli</i>	60	59	98.3%
<i>Klebsiella pneumoniae</i>	40	39	97.5%
<i>Staphylococcus aureus</i>	40	39	97.5%
Coagulase-negative staphylococci	28	27	96.4%
<i>Enterococcus</i> spp.	22	21	95.5%
<i>Pseudomonas aeruginosa</i>	14	13	92.9%
<i>Acinetobacter baumannii</i> complex	6	5	83.3%
Total on-panel organisms	210	203	96.7%

The 30 culture-negative-for-panel episodes comprised 20 off-panel isolates and 10 specimens without reproducible growth. Of these, 2 gave an unconfirmed panel-positive PCR result.

The difference in diagnostic workflow was reflected in mean times of 2.3 ± 0.7 hours for valid PCR organism identification and 25.8 ± 5.4 hours for conventional culture identification after positivity (mean paired difference, 23.5 hours; $p < 0.001$). PCR marker reporting required 2.9 ± 0.9 hours, while complete phenotypic susceptibility was available after 49.6 ± 8.7 hours. These endpoints are not interchangeable: a negative gene panel does not exclude every clinically important resistance mechanism.

Within appropriate taxonomic strata, 15 of 16 methicillin-resistant *S. aureus* isolates carried detectable *mecA*; all five vancomycin-resistant enterococcal isolates carried *vanA* or *vanB*. Among Enterobacterales, *bla*CTX-M was identified in 34 of 39 isolates with an extended-spectrum beta-lactamase phenotype and in two of 61 without that phenotype; *bla*NDM or *bla*KPC was detected in 12 of 14 carbapenem-nonsusceptible isolates and none of 86 carbapenem-susceptible isolates (Table 3). Resistance-gene detection therefore gave timely mechanistic evidence but remained incomplete as a phenotypic resistance screen.

Table 3. Resistance gene signals in organism groups with applicable phenotypic comparators

Resistance marker	Phenotype-positive, n	Gene detected in positive, n (%)	Gene detected in phenotype-negative, n
<i>mecA</i> in <i>S. aureus</i>	16 / 40	15 (93.8%)	0 / 24
<i>vanA/vanB</i> in enterococci	5 / 22	5 (100.0%)	0 / 17
<i>bla</i> CTX-M in Enterobacterales	39 / 100	34 (87.2%)	2 / 61
<i>bla</i> NDM or <i>bla</i> KPC in Enterobacterales	14 / 100	12 (85.7%)	0 / 86

Resistance phenotypes are based on the relevant drug/class comparator. Enterobacterales strata overlap because ESBL and carbapenem nonsusceptibility can coexist. A negative gene finding does not establish phenotypic susceptibility.

DISCUSSION

The paired analysis indicates that multiplex real-time PCR could significantly shorten the time between automated blood-culture positivity and preliminary pathogen identification. The high overall sensitivity is due to the 7-group assay which covers the most common bloodstream organisms, and the 96.3% total agreement is due to the detection of organisms, as well as a relatively small but clinically relevant number of discordant cases. The outcome should not be interpreted as a 96.7% sensitivity for the diagnosis of bloodstream infection in all patients; it was tested in bottles that had already tested positive and the assay was tested against an organism panel, not against all causes of sepsis.

These observations are in line with the results of expanded multiplex blood-culture panels, which highlight the high performance for covered organisms, but a continued limitation due to organisms not included in the assay menu [8]. In rare cases, a negative result may be obtained even if the organism is growing, due to the molecular method. Likewise, the small *Acinetobacter* subgroup in the present material does not give sufficient precision for independent clinical validation. Unexpected pathogens, mixed-species signals, detailed characterization and epidemiologic investigation require routine culture.

The second contribution is the resistance-marker comparisons. There was a good phenotypic correlation between *mecA* and *vanA/vanB* in these very specific strata, which could be used in early antimicrobial review. However, for Enterobacterales, *bla*CTX-M does not detect all the extended-spectrum beta-lactamase (ESBL) genotypes and *bla*NDM/*bla*KPC is only a part of the families of carbapenemases; other mechanisms can also lead to reduced carbapenem susceptibility. The two *bla*CTX-M-positive isolates that were not an ESBL phenotype illustrate the fact that molecular detection and observed MIC categories represent different but related properties. Narrowing therapy should therefore be based on the isolate, full antibiogram (if available), the severity of the infection and local stewardship guidelines.

Earlier identification has been shown to be beneficial in terms of better opportunities for targeted therapy in published evaluations, although the extent of benefit to the patient will depend on the communication pathway between the laboratory and the prescriber [9]. Rapid identification has also been correlated with early optimization opportunities, particularly when alerts are sent to antimicrobial stewardship staff, in multicentre clinical observations [10]. A pre/post study implementation study found that more patients were receiving appropriate therapy at an earlier decision point after the introduction of BCID2 [11]. The shorter laboratory turnaround time seen here does not necessarily imply a decrease in mortality, length of stay or cost, as none of these were endpoints in these studies.

Preanalytical carryover, contamination, extraction efficiency, assay inhibition, and Ct thresholds need to be considered for practical deployment. The seven cases that were missed on the panel should be retested and audit of bottle broth handling should be conducted. On the other hand, a false positive target could be due to low level microbial DNA, contamination or

suppressed viability following antibiotic exposure. The 93.3% estimate for specificity is very uncertain because positive blood-culture signal without growth was included in the denominator. Economic modelling indicates that the implementation costs and stewardship capacity influenced by workflow will influence the value of rapid testing [12].

There is more published evidence to support separating the analytic accuracy from the clinical utility of a result. A diagnostic-accuracy meta-analysis of the BCID2 platform combined the data from nine studies including 2,005 blood cultures, and revealed high specificity for most of the pathogen targets, with the caveat that per-target precision is dependent on the number of positive blood cultures [13]. The seven group panel used in this assessment is not representative of a larger commercial panel and should not be interpreted as being as sensitive as a larger commercial panel. A later network meta-analysis of 88 studies and 25,682 encounters confirmed that blood culture alone was not associated with better outcomes than rapid diagnostics and antimicrobial stewardship, but that there was an equivalent survival benefit from rapid testing and antimicrobial stewardship [14]. These findings support pathways for testing implementation, rather than turnaround time of the instrument.

There's context to resistance detection, too. El Sherif et al. evaluated the commercial multiplex blood-culture identification panel versus VITEK-2 for central-line-associated infections, and showed the potential utility of an early report of both the organism and the resistance genes [15]. In another prospective study, Peri et al. found good agreement with traditional identification for covered pathogens and identified missed off-panel organisms and limitations in polymicrobial samples [16]. These reports should all be viewed in favor of a tiered laboratory policy: molecular reporting should clearly list the analytes being tested, explain the limitations of a negative resistance-marker result, and should not replace culture-based identification and full susceptibility testing.

The advantages of the design are the same-bottle paired testing, the use of a prespecified diagnostic comparator, the separation of the detection of the organism from the agreement between the organism and the gene, and the measurement of reporting time from a reproducible laboratory event. Some of the main limitations are the single-centre case mix, small numbers of individual pathogens, only positive-alarm bottles are included, there may be mixed-specimen contamination, sequencing may not be able to resolve all discrepant molecular calls, and there are no patient-level treatment outcomes. The endpoint timing also does not include the frequently long time interval between blood sampling and a culture bottle becoming positive, so the assay is actually an accelerated post-positivity identification strategy, rather than a culture independent early sepsis test.

CONCLUSION

A high level of concordance was found between multiplex real-time PCR and conventional organism identification, and there was a significant decrease in the time to preliminary reporting after a blood-culture positivity alert. The results of targeted *mecA*, *vanA/vanB*, *blaCTX-M* and *blaNDM/blaKPC* were useful for providing early resistance clues but were not a substitute for full phenotypic susceptibility testing. Prospective clinical evaluation should be integrated with Gram stain, subculture and antimicrobial stewardship.

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