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Multiplexed Molecular Diagnostics for Respiratory, Gastrointestinal, and Central Nervous System Infections

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The development and implementation of highly multiplexed molecular diagnostic tests have allowed clinical microbiology laboratories to more rapidly and sensitively detect a variety of pathogens directly in clinical specimens. Current US Food and Drug Administration–approved multiplex panels target multiple different organisms simultaneously and can identify the most common pathogens implicated in respiratory viral, gastrointestinal, or central nervous system infections. This review summarizes the test characteristics of available assays, highlights the advantages and limitations of multiplex technology for infectious diseases, and discusses potential utilization of these new tests in clinical practice.

Keywords. molecular diagnostics; respiratory; gastrointestinal; meningoencephalitis.

The ability to simultaneously detect and identify the most frequent causes of infectious diseases directly from clinical specimens is useful for patient care, hospital infection control practices, and epidemiologic studies. A variety of commercially available nucleic acid amplification platforms are capable of targeting multiple microorganisms in a single test reaction. This approach, known as multiplexing, is being increasingly applied for the diagnosis of a variety of different infectious diseases. The focus of this review is on the current US Food and Drug Administration (FDA)–cleared or approved multiplex polymerase chain reaction (PCR) panels designed to aid in the diagnosis of respiratory virus (RV), gastrointestinal (GI), and central nervous system (CNS) infections (Table 1).

Respiratory, GI, and CNS infections are similar in that the clinical signs and symptoms of these syndromes are often not pathogen specific and the infectious differential diagnosis is broad. Historically, making a microbiologic diagnosis required a combination of microscopy, antigen tests, culture, and pathogen-specific PCR assays. A multiplex molecular approach potentially simplifies testing algorithms and laboratory workflow, as well as increases the likelihood that an actionable diagnosis will not be missed. Multiplex assays significantly increase diagnostic yield (ie, number of organism detections), largely due to the fact that they target potential pathogens not routinely identified by traditional methods. In addition, multiplex PCRs are generally more sensitive than routine culture or antigen detection. The limitations of current multiplex tests are that they

no not detect all potential pathogens and that a negative panel result does not entirely exclude infection. Furthermore, panel-based testing cannot be tailored to the individual patient because current platforms offer few or no options for selecting which organism targets will be tested.

FDA-approved panels for microorganism detection vary in the organisms that they detect (Tables 2 and 3) and turnaround time (TAT) to results; all are considered to be of moderate or high complexity. Laboratories performing these tests must meet Clinical Laboratory Improvement Amendments quality standards, such as those for proficiency testing, quality control, and personnel requirements. Regulations for moderate vs high complexity testing differ only in the personnel requirements, which ultimately influence whether individual laboratories can perform multiplex testing and where the testing is performed (eg, in a rapid-response laboratory located near the patient vs a specialized molecular section in the main laboratory).

RESPIRATORY TRACT INFECTION

The first large multiplex PCR panel for infectious diseases targeted 12 respiratory viruses and was FDA approved in 2009. Since then, multiple other RV panels have been FDA approved (Table 2). These assays are all intended for use with nasopharyngeal (NP) swabs. Clinical laboratories may also validate other specimen types such as bronchoalveolar lavage fluid or nasal washes/aspirates to run off-label. Testing lower respiratory tract specimens is required to confirm whether or not a virus detected in an NP sample is also the cause of lower respiratory tract. Additionally, testing a lower tract specimen may be indicated despite a negative nasal result when the clinical suspicion for viral pneumonia is high.

The various commercially available RV panels have been compared to one another and to laboratory-developed monoplex PCRs. Significant sensitivity differences have been observed for

	Test System					
Characteristic	BD MAX	FilmArray	eSensor	Prodesse	Verigene	Luminex
Method	Real-time PCR	Nested PCR with melt curve analysis	PCR with electrochemical detection	Real-time PCR	PCR with low-density nucleotide array	PCR with liquid phase bead array
Degree of multiplexing	4 targets	14–22 targets	13 targets	3–4 targets	1–16 targets	9–20 targets
Panels	GI	Respiratory, GI CNS	Respiratory	Respiratory, GI	Respiratory, GI	Respiratory, GI CNS
Testing location	Clinical laboratory	Near patient facility or clinical Laboratory	Clinical laboratory	Clinical laboratory	Near patient facility or clinical laboratory	Clinical laboratory
Complexity	Moderate	Moderate	High	High	Moderate	High
automation	Full	Full	Partial	Partial	Full	Partial
throughput	Low-medium	Low-medium	Medium	Medium	Low	Medium-high
Time to results	~3 h	~1 h	~6 h	3–4 h	~2 h	~5–8 h

Abbreviations: CNS, central nervous system; GI, gastrointestinal; PCR, polymerase chain reaction.

adenovirus and influenza virus detection in particular [1–3]. A recent comparative study reported that the sensitivity of different influenza A targets ranged from 73% to 100% and influenza B from 46% to 100% across various platforms [1]. It is essential that laboratorians and clinicians understand the performance characteristics of all the members in a panel as well as recognize that the prevalence of individual pathogens in a given patient population will affect the predictive value of their test.

Pathogens	FilmArray	eSensor	Verigene	Luminex xTAG		
				RVP	RVP Fast	NxTAG
Viral						
Adenovirus	•	•	•	•	•	•
Coronavirus HKU1	•					•
Coronavirus NL63	•					•
Coronavirus 229E	•					•
Coronavirus OC43	•					•
Human bocavirus						•
Human metapneumovirus	•	•	•	•	•	•
Influenza A	•	•	•	•	•	•
Subtype H1	•	•	•	•	•	•
Subtype H3	•	•	•	•	•	•
Subtype 2009 H1N1	•	•				
Influenza B	•	•	•	•	•	•
Parainfluenza 1	•	•	•	•		•
Parainfluenza 2	•	•	•	•		•
Parainfluenza 3	•	•	•	•		•
Parainfluenza 4	•		•			•
Respiratory syncytial virus	•				•	•
Respiratory syncytial virus A		•	•	•		•
Respiratory syncytial virus B		•	•	•		•
Rhinovirus/enterovirus		•	•	•	•	•
Bacteria						
Chlamydia pneumoniae	•					•
Mycoplasma pneumoniae	•					•
Bordetella pertussis	•		•			
Bordetella parapertussis/Bordetella bronchiseptica			•			
Bordetella holmesii			•			

Abbreviation: RVP, respiratory viral panel.

Table 3. Comparisons of US Food and Drug Administration–Approved Gastrointestinal Panels

Pathogens	FilmArray	Verigene	Luminex	BDMax	Prodesse
Bacterial					
Campylobacter	•	•	•	•	•
Salmonella	•	•	•	•	•
Shigella	•	•	•	•	•
Shiga-like toxin 1 and 2	•	•[a]	•	•	•
Enterotoxigenic *Escherichia coli*	•		•		
Enteropathogenic *E. coli*	•				
Enteroaggregative *E. coli*	•				
E. coli O157	•		•		
Vibrio	•	•			
Yersinia enterocolitica	•	•			
Plesiomonas shigelloides	•				
Clostridium difficile	•		•		
Viral					
Norovirus GI and GII	•	•	•		
Adenovirus 40/41	•		•		
Rotavirus	•	•	•		
Astrovirus	•				
Sapovirus	•				
Parasitic					
Giardia	•		•	•	
Cryptosporidium	•		•	•	
Cyclospora cayetanensis	•				
Entamoeba histolytica	•		•	•	

[a] Verigene detects and reports each Shiga-like toxin gene separately.

The potential for false-positive results from PCR amplicon carryover contamination is a major concern for the high-complexity platforms that involve manipulation of amplified PCR products. Even with fully integrated, closed systems, there is potential to contaminate raw specimens with viruses shed by the respiratory tract of the operator. Laboratories must carefully follow operating procedures designed to minimize the contamination of specimens, work surfaces, and equipment. This can be a major challenge when testing is performed outside of a dedicated molecular diagnostics section. For near patient testing, consideration should be given to manipulating specimens in a biological safety cabinet that can be cleaned and kept separate from positive control material. At a minimum, wearing a mask or face shield should be considered for specimen processing. Laboratories should also monitor positivity rates of the individual targets as a way to quickly detect potential contamination problems.

Despite years of experience with RV panels, there are relatively few data on the clinical impact of multiplex testing. Previous RV diagnostic studies have shown that rapid direct fluorescent antibody staining from NP specimens can shorten the length of hospital stay, minimize ancillary testing, and reduce unnecessary antibiotic use for pediatric patients [4, 5]. Is there an advantage to more-sensitive molecular tests that detect a broader spectrum of potential pathogens? An inpatient pediatric cost analysis assessed the price of hospital admission, time in respiratory isolation, antibiotic usage, and diagnostic procedures to conclude that multiplex PCR testing was the least expensive test strategy as long as the prevalence of RV disease was ≥11% [6]. Multiplex panel testing may also be cost-effective for children with influenza-like illness evaluated in the emergency department [7].

In contrast to pediatric studies, most hospitalized adult patients with suspected lower respiratory tract infection receive antibiotics even when a virus is detected out of concern for bacterial coinfection [8]. This approach leads to the overuse of antibiotics and has potential for unnecessary drug toxicity. Comprehensive molecular testing for viral and bacterial pathogens from sputum significantly increases the likelihood of making an etiologic diagnosis in community-acquired pneumonia (CAP) [9, 10]. However, currently there are no FDA-approved molecular tests for "typical" bacterial CAP pathogens. An alternative strategy has been to combine RV multiplex testing with the results of cultures and urine pneumococcal and *Legionella* antigens. Going forward, combining RV panel results with serum biomarkers [11, 12] and/or measures of host immune response [13] may hold promise for ruling in or out viral infection.

A recent example of the utility of multiplex testing for epidemiology was the enterovirus D68 outbreak that occurred in the summer of 2014. Hospitals in Kansas City and Chicago notified the Centers for Disease Control and Prevention (CDC) due to

an increase in severe respiratory illness among children in those 2 cities [14]. Respiratory specimens initially tested positive for rhinovirus/enterovirus. Identifying these viruses as the etiologic agent of disease was possible because the clinical laboratories were using multiplex panels for routine patient testing.

GASTROINTESTINAL INFECTIONS

The use of multiplex molecular panels for the detection of GI pathogens has also been a hot area of commercial product development in recent years. Some GI assays are designed to detect subsets of parasites or bacteria only, whereas others combine bacterial and viral targets, or target broader combinations as syndromic panels (Table 3). The various platforms have different TATs, but even the most time-intensive assays (>4 hours) are significantly faster than stool culture (2–5 days). The rapidity of multiplex tests compared to that of conventional methods is one of the major advantages of molecular testing.

Available evidence suggests that the analytical performance of current GI panels is essentially equivalent. Analytical specificity has exceeded 98% for all targets with rare exceptions [15–19]. Sensitivity characteristics have been more variable, however, with values ranging from 90% to 100% [15–19]. Sensitivity differences may be due in part to the low prevalence of individual pathogens in some studies—a situation where a single missed organism detection can dramatically affect calculations of target test performance.

It is also important to recognize the lack of a robust diagnostic gold standard for use as the comparator test for multiplex panel studies. For example, multiple GI studies have observed a significant proportion of multiplex PCR *Campylobacter*–positive, culture and monoplex PCR- or DNA sequencing – negative stool specimens [15, 18–21]. An important consideration here is that *Campylobacter* culture is insensitive [21] and the analytic sensitivity of arbitrator PCRs and/or nucleic acid sequencing was poorly defined. It is likely that many of the multiplex *Campylobacter* DNA detections were true positives given the high specificity of these assays. Similar, though less dramatic, trends were seen with other targets across multiple platforms including *Salmonella*, *Shigella*/enteroinvasive *Escherichia coli*, and Shiga-like toxin–producing *E. coli*.

Clinicians typically formulate a discrete list of pathogens at the top of their differential diagnosis, and a multiplex panel may or may not detect those organisms. A potential challenge comes when a patient tests positive for *Clostridium difficile*, for example, simply because it is included in the panel but not necessarily because the organism is the causative agent of disease. With increasing incidence of community-acquired *C. difficile* infections, the identification of this target in the stool of a symptomatic outpatient may be difficult to interpret [22]. Children <1 year of age can be colonized with toxigenic *C. difficile*, but clinical disease in this age group is rare [23]. Laboratories that implement GI panel testing with *C. difficile* toxin A/B targets should consider how positive results will be reported for infants and children. Some laboratories may choose not to release these results or potentially use disclaimers in their reporting. Healthcare providers will also need to interpret the significance of other unexpected detections, such as for sapovirus and enteroaggregative or enteropathogenic *E. coli*. These are examples for organisms not routinely identified by clinical laboratories in the past.

Prolonged shedding of GI pathogens also presents a potential conundrum. Both *Salmonella* [24] and norovirus [25] can be excreted in the stool for weeks or months after symptoms have resolved, and children may be asymptomatically infected with *Cryptosporidium* [26] or *Giardia lamblia* [27]. Prolonged shedding events could complicate acute diagnosis when >1 enteropathogen is detected. Recent studies have shown that 27%–33% of GI panel–positive specimens contained >1 potential pathogen [19, 28], but the clinical implications of coinfection with specific pathogen combinations have not been defined. These nuances will force clinicians and laboratorians to evaluate panel results carefully in the context of the host and the season.

Unexpected organism detections also provide unique opportunities to expand our understanding of rare pathogens and for seasonal outbreaks. In the clinical trials of one of the most comprehensive GI panels, investigators in Iowa and Nebraska detected *Cyclospora cayetanensis* in multiple patient specimens well in advance of an outbreak being recognized by laboratories employing classical methodology [29].

Conventional microbiologic testing for the evaluation of diarrheal illness has historically been viewed as low cost. Thus, the threshold for ordering stool cultures, stool antigens, and/or ova and parasite examinations has been low despite limited clinical utility in the acute care setting [30, 31]. Given the difference in cost for newer rapid molecular tests vs traditional methods, institutions should not consider all cases of acute diarrhea as immediate candidates for molecular GI testing. In the outpatient setting, clinicians should think about what, if any, impact a positive or negative test result will have on patient care, especially when the illness may be self-limited. On the inpatient side, Goldenberg and colleagues calculated potential cost savings to the hospital through a reduction of unnecessary isolation practices, but with an increase in laboratory expenses vs conventional methods [32].

Although GI panels may replace traditional testing in certain situations, there is still a need to maintain culture capabilities for repeat testing if the molecular test is negative and clinical suspicion is high or to have an isolate for susceptibility testing. For example, routine susceptibility testing is indicated for typhoidal *Salmonella* and *Shigella* isolated from intestinal sources. Isolation and submission of cultured isolates from ill patients to public health laboratories (PHLs) for typing helps to identify clusters of disease caused by bacterial pathogens such as *E. coli* 0157, *Salmonella*, and *Listeria*. Working with the CDC,

the Association of Public Health Laboratories and the American Society for Microbiology have published interim recommendations in the new era of culture-independent tests [33]. These recommendations include contacting the PHL prior to implementing molecular testing and to continue to obtain isolates as much as possible. If clinical laboratories are unable to culture isolates, the original nucleic acid test–positive specimens should be submitted in transport medium within 24 hours.

MENINGITIS AND ENCEPHALITIS

In October 2015, the FDA cleared the first fully automated multiplex PCR panel for CNS pathogens. The FilmArray meningitis/encephalitis (ME) panel (BioFire Diagnostics) tests for 14 bacterial, viral, and yeast pathogens using a 200-µL sample of cerebrospinal fluid (CSF) in about an hour. The bacteria and yeast pathogens identified by the panel are listed in Table 4.

There have been 3 published reports evaluating the test performance of the ME panel [34–36]. The largest was a prospective study that enrolled 1560 residual CSF specimens and was designed to support the company's 510(k) submission to the FDA [35]. Culture was used as the diagnostic gold standard for bacterial target comparisons, and monoplex PCR assays followed by bidirectional sequencing were used for the viruses and yeast. Discrepancies across methods were resolved by repeat molecular testing (when possible) combined with a blinded review of available medical records. After adjudication of discordant results, there was 84.4% positive and >99.9% negative agreement between the ME panel and conventional methods [35].

Several important messages are evident in the report by Leber and colleagues [35]. First, there were relatively few cases of bacterial or cryptococcal meningitis, which precluded statistically significant assessments of these targets. Previous reports observed that the *Cryptococcus* targets were less sensitive than antigen testing [34], and the ME panel had a limit of detection on the order of 100 colony-forming units/mL [36]. Next, there were as many false-positive or unconfirmed ME panel results (n = 22) as there were additional confirmed detections (n = 21) made with the comprehensive panel. The authors hypothesized that contamination could have played a role in the false-positive ME panel results, either from the positive control material or from the normal flora of the test operators. The ME test system is closed (ie, the likelihood of amplicon carryover should be low); but like the RV panels, environmental contamination is still possible and laboratories must work diligently to prevent and monitor for this. It is also important to note that study subjects did not necessarily have a high pretest probability for infectious meningoencephalitis, which would have also reduced the pretest probability for disease in some cases. Last, the detection of latent or reactivated Herpesviridae was not uncommon, and the significance of these identifications must be evaluated in the context of the patient.

Available data suggest that the ME panel test performance is acceptable for patient care but that the test cannot replace current routine testing. The CSF Gram stain remains critical for interpreting PCR results, and bacterial culture is required to detect organisms not covered by the panel as well as to have an isolate for susceptibility testing. Furthermore, cryptococcal antigen with fungal culture remains the preferred method for diagnosing cryptococcal meningitis. Because the panel does not detect all potential pathogens and false-negative results are possible, patients with a very high clinical suspicion for bacterial meningitis or herpes encephalitis should still receive empiric therapy even when the ME panel is negative.

DISCUSSION

The potential advantages and disadvantages of large multiplex panels for infectious diseases are summarized in Table 5. These assays have substantially increased our ability to detect potential pathogens in a variety of different clinical specimens. Furthermore, moderate-complexity platforms have enabled many more clinical laboratories to perform molecular diagnostic testing in-house, which substantially improves TAT relative to conventional methods. Multiplex molecular capabilities also come with several important caveats. Just because a new test is FDA approved does not necessarily mean it is the right test for all patients. Multiplex reagents and instrumentation is expensive and the pretest probability for infection differs significantly according to patient age, host immunocompetence, time of year, and

Table 4. The FilmArray Meningitis/Encephalitis Panel

Bacteria	
• *Escherichia coli* K1	• *Neisseria meningitidis*
• *Haemophilus influenzae*	• *Streptococcus agalactiae*
• *Listeria monocytogenes*	• *Streptococcus pneumoniae*
Viruses	
• Cytomegalovirus	• Human herpesvirus 6
• Enterovirus	• Human parechovirus
• Herpes simplex virus 1	• Varicella zoster virus
• Herpes simplex virus 2	
Yeast[a]	
• *Cryptococcus neoformans*	• *Cryptococcus gattii*

[a] The assay does not differentiate *C. neoformans* from *C. gattii*.

Table 5. Potential Advantages and Limitations of Large Multiplex Panels

Pros	Cons
• Convenience	• Cost
• Rapid turnaround time to results	• Not tailored to the individual patient
• Guide treatment	• Nucleic acid detection ≠ viable organism
• Impact isolation practices	• Detects asymptomatic carriers, prolonged shedding, or latent/reactivated viruses
• Patient satisfaction	• May still need culture, additional PCRs, antigens, and/or stool O&P
• Identify outbreaks	• Potential for contamination and false-positive results
• Epidemiologic studies	

Abbreviations: O&P, ova and parasite; PCR, polymerase chain reaction.

geographic region. In the absence of cost-effectiveness data, we recommend the following targeted approach to panel testing:

1. RV panels have the highest potential impact for immunocompromised patients, critically ill patients, and hospitalized children.

2. GI panels should primarily be used in cases of dysentery, for moderate or severe disease, for symptoms lasting more than a week [37], and for the immunocompromised host with community-onset symptoms.

3. The ME panel targets pathogens that are the most problematic for immunocompromised patients. Testing may also speed time to diagnosis when the clinical suspicion for bacterial meningitis is high or when the patient has already received antibiotics. Outside of these selected scenarios, laboratory consultation with acceptance criteria based in part on elevated CSF cell counts should be considered for immunocompetent adults as a way to limit unnecessary testing [38].

CONCLUSIONS

Highly multiplexed molecular diagnostics are powerful tools for patient care, epidemiologic studies, and, potentially, infection control. It is highly likely that panel testing will be increasingly used in clinical microbiology in the future. However, these assays cannot replace culture, and additional testing for organisms not included in the panel will be required in some cases. Isolation of microorganisms will still be required to perform phenotypic antimicrobial susceptibility testing and to provide isolates for public health investigations.

The optimal use of large panels for infectious diseases has not been established. Clinical utilization and outcome studies are ultimately required to determine whether a syndromic diagnostic approach is cost-effective. Ideally, randomized trials should be conducted to determine the potential impact of rapid and comprehensive molecular diagnostic tests. Outcomes of interest include antimicrobial use, time to optimal therapy, length of hospital or emergency department stay, mortality, and costs. However, prospective studies may not be feasible due to prohibitive costs or time required to accrue enough patients with a rare disease (eg, meningoencephalitis). Implementation research and cost-effectiveness decision analyses could also provide valuable information to help guide the rational use of these technologies. In the meantime, it is essential that local implementation of panel testing be done in partnership with clinicians to assure that there is a clear understanding of test characteristics, results interpretation, and appropriate utilization. Consultation with the clinical laboratory is also useful to help interpret unexpected or confusing results.

Notes

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