



# Impacts and Challenges of Advanced Diagnostic Assays for Transplant Infectious Diseases

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## Introduction

The administration of immunosuppressive therapy to prevent rejection of allografts and graft-versus-host disease, although necessary, renders transplant recipients susceptible to opportunistic infections [1, 2]. As a group, transplant patients present a real challenge for initial diagnosis of infection partly due to lowered or absent markers of inflammation [2]. Traditionally, these infections have been diagnosed using bacterial, fungal, and viral culture as well as a variety of immunological assays. These methods still remain the standard of care for diagnosis of most infections. However, a series of advanced detection techniques led by nucleic acid amplification have now become prominent in most clinical microbiology laboratories, and novel proteomic assays are

currently being added in the list of diagnostic tools available for infectious pathogen detection and identification.

The goal of this chapter is to review traditional and molecular methods used for the diagnosis of infectious diseases in transplant patients and discuss novel methodologies currently in development and their potential impact on clinical decisions.

## Traditional Diagnostic Assays

### Culture-Based Assays

Culture remains the gold standard for the diagnosis of most infectious diseases including those caused by bacteria, mycobacteria, and fungi [3, 4]. One of the advantages of culture is that it does not require a priori knowledge of the specific pathogen responsible for the infection as it casts a wide search net by using multiple growth media and incubation conditions. For example, diarrhea and vomiting are common symptoms in transplant patients, and determining the infectious cause for those symptoms can be challenging due to confounding factors such as intestinal graft-versus-host diseases (GVHD) in hematopoietic stem cell transplantation (HSCT) recipients, neutropenic enterocolitis, and immunosuppressive drugs [5]. A request for a bacterial stool culture will allow detection of the most common cause of bacterial gastroenteritis, namely, *Salmonella* species, *Shigella* species, *Campylobacter* species, and *E. coli* O157 with a recovery rate ranging from 0.2% to 2.4% [3, 6–8]. In addition, any other bacterial organisms growing on the culture media, with potential for causing gastrointestinal symptoms, will be detected and reported. However, culture of certain organisms, including *C. difficile*, the most common cause of bacterial diarrhea in hospitalized patients, requires special culture media and setup and has been replaced in most part by non-culture-based methods. Similarly, viral causes of diarrhea including cytomegalovirus (CMV), norovirus, adenovirus, rotavirus, and other small round viruses are better detected

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by nucleic acid amplification tests (NAAT) and antigen tests [9–11].

The diagnostic yield of culture for various specimen types remains low. In one retrospective study, the yield of bronchoalveolar lavage (BAL) fluid culture for diagnosis of pneumonia in bone marrow transplant patients was reported at 2.2%, 3.0%, and 16.4% for bacteria, fungi, and viruses, respectively [4]. In contrast, the use of PCR tests and antigen testing increased the overall diagnostic yield to 22%, although the increased detection of CMV by PCR was not deemed clinically significant for all cases. The diagnostic yield of BAL in other studies was higher with one study reporting rates of 31%, 12.7%, and 23.6% for detection of bacteria, fungi, and viruses, respectively, in cancer patients [12]. For solid organ transplants (SOT), the overall recovery of pathogens by culture can be higher, up to 58.9% in lung transplants [13–15].

Blood culture is important for diagnosis of bacteremia and fungemia [16]. Unfortunately, the yield of blood culture remains low for both conditions [17–20]. Current blood culture systems are automated and set up to continuously monitor blood culture for the detection of microorganisms. An exception to continuous monitoring is the use of the isolator tube system, a manual lysis-centrifugation system (Wampole Laboratories, Cranbury, NJ). Performance of the isolator tube has been evaluated extensively against other automated blood culture systems designed to improve recovery of fungi such as the MYCO/F Lytic bottle (BD Diagnostics, Sparks, MD) with some studies showing increased recovery of *H. capsulatum* and *C. neoformans* [21, 22], while other concludes that the two systems performed equally well [23, 24]. A study by Creger et al. retrospectively analyzed the performance of the isolator tube system specifically in a cancer population and did not observe an advantage over conventional blood culture methods [25]. Even with the use of the isolator tube, the detection of fungi in blood culture is low, and in biopsy-proven candidiasis, only 50% of patients had a positive blood culture [26].

In transplant patients, the yield of blood culture varies with the type of transplant and the degree of immunosuppression. In lung transplant patients, the yield of blood culture can be as high as 25% with *S. aureus*, *P. aeruginosa*, and *Candida* species being the most common isolates recovered [27, 28], while in HSCT, the yield varies greatly from 4.9% to 8.7% and is dominated by Gram-positive bacteria [29–32].

The majority of fungal isolates from blood culture are *Candida* species, with *C. albicans* being the most common species isolated [18]. Although rare, other non-*Candida* yeasts including *Trichosporon* species, *Rhodotorula* species, and *Saccharomyces cerevisiae* are being recovered with increased frequency from blood cultures of immunocompromised patients [33, 34]. Fungemia caused by molds, including *Aspergillus* species, is rarely detected by blood culture

[35–37]. In patients with indwelling devices, molds such as *Fusarium*, *Paecilomyces*, *Scedosporium*, and *Wangiella* have been recovered from blood culture [38–40].

A recent study by Limmathurotsakul et al. highlighted the limitation of culture as a diagnostic tool and an imperfect gold standard [41]. The authors applied Bayesian latent class models (LCM) to establish the true sensitivity of culture and the true specificity of four serological tests for detection of pathogens using *Burkholderia pseudomallei* and melioidosis as a model system. Using Bayesian LCM with either conditional independence (i.e., no single test considered gold standard and no correlation among tests) or conditional dependence (i.e., correlation among all tests), the sensitivity of culture was estimated to be 61% with a negative predictive value of 62.1% [41]. The specificity and positive predictive value of the four serological tests increased significantly using both Bayesian LCM models, emphasizing the limitation of using the culture as an imperfect gold standard.

Recent studies defining microbial populations of various organs using deep sequencing and high-density sequencing methods have now revealed the complexity of microbial organisms, many of them non-culturable, present in various tissues and the difference in composition for transplants versus healthy patients [42, 43]. The significance of detecting these non-culturable organisms for infectious diseases management remains to be established.

## Antigens and Antibody Assays

Depending on the degree and type of immunosuppression, transplant patients may not be able to mount a sufficient antibody response to pathogens limiting the use of serological assays to detect antibodies [2]. On the other hand, antigen testing can be beneficial, especially for fungal infections where results of these tests are used as one of the mycological criteria to define invasive fungal disease (IFD) [44]. Some of the most commonly used antigen tests include the galactomannan (GM) antigen produced by members of the *Aspergillus* family and the (1,3)  $\beta$ -D (BD) glucans, present in the cell wall of *Aspergillus* and a variety of other molds and yeasts [45].

The serum GM assay (Platelia *Aspergillus* EIA, Bio-Rad Laboratories) was approved by the United States Food and Drug Administration (FDA) in 2003. The assay is an enzyme immunoassay that uses rat monoclonal antibody EBA-2 to detect circulating GM antigen in serum. The GM assay has been evaluated extensively in various patient populations with sensitivity ranging from 30% to 100% and specificity ranging from 38% to 98% in serum with greater utility in HSCT patients than in SOT recipients [46]. In a study by Jathavedam and colleagues, the GM assay was shown to have limited utility within the first 100 days after auto-SCT

and therefore not useful for patient management decision [47]. In another study conducted in patients with hematologic malignancies, the sensitivity of the GM assay was 49% for invasive fungal infections caused by *Aspergillus* species other than *A. fumigatus* and only 13% for IFD caused by *A. fumigatus* [48]. Results of these various studies suggested that the performance of the GM assay depends on several factors including the infecting species of *Aspergillus*, the type of transplant populations, the frequency of testing, and the duration of antifungal therapy [45, 49]. The sensitivity of the GM assay in BAL of HSCT recipients and patients with hematological malignancies is higher than that reported for serum and ranges between 88% and 100% using the same optical density cutoff value used for serum [45, 50, 51]. In solid-organ transplant patients, the sensitivity and specificity of the GM assay in BAL ranged from 60% to 100% and 84–98%, respectively, depending on the optical density cutoff value used [50, 52–56]. Thus, the GM assay in BAL is a useful additional test for diagnosing IFD. False-positive results were observed in patients receiving piperacillin, amoxicillin, or ticarcillin with or without a beta-lactamase inhibitor, in patients being administered electrolyte replacement fluids (i.e., PlasmaLyte), and in patients infected with molds other than *Aspergillus* for a low specificity and positive predictive value [45, 57]. However, a study by Vergidis et al. showed that the current formulation of piperacillin-tazobactam do not appear to be contaminated with galactomannan [58].

Four assays, the Fungitell (Associates of Cape Cod Inc., East Falmouth, MA, cutoff, 60–80 pg/mL), the Fungitec-G (Seikagaku, Tokyo, Japan, cutoff, 20 pg/mL), the Wako (Wako Pure Chemical Industries, Tokyo, Japan, cutoff, 11 pg/mL), and Maruha (Maruha-Nichiro Foods, Tokyo, Japan, cutoff, 11 pg/mL) are commercially available for the detection of (1,3)- $\beta$ -D (BD) glucans, a cell wall antigen found in most fungal species cell wall excluding *Mucormycetes* and *Cryptococcus* species [59]. A recent meta-analysis review of studies conducted in adult hemato-oncology patients showed similar performance for all four assays in the diagnosis of IFD, a higher diagnostic yield for performance of two consecutive tests, and an overall low sensitivity (52%) and high specificity (99%) for proven or probable IFD [59]. In another meta-analysis study, which included reports with various patient populations, the sensitivity and specificity of the BD glucans test were 77% and 85%, respectively [60]. Both studies concluded that the BD glucans assay was a useful adjunct test, especially for diagnosis of IFD due to *Candida* and *Aspergillus*. However, a recent report of high-false positive in patients with hematologic malignancies puts in question the value of this test as a stand-alone test for diagnosis of IFD [61].

The sensitivity of BD glucans is highest (90–100%) for the diagnosis of *Pneumocystis jirovecii* pneumonia (PCP), although its specificity in non-HIV immunocompromised

patients varies widely (42–98%); therefore, results of the test taken alone are not conclusive for making a diagnosis of PCP [62, 63]. However, studies have shown that serum BD glucan levels correlate well with *P. jirovecii* fungal load in BAL as determined by *Pneumocystis* PCR, supporting the use of the assay to monitor response to therapy [62, 63].

Other useful antigens tests used for diagnosis of fungal infections include the latex agglutination cryptococcal antigen, which has higher sensitivity for central nervous system infection than disseminated disease, and the urine and serum antigen for endemic mycoses (*Blastomyces dermatitidis* and *Histoplasma capsulatum* antigens), although some cross-reaction occurs among targets [64].

The diagnosis of viral infections has been replaced in most instances by nucleic acid-based tests. Antigens testing and serological assays by methods such as direct fluorescent antibody (DFA) staining and enzyme immunoassays (EIA) do still play a part in the diagnosis of certain infections including diagnosis of acute or chronic hepatitis, infectious mononucleosis, and HTLV-1-/HTLV-2-associated T-cell leukemia [65]. One of the most common viral antigens tested in transplant patients is the CMV pp65 antigen for monitoring of viral loads [66]. The reported sensitivity and specificity of the CMV antigenemia test varies greatly due to lack of standardization in protocols including specimen processing, monoclonal antibody used, slide processing, and quantification [67]. Advantages of the antigenemia assay include its low cost in terms of reagents and equipment, but due to its disadvantages including the need for rapid specimen processing, the labor-intensive nature of the assay, and the subjectivity in reading of the slides, the antigenemia test has been replaced in many institutions by molecular tests for monitoring of CMV viral loads [67–69].

Bacterial antigen tests of importance for transplant patients include the urinary antigen tests for *Legionella pneumophila* serotype 1 (Binax, Scarborough, Maine, USA) and *Streptococcus pneumoniae* (Binax, Scarborough, Maine, USA), which are rapid, noninvasive tests useful in the diagnosis of both community- and hospital-acquired pneumonia [70, 71].

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## New Generation Diagnostic Assays

Although the use of culture and serological assays provides important information, their shortcomings created a need to develop faster and more sensitive assays. The following sections will cover the more rapid methods currently in use in most laboratories for diagnosis of infection and the newer methods being developed and conclude with the impact of these methods on the diagnosis and management of transplant patients.

## Genomic Assays

The first published polymerase chain reaction (PCR) report described the amplification of specific target sequences of the  $\beta$ -globin gene for diagnosis of sickle cell anemia [72, 73]. Several modifications and improvements have occurred since that first report, ultimately resulting in the transfer of PCR from research laboratories to clinical diagnostic laboratories [74, 75]. Alternative nucleic acid amplification formats have since

been developed including ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), branched DNA (b-DNA) signal amplification, strand displacement amplification (SDA), helicase-dependent amplification (HDA), and loop-mediated amplification (LAMP) [76, 77]. Numerous commercial molecular assays have been approved by DFA for diagnosis of microbial infections in transplant patients (Table 47.1).

The development of real-time PCR, combining rapid thermal cycling and real-time monitoring of amplification

**Table 47.1** List of US FDA-cleared commercial molecular tests

Manufacturer	Test name	Targets	Complexity level
BD diagnostics	BD MAX MRSA assay	MDRO surveillance	√
	BD GeneOhm MRSA ACP assay	MDRO surveillance	√
	BD GeneOhm StaphSR assay	Bacteremia	√
bioMérieux	BioFire FilmArray blood culture identification panel	Bacteremia	√
	NucleiSENS EasyQ MRSA assay	MDRO surveillance	√
Cepheid	Xpert MRSA	MDRO surveillance	√
	Xpert SA nasal complete	MDRO surveillance	√
	Xpert MRSA/SA SSTI	Skin and soft tissue infections	√
	Xpert MRSA/SA BC	Bacteremia	√
Nanosphere, Inc.	Verigene gram-positive blood culture test	Bacteremia	√
Roche molecular diagnostics	LightCycler MRSA advanced test	MDRO surveillance	√
AdvanDx, Inc.	<i>E. faecalis</i> /OE PNA FISH	Bacteremia	
	<i>E. faecalis</i> PNA FISH	Bacteremia	
BD diagnostics	BD GeneOhm VanR assay	MDRO surveillance	√
bioMérieux/BioFire	BioFire FilmArray blood culture identification panel	Bacteremia	√
Cepheid	Xpert vanA	MDRO surveillance	√
Intelligent medical devices, Inc.	IMDx VanR for Abbott m200	MDRO surveillance	√
Nanosphere, Inc.	Verigene gram-positive blood culture test	Bacteremia	√
BD diagnostics	BD MAX <i>C. diff</i> assay	<i>C. difficile</i> infection	
	BD GeneOhm <i>C. diff</i> assay	<i>C. difficile</i> infection	
bioMérieux/BioFire	FilmArray gastrointestinal panel	Gastrointestinal tract infection	
Cepheid	Xpert <i>C. difficile</i>	<i>C. difficile</i> infection	
	Xpert <i>C. difficile</i> /epi	<i>C. difficile</i> infection	
Focus diagnostics, Inc.	Simplexa <i>C. difficile</i> universal direct assay	<i>C. difficile</i> infection	
Great Basin scientific, Inc.	Portrait Toxigenic <i>C. difficile</i> assay	<i>C. difficile</i> infection	
Intelligent medical devices, Inc.	IMDx <i>C. difficile</i> for Abbott m200	<i>C. difficile</i> infection	
Luminex molecular diagnostics, Inc.	xTAG gastrointestinal pathogen panel (GPP)	Gastrointestinal tract infection	
Meridian biosciences, Inc.	Illumigene <i>C. difficile</i> DNA amplification	<i>C. difficile</i> infection	
Nanosphere, Inc.	Verigene <i>C. difficile</i> test	<i>C. difficile</i> infection	
PrimeraDx	ICEPlex <i>C. difficile</i> kit	<i>C. difficile</i> infection	
Prodesse, Inc.	ProGastro Cd assay	<i>C. difficile</i> infection	
Quidel Corp.	Quidel molecular Direct <i>C. difficile</i> assay	<i>C. difficile</i> infection	
AdvanDx, Inc.	GNR traffic light PNA FISH	Bacteremia	√
	<i>E. coli</i> / <i>P. aeruginosa</i> PNA FISH	Bacteremia	
	EK/ <i>P. aeruginosa</i> PNA FISH	Bacteremia	
	<i>E. coli</i> PNA FISH	Bacteremia	
bioMérieux	BioFire FilmArray blood culture identification panel	Bacteremia	√
Nanosphere, Inc.	Verigene gram-negative blood culture test	Bacteremia	√
AdvanDx, Inc.	<i>C. albicans</i> PNA FISH	Bacteremia	√
	<i>C. albicans</i> / <i>C. glabrata</i> PNA FISH	Bacteremia	

**Table 47.1** (continued)

Manufacturer	Test name	Targets	Complexity level
	Yeast traffic light PNA FISH	Bacteremia	
bioMérieux	BioFire FilmArray blood culture identification panel	Bacteremia	
Alere Scarborough, Inc.	Alere I influenza A and B	Pneumonia	
bioMérieux/BioFire	FilmArray respiratory panel	Pneumonia	
Cepheid	Xpert flu/RSV	Pneumonia	√
	Xpert flu	Pneumonia	
Focus diagnostics, Inc.	Simplexa flu A/B & RSV	Pneumonia	
	Simplexa influenza A H1N1	Pneumonia	
GenMark diagnostics, Inc.	eSensor respiratory viral panel	Pneumonia	
Intelligent medical devices, Inc.	IMDx flu A/B and RSV for Abbott m200	Pneumonia	
IQuum/Roche molecular Inc.	Liat influenza A/B assay	Pneumonia	
Luminex molecular diagnostics, Inc.	xTAG respiratory viral panel (RVP)	Pneumonia	
	xTAG respiratory viral panel FAST (RVP FAST)	Pneumonia	
Meridian biosciences, Inc.	Illumigene mycoplasma DNA amplification	Pneumonia	
Nanosphere, Inc.	Verigene respiratory virus + test	Pneumonia	
	Verigene respiratory pathogens flex nucleic acid test (RP flex)	Pneumonia	
Prodesse, Inc.	Pro hMPV assay	Pneumonia	
	ProFAST assay	Pneumonia	
	ProParaflu assay	Pneumonia	
	ProFlu+ assay	Pneumonia	√
QIAGEN GmbH	Artus Infl A/B RG RT-PCR kit	Pneumonia	
Quidel Corp.	Quidel molecular RSV + hMPV assay	Pneumonia	
	Quidel molecular hMPV assay	Pneumonia	
	Quidel molecular influenza A + B assay	Pneumonia	
BD diagnostics	BD MAX enteric parasite panel		
BD diagnostics	BD MAX enteric bacterial panel		
bioMérieux	BioFire FilmArray gastrointestinal panel	Gastrointestinal tract infection	
Cepheid	Xpert norovirus	Gastrointestinal tract infection	
Luminex molecular diagnostics, Inc.	xTAG gastrointestinal pathogen panel (GPP)	Gastrointestinal tract infection	
Luminex molecular diagnostics, Inc.	xTAG gastrointestinal pathogen panel (GPP)	Gastrointestinal tract infection	
Prodesse, Inc.	ProGastro SSCS assay	Gastrointestinal tract infection	
Prodesse, Inc.	ProAdeno+ assay	Gastrointestinal tract infection	
Nanosphere, Inc.	Verigene enteric test	Gastrointestinal tract infection	

products [78, 79], completely revolutionized the practice of clinical microbiology [80, 81]. Today, real-time nucleic acid amplification methods are mainstream in most sections of clinical microbiology, and their impact on care of transplant patients is significant.

Several real-time PCR laboratory-developed tests (LDT) as well as a few FDA-approved assays are used for the diagnosis of bacterial infections in transplant patients. Bacteria targeted for assays development have traditionally been those related to nosocomial infections. For example, until recently, most PCR assays for detection of *C. difficile* were LDT assays

that resulted in an increased sensitivity and turnaround time for results [82–85]. At the time of this chapter preparation, the FDA had cleared over eight molecular assays for the diagnosis of *Clostridium difficile* infection (CDI). Similarly, rapid molecular assays have been developed for a variety of bacterial targets including difficult-to-culture or slow-growing organisms (i.e., *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, *Borrelia burgdorferi*), targeted diagnosis (i.e., group A *Streptococcus* in throat swabs), and nosocomial pathogens (methicillin-resistant *S. aureus*, methicillin-sensitive *S. aureus*, and vancomycin-resistant *Enterococci*) [80].

The turnaround time and identification of the most common *Mycobacteria* species were greatly improved with the introduction of nucleic acid hybridization probes in the laboratory. Nucleic acid hybridization probes are single-stranded or double-stranded DNA/RNA fragments complementary to a sequence in the target organisms and most commonly labeled with a fluorescent or chemiluminescent marker for detection [86]. Probes for same-day identification of *M. tuberculosis* complex, *M. kansasii*, *M. avium* complex, and *M. goodii* from either solid or liquid cultures have been commercially available since the early 1990s (Gen-Probes, San Diego, CA). These probes show excellent sensitivity and specificity, although cross-reaction has been reported between *M. tuberculosis* complex and *M. terrae* [87–90]. Similar probes are available for identification of medically important filament fungal species.

The current trend for molecular diagnosis is a move toward syndromic, highly multiplexed real-time PCR assays and newer technologies including various solid and liquid microarray formats. Currently, FDA-cleared molecular syndromic panels are available for the diagnosis of respiratory tract infections, bloodstream infections, gastrointestinal infections, and meningitis/encephalitis (Table 47.2). These panels differ on the numbers of pathogens they can detect (5–27 targets), the type of pathogens included (e.g., bacteria, viruses, or yeasts), the level of complexity (low versus high), and the turnaround time to results (from 1 h to 12 h). Performance characteristics, however, are comparable with sensitivity and specificity greater than 90% when compared to culture or bi-directional sequencing as the gold standard [91].

Other multiplexed bacterial assays are available outside of the United States, for example, the LightCycler SeptiFast (Roche Diagnostics GmbH, Wien/Austria), a multiplexed real-time PCR-based assay that can detect bacteria and yeasts directly from whole blood. An agreement of up to 83% between SeptiFast and blood culture results has been reported with the overall conclusion that in its cur-

rent form, the assay can be used to supplement rather than replace blood culture methods [92–94]. The SeptiFast assay has been shown to be especially useful in providing additional information for immunocompromised patients including liver transplants, septic ICU patients, and neutropenic patients, for fungal infection and in cases of prior antibiotic administration [93–96].

Other potential molecular methods have been developed and evaluated for the diagnosis of bacterial infections including sequencing [97], quantitative loop-mediated isothermal amplification [98], PCR hybridization [99], and mass spectrometry [100].

As described in the previous section, the diagnosis of IFD currently relies on microscopic examination, recovery of molds or yeasts in culture, detection of fungal antigens including galactomannan and BD glucans, and various radiological findings of pulmonary infiltrates [45, 101]. Although useful, these methods can lack specificity, be time-consuming, or result in inconclusive findings. A study by Lin et al. [102] suggested that earlier diagnosis of fungal infection could result in decreased mortality in neutropenic and cancer patients. Molecular diagnosis of fungal infections has relied mostly on the identification of organisms growing in culture. Nucleic acid hybridization probes for identification of *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Coccidioides immitis* from culture isolates have been available since the early 1990s (AccuProbes, Gen-Probe, San Diego, CA) with sensitivity ranging from 87.8 to 100% and specificity nearing 100% [103, 104]. The hybridization probes are rapid and demonstrate good sensitivity and specificity from culture, although some cross-reactivity with uncommon fungal organisms has been reported [104, 105]. More recently, peptide nucleic acid fluorescent in situ hybridization (PNA FISH) probes and syndromic panel for bloodstream infections (FilmArray Blood Culture ID panel) have become available for rapid identification of *C. albicans/Candida*

**Table 47.2** US FDA-cleared syndromic molecular tests

Manufacturer	Test name	Syndrome	# targets	Date cleared
Luminex	xTAG respiratory viral panel (RVP)	Respiratory	12	01/2008
	NxTAG respiratory pathogen panel (RPP)	Respiratory	20	12/2015
	xTAG gastrointestinal pathogen panel (GPP)	GI tract	14	01/2013
Nanosphere	Verigene respiratory virus + test	Respiratory	8	01/2011
	Verigene gram-positive blood culture test	Bacteremia	15	06/2012
	Verigene enteric test	GI tract	9	06/2014
	Verigene gram-negative blood culture test	Bacteremia	14	11/2014
	Verigene respiratory pathogens flex NA test	Respiratory	16	09/2015
bioMérieux/BioFire	FilmArray respiratory panel	Respiratory	20	05/2011
	FilmArray blood culture identification panel	Bacteremia	27	06/2013
	FilmArray gastrointestinal panel	GI tract	22	05/2014
	FilmArray meningitis/encephalitis panel	CNS	14	10/2015
GenMark	eSensor respiratory viral panel	Respiratory	14	02/2012
Prodesse	ProGastro SCS assay	GI tract	5	01/2013

*parapsilosis*, *C. glabrata*/*Candida krusei*, and *Candida tropicalis* from positive blood cultures [91, 106–108].

Several real-time PCR assays have been developed over the last few years with varied level of sensitivity and specificity and often with limited range, only targeting a few *Candida* or mold species [109–113]. A recent shift toward development of pan-fungal assay can be observed in the literature and reflect the need for tools that detect most of the clinically relevant fungal pathogens in patient specimens [114–117].

More recently, a few commercial assays and reagents have become available for the detection of mold directly from specimens. Several MycArray™ assays (Myconostica Ltd., UK) targeting yeasts, *Aspergillus* species, and *Pneumocystis jirovecii* are commercially available outside of the United States and demonstrates high sensitivity and specificity compared to culture or LDT assays [118–120]. Other molecular methods used for fungal diagnosis include sequence-based identification using the ITS1 and ITS2 regions between the 18S and 28S rRNA subunits and the D1/D2 region of the 25–28S large ribosomal subunit [121]. Several studies have been published showing the utility of sequencing for fungal identification, and in some laboratories, sequencing has completely replaced the use of phenotypic methods to identify fungi growing in culture [122–126].

Unlike bacteria and fungi, molecular methods for detection of viruses are well established and for most pathogens are considered the gold standard. As such, there is extensive literature on the development and applications of molecular assays for the detection of viruses of importance to transplant patients including herpesviruses (*Cytomegalovirus* and Epstein-Barr viruses), polyomavirus (BK and JC virus), hepatitis viruses, and respiratory viruses [80, 127].

One of the first application of molecular assays in virology included qualitative and quantitative real-time PCR assays for the diagnosis and monitoring of human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). These assays have been extensively evaluated and shown to be useful for the management and monitoring of patients with these infections [128–132]. A variety of commercial tests based on PCR (or RT-PCR) combined with sequencing (i.e., TRUGENE HIV-1 Genotyping Kit and ViroSeq genotyping system) or hybridization (i.e., INNO-LiPA HBV DR v2) are available for genotypic resistance testing of HIV [133, 134], HBV [135, 136], and HCV [137, 138].

Similarly, quantitative viral load testing has been developed for monitoring of viruses of importance to various transplant groups including cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK virus, JC virus, and adenoviruses [80]. However, the biggest challenge associated with the use of these laboratory-developed quantitative assays is the inability to compare viral load results obtained across

laboratories due to differences in genomic target (single vs multi-copy genes), extraction methods (manual vs automated), detection platforms, and lack of international standards and calibrators [139]. These limitations of quantitative assays have made the establishment of useful quantitative threshold for treatment difficult to establish [140–145]. The recent introduction of the first World Health Organization (WHO) international standards for cytomegalovirus [146] and Epstein-Barr viruses [147] as well as the availability of the first FDA-approved commercial real-time quantitative assay for monitoring of CMV viral loads was aimed at decreasing the variability in viral loads measured across methods, but a recent study by Hayden et al. showed that although improved, the standardization challenge remains in the field [148].

Other useful molecular assays for transplant patients include genotypic assays for drug resistance testing. Because transplant patients are often on prolonged antiviral therapy, these patients tend to develop mutations. These mutations can be detected by real-time PCR assays targeting known existing mutations that confer resistance to certain drugs, i.e., CMV UL97 mutations for ganciclovir or sequencing assay to detect all wild-type variants [149].

Several molecular assays have received FDA clearance for detection of respiratory viruses (Tables 47.1 and 47.2). The configuration of these assays varies from single target to highly multiplexed assays. The first FDA-cleared multiplexed molecular assay for respiratory viruses, the xTAG® Respiratory Viral Panel (RVP) (Luminex Molecular Diagnostics, Toronto, Canada), targets 12 viruses and subtypes (respiratory syncytial viruses A and B; influenza A (H1 subtype, H3 subtype, and untypeable); influenza B; parainfluenza 1, 2, and 3; human metapneumovirus; adenovirus; and enterovirus/rhinovirus). This assay provided a significant improvement in the diagnosis of respiratory viral infections compared to conventional method and was instrumental in the rapid diagnosis of influenza A H1N1 during the 2009 outbreak in New York City [150, 151]. Additional multiplex molecular assays have since been approved including the FilmArray Respiratory Viral Panel (FA RVP) (BioFire Diagnostic Inc., Salt Lake City, Utah) FDA cleared for the detection of 17 viruses and subtypes including the virus targets in xTAG RVP plus human coronaviruses (NL63, HKU1, 229E, and OC43) and parainfluenza 4 as well as three bacterial targets: *Bordetella pertussis*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*. Multiple studies have been published comparing these highly multiplexed assays against each other, against monoplexed assays, and against traditional methods in various patient populations [152–157]. Results have shown comparable performance with overall sensitivity and specificity between 90% and 100%, although differences were detected for specific targets including adenoviruses, which are detected with higher sensitivity by

single target assays than by highly multiplexed PCR [156]. Other molecular devices for detection and identification of a panel of respiratory viral pathogens are also commercially available from several manufacturers including Gen-Probes (Prodesse assays), Focus Diagnostics (Simplexa assays), and Nanosphere, Inc. (Verigene assays) [158, 159].

A parasite of interest for transplant patients, especially those undergoing heart transplantation, is *Toxoplasma gondii*, which can be due to either reactivation of latent infection or acquisition of parasites from transplanted organs [160]. Unlike immunocompetent hosts, the diagnosis of toxoplasmosis in immunocompromised patients, including transplant recipients, is most effectively done using PCR on the appropriate specimens [160, 161].

### Transcriptomic Assays

Genomic assays detect microbial organism-specific nucleic acids; therefore, a positive result can occur with both alive and dead microorganisms, which is particularly true for those pathogens that have protective cell wall. The best example of this is the detection of *Mycobacterium tuberculosis* DNA in sputum where the dead microbial pathogen DNA can remain un-degraded due to the fatty acid-rich cell walls [162, 163]. Unlike the results of a function-based testing method, such as mycobacterial cultures, in the clinical setting, a positive PCR result after antituberculosis therapy does not necessarily mean treatment failure. Therefore, DNA-targeted molecular assays are usually not considered to be tests of cure. This is also true for sexually transmitted pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [164]. A positive result may reflect treatment failure with persistent infection but may also reflect resolved infection by detecting the mere presence of ribosomal RNA debris and nonviable *C. trachomatis* DNA [165].

To overcome this disadvantage, transcriptomic assays have been explored. The ability of mRNA-based assays to distinguish viable from nonviable organisms suggests that such assays should be useful in monitoring the efficacy of antituberculosis therapy [166–170]. For monitoring efficacy of therapy, mRNA RT-PCR results paralleled well with those of culture at the follow-up time points [163]. Another study further demonstrated sputum *M. tuberculosis* mRNA is a reliable marker of bacteriologic clearance in response to several mono or combined antituberculosis therapies [162]. Nucleic acid amplification assays targeting microbial mRNA have also been used for diagnosis and assessment of human papillomavirus (HPV) infections. Several reports have shown not only the ubiquitous presence of E6 and E7 mRNA in cervical cancer but also a quantitative difference in the overexpression of E6/E7 depending on the severity of the cervical lesion [171]. Several E6 and E7 mRNA qualitative

assays including Aptima (Gen-Probe), NucliSENS EasyQ HPV (bioMérieux), and PreTect HPV-Proofer (NorChip) have been reported to improve the low specificity and positive predictive value of HPV DNA assays [172].

Advances in molecular biology technologies, especially the real-time quantitative PCR formats, have made the implementation of mRNA-based assay relevant and accurate. Another novel approach known as RNA-seq, which uses next-generation sequencing technologies to generate transcriptome profiling [173], is starting to come into the diagnostic microbiology field [174, 175]. Using dual-species transcriptional profiling in a murine model of systemic candidiasis, Hebecker et al. observed a delayed transcriptional immune response accompanied by late induction of fungal stress response genes in the kidneys. In contrast, early upregulation of the proinflammatory response in the liver was associated with a fungal transcriptome resembling response to phagocytosis, suggesting that phagocytes contribute significantly to fungal control in the liver [176]. Rasmussen et al. combined longitudinal, dimensionality reduction and categorical analysis of the transcriptome from 111 liver biopsy specimens taken from 57 HCV-infected patients over time and identified alterations in gene expression that occur before histologic evidence of liver disease progression, suggesting that events that occur during the acute phase of infection influence patient outcome [177].

In contrast to these “fancy” and advanced technologies, transcriptomic assays face basic specimen source-related challenges. Currently, there are limited methods which can be used to differentiate and overcome the DNA contamination when mRNA targets are tested. Theoretically, specimens can be pre-treated with DNAase prior to the mRNA amplification and detection [178, 179]. However, absolutely RNase-free DNAase is rarely available to actually do the job. Designing primers/probes to cover RNA splicing sites has been demonstrated efficient if relevant RNA splicing sites are available in targeted bacteria and viruses [180, 181]. Indirect methods have been reported to determine antimicrobial susceptibility by selectively detecting viable microorganisms. This assay uses a DNA-binding dye that penetrates damaged bacterial cells and renders DNA un-amplifiable, thereby decreasing background amplification from killed organisms [182, 183].

### Proteomic Assays

One leading proteomic technology, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), has emerged as a rapid and powerful tool for microbial species identification [100]. The analyte molecules embedded within the saturated matrix on the target plate are irradiated by a laser of special wavelength and intensity, inducing desorption and ionization; the charged



analytes then are accelerated by an electric field in a flight tube to a detector, where they are captured. The separation of various molecules depends on the time of flight, which is reversely proportional to the mass of molecules. After detection signals are processed and interpreted into the mass spectra, the characteristic mass peaks are used to characterize and eventually identify the microorganisms. By measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each bacterial species, it is possible to determine the species within a few minutes of when the analysis is started with whole cells, cell lysates, or crude bacterial extracts [184–186].

Numerous reports have shown that MALDI-TOF MS has revolutionized the routine identification of microorganisms in clinical microbiology laboratories by introducing an easy, rapid, high-throughput, low-cost, and efficient identification technique [187–190]. Two such systems, the Bruker Biotyper (Bruker Daltonics Inc., Billerica, MA) and Vitek MS (bioMérieux Inc., Durham, NC), have been successfully used in the routine clinical microbiology laboratory [191, 192]. A recent comparative study was performed on five methods for differentiation of coagulase-negative staphylococci (CoNS), i.e., Vitek2 (Gram-positive card REF 21342; bioMérieux), the ID 32 Staph strip (bioMérieux), partial 16S rRNA gene sequencing (MicroSeq; Applied Biosystems), partial *tuf* gene sequencing (in-house), and MALDI-TOF MS (Bruker Daltonics), on 142 CoNS clinical isolates. MALDI-TOF MS showed the best results for rapid and accurate CoNS differentiation with 99.3% of strains correctly identified [193]. In addition to microbial identification from purified colonies, the MALDI-TOF MS has been successfully used directly from urine and positive liquid culture media [194–197].

In addition to rapid identification of microorganisms, MALDI-TOF MS has been explored for determining epidemic relatedness and antibiotic resistance of microbial isolates. The utility of MALDI-TOF MS for microbial typing was investigated in *Staphylococcus aureus* in two recent studies. The composition correlation index analysis of the MALDI-TOF MS data demonstrated the similar inter-strain relatedness found with the standard typing methods used to confirm the outbreak [198, 199]. These data indicated that this technology is a potential rapid screening tool for nosocomial infection investigations. The MALDI-TOF MS was capable of rapidly and accurately identifying *mecA*-positive *S. aureus* and *vanB*-positive *Enterococcus faecium* from susceptible isolates [200, 201]. The MALDI-TOF MS has been directly used to determine mechanisms of antibiotic resistance [202]. Bittar et al. described the use of a MALDI-TOF MS profile and a ClinPro Tools software to detect and identify staphylococcal Panton-Valentine leukocidin [203]. The detection and identification of a series of  $\beta$ -lactamases from Gram-negative bacilli by MALDI-TOF MS seem to be a powerful, quick, and cost-effective method for clinical

microbiology laboratories [204–207]. These studies represented a proof of concept for the use of MALDI-TOF MS technology as a rapid method to timely monitoring microbial infections.

Numerous proteomic biomarkers have been used to diagnosis and monitoring of microbial infections. One of the most promising biomarkers in recent years is procalcitonin (PCT). PCT has many favorable properties as it is rapidly induced during infections and has a long half-life with capacity to differentiate bacterial from viral etiologies [208]. For the use and value of procalcitonin in SOT transplantation, the existing literature suggests reasonable sensitivity and specificity for the PCT test in identifying infection complications among patients undergoing transplantation. Monitoring PCT in the early posttransplant period seems to be a promising method for early detection of infectious complications; however, given the imperfect sensitivity and specificity of the PCT test, medical decisions should be based on both PCT test results and clinical findings [209, 210]. Recently, van Houten et al. reported the use of a three-host protein (TRAIL, IP-10, and CRP)-based assay to differentiate between bacterial and viral infections in children with lower respiratory tract infection or fever without source [211].

## Metabolic Assays

Diagnosing bacterial infections by smell has been practiced for millennia. Volatile organic compounds (VOCs), produced by bacteria as metabolites, may be produced in different quantities and combinations by each bacterial species or serovar, generating characteristic odors. These compounds, in combination with other VOCs, could be used as a volatile fingerprint of each bacterium. Recently, fast and sensitive techniques, led by a variety of mass spectrometry platforms, have been developed and implemented to detect and characterize microbial pathogens based on microbial metabolite analysis [212]. In addition, metabolic analysis can be used for functional characterization including virulence and resistance determination. Gilreel et al. recently examined the metabolic potential of multidrug-resistant uropathogenic *Escherichia coli* and demonstrate metabolic activity of members of the ST131 lineage correlated with antibiotic susceptibility profiles [213].

Direct detection of exogenous fungal metabolites in breath may be used as a novel, noninvasive, species-specific approach to identify patients with invasive aspergillosis (IA), potentially allowing more precise targeting of antifungal therapy and fewer invasive diagnostic procedures. Gas chromatography coupled with mass spectrometry (GC-MS) has been the mainstay for the detection and characterization of VOCs produced by a panel of Gram-negative bacilli [214–216]. Unique GC-MS VOCs were found to be produced by

five *Aspergillus* species such as *A. fumigatus*, *A. versicolor*, *A. sydowii*, *A. flavus*, and *A. niger* cultivated on malt extract agar and gypsum board [217]. In another study, 2-Pentylfuran (2PF) was consistently detected in the media of *A. fumigatus*, *Fusarium* spp., *A. terreus*, and *A. flavus* and to a lesser extent by *A. niger*. 2PF was detected in breath samples from 4/4 patients with cystic fibrosis and *A. fumigatus* colonization, 3/7 patients with cystic fibrosis but no microbiological evidence of *A. fumigatus*, and none of the 10 healthy controls [218]. Using thermal desorption-GC-MS, Koo et al. characterized the in vitro volatile metabolite profile of *A. fumigatus*. A pathogen-specific metabolic signature combined with  $\beta$ -trans-bergamotene,  $\alpha$ -trans-bergamotene, a  $\beta$ -vatiirene-like sesquiterpene, and trans-geranylacetone accurately discriminated patients with IA from patients with other pneumonia [219]. Besides *Aspergillus* species, VOCs such as nicotinic acid have been found to be promising biomarkers for *Mycobacterium tuberculosis* infections [220].

## Clinical Perspective

During the last 10 years, mortality related to infection after HSCT has declined substantially [221]. Nonetheless infection remains a substantial cause of non-relapse mortality. Use of alternative donors such as cord blood and haploidentical donors, older age at transplant, and increased comorbidities continue to increase [222, 223]. These transplant characteristics have been associated with increased infection risk. Furthermore neutropenia, T-cell depletion, GVHD, and immunosuppressive agents continue to shape the spectrum and period of risk for specific infections. Our expanding knowledge of the role of the human microbiome in the outcomes of transplantation provides new challenges and opportunities for clinical interventions.

Management of infections in the immunocompromised host poses several challenges. Inflammatory host responses are usually reduced or absent. Patients with life-threatening infections may present with minimal signs and symptoms and deteriorate rapidly often developing disseminated disease. Organisms of little or no pathogenicity for healthy individuals may cause life-threatening infections, and multiple pathogens may coexist in the same patient. Invasive procedures needed to maximize diagnostic accuracy may be not feasible due to thrombocytopenia or other conditions. Timely institution of broad empiric therapy is essential to improved outcomes; yet polypharmacy may lead to substantial toxicities and serious drug interactions.

The increasing implementation of nucleic acid-based assays in clinical practice has enabled rapid and often quantifiable diagnosis of an expanding list of organisms. Clinical decision-making is complex as quantification enables real-time monitoring of pathogen replication dynamics.

**Table 47.3** Clinical applications of diagnostic assays in immunocompromised patients

Level	Goal
Prevention	Risk assessment
Preemptive	Testing asymptomatic patients at risk for disease
Diagnostic	Testing patients with clinical signs and symptoms of infection for specific pathogens
Therapeutic	Testing patients with established infection to direct treatment, assess response to therapy, and evaluate prognosis
Prognostic	Test patients at risk for recurrence of disease

Diagnostic assays are used by the clinicians to predict risk of infection in asymptomatic patients, monitor patients at risk for disease, diagnose disease in symptomatic patients, or monitor response to therapy or predict outcomes in patients with established disease (Table 47.3).

## Prediction of Risk for Infection

The pretransplant evaluation of donors and recipients of HSCT includes serology to determine prior exposure to pathogens. The Federation for Accreditation of Cellular Therapies (FACT) requires donors and recipients to be tested for antibodies to HIV, human T-cell lymphotropic viruses I and II, HBV, HCV, and herpes viruses (HSV, VZV, CMV, EBV). Donors and recipients are tested for exposure to West Nile virus and *Trypanosoma cruzi*. Donors should be tested within 30 days prior to collection. Emergence of pathogens with potential for transmission through cellular products requires development of new diagnostic assays. A recent example is Zika virus. A non-FDA approved test is currently used to screen blood donors (<https://www.cdc.gov/zika/transmission/blood-transfusion.html>). However the current CDC recommendations to reduce transmission of Zika through human cells and cellular-based products are based on epidemiologic history (<https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM488582.pdf>).

Based on the results of the pretransplant tests, clinicians assess risks, benefits, and alternatives to HSCT or implement preventive treatment. For example, recipients with positive IgG antibody for hepatitis B (HBV) core antigen (with negative HBV surface Ag and negative HBV PCR) are treated with entecavir to prevent reactivation of HBV posttransplant.

CMV serology of the donor and recipient has major implications. For recipients with acute CMV infection indicated by positive CMV IgM (negative IgG), transplant may be delayed, and treatment may be required. The CMV serostatus of the recipient is the most important predictor for development of CMV infection posttransplant. Combined results of donor and recipient serology is used to optimize donor selection [224]. Given the availability

several donors with similar degree of HLA match, preference is given to donor matching the CMV serostatus of the recipient. For CMV-seronegative patients, the use of a CMV-seronegative donor alleviates the risk of CMV transmission through the allograft [225]. CMV-seropositive recipients who receive conventional allografts from CMV-seropositive donors are receiving CMV-specific cytotoxic T lymphocytes (CTL) contained in the allograft. Lymphocytes from CMV-seropositive donors can also be used to generate *ex vivo* CMV CTL for adoptive immunotherapy posttransplant [226]. The CMV serostatus of donor and recipient also determines the need for posttransplant serial monitoring for CMV. Recipients who are CMV positive or receive grafts of CMV-seropositive donors are monitored by CMV PCR, and preemptive therapy is initiated, if CMV infection occurs [227]. Another approach is antiviral prophylaxis for CMV for high-risk groups such as recipients of mismatched or T-cell-depleted allografts [228]. It will be interesting to assess whether CMV monitoring by the PCR will eliminate survival differences between CMV-seropositive and CMV-seronegative recipients.

Additional screening may be indicated for donors and recipients of T-cell-depleted grafts. Toxoplasma serology is not required by FACT and is tested per institutional practices. Patients receiving T-cell-depleted allografts are at higher risk of toxoplasmosis compared to patients who receive conventional allografts. Thus recipients of T-cell-depleted allografts may be candidates for prophylaxis against toxoplasma posttransplant. At present the interpretation of serology is qualitative (positive vs negative). Recent studies suggest that the magnitude of titers may be relevant in predicting disease risk. Meers et al. reported that high titers of toxoplasma IgG pretransplant were associated with increased risk of toxoplasmosis after HSCT [229]. Given the low frequency of toxoplasmosis in HSCT, a multicenter study would be required to confirm these findings.

The notion that the magnitude of IgG titers may be useful as a predictor for infection posttransplant was also supported by a pilot study assessing pretransplant antibodies to adenovirus (ADV). In that study, patients with high pretransplant IgG titers to a specific ADV serotype were more likely to develop ADV infection with the same ADV serotype after HSCT [230].

Patient exposures may also indicate the need for additional testing. For example, QuantiFERON Gold™ testing for detection of latent tuberculous infection is pertinent for transplant candidates from endemic areas for *M. tuberculosis* [231, 232]. Patients with latent tuberculous infection pretransplant will require treatment posttransplant. Pretransplant stool examination for ova and parasites for transplant candidates coming from endemic areas of *Strongyloides stercoralis* or empiric treatment for *Strongyloides stercoralis* pretransplant could be employed for such individuals [233].

## PCR Assays for Detection of Double-Stranded (ds) DNA Viruses

The availability of commercially available quantitative PCR assays for many dsDNA viruses has enabled the detection of these viruses in body compartments such as blood, urine, stool, bronchoalveolar lavage (BAL), or cerebrospinal fluid. While PCR assays provide accurate and rapid identification and quantification, several challenges remain regarding their optimal use and interpretation of results.

### Cytomegalovirus (CMV)

CMV is an important cause of morbidity and mortality in transplantation. The biologic properties and natural history of CMV are well defined [234]. CMV viremia occurs frequently after HSCT and in most instances precedes development of end-organ CMV disease. Effective antiviral treatment is available, and preemptive treatment of CMV infection has been shown to be effective in preventing end-organ disease [235]. Routine monitoring is recommended for patients at risk for CMV disease [236, 237]. Currently PCR-based assays for CMV have replaced pp65 antigenemia assay in most centers. Green et al. reported that transition of preemptive therapy strategy from antigenemia to PCR-based monitoring and host risk factors successfully prevented CMV disease without increasing the proportion of patients receiving preemptive therapy and attributable toxicity [238]. The performance characteristics of individual CMV PCR assays vary; thus cutoff values and thresholds for treatment are not comparable among laboratories [145]. The availability of the World Health Organization (WHO) International Standard (IS) for CMV for nucleic acid amplification techniques is an important development for decreases variance between laboratories and enables to develop international clinical practice guideline [227]. Even with the WHO standardized assay, there is considerable variability (up to 1.5 log<sub>10</sub> IU/mL) in different determinations of viral load from the same specimen [239].

### Other Double-Stranded (ds) DNA Viruses

BK polyomavirus (BKV), adenovirus (ADV), and human herpesvirus 6 (HHV-6) are detected with variable frequencies in HSCT patients, yet their natural history is not fully understood. All these viruses have been associated with potentially serious end-organ disease and adverse transplantation outcomes. Yet the utility of routine monitoring and preemptive intervention have not been evaluated in prospective clinical trials. Because of the relatively low frequency of end-organ disease caused by these viruses, multicenter studies would be better suited to address such questions. Differences in diagnostic assays and clinical practices among institution and lack of approved treatments for these pathogens pose logistical difficulties.

### BK Polyomavirus (BKV)

BK polyomavirus (BKV) is identified as a cause of allograft nephropathy in kidney transplants (BKVAN) and a cause of hemorrhagic cystitis in HSCT recipients [233, 240]. In renal transplant recipients, several studies have directly linked BKV replication to BKV nephropathy (BKVN), and BKV viremia is a predictor of BKVN in renal allografts [241]. Furthermore an association between the magnitude of BKV viral load in the blood and development of BKVN has been well described, and appropriate cutoffs have been established for the clinical significance of BKV viremia. BKVN cases have been reported in HSCT recipients [242–244]. The diagnosis for BKVN in HSCT recipients is challenging as kidney biopsy is oftentimes not feasible due to thrombocytopenia and bleeding risk.

The exact biologic relationship between BKV and hemorrhagic cystitis in HCT recipients is not well understood [245–252]. Some studies have shown a relationship between the magnitude of urine BKV viral load and development of hemorrhagic cystitis [253–255]. However the concentrations of virus vary widely and often overlap with patients who do not develop hemorrhagic cystitis. Unfortunately, no effective therapy is currently available for the prevention or treatment of symptoms associated with BKV, in large part due to a lack of understanding about its etiology and pathogenesis [256–261]. It is likely that the pathophysiology of cystitis in this setting is multifactorial with BKV reactivation as a contributing factor. The level of BKV viremia in HSCT exceeds by several logs the levels observed in renal transplants [250, 251, 254, 262]. Reduction of immunosuppression, the mainstay for management in renal transplantation, is not an option in the allogeneic HSCT due to the risk of triggering or exacerbating graft-versus-host disease. Despite the lack of established guidelines for the interpretation of BKV PCR results in HSCT and paucity of therapeutic measures for BKV in HSCT, BKV PCR is frequently ordered in symptomatic patients.

At our institution we prospectively monitored in 100 adult HSCT recipients for BKV in the urine by Q-PCR every 2 weeks from beginning of conditioning until week +15 posttransplant [252]. We found that 50% of patients had BKV viremia by day +30, and the rate remained stable for the duration of the study. Ten (10%) patients developed hemorrhagic cystitis (grade  $\geq 2$  by Bedi et al. [245]). Seven (70%) patients with hemorrhagic cystitis had BKV in the urine (two with concomitant adenovirus). In univariate analyses, high BKV viral load ( $\geq 1.0 \times 10^7$  copies/mL) and older age were predictors of hemorrhagic cystitis. During the study period, 36 patients died and 8 patients had autopsies performed. One patient was found to have BKVN at autopsy. Our findings suggest that factors in addition to BKV are likely involved in the pathogenesis of hemorrhagic cystitis posttransplant. At present, we do not recommend monitoring asymptom-

atic patients for BKV in urine. In patients with symptoms of cystitis and no other identified etiology, we suggest checking BKV PCR once. We discourage monitoring of BKV viral load in the urine in patients with known BKV viremia. BKV nephropathy should be considered as a cause of renal dysfunction in severely immunosuppressed HSCT patients without any other obvious etiology.

### Adenovirus (ADV)

Adenovirus infection occurs in <5–20% of HCT recipients depending on patient age, type of transplant, and degree of immunosuppression [263–265]. ADV-associated hepatitis, pneumonitis, and encephalitis are frequently fatal, while colitis and hemorrhagic cystitis cause substantial morbidity and may contribute to mortality [266–268]. More than 50 ADV serotypes are identified and differ in terms of frequency, tropism, and potential for disease severity [269]. ADV viremia has been associated with decreased overall survival after HSCT [270, 271].

Quantitative PCR assays for ADV have replaced for most part culture or antigen assays. Routine surveillance for ADV is suggested for high-risk patients such as recipients of T-cell-depleted transplant (TCD), cord blood transplant, or haploidentical transplant or for patients with refractory GVHD [272–274]. The American Society of Bone Marrow Transplant recommends serial monitoring for ADV by PCR during the first 6 months after HSCT or for the duration of severe immunosuppression and/or lymphopenia for patients at highest risk [275]. These recommendations are based on single-center experience and expert opinion but not validated in controlled trials. Ohrmalm et al. found little utility in serial monitoring of plasma ADV PCR in a cohort of 97 HSCT comprised of 64% T-cell-depleted allografts [276]. High level or rising ADV viremia has been reported to predict disseminated ADV disease and death [277–280]. Rising ADV viral load in the stool has also been reported as a useful predictor of ADV disease [279]. T-cell depletion, younger age, and GVHD have been associated with invasive ADV disease [266, 277–279]. Cidofovir has been used in established ADV disease and ADV viremia, yet its efficacy is based on small noncontrolled studies and case series [272–274]. Brincidofovir, a novel, orally administered, broad-spectrum antiviral active against ADV, has shown promising results in case reports [277, 281, 282]. A small randomized placebo-controlled clinical trial of preemptive treatment of ADV viremia with brincidofovir confirmed the antiviral activity in HCT patients however [283]. A subsequent open-label phase III study evaluated brincidofovir treatment for localized or disseminated ADV infection in adult and pediatric HSCT recipients. Virologic response was correlated with lower ADV viral load at start of treatment and earlier start of brincidofovir after ADV diagnosis. Gastrointestinal-related (abdominal pain, diarrhea, nausea, vomiting) symptoms

were most common adverse events and led to treatment discontinuation especially in adult HCT.

Since 2012, we have implemented routine blood PCR monitoring from day+14 until day+100 posttransplant in TCD and cord blood HSCT recipients. The rate of ADV viremia was 8%, and 33% of viremic patients developed ADV disease in TCD HSCT recipients. ADV disease was diagnosed within 60 days posttransplant, and 85% of patients with ADV diseases died. The benefit of preemptive therapy for ADV for prevention of ADV disease in recipients of TCD grafts should be evaluated in prospective clinical trials.

### Human Herpesvirus 6 (HHV-6)

HHV-6 infects over 90% of individuals in the first 18 months of life. After resolution of the primary infection, the virus establishes latency mainly in CD34+ cells including monocytes and macrophages. An alternative form of HHV-6 persistence is integration of viral sequences into host cell chromosomes [284]. Approximately 40% of all HSCT recipients develop HHV-6 reactivation, and the cords rates may be >90% [285]. At our Institution 61% CD34+ selected HCT and 94% cord blood recipients (without ATG) developed early HHV6 viremia. Rates of HHV6 encephalitis were low in our patients, 0.7% and 1.6% in Cd34+ and cord blood, respectively [286].

HHV6 has been associated with a host of indirect consequences such as acute GVHD, CMV reactivation, and mortality after HSCT [287]. Zerr et al. suggest HHV-6 reactivation is associated with delirium and neurocognitive decline after HSCT [288]. The most recognized and severe form of HHV-6 is posttransplant acute limbic encephalitis (PALE). Hill et al. examined a cohort of 1243 adult donor HSCT and 101 umbilical cord transplants to identify risk factors for PALE. In multivariate analyses cord blood transplant, grade II-IV GVHD and adult mismatched donor were significant. While viral loads for HHV-6 were higher in patients with PALE, values greatly overlapped. Furthermore, peak values were detected a median 1 day to 9 days from symptom onset [289]. Foscarnet, cidofovir, and ganciclovir are available antiviral agents that demonstrated *in vitro* activity against HHV-6, but there are no controlled trials to study these agents for HHV-6 therapy. A few studies evaluating the efficacy of preemptive or prophylactic therapy to prevent PALE have been disappointing [290–292].

## Diagnostic Evaluation of Specific Syndromes

### Challenges

Infectious complications in transplant patients are often extremely complex to assess since there is a wide array of pathogens that can cause infections, including bacteria, viruses, fungi, and parasites. Further, patterns of pathogen

infectivity vary tremendously, particularly in the setting of HSCT in which immune recovery plays a major role in defining the type and clinical presentation of many infections. Infections may occur as acute events such as a pneumonia or bloodstream infection, reactivation of latent organisms as in the case of herpesvirus infections, and colonization without true invasive infection or as recurrent, nonresponding, or resistant infections. Furthermore, sites of infections may be localized to a single body area or tissue or may be disseminated. HSCT recipients may be suffering from immune incompetence that can last for years.

Multiplex assays offer the advantages when the quantity of sample is limited as they provide information on multiple pathogens. Combination of multiple diagnostic platforms in the same sample and testing of several body compartments cast a wider net and expand diagnostic capabilities. We present specific challenges in clinical evaluation of pulmonary syndromes in HSCT patients.

## Evaluation of Pulmonary Syndromes

### Viral Infections

The use of PCR to analyze samples from HSCT recipients may facilitate early detection of respiratory viruses, even prior to onset of symptoms when viral loads are likely to be low. For symptomatic patients, PCR testing provides a sensitive diagnostic approach to identify the etiology of respiratory symptoms and an appropriate isolation of the ill patient. Additionally, quantitative RT-PCR assays can be used to initiate appropriate treatment and monitor changes in viral load during therapy.

Some respiratory viruses such as RSV, parainfluenza viruses, adenovirus, and influenza viruses are known to cause low respiratory infections associated with substantial morbidity and mortality in immunosuppressed patients. In contrast the correlation of the presence of rhinovirus or coronavirus in the upper respiratory tract with development of lower respiratory infection in HSCT is not clear [293]. The correlation between magnitude of viral load in bronchoalveolar lavage fluid and pneumonia or transplant outcomes is currently being investigated for a variety of viruses [294, 295].

The use of nucleic acid assays may contribute to identification of organisms not previously associated with pulmonary disease. Enterovirus D68 was recently associated with acute respiratory distress syndrome in infants and HSCT [293, 296]. Human metapneumovirus (hMPV) and human bocavirus have been reported as a cause of severe lower respiratory tract infection [297–299]. Two new human polyomaviruses, KI polyomavirus (KiPyV) and WU polyomavirus (WUPyV), are found in one third of allogeneic HSCT recipient's respiratory specimens during the first year posttransplant, but the associations with respiratory symptoms are unclear [300].

## Invasive Fungal Infections

Diagnosing invasive pulmonary aspergillosis (IPA) remains a challenge. Tissue diagnosis is ideal, yet invasive procedures may not be feasible in critically ill patients especially those with cytopenia. Isolation of *Aspergillus* species from BAL may represent colonization or invasive infection depending on species and clinical context. For example, *Aspergillus versicolor* and *Aspergillus niger* are often not associated with disease when they were isolated from BAL specimens [301, 302]. Cytology in combination with traditional culture techniques may improve diagnostic yield. In a retrospective study comparing diagnostic yield of cytology and culture for septate, mold infections (cytology of BAL and bronchial wash specimens) had higher yield compared to culture of tissue (autopsy and biopsy) samples (58% vs 30%,  $P < 0.03$ ) [303].

Noninvasive sensitive tests are needed for the diagnosis of mold infections. Detection of an *Aspergillus* secondary metabolite signature in a simple breath test showed 94% sensitivity and 93% specificity in diagnosis of IPA in a small preliminary study [219]. Such tests offer promising alternatives for patients that cannot undergo bronchoscopy.

Molecular-based assays are expected to allow a rapid diagnosis of *Aspergillus* and non-*Aspergillus* invasive fungal infections with a high sensitivity. In a recent multicenter prospective study evaluation, addition of PCR to GM in BAL sampling improved the diagnosis of invasive aspergillosis [51]. Initial validation studies of the serum GM assay reported 61% sensitivity and 93% specificity in probable and proven IPA; however, the sensitivity of serum GM is considerably lower in setting of mold-active azole prophylaxis [304, 305]. Determination of GM in the BAL fluid may improve the diagnostic utility of this assay. In a prospective cohort study including 530 patients with hematologic malignancy, the sensitivity and specificity of BAL GM was 50% and 73% for detecting probable and proven IPA [306]. Further prospective studies are needed for the combination of these two diagnostic modalities for the diagnosis of proven and probable aspergillosis.

Fungal PCR has been useful in confirming diagnosis of invasive fungal infections when traditional cultures are negative especially in patients previously treated with antifungal agents. At our institution among 46 patients participating in a randomized trial for antifungal prophylaxis of fungal infection in neutropenic patients undergoing induction or re-induction chemotherapy, six patients underwent bronchoscopy for evaluation of pulmonary infiltrates. BAL was tested by cytology, traditional fungal cultures, GM, and universal fungal PCR. None of the patients had positive fungal cultures or positive GM in the BAL. Fungal PCR identified *Rhodotorula nogopathi* and *Cryptococcus saitoi* in one patient each. While these fungi are not recognized previously as pathogens in humans, our patients responded clinically when antifungal therapy was adjusted to target these organisms.

Traditional culture techniques are routinely used for diagnosis of candidemia. The clinical relevance of non-*Candida* species isolated from blood has to be interpreted with caution. In a retrospective study of non-*Candida* fungemia episodes in allogenic HSCT recipients, 42% of patients did not have clinically significant fungemia [307].

## Therapeutic Monitoring

### Viral PCR

Monitoring of the viral load to assess response to treatment is a well-established practice for CMV. CMV viral replication in the blood usually correlates with disease activity. Depending on CMV viral load, clinicians may continue treatment, change dose or type of antiviral, or discontinue treatment. Less evidence exist on the correlation of ADV or HHV-6 viral loads with disease activity, yet clinicians routinely use viral loads as an aid to treatment decisions.

Monitoring of viral load of respiratory viruses as a prognostic indicator of lower respiratory tract infection in HSCT patients is not a routine clinical practice at present. Recent studies suggest that this approach may be of value [294, 308].

### Genotypic Assays for Mutations Conferring Resistance to Antivirals

Genotypic assays for antiviral resistance may offer clinical guidance in a timely fashion. Commercially available assays are available for cytomegalovirus. Resistance usually emerges after prolonged or subtherapeutic exposure to antivirals in the setting of immunosuppression [309]. CMV resistance to current antiviral agents is mediated by alterations in either the UL97 kinase or DNA polymerase, encoded by the UL97 and UL54 genes, respectively. UL97 mutations are capable of conferring resistance to ganciclovir, while UL54 mutations can impart resistance to ganciclovir, cidofovir, and foscarnet [310].

Studies correlating CMV genotypes and drug susceptibility phenotypes may further guide treatment decisions. This will improve the interpretation of sequence-based assays currently used for clinical diagnosis and guide the development of new antiviral drugs [311].

Resistance of influenza virus to antiviral agents is a concern in immunocompromised HSCT patients due to high grade and prolonged viral replication and prolonged exposure to antivirals. Rapid identification of emerging resistance during treatment would be helpful in modifying treatment [312, 313].

### Serial Monitoring of Fungal Burden Markers

In patients with invasive aspergillosis and positive serum GM at baseline, serial monitoring of serum GM provides useful information on response to treatment and prognosis. Koo et al.

reported that the combination of GM at baseline and at 1 week was predictive of all-cause mortality independent of other traditional risk factors for mortality and antifungal exposure [314]. In a prospective study, Bergeron et al. showed that (i) a poor day 45 outcome was strongly associated with a high baseline serum GM index; (ii) a consistently negative serum GM index during the follow-up was associated with a good outcome, in contrast to either a steady or an emerging positive GM index; and (iii) the day 14 clinical evaluation was predictive of the day 45 outcome [315]. In patients with treated *Aspergillosis*, rising GM levels after initial normalization raise concern for breakthrough infection and inadequate exposure of development of resistance to ongoing antifungal therapy.

## Summary

In the last decade, nucleic acid-based assays have enhanced diagnostic sensitivity and specificity, shortened test turnaround time, provided automatic and high-throughput processing, and enabled quantification of microbial pathogens. A positive molecular test result indicates that targeted pathogen-specific nucleic acids are detected. For opportunistic pathogens in particular, clinical interpretation is crucial in determining the clinical significance of a positive test. Evolving genomics, transcriptomic, proteomic, and metabolomic technologies are being translated into clinical applications at a fast pace. Collaboration between laboratory and clinical medicine is paramount to ensuring optimal utilization and interpretation of diagnostic modalities.

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