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Molecular diagnostics of infectious disease: Detection and characterization of microbial agents in cytology samples

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Abstract

Background: Cytology samples are widely used to diagnose various infectious diseases by detection and identification of causative infectious agents, including bacteria, fungi, and viruses. The role of cytopathology in infectious disease has expanded tremendously in the past decades with the advances in molecular techniques. Molecular diagnostic methods, compared to conventional methods, have shown improved patient outcome, reduction in cost, and shortened hospital stay times. The aim of this article is to review molecular testing in cytology samples for diagnosis of infectious diseases.

Methods: The literature search for molecular testing in common cytology samples for diagnosis of infectious diseases was performed. The findings of the studies were summarized. The common cytology samples included in this article were gynecologic specimens, cerebrospinal fluid, bronchoalveolar lavage, and urine samples.

Conclusions: There are a number of molecular diagnostic tests that are available to be used in common cytology samples to detect infectious agents. Each test has its own advantages and limitations. It is our hope that upon reading this review article, the readers will have better understanding of molecular diagnostic testing of infectious diseases utilizing commonly sampled cytology specimens in daily practice.

KEYWORDS
cytology samples, infectious disease, molecular diagnostic testing

1 | INTRODUCTION

It is well known that cytology samples can be used to diagnose various infectious diseases by detection and identification of causative infectious agents, including bacteria, fungi, and viruses. However, the detection of specific infectious agents solely based on the cytomorphology and traditionally ancillary studies, such as special stains, has limitations. The role of cytopathology in infectious disease has expanded tremendously in the past decades with the advances in molecular techniques.[1] It has been shown that there are advantages by using molecular diagnostic methods compared to conventional methods in the diagnosis of infectious disease, including improved patient outcome, reduction in cost, and shortened hospital stay times.[1,2]

Various cytology specimens can be used for screening and diagnosis of infectious diseases and these include exfoliative cytology samples and fine-needle aspiration (FNA) material. Advances in molecular diagnostics, such as human papillomavirus (HPV) testing using liquid-based cytology samples, has led to an increasing menu of diagnostic tests for micro-organism identification in cytology samples.[3] This review article is focused on application of molecular diagnostic testing in commonly sampled cytology materials, including gynecologic specimens, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL), and urine samples, for diagnosis of infectious diseases.

2 | MOLECULAR TESTING OF HPV IN GYNECOLOGIC CYTOLOGY SAMPLES

Cervical cancer is one of the major causes of morbidity and mortality among women worldwide.[4] Pap screening significantly decreased

TABLE 1 Comparison of the 5 commercially available FDA-approved HPV molecular diagnostic tests

Test	Hybrid capture 2	Cervista	cobas	Aptima	BD onclarity
Assay type	DNA-based signal amplification	DNA-based signal amplification	DNA-based target amplification	mRNA-based target amplification	DNA-based target amplification
Manufacturer	Qiagen	Hologic	Roche	Hologic Gen-Probe	Becton Dickinson
Year FDA approved	1999	2009	2011	2012	2018
Internal control	No	Yes	Yes	Yes	Yes
Genotypes detected	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68
Target regions	Multigene probes	L1, E6, E7 genes	L1 gene	E6/E7 gene transcripts	E6/E7 DNA
Collection medium	PreservCyt or Qiagen transport medium	PreservCyt	PreservCyt or cobas PCR collection medium	PreservCyt or Aptima cervical specimen collection medium	BDSurePath preservative fluid collection medium
Clinical performance (compared to HCII)	Sensitivity for CIN2+ 84.9%–100%[8,25] Specificity for CIN2+ 69.5%–95.8%[8,25]	At least comparable to HC2 with lower cross-reactivity to other HPV types[8,30–36]	Sensitivity comparable to that of HC2 and better specificity[8,38,39]	Significant higher specificity than HC2[8,40–46]	Comparable to HC2[9,48–50]
Genotyping	n/a	16, 18	16, 18	16, 18/45	16, 18, 45

Abbreviation: n/a, not applicable.

cervical cancer incidence and mortality worldwide in the past decades.[5] HPV, the most prevalent sexually transmitted infection in the United States, plays the critical role in pathogenesis of cervical cancer.[6,7] HPV is a non-enveloped, double-stranded DNA virus with more than 200 known genotypes. The size of the viral genome is about 8 kb and the genome is divided into three major regions including early genes, late genes, and an upstream regulatory region. The early genes include E1, E2, E4, E5, E6, and E7. E1 and E2 genes are involved in initiation of viral DNA replication and regulation of early transcription. The major transforming activity of high-risk HPV is due to E6 and E7 genes.[8] The E6 and E7 genes work by inhibiting Rb, P53, and P21, leading to an accumulation of p16 in infected cells. Overexpression of viral E6 and E7 oncogene mRNA is highly associated with squamous intraepithelial lesion (SIL) development and is necessary and sufficient for cell immortalization, neoplastic transformation, and development of invasive cancer.[9–14] The late genes L1 and L2 encode the major and minor viral capsid proteins used in the construction of new viruses.[8] The most common high-risk types of HPV (hrHPV) include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68,[9] and they have the potential to transform to malignancy in the cervix, anogenital region, and head and neck sites, especially oropharynx.[9] HPV tests have been mostly used for reflex testing in patients with atypical squamous cells of undetermined significance (ASC-US) cytology results or co-testing with routine cytology Pap tests in women over age 30 to determine if a referral to colposcopy is needed.[15,16] The updated cervical cancer screening guidelines endorsed by American College of Obstetricians and Gynecologists/American Society of Colposcopy and Cervical Pathology (ACOG/ASCCP) recommend primary hrHPV testing as one of stand-alone screening methods in the average-risk women over 30 years of age.[17–19]

There are a number of molecular tests available to detect HPV DNA or mRNA.,[8,9,20–23] Most of the tests are designed to detect nucleic acids of 13 International Agency for Research on Cancer (IARC) HPV group 1 carcinogens (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and HPV 66.[8,9] Genotyping tests to distinguish individual hrHPV types, mostly HPV 16 and 18, can be performed on some assay platforms.[8,9,24] In this review, we will be focused on five commercially available Food and Drug Administration (FDA)-approved molecular tests in the market for detection of hrHPV in cytology samples. (Table 1) These DNA- or RNA-based assays use different test platforms with different analytical and clinical sensitivities and specificities.[8,9]

2.1 | Hybrid capture 2 HPV DNA test

Hybrid Capture 2 (HC2) HPV DNA test was developed by Digene Corporation (Gaithersburg, MD) and is now marketed by Qiagen (Germantown, MD). The HC2 test was first approved by the FDA in 1999 and replaced the original HC1 tube-format assay. The HC2 test was initially approved for reflex testing of patients with ASC-US cytology results and was expanded to include co-testing in conjunction with routine cytology testing for women over age 30 in 2003.[25] The HC2 test is a microtiter format nucleic acid hybridization assay using signal amplification and detects 13 hrHPV DNA strains including genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.[8,9]

The acceptable samples for this test should be collected in either Qiagen transport medium or PreservCyt ThinPrep Pap test solution. After DNA is extracted, a mixture of multigene RNA probes is added. If the hrHPV DNA is present, it will bind to the RNA with the probes and the resultant DNA–RNA hybrids are captured onto the wells that are coated with monoclonal antibodies to DNA–RNA hybrids. A second monoclonal antibody conjugated with alkaline phosphatase will then be added to the well which can bind to the captured hybrids. The alkaline phosphatase dephosphorylates a chemiluminescent substrate which produces light. The emitted light is measured in relative light units (RLU) on a luminometer.[8] This test has an analytical sensitivity of 0.2 to 1 pg/ml, equivalent to 1000 to 5000 genome copies of HPV/ml.[8] The clinical sensitivities for the detection of cervical intraepithelial neoplasm grade 2 (CIN2) or greater lesions ranged from 84.9% to 100% and specificities from 69.5% to 95.8%.[8,26] The negative predictive values were high and ranged from 99.8% to 100%.[8,26,27] One of the limitations of this test is cross-reactivity of the probe mixture with non-hrHPV types.[8,28,29] In addition, the HC2 test does not contain an internal control, therefore, it is not possible to determine the adequacy of the sample.[8]

2.2 | Cervista HPV HR test

Cervista HPV HR test (Hologic, San Diego, CA) was approved by the FDA in 2009. This is a DNA-based test using invader signal amplification chemistry to detect HPV DNAs from 14 hrHPV types, including the same 13 types in the HC2 test and HPV 66.[8,9] This assay is intended to use as a reflex testing in patients with ASC-US cervical cytology results and co-testing in conjunction with routine cytology testing for women 30 years and older.[30] Cervista HP 16/18 assay can be used adjunctively with Cervista HPV HR test in above patients to assess the presence or absence of HPV 16 and 18.[31] The samples should be collected in PreservCyt solution for this test. The test starts with extraction of DNA using the Genfind DNA extraction kit (Hologic/GenProbe, San Diego, CA). The subsequent invader assay uses two simultaneous isothermal reactions in a single tube. The first reaction uses three mixtures of sequence-specific probes that target the L1, E6, and E7 genes to detect four groups of hrHPV based on phylogenetic relatedness. The second reaction produces a fluorescent signal. Both internal and external positive controls are included in this assay.[8] The analytic sensitivity of this test ranges from 1250 to 7500 genome copies per reaction.[8,32] A number of clinical trials have shown that the clinical performance of Cervista HPV HR test is at least comparable to that of HC2 and the cross-reactivity to other HPV types is lower.[8,32–39]

2.3 | Cobas 4800 HPV test

Cobas 4800 HPV test (Roche Molecular Diagnostics, Pleasanton, CA) was approved by the FDA in 2011. The test is intended to use as a reflex test for ASC-US cervical cytology results in patients 21 years and older and co-testing in conjugation with routine cervical cytology testing in patients 30 years and older, In addition, this test can be used as a genotyping test to detect HPV 16 and 18 in above patients. In women 25 years and older, this test can also be used (in specimens collected only in ThinPrep PreservCyt Solution) as a first-line primary cervical cancer screening test to detect hrHPV, including genotyping for 16 and 18.[40] The samples can be collected in either PreservCyt solution or cobas PCR cell collection medium. This test uses multiplex real-time PCR and nucleic acid hybridization with different reporter probes to concurrently detect the L1 gene of HPV 16 and 18 as individual reactions and other hrHPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) as a pooled result.[8] This test includes β-globin as internal control and is automated using cobas 4800 system that can be interfaced with laboratory information systems. The analytical sensitivity of the test per the manufacturer report is 150 to 2400 genome copies/ml.[40] Studies show that cobas 4800 test has clinical sensitivity comparable to that of HC2 and better specificity due to lower cross-reactivity with low-risk HPV types.[8,41,42]

2.4 | Aptima HPV assay

Aptima HPV assay (Hologic Gen-Probe, Inc., San Diego, CA) was approved by the FDA in late 2012. This is an mRNA-based test for detection of E6/E7 mRNA transcripts of 14 hrHPV types. The intended use is to screen patients 21 years and older with ASC-US cytology results and co-testing in conjunction with routine cytology test in women 30 years and older.[43] Aptima HPV 16 18/45 genotype assay is intended to use in the above patients with positive Aptima HPV assay results to assess the presence or absence of HPV genotypes 16, 18, and/or 45.[44] There is very little expression of E6/E7 mRNA in transient HPV infection and there is overexpression of E6/E7 mRNA in persistent infections. Therefore, comparing to the DNA-based tests, detection of overexpression of E6/E7 mRNA may be more directly associated with disease progression. Cervical samples can be collected either in PreservCyt solution or Aptima cervical specimen collection medium. The Aptima assay is performed in a single tube. It uses complementary oligomers to isolate the target mRNA onto magnetic microparticles. The captured mRNA is then amplified using transcription-medicated amplification (TMA). The amplified products are eventually detected by hybridization protection assay using chemiluminescent labels. A noninfectious RNA transcript serves as an extrinsic process control. The test is fully automated on either the Panther system or the Tigris DTS system.[8] The analytic sensitivity of this test is high with limit of detection of 24 to 488 copies per reaction.[45] In addition, the Aptima test shows no cross-reactivity with any tested low-risk HPV types and, therefore, has significantly higher specificity for high-grade lesions than the HC2 test.[8,45–51]

2.5 | BD onclarity HPV assay

BD Onclarity HPV assay (Becton Dickinson, Franklyin Lakes, NJ) was most recently FDA approved in early 2018. The test is intended to

use as a reflex test for ASC-US cervical cytology results in patients 21 years and older and co-testing in conjugation with routine cervical cytology testing in patients 30 years and older. In addition, this test can be used as a genotyping test to detect HPV 16, 18, and 45 in above patients. In women 25 years and older, this test can also be used as a first-line primary cervical cancer screening test to detect hrHPV, including genotyping for 16 and 18.[52] This test utilizes real-time PCR, targeting the E6 and E7 DNA regions of the HPV genome, followed with nucleic acid hybridization to detect 14 hrHPV types. The assay harbors an internal human β-globin control for sample adequacy. The test is fully automated and is performed on the Viper Lt platform. The sample can be collected in BD SurePath Preservation Fluid Collection Vial for this assay. The analytic sensitivity of this assay is 251 genome copies/ml for HPV 16 and 814–2367 genome copies/ml for other types.[52] The clinical performance of the BD Onclarity assay is comparable to that of the HC2 test in various clinical studies.[9,53–55]

2.6 | HPV genotyping

It has been known that the majority of invasive cervical cancers worldwide are associated with HPV 16 or 18.[8] Large trial studies suggest that by detection of certain individual HPV genotypes, especially HPV 16 and 18, a subset of women who have a markedly greater risk of having CIN2 or worse can be identified.[8,41,56–61] HPV 16 was shown to provide the biggest risk stratification, whereas the additional benefit of testing for HPV 18 and 45 was limited. In addition, the prevalence of HPV 16 and 18 is rapidly decreasing in populations who are being vaccinated.[8,62–67] HPV type replacement (with HPV 51, 52, 53, 56, and 58) is occurring. There is, therefore, a need for HPV genotyping tests to detect additional HPV types other than HPV 16 and 18. Currently the genotyping test is used to identify the presence or absence of HPV 16 and/or 18 in women 21 years of age and older who have ASC-US cervical cytology results. Genotype results in this population can be used to guide management along with other information. The second application is to evaluate women 30 years of age and older who are tested positive for hrHPV but have normal corresponding cervical cytology results.[8] The current recommendation is that women in this population with positive HPV 16 and/or 18 should be referred to colposcopy, while women with negative HPV 16 or 18 have repeat cytology and hrHPV testing in 12 months.[15,17]

HPV genotyping tests are done by targeting a type-specific DNA sequence to amplify a single genotype of HPV. There are several HPV genotyping tests that are commercially available on the market. Cobas HPV test (Roche Molecular Diagnostics, Pleasanton, CA) is a real-time PCR that can identify HPV 16 or 18 in addition to detection of 14 hrHPV types as a group. The analytical sensitivity of Cobas HPV test is 600 copies/ml for both HPV 16 and 18.[40] Cervista HPV 16/18 test (Hologic, San Diego, CA) uses the same Invader chemistry as the corresponding hrHPV screening test described above but includes oligonucleotide probes that bind specifically to target sequences of HPV 16 and 18.[8] The analytical sensitivity of the HPV 16/18 genotyping test is 625 to 1250 copies per reaction for both types.[68] One limitation of this test is cross-reactivity with high levels of HPV 31. Another genotyping test is the Aptima HPV 16 18/45 genotype assay (Hologic Gen-Probe, Inc., San Diego, CA) which was approved by FDA in late 2012 for use with the Tigris DTS system and in 2013 for use with the Panther system to identify RNA from HPV 16, 18, and 45. This test includes HPV 45 genotype which is the third most common HPV type associated with invasive cervical cancers in some series as well as 12% of adenocarcinoma.[8,69] The incidence of adenocarcinoma has risen approximately 32% despite the incidence of cervical cancer has been decreasing in the past few decades.[8,70] The analytical sensitivity of Aptima HPV 16 18/45 genotype assay reported by the manufacturer is 57.3 copies per reaction for HPV 16, 84.8 copies per reaction for HPV 18, and 60.0 copies per reaction for HPV 45.[44] BD OnClarity HPV test is a real-time PCR test that reports individual results for 6 of 14 hrHPV genotypes (16, 18, 31, 45, 51, 52) and grouped results for the remaining eight hrHPV genotypes. The analytical sensitivity of the BD Onclarity HPV assay reported by the manufacturer is 251 copies/ml for HPV16, 1083 copies/ml for HPV18, and 1261 copies/ml for HPV45.[52]

2.7 | Other ancillary molecular tests

Most of the cervical cancer screening guidelines are considering reflex cytology a valuable triage tool for hrHPV-positive women. Nevertheless, there are up to 8% of hrHPV-positive women with normal cytology have or will develop in the subsequent years a CIN2+ lesion. Therefore, there is a strong need for biomarkers that may allow risk stratification of hrHPV-positive women with normal cytology.[71–74] Several ancillary techniques are potentially useful in triaging hrHPV-positive women to increase the specificity of the test. Other than HPV genotyping that was described above, other molecular markers include those involved in cell cycling (overexpression of p16, HPV viral load, and integration) and those related to epigenetic change (methylation of human and viral genes, and microRNA).[71,75] These tests can be applied in cervical cytology samples. Although it is promising, most of these molecular tests are still in the research study phase and need clinical validation.[71]

2.8 | HPV testing in other non-gynecologic specimens

Other than cervical cancer, HPV is known to cause other types of cancer, including oropharyngeal squamous cell carcinoma (OPSCC) and anal cancer. Studies have shown that most OPSCC are mediated by HPV 16 (76–95%), followed by HPV 18 (1%–8%) with HPV 33, 35, 56, 58, and 67 found infrequently.[76–78] HPV testing in the head and neck region is important for the following reasons. (1) HPV testing can be a prognostic indicator as HPV-positive tumors have better clinical outcomes compared to the conventional SCC in the head and neck region. (2) HPV testing can help with localization of the primary site of

tumor in patients with metastatic disease. (3) HPV testing can help predict distant metastases as HPV-negative and HPV-positive tumors tend to metastasize in different fashions. (4) HPV testing can help with patient management as patients with HPV-positive OPSCC can be treated less aggressively than smoking-related tumors.[8] Even though the testing is suggested, there are no recommendations as to the types of tests to be used to identify HPV-associated OPSCC. Furthermore, there are no FDA-approved molecular tests for OPSCC screening in the United States. Any commercially available test approved for cervical cytology could be validated by a clinical laboratory to detect HPV in oropharyngeal biopsy specimens.[8] Currently HPV DNA in-situ hybridization (ISH) and p16 immunohistochemistry (IHC) are the most useful tests. HPV DNA ISH has been found to be highly specific but not entirely sensitive, and p16 IHC as a surrogate marker of transcriptionally active HPV infection has been found to be highly sensitive but not entirely specific.[8] DNA ISH assays for hrHPV allow for direct visualization of the virus in the nuclei of tumor cells in the tissue, better demonstrating HPV as a causal agent. Mixture of type-specific probes in a single reaction mixture to cover an extended range of HPV types are available. Tumor suppressor protein p16 is upregulated in HPV-related tumor as a result of transcription of the E6, E7, and/or E5 viral oncogene.[79] P16 IHC alone is the most useful prognostic indicator for patients with known OPSCC, regardless of HPV status.[80,81]

Squamous cell carcinoma of the anal canal is clinically and histologically similar to that of the cervix. The most common HPV types that are linked to anal cancer include HPV 16, 6, 42, 18, 11, 31, and 52. HPV 16 is found in 85% of anal cancer and precancerous lesions.[82–85] Similar to the cervical cancer, the high-grade anal intraepithelial neoplasm (AIN) is a precursor to invasive anal cancer. Many experts have advocated for routine screening to detect precancerous anal lesions in high-risk individuals. However, there are currently no formal recommendations in the United States for routine screening in the general population or for any subgroup.[8] Cytologic screening for precancerous anal lesions can be done using Papanicolaou-stained smears. The criteria used to evaluate anal cytology smears are similar to those for cervical cytology. Testing for HPV has limited utility for anal cancer screening because of the high prevalence of HPV and presence of multiple HPV types in the anal canals of the high-risk population.[8]

3 | MOLECULAR TESTING IN CEREBROSPINAL FLUID

There are many infectious agents that can cause infections of central nervous system (CNS). Patients with CNS infections can show different clinical signs, such as myelitis, encephalitis, meningitis, or cerebral abscesses, that are associated with different micro-organisms, including bacteria, fungi, and viruses.[86] It is critical to make the accurate diagnosis and to detect the organisms in a timely fashion so that patients can be properly managed. Almost all acute infections of the CNS produce some degree of meningeal as well as parenchymal inflammation.[87] The CSF is usually abnormal in the majority of cases with CNS infections.[87] For example, the CSF typically consists of a lymphocytic pleocytosis, mildly elevated protein, and normal glucose in >90% of cases with viral infections.[87] There are over 100 viruses that are known to cause acute viral encephalitis in humans.[87,88] The PCR technique has been widely used for detection of both DNA and RNA viruses in the CSF. Less commonly used nucleic acid amplification techniques that have been applied in the CSF includes nucleic acid sequence-based amplification (NASBA) and branched-DNA assay.[87,89] Qualitative and quantitative PCR tests can detect as few as 1 to 10 copies of target DNA from the original sample, identify etiologic agents in quantitation of viral load to monitor duration and adequacy of antiviral drug therapy, differentiate productive viral infection versus postinfectious immune-mediated disease as the nature of relapses following viral infections, and identify determinants of drug resistance.[87] Compared with traditional culture or serology tests, molecular testing with PCR has advantages. PCR is ideally suited to identify fastidious organisms that may be difficult or impossible to culture. In addition, PCR test can be performed rapidly and inexpensively with a turnaround time of 24 hours or less rather than the standard of 1 to 28 days required for culture. CSF PCR is more sensitive compared to culture, especially in patients who have been treated with antiviral drugs.[87]

Most CSF PCR test assays can be run with a minimum of 30 ul of sample even though 100–200 ul may yield better results. A freshly obtained CSF sample is preferred for molecular testing.[87] Nuclei acids in CSF can be extracted by simple methods such as exposure to high temperature or repetitive freeze-thawing, or preferably nucleic acid extraction and purification techniques, such as phenol-chloroform or spin column-based techniques which provides pure nucleic acid along with removal of potential inhibitors of PCR reaction.[87]

3.1 | Herpes simplex virus

Herpes simplex virus (HSV) is a large, enveloped DNA virus belonging to the *Herpesviridae* family with two subtypes, HSV-1 and HSV-2. HSV encephalitis is the most common cause of acute sporadic focal encephalitis in both the United States and the Western world. The majority of adult cases result from HSV-1, with the remainder due to HSV-2.[87,90] In cases of HSV encephalitis, the virus is only cultured from CSF in fewer than 5% of cases.[87] In comparison, multiple studies have shown that the sensitivity of CSF PCR test for HSV DNA ranges from 75% to 98%, a specificity of 94%–100%, a positive predictive value (PPV) of 95–100%, and a negative predictive value (NPV) of 98%.[87,91–93] The possible causes for false negative results are inclusion of compounds in the CSF that can be potentially inhibitory for PCR reactions, such as porphyrin compounds derived from the degradation of heme in erythrocytes.[87,91,94,95] The incidence of false-positive CSF PCR results for HSV infection is exceedingly low. Isolated cases of false-positive results have been documented due to improper procedures resulting in sample-to-sample contamination.[87,96]

3.2 | Enterovirus

Enteroviruses are RNA viruses of the *picornavirus* family. Enteroviral encephalitis occurs as either a focal or a diffuse process and is the third most commonly identified cause of encephalitis, after HSV and arboviruses.[87,97] The enteroviral RT-PCR test allows rapid detection (<24 h) of enterovirus in CSF compared to the 4–8 days that are required for culture.[87,98,99] Studies show a sensitivity of 96%–100% and a specificity of 96%.[87,100–103] The sensitivity of enteroviral RT-PCR test greatly exceeds that of culture, and it has replaced culture as the gold standard for detection of enteroviral infection of the CNS.[87] In addition, the sensitivity of CSF RT-PCR is higher than that of serum RT-PCR (81%–92%) and urine RT-PCR (62%–77%).[87]

3.3 | JC virus

JC virus is a type of human polyomavirus and is the causative agent of progressive multifocal leukoencephalopathy (PML), a demyelinating CNS infection primarily affecting patients with acquired immunodeficiency syndrome (AIDS). CSF PCR for JCV has a sensitivity of 50%–75% and a specificity of 100% for the diagnosis of PML in human immunodeficiency virus (HIV)-infected patients with focal neurologic signs and symptoms.[87,104,105]

3.4 | Epstein–Barr virus

Epstein–Barr virus (EBV), also known as human herpesvirus 4, is a double-stranded DNA virus belonging to the *Herpesviridae* family. EBV has been implicated in both immunocompetent and immunocompromised patients. CSF PCR has been extremely useful in diagnosis of EBV infection of the CNS.[87,106–108] CSF PCR is not positive in patients with latent EBV infection. EBV infection is associated with AIDS-related CNS lymphomas in HIV-infected patients. Patients with these conditions often have positive EBV CSF PCR results and this test appears to be a sensitive indicator for the presence of the neoplasm, with nearly 100% sensitivity and 98.5% specificity.[87,109–114] In addition, PCR has also been used to monitor the response to therapy in patients with AIDS-related CNS lymphomas.[87]

3.5 | Cytomegalovirus

Cytomegalovirus (CMV) is a double-stranded DNA virus and is a member of the *Herpesviridae* family. CMV meningoencephalitis is largely a disease of congenitally infected newborns and immunocompromised individuals. CSF PCR test has proven to be a very useful technique to detect CMV infection in CSF in immunocompromised patients. The sensitivity is 82%–100% and the specificity is 86%–100% in HIV-infected patients.[87,115–119] Quantitative CMV PCR is available in a number of laboratories and has been shown to provide a better index of disease severity.[87,120–122] In addition, CMV PCR in CSF has been utilized to identify the presence of antiviral-resistant strains from patient isolates.[87,118,123] As with EBV PCR, CMV-seropositive individuals with latent infection do not have detectable viral DNA based on PCR testing.[87]

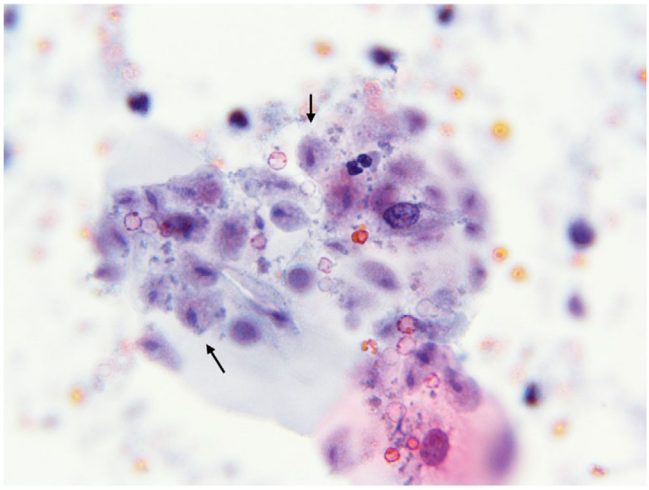

FIGURE 1 Incidental finding of *Trichomonas vaginalis* in a male voided urine sample submitted for high grade urothelial carcinoma screening. Numerous microorganisms are present on top of benign squamous cells present in the specimen (x600).

3.6 | Other micro-organisms

CSF PCR testing has been utilized to detect other organisms but these tests are not being widely used mostly due to the limited sensitivity of these tests. However, PCR test can be used to help with diagnosis in certain situations.[87] For example, the diagnostic sensitivity of PCR in CSF for detection of *Borrelia burgdorferi*, the causing agent for Lyme disease, is only 17%.[87] Serologic detection of intrathecal IgM is routinely used as the diagnostic method of choice, because specific intrathecal antibody production can be found in 90% of the patients with neuroborreliosis. However, IgM may not be present at the time of clinical symptoms. In patients with a short duration of disease (<14 days), CSF PCR may be a useful diagnostic method.[87] In regards to *Toxoplasma* infection, serologic detection methods are the standard for clinical diagnosis. However, PCR test has been applied for prenatal diagnosis of congenital toxoplasmosis and the detection of acute disease in immunocompromised patients.[87] Early detection of *Mycobacterium tuberculosis* meningitis (TBM) by PCR and other molecular tests has been attempted with mixed success. Most studies show that sensitivity for detection of TBM by PCR testing is low (33%–91%) despite the high specificity (88%–100%).[87,124–126]

4 | MOLECULAR TESTING IN URINE SPECIMENS

4.1 | Trichomonas vaginalis

Trichomonas vaginalis (TV) is the protozoa causing Trichomoniasis, a usually asymptomatic and treatable sexually transmitted disease

affecting approximately 3.7 million persons in the United States. (Figure 1) Females are the most affected patients.[127] The symptomatic patients present with foul-smelling vaginal or penile discharge, itchiness, dysuria and/or irritation. Irritation of the uterine cervix ("strawberry cervix") is particularly important since it predisposes to HIV co-infection. Wet mount test remains as the most inexpensive and rapid test for TV detection in vaginal, cervical, or penile discharge; however, its sensitivity remains low (44%–68%).[128] Before the molecular era, the gold standard method for diagnosis was culture with higher sensitivity and specificity than the wet mount test. Unfortunately, the samples might have cross-contamination with the vaginal flora and it is time consuming.[129] Currently, the gold standard test for TV diagnosis is nucleic acid amplification test (NAAT) with superior sensitivity and specificity. The FDA has approved several NAAT for the diagnosis of trichomoniasis. *Aptima Trichomonas vaginalis assay*, a ribosomal RNA (rRNA) detection test via transcription-mediated amplification and hybridization, was the first approved NAAT assay for this purpose in females, with a sensitivity of 95.2%–100% and specificity of 98.9%–99.6%. The approved specimens for this test are vaginal and endocervical swabs, ThinPrep and urine samples. *BD MAX CT/GC/TV assay* is another approved test for TV diagnosis, which is also capable of simultaneously detect co-infection with *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) in females. This assay is based on DNA extraction followed by amplification by real-time PCR, and has a high sensitivity (92.9%) and specificity (96.1%). Urine, vaginal and endocervical swabs are the accepted specimens in females. This same assay has been also approved for NG and CT diagnosis only in males using urine specimens. Both Aptima and BD MAX CT/GC/TV assays give results within 8 h. Another FDA cleared NAAT for TV diagnosis is the DNA based *Solana Trichomonas assay*. This test uses only vaginal swabs and urine samples from females and gives results in 30 min. The downside of this test is the requirement of specific Helicase-Dependent Amplification (HAD) technology and Solana instrument for operation. Another revolutionary NAAT is the *Xpert TV assay*, since urine from both females and males are acceptable specimens for TV diagnosis. Vaginal and endocervical swabs are also valid samples. *Solana and Xpert TV assay* are the fastest NAAT assays for TV diagnosis with a turnaround time of 30–35 min. Lastly, *Affirm VP III* and *AmpliVue Trichomonas* assays are also FDA cleared tests for TV detection. The first one is a nucleic acid probe test with turnaround time of 45 min, while the second one is a NAAT with results in 50 min. Neither works in urine samples, and only vaginal swabs have been validated as acceptable specimens.[130–134]

4.2 | Polyomavirus (JC and BK virus)

Polyomavirus is a subgroup of non-enveloped double stranded, circular, and super-coiled viruses belonging to the *Papovaviridae* family. The viral structure consists of a non-coding regulatory region (NCCR), early and late coding regions. The NCCR regulates the transcription of the early and late coding regions. The early coding region encodes the large "T antigen," associated with the infection process, and the small "t antigen." The late coding region encodes the VP1, VP2, VP3 and agnoprotein viral proteins. The VP1 is the major capsid protein.[135] The nomenclature comes from the patients name initials where the viruses were originally detected. BK and JC virus were the first isolated Polyomaviruses in humans in 1971. The first one was found in the urine from a patient with renal transplant and ureteral stenosis, while the second one was identified in a patient with clinical history of Hodgkin's lymphoma and PML. BK virus primary infection usually occurs during early childhood and remains latent in the renal tubular epithelial cells. JC virus primary infection usually occurs during late childhood, between 10 and 14 years, and can remain latent in tonsils, bone marrow, brain, and renal tubular cells. When polyomaviruses are detected beyond the period for primary infection (older age), most likely represents a reactivation process. Immunosuppressed status, such as kidney or bone marrow transplants, high pre-transplant virus seropositivity, anti-BK virus antibodies, and older age, are major risk factors for reactivation and disease development.[136] BK virus reactivation has been linked with non-hemorrhagic and hemorrhagic cystitis in allogenic bone marrow transplant, asymptomatic hematuria, tubulointerstitial nephritis (known as BK virus associated nephropathy or BKVAN) and ureteral stenosis resulting in kidney transplant failure. Increased risk of prostate and bladder cancer has been also associated with BK infection.[137] On the other hand, JC virus can cause PML in patients treated with immunosuppressive therapy and HIV.[138] In voided urine, the infected urothelial cells, known as Decoy cells, have an increase nuclear to cytoplasmic ratio with occasional eccentric nuclei, mimicking high-grade urothelial carcinoma. A total or four types of Decoy cells have been described based on the virus cytopathic effect. Type 1, the most common one, shows a large ground glass nuclear inclusion with a dense chromatin rim. (Figure 2) Type 2 mimics CMV infection due to granular nuclear inclusion surrounded by an incomplete irregular halo. Decoy cells type 3 demonstrate a granular chromatin pattern and sometimes are multinucleated. Type 4 Decoy cells demonstrate vesicular nucleus with coarse and crumpled chromatin and at times prominent nucleoli.[139] Quantitative PCR (qPCR) plays a major role in diagnosis and surveillance. The early detection of viremia and viruria helps to reduce the risk of transplant failure. PCR is a highly sensitive screening method (100% for urine and plasma) that allows viral load quantification, differentiates between BK and JC virus infection, is not affected by interpreter experience, and expedites turnaround time.[140] Plasma qPCR instead of urine qPCR has been proposed in the guidelines as the preferred screening method in transplant patients, since the specificity (45% for urine and 66% for plasma which can increase to 90% when viral load is >10,000 copies/ml of blood), positive predictive value (low in urine and high in plasma), and negative predictive value (high in urine and low in plasma) are superior. Additionally, only a third of patients with BK viruria actually develop viremia or BKVAN.[141] Kalorinska Institute Polyomavirus (KI), Washington University Polyomavirus (WU) and Merkel cell polyoma (MCP) are also clinically relevant polyomaviruses for humans KI and WU were identified in respiratory secretions from symptomatic patients in 2007,[142] while Merkel cell polyomavirus (MCP) was isolated in 2008. Approximately 80% Merkel cell

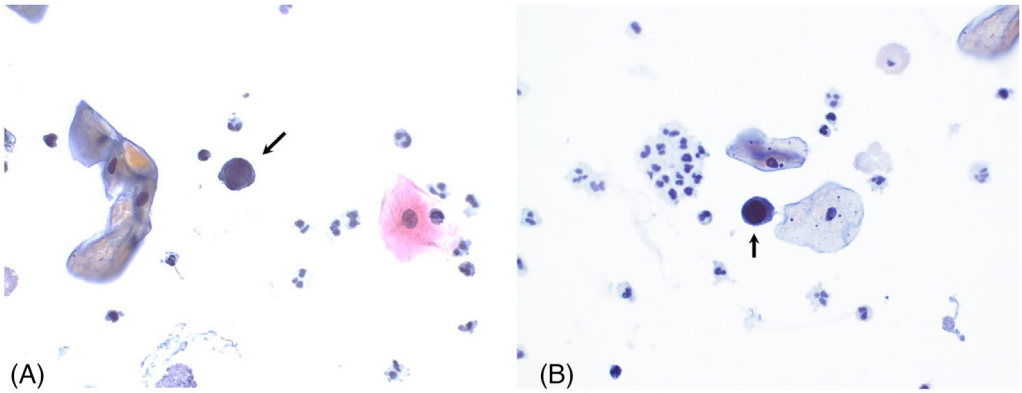

FIGURE 2 Polyomavirus in a voided urine sample. Isolated cells with increased nuclear cytoplasmic ratio, ground-glass nuclear opacities and a dense chromatin rim, compatible with a Decoy cell type 1 (x600).

carcinoma cases have MCP integrated into the host genome.[143] KI and WU do not have significant viral cytopathic effect in cytology samples, while Merkel cell carcinoma is commonly first diagnosed in histologic specimens.

5 | MOLECULAR TESTING IN RESPIRATORY TRACT SAMPLE

5.1 | Pneumocystis jirovecii

Pneumocystis jirovecii, the pathogen causing the life-threatening Pneumocystis pneumonia (PCP), is an ascomycete originally classified as a protozoan and called *Pneumocystis carinii*. It was reclassified as a fungus in 1988 after the small subunit ribosomal RNAs (16 S-like rRNAs) sequence demonstrated similarities with the fungi kingdom.[144] *Pneumocystis jirovecii* remains as the most common opportunistic infection in patients with HIV without antiretroviral therapy or prophylaxis and when CD4 count is <200 cells/ml.[145] PCP symptoms include low-grade fever, non-productive cough, and dyspnea. Bilateral pulmonary infiltrates are identified by imaging studies.[146] *P. jirovecii* has two life forms denominated cystic and trophozoite life-forms.[147] The trophozoite, which originates from the cyst and contains up to eight intracystic bodies, interacts with the host pneumocytes, making it a near obligate alveolar pathogen.[148,149] Multiple diagnostic methods are currently available for *Pneumocystis jirovecii* detection in bronchoalveolar lavage fluid (BALF), sputum, bronchial washing and even tissue samples. Before describing current molecular tests, the classic and non-molecular methods are imperative to be mentioned since they are the guidelines-recommended first steps for diagnosis. One of the most common tests in cytology is the direct microscopic examination of BALF and detection of microorganisms by conventional staining methods. The cup-shape *P. jirovecii* cysts are visualized within a characteristic foamy proteinaceous sphere (alveolar casts) on Grocott-Gomori's methenamine silver stain (GMS). Giemsa, Diff-Quik, and Wright stains highlight cyst and trophozoite forms. Papanicolaou stain is negative.[146] (Figure 3) The sensitivity and specificity of this method relies on the quality of the sample, the pathogen load, and experience of the cytotechnologists and cytopathologists reviewing the case. Another staining method is the direct fluorescent antibody (DFA) test. DFA has a higher sensitivity (91%) than GMS stain. In HIV-positive patients, expectorated sputum is the preferred specimen.[150] Other validated non-molecular methods for *Pneumocystis jirovecii* infection detection are the biomarker assay detecting the cell wall component 1,3-β-D-Glucan (the only FDA approved method), flow cytometry, and antibody assays by enzyme-linked immunosorbent assay (ELISA). Regarding NAAT assays, qPCR, and loop-mediated isothermal amplification (LAMP) are the available diagnostic tools in the market. Unfortunately, no PCR method for *Pneumocystis jirovecii* diagnosis has yet been FDA approved. PCR is highly sensitive (97%–99%) and specific (90%–94%) for detecting *Pneumocystis*; however, it cannot distinguish between colonization from actual disease. LAMP has 87.5%–95.4% sensitivity and is the PCR alternative. As the name implies, the nuclear acid amplification occurs rapidly under isothermal conditions using four primers recognizing six specific regions. Moreover, the whole process time can be expedited by adding two primers known as the loop primers. Overall, in comparison with PCR, LAMP is more cost-effective due to unnecessary expensive equipment and lacks of cross-reaction with other fungi present in the samples.[151–153] Current guidelines recommend the performance of DFA or NAAT assays to confirm or rule out *Pneumocystis jirovecii* infection after the levels of 1,3-β-D-Glucan in BALF or serum are elevated.[154]

5.2 | Angioinvasive molds

Pan-fungal PCR assays, using universal fungal primers, became an important tool for the rapid diagnosis of invasive fungal disease with high specificity and sensitivity. BALF, CSF, fresh and formalin-fixed paraffin embedded tissue (including cell blocks), blood, and vitreous fluid are valid specimens for these tests.[155] This section will cover *Aspergillus spp* and *Mucorales* since these are the most common angioinvasive molds causing pulmonary disease. PCR for invasive aspergillosis (IA) allows identification and quantification of *Aspergillus*

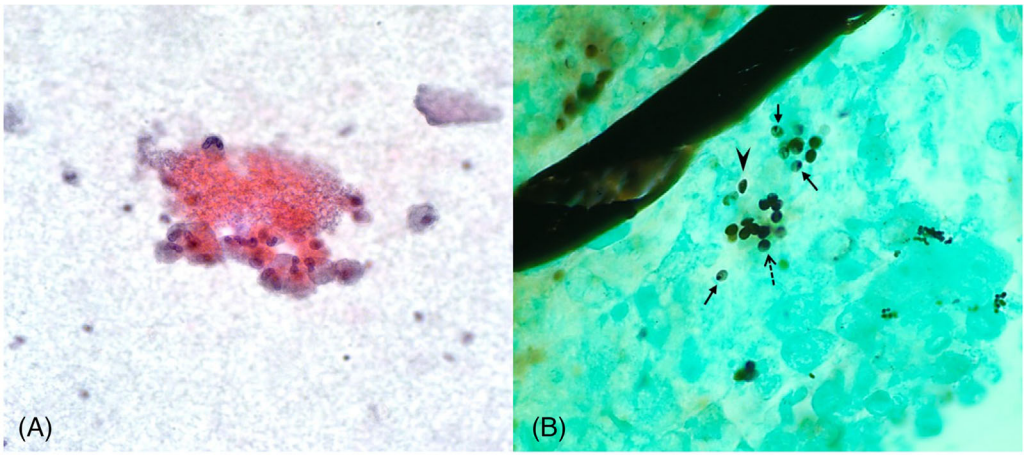

FIGURE 3 Bronchoalveolar fluid from HIV positive male without antiretroviral therapy (case diagnosed in 1991). (A). *P. jirovecii* foamy proteinaceous sphere (Papanicolaou stain, x600); (B). *P. jirovecii* cup-shape cysts (arrowhead and incomplete arrow) and trophozoites within the cysts (complete arrows), (GMS stains, x600).

DNA in serum and BALF. Two positive tests are needed in order to report positive results.[156] Due to the high negative predictive value of this assay (94%–96%), a negative result allows to rule out IA. Unfortunately, PCR cannot differentiate between real invasion and colonization when non-sterile specimens are utilized deceasing the positive predictive value of the assay. This disadvantage makes non-molecular methods such as antigen detection (Galactomannan and 1,3-β-D glucan) more clinically relevant.[157] From all the commercially available PCR-based assays, only MycAssay Aspergillus and AsperGeinus have been recommended for routine testing of respiratory samples.[158]

Immunocompromised patients, especially those with poorly control diabetes mellitus, are always at high risk for invasive fungal disease caused by *Mucorales*. Conventional and real-time PCR assays for *Mucorales* DNA detection for clinical purposes have been proposed in the literature.[159] However, to the date no FDA approved tests are available for clinical use.[160]

5.3 | Blastomyces dermatitidis and histoplasma capsulatum

Blastomyces dermatitidis and *Histoplasma capsulatum* are dimorphic fungi causing blastomycosis and histoplasmosis respectively. Cultures remain as the gold standard diagnostic methods. There is a currently available real-time PCR test for rapid and simultaneous detection of both fungal DNA. Fresh tissue, CSF, BALF, bone marrow, and other body fluids but urine are acceptable specimens for this assay.[161,162]

5.4 | Mycobacterium tuberculosis

Mycobacterium tuberculosis (MTB) is the aerobic and acid-fast bacteria causing tuberculosis (TB). Its unique characteristic is the presence of mycolic acid in the cell wall, making it very difficult to treat. The development and utilization of accurate diagnostic techniques such as NAAT have become a critical clinical tool capable of addressing the challenges posed by multi-drug resistance organism. Current molecular methods for MTB diagnosis and multi-drug resistance characterization include line probe assay (LPA), LAMP, *Xpert MTB/RIF*, and whole genome sequencing (WGS)[163] LPA was the first molecular tests recommended by the WHO in 2008 for the diagnosis of MTB, and rifampin and isoniazide drug-resistance mutations.[164] This method consists of DNA amplification by PCR with biotinylated primers. Once the target DNA binds to the correct probe, a colorimetric indicator is observed. *GenoType MTBDRplus (version 1.0)* and *INNO-LiPA* are part of the LPA first generation, and they are useful if the smear is positive. These two assays are commercially available,[165] but have been replaced in clinical practice by newer generations. The LAMP-PCR method is good for the diagnosis of active tuberculosis with a specificity and sensitivity of 97–98% and 76–80% respectively.[166] *Xpert MTB/RIF* is an automated amplification assay that uses real time multiplex PCR and is good for detection of active TB and rifampicin resistance. Its FDA approval is only for sputum from non-treated patients. The method consists of mixing the sputum with the specific reagent provided by the assay and then placing the mixture in a disposable cartridge that goes into the GeneXpert Instrument System, whit results in 90 minutes. Xpert has high specificity even in cases with negative smears. The sensitivity is affected by the smear results.[167] Lastly, the WGS method is based on next generation sequencing and the advantages are the detections of all the mutations with their functional categorization and possible MTB resistance to new drugs.[168]

5.5 | Multiplex PCR respiratory viral panel assays

Community-acquired respiratory viruses are common causes of respiratory infection in immunocompromised patients. Diagnosis of these

viral infections is best accomplished with one of the available multiplex PCR respiratory viral panel (RVP) assays in respiratory samples. These RVP assays are able to simultaneously evaluate for molecular targets of multiple viruses and have shown increased sensitivity over shell viral cultures and viral direct fluorescence antibody studies.[169] There are four major FDA-approved respiratory multiplex assays: Luminex NxTAG Respiratory Pathogen Panel (RPP), Nanosphere Verigene Respiratory Panel (RP) Flex, BioFire Film Array Respiratory Panel (RP), and sensor Respiratory Viral Panel (RVP).[170] The commonly tested viruses in these panels include adenovirus, human metapneumovirus, influenza A, B, parainfluenza, RSV, and rhinovirus/enterovirus, among others. These assays are all intended for use with nasopharyngeal (NP) swabs. Clinical laboratories may also validate other specimen types such as BALF to run off-label.[171] One study compared analytical performance of the BioFire FilmArray RP, GenMark eSensor RVP, Luminex xTAGv1, and Luminex RVP FAST. The study showed that the eSensor RVP had the highest sensitivity (100%) for nearly all targets (rhinovirus/enterovirus, 90.7% the only exception). Low sensitivity for adenovirus was a weakness for most assays: FilmArray RP, 57.1%; xTAG RVPv1, 74.3%; xTAG RVP FAST, 82.9%; and eSensor RVP, 100%. Detection of influenza B was also difficult: xTAG RVP FAST, 45.5%; FilmArray RP, 77.3%; xTAG RVPv1, 95.5%; and eSensor RVP, 100%.[172]

5.6 | Coronavirus

In 2020, the World Health Organization (WHO) recognized coronavirus as the pathogen causing the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), and the outbreak was named Coronavirus Disease-2019 (COVID-19).[173,174] Coronavirus is an enveloped single-stranded positive-sense RNA virus part of subfamily Coronavirinae (Betacoronavirus 2B linage) in the *Coronaviridae* family.[175] The nucleocapsid protein (N) forms the capsid, while the genome is contained in an envelope associated with the structural protein spike (S), membrane (M), and envelope (E). The S protein grants the entrance of the virus into the host cells. Additional 16 viral non-structural proteins were recognized.[176–179] Overall findings in BALF specimens from patients with SARS-CoV-2 are non-specific, including edema, increase neutrophils and macrophages infiltration in critically ill patients, necrosis, and exfoliation of alveolar epithelial cells. Histopathologic findings are similar, in addition to alveolar septal widening, and damage of alveolar septa and pulmonary interstitial arteriolar walls.[180] Some viral particles have been identified in the cytoplasm of epithelial cells and macrophages by electron microscopy only.[181–183] NAAT for viral genome amplification, viral antigen and antibody tests are the FDA approved methods for COVID-19 detection. The NAAT category includes PCR, LAMP, recombinase polymerase amplification (RPA), and regularly interspaced short palindromic repeat (CRISPR)-based assays. Nasopharyngeal swabs are the most commonly submitted sample in the non-admitted population due to the easiest access to the nasopharyngeal tract, are less invasive than BALF, and have more viral loads. In critically ill patients, BALF is the preferred sample for diagnosis. Real-time reverse transcriptase quantitative polymerase chain reaction (rRT-qPCR) is highly specific and is currently the most reliable method for diagnosis. It usually takes few hours for results and requires the creation of a DNA template by a reverse transcriptase for later amplification. The reverse transcription loop-mediated isothermal amplification (RT-LAMP) is faster than the PCR assay as we previously described, since it synthesizes up to 109 copies of the targeted gene in less than an hour. It also has a high sensitivity and specificity for SARS-CoV-2. RPA detects DNA sequences through a recombinase enzyme that amplifies viral genes. RPA turnaround time may take hours like PCR. Lastly, CRISPR-based diagnostics uses a highly specific targeting and cleaving action of CRISPR-Cas systems to identify and cut a specific part of SARS-CoV-2 RNA sequence, generating a visual signal if the sample is positive for coronavirus.[184,185]

6 | CONCLUSIONS

Cytopathology plays the critical role in diagnosis of infectious diseases. Cytology samples are usually easy to obtain with minimally invasive procedures. The application of molecular diagnostic testing in cytology samples can provide improved and more specific diagnosis of infectious diseases and in some cases more rapidly for life saving treatment. It is essential for cytopathologists to understand what the clinical indications for molecular testing of infectious diseases are, what cytology samples can be used in molecular testing for infectious diseases, and the advantages and the limitations of each diagnostic test has. The effort was made in writing this article to address the above questions in a comprehensive way. We acknowledge that we are unable to cover all molecular diagnostic tests in all cytology samples. Our hope is to make the readers understand the commonly used molecular testing methods in commonly sampled cytology specimens in daily practice.

AUTHOR CONTRIBUTION

Carla R. Caruso contributed to conceptualization, writing, reviewing and editing. Zhongbo Yang contributed to supervision, conceptualization, writing reviewing & editing.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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