

# Advanced Pathology Techniques for Detecting Emerging Infectious Disease Pathogens



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## Introduction

Detection and surveillance for emerging and reemerging pathogens require a multi-disciplinary approach. The intertwining complexity of these pathogens with their diverse tissue tropisms, direct effects on host cells, multiphasic immunological responses, and additional influence of superimposed secondary agents is beyond the expertise of any single discipline in modern medicine. A combined evaluation of patient's history, clinical manifestations, and physical examination may suggest a list of differential diagnosis, but it is often insufficient to determine the specific infectious etiology. Laboratory methods are essential to identify an etiologic agent from testing clinical samples, such as blood, serum, nasopharyngeal swab, etc. These methods, including traditional microbiological techniques, conventional immunological assays, and modern molecular methods, remain the mainstay in today's practice of clinical microbiology and infectious disease medicine. Nevertheless, there are technical and logistic issues associated with these methods, and the test results often lack a clinicopathologic correlation that can confound the interpretation of their clinical significance. For example, microbiological culture may fail to grow a causative organism, while the organism isolated by the laboratory in vitro may arise from contamination and does not represent the actual infective agent in vivo.

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Pathology plays a key role as a bridging subspecialty in such multidisciplinary approach. Pathologic examination, if available, can establish a more specific diagnosis correlated with clinical manifestations. Although general practice of pathology is largely oriented toward diagnosis of neoplastic diseases, pathologists have been increasingly called upon to make diagnoses from tissue samples collected by cytology, biopsy, and autopsy procedures in response to the challenge of emerging infections [1–4]. Using these tissue samples as the source for laboratory workup, pathologists have made various contributions to our understanding of emerging infectious diseases in diagnostics, pathogenesis, epidemiology, and clinical aspects of these diseases (Table 1). In addition, results from pathologic studies can help design better strategies for control and prevention of these emerging infectious diseases, especially when they occur as an outbreak [5, 6]. Furthermore, pathologic studies also play an essential role in identifying the effects of secondary pathogens that commonly complicate the primary disease syndrome [7, 8].

Recent advances in molecular biology have revolutionized the practice of medicine, especially in the arena of diagnostic pathology and laboratory medicine [9–11]. The practice of pathology has evolved from using morphologic pattern recognition as the main tool to a sophisticated medical subspecialty by applying a wide array of advanced immunologic and molecular techniques on top of the traditional methods. The so-called traditional methods include routine hematoxylin and eosin (H&E) stain, histochemical (special) stain, and electron microscopy (EM). The more commonly used advanced techniques include immunohistochemistry (IHC), in situ hybridization (ISH), polymerase chain reaction assay (PCR), and tissue microarrays. Other advanced techniques that are less standardized as diagnostic utilities for infectious diseases include confocal microscopy, proteomics, laser capture microdissection (LCM), in situ PCR, pyrosequencing, and next-generation sequencing (NGS). The results from these techniques provide different information regarding the infectious agents in the organ systems they involve (Table 2). Each technique has its respective advantages and limitations, and there is no single technique that can stand alone as the only method for etiologic diagnosis. The advanced techniques complement the traditional methods to confirm the diagnosis; therefore, it is always necessary to apply these techniques as an integrated laboratory utility to take full advantage of the pathology approach. A good example to illustrate such approach is the identification of a novel coronavirus during the global epidemic of severe acute respiratory syndrome (SARS) in 2003 [12–17]. By using traditional culture (Fig. 1a) and EM examinations (Fig. 1b) on clinical samples and tissue specimens, the morphologic evidence of coronavirus leads to subsequent anatomic localization of this novel virus in lung tissues by using a combination of IHC (Fig. 1c), ISH (Fig. 1d), and PCR. Ultimately, correlations of these data with serological and clinical findings confirmed the SARS-associated coronavirus (SARS-CoV) as the etiologic pathogen of the outbreak. This is a prime example of the contributions made by infectious disease pathology as part of a multidisciplinary approach to investigate emerging infections and disease outbreaks.

**Table 1** Examples of outbreaks caused by emerging pathogens initially identified or confirmed by pathologic studies

Year(s)	Disease outbreak	Country or geopolitical region
1993	Hantavirus pulmonary syndrome	USA
1995	Ebola hemorrhagic fever	Zaire
1995	Leptospirosis associated with pulmonary hemorrhage	Nicaragua
1996	Lassa hemorrhagic fever	Sierra Leone
1997	Enterovirus 71 hand-foot-and-mouth disease with encephalitis	Malaysia
1997	H5N1 influenza	Hong Kong
1998	Enterovirus 71 hand-foot-and-mouth disease with encephalitis	Taiwan
1998–1999	Marburg hemorrhagic fever	Democratic Republic of the Congo
1999	Nipah virus encephalitis	Malaysia
1999	West Nile encephalitis	USA
2000	Rift Valley fever	Saudi Arabia/Yemen
2000	Ebola hemorrhagic fever	Uganda
2001	Inhalational and cutaneous anthrax	USA
2002	Transplant-associated West Nile encephalitis	USA
2003	Sever acute respiratory syndrome	Global
2003	Monkeypox	USA
2003, 2005, 2007, 2010	Transplant-associated lymphocytic choriomeningitis virus	USA
2004	Transplant-associated rabies	USA
2006/07	Rift Valley fever	Kenya/Somalia
2008	Lujo virus hemorrhagic fever	Zambia/South Africa
2009	H1N1 pandemic Influenza	Global
2009	Transplant-associated Balamuthia mandrillaris meningoencephalitis	USA
2010	Dengue hemorrhagic fever	Puerto Rico
2011	Leptospirosis	Puerto Rico
2012	Transplant-associated microsporidia infection	USA
2012	Multistate steroid injection-associated fungal meningitis	USA
2013	Ferret-badger rabies	Taiwan
2014–2015	Chikungunya virus fatal cases	Puerto Rico
2015–2016	Congenital Zika syndrome	Brazil/Colombia/Puerto Rico/Caribbean

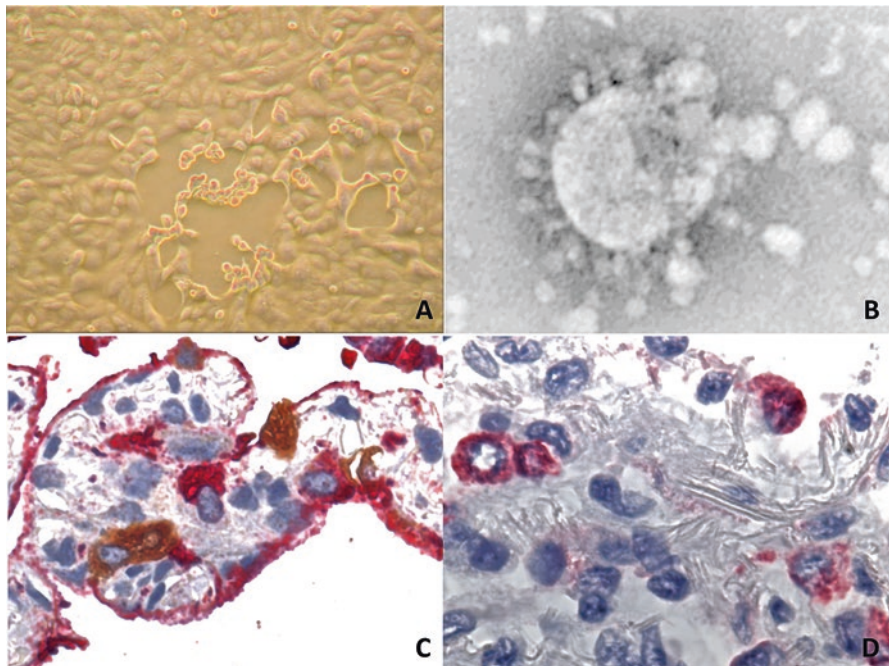
**Table 2** Pathology techniques and their utilities for infectious disease diagnosis

Technique	Main utility	Remarks
Hematoxylin and eosin Stain (H&E)	Shows histopathologic features of infectious process	Illustrates the evidence of a microbial infection and provides guidance to subsequent laboratory testing Does not highlight the pathogen per se Can only suggest certain infections and not a specific etiologic organism
Histochemical stain (special stain)	Highlights organisms	More useful for bacterial, mycobacterial, and fungal organisms Only categorizes organisms within a broad classification but not a specific species Can be difficult to interpret
Electron microscopy (EM)	Illustrates microbial ultrastructure	The most direct evidence to show an infectious agent Timeconsuming and limited to small areas of interest
Immunohistochemistry (IHC)	Localizes microbial antigens	Demonstrates antigens regardless the organism is intact or not Provides histomorphologic correlation of infectious process Many commercially available antibodies for common pathogens Antibodies of novel pathogens may not be readily available Formalin fixation may decrease sensitivity
In situ hybridization (ISH)	Localizes microbial nucleic acids	Probes can be synthesized in house with known sequence Provides histomorphologic correlation of infectious process Usually more specific but less sensitive than IHC Formalin fixation may decrease sensitivity
Polymerase chain reaction assay (PCR)	Amplifies small amount of microbial nucleic acids	Usually more sensitive than IHC and ISH Contamination issues frequently encountered Does not provide histomorphologic correlation of infectious process Formalin fixation may decrease sensitivity
Tissue microarray	Detects multiple microbial nucleic acids	Facilitate sequence analysis and pathogen identification Can detect microbes and assess related host responses simultaneously Biosafety concerns using frozen tissues Less sensitive than conventional PCR
Next-generation sequencing (NGS)	Analyze individual genome and large-scale sequencing	Can analyze individual near-complete exome or genome to assist in the diagnosis Reduce the cost of large-scale sequencing Current limitations, including nonstandardized platforms, long turnaround time, and the need of powerful bioinformatics to analyze large amount of data

(continued)

**Table 2** (continued)

Technique	Main utility	Remarks
Confocal microscopy	Increases morphologic dimension	Provides wider spectrum for histopathologic or cytologic interpretation Limited diagnostic utility for emerging pathogens
Laser capture microdissection (LCM)	Dissect special target cells for PCR or proteomic studies	Useful in studies of pathogenesis Limited diagnostic utility for emerging pathogens
In situ polymerase chain reaction assay	Localizes microbial nucleic acids with amplification process	Combines amplification and in situ localization methods Inherent technical issues with nonstandardized protocols Formalin fixation may decrease sensitivity Limited diagnostic utility for emerging pathogens
Proteomics	Detects microbial and host peptides	Useful in studies of pathogenesis Formalin fixation may decrease sensitivity Limited diagnostic utility for emerging pathogens



**Fig. 1** (a) Vero E6 cells show early cytopathic effect with coronavirus isolates from patients with SARS (Courtesy of Dr. Thomas G. Ksiazek). (b) Negative-stain (methylamine tungstate stain) electron microscopy shows coronavirus particle with an internal helical nucleocapsid-like structure and club-shaped surface projections (Courtesy of Dr. Charles D. Humphrey). (c) Double-stain IHC (immunoalkaline phosphatase polymer and peroxidase polymer) shows SARS-CoV (red) and surfactant antigens (brown) in type II pneumocytes. (d) ISH shows SARS-CoV nucleic acids in pneumocytes

## Highlights of Techniques

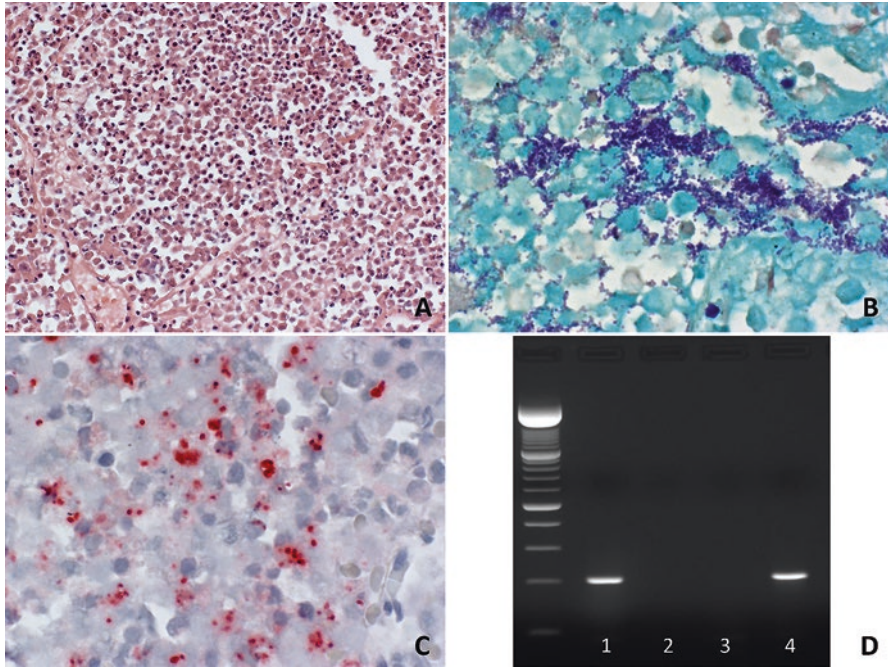
### *Hematoxylin and Eosin Stain*

Any pathology laboratory dealing with clinical diagnosis routinely performs hematoxylin and eosin (H&E) stain. It demonstrates the histologic and cytologic features in a tissue section and allows the pathologists to examine the microscopic changes related to infectious processes. Although it cannot highlight the pathogen per se, microscopic examination of H&E-stained slides is the most unequivocal method to illustrate the evidence of a microbial infection and its consequence in the tissue. For example, the presence of abundant neutrophils in the pulmonary alveoli is indicative of pneumonia (Fig. 2a), while neutrophils in the meninges support the diagnosis of meningitis. In some instances, the histopathologic features may be suggestive of infection caused by a specific pathogen; for example, the presence of smudge cells with necrotizing pneumonitis is indicative of an adenovirus infection in the lung. Nevertheless, most of the histopathologic findings shown by H&E stain are not specific because they can be caused by a variety of organisms. Their importance is to pave the first step leading to further laboratory assays for detecting the causative agent.

### *Histochemical Stains (Special Stains)*

Many histochemical stains have been developed to highlight a variety of microbial organisms. Some of the common ones are tissue Gram stain (for bacteria), Grocott's methenamine silver stain (for fungi), acid-fast stain (for mycobacteria), periodic acid-Schiff stain (for organisms with high content of carbohydrate macromolecules), Warthin-Starry silver stain, or Steiner's silver stain (for spirochetes and other bacteria). Interpretation of these special stains performed on tissue sections is usually more difficult than those performed on cultures because the coexistence of host tissue responses and accompanied histopathologic changes in the sections can confound the interpretation. It needs more expertise and effort to examine these special stains and usually requires a trained pathologist to carry out such examination. For example, *Streptococcus pneumoniae* can appear as gram-negative cocci in tissue sections because the host inflammatory responses, antibiotic treatment, or autolysin produced by the bacteria per se can damage the bacterial cell wall and render the Gram stain appear negative. Even when these special stains properly highlight organisms of interest, they can only categorize them within a broad classification but not a specific species. For example, gram-positive cocci demonstrated by tissue Gram stain in a lung section (Fig. 2b) could represent different species of *Streptococci* or *Staphylococci*, and further testing with more specific assays is needed to reveal the true identity of these cocci.





**Fig. 2** (a) H&E stain shows abundant polymorphonuclear inflammatory cells in alveoli indicative of an acute pneumonia. (b) Gram stain highlights numerous gram-positive cocci mixed with inflammatory cells. (c) IHC with anti-*S. pneumoniae* antibody shows abundant extracellular and intracellular bacterial antigens. (d) PCR targeting pneumolysin gene of *S. pneumoniae* shows positive amplicon (lane 1, positive control; lane 2, negative control; lane 3, water control; lane 4, lung sample tested)

### *Electron Microscopy*

Four decades ago, electron microscopy (EM) was the only ancillary technique available to the pathologists when routine H&E and special stains failed to reveal diagnostic features in histopathology [18]. EM examination provides a direct visualization of microbial organisms at a high magnification. Ultrastructural finding is the most direct evidence to show the presence of an infectious agent in clinical specimens. Thin section and negative stain are two common EM methods used to study pathogen morphology and morphogenesis of the microorganisms with recognition of their cytoplasmic organelles and matrix constituents. Therefore, correlation of light and electron microscopic findings not only improves pathologist's diagnostic acumen but also allows for a more coherent explanation of the pathogenesis. Since the advent of immunohistochemical and molecular techniques, EM has been less often used for identifying infectious agents. However, EM still played an essential role in determining the specific family of the pathogen involved in several

outbreaks caused by novel viruses, such as Sin Nombre virus [19, 20], Nipah virus [21, 22], SARS-CoV [12, 23], and monkeypox virus [24]. In these outbreak investigations, negative stain of virus isolated from tissue culture and thin-section preparation of tissue specimen facilitated the ultrastructural examination. The determination of etiologic agents guided subsequent laboratory, clinical, and epidemiologic investigations. Advanced EM methods, such as immuno-EM or EM in situ hybridization using colloidal gold labels, have been developed for a more specific ultrastructural diagnosis.

### ***Immunohistochemistry***

Immunohistochemistry (IHC) has been widely used in all aspects of pathology diagnosis in the past three decades [25–27]. A large number of IHC are available that can be helpful in the identification of microorganisms. By using a variety of antibodies, IHC can detect the presence of microbial antigens in tissue specimens, whether they represent the intact or degraded pathogens and whether they are intracellular or extracellular (Fig. 2c). Therefore, IHC has become a powerful technique used by pathologists for tissue diagnosis of infectious diseases. There are many ways to visualize an antibody-antigen interaction. The most common method is to apply an antibody conjugated to an enzyme, such as peroxidase [28–30] or alkaline phosphatase [31, 32], which can further catalyze a reaction for colorimetric detection. The antibodies used for specific detection can be polyclonal or monoclonal. Polyclonal antibodies are a heterogeneous mixture of antibodies that recognize several epitopes of a specific organism or more commonly, a group of related organisms. Monoclonal antibodies are generated against a single epitope and hence more specific to the target antigen than polyclonal antibodies. Many of these antibodies are commercially available and are widely used in diagnostic pathology laboratories. Others, especially those antibodies for detecting novel emerging pathogens, are available only at highly specialized centers such as the Centers for Disease Control and Prevention. Development of new IHC is a worthwhile but usually labor-intensive task. Similar to all other laboratory assays, the sensitivity and specificity of any IHC always need a careful evaluation before establishing its status as a diagnostic assay.

Detection of two or more target antigens on one slide can be achieved with multiple staining IHC assays [33–35]. These assays can expand the information obtained from each slide and reduce turnaround time compared to single staining or sequential staining methods. It is possible to assess the topographic relationship of the targets by using multiple staining IHC assays for determining the cellular tropism of viral infection with antibodies raised against virus and specific cellular markers, respectively (Fig. 1c). These multiple staining methods not only help confirm the immunolocalization of pathogens but also enhance further understanding of pathogenesis in many emerging infections [7, 16, 19, 36].

There are many advantages of using formalin-fixed tissues and IHC to detect etiologic pathogens. It is particular useful in detecting those fastidious or slow-



growing organisms, such as mycobacteria [37, 38] or *Tropheryma whipplei* [39], and can improve the speed, sensitivity, and specificity of microbial diagnosis. It is also valuable for characterizing emerging infections, whose causes are initially unknown, such as those caused by Nipah virus [21], SARS-CoV [12], or Zika virus [40]. Immunolocalization of antigens by IHC provides histomorphologic correlation between the infectious pathogen and host tissue responses, which is not only crucial for diagnosis but also important to study the pathogenesis of those emerging infections [19, 21, 41, 42]. Additionally, IHC performed on fixed tissues can minimize laboratory worker's potential risk of exposure to infectious agents because of the deactivation of pathogens by formalin fixation. Another advantage of using IHC is its capability of detecting well-preserved microbial antigens in archived formalin-fixed, paraffin-embedded (FFPE) tissues, which allows retrospective studies of many emerging pathogens even after decades of archive [43, 44].

### ***In Situ Hybridization***

In situ hybridization (ISH) is a technique that uses fluorescent or radiolabeled nucleic acid probes comprising complementary DNA or RNA strand to localize specific sequences in tissue sections [45, 46]. It has been applied in many medical diagnostics, such as gene expression profiling, chromosomal integrity, and karyotyping, etc. There are also many ways to perform ISH in diagnosis of infectious pathogens with a variety of probes [47–52], including double-stranded DNA (dsDNA) probes, single-stranded DNA (ssDNA) probes, RNA probes (riboprobes), and synthetic oligonucleotides (oligoprobes). ISH can localize nucleic acids of microorganisms in tissues and provides histomorphologic correlation between the infectious pathogen and host tissue responses (Fig. 1d). ISH can utilize in-house probes synthesized in a well-equipped laboratory with known sequences of the target nucleic acids, minimizing the need to depend on commercial resources. The advantages of using formalin-fixed tissues and ISH to detect etiologic pathogens are similar to IHC, except it is usually less sensitive than IHC because of the potential fragmentation of target nucleic acids by formalin fixation [53, 54]. Therefore, clinical utility of ISH for infectious disease diagnosis is much more limited than IHC, especially for in situ RNA analysis. This disparity is particularly notable when considering the abundance of RNA biomarkers discovered through whole-genome expression profiling. The reasons are mainly due to the high degree of technical complexity and insufficient sensitivity and specificity of conventional RNA ISH techniques. A novel RNA ISH technology, RNAscope, has been developed and utilized in the past few years. The technology applies a unique probe design strategy that allows simultaneous signal amplification and background suppression to achieve single-molecule visualization while preserving tissue morphology. RNAscope can be applied on routine formalin-fixed, paraffin-embedded tissue specimens and can use either conventional chromogenic dyes for bright-field microscopy or fluorescent dyes for multiplex analysis. Unlike conventional RNA

analysis methods such as real-time RT-PCR, RNAscope brings the benefits of in situ analysis to RNA biomarkers and may enable rapid development of RNA ISH-based molecular diagnostic assays [55, 56].

### ***Polymerase Chain Reaction Assay***

Polymerase chain reaction (PCR) assay amplification undoubtedly is the most sensitive method available to detect microbial organisms in tissue specimens and has become a common practice in many pathology laboratories. PCR can be performed on FFPE samples [57–60]; therefore, diagnoses can be made even if cultures were not obtained initially from biopsy or autopsy at the time of processing. In addition, molecular identification can accelerate definitive diagnosis of fastidious organisms that either grow slowly or do not grow at all with culture methods. When combined with other techniques mentioned above, PCR has markedly improved the capabilities of providing rapid and accurate detection of many emerging and reemerging pathogens [61] as well as pathogens commonly encountered in medical practice [58, 59].

PCR requires the isolation of nucleic acids from microorganisms in clinical samples and needs to apply adjunct techniques with restriction endonuclease enzymes, gel electrophoresis (Fig. 2d), and other nucleic acid hybridization methods. Degenerate primers can be employed in PCR assays at reduced stringency to facilitate detection of related but unknown organisms [12, 62, 63]. A vast number of PCR-based techniques have been developed in the past two decades and have been increasingly applied to clinical samples. For instance, multiplex PCR has been shown to increase the diagnostic yield in acute respiratory tract infections and contribute to overall improved outcome in patient care [64, 65]. New platforms such as real-time polymerase chain reaction (rt-PCR) combine nucleic acid amplification and fluorescent detection of the amplified product in the same closed system, resulting in an excellent technique that can diagnose a wide spectrum of infectious pathogens with tremendous flexibility, rapidity, and accuracy [59, 64, 66–68]. Nucleic acid sequence analysis has become highly automated and is now practical for use in many diagnostic and reference laboratories for the identification of a large number of microorganisms, whether they are cultivatable or not.

One particularly prevalent utility of PCR is the usage of the wide-range pan-eubacterial 16S ribosomal RNA (16S rRNA) PCR for detecting unknown bacterial organisms in tissue specimens. 16S rRNA is 1542 nucleotides in length and is a component of the 30S subunit of prokaryotic ribosomes. The 16S rRNA gene in bacteria contains well-conserved sequences that can be used as binding sites for universal PCR primers adjacent to variable sequences [69–71]. Subsequent analyses and comparisons of the sequences from amplicons to databases of known sequences can provide valuable information for etiologic diagnosis and further speciation. A set of broad-range PCR primers directed against conserved regions in the 16S rRNA gene was designed to specifically amplify either gram-positive or gram-negative

bacteria [72]. These differential 16S rRNA gene PCR assays provide more specific information regarding the bacteria identity, which are very useful for detecting bacterial pathogens in tissue samples in conjunction with histopathologic evaluation, special stains, and IHC.

Despite their high sensitivity, PCR techniques often face challenges from potential contamination issues. Processing of tissue samples, especially autopsy tissues, is often performed under a rather lax sterile condition and may enhance the chance of contamination. Many infectious pathogens can be present in the environment as commensals and their clinical relevance from PCR testing results can be confounded by such nature. Therefore, the PCR results should always be evaluated within the context of other diagnostic criteria. Moreover, any PCR testing of formalin-fixed tissues may be compromised by damage to DNA caused by the fixative. It is also important to know that identification to the species level may not be rigorous because the target gene may contain limited amount of sequence data available for comparison.

### ***Microarrays***

Microarrays can be performed on frozen tissue samples and may be helpful when multiplex PCR or other nucleic acid methods fail [73–75]. However, the sensitivity is generally lower than those multiplex PCR methods. Viral microarrays can be roughly divided into those targeting 10–100 agents and those designed for detection of thousands of agents, including unknown pathogens. Arrays designed to address a limited number of agents may employ multiplex consensus PCR to amplify specific genetic targets. Oligonucleotide microarrays with probes of up to 70 nt can offer a considerable advantage for detection of rapidly evolving targets, such as RNA viruses because these arrays are less likely to be confounded by minor sequence variation. Viral microarrays can facilitate sequence analysis and pathogen identification [73, 76–78]. Additionally, both microbial and host gene targets can be incorporated in these high-density arrays, thus allowing an opportunity to detect microbes and assess related host responses simultaneously for pathogenic features consistent with various classes of infectious agents.

### ***Next-Generation Sequencing***

Next-generation sequencing (NGS) is a powerful technique that can be applied on the FFPE tissue samples and has been rapidly spreading in the clinical and research arena [79, 80]. NGS can significantly reduce the cost of large-scale sequencing and is feasible to analyze an individual's near-complete exome or genome to assist in the diagnosis of a wide array of clinical scenarios. It can also facilitate further advances in therapeutic decision-making and disease prediction for at-risk patients. Currently,

there are still multiple factors that limit the diagnostic use of NGS in clinical laboratories, such as nonstandardized platforms, long turnaround time, large amount of data, and the need of powerful bioinformatics for data analysis. Targeted NGS investigates specific areas of interest rather than an entire gene or exon and thus produces smaller, more manageable datasets, reduces turnaround time, and decreases sequencing costs. Also, as it focuses on specific regions of interest, it leads to greater depth of coverage and increases the confidence of detecting a low-level variant in clinical samples [81, 82].

### *Other Advanced Techniques*

Other advanced pathology techniques, such as confocal microscopy [83], proteomics [84–86], laser capture microdissection (LCM) [87, 88], in situ PCR [89], and pyrosequencing [90, 91], have been used sparingly for detecting novel pathogens in a few specialized laboratories. Although they can become potentially powerful tools for diagnosis of emerging infections, most of them remain as pilot utilities and need further optimization to gain wide acceptance as mainstream techniques in practice of infectious diseases pathology.

### **General Guidelines of Using Pathology Techniques**

Appropriate clinical specimen collection, transport, and processing are crucial to establish an accurate laboratory diagnosis of infectious diseases. Similarly, adequate tissue sampling is the first and the most important step to obtain an organism-specific diagnosis of infectious diseases by using pathology techniques. The pathology laboratory must have practical guidelines for optimal specimen collection and handling and should communicate this information to the clinical staff and patient care sites. It is prudent to obtain biopsy or surgical samples from the precise site of infection and preferably before initiation of therapy to minimize the impact of treatment on subsequent diagnostic tests. This is particularly true for bacterial or fungal infections. Tissue specimens obtained surgically are acquired at great expense and pose considerable risk to the patient; therefore, they should be procured with an amount of material adequate for both histopathologic and microbiological examination. Swabs are rarely adequate for this purpose. Representative samples from all major organs should be collected in autopsy cases, especially those unexplained fatal cases due to infectious causes.

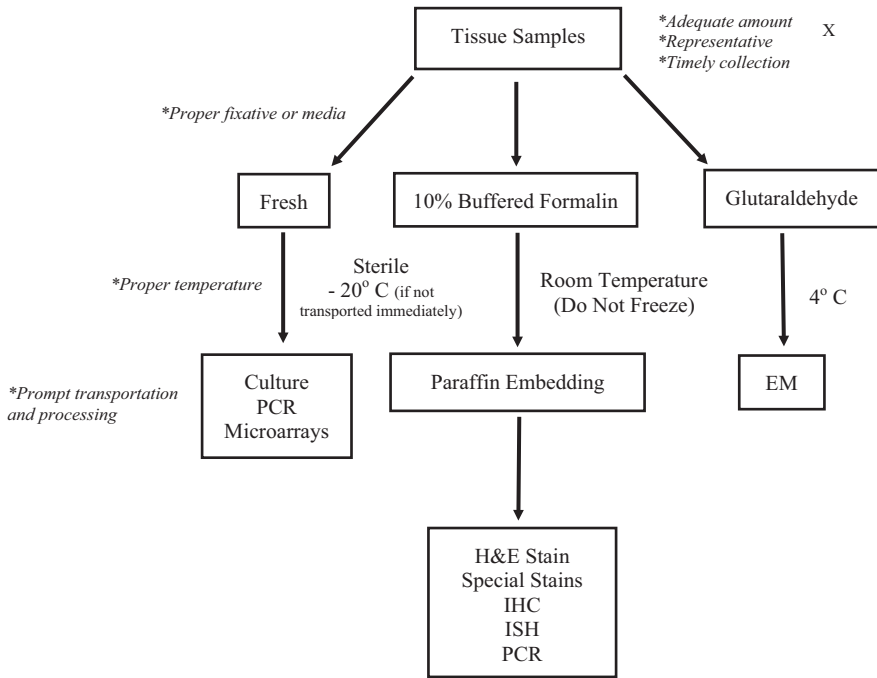
Etiologic pathogens may be focally or sparsely present in involved organs, and only a complete postmortem examination can attentively localize the causative organisms, as well as the full spectrum of their pathologic effects. In addition, the predilection site for infection may vary among different organisms. For example, herpes simplex virus tends to involve the temporal lobe in the brain more frequently,

**Table 3** Tissue sample collection with syndrome-based approach

Target system (syndrome)	Representative tissue sample collection
Central nervous system (meningitis, encephalitis, myelitis)	Cerebral cortex (frontal, parietal, temporal, and occipital), brain stem (midbrain, pons, medulla), spinal cord, cerebellum, basal ganglia, thalamus, hypothalamus, hippocampus, and meninges
Respiratory system (laryngitis, tracheitis, bronchitis, pneumonia, pulmonary hemorrhage)	Larynx, trachea, left and right main bronchi, hilar lung with segmental bronchi, and peripheral pulmonary parenchyma from both lungs
Cardiovascular system (myocarditis, endocarditis, pericarditis)	Ventricles, interventricular septa, and atria, including endocardium, epicardium, and pericardium
Hepatobiliary system (hepatitis, cholecystitis, hepatic failure)	Different areas of the liver, gall bladder
Gastrointestinal system (gastritis, enteritis, intestinal perforation, intussusception)	Esophagus, stomach, small intestine, large intestine, appendix, and mesenteric lymph nodes
Urinary system (nephritis, cystitis, renal failure)	Renal cortex and medulla, urinary bladder, and adrenal gland
Reproductive system (cervicitis, endometritis, pelvic inflammatory diseases, funisitis, chorioamnionitis, orchitis)	Cervix, uterus (endometrium and myometrium), ovary, fallopian tube, umbilical cord, placenta, testicles
Cutaneous system (skin rashes, including macule, papule, vesicle, pustule, ulceration, and eschar)	Minimally, a 3 mm punch, deep shave, or excisional biopsy specimen from the representative rash lesion. Multiple biopsies should be obtained if multiple stages or forms of cutaneous lesions are identified

while West Nile virus usually causes more severe infection in the brain stem and spinal cord. Moreover, since multiple organs can be involved in the context of systemic diseases, collecting multiple representative portions of target organs with syndrome-based approach (Table 3) and tissue samples from any other organ system with findings suggestive of infection ensures the best chance of detecting the causative agent. Influenza-associated myocarditis is a good example to show the difficulty of identifying influenza virus in the heart tissue even with prominent histopathologic changes of myocarditis, while the evidence of infection is usually present in the respiratory tissues [92].

FFPE tissue samples are usually adequate for routine H&E stain, special stains, IHC, and ISH assays. However, prolonged formalin fixation can cause cross-linking of proteins and nucleic acids in tissues and hence decrease the sensitivity of IHC, ISH, or PCR assays. In general, antigens and nucleic acids in tissue samples can be well preserved in paraffin-embedded blocks if formalin fixation does not exceed 2 weeks. It is highly recommended to embed tissue samples in paraffin no longer than 72 h after adequate formalin fixation. Although FFPE blocks can also be used for ultrastructural examination, it is preferably to dissect tissue samples into small thin pieces (1 mm<sup>3</sup>), placed in glutaraldehyde fixative, and stored in a refrigerator for optimal EM studies.



**Fig. 3** Optimal tissue collections for pathologic studies

Sterile techniques are mandatory to obtain target tissue samples for microbiologic culture and PCR assays. While biopsy procedure is usually performed under a stringent sterile condition, autopsy is not. In addition, delay of postmortem examination will facilitate colonization by normal flora or contamination by environmental organisms and interfere subsequent diagnostic assays. Therefore, autopsy should be performed as soon as possible (preferably within 12 h after death) to minimize these postmortem confounding factors. Representative tissue samples for potential PCR assay should be obtained with sterile technique and frozen at  $-20^{\circ}\text{C}$ . It is noteworthy that FFPE can also be used for PCR testing if frozen samples are not readily available, but the sensitivity is usually lower because of the chemical property of formalin fixative mentioned earlier.

A diagram of optimal tissue collection for pathologic studies is shown in Fig. 3.

## Summary

Diagnosis with pathologic techniques provides histomorphologic correlation for a specific infectious agent with the disease it causes and is essential for identifying the cause of death. It helps identify or confirm the etiology of an outbreak caused by a



novel pathogen, especially from severe or fatal cases. It is crucial for the management of the clinical patient with unknown etiology of infection, control, and prevention for emerging disease outbreak, epidemiologic surveillance, and study of pathogenesis. Tissue samples, especially postmortem specimens, should be collected adequately and promptly. They should be preserved in proper media and processed in a timely fashion. The histopathologic features identified in the tissue specimens in conjunction with relevant clinical and epidemiologic information should determine the performance of specific IHC, ultrastructural, molecular, or other assays.

There are limitations of using pathologic techniques despite the advantages. Because immune mechanisms can greatly amplify the host response, the actual numbers of pathogens present in tissues can be relatively small. This means that many sections may need to be examined before a pathogen is identified. Topographic issues related to tissue sampling can also affect the outcome of tests. If the tissue specimens are not obtained from relevant lesions or areas with histopathologic changes, the subsequent tests performed on such specimens can all result in false-negative outcomes. Timing of tissue sampling, as mentioned earlier, is another crucial element that can affect test results. Delayed autopsy procedure increases the chance of tissue autolysis and postmortem contamination, which can significantly interfere with histopathologic evaluation and all related pathologic tests. Technical issues, such as sensitivity and specificity, are universally present for each IHC, ISH, or PCR testing. A negative result cannot exclude the possibility of an infection caused by certain organisms because duration of illness, modalities of treatment, tissue sampling, and fixation may affect the outcome of these assays. Therefore, a correlation of the test results with clinical history, epidemiological information, and other laboratory assays is highly recommended for a more accurate interpretation involving inpatient care and public health management.

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