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Review

Emerging Pathogens: Challenges and Successes of Molecular Diagnostics

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More than 50 emerging and reemerging pathogens have been identified during the last 40 years. Until 1992 when the Institute of Medicine issued a report that defined emerging infectious diseases, medicine had been complacent about such infectious diseases despite the alarm bells of infections with human immunodeficiency virus. Molecular tools have proven useful in discovering and characterizing emerging viruses and bacteria such as Sin Nombre virus (hantaviral pulmonary syndrome), hepatitis C virus, *Bartonella henselae* (cat scratch disease, bacillary angiomatosis), and *Anaplasma phagocytophilum* (human granulocytotropic anaplasmosis). The feasibility of applying molecular diagnostics to dangerous, fastidious, and uncultivated agents for which conventional tests do not yield timely diagnoses has achieved proof of concept for many agents, but widespread use of cost-effective, validated commercial assays has yet to occur. This review presents representative emerging viral respiratory infections, hemorrhagic fevers, and hepatitides, as well as bacterial and parasitic zoonotic, gastrointestinal, and pulmonary infections. Agent characteristics, epidemiology, clinical manifestations, and diagnostic methods are tabulated for another 22 emerging viruses and five emerging bacteria. The ongoing challenge to the field of molecular diagnostics is to apply contemporary knowledge to facilitate agent diagnosis as well as to further discoveries of novel pathogens. ***(J Mol Diagn 2008, 10:185–197; DOI: 10.2353/jmoldx.2008.070063)***

In reality every infectious disease has emerged at some time in the past. Smallpox most likely jumped from a rodent host to humans and was maintained by human-to-human transmission when agriculture and urban centers supported large enough populations to sustain continuous spread without exhausting susceptible hosts. *Mycobacterium tuberculosis* may have evolved from *M. bovis*, causing human infections after the domestication of cattle. Extensive human-to-human transmission of such diseases did not occur until the conditions of human crowding during the Industrial Revolution. Despite the optimistic statement of the U.S. Surgeon General in 1967 that the war on infectious diseases was over and we had won, emergence of infectious diseases has not ceased and never will. Indeed, Marburg hemorrhagic fever appeared in 1967 and Lassa fever 2 years later. Approximately 50 new infectious disease agents have been identified during the last 40 years. Their identification reflects both conditions that favored spread of infectious diseases and scientific advances enabling discovery and characterization of microbes. Some of the conditions favoring emergence include human behavior (eg, cyclosporiasis), new technical products (eg, toxic shock syndrome), blood transfusion (eg, hepatitis C virus), movement of exotic animals (eg, monkeypox), nosocomial transmission creating large outbreaks (eg, Ebola hemorrhagic fever), deforestation (eg, Venezuelan hemorrhagic fever), and increased populations of reservoir and vector species (eg, white-tailed deer and *Amblyomma americanium* ticks transmitting human monocytotropic ehrlichiosis). The discovery of *Bartonella henselae* allowed its association with a long-known illness, cat scratch disease. None of the agents are truly new, except to our own knowledge. Emerging agents comprise viruses (Table 1), bacteria (Table 2), and protozoa. Emerging infectious diseases include respiratory, central nervous system, and enteric infections, viral hemorrhagic fevers, hepatitides, systemic bacterial infections, and human retroviral and novel herpes viral infections.

The use of molecular methods for detection, identification, and characterization of infectious agents in general is gaining importance in clinical microbiology laboratories. Emerging and re-emerging pathogens pose several chal-

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Table 1. Emerging Viral Infectious Agents, Epidemiology, Clinical Illness, and Methods of Laboratory Diagnosis

Viruses	Agent characteristics	Epidemiology	Clinical manifestations	Laboratory diagnosis
Sin Nombre virus (SNV)	Genome comprises three negative-sense RNA segments, S, M, and L. Two distinct lineages in rodents. Sequence differences consistent with gene reassortment between genetic variants	Affects healthy adults in rural settings through exposure to aerosols of deer mouse excreta. Many cases in southwestern U.S. Majority of cases in spring and summer; but also throughout the winter and early spring	Cardiopulmonary syndrome with temperature >38.3°C, respiratory distress, and cardiogenic shock developing within 72 hours of hospitalization in a previously healthy person. Case fatality ratio of 30–40%. Prodromal fever, headache, cough, dyspnea, nausea, vomiting, and myalgia. Thrombocytopenia	Hantavirus-specific IgM or rising titers of hantavirus-specific IgG, detection of hantavirus-specific ribonucleic acid sequence by RT-PCR or hantavirus antigen by immunohistochemistry. Never cultivated from any human source
Junin virus	Single-stranded, three-segment genome, lipid-enveloped, ambisense RNA arenavirus	Vector/reservoir is field rodent *Calomys musculinus*. Main infectious route via aerosols and urine. Argentine plains	Argentine hemorrhagic fever. Severe constitutional symptoms and signs, systemic microvascular leakage and hemorrhage. Neurologic syndromes	Viral isolation in BSL-4 environment. IgG and IgM detection by ELISA or IFA. Antigen detection in serum. RT-PCR. Targets are S and L RNA segments
Machupo virus	Single-stranded, three-segment genome, lipid-enveloped, ambisense RNA arenavirus	Vector/reservoir is field rodent *Calomys callosus.* Beni Department, Bolivia. Urine and aerosols	Bolivian hemorrhagic fever. Similar to other arenaviruses	Viral isolation in BSL-4 environment. IgG and IgM detection by ELISA or IFA. Antigen detection in serum. RT-PCR
Lassa virus	Single-stranded, three-segment genome, lipid-enveloped, ambisense RNA arenavirus	Field rodent of genus *Mastomys*. Urine and aerosols	Lassa fever, West Africa. Similar to other arenaviruses. Less severe than South American HF	Viral isolation in BSL-4 environment. IgG and IgM detection by ELISA or IFA. Antigen detection in serum. RT-PCR
Sabia virus	Single-stranded, three-segment genome, lipid-enveloped, ambisense RNA arenavirus	Rural areas around Sao Paulo, Brazil. Rodent reservoir unidentified. Urine and aerosols	Brazilian hemorrhagic fever. Similar to other arenaviruses	Viral isolation in BSL-4 environment. IgG and IgM detection by ELISA or IFA. Antigen detection in serum. RT-PCR
Marburg virus	Single-stranded, lipid-enveloped, negative-sense RNA filovirus	Africa. Reservoir possibly bats	Most severe of all HF. Prominent microvascular leakage and hemorrhages	Virus isolation, antibody and antigen detection. PCR targets include the polymerase gene (highly conserved), and the nucleoprotein gene
Ebola virus	Single-stranded, lipid-enveloped, negative-sense RNA filovirus	Africa. Reservoir possibly bats	Prominent microvascular leakage and hemorrhages	Virus isolation, antibody and antigen detection. PCR targets include the polymerase gene (highly conserved), the glycoprotein gene (detects all subtypes of Ebola), and the nucleoprotein gene
Alkhurma virus	Single-stranded, enveloped, positive-sense RNA flavivirus related to Kyasanur Forest virus	Middle East. Possible tick-domestic livestock-tick cycle. Unknown route of infection. Related to livestock occupations	Severe constitutional signs and symptoms. Hemorrhagic period followed by neurologic involvement	Antigen and antibody detection by ELISA. RT-PCR

(table continues)

Table 1. *Continued*

Viruses	Agent characteristics	Epidemiology	Clinical manifestations	Laboratory diagnosis
Hendra virus	RNA virus of family Paramyxoviridae genus *Henipavirus*	Australia. Bats are reservoirs. Mode of transmission unknown	Severe pneumonitis and meningitis	Virus isolation in BSL-4 laboratory. IgM and IgG detection. RT-PCR
Nipah virus	RNA virus of family Paramyxoviridae genus *Henipavirus*	Malaysia, Singapore, Bangladesh. Bats are reservoirs. Mode of transmission unknown	Encephalitis	Virus isolation in BSL-4 lab. IgM and IgG detection. RT-PCR
Hepatitis C virus (HCV)	Positive-sense, single-stranded RNA virus of the *Flaviviridae* family. Six genotypes with differences in the treatment response and prognosis	170 million people worldwide are chronically infected. Transmitted parenterally (blood products and needles shared by intravenous drug users)	Hepatitis, hepatic fibrosis, cirrhosis, and hepatocellular carcinoma	ELISA; RIBA to identify false-positive results. False-negative serologic results can occur in immunocompromised individuals. Nucleic acid assays confirm active HCV infection in immunocompromised patients, diagnose post-exposure infection prior to seroconversion (usually within 3 months of HCV infection), monitor the efficacy of antiviral therapy (viral load), and direct therapy and predict prognosis (HCV genotyping)
Rotavirus	Eleven segments of double-stranded RNA. Five groups (A, B, C, D, and E); group A is the main human pathogen	Worldwide year-round. Fecal-oral transmission. Primary infection in infants and young children. All ages can be infected	50% of severe diarrhea. Self-limiting disease. Sudden onset of watery diarrhea with or without vomiting for 3–8 days	Rapid antigen detection in stool by enzyme immunoassay. RT-PCR provides increased diagnostic sensitivity and genotyping of strains
Norovirus	Single-stranded RNA virus	Worldwide outbreaks of gastroenteritis after ingestion of contaminated food and water	Acute vomiting with cramps and diarrhea	RT-PCR of emesis, stool, and food
Parvovirus B19	Single-stranded DNA genome. Tropism for immature red blood cells	Worldwide year-round; 50% infected some time during childhood or adolescence. Transmitted by respiratory secretions and occasionally blood products	Erythema infectiosum (fifth disease) with characteristic slapped-cheek rash, arthropathy, anemia, miscarriage	IgM-specific antibodies during acute infection. DNA assays using serum, leukocytes, respiratory secretions, urine, or tissue specimens in immunocompromised patients unable to produce antibodies
Human T lymphotropic virus-1 (HTLV-1)	Positive-sense, single-stranded RNA virus with tropism for CD4-bearing cells. The retroviral genome is reverse transcribed into provirus DNA, which integrates into the host genome at random sites	Worldwide. Sexual, parenteral (eg, blood transfusion, shared injection equipment), and vertical (mother to child through breastfeeding) transmission	T-cell leukemia/ lymphoma, myelopathy/tropical spastic paraparesis	Immunoassays, nucleic acid assays of provirus in infected cells (PCR/ RT-PCR, *in situ* hybridization) or viral culture
Human T lymphotropic virus-2 (HTLV-2)	Positive-sense, single-stranded RNA virus with tropism for CD4-bearing cells. DNA of HTLV-2 integrates in the host genome	Worldwide. Sexual, parenteral (unscreened blood, shared injection equipment), and vertical (mother to child through breastfeeding) transmission	Rare reports of possible tropical spastic paraparesis	Immunoassays, nucleic acid assays

(table continues)

Table 1. *Continued*

Viruses	Agent characteristics	Epidemiology	Clinical manifestations	Laboratory diagnosis
Human immune deficiency virus-1 (HIV-1)	ssRNA in the *Lentivirus* genus with tropism for CD4-bearing cells	Over 40 million people are infected worldwide. Transmission via infected blood or other body fluids (unscreened blood, shared injection equipment, sexual activity), transplacental, and breast-feeding	Acquired immunodeficiency syndrome (AIDS). Severe opportunistic infections and malignancies	ELISA and rapid HIV antibody screening tests confirmed by Western blot or HIV RNA tests. PCR detection of proviral DNA in peripheral blood mononuclear cells, or HIV RNA in plasma for diagnosis of early, post-exposure infection prior to seroconversion (usually within 3 months of HIV infection). HIV viral load test to monitor treatment response and predict the course. HIV antiviral drug resistance testing (genotyping, phenotyping, virtual phenotyping assays) to direct therapy
Human immunodeficiency virus-2 (HIV-2)	Retrovirus in the *Lentivirus* genus with 60% nucleotide homology to HIV-1 and tropism for CD4-bearing cells	Mostly West and Central Africa. Transmitted in the same manner as HIV-1	AIDS	Serologic and RNA detection assays as for HIV-1
Human herpesvirus-6 (HHV-6)	Double-stranded DNA genome. Types A and B are 95% homologous. Integrates into the chromosome and establishes latency	Almost all children worldwide are HHV-6 seropositive by 2 years of age. Believed to be transmitted through saliva	Roseola infantum (exanthema subitum) febrile illness in young children, and mononucleosis in adults. Major cause of opportunistic viral infections in the immunosuppressed, typically AIDS and transplant recipients. Re-activated in half of hematopoietic stem cell and solid organ transplantation recipients associated with encephalitis, bone marrow suppression, and graft-versus-host disease	Detection of HHV-6 DNA or RNA in plasma/serum or PBMC by PCR or RT-PCR indicates active infection. Culture and serologic tests
Human herpespvirus-7 (HHV-7)	Double-stranded DNA genome. After primary infection, latent infection in lymphocytes	Almost all children worldwide have HHV-7 antibodies by 6 years of age. Believed to be transmitted through saliva	Febrile illness in children and exanthema subitum	Detection of HHV-7 DNA or RNA in plasma/serum or PBMC by RT-PCR indicates active infection. Culture and serologic tests
Human herpesvirus-8 (HHV-8)	Double-stranded DNA virus with tropism for lymphoid, endothelial, and epithelial cells. Mostly a latent infection	Sexual transmission. Blood-borne transmission rare	Kaposi sarcoma, primary effusion B-cell lymphoma, and multicentric Castleman's disease	Immunological and molecular assays of blood and tissue specimens
West Nile virus (WNV)	Single-stranded RNA flavivirus	Transmitted by *Culex* mosquito bite, blood product transfusion, organ transplantation and occupational exposure in laboratory workers. Worldwide	Meningoencephalitis. Acute fever, severe myalgias, headache, conjunctivitis, lymphadenopathy, and roseolar rash	Detection of IgM (CSF) and IgG (serum) antibodies, RT-PCR (CSF or serum)

lenges to diagnosis, treatment, and public health surveillance. Identification of an emerging pathogen by conventional methods is difficult and time-consuming due to the "novel" nature of the agent, requiring a large array of techniques including cell cultures, inoculation of animals, cultivation using artificial media, histopathological evaluation of tissues (if available), and serological techniques using surrogate antigens. Looking back at past epidemics or outbreaks caused by previously unknown infectious agents, we realize that identification and characterization of a new infectious agent can take years to decades or even centuries. Such time frames have been decreased to weeks or months by the use of powerful molecular techniques, as illustrated by the identification of severe acute respiratory syndrome coronavirus (SARS-CoV) within weeks of the first cases reported, the discovery of a new hantavirus in North America in 1993, and the detection of bacteria as etiological pathogens of human infections such as *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* in human monocytotropic ehrlichiosis and human granulocytotropic anaplasmosis, respectively. Molecular techniques offer several advantages over conventional methods, including high sensitivity and specificity, speed, ease of standardization and automation. Other advantages include identification of novel, noncultivable or very slowly growing organisms, strain typing in epidemiological studies, antimicrobial susceptibility determination, and monitoring treatment by measuring bacterial or viral loads. Examples are provided in this review for representative emerging viral respiratory infections, hemorrhagic fevers, and hepatitides, as well as bacterial and parasitic zoonotic, gastrointestinal, and pulmonary infections.

Emerging and Re-Emerging Viral Respiratory Infections

Six new respiratory viruses have emerged since 2000, including H5N1 avian influenza, SARS-CoV, human coronaviruses NL63 and HKU1, metapneumovirus, and human bocavirus. Molecular assays have played critical roles in the discovery, surveillance, and routine clinical laboratory diagnosis of these agents.

Influenza A

Seasonal and pandemic influenza A represents one of the greatest threats to global health. Influenza A viruses undergo constant antigenic changes in the viral surface glycoproteins hemagglutinin (H) and neuraminidase (N). Influenza A has 15 H and nine N subtypes. Only subtypes H1, H2, H3, N1, and N2 have stable lineages in human population, whereas all 15 H and nine N subtypes occur in birds.[1,2] H and N antigenic variants determined by

Table 2. Emerging Bacterial Agent Characteristics, Epidemiology, Clinical Manifestations, and Preferred Method for Laboratory Diagnosis

Bacteria	Agent characteristics	Epidemiology	Clinical manifestations	Laboratory diagnosis
Campylobacter jejuni/coli	Motile, thermophilic gram-negative curved or spiral bacilli	Worldwide sporadic gastrointestinal infections in infants and children; more frequent in summer and fall	Range from asymptomatic or self-limiting infections to severe diarrhea	Direct microscopy and isolation by conventional selective media culture
Clostridium difficile	Obligately anaerobic gram-positive, spore-forming, toxin-producing enterobacilli, normal intestinal flora	Normal flora in healthy neonates, but common nosocomial, antibiotic-associated enterocolitis in patients of advanced age	Asymptomatic in healthy neonates; severe infections include toxic megacolon, pseudomembranous colitis, sepsis and death	Two-step algorithm consisting of cell culture assay for toxin detection and EIA glutamate dehydrogenase (GDA)
Escherichia coli O157	Gram-negative Shiga toxin-producing enterobacillus; motile with peritrichous flagella	Most outbreaks are associated with ingestion of contaminated beef	Mild to severe diarrhea; hemolytic-uremic syndrome	Culture and biochemical identification; confirmation with immunoassays for toxin, lipopolysaccharide or flagella
Vibrio cholerae O139; *V. vulnificus*	Motile gram-negative facultative anaerobic bacilli endemic in warm marine environments	*V. cholerae*: Outbreak in India spread through southeast Asia and Latin America; *V. vulnificus*: seasonal outbreaks in U.S. and Taiwan; leading cause of seafood-associated fatalities in the U.S.	Manifestations range from asymptomatic and self-limiting diarrhea to severe massive diarrhea (rice water stools), dehydration and death; wound infections and sepsis	Biochemical identification and serological typing
Legionella pneumophila	Thin facultatively intracellular gram-negative bacilli	Widespread in surface and potable water; immunocompromised individuals or those with chronic diseases have an higher risk of infection	Mild to severe pneumonia that cannot be distinguished from other forms of community-acquired pneumonia	Direct culture and/or immunofluorescent microscopy; EIA for urine antigen

point mutations cause seasonal influenza epidemics, whereas new antigenic H and N subtypes introduced by reassortment of virus genes cause pandemics.[1,2] Avian influenza A virus crossed the species barrier to infect humans in the 1918 pandemic[3] and in recent human infections by avian H5N1 influenza virus.[4]

Molecular assays are the preferred method for identification of new strains of influenza A infections. Nucleotide sequence analyses of the H and N genes confirmed that a virus isolated in 1999 was the avian H5N1 subtype.[5] Reverse transcriptase-polymerase chain reaction (RT-PCR) using specific hemagglutinin gene sequence of influenza A H5N1 has been used subsequently.[6]

Human H5N1 cases were reported during the period 1998 to 2007[2,7] with limited human-to-human transmission.[6] H5N1 virus has the potential to mutate and reassort to generate viruses that could be highly lethal with the capability to be transmitted efficiently from person to person, as is believed to have occurred in the influenza pandemics of 1957 and 1968.[1,6,7]

Influenza A has no pathognomonic symptoms, and diagnosis based on clinical signs is correct in only two-thirds of patients[8,9]; therefore, sensitive, rapid laboratory tests are required to guide antiviral use. Influenza virus replication can be detected in respiratory secretions, and the viral load remains high for 72 hours. RT-PCR increasingly provides a definitive diagnosis especially early in the infection, along with antigen detection. Recently, multiplex strategies for respiratory viruses including influenza viruses have been developed.[10,11] These assays provide rapid and sensitive tests for respiratory viral infections.

SARS-CoV

The first pandemic of the 21st century was caused by the SARS-CoV, which emerged in southern China in November 2002 and spread to 29 countries in five continents with 8096 confirmed cases and 774 deaths in 3 months.[12] RT-PCR, cloning, and sequencing contributed to identification of the novel coronavirus.[13–16] SARS-CoV spreads by respiratory secretions and airborne transmission. Although market animals are believed to have been the source of human transmission,[17] bats are suspected to be the sylvatic reservoir of the virus. Early in the illness, SARS cannot be distinguished from common respiratory infections based on clinical symptoms.[18] SARS-CoV causes high mortality; thus, timely, sensitive, and specific diagnosis is needed to prevent the spread of this contagious disease. During the SARS epidemic, PCR-based molecular testing was helpful because of its ability to rapidly screen for many viruses. After the identification of SARS-CoV, specific RT-PCR and serological assays were developed to detect infection before the appearance of antibodies when the risk of transmission is greatest.[19–22] SARS-CoV was detected by RT-PCR in nasopharyngeal aspirates in 32% of patients at the initial presentation and in 68% by day 14.[21] Viral RNA was also detected by RT-PCR in 97% of stool samples 14 days after disease onset. In contrast, SARS-CoV IgG seroconversions began on day 10 with detection of about 60% by day 18 and 100% by day 30 after onset. Combined RT-PCR and immunoassays diagnosed 95% and 100% of patients on day 10 using nasopharyngeal aspirate and stool samples, respectively.[21]

Human Coronaviruses (HCoVs)

Coronaviruses are enveloped positive-strand RNA viruses.[23] In the 1960s, HCoV subtypes 229E and OC43 were identified to cause common colds.[23,24] Shortly after the outbreak of SARS, two additional HCoVs, NL63 and HKU1, were identified.[25,26] These four HCoVs mostly cause mild upper respiratory illness with sore throat, rhinorrhea, cough and fever, accounting for an estimated one-third of common cold cases.[27] However, in young children and immunocompromised adults, HCoVs can cause more severe, although usually not life-threatening, lower respiratory tract infections including bronchiolitis and pneumonia.[23,28,29]

Traditionally, viral culture and immunological assays are used in the detection of HCoV-229E and HCoV-OC43. However, viral culture often takes 2 to 3 weeks to complete, HCoV-NL63 and HCoV-HKU1 have fastidious growth requirements, and immunological assays are relatively insensitive. Molecular assays have become an essential diagnostic tool for sensitive and specific detection of HCoVs. Several consensus and subtype-specific molecular assays have been developed for either all or each of the four HcoVs.[27,28,30] The genetic variability of HCoVs makes the detection of all circulating strains technically challenging. For example, when panCoV primers were used, Gerna et al failed to detect approximately 30% of circulating strains.[28] Thus to detect the maximal number of HCoVs, specific primers are used, and results are confirmed by a second set of primers.[28] False-negative results occur when assay sensitivity is below the actual viral load in the specimen. Another potential technical issue is false identification because of cross-reactivity between HCoV strains.

Human Metapneumovirus (hMPV)

The etiological agents of respiratory infections are identified in only half of the cases. Identification of hMPV exemplifies how molecular diagnostic assays aid in the identification of new pathogens. Using randomly primed PCR, cloning and sequencing, van den Hoogen obtained genomic sequence of hMPV, a previously unknown paramyxovirus, from nasopharyngeal aspirates of children with respiratory tract illness.[31] RT-PCR detects hMPV in 10 to 20% of subjects with respiratory symptoms, and by the age of 5 years nearly all individuals have antibodies to hMPV.

hMPV has a worldwide distribution and circulates principally in the winter and spring in temperate climates. The virus is associated with flu-like syndromes, otitis media, croup, exacerbation of asthma and obstructive pulmonary disease, and pneumonia.[32,33] Among immunocompromised patients including transplant recipients, the elderly, and infants, hMPV infection commonly causes

severe disease. RT-PCR is the method of choice for the diagnosis of hMPV infection. hMPV grows poorly in cell culture, and serological tests have low specificity because hMPV infection is almost universal in childhood.

Human Bocavirus (HBoV)

As more infectious agents are identified and multiplexing methods are developed for comprehensive detection of infectious agents, clinical laboratories will increasingly face the question of whether the identified agent is responsible for the disease or not. HBoV provides such an example. HBoV is a newly discovered human parvovirus that was first identified by random PCR/cloning techniques on respiratory samples.[34] HBoV has a worldwide distribution and a high prevalence of 2 to 18%.[35,36]

Although detected primarily in infants and children with acute respiratory tract infection, HBoV is found concurrently with other infectious agents in up to 80% of cases.[35,37] Because similar symptoms can be produced by the coinfecting agents, it has been questioned whether HBoV causes respiratory tract disease or is only a coincidental finding. Detection of HBoV is only possible with PCR or other molecular assays. No animal model or method for virus culture is available to fulfill Koch's postulates for proving disease causation.[38]

HBoV was previously found to be prevalent among children with acute wheezing and exacerbation of asthma.[34,39] Regamey and colleagues prospectively analyzed the first acute respiratory infection in a birth cohort of healthy Swiss neonates between April 1999 and December 2004.[35] HBoV was identified in five of 112 (4.5%) infants, with mild disease, and none was hospitalized. In four of the five infants, HBoV was associated with other respiratory viruses. In another study, Volz et al investigated HBoV infection in hospitalized pediatric patients during one winter.[36] HBoV DNA was detected in 11 of 389 (2.8%) nasopharyngeal aspirates from symptomatic hospitalized children. The majority of the patients had severe illness with radiologically confirmed pneumonia and required oxygen supplementation. In five of six patients with pneumonia, HBoV was the only agent detected, suggesting that HBoV was not an innocent bystander but rather can cause severe respiratory tract infections in infants and young children. Nevertheless, future studies are necessary to characterize the clinical illness and establish the pathological role of this virus.

Emerging Viral Hemorrhagic Fevers

The viral hemorrhagic fever syndrome is caused by a heterogenous group of viruses, the common features of which are a single-stranded, negative-sense RNA genome and a lipid envelope.[40] The viruses belong to the families *Arenaviridae, Bunyaviridae, Filoviridae,* and *Flaviviridae*. Infections in humans usually have very high case/fatality ratios. Infections are geographically limited owing to ecological factors and specific viral hosts.[40,41] Patients develop high fever, malaise, severe headache, and myalgias followed by microvascular leakage that progresses to bleeding, cardiovascular compromise with hypovolemic shock, pulmonary edema, acute renal failure, severe hepatic injury, and neurological involvement (Table 1).

Diagnosis

Viral isolation from serum, plasma, or other body fluids can only be performed safely in a BSL-4 laboratory.[40–43] Viremias are usually high at presentation (except for hantaviruses). Isolation is performed in cell culture or by initial recovery of the isolate in a laboratory animal. Specific identification of isolates is performed by PCR or immunofluorescence using specific antibodies. Detection by electron microscopy in organs requires high concentrations of viral particles ($>10^7$ particles/ml). Antigen detection is possible, and IgM antibodies are usually elevated at presentation and often detectable by enzyme-linked immunosorbent assay. Immunohistochemical demonstration of viral antigens allows diagnosis on tissue samples obtained pre- or postmortem.

RT-PCR has been successfully applied to all of these pathogens. Detection utilizes regular or real-time PCR. However, the viruses are heterogenous, and "universal" targets are not available. Therefore, primers are specific for each family, and the possibility of multiplexing in a real-time format is limited. Modified nucleic acid amplification techniques such as nested or seminested PCR can increase sensitivity and specificity. Real-time PCR assays have also been developed recently but are not as numerous as conventional PCR assays. Advantages of real-time PCR include less risk of amplicon contamination and use of detection probes instead of SYBR Green or SYBR Gold (increased specificity). The clinical sensitivity of PCR ranges between 60% and 100% depending on the pathogen (lowest for Crimean-Congo hemorrhagic fever), and the analytical sensitivity of real-time PCR is estimated as low as 5–8 pfu/ml for filoviruses.

PCR targets for filoviruses include the polymerase gene (highly conserved), glycoprotein gene (for detection of all subtypes of Ebola but not Marburg virus), and the nucleoprotein gene. The bunyavirus Crimean-Congo hemorrhagic fever virus is detected by targeting the S RNA segment and Rift Valley fever virus by targeting the M and S segments. The clinical sensitivity for the bunyaviruses (66 to 70%) is not as high as for filoviruses (close to 100%). For arenaviruses the target is the S RNA segment. However, this segment is highly variable, and several PCR primers are required to detect all strains. Primers for the L segment have also been designed. Primers for yellow fever virus (a flavivirus) target the highly conserved NS5 gene. Dengue virus primers have been designed to detect all subtypes or to differentiate among the four subtypes.

Emerging Viral Hepatitides

Molecular assays have played important roles in the identification, surveillance, and routine clinical diagnosis and management of hepatitis C, delta, and E viral infections.

Hepatitis Delta Virus (HDV)

HDV requires hepatitis B virus (HBV) to complete its life cycle in the eukaryotic host cell since HDV replication depends on HBsAg for packaging of the HDV genome. The exact classification of the virus remains elusive, but it is related to viroids, plant satellite viruses, and simple infectious RNA molecules. Infection with HDV occurs either as coinfection with HBV or superinfection in a patient already infected with HBV. When both viruses are present, the risk of severe disease is fourfold greater than in patients infected with HBV alone. The spectrum of disease ranges from asymptomatic to end-stage liver disease (cirrhosis). In addition, superinfected patients are at risk for developing acute fulminant hepatitis.

Measurements of HDV antibodies employ radioimmunoassay or enzyme-linked immunosorbent assay. In superinfected patients both IgG and IgM responses are usually very strong. Some coinfected patients produce only IgG or IgM. The main marker for HDV infection is anti-HDV IgM, which correlates with active liver inflammation and progression to cirrhosis. HDV antigen is only detected in the early stages of the disease. Once anti-HDV antibody titer rises, antigens are masked. Nuclear HDV is detected in tissue by immunohistochemistry or *in situ* hybridization. The more prolonged the infection, the less likely the virus will be detected in tissues.

Conventional and real-time reverse transcription amplification assays are performed for detection of HDV in serum in research laboratories only. The primers amplify all known clades or types (seven) and target the ribozyme regions of the genome and antigenome of HDV. The analytical sensitivity of the real-time assays is approximately 100 copies/ml. Quantitative analysis can be used to follow patients during α-interferon treatment.

Hepatitis E Virus (HEV)

HEV is transmitted enterically and is common in tropical and subtropical regions. No chronic sequelae develop after infection, but fulminant hepatitis occurs especially in pregnant women. The disease is most severe during the third trimester, and mortality approaches 20%. HEV is a non-enveloped, single-stranded, positive-sense RNA virus. Morphologically, the closest related viruses are the *Caliciviridae* family; however, the genomic organization is closer to rubellavirus.

Detection of specific antibodies (both convalescent phase IgG and acute phase IgM) employs enzyme-linked immunosorbent assay or Western immunoblotting. An elevated IgM titer or rising IgG titers suggest acute infection. Amplification of the viral RNA from serum, feces, or environmental samples by RT-PCR in regular or real-time formats is available at selected research centers. PCR testing should be performed as early as possible since fecal excretion and viremia are short-lived. Primers target the viral helicase, polymerase, and parts of the 3′ end of open reading frame 2. The assays are sensitive and specific. These methods offer effective diagnosis for an illness that, unfortunately, occurs in developing countries where these research methods are usually unavailable.

Emerging Bacterial Pathogens

Molecular approaches to the detection of emerging bacterial pathogens are steadily evolving, and although there are many laboratory-developed LLI molecular assays for most agents, primary laboratory diagnosis of these emerging bacterial agents still relies mainly on conventional diagnostic methods (Table 2). Molecular diagnostics are most often used to confirm conventional detection methods such as direct culture, serological and biochemical identification, and microscopic visualization. Laboratory-developed molecular assays have been developed for nearly all emerging bacterial pathogens, although sensitive FDA-approved assays may not be available. However, there remain challenges involving ideal diagnostic specimens, standardization of PCR protocols, laboratory contamination, variability in pathogen DNA, sensitivity compared to conventional assays, and cost effectiveness that must be addressed before molecular detection will become the preferred diagnostic method. Nevertheless, molecular assays would be advantageous for several emerging pathogens that cannot be isolated in the conventional laboratory, cause diseases that are clinically difficult to diagnose, require conventional diagnostic methods that are laborious and technically challenging, or require special biocontainment facilities.

Ehrlichia and Anaplasma Species

Ehrlichia and Anaplasma are tick-transmitted obligate-intracellular gram-negative bacteria that cause three distinct emerging human zoonoses described in the last two decades. *E. chaffeensis* and *E. ewingii* are the etiological agents of human monocytotropic ehrlichiosis and ehrlichiosis ewingii, respectively, and *A. phagocytophilum* causes human granulocytotropic anaplasmosis.[44] Human monocytotropic ehrlichiosis, ehrlichiosis ewingii, and human granulocytotropic anaplasmosis have a seasonal (May through July) peak incidence and manifest as undifferentiated febrile illnesses. Human monocytotropic ehrlichiosis is a life-threatening disease that results in hospitalization for a large proportion (40 to 60%) of immunocompetent patients and a case fatality rate of 3%, whereas ehrlichiosis ewingii is reported primarily in immunocompromised individuals and is a milder disease.[45–47] The severity of human granulocytotropic anaplasmosis is age-dependent, and many patients require hospitalization.[48]

Microscopic visualization of morulae in peripheral blood leukocytes may be the simplest test, but it is also the least sensitive.[49] Currently serological testing is the most used method with excellent sensitivity, specificity, and reliability. Unfortunately, antibodies are usually absent during the first 2 weeks of symptom onset.[49] Conversely, PCR using multiple gene targets is a sensitive method for culture-confirmed cases of human monocytotropic ehrlichiosis in the early acute phase. Therefore, the advantages of molecular detection of *Ehrlichia* and *Anaplasma* are diagnosis before the development of antibodies and detection of *Ehrlichia* species that are uncul-

tivable (*E. ewingii*). Although commercial PCR assays for the ehrlichioses have not been developed, real-time PCR assays capable of simultaneous, rapid, sensitive detection (10–50 copies) of medically important *Ehrlichia* and *Anaplasma* in the acute phase of disease have been developed and could readily be used in clinical molecular diagnostic laboratories.[50,51]

Spotted Fever Group Rickettsiae

Rickettsiae are arthropod-borne, obligate-intracellular bacteria that primarily infect the microvasculature. Spotted fever group rickettsiae are mainly tick-transmitted pathogens, including *Rickettsia rickettsii, R. japonica, R. honei, R. africae, R. parkeri, R. slovaca,* and *R. felis* (transmitted by fleas).[52] Spotted fever group rickettsiae include life-threatening infections with symptoms including fever, severe headache, malaise, myalgia, nausea, vomiting, and abdominal pain.

Diagnosis of rickettsial infection can be accomplished by a number of conventional techniques including isolation and serology. Serology is the most used diagnostic assay.[53] However, most patients do not develop antibodies in the early stages of the disease, and thus serology is not useful when important therapeutic decisions are required. Isolation procedures are laborious, time-consuming, and require appropriate biocontainment in a reference laboratory with specially trained rickettsiologists. Immunohistochemical detection of spotted fever group rickettsiae in eschars and rash has a sensitivity of 70% and specificity of 100%.[53] Molecular diagnostics are advantageous during acute infection before the development of specific antibodies. However, molecular detection is available in only a few reference laboratories. Laboratory-developed PCR assays have been developed to detect spotted fever group rickettsiae, and real-time PCR using several gene targets (*gltA*, 17-kd, *ompA* and *ompB*) appears to have improved analytical sensitivity.[54,55] However, the sensitivity of these assays remains to be validated in a clinical context.

Chlamydophila pneumoniae

Chlamydiae are obligate-intracellular gram-negative bacteria that replicate within endosomal vacuoles of host cells. *Chlamydophila pneumoniae* emerged in the early 1980s as a new cause of 10% of community-acquired pneumonias and 5% of bronchitis and sinusitis cases, primarily in children 5 to 14 years of age.[56] Serological evidence indicates that *C. pneumoniae* infections occur worldwide with a seroprevalence greater than 50% among adults.

Diagnosis of *C. pneumoniae* infection is usually accomplished by serological testing. The Centers for Disease Control and Prevention considers the microimmunofluorescence test the reference standard, but it is technically difficult and time-consuming and lacks sensitivity compared with PCR and cell culture isolation.[57] Cell culture isolation of chlamydiae is also technically complex and requires weeks to obtain a result. Furthermore, cell culture isolation and microimmunoflourescence are not useful for high-throughput screening. Thus, molecular assays and other nonculture methods would be clinically advantageous. Numerous laboratory-developed PCR assays have been reported, but none has been fully validated in a clinical context.[57] Furthermore, lack of standardization and interlaboratory variation of *C. pneumoniae* PCR assays makes it difficult to determine their comparative sensitivities and specificities. One commercial PCR assay has been developed with a reported high analytical sensitivity (100% with more than one copy of DNA) and specificity.[57]

Borrelia burgdorferi

Borrelia are tick-transmitted spirochetes that are maintained in nature in diverse mammalian reservoirs, with rodents being the primary host. *B. burgdorferi* emerged as a tick-borne disease of public health importance in 1975 after an outbreak of epidemic juvenile rheumatoid arthritis in children from three contiguous towns in Connecticut.[58] Lyme disease has its highest incidence in the northeast (Connecticut, New York, Rhode Island, Massachusetts, New Jersey) and the upper midwest where *Ixodes scapularis* ticks are the primary vector. Lyme disease is seasonal (summer to early fall), and most patients (60 to 80%) present with a distinctive rash (erythema migrans), headache, stiff neck, myalgias, arthralgias, malaise, lymphadenopathy, and fatigue.[59] Early disseminated disease occurring months later includes neurological and musculoskeletal manifestations. Some patients develop disease years later with chronic neurological and musculoskeletal manifestations.[59]

Diagnosis of Lyme borreliosis can be accomplished using culture, microscopy, and detection of DNA, proteins, or anti-*B. burgdorferi* antibodies.[59] Antibody detection is insensitive early in infection and does not allow for distinguishing active from inactive infection. Direct culture in liquid medium can be used to detect active infection, but the sensitivity is low. Numerous PCR assays have been developed, but a gold standard molecular assay has not emerged. The sensitivity of PCR assays is dependent not only on the assay but also on the clinical specimens evaluated.[59,60] The highest PCR sensitivities have been reported with skin biopsy and synovial fluid.[59] In contrast, the sensitivity of PCR with blood or cerebrospinal fluid is low. Thus, PCR assays have not been widely embraced for laboratory diagnosis of Lyme disease.

Helicobacter pylori

Helicobacter (23 named species) are motile spiral or curve-shaped, gram-negative bacteria that reside in the gastrointestinal tract of humans and animals. By 1994 *H. pylori* was accepted as the major cause of peptic ulcer disease and was designated as a human carcinogen by the World Health Organization. Most adults (70%) have serological evidence of infection by age 50 years. *H. pylori* infections cause acute gastritis, but chronic infec-

tions occurring in many individuals produce gastritis, duodenitis, gastric and duodenal ulcers, multifocal atrophic gastritis, and gastric adenocarcinomas.

H. pylori infections can be diagnosed using a variety of conventional and molecular techniques, all of which fall short of being considered a gold standard, and invasive tests are usually considered the reference methods.[61] Direct culture, a reference method, is often unavailable and difficult, and specimen transport conditions are strict.[61] Infections are also diagnosed by histological examination, urease tests, or noninvasive tests such as urease breath test, serology, and detection in stool by immunoenzymatic and molecular techniques. Immunoassays for direct detection of *H. pylori* antigens in fecal samples are U.S. Food and Drug Administration-approved, have high sensitivity (91 to 96%) and specificity (93 to 97%), and are very cost effective for routine screening.[62] Serology has high sensitivity (>90%) and specificity (>90%), and urease breath test is widely used with greater than 95% sensitivity and specificity.[61] Molecular tests for *H. pylori* are not commercially available, but many PCR assays including real-time PCR provide rapid detection and quantification of the organism and identification of mutations associated with antibiotic resistance. However, standardization is lacking, and molecular diagnostics may not be a cost-effective alternative to the highly sensitive and specific immunoassays or urease breath tests.[61]

Emerging Parasitic Infections

Cryptosporidia

Cryptosporidium parvum was first diagnosed as a human pathogen in 1976 in two immunocompromised patients with persistent diarrhea. The largest outbreak involved 400,000 persons in Milwaukee in 1994.[63] In immunocompromised patients (eg, acquired immunodeficiency syndrome) debilitating diarrhea persists for weeks or months. Cryptosporidiosis, a cause of childhood diarrhea, is endemic in developing countries.

Conventional diagnosis is by examination of direct wet mounts of fecal specimens with increased sensitivity after concentration. Oocysts are detected using modified acid fast stains or auramine-O stain. The analytical sensitivity of immunoassays after concentration of fecal samples is approximately 5000 oocysts/g of stool.[64–66] Several laboratory-developed standard and real-time PCR assays target the 18S rRNA and outer wall protein genes. Sensitivity ranges from 95% to 97%. Specificity approaches 100%. The analytical sensitivity has been estimated at 50 to 500 oocysts/ml of liquid stool.[65,67–69]

Microsporidia

Microsporidia are spore-forming, obligate-intracellular protozoa that infect the intestine, liver, kidney, brain, and other tissues. Of 144 genera (containing more than 1000 species) *Encephalitozoon, Enterocytozoon, Pleistophora, Brachiola, Nosema, Trachipleistophora, Vittaforma,* and *Microsporidium* are human pathogens, most commonly *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*. Microsporidioses have increased incidence in patients with acquired immunodeficiency syndrome.[70–72]

The most accepted conventional method for stool examination is the Weber's chromotrope-based stain. Ultraviolet visualization is achieved using Calcofluor white M2R or Uvitex 2B. The analytical sensitivity is around 50,000 spores/ml. Fluorescent techniques are slightly more sensitive but slightly less specific than chromotrope-based techniques. Cytological and histological diagnosis of extraintestinal microsporidiosis employs modified trichrome, gram, Giemsa, or tissue-based gram stains (Brown-Hopps), and chemofluorescent agents. Immunofluorescence techniques using either polyclonal or monoclonal antibodies directed against spore antigens and polar tube proteins have also been used for body fluids and biopsies, but their sensitivity appears somewhat lower than routine staining methods.[72]

Commercial molecular tests for microsporidiosis are not available, but molecular assays are used in reference laboratories. PCR-based tests identify microsporidia at the species level in stool specimens, intestinal biopsies, and other tissues.[71] Microsporidia are eukaryotes with genome sizes overlapping those of bacteria (5.3 to 19.5 Mb). Furthermore, their rRNA genes are more closely related to prokaryotes with 16S rRNA and 23S rRNA separated by an intergenic spacer. PCR targets are usually the small and large subunit rRNA gene and the intergenic spacer region.[71] The overall sensitivity of PCR is 67% (range, 36–96%), and light microscopic methods with routine staining procedures have an overall sensitivity of 54% (25–71%). Specificities are 98% and 95%, respectively. The analytical sensitivity of microscopy is between 10^4 and 10^6 spores/g of feces and for PCR around 10^2 spores/g.[71]

Cyclospora

Cyclospora cayetanensis was first described as a human pathogen in 1994. Food-borne outbreaks have occurred around the world.[73,74] Infection occurs via the fecal-oral route a by ingestion of contaminated water or produce. Human-to-human infection is made less likely because of the long sporulation time (at least 7 days) after shedding in feces. Most cases occur in tropical and subtropical countries. The disease is characterized by watery diarrhea, cramping, and bloating, as well as weight loss if prolonged. If not treated the diarrhea persists for weeks to months. In immunocompromised patients the disease lasts for several months.

Oocysts, which are visualized using modified acid-fast stains or a modified "hot" safranin technique, are similar to *Cryptosporidium* but are twice the diameter. Oocysts are also visualized under ultraviolet light due to autofluorescence. When compared to ultraviolet detection, the sensitivity of the acid-fast technique is about 78%. *C. cayetanesis* is observed intracellularly in small-bowel biopsies.[75,76] PCR detection in human feces, produce, and water employs primers that target the internal transcribed

spacer region, with analytical sensitivity of 10 oocysts/g of feces.[75]

Balamuthia mandrillaris

Acanthamoeba and Balamuthia cause granulomatous amebic encephalitis, and *Naegleria fowleri*, primary amebic meningoencephalitis. Granulomatous amebic encephalitis is more common in immunocompromised patients and occurs worldwide. *Balamuthia* was isolated first in 1986 from a baboon that died of granulomatous amebic encephalitis.[77] Isolation is performed in mammalian cell cultures. Initial sites of infection are possibly the lower respiratory tract, respiratory sinuses, or skin with dissemination to the central nervous system.[78] Diagnosis is based on histological identification in brain tissue.[78] Differentiation between *Acanthamoeba* and *Balamuthia* is difficult, but specific antibodies are applied in research laboratories for immunohistochemistry.[78] PCR tests target the mitochondrial 16S rRNA genes and 18S rRNA genes. The analytical sensitivity using the mitochondrial target is as low as 0.2 amoebas per reaction mixture due to the abundant targets present in each amebic cell, which contains hundreds or thousands of mitochondria. This assay has been effective in three cases using brain tissue and three cases using cerebrospinal fluid. A multiplex approach on a real-time PCR platform differentiates among the three species responsible for amebic encephalitis using TaqMan probes specific for each of the species. The test is rapid and specific with analytical sensitivity of one amebic cell per reaction mixture.[79,80]

Future Prospects

The future is bright for molecular testing for emerging pathogens, and availability of molecular diagnostic tests as point-of-care testing is not far away. This technology will result in timely, accurate, and inexpensive diagnosis and effective treatment of these infectious diseases. Public health implications are unlimited. All companies developing point-of-care testing instruments for molecular diagnosis of infectious diseases are aiming to miniaturize the analytical methods and build small "walk-away" instruments that require minimal operation. Therefore the high complexity associated with molecular testing would diminish as a limiting factor in hospitals and diagnostic laboratories. In fact, many of these instruments would be available for deployment in remote areas. Promising detection systems used in experimental point-of-care testing instruments include real-time PCR, nanoparticle probe-based, bioluminescence real-time amplification, microarray, and micropump technologies.

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