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CHAPTER

7

Scientific Advances in the Diagnosis of Emerging and Reemerging Viral Human Pathogens

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ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
DNA	deoxyribonucleic acid
EBOV	Ebola virus
EVD	Ebola virus disease
FDA	Food and Drug Administration
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus

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ICU	intensive care unit
LRN	Laboratory Response Network
miRNA	microRNAs
PCR	polymerase chain reaction
RNA	ribonuleic acid
RT-PCR	reverse transcription-polymerase chain reaction
TB	tuberculosis
VZV	varicella-zoster virus

CLINICAL APPLICATION OF MOLECULAR METHODS

The diagnosis of any infectious disease requires active communication between clinicians and clinical laboratory personnel, usually in the form of "consultations" from clinicians to the laboratory director "on call" or directly with the laboratory technologists. Arriving at a correct diagnosis in a timely fashion begins with the acquisition of an adequate clinical specimen (blood, cerebrospinal fluid, urine, respiratory secretions, bronchoalveolar lavage, feces, etc.) and its transportation to the laboratory in an appropriate container. Specimens for molecular diagnostic tests sometimes require different methods of transportation/preservation from those of regular specimens so as to ensure that the techniques, used efficiently, will detect the suspected infectious agent. The classic diagnostic principles in clinical medicine still apply when using molecular methods of diagnosis. Patients and populations are evaluated clinically and epidemiologically, and case definitions are created so that test parameters, such as sensitivity, specificity, and predictive values, can be defined. Likewise, understanding the concept of screening versus confirmatory testing is also important. The three major steps performed in molecular assays are specimen processing, nucleic acid amplification or hybridization, and product detection (Nolte and Caliendo, 2007; Persing et al., 2004). Processing of the specimen is one of the most important steps for the successful detection of nucleic acids. Protocols vary, depending on the specimen received, and the specimen "matrix" plays an important role in nucleic acid extraction, owing to the presence of extraneous material in some matrices, that could potentially interfere with the molecular assays being performed. The most widely known example of such interference is the presence of *Taq* polymerase inhibitors in nucleic acid extracts, leading to false-negative results. Nucleic acid extraction protocols are usually cumbersome, and automation has been relatively challenging. However, several instruments have been designed for the clinical laboratory in which automated extraction protocols have been incorporated. Automation has also been incorporated for nucleic acid amplification methods, including conventional and real-time polymerase chain reaction (PCR), branched DNA, and isothermal technologies that

obviate the usual thermal cycling and potentially speed up the time required for amplification. Automated platforms, however, have decreased the cycling time by changing more quickly the temperatures required for different PCR cycles. Real-time PCR systems are usually coupled with automated detection systems that also vary in complexity. A few instruments are also capable of complete automation, including extraction, nucleic acid amplification, and detection systems. Tables 7.1 and 7.2 present a summary of the techniques available for the detection of nucleic acids from infectious agents.

TABLE 7.1 Classification of Nucleic Acid–Based Molecular Testing

Type	Advantages	Disadvantages
NONAMPLIFICATION TECHNIQUES		
Nucleic acid	Simple to perform	Low analytic sensitivity
Peptide nucleic acid probes	Bind to target more avidly	Low analytic sensitivity
SIGNAL AMPLIFICATION		
Hybrid capture assays	Simple to perform	Insensitive
Branched-DNA assays	Quantitative or qualitative—no risk of amplicon contamination	Potentially less sensitive than target amplification assays
TARGET AMPLIFICATION		
PCR and variants	High sensitivity, specificity depends on primers and amplicon contamination	Risk of amplicon contamination. Need thermocyclers
Reverse transcriptase–PCR	RNA targets susceptible to ribonucleases Multiple detection of pathogens from single specimen	Requires thermostable DNA polymerase Primer design is critical
PCR multiplex	Powerful technology when coupled with liquid-based microarray beads (Luminex, Toronto, Ontario, Canada) Highly sensitive and specific	Potentially lower sensitivity owing to competition for reagents in the PCR mixture High risk of amplicon contamination if transfer occurs
Nested PCR	Target amplification and detection in the same tube	Technically complex

(*Continued*)

TABLE 7.1 (Continued)

Type	Advantages	Disadvantages
Real-time (kinetic) PCR	Multiple detection systems: SYBER Green, FRET probes, dual hybridization probes, molecular beacons, dark quencher probes Low risk of amplicon contamination Decreased time of analysis	Probe design is critical for success Limiting multiplexing capabilities
Transcription-based amplification methods	Isothermal, rapid kinetics, RNA product single stranded: no denaturation for detection	Poor performance for DNA targets
Nucleic acid sequence–based amplification	No amplicon contamination (labile RNA) Isothermal, rapid kinetics, RNA product single stranded: no denaturation for detection No amplicon contamination (labile RNA)	Multienzyme system (stability) Poor performance for DNA targets
Transcription-mediated amplification	Isothermal. Fast amplification time	Multienzyme system (stability) Nonspecific primer hybridization owing to low temperature used
Strand displacement amplification		
PROBE AMPLIFICATION		
Cleavase invader assays (Hologic Inc. Third Wave Technologies, Bedford, MA)	Detection of point mutations based on primer design	Less prone to amplicon contamination
Cycling probe assays[a]		
Ligase chain reaction[a]		

[a]*Not available in the United States.*
DNA, Deoxyribonucleic acid; *PCR*, polymerase chain reaction.

TABLE 7.2 Detection and Analysis Platforms for Amplification Tests

Agarose and polyacrylamide gels
Colorimetric microtiter plates
Conventional hybridization
Nucleic acid sequencing • Conventional • Pyrosequencing • CLIP (integrated amplification and sequencing)
Hybridization microarrays
Bead-based flow cytometric assays
Electrospray ionization and mass spectrometry

FIRST-TIME DIAGNOSIS

Fortunately, the biotechnology boom of the late 1990s and early 2000s fueled the development of highly automated nucleic acid–based testing methods, which had important implications for the identification of infectious pathogens in human specimens (Monecke and Ehricht, 2005). One of these technologies, commonly referred to as real-time PCR, has gained considerable popularity. This method combines nucleic acid amplification and fluorescent detection of the amplified product in the same closed system (Morse, 1995; Relman et al., 1990; Hjelle et al., 1994; Aragon et al., 2006). The promulgation of real-time PCR as an important testing platform in clinical microbiology was catapulted by US homeland security efforts to produce rapid, reliable testing methods for identifying potential agents of bioterrorism. The Laboratory Response Network (LRN), an integrated group of public health, armed forces, and private referral laboratories, was created by the Centers for Disease Control and Prevention (CDC) to serve as a reference laboratory network for identifying and confirming agents of bioterrorism. In a very short period, scientists at CDC successfully developed a number of real-time PCR assays for the detection of agents of bioterrorism, and these assays are now available at many of the LRN laboratories.

Therefore molecular tools have improved the initial diagnosis of emerging and reemerging infectious diseases especially for pathogens that are uncultivated or difficult to isolate in clinical laboratories (Nolte and Caliendo, 2007; Persing et al., 2004). In addition, conventional diagnostic tests for infectious agents are slow, sensitive, expensive, or unavailable. Therefore since 1990 the identification of several pathogens has been obtained with molecular techniques that have contributed primarily to the diagnosis of these infectious agents (Persing et al., 2004; Relman, 1998). Nucleic acid–based assays are now gold standards for several infectious agents, including hepatitis C virus (HCV), *enterovirus*, *Bordetella pertussis*, and *herpes simplex* virus (HSV) (in the context of herpes encephalitis, *Chlamydia trachomatis*, and others). Cost-effectiveness is undoubtedly an extremely important factor when introducing molecular diagnostics into the clinical laboratory to replace conventional techniques. The best example is that of common bacterial pathogens, some of which require, in addition to identification, sensitivity tests to guide clinicians in choosing the most effective antibiotic.

One of the most promising platforms in clinical microbiology laboratories is that of molecular diagnostics using multiplexing technology (Loeffelholz, 2004; Smithn et al., 1998). The ability of real-time PCR to amplify and detect the product to be amplified, using specific probes at the same time, allows for multiple amplifications with the same clinical sample. The design of primers is the most critical step in multiplex PCR so

that efficiency and specific amplification are not compromised. Real-time multiplex coupling PCR with liquid-stained microarrays provides the multiplex detection of pathogens from clinical syndromes. FDA-approved diagnostic products are already available for viral respiratory pathogens.

We could imagine the use of multiplex systems to diagnose enteric pathogens, nosocomial infections, including identification of genes responsible for antibiotic resistance (according to the hospital's epidemiological data), pathogens of the central nervous system, hemorrhagic fever, and sepsis. We could also consider the inclusion of emerging and reemerging infectious agents in some of the syndrome-based diagnostic products to establish true incidence and prevalence of these highly neglected infectious diseases.

Furthermore, numerous reports have described the utility of this user-friendly technology for the rapid (same-day) and accurate detection of many emerging (new) and reemerging pathogens as well as pathogens commonly encountered in medical practice. A search for all articles published in the *Journal of Clinical Microbiology* from 2000 through 2003, which evaluated real-time PCR as a test method for pathogen detection and/or identification of genes or mutations associated with antimicrobial resistance in pathogens, revealed in 109 articles. Among these articles, 84 described assays with the LightCycler instrument (Roche Diagnostics Corporation, Indianapolis, IN); 21 described assays with the ABI PRISM 7000, 7700, or 7900H instrument (Applied Biosystems, Foster City, CA); 2 described assays with the SmartCycler instrument (Cepheid, Sunnyvale, CA); and 2 described assays with the iCycler instrument (Bio-Rad Laboratories, Hercules, CA). The availability of nucleic acid–based technology, such as real-time PCR, along with conventional staining and culture methods and immunoassays, can provide laboratories of many sizes with a comprehensive and responsible approach for the detection of both commonly encountered and emerging or reemerging pathogens.

EXAMPLES OF MOLECULAR DETECTION OF VIRAL EMERGING AND REEMERGING DISEASES

Variola Virus

The clinical presentations of concerned patients infected with common viruses that cause cutaneous vesicular lesions [HSV, varicella-zoster virus (VZV), enterovirus, or disseminated vaccinia virus following smallpox vaccination] might mimic those of patients with smallpox. Another complicating feature is that some recipients of the smallpox vaccine may develop erythema multiforme, which can also present as

vesicular lesions. It has been our experience that by using real-time PCR assays, one can rapidly discriminate among these possibilities. Analyte specific reagents (ASRs) or kits for the detection of HSV or VZV with the LightCycler instrument are available from at least two vendors [RealArt HSV 1/2 kit and RealArt VZV PCR kit (Artus); LightCycler Herpes Simplex Virus 1/2 and LightCycler VZV ORF29 (Roche Diagnostics Corporation)]. Kits are also available for testing for VZV (RealArt VZV PCR kit; Artus) with the ABI PRISM 7000, 7700, and 7900H instruments. Researchers have used the assays with the Roche LightCycler instrument to routinely detect HSV and VZV and have developed an in-house real-time PCR assay for poxviruses, including variola virus that uses the LightCycler instrument. These assays have been invaluable for providing a rapid result, especially for military personnel who have developed cutaneous vesicular lesions as a complication of receiving the smallpox vaccine and who have been on assignment in areas of the world at significant risk for bioterrorism events. Importantly, the home-brewed real-time assay that they have developed can discriminate among several poxviruses and was useful in the identification of viremia in a recent case of monkey poxvirus disease in a patient from the Upper Midwest. An ASR for the detection of variola virus with the LightCycler instrument is also available from Artus (RealArt Orthopox PCR kit).

West Nile Virus

West Nile virus, a RNA virus of the family Flaviviridae, has a predilection for the central nervous system and can be associated with significant morbidity and mortality. The first human cases of West Nile virus infection occurred in the northeastern United States in the summer of 1999; since then the disease has progressed relentlessly from east to west across the continental United States. Yet, no effective therapy has been defined.

Traditionally, during the summer and early fall in the United States, viral central nervous system disease is most frequently caused by enterovirus. In most regions of the United States, West Nile virus infection must now also be considered during this time of the year. HSV can cause encephalitis at any time of the year, and antiviral therapy is available and effective. Therefore ruling out HSV infection should be a priority, especially when encephalitis is encountered. Real-time PCR has replaced viral culture as the gold standard for the rapid and accurate detection of HSV in cerebrospinal fluid. As mentioned previously, ASRs or kits for the detection of HSV are available from Artus and Roche. Artus also has a kit that can be used to test for enterovirus (RealArt Enterovirus RT-PCR kit) with the LightCycler instrument.

Limited studies have shown that the PCR detection of West Nile virus in cerebrospinal fluid is less sensitive than immunoassay for immunoglobulin M antibodies. Currently, only a few referral and public health laboratories have the capability to perform immunoassays. At least two companies offer ASRs or kits for real-time PCR [RealArt WNV RT-PCR kit (Artus); LightCycler WNV Detection Kit (Roche Applied Science)] with the LightCycler platform. If effective antiviral therapy becomes available, the rapid on-site diagnosis of West Nile virus disease in areas of endemicity may be desirable.

SARS CoV

One important lesson learned from the 2002–03 winter outbreaks of severe acute respiratory syndrome (SARS) was that the early identification and quarantine of individuals with suspected cases of SARS were essential for controlling the disease, especially in institutional settings. This effective approach toward the control of a communicable infectious disease adds credence to the concept that similar measures can be effective for controlling and preventing nosocomial VRE and methicillin-resistant *Staphylococcus aureus* (MRSA) outbreaks. No laboratory tests were available for the detection of SARS coronavirus (SARS CoV) during much of the outbreak, as the etiological agent was not confirmed until early March 2003. Eventually, real-time PCR tests were developed and were available commercially from at least two manufacturers for use with several real-time PCR testing platforms [RealArt HPA-Coronavirus RT-PCR Kits (Artus) for use with the LightCyler instrument, the ABI PRISM 7000, 7700, and 7900H instruments, and the Rotor-Gene instrument; and LightCycler SARS CoV (Roche Diagnostics Corporation) for use with the LightCycler instrument]. During the outbreak, it was important to rule out treatable influenza virus types A or B infections, whose clinical presentations can mimic those of SARS CoV.

Rapid antigen tests for the detection of influenza virus (both types A and B) are relatively easy to perform and may be useful in the local setting for the detection of cases of influenza; however, these tests lack sensitivity. As infections due to both influenza virus types A and B are now treatable, rapid on-site diagnostic capabilities are important. Recently, a real-time PCR assay that uses the LightCycler platform was demonstrated to have much greater sensitivity than antigen detection (100% and 44%, respectively) for the detection of influenza virus type A infections.

Following the 2002–03 SARS outbreak, many LRN member laboratories developed the capability to detect SARS CoV. Should another outbreak occur, this public health laboratory network should facilitate the laboratory diagnosis of cases, especially when testing at the local level is not available.

DISEASE PROGNOSIS

The prognosis of infectious diseases has also been significantly affected by the availability of molecular techniques. Viral load in several chronic infections, such as human immunodeficiency virus (HIV), human papillomavirus, hepatitis C, BK virus, and cytomegalovirus, plays an important role in predicting better or worse outcomes and the need to start specific antiviral therapy to improve survival (Humar et al., 1999; Mellors et al., 1996; Reidn et al., 1987; Walsh et al., 1997). Viral and bacterial quantification by molecular methods could also be used in the future to differentiate a mild subclinical infection from a disease caused by the agent under certain conditions.

DIAGNOSIS BY MICROARRAYS

A microarray is a "collection of microscopic features," usually DNA, which can be probed with target molecules to produce quantitative or qualitative data (Miller and Tang, 2009). Indeed, the diagnosis of infectious diseases is most often qualitative (presence or absence of the pathogen), although the quantitative data become more numerous under certain conditions, such as the HIV/immunodeficiency syndrome acquired and hepatitis C.

The microarray platforms can be classified as follows: printed, synthesized in situ, high-density bead networks, electronic suspension, and liquid bead (Loeffelholz, 2004; Miller and Tang, 2009). All microarray platforms have the ability to multiplex, a characteristic that becomes indispensable in the diagnosis of infectious diseases.

For all approaches the probe is the DNA sequence bound to the solid surface of the array, and the target is the nucleic acid to be detected. The probes are synthesized and immobilized on microscopic spots.

It has been reported that of all platforms, suspension beads probably offer the most practical technology to clinical microbiology labs (Loeffelholz, 2004; Miller and Tang, 2009). Universal heel sets are available, and user-defined applications are easily implemented because of their flexibility. Therefore the use of wide-range or multiplex PCR followed by microarrays provides an excellent platform for the rapid and efficient identification of bacterial, viral, or fungal pathogens (Mikhailovich et al., 2008).

For the diagnosis of bacterial infections, microarrays are also incorporated in clinical laboratories for the rapid detection and characterization

of MRSA, determination of antimicrobial drug resistance in several pathogens, such as *Enterococcus* and *Mycobacterium tuberculosis*, and the diagnosis of sepsis (Mancini et al., 2010; Aragon et al., 2006; Monecke and Ehricht, 2005). Several difficult problems remain before the microarrays can be widely used in the clinical setting, including pre- and postanalytical variables, such as the type of clinical specimens (matrices), extraction techniques, labeling, and quality controls (Miller and Tang, 2009). The introduction into microbiology laboratories of additional diagnostic tests based on molecular technology will depend heavily on automation. Many molecular tests remain difficult because of complex and lengthy problems of processing and interpretation of samples.

MOLECULAR DIAGNOSIS AND LATEST GENERATION SURVEILLANCE SYSTEMS

Biosurveillance has become an important health priority because of the increased risk of emerging and reemerging pathologies and their effects on human populations (Sintchenko and Gallego, 2009). The potential consequences of large-scale epidemics or epidemics can be economically, socially, and environmentally profound, as evidenced by the global spread of coronavirus associated with severe acute respiratory syndrome. Traditional biosurveillance relies heavily on disease reporting by clinical laboratories to local, state, and federal health-care organizations (Sintchenko and Gallego, 2009).

Most clinically diagnosed cases are confirmed using standard microbiological methods, such as serologic markers and cultures, followed by biochemical identification, if necessary, or DNA-based typing methods, once a pure isolate is obtained. As a result, these methods cannot be described as slow and insensitive. Traditional biosurveillance has improved through electronic reporting to some extent, with an increase in the proportion of diagnosed cases reported in all cases occurring in the community (Panackal et al., 2002; Effler et al., 1999).

On the other hand, other factors include variability in case definitions (clinical and laboratory criteria), screening practices, contact tracking methods, and the quality of performance of the diagnostic tests used.

The second surveillance system is said to be syndromic and is based on the collection of vast amount of data from the various services, including emergency departments, intensive care units (ICUs), admission and hospitalization systems, and clinical laboratories (Sintchenko and Gallego, 2009; Bravata et al., 2004; Lewis et al., 2002). Indeed, the purpose of this system is to play the role of an alarm signal when the models

obtained over time move away from the "normal" state. The normal state is determined by the collected data and by creating "baselines" for a given area or community (Wang et al., 2005; Berger et al., 2006).

As expected, the specificity of these systems is rather low but can be improved by combining syndromic surveillance systems and laboratory surveillance (Sintchenko and Gallego, 2009; Buehler et al., 2004; Weber and Pitrak, 2003).

The latest generation of surveillance is based on the genomics of surveillance systems and is essentially based on the development of rapid molecular tests applied to the diagnosis of infectious diseases. A technique based on molecular subtyping has increased sensitivity and specificity in the investigation of epidemics at all levels (hospital, local, state, and national) (Hedberg and Besser, 2006; Monecke et al., 2007; Mellmann et al., 2006). Similarly, modern typing methods, based on direct data, such as sequencing and powerful software, to analyze gene clusters and genetic evolution, have also made it possible to detect possible epidemics by new pathogens or reemerging agents (Sintchenko and Gallego, 2009). Typical examples include surveillance systems for influenza A viruses and severe acute respiratory syndrome. Powerful molecular techniques cannot be used in isolation. Modern biomonitoring systems are important elements, including traditional epidemiological tools and newly developed "computer-based" systems in which complete integration of microbial profiling is merged with epidemiological surveillance and spatial surveillance (depending on the use of systems geographical information). Such a "network" or "global laboratory" would provide an exquisitely comprehensive view of potential biotherapies (Urwin and Maiden, 2003; Casadevall and Relman, 2010; Reis et al., 2007; O'Connor et al., 2003; Layne and Beugelsdijk, 1998; Heymann and Rodier, 2001).

NANOTECHNOLOGY DIAGNOSIS

Nanotechnology is a broad term that encompasses several disciplines and techniques, some of which currently have or will have a major impact on health care. For the purpose of this review, nanobiotechnology and its applications in molecular diagnostics are discussed. Nanotechnology is the study of the control and manipulation of matter at the atomic and molecular scales (Johnson et al., 2008), the materials used are 100 nm or less in at least one of the dimensions of the material. On the other hand, molecular diagnosis is an essential element in the development of personalized medicine, thus presenting punctual performance of diagnostic procedures. This report focuses on the application of several technologies in the clinical laboratory setting. The

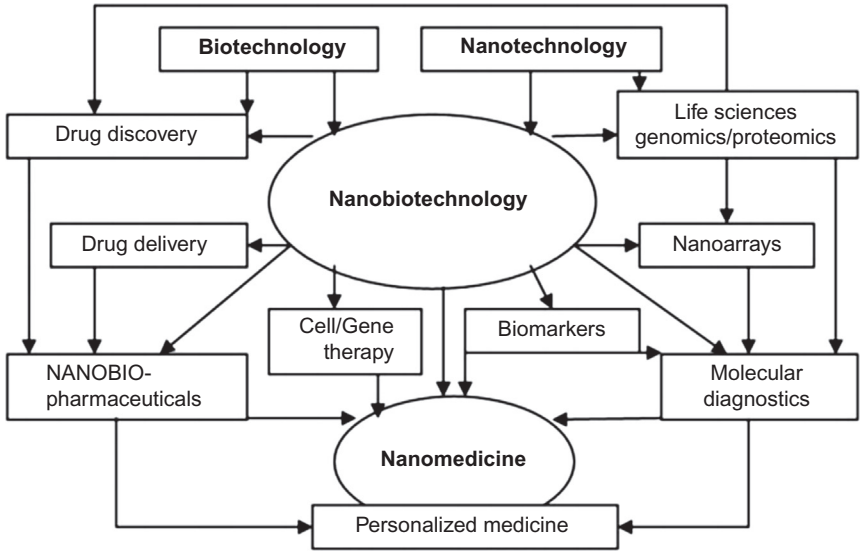

FIGURE 7.1 The interdependence of various technologies that contribute to clinical nanodiagnosis.

different interrelationships between nanotechnology and molecular diagnostics and their role in nanomedicine and personalized medicine are shown in Fig. 7.1.

Array Based on Nanotechnology

These are nanofluidic networks for the isolation and analysis of biomolecules, such as nucleic acids or proteins. The networks use nanotubes for the isolation of molecules and the detection of molecules trapped with electrode-based systems. The volumes required for such tests are very small, compared to those required with conventional instruments, and turnaround times are significantly reduced (Jain, 2007).

Whenever a single molecule of DNA moves in the nanotube, the electric current changes abruptly. The current returns to its reference value when the DNA molecule exits the nanotube. Nanofluidic technology is expected to have wide applications in systems biology, personalized medicine, pathogen detection, drug development, and clinical research.

Nanoparticles

Gold nanoparticles, nanocrystals (also called quantum dots) and magnetic nanoparticles are the main examples in this category. Gold

nanoparticles range from 0.8 to 250 nm and can be functionalized with a variety of biomolecules including antibodies, nucleic acids, peptides, proteins, and carbohydrates. Thus gold nanoparticles have better optical absorption and scattering properties because of a unique property known as plasma resonance. Like nanocrystals (see the next section), their optical properties can be modified by changing size, shape, and composition.

On the other hand the surface of nanocrystals can also be functionalized with nucleic acids, antibodies, proteins, and peptides, allowing adaptation to several diagnostic platforms (Bruchez et al., 1998; Hotz, 2005; Michalet et al., 2005). The magnetic nanoparticles are made of iron and incorporated into copolymer beads. The surface charge of the beads can be manipulated with the polymer coating to enhance nonspecific protein adsorption to the surface of the beads, increasing the specificity of the assay (Johnson et al., 2008; Jain, 2007).

Nanobiosensors

Nanobiosensors are nanosensors that are allowed for the detection of chemical or biological materials. These materials are extremely sensitive (Jain, 2003). Prototype sensors have demonstrated the detection of nucleic acids, proteins, and ions.

These sensors can operate in liquid or gaseous phase, which allows them to be used for different downstream applications. These sensors are inexpensive in their manufacture and are portable. Thus they can serve even as bases for the implementation of implantable devices for detection and monitoring.

Cantilever Biosensors (Cantilever)

This technology provides an alternative approach to PCR and complements current DNA and protein microarray methods. Using this method, it is not necessary to label or copy the target molecules. The advantages of cantilevers are that they provide fast, unlabeled recognition of specific DNA sequences for single-nucleotide polymorphisms, oncogenes, and genotyping. Nanocantilevers could be crucial in the design of a new class of ultrasonic sensors to detect viruses, bacteria, and other pathogens (Gupta et al., 2006). Finally, a real-time cantilever biosensor can provide continuous monitoring of clinical parameters in personalized medicine.

Viral Nanobiosensors

The virus particles are biological nanoparticles. Giving examples of HSV and adenovirus that can be used to trigger the assembly of nanomagnetic beads as nanobiosensors for clinically relevant viruses

(Perez et al., 2003). Thus this technique offers the possibility of detecting up to five virus particles in a 10 mL serum sample. This system has the advantage of having an increased sensitivity compared to the enzyme-linked immunosorbent assay (ELISA)-based methods as well as an improvement over the PCR-based detection because it is less expensive and faster and has fewer artifacts.

MICRORNAS AND EMERGING VIRAL INFECTIONS

History of MicroRNA

MicroRNAs (MiRNAs) represent a recently uncovered class of small noncoding RNAs from 20 to 22 nucleotides that function as posttranscriptional regulators of gene expression. These miRNAs are coded by separate genes that are localized in the nonprotein coding part of the genome. MicroRNAs predict to regulate about 30%–60% of human genes (Griffiths-Jones et al., 2008; Malumbres, 2013).

These small noncoding RNAs bind their target messenger RNAs (mRNAs) in the untranslated region (UTR) and coding sequence (CDS) regions and act as negative regulators. This action affects various biological processes including cell growth, differentiation, signal transduction, metabolism, and development (Inui et al., 2010).

Dysregulation of miRNA expression has been described in several diseases, and their role appears to be pivotal in driving tumorigenesis (Iorio and Croce, 2012). MiRNA dysregulation is considered to be an early event in tumorigenesis (Cortez et al., 2011).

It was discovered in 1993 in the *Caenorhabditis elegans* nematode of a DNA fragment containing a small sequence that encodes a 22-nucleotide RNA, Lin4 that regulates the transition from larval stage L1 to stage L2.

This regulation is achieved by repressing the expression of the proteins LIN14 and LIN28 by this miRNA by binding to the 3′ noncoding regions (3′-UTR) of the corresponding mRNAs.

In 2000 another miRNA Let7 was identified, which regulates the transition from larval stage L4 to adult stage by the same mechanism of action. Since then, several discoveries have been made and have demonstrated the conservation of these miRNAs in several species. Studies have revealed that more than 500 miRNAs can potentially target nearly 30% of mRNAs in humans (Friedman et al., 2009).

Structure and Biogenesis

In the nucleus the gene of miRNA is transcribed into a pri-miRNA by the action of a RNA polymerase type II or III (Borchert et al., 2006;

Yoontae et al., 2003). Pri-miRNA will then be cleaved by the nuclear microprocessor complex formed by the association of the enzyme Drosha and the DGCR8 (DiGeorge critical region 8) protein into an intermediate precursor called a pre-miRNA (Han et al., 2004).

Once the complex is attached to primer-miRNA, the DGCR8 protein will allow to define the cleavage distance that generally measures 11 base pairs from this junction, while the two Drosha domains will cleave the 3′ and 5′ ends of the pri-miRNA. The resulting pre-miRNA is a loop stem having two unmatched nucleotides at the 3′ end and a phosphate at the 5′ end, this asymmetry is specific for type III RNAses. The pre-miRNA will migrate to the cytoplasm by the exportin 5 (Huang et al., 2011; Garza, 2011; Sotillo and Thomas-Tikhonenko, 2011).

In the cytoplasm the pre-miRNA undergoes another stage of maturation where the RLC (RISC loading complex) is composed by RNaseDICER acytoplasmic endonuclease that performed the second stage of processing, which includes a leading strand or miR and a passenger strand or miR*.

For miRNAs with a high degree of complementarity throughout the loop stem, an additional cleavage at the middle of the strand is effected by the triteness activity of the Ago2 protein before that cleaved by the enzyme dicer in order to generate an ac-pre-miRNA (Ago2-cleaved precursor miRNA) (Eulalio et al., 2008).

Then, the resulting miRNA will form a RISC complex, one of the two strands called the "passenger strand" is degraded, whereas the strand having the least stable 5′ end called the "guide strand" is retained—it is the mature miRNA. The evidences have shown that the strand with more unstable 50 (weaker base pairing) has lower chance of degradation (Sotillo and Thomas-Tikhonenko, 2011) (Fig. 7.2).

MicroRNAs in Emerging Disease

Although new established methods such as reverse transcription quantitative PCR (RT-qPCR) have been used to detect viral infections, there is still a lack of robust biomarkers for early diagnosis and prognosis of the infectious disease. Increasing evidence indicates that cell free miRNAs are present in body fluids including blood and saliva. They are produced endogenously in response to the molecular change in cells, and therefore they can be used as diagnostic reporters for various diseases such as cancer and viral infections (Zhu et al., 2014). MiRNA is a class of small (18−24 nucleotides in length) noncoding RNAs, which involves in gene regulation and plays important roles in cell proliferation, differentiation, apoptosis, and tumorigenesis (Kaladhar, 2015). From the previous reports, miRNAs have distinct expression profiles in virus-infected cells in comparison to their healthy counterparts

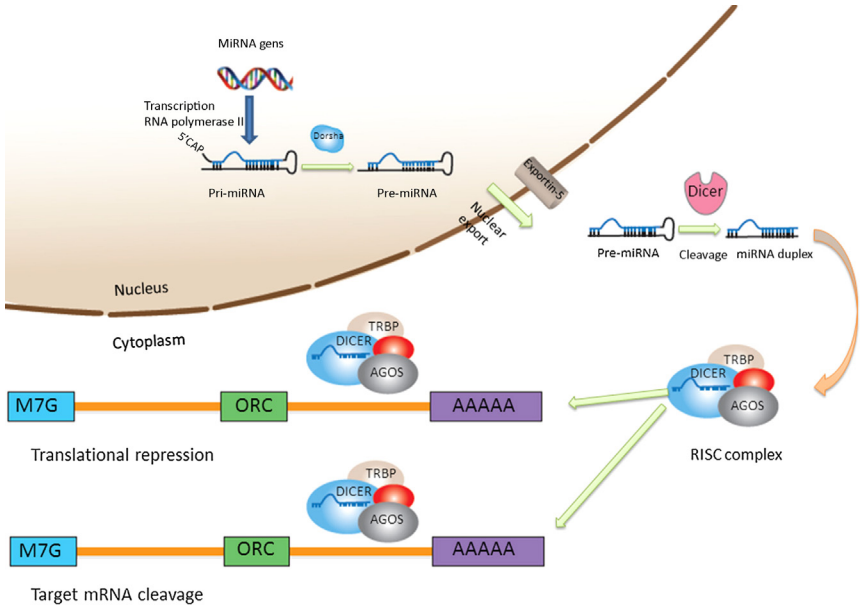

FIGURE 7.2 An overview of the miRNA biogenesis and its functional mechanism. RNA polymerase II produces a 500–3000 nucleotide transcript, called pri-miRNA, that is mature to pre-miRNA hairpin by a DROSHA (_60–100 nucleotides). This double-stranded hairpin structure is exported from the nucleus into cytoplasm by exportin 5 (RAN GTPase). Lastly, the pre-miRNA is processed by DICER1 and produces sense and antisense strands, approximately 20 nucleotides in length, the effective strand called antisense and known as mature miRNA and short-lived complementary sequence called passenger strand (miR*). The antisense-stranded miRNA is combined into RISC, which then targets it to the target 30 untranslated region mRNA sequence to facilitate repression and cleavage. *AA*, Poly A tail; *m7G*, 7-methylguanosine cap; *miRNAs*, microRNA; *ORF*, open reading frame; *RNA*, ribonucleic acid.

(Tambyah et al., 2013; Zhu et al., 2014). Moreover, the expression levels of many miRNAs in virus-infected samples and normal controls exhibit fold change difference (Song et al., 2010).

This studies show that in addition to regulating multiple processes, miRNAs themselves may be independent effectors of innate immunity by directly targeting viral transcripts. In vitro studies show miRNA target influenza; vesicular stomatitis virus, human T-cell leukemia virus 1; human papillomavirus; and enterovirus 71, and they inhibit viral replication (Fig. 7.3), which facilitates the clearance or potentiating viral latency of the pathogen (Bai and Nicot, 2015; Hen et al., 2014; Zheng et al., 2013). MiRNAs were also essential to show malaria transcripts translocation into the parasite (150). Indeed, due to their targeting of viral transcripts, miRNAs have the potential to partially dictate the cell tropism of a virus, the resistance of resting T-cells to human T-cell leukemia virus appears to be due to their expression of mir-28-3p

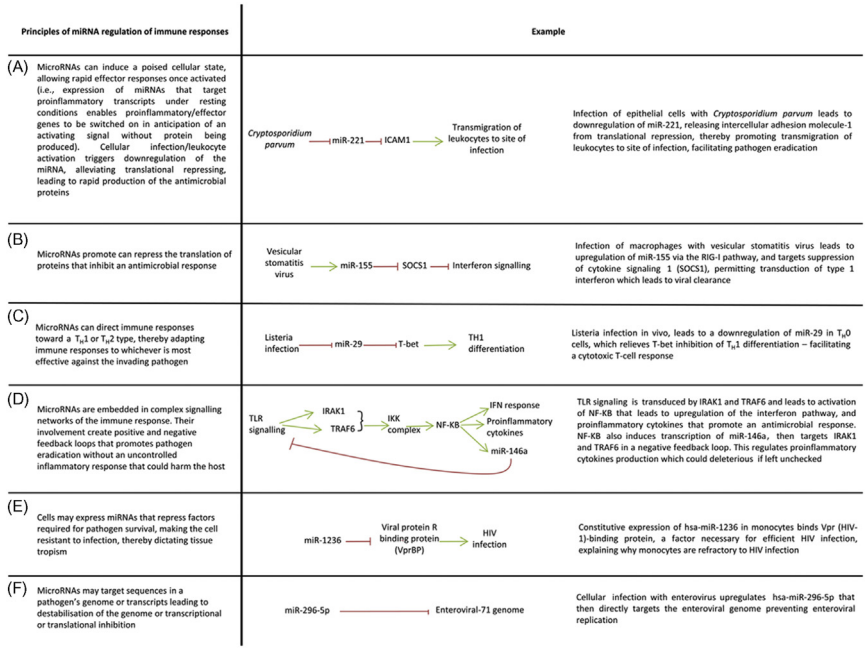

FIGURE 7.3 Key ways miRNAs mediate immune responses to pathogens. (A–F) A variety of ways in which miRNAs regulate immune responses. *miRNAs*, microRNAs.

(Bai and Nicot, 2015). Although a few scientists agree on the direct interaction between miRNAs and viral transcripts, Bogerd et al. (2014) argue that cellular miRNAs do not target viruses as global downregulation of host cell miRNAs (via DICER knockout) does not lead to the enhancement of 11 viruses in human embryonic kidney cell line 293T. However, Bogerd et al.'s model is problematic as viruses may be dependent on cell mechanisms that are controlled by miRNAs, and the usual host cell of the viruses in their study is not human embryonic kidney cells. Contrary to Bogerd et al.'s study, there is evidence that direct targeting of viral genome/transcripts occurs in vivo as several groups have successfully attenuated viral vaccines by incorporating human miRNA seed sites in viral genome (see The Clinical Applications of miRNAs: Improving Vaccines) (Barnes et al., 2008). The relative importance of miRNA direct targeting of viruses in innate immunity remains to be seen however as in vivo and in vitro studies show viral mutation of miRNA seed sites in viral genomes means viruses can quickly evolve to avoid being targeted by miRNAs (Zheng et al., 2013; Heiss et al., 2012).

There is another category of miRNAs, which are secreted from cells and called extracellular miRNAs (ex-miRNAs, circulating miRNAs).

This ex-miRNAs can be isolated from most biological fluids (de Candia et al., 2013; Irmak et al., 2012; Weber et al., 2010).

Often, we find these ex-miRNAs in extracellular vesicles (exosomes, microvesicles, and apoptotic bodies), and through their association with Argonaute protein (a component of the RISC complex—see Fig. 7.2) and high-density lipoprotein (de Candia et al., 2013; Arroyo et al., 2011; Vickers et al., 2011; Zernecke et al., 2009). Their biological function is debated since their secretion may be activated as intercellular communicators of gene regulation, or as cellular waste disposal method, or passively secreted as a by-product of cell death (Turchinovich and Cho, 2014). Although these three theories can be valid, there is increasing evidence that ex-miRNAs are functional, can be passed between leukocytes in vitro and in vivo, and play a role in disease (Alexander et al., 2015; Bell and Taylor, 2017; Lehmann et al., 2012; Mittelbrunn et al., 2011). Regardless of their functions, one clinical application of ex-miRNA is the use of these as biomarkers of infectious disease.

Furthermore, these miRNAs have shown the potential to be used as a biomarker for the prognosis and therapy of infectious diseases. miRNAs have also shown a significantly altered expression during infection. The altered expression of miRNA level in an infected human can be identified by the use of advanced diagnostic tools. In addition to their availability in numerous body fluids, miRNAs are highly stable in these fluids. These features make miRNAs, in single or in a combination (Peng et al., 2016), ideally suited as biomarkers for disease diagnosis.

Presently, only few standardized procedures are available for the isolation and characterization of specific miRNA. Experimental research and its observation have shown that small interfering RNA, premature miRNAs, and transfer RNA may interfere with specific miRNA during the process of isolation and characterization. Therefore this interference leads to the false-positive result, which should be taken care of during diagnosis. The necessity of the large amount of RNA input for the Northern blot technique can generate difficulties in the quantification of the miRNA. RT-qPCR, microarray profiling, and next-generation sequencing have been found to be useful for the identification of novel miRNA. An experienced researcher having a good knowledge of molecular biology as well as bioinformatics should do the characterization of miRNA.

Applications of MicroRNAs in the Treatment of Infectious Diseases

The immunomodulatory functions of miRNAs represent a promising application of miRNAs in the target of promoting antimicrobial pathways during infection and controlling dysregulated inflammatory

responses during sepsis. In the lungs of mice infected with nontypeable *Haemophilus influenza*, Hock et al. noted that the physiological downregulation of miR-328-3p promotes phagocytosis by neutrophils and macrophages and bacterial killing and found that it boosts this downregulation by intratracheally administering an antagomiR of miR-328-3p enhanced bacterial killing when they made challenge with nontypeable *H. influenza*. In another case, Alexander et al. observed that mice inflammatory response to endotoxin in vivo was ameliorated and enhanced with administration of exosomes containing miR-146a and miR-155, respectively, which prompts the authors to conclude that such treatments could be useful adjuncts in managing sepsis (in the case of miR-146a) or vaccination (in the case of miR-155). Moreover, another work by Wang et al. (2015) supports the idea that miRNAs can be used in diseases therapy, especially sepsis, when they administrate mesenchymal stem cell exosomes containing miR-223, which confer cardiac protection in septic mice.

Overall, using miRNA-based therapies to leverage immune response may prove useful adjuncts to standard antimicrobial therapies, for example, in multidrug-resistant Gram-negative infections, or chronic viral infections such as hepatitis C. Nevertheless, there are significant challenges in implementing miRNA-based antimicrobial therapeutics, which include devising methods of administration, and drug design that will protect miRNA mimics/antagomiRs from circulating RNAse enzymes. Delivery systems have to ensure targeted efficient delivery of miRNAs to the site of infection, because, as noted earlier, a miRNA may appear in many cell types, serving very different functions making off-target effects a real possibility, limiting efficacy, and safety (Chen et al., 2015).

A detailed analysis of the outcomes of phase 1 trials of two miRNA-based cancer treatments will provide more important data on the feasibility of miRNA-based therapeutics generally (Beg et al., 2017; Reid et al., 2013). As noted in the earlier RG-101 trial, viral mutation and resistance is an issue that will need tackling (Van der Ree et al., 2017).

Applications of MicroRNAs as Biomarkers of Infectious Disease

The class of ex-miRNAs is ideal biomarker candidates due to their possible isolation from biological fluids (Boon and Vickers, 2013). Molecular methods such as RT-PCR are already used routinely in the clinical setting to quickly identify infections (e.g., respiratory infections in babies with bronchiolitis) and could be used to quantify ex-miRNAs in patient samples.

A search of the literature identified 57 studies assessing ex-miRNAs in infectious diseases through whole micronome profiling and candidate miRNA approaches. Thus the huge majority of these works are based on serum and plasma, but ex-miRNAs in cerebrospinal fluid (CSF), saliva, and sputum have also been implicated. Until now, most of the studies have focused on HCV, hepatitis B virus, HIV, TB, and sepsis with the aim to improve diagnosis and prognosticate infection outcome (e.g., death in sepsis, liver cirrhosis in hepatitis) or treatment response.

These infection studies are based on comparison of ex-miRNA profile of patients and healthy controls. Then, many studies identify that expressions of ex-miRNA are highly predictive of infection. In the study by Zhang et al. (2013), they found a differentiated expression of miR-378, miR-483-5p, miR-22, miR-29c, miR-101, and miR-320b between pulmonary TB and healthy controls with a high sensitivity and specificity going up to 95% and 91.8%, respectively. The limitation of healthy controls used for comparison is that differentially expressed ex-miRNAs may represent a nonspecific marker of infection, and this leads to limited clinical translatability of these studies given most people undergoing tests are symptomatic of some disease process. Although, to solve this problem, a handful of studies have chosen more pragmatic comparator groups and promisingly suggest ex-miRNA signatures can differentiate particular infectious disease from other differential diagnoses.

Furthermore, a promising application of ex-miRNA biomarker work may be to differentiate viral from bacterial infection, identify or prognosticate sepsis, and in monitoring of response to antimicrobial treatment. There are substantial interstudy discrepancies in miRNAs identified as potential biomarkers. This may be due to heterogeneity in study design (e.g., data normalization methods) and confounders such as hospital differences in defining sepsis and ICU admission criteria. Differences in the lengths of illness between patients create noise in the data; longitudinal studies that measure serial miRNA levels would provide temporal information on miRNA expression in sepsis and may resolve some conflicting findings.

However, there are challenges in using miRNAs as biomarkers of infectious disease, and this is underlined by a lack of interstudy cross validation of many results. Conflicting study results may arise from heterogeneity in study design including differences in populations and control groups, methods of miRNA extraction and the circulating fraction under investigation (serum, plasma, microvesicles, or exosomes), micronome expression profiling platforms (next-generation sequencing, probe-based hybridization microarrays, or RT-PCR arrays) and the dearth of miRNAs assessed, the limited statistical power of many studies at the profiling stage, data normalization methods, whether *P*-values were adjusted to take account of multiple testing issues (usually not

done), and whether confirmatory cohorts were used to validate results. Given there is good evidence that miRNA contained in exosomes is functionally secreted as intercellular mediators of gene regulation, it is tempting to speculate that biomarker studies which profile miRNAs in exosomes rather than ex-miRNA in total plasma/serum (which will include a background of miRNA present from dead cells) could be more sensitive or specific biomarkers; comparisons of different extraction methods within the same biomarker study could help resolve this possibility.

Examples of MicroRNAs in Diagnosis of Emerging Diseases

Case of H7N9

In the previous studies, it was reported that miRNAs play an important role in influenza virus–host interaction. In a study by Fang Peng et al., the miRNA expression profiles in the sera of H7N9-infected patients and healthy controls were analyzed using miRNA microarray. Among the 94 miRNAs that were significantly differentially expressed in H7N9 serum samples when compared with that of healthy controls, 53 miRNAs were upregulated and 41 downregulated. Five serum miRNA candidates (hsa-miR-197-5p, hsa-miR-320a, hsa-miR-320d, hsa-miR-320e, and hsa-miR-765) were further verified by RT-qPCR. Receiver operating characteristic curve analysis was performed to evaluate the potential use of these miRNAs for the H7N9 infection diagnosis from the serum samples.

In this study, miRNA microarray assays revealed differential expression of 94 miRNAs in H7N9 patients' serum samples when compared to that in healthy controls. They identified five miRNAs that can be used for the diagnostic biomarkers for the early detection of the H7N9 infection, and this miRNA signature will advance our understanding of the molecular mechanisms involved in the influenza H7N9 infectious disease.

Ebola Virus

Rapid and accurate diagnosis of highly transmissible, lethal illnesses such as Ebola virus (EBOV) disease (EVD) is critical for restricting pathogen spread and for applying appropriate therapeutic strategies. As demonstrated by the recent EVD outbreak in Western Africa, early detection and confirmation of suspected cases are essential to halting disease spread (Blackley et al., 2014).

Current diagnostics rely on identifying EBOV in blood samples by targeting viral antigens using enzyme immunoassays or by amplifying

specific viral sequences through quantitative reverse transcriptase PCR (RT-PCR) (Trombley et al., 2010).

In the study by Janice Duy et al., the authors analyzed the expression of 752 circulating miRNA sequences in archived plasma from rhesus macaques exposed to EBOV infected either through intramuscular injection or through aerosol inhalation. We identified miRNAs that showed significant changes in abundance during lethal EBOV infection for each group. They found 15 miRNAs correlated with viral titer in both rhesus macaque as well as human samples. As a proof of concept for a host miRNA–driven diagnostic, they identified eight miRNA classifiers predictive of acute infection with high accuracy in both nonhuman primates and humans, and this classifier identified half of the presymptomatic macaque hosts.

Keeping these caveats aside, this work shows that miRNAs are potential diagnostic candidates via a proof of concept acute EVD classifier while also establishing the potential basis for presymptomatic or asymptomatic diagnosis of the disease.

CONCLUSION

Clinical microbiology laboratories at the local level have an increasing responsibility to provide rapid and accurate diagnostic services for emerging (new) and reemerging infectious diseases, especially those diseases for which significant mortality or morbidity may occur as the result of a delay in diagnosis. Rapid, accurate diagnosis of emerging and reemerging infectious diseases may also be critical at the local level to ensure optimal infection control. Detection of these pathogens has often required esoteric procedures such as conventional PCR, which could be performed only at referral laboratories or, recently, at public health laboratories.

Recent technical advances in molecular diagnostics have resulted in the development of user-friendly automated testing platforms, such as real-time PCR. These novel-testing methods can be used to detect emerging and reemerging pathogens as well as common pathogens and have the potential for broadscale use in smaller laboratories in close proximity to the delivery of care.

While writing this review, a large outbreak of influenza virus type A (H3N2) was peaking in the United States, and new influenza virus type A strains (H5N1, H9N2) have been associated with both avian and human influenza in regions of the Far East. The apparent significant morbidity and mortality associated with these new influenza virus strains emphasize the need for rapid, accurate laboratory diagnostic capabilities at the local level. As is the case for SARS, agents of

bioterrorism, and the other pathogens, rapid diagnostic methods, such as real-time PCR, and microarray will likely play a major role in the early and sensitive detection of emerging and reemerging infectious diseases encountered in the future.

Otherwise, a class of small RNAs implicated in the diagnosis of these diseases is miRNAs and is considered an essential mediator of host response to pathogens. Since several microbes have evolved to exploit their pleiotropic characteristics, identification of key genes and pathways in terms of activation, enhancement, repression, or silent, which are essential to facilitate the immune response, is based on the elucidation of the roles of miRNAs in host response to infectious disease. The complex regulatory network within which miRNAs are embedded makes unpicking the roles of miRNAs tough but not impossible. Integrating large miRNA and mRNA datasets using advanced statistical techniques (in a "systems biology" approach) will facilitate the unpicking of these complex networks.

Overall, miRNAs have multiple targets, and therefore any vaccines or treatments that harness miRNAs may produce off-target effects compromising safety; however, there are challenges that must be overcome. With the objective to improve the cross-study reproducibility of the findings, especially in the context of ex-miRNA biomarkers identification, universal endogenous controls are needed, and a more standardized approach to biomarker studies may also help. Initiatives devoted to harnessing the diagnostic and therapeutic potential of extracellular RNAs such as The National Institute for Health Extracellular Communication Consortium can facilitate this.

As the literature and experimental studies on miRNAs are developing, the potential for new miRNA therapeutics, diagnostics/prognostics, and vaccines becomes tangibly closer. Translating the insights of miRNA studies into improving the lives of patients is the critical next step.

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