Recent progresses and remaining challenges for the detection of Zika virus

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Abstract

Zika virus (ZIKV) has emerged as a particularly notorious mosquito-borne flavivirus, which can lead to a devastating congenital syndrome in the fetuses of pregnant mothers (e.g., microcephaly, spasticity, craniofacial disproportion, miscarriage, and ocular abnormalities) and cause the autoimmune disorder Guillain-Barre’ syndrome of adults. Due to its severity and rapid dispersal over several continents, ZIKV has been acknowledged to be a global health concern by the World Health Organization. Unfortunately, the ZIKV has recently resurfaced in India with the potential for devastating effects. Researchers from all around the world have worked tirelessly to develop effective detection strategies and vaccines for the prevention and control of

Abbreviations: 25HC, 25-hydroxycholesterol; 3′-UTR, 3′-untranslated region; Au-SHIN, gold shell-isolated nanoparticle; AZBTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic-acid); BART-LAMP, bioluminescent assay in real-time loop-mediated isothermal amplification; BPD, bioplasmonic paper-based device; BPE, 1,2-bis (4-pyridyl) ethylene; CDC, Centers for Disease Control and Prevention; CHIKV, chikungunya virus; CPE, cytopathic effect; CSF, cerebrospinal fluid; DENV, dengue virus; ECL, electrogenerated chemiluminescence; EDIII, domain III of the envelope protein; EIS, electrochemical impedance spectroscopy; EP, envelope protein; EUA, Emergency Use Authorization; FEB, field effect biosensing; flaviMIA, flavivirus IgM MIA; GBS, Guillain-Barré syndrome; HAD, helicase-dependent amplification; hCNPC, human cortical progenitor cell; HRP, horseradish peroxidase; IFA, immunofluorescence assay; IgG, immunoglobulin G; IgM, immunoglobulin M; JEV, Japanese encephalitis virus; LCV, leuco crystal violet; LFA, lateral flow assay; MAC-ELISA, IgM antibody capture enzyme-linked immunosorbent assay; MB, molecular beacon; MABS, 4-mercaptobenzoic acid; MIA, multiplex microsphere immunoassays; MMI, multi-mode interference; MOP, metal-organic framework; NASBA, nucleic acid sequence-based amplification; NAT, nucleic acid test; NB, Nile blue; NBC, nanomotor-based bead-motion cellphone; NIRS, near-infrared spectroscopy; NP, nanoparticles; NPC, neural precursor cell; NS1, nonstructural protein 1; OPD, o-phenyl-enediamine; PAHO, Pan America Health Organization; PBS, phosphate-buffered saline; PFU, plaque forming units; PRNT, plaque reduction neutralization test; PSB, polystyrene beads; Pt, platinum; Qdot, quantum dot; QUASR, quenching of unincorporated amplification signal reporters; RDB, reverse dot-blot; RDP, Recombination Detection Project; RGS, radial glia cells; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; RT-PCR, reverse transcriptase polymerase chain reaction; RT-SIBA, reverse transcription strand invasion based amplification; RVNT, reporter virus neutralization test; RVP, reporter virus particle; SCC, smart-connected cup; SDA, strand-displacement amplification; SERS, surface-enhanced Raman spectroscopy; SWV, square wave voltammetry; TMB, 3,3′,5,5′-tetramethyl benzidine; UTR, untranslated region; VLP, virus-like particle; WHO, World Health Organization; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

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ZIKV infection. In this review, we comprehensively summarize the most recent research into ZIKV, including the structural biology and evolution, historical overview, pathogenesis, symptoms, and transmission. We then focus on the detection strategies for ZIKV, including viral isolation, serological assays, molecular assays, sensing methods, reverse transcription loop mediated isothermal amplification, transcription-mediated amplification technology, reverse transcription strand invasion based amplification, bioplasmonic paper-based device, and reverse transcription isothermal recombinase polymerase amplification. To conclude, we examine the limitations of currently available strategies for the detection of ZIKV, and outline future opportunities and research challenges.

KEYWORDS molecular assay, sensing methods, serological assay, viral isolation, ZIKV infection

1 INTRODUCTION
Zika virus (ZIKV), a mosquito-borne virus of the Flaviviridae family, is associated with high mortality rates in fetuses, neurological impairments and microcephaly in neonates, and the autoimmune disorder Guillain-Barre’ syndrome (GBS) in adults. Due to its fast dispersal over several continents and recent outbreaks in the Americas, ZIKV has caused significant concern in susceptible populations.1-7 In view of the severity of associated birth defects and the large-scale outbreaks, ZIKV has been acknowledged as a public health emergency of international concern by the World Health Organization (WHO) on February 1, 2016.8-13 Recently, it has been reported that ZIKV has resurfaced in India with a significant outbreak of cases reported in Jaipur city, Rajasthan, India during the period of September 2nd, 2018, to October 29th, 2018. Complete genome phylogenetic analysis of Jaipur city sequences with the known GenBank ZIKV sequences revealed that the outbreak in Jaipur city was being caused by the ZIKV belonging to Asian lineage.14 Therefore, with the increase of ZIKV infections worldwide, the monitoring of ZIKV infections presents a huge and currently unmet challenge.15,16

1.1 Structural biology and evolution of ZIKV
ZIKV is an arthropod borne virus that belongs to the flavivirus genus within the flaviviridae family. ZIKV is predominantly single-stranded positive-sense RNA virus with a 10.7-kb genome. The ZIKV RNA genome consists of 10,794 nucleotides, with a long single open reading frame (ORF) flanked by 5’- and 3’-noncoding region. The ORF encodes for all of structural and nonstructural components of ZIKV, including three structural proteins (capsid [C], premembrane [prM], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 1).17-27 E protein plays an important role in the host surface binding and membrane fusion, and the prM averts premature fusion, and the C protein can help package new RNA genomes into progeny
virions. While seven nonstructural proteins play a pivotal role in regulating viral replication and transcription together with the activation of host antiviral responses. Based on previous reports, ZIKV evolved in Uganda probably between 1892 and 1943. A nucleotide sequence-based study of the NS5 gene indicated that three lines of ZIKV exist: Asian (one strain analyzed), East African (one strain analyzed), and West African (three strains analyzed). Through the analysis of entire genomes (phylogenetic), there are two genetic lines of ZIKV originating from Africa and Asia. The Asian lineage was further classified into two factions: Micronesian and Malaysian strains.

**FIGURE 1** (A) Polyproteins of ZIKV, including three structural proteins and seven nonstructural proteins. (B) ZIKV virions are enveloped, spherical, approximately 50 nm in diameter and the surface prM/M and E proteins are arranged in an icosahedral-like T = 3 symmetry. The pictures are modified from ViralZone, SIB Swiss Institute of Bioinformatics. (C) Genome expression of ZIKV. [Color figure can be viewed at wileyonlinelibrary.com]
African strains were grouped into a Nigerian cluster and MR766 prototype cluster (discovered in Senegal and Uganda). The full length of the ZIKV genome was identified and published in 2007. At that time, the ZIKV primarily infected wild primates and caused sporadic infections in humans. The virus is believed to adapt to humans by losing an NS1 codon of the genome or by molecular evolution. Unlike other flaviviruses, ZIKV had experienced several recombination events. There are around 13 reported recombination events obtained from primary analysis via the Recombination Detection Project sequencing tool and Genomic breakpoints at several different alignment positions were evaluated using Rec-HMM (e.g., 1044–1095, 5181–5238, 9580–9631, and 9007–9132). But genetic breakpoints are only observed in NS5 and E regions within the genome. While one event in the E sequences was observed near the 1065th and 414th site regions of the E gene producing nine viral strains (including ArA982, ArA986, ArA27096, ArA27106, ArA27290, ArA27101, ArA27407, ArA27443, and HD78788). Similarly, in the NS5 gene of strains (i.e., ArD158084, ArB1362, and ArD157995), one recombination event was evaluated near 2152 and 1581.

1.2 | Historical overview

In 1947, ZIKV was recognized in a sentinel rhesus monkey captured from the Zika Forest of Uganda, by the Yellow Fever Research Institute in Entebbe. Shortly after, ZIKV was isolated from *Aedes africanus* mosquitoes. However, it was not confirmed in humans until 1952 when human infection was first detected in Nigeria. Owing to an interconnected world, ZIKV quickly migrated to other African countries over the next two decades, including the Central African Republic, Tanzania, Sierra Leone, Gabon, and Egypt. Then, the virus migrated to Asia and was discovered in India, Pakistan, Thailand, Vietnam, Cambodia, Indonesia, Malaysia, and the Philippines. Up until 2007, there were just 14 reported clinical cases of human infection worldwide, including 13 natural infections and one laboratory-acquired infection. In 2007, the first large outbreak occurred in Yap Island, Micronesia. Over a 3 month period from April to July, approximately 5000 infections of ZIKV occurred. This outbreak in Yap Island, Micronesia was confirmed as the first ZIKV transmission outside Africa and Asia. French Polynesia reported a second outbreak of ZIKV in October, 2013, and for the first time neurological (GBS) was associated with ZIKV infection. Over a period of 5 months, there were about 30,000 symptomatic cases as well as 42 cases of GBS associated with ZIKV infection. Subsequently, the virus quickly spread throughout the Pacific. However, when an increase in congenital ZIKV syndrome (CZS) cases related to ZIKV was observed in August, 2015, it began to attract global attention. ZIKV rapidly spread throughout northeastern Brazil, resulting in birth defects (e.g., microcephaly). Large outbreaks have since spread to other countries such as America and Mexico. In Florida and Texas, there are two documented foci of autochthonous ZIKV transmission. On October 16, 2018, the Government of the Indian Ministry of Health and Family Welfare reported more than 80 positive cases of ZIKV infection in Jaipur, Rajasthan, India. To understand the circulating ZIKV strain in Rajasthan state 10 human serum specimens were collected from positive ZIKV patients in Jaipur city, a complete genome phylogenetic analysis of the Jaipur sequences with the known GenBank ZIKV sequences showed that the outbreak was caused by ZIKV belonging to an Asian lineage.

1.3 | Pathogenesis and symptoms

1.3.1 | Pathogenesis

There are few reports on the pathogenesis of ZIKV infection, and as such remains largely unknown. However, most arboviruses have been reported to replicate within the skin dendrites at the primary inoculation site, then they spread to the regional lymph nodes and subsequently to the blood stream. Immature dendritic cells, human
dermal fibroblasts, and epidermal keratinocytes are known to be accommodating to ZIKV infection. The TIM-1, DC-SIGN, AXL, and Tyro entry/adhesion factors permitted the entry of ZIKV. The replication of ZIKV could trigger an immune antiviral response and in infected cells produced type I interferon. Similarly, T cells (e.g., Th1, Th2, Th9, and Th17) are activated during the acute phase of ZIKV infection. Animal models can be used to help understand the pathogenesis of ZIKV infection. For example, Li et al. have used a mouse model to demonstrate that 25-hydroxycholesterol (25HC) could protect hosts against ZIKV infection. The addition of 25HC inhibited ZIKV infection in vitro by blocking the entry of the virus. Moreover, treatment with 25HC could reduce viremia and could provide protection against ZIKV in rhesus macaques and mice. The results indicated that 25HC could be used as a potential natural antiviral agent to defeat ZIKV infection and prevent ZIKV-associated birth defect (such as microcephaly). The primary neural progenitor cells for cortex development in the fetus are radial glial cells (RGs). Wu et al. found that when the abdominal cavities of pregnant mice were injected with a contemporary ZIKV strain, the RGs of the dorsal ventricular zone were infected, causing a remarkable reduction of these cortex founder cells in fetuses. Therefore, the study reinforced the conclusion of ZIKV vertical transmission and infection affecting fetal brain development. Moreover, the study also provided a promising animal model for the evaluation of therapeutic regimes and preventative measures. Cugola et al. demonstrated that Brazilian ZIKV (ZIKVBR) could infect fetuses and could lead to birth defects, including intrauterine growth restriction and microcephaly in mice. In addition, the virus could infect human cortical progenitor cells (hCNPCs) and even result in the increased cell death. It was verified that ZIKVBR could cross the placenta and lead to microcephaly by targeting cortical progenitor cells, and could induce cell death and impair neuronal development. It has been confirmed that ZIKV infection can increase cell death by the dysregulation of cell-cycle progression and lead to the death of hCNPCs, causing cortical thinning and microcephaly. Rana et al. demonstrated that a prototype strain of ZIKV, MR766, which could efficiently infect organoids and lead to a decrease in overall organoid size correlated with the kinetics of viral copy number. The human embryonic stem cell derived organoid was successfully developed for recapitulating first trimester growth of fetal brain. Moreover, it was found that in neurosphere models that ZIKV could infect neural progenitor cells, and the ZIKV activated toll-like receptor 3 (TLR3) in cerebral organoids attenuating neurogenesis. In another study, Kriegstein's group clarified the fundamental cellular and molecular mechanisms of neurological defects linked to ZIKV infection. Moreover, the expression of receptors during the entry of enveloped viruses such as ZIKV into various cell types of the developing brain has been investigated. Importantly, the strong neurotropism of ZIKV was confirmed to be associated with microcephaly and other neurological complications, including myelitis, meningoencephalitis, and GB syndrome. Based on inherited syndromes contacting microcephaly with ocular findings comparable to those associated with ZIKV infections, Bullerdiek et al. hypothesized that ZIKV could impair proper mitotic apparatus function and lead to teratogenic effects. Similarly, Dang et al. confirmed that the gangliosides could play a huge role in ZIKV associated neurological complications. In addition, Garcez et al. confirmed that ZIKV could target human brain cells (which were used as neurospheres and brain organoids) and reduce the viability and growth. Moreover, it was verified that ZIKV could eliminate neurogenesis during the development of the human brain. Importantly, the strong neurotropism of ZIKV was confirmed to be associated with microcephaly and other neurological complications, including myelitis, meningoencephalitis, and GB syndrome. Based on the study of murine models, the placenta has been confirmed to be a portal for infection as well as replication. Aagaard et al. reported that in human placental trophoblasts the ZIKV-FLR strain could replicate and not destroy the host cell, which served as a possible pool and gateway for fetal transmission with the risk of latent malformations and microcephaly.
### 1.3.2 Symptoms

In 1952, the first human infection by ZIKV was observed, and the symptoms of ZIKV infection were asymptomatic or mildly symptomatic with retro-orbital headache, conjunctivitis, mild fever, and small joint pain. The temperature of the patient infected by ZIKV rises after about 3–5 days, and the patients develop a maculopapular rash. With the global spread of ZIKV, the symptoms of ZIKV infection have become diverse. After ZIKV has incubated for around 3–12 days, there are some common symptoms of ZIKV infection, such as fever, headache, arthralgia, conjunctivitis, myalgia, and rash. In addition, there are some other less common symptoms, including abdominal pain, diarrhea, anorexia, dizziness, and constipation. Fortunately, most individuals infected by ZIKV only suffer from mild or no symptoms. About 25% of patients infected by ZIKV show some symptoms, such as joint pain, fever, rash, headache, and red eyes. Moreover, the ZIKV rarely results in death, and the patients on the whole completely recover. Based on the reports of the Pan America Health Organization (PAHO), there are some common clinical symptoms in patients infected by ZIKV, including mild fever, exanthema (skin rash), muscle, or joint pain, as well as conjunctivitis. However, compared with the epidemics in Polynesia and Brazil, the neurological and autoimmune complications are quite rare. Most ZIKV symptoms are unlike other flaviviruses, the ZIKV can enter the fetal blood stream to gain access to fetal tissue and cause microcephaly in neonates by efficiently using the AXL cell-surface receptor.

During a ZIKV outbreak in French Polynesia, GBS in 73 cases was suggestive of a possible connection between the ZIKV and serious neurological complications. Likewise, it has been reported that the frequency of GBS increased markedly, and there was a 20-fold increase in the rate of microcephaly in the newborn population during a ZIKV outbreak in Brazil in April 2015. However, there is no strong link between ZIKV infection and GBS, with the strongest evidence coming from a study that identified 42 cases of GBS. Among the 42 cases of GBS, 88% of them had a recent history of acute viral infection during a ZIKV outbreak in French Polynesia. Soon after this, it was shown that ZIKV infection in the first trimester of pregnancy resulted in microcephaly as well as other congenital anomalies. In addition, it has been reported that the ZIKV can cross the placenta and result in congenital anomalies.

CZS as the one of the clinical syndromes of symptomatic ZIKV infection has been widely investigated. Microcephaly is defined as a head circumference (HC) below the third percentile (<2 SD), which is the most prominent and common clinical feature of suspected CZS. In addition to microcephaly, fetuses and neonates with the suspected congenital ZIKV infection also exhibited other malformations. Common features mainly involve arthrogryposis, anasarca, polyhydramnios, low birth-weight, and redundant scalp skin. Neurologic impairments include swallowing dysfunction, epilepsy, cerebral lesions, brainstem dysfunction, movement abnormalities, and polymalformative syndromes. Eye abnormalities include coloboma of the retina, asymmetrical eye sizes, lens subluxation, intraocular calcifications, cataract, optic nerve hypoplasia, and macular atrophy. Using ultrasonographic examination, cerebral atrophy, intracranial calcifications, enlarged cisterna magna, severe unilateral ventriculomegaly, asymmetrical cerebral hemispheres, the midline displacement, and the thinning of the parenchyma on the dilated side, brainstem and pons have been observed.

### 1.4 Transmission

Based on previous reports, the transmission of ZIKV between humans is mainly through vector transmission, sexual transmission, maternal fetal transmission, and body fluid transmission (e.g., blood, urine, and saliva). We will now discuss in detail the different routes (Figure 2) for the person to person transmission of ZIKV.

#### 1.4.1 Vector transmission

It has been reported that the ZIKV incubates for a period of 4–5 days in the human host and can then infect other vectors during blood feeding. Then, the virus spends an extrinsic incubation period of 8–12 days in the vectors
infected by the virus and is then transported in the saliva of vectors to infect other hosts.\textsuperscript{34,114} Based on previous reports, the mosquitoes have been known as one of the most important carriers that spread and transport ZIKV. However, \textit{Aedes} are the most common mosquito species found as vectors of ZIKV transmission, including \textit{A. africanus}, \textit{A. aegypti}, \textit{A. furcifer}, \textit{A. vitattus}, \textit{A. apicoargenteus}, \textit{A. vittatus}, \textit{A. hensilii}, and so forth.\textsuperscript{19,114,124,166} For example, in the tropics and subtropics, \textit{A. aegypti} is the main mosquito vector associated with ZIKV transmission. In addition, \textit{A. aegypti} and the \textit{A. albopictus} species are known to spread ZIKV within the Americas.\textsuperscript{167} When \textit{Aedes} mosquitoes bite people infected by ZIKV, they can be infected by the ZIKV from humans. Then, healthy individuals can be infected by infected mosquitoes during the process of blood feeding.\textsuperscript{105,168} Since, ZIKV is associated with multiple mosquito species, the transmission dynamics of ZIKV still remain largely unknown.\textsuperscript{169} In recent years, the vertical transmission of ZIKV between mosquitoes has been widely studied. To test whether ZIKV could be vertically transmitted, Thangamani et al. injected ZIKV into the female \textit{A. albopictus} and \textit{A. aegypti}. The results indicated that the vertical transmission of ZIKV in \textit{A. aegypti} mosquitoes could offer a potential mechanism for the virus to survive under adverse conditions.\textsuperscript{170} Moreover, to measure the potential role of vertical transmission in ZIKV expansion, the larval pools of perorally infected \textit{A. aegypti} and \textit{A. albopictus} adult female mosquitoes were evaluated by Ciota et al. Approximately 1/84 larvae tested were ZIKV-positive and rates varied among mosquito populations, which indicated that the vertical transmission of ZIKV may play a role in ZIKV spread and maintenance.\textsuperscript{171} In addition, vertical transmission of ZIKV in \textit{Culex quinquefasciatus} Say, \textit{A. aegypti} (L.), larval \textit{A. aegypti} (Morelos, Mexico), and Jiegao and Mengding \textit{A. aegypti} strains in Yunnan province in China were

**FIGURE 2** The routes of ZIKV transmission. The picture is modified from Ref.\textsuperscript{165} Copyright 2018, Elsevier. ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
also studied. These studies suggested that vertical transmission of ZIKV in mosquitoes played potential roles in ZIKV spread and maintenance, and thus more mosquito control strategies should be proposed to regulate mosquitoes when a ZIKV epidemic occurs.

1.4.2 | Sexual transmission

Sexual transmission of ZIKV has been reported, and significant effort has been given to explore that transmission mode. The first report of the sexual transmission of ZIKV was in Colorado, USA (2008). Similarly, in 2011, another report indicated sexual transmission of ZIKV. While, another similar study indicated that a female diagnosed through the reverse transcriptase polymerase chain reaction (RT-PCR) was infected by ZIKV. The woman had sexual intercourse with an infected man who had recently returned from the Caribbean, and serologic testing confirmed that they were both infected by ZIKV. In addition, viral particles were discovered in the semen of a man who had been infected by ZIKV. Shortly afterward, the female-to-male sexual transmission of ZIKV was reported for the first time, which indicated that ZIKV could be transmitted through anal sex. The infectious viral load and ZIKV RNA detected in the semen of a male indicated the potential of sexual transmission. To date, ZIKV is the first arbovirus to be detected in the semen of humans. To study ZIKV sexual transmission and vaginal viral replication, Shresta et al. constructed a mouse model of ZIKV. Where, it was found that the infected mice were resistant to vaginal infection during the estrus-like phase. While, when the mice were infected during the diestrus-like phase, they succumbed to infection or experienced transient illness. Based on these lethal and sublethal mouse models, it was shown that intravaginal deposition of ZIKV could lead to transgenital transmission, hormonal changes in the reproductive tract (FRT) of female mice, and the replication of ZIKV could persist in the FRT of female mice for several days. Recently, some reports indicate that the RNA and infectious viral load of ZIKV can be detected in the urine and saliva of infected patients. Due to the related properties of behavior connected with sexual activity, it was quite difficult to distinguish between sexual transmission and urinary/salivary transmission.

1.4.3 | Maternal fetal transmission

Significant evidence exists to confirm that mothers infected by ZIKV can transmit the virus to their foetuses during pregnancy. In addition, it has been shown that ZIKV can cross the placenta and infect the nervous tissues of foetuses, the mechanism of which is supported by detecting ZIKV RNA and antigens in the placenta, amniotic fluid, and the brain tissues of babies. Moreover, ZIKV could damage the human placenta and display significant fetal neurotropism. To further study the mechanism of action, Miner et al. found that ZIKV infection could lead to placental damage and fetal death during pregnancy using mouse models, which further supported the transmission by a trans-placental route. Babies may be born with congenital teratogenic disorders when mothers were infected by ZIKV during pregnancy. For example, in Brazil, infected pregnant women have reported that their babies are born with congenital teratogenic disorders, such as microcephaly. Similarly, the ZIKV has been detected in the amniotic fluid of pregnant woman during the 17th week of pregnancy in Spain. Using an ultrasound scan, fetal malformations were observed, such as arthrogryposis and brain microcalcifications. It has been reported that the RNA and infectious viral particles of ZIKV can be detected with high loads in the breast milk of infected mothers. On this basis, the result indicated that the transmission of ZIKV can occur from a mother to her baby. For example, Besnard et al. evaluated two cases of mother-to-child ZIKV transmission.
and the RNA of ZIKV was found in the breast milk and serum of the two mothers. In addition, ZIKV RNA was also detected in the serum of their babies. However, when the breast milk was inoculated with Vero cells, the replication of ZIKV could not be detected, which made the transmission by breast milk uncertain yet plausible. Therefore, more evidence is required to support the ZIKV transmission through breast milk.

1.4.4 | Transmission through body fluids

It is known that the ZIKV can be transported by body fluids (e.g., blood, urine, and saliva), which has been confirmed through the detection of ZIKV RNA in urine, blood, saliva, and other body fluids using the RT-PCR. During a ZIKV outbreak in French Polynesia, there were about 3% asymptomatic blood donors detected with ZIKV RNA, and the potential risk of ZIKV transmission through blood transfusion received from infected patients has been reported by Musso et al. In addition, there have been two cases of blood transfusion-related ZIKV transmission in Brazil. To solve this problem, strict guidelines were issued by the WHO for blood donation/transfusion in regions where ZIKV was endemic in February 2016. In addition, the American Association of Blood Banks indicated that ZIKV represented a high risk and took preventative measures to control the ZIKV transmission through blood transfusion, such as pathogen inactivation, and nucleic acid test (NAT) of blood products. There were two methods put forward to prevent ZIKV transmission through blood transfusion, one was the detection of ZIKV in donated blood by a simple yet precise test, another was avoiding blood donations from infected individuals in ZIKV endemic regions or those who have traveled to those regions. To improve the safety of transfusion, rendering the pathogens in blood inactive through the pathogen reduction technologies is an extremely efficient approach. However, detection strategies and the transmission of ZIKV from donated blood require more investigation.

ZIKV has been detected in saliva and urine using RT-PCR, and ZIKV is more frequently detected in saliva and urine than that in blood using the RT-PCR tests. Infected individuals display the highest concentrations of ZIKV in their saliva at disease onset. Therefore, the saliva can be used as a suitable sample for the routine detection of ZIKV RNA when blood collection is difficult, particularly in neonates and children. In January 2016, Barzon et al. demonstrated that ZIKV could be detected in the saliva of patients who had recently returned from the Dominican Republic with febrile illness. Urine samples have been used for the detection of ZIKV infection, particularly during the later phases of ZIKV infection. Kutsuna et al. reported that the ZIKV was detected in the urine of a Japanese traveler who had travelled to Bora Bora in January 2014. In addition, there is evidence indicating that ZIKV can be transported through urine. For example, in June 2016, Bonaldo et al. reported that infectious ZIKV particles were isolated from the urine and saliva of patients with acute symptoms in Brazil. The urine and saliva samples were inoculated with Vero cells, and were detected through RT-PCR, NAT, and quantitative RT-PCR. However, the viral load found in urine samples was higher than that in saliva samples. It was shown that urine samples were important for the diagnosis of ZIKV infections within 10 days after the onset of the disease.

ZIKV is a huge global threat for humans due to rapid worldwide dispersal. Researchers from around the world have made significant effort to develop effective strategies for the detection of ZIKV infection (Figure 3). Unfortunately, recently, ZIKV has resurfaced in India with the potential for devastating effects. Therefore, novel, rapid, accurate, and sensitive strategies for the detection of ZIKV are urgently required to effectively prevent and control outbreaks of ZIKV. The discovery of ZIKV can be performed by detecting the viral components of ZIKV (e.g., viral proteins, RNA, or virus isolation), or measuring the antibody concentration against viral proteins of ZIKV (host immune response) through serological assays. In recent years, there have been many strategies (Table 1) developed for the detection of ZIKV, including viral isolation, serological assays, molecular assays, and sensing methods. Recently, there have been some novel detection strategies developed for the detection of ZIKV, such as reverse transcription loop mediated isothermal amplification (RT-LAMP), transcription-mediated amplification (TMA) technology, reverse transcription strand invasion based amplification (RT-SIBA), bioplasmonic
paper-based device (BPD), reverse transcription isothermal recombinase polymerase amplification, near-infrared spectroscopy (NIRS), nanoparticle (NP)-enhanced viral lysate electrical sensing assay, and smartphone-based fluorescent lateral flow immunoassay. In addition, there are a great many kits developed for the detection of ZIKV, such as the kits based on nucleic assay and serological assays. The ZIKV diagnostic kits currently authorized by Emergency Use Authorization and commercially available kits for ZIKV detection have recently been evaluated. While may reviews summarize the methods for the detection of ZIKV, they are not comprehensive, and just cover viral isolation, serological assays, and molecular assays. Herein, we comprehensively summarize the strategies for detecting ZIKV (i.e., the viral isolation, serological assays, molecular assays, advanced sensing strategies, and other current state of the art methods).

2 | VIRAL ISOLATION

The viral isolation from cell cultures has been considered as the "gold standard" for the detection of viruses, and has been widely and universally performed since the early 1960s. Moreover, viral isolation has been the standard strategy that all other strategies are compared with. Viral isolation is capable of being undertaken through the intracerebral inoculation of mice, AP 61 cell line (originated from Aedes pseudocutellaris), C6/36 cells (originating from A. albopictus), or Vero cells (originating from the kidney epithelial cells of the African green monkey) with the appropriate media and supplements for growth. Currently, C6/36 cell, AP 61 cell, and Vero cell lines are used more frequently than the intracerebral inoculation. Numerous types of cells grown in vitro (in flasks and test tubes) have been used as the living hosts for infection by human viruses. Compared with eggs and animals, such cell cultures are cheap and convenient to examine through a microscope for the evidence of viral proliferation. Thus, cell cultures have been recognized as a desirable environment for viral isolation. In brief, the samples are added into cell cultures and incubated at a controlled temperature for a specific period. The infectious capability of the virus can then be studied through series passages. The microscopic identification of the unstained cell culture as a standard method is used for the examination of viral proliferation. The existence of the virus can lead to the
| Method                  | Sample type     | Target                          | Sensitivity/LOD                  | Specificity   | Ref. |
|------------------------|-----------------|---------------------------------|---------------------------------|---------------|-----|
|                        |                 |                                 |                                 |               |     |
| **Serological assays** |                 |                                 |                                 |               |     |
| MAC-ELISA              | Serum           | ZIKV                            | Negative/quivocal/positive      | /             | 48  |
|                        | Human serum     | ZIKV antibodies                 | Negative/positive               | Highly specific | 236 |
|                        | Serum, CSF      | ZIKV                            | Negative/positive               | /             | 237 |
|                        | Virus cell culture | ZIKV antibodies           | Negative/positive               | /             | 238 |
|                        | Human Serum, CSF | ZIKV NS1                         | Less sensitive during initial 5 days | Highly specific | 239 |
|                        | Serum, CSF      | ZIKV antibodies                 | 93.98%                          | 100%          | 240 |
|                        | Serum           | ZIKV antibodies                 | 100%                            | 92.5%         | 241 |
|                        | Serum           | ZIKV antibodies                 | 82%                             | 100%          | 242 |
|                        | Serum           | ZIKV antibodies                 | 100%                            | 99.6%         | 243 |
|                        | Serum, CSF      | ZIKV antibodies                 | Serum: 90.5%, CSF: 97%          | /             | 244 |
|                        | Whole blood, EDTA, serum | ZIKV antibodies | Whole blood: 96.1% | 100% | 245 |
|                        | Serum and plasma |                                 | Serum: 95.1%                    |               |     |
|                        |                 |                                 | Plasma: 98.1%                   |               |     |
| PRNT                   | Serum           | ZIKV antibodies                 | Negative/positive               |               |     |
|                        | CSF, serum      | ZIKV antibodies                 | Negative/positive               | /             | 244 |
|                        | ZIKV infected cells (U2OS-GFP-IRF3) | ZIKV E protein   | /                               | /             | 247 |
| MIA                    | Serum           | ZIKV E, NS1 and NS5 proteins    | Negative/positive               | /             | 248 |
|                        | Serum           | ZIKV antigens                   | Acute sera: 47.4%               | 100%          | 249 |
|                        |                 |                                 | Convalescent sera: 100%         |               |     |
| IFA                    | Human serum     | ZIKV                            | Negative/positive               | /             | 112 |
| RVNT                   | Sera            | ZIKV                            | /                               | /             | 246 |
|                        | Serum           | ZIKV                            | 100%                            | 98.1%         | 250 |

(Continues)
| Method                                                      | Sample type                      | Target                              | Sensitivity/LOD | Specificity | Ref. |
|-------------------------------------------------------------|----------------------------------|-------------------------------------|-----------------|------------|------|
| Immunochromatography-aphy approach                          | Serum                            | ZIKV NS1 protein antigen            | 81%             | 86%        | 251  |
| ZIKV NS1 blockade-of-binding assay                           | Serum                            | ZIKV NS1 protein antigen            | ~92%            | 80.4–95.9%| 252  |
| Biosensor-based detection                                    | Serum, saliva                    | ZIKV NS1 protein, domain III of the E protein (EDIII) | Highly sensitive | Highly specific | 253  |
| Nanomotor-based bead-motion cellphone system                 | Urine, saliva, phosphate buffer  | ZIKV E protein                      | Urine and saliva: 1 particle/μl | /           | 254  |
|                                                            |                                  |                                     | PB: 1 and 10 particles/μl          |             |      |
| ZIKV NS1-based ELISA                                        | Sera                             | ZIKV NS1 protein                    | IgM: 58.8%; IgG: 88.2%; IgM/IgG: 100% | 99.8%       | 255  |
| Molecular assays                                             |                                  |                                     |                 |            |      |
| Method                                                      | Sample type                      | Target                              | Sensitivity/viral RNA load/LOD     | Specificity | Ref. |
| RT-PCR                                                      | Breast milk                      | ZIKV RNA                            | Breast milk: 2.9 × 10⁴ and 205 × 10⁴ copies/ml; Serum: about 60 × 10⁴ copies/ml | /           | 195  |
|                                                            | Newborns serum                   | ZIKV RNA                            | 0.7–220.10⁶ copies/ml              | /           | 256  |
|                                                            | Urine                            | ZIKV RNA                            | Up to 2.1 × 10⁶                   | /           | 257  |
|                                                            | Breast milk                      | ZIKV RNA                            | 30–8.1 × 10⁶                  | /           | 215  |
|                                                            | Blood                            | ZIKV RNA                            | 90–3 × 10⁶                   | /           | 258  |
|                                                            | Saliva                           | ZIKV RNA                            | 1.1 × 10⁸–4 × 10⁸               | /           | 259  |
|                                                            | Serum                            | ZIKV RNA                            | 91%                          | 97%         | 260  |
|                                                            | Serum, urine                     | ZIKV RNA                            | 100%                         | 96.6%       | 261  |
|                                                            | Serum, plasma, urine             | ZIKV RNA                            | Serum: 94.2%                   | 100%        | 262  |
|                                                            |                                  |                                     | Plasma: 94.9%                  |             |      |
|                                                            |                                  |                                     | Urine: 100%                    |             |      |
| Real-time RT-PCR                                             | Human plasma                     | ZIKV RNA                            | 0.05 PFU/reaction              | High specificity | 263  |
|                                                            | Virus cell culture, human serum  | ZIKV RNA                            | 140 copies viral RNA/reaction    | /           | 264  |
TABLE 1 (Continued)

| Method | Sample type | Target            | Sensitivity/LOD | Specificity | Ref. |
|--------|-------------|-------------------|-----------------|-------------|-----|
| VZIKV cell culture, human serum | ZIKV RNA | $10^{0-10}$ copies/µl | 100% | / | 265 |
| Serum | ZIKV RNA | 100% | 100% | / | 266 |
| Serum, plasma and urine | ZIKV RNA | Serum: 100% Plasma: 92% Urine: 100% | Serum: 96.4% Plasma: 87.5% Urine: 93.9% | / | 267 |
| Serum, urine | ZIKV RNA | Serum: 94.6% Urine: 100% | Serum: 100% Urine: 100% | / | 268 |
| Serum, whole blood, and urine | ZIKV RNA | $10^3$ genome copy equivalents per milliliter (GCE/ml) | Highly specific | / | 269 |
| Serum, urine | ZIKV RNA | Serum: 25.162 GCE/ml Urine: 7.957 GCE/ml | / | / | 270 |
| Serum, urine, serum, semen, urine, and mosquito pool samples | ZIKV RNA | 10–100 transcribed RNA copies/µl | / | / | 272 |

**Sensing methods**

| Method | Sample type | Target | LOD (sensitivity) | Range of detection | Ref. |
|--------|-------------|--------|-------------------|-------------------|-----|
| Electrochemical sensing | Real samples | ZIKV E protein | 10 pM (12 kΩM$^{-1}$) | 10 pM–1 nM | 273 |
| PBS, serum | ZIKV | PBS: 1 RNA copy/ml Serum: 10–250 RNA copies/ml | / | / | 274 |
| Samples | RNA (NS5 protein) | 54–340 nM | 25 nM | 275 |
| Samples | ZIKV RNA | / | / | 276 |
| Colorimetric sensing | Serum | ZIKV RNA | 1 fM | 3 fm–30 pM | 277 |
| Saliva | ZIKV E protein RNA | 50–100 PFU/ml | 50–5 × 10^4 PFU/ml | 278 |
| Serum, urine | ZIKV RNA | 1.2 RNA copies/µl (80.6%) | / | / | 279 |
| Fluorescence sensing | PBS, urine, plasma | ZIKV E protein | PBS: 10 PFU/ml Urine, plasma: 10 PFU/ml | PBS: 10–10^5 PFU/ml | 280 |
| | | | Urine, plasma: 10–10^4 PFU/ml | (Continues) | 280 |
| Method                                      | Sample type                  | Target        | Sensitivity/LOD                      | Specificity | Ref. |
|---------------------------------------------|------------------------------|---------------|-------------------------------------|-------------|------|
| Blood, saliva, urine                        | ZIKV RNA                     | LOD<sub>25</sub> = 22 PFU/ml  
|                                             |                              | LOD<sub>50</sub> = 4.9 PFU/ml | /         | 281  |
| Buffer, human blood serum                   | ZIKV-artificial DNA and RNA  | /             | /                                  | /           | 282  |
| Infected mosquitoes, urine                  | ZIKV RNA                     | 0.71 PFU equivalent viral RNA | /         | 283  |
| Tris-HCl buffer solution                    | ZIKV RNA                     | Single fluorescence analysis: 332/192 pM;  
|                                             |                              | Synchronous fluorescence analysis: 184/121 pM | /         | 284  |
| Sample                                      | ZIKV NS1                     | 100 nM        | /                                  | /           | 285  |
| Serum                                       | ZIKV NS1                     | 0.72 ng/ml    | /                                  | /           | 286  |
| PBS buffer                                  | ZIKV NS1                     | 10 ng/ml      | 50 μg/ml–100 ng/ml                 | /           | 287  |
| SERS sensing                                | PBS, serum                   | PBS: 3 ng/ml  | PBS: 10–2000 ng/ml                 | /           | 288  |
|                                             | (impedimetry mode)           | Serum: 30 ng/ml | Serum: 10–2000 ng/ml              |             |      |
|                                             | PBS, serum (capacitance mode)| PBS: 0.2 ng/ml | PBS: 5–1000 ng/ml                 | /           | 288  |
|                                             | Serim: 0.5 ng/ml             | Serum: 5–1000 ng/ml |             | /           | 289  |
| Simulated serum, PBS                        | ZIKV NS1                     | 450 pM        | /                                  | /           | 289  |
| Cultured virus, clinical sample             | ZIKV                         | Cultured virus: 500 copies | /         | 290  |
| (blood)                                     |                              | Clinical sample: 1000 copies |             |      |
| Buffer                                      | ZIKV RNA                     | 1.7 copies/ml  | 6.73–673 copies/ml |             | 291  |

**Other strategies**

| Method                   | Sample type          | Target                  | LOD (sensitivity) | Range of detection (specificity) | Ref. |
|--------------------------|----------------------|-------------------------|-------------------|----------------------------------|------|
| RT-LAMP (LAMP)           | Serum                | ZIKV NS5 oligonucleotide| 1 aM              | 1 aM–10 fM                       | 292  |
|                          | Serum, urine         | ZIKV RNA                | RNA standard: 4000 copies/ml | Simulated ZIKV clinical samples: 4 PFU/ml | 293  |
| Method | Sample type | Target | Sensitivity/LOD | Specificity | Ref. |
|--------|-------------|--------|----------------|-------------|-----|
|        | Human whole blood, pure water | ZIKV RNA | A single copy | / | 294 |
|        | Human blood, plasma, saliva, urine, and semen; mosquitoes; Infected cells | Asian/African-lineage ZIKV RNA | Plasma was <2.1 × 10^2; blood was <1.26 × 10^3; urine was <6.3 × 10^3; saliva was <4.28 × 10^3; semen was <3.04 × 10^5 (PFU/ml) | / | 295 |
|        | Saliva | ZIKV RNA | 2 × 10^5 RNA copies/ml | / | 296 |
|        | Urine, serum; mosquito lysates | ZIKV RNA | 1 genome | / | 297 |
|        | Saliva, urine | ZIKV | 5 PFU per saliva/urine sample | / | 298 |
| TMA    | Serum, urine | ZIKV RNA | Serum: 11.5 GCE/ml; Urine: 17.9 GCE/ml (94.7%) | Specificity: 94.8% | 299 |
| RT-SIBA| Lysis buffer | ZIKV RNA | 5000 copies/ml | 5 × 10^3–5 × 10^6 copies/ml | 300 |
| BPD    | Serum, PBS buffer | Anti-ZIKV-NS1IgG | Serum: 200 ng/ml; PBS: 1 ng/ml | / | 301 |
| Flow cytometry | Whole blood samples | ZIKV NS3 | 0.5 MOI infection | / | 302 |
| RT-isothermal-RPA | Urine | ZIKV RNA | NS1/NS2 RNA diluted standard: 21 RNA copies; Dilution range of 10^7–10^5/µl: 10 RNA copies; (92%) | 100% | 303 |
|        | Serum, urine | ZIKV RNA | High sensitivity (1 PFU of ZIKV) | High specificity (no false positives or negatives) | 304 |
|        | Saliva, serum | ZIKV RNA | 0.5 PFU/extraction | / | 305 |
| NIRS   | Aedes aegypti mosquitoes (heads/thoraces) | ZIKV | Prediction accuracies: 94.2–99.3% | / | 306 |
| Nanoparticle- enhanced viral lysate electrical sensing assay | PBS, plasma, urine, and semen | ZIKV E protein | 10 copies/µl | / | 307 |

(Continues)
| Method                                      | Sample type          | Target             | Sensitivity/LOD                      | Specificity | Ref. |
|--------------------------------------------|----------------------|--------------------|-------------------------------------|-------------|------|
| Smartphone-based fluorescent LFIA          | Buffer, serum        | ZIKV NS1           | Buffer: 0.045 ng/ml Serum: 0.15 ng/ml| /           | 308  |
| Polydimethylsiloxane-based microfluidic device | PBS, calf blood  | ZIKV envelope protein | PBS: 1 pM Calf blood: 10 pM       | /           | 309  |
degeneration of cells, and changes of cells are collectively called the cytopathic effect (CPE). Unfortunately, viral isolation methods suffer from some disadvantages, such as requiring a long incubation period and the expertise of evaluating the CPE. In addition, when evaluating the CPE, it is worth noting that the viral load in the serum often presents at a low level with a fleeting viremic stage. Moreover, the viremic stage may cease after the onset of symptoms.\textsuperscript{311} With technological advances, the development of monoclonal antibodies and introduction of molecular diagnostics have offered powerful strategies for the detection of viral infections. Currently, viral isolation in cell culture serves as an unpractical tool for most clinical situations, and as such is primarily used for research purposes.\textsuperscript{226,312}

3 | SEROLOGICAL ASSAYS

Serological assays based on the interaction between antigens and antibodies are an important strategy for the detection of ZIKV, while the cross-reactivity of the ZIKV antibodies with the antibodies against other homologous flaviviruses (such as dengue virus [DENV]) may occur.\textsuperscript{313} When people are infected by ZIKV, their body will begin to produce antibodies (immunoglobulin M [IgM]) against the ZIKV during the later period of infection, especially the first week of symptoms. Therefore, there is a huge need for specific and sensitive serological assays that can meet the demand in endemic areas. Owing to the high percentage of asymptomatic infections and wide time window for ZIKV detection, serological assays play a key role for the detection of ZIKV. Currently, a great many serological assays have been developed for the detection of ZIKV. Herein, some commonly used strategies are presented (e.g., IgM antibody capture enzyme-linked immunosorbent assay [MAC-ELISA], plaque reduction neutralization test [PRNT], immunofluorescence assay [IFA]), and some recently developed methods (e.g., reporter virus neutralization test [RVNT], multiplex microsphere immunoassays [MIA]).\textsuperscript{112,114,224,225,236,248,314-324} However, there are some performance limitations of serological assays. Because of the cross-reactivity between the antibodies of ZIKV and the antibodies against other homologous flaviviruses (e.g., dengue virus), low viral loads, and nonspecific binding, the serological assays of ZIKV are difficult.\textsuperscript{325-329} Though they possess some disadvantages, serological assays still need to be further developed for overcoming the shortcomings of other methods.

3.1 | IgM antibody capture enzyme-linked immunosorbent assay

Due to its high specificity, sensitivity, and ability to directly detect complex biological samples, the ELISA technique has been viewed as an effective strategy for the detection of IgM in the serum of patients, and has been widely applied in multiple fields.\textsuperscript{330-342} Over the past few decades, the outbreak of ZIKV represents a huge global threat for humans, especially for pregnant women.\textsuperscript{343-345} The ELISA technique has been widely applied for the detection of ZIKV, because the IgM antibodies in the infections of ZIKV are generally generated during the later stages of infection and detected for up to 3 months. Moreover, after the development of IgM antibodies, the immunoglobulin G (IgG) antibodies are typically produced within a few days, and can be constantly detected for several months.\textsuperscript{231,236,346-348} As reported previously, the first step in MAC-ELISA (Figure 4A) is adding the sample from patients into a well plate precoated with antibodies against human IgM. Following that, the virus-specific antigen is added into a well plate, if IgM is present in the patient sample, strong binding to antibodies in the well plate will occur. Otherwise, the sample from the patient will be washed away and there is no detectible signal when the secondary antibody (the antibody tagged with the enzyme like horseradish peroxidase) is added. After the secondary antibody is added, chromogenic substrates (e.g., 3,3’5,5’-tetramethyl benzidine; 2,2’-azinobis(3-ethylbenothiazoline-6-sulfonic-acid); and o-phenyl-enediamine) are employed for quantification. The samples from infected patients will lead to an optically detectible signal (e.g., fluorescence and absorbance), which can be measured and read using a spectrophotometer to determine the IgM concentration for the patient’s samples.\textsuperscript{235} At
on February 26, 2016, a letter was issued by the FDA to authorize the urgent use of the ZIKV MAC-ELISA, which was employed for detecting the specific IgM of ZIKV in human cerebrospinal fluid or sera. Moreover, the detection of ZIKV has been performed based on the epidemiological and clinical criteria of CDC for ZIKV (e.g., the clinical signs associated with ZIKV infection, the symptoms associated with ZIKV infection, and other epidemiologic criteria that can indicate ZIKV infection). In June 2007, during the ZIKV outbreak in Yap State, Micronesia, Lanciotti et al. reported that the serologic and genetic properties of the ZIKV were associated with this epidemic. At that time, the immune responses due to the infection by ZIKV had been rarely described. When the MAC-ELISA was employed for the assays of IgM with whole viral antigen (inactivated virus) and the monoclonal antibody (MAbs)-based capture ELISA was used for the detection of IgG, it was found that the IgM appeared as early as 3 days after the onset of symptoms but the IgG emerged after 10 days in an infected patient without a history of flavivirus infections. As such, the detection of neutralizing antibodies against ZIKV as early as 5 days after the onset of symptoms is possible.
In 2016, the use of putative cross-reactive sera from infected patients with Euroimmun (Euroimmun, Lübeck, Germany) IgM antibodies against ZIKV and anti-ZIKV IgG in ELISA tests was reported by Huzly et al., which exhibited a high specificity and confirmed the applicability of Euroimmun ZIKV ELISA for specifically detecting the virus from individuals with suspected previous exposure to flavivirus or vaccine. Moreover, it demonstrated this ELISA strategy as an effective diagnostic tool that could be used for the counseling and screening of patients who had potentially been infected by ZIKV (e.g., the travelers and pregnant women commuting from the endemic regions of ZIKV). However, in most assays of IgM, given the period of illness and the specificity and sensitivity of the test, a negative result indicates that there is no recent infection while a positive MAC-ELISA result can be a sign of recent flavivirus infection (presumptive positive). In addition, the cross-reactivity with closely related flaviviruses could increase the possibility of false positives. To validate inconclusive results, the PRNT was recommended by the CDC for the validation of the previous assumptions caused by an inconclusive MAC-ELISA.

### 3.2 Plaque reduction neutralization test

As a result of the uncertainty of results of MAC-ELISA (Including false negatives and false positive results), the PRNT as a confirmatory tool can be employed for a conclusive indication of recent ZIKV exposure in areas with dengue or other flaviviruses epidemics. The first step in PRNT requires serial dilutions of a patient's serum samples. Following dilution the samples are added to ZIKV suspensions, mixed and incubated with confluent host cell cultures like Vero cells. When the antibodies against ZIKV are present in the serum of patients, the amount of plaque forming units (PFU) in those cell cultures will be reduced, and the antibody titer can be detected after a series of dilutions. Compared with MAC-ELISA, the PRNT exhibits a higher specificity and sensitivity for the detection of ZIKV. Moreover, the PRNT possesses a higher throughput so that 12 specimens of patients can be analyzed in 2 six-well plates but the MAC-ELISA can only analyze eight specimens of patients per 96-well microtiter plate. The PRNT shows an additional merit of ZIKV antibody assay over the normal window of IgM (the concentration of IgM is lowest after 12 weeks onset of symptoms). PRNT possesses better specificity and sensitivity than MAC-ELISA. However, there are some disadvantages with PRNT. For example, the PRNT is labor-intensive and expensive, and requires live virus. Moreover, antibodies against the conserved flavivirus domain can reduce detection specificity, especially during the acute stage and early convalescent period. In a recent study, Collins et al. evaluated the late convalescent serum samples from the patients with previous exposure to ZIKV or DENV. It was found that most DENV infections could not induce lasting, high-level, and cross-neutralizing ZIKV antibodies. Moreover, ZIKV-specific antibody populations develop after ZIKV infection irrespective of prior DENV immunity. The development of ZIKV-specific antibody populations after the ZIKV infection was unrelated to prior DENV immunity. Additionally, data from Puerto Rico demonstrated that PRNT provided an inaccurate confirmatory test, for populations with a high pre-exposure to DENV.

### 3.3 Immunofluorescence assay

IFA as one the most commonly used tests has been employed for the detection of ZIKV. The first step of IFA is that the BHK21 cells are infected by various strains of flavivirus and fixed by ice-cold acetone. Then, the infected cells are incubated in the normal mouse serum or a 100-fold dilution of mAb 2A10G6, and the cells need to be washed three times with phosphate-buffered saline (PBS) after incubation for 60 min at 37°C. Subsequently, cells were treated at 37°C using a 200-fold dilution of FITC-conjugated anti-mouse IgG in 0.02% (w/v) Evans blue for 30 min. Finally, after being rinsed with PBS five times, the positive cells can be determined with a fluorescence microscope. For example, Hamel et al. reported that IFA was used for fibroblasts infected by ZIKV (Figure 4B).
Moreover, it was found that the skin immune cells (e.g., immature dendritic cells, dermal fibroblasts, and epidermal keratinocytes) were all permissive to the infection of ZIKV. To conduct the laboratory confirmation of ZIKV infection in infected patients, Huzly et al.\textsuperscript{112} used an indirect IFA to determine the ZIKV infection of infected patients as previously described. It was found that the anti-ZIKV IgM was determined by indirect IFA titers ranging from 1:1280 to 1:>20,480, and the anti-ZIKV IgG was determined by indirect IFA titers ranging from 1:320 to 1:>20,480.\textsuperscript{236} Deng et al. studied the Asian lineage ZIKV imported to China, including its isolation, identification, and genomic characterization. Moreover, they further identified the newly isolated ZIKV by IFA by using the convalescent phase serum of a patient infected by imported ZIKV. It was found that the serum of the convalescent phase with 1:320 dilution could strongly react with the SZ01 virus which was newly isolated.\textsuperscript{368} Recently, Li et al. found that 25HC could protect host against ZIKV infection. It was found that the Vero cells could be protected from ZIKV with a 0.4 mM concentration of 25HC.\textsuperscript{369} However, it was reported that the IFA also exhibited cross-reactivity related to various flaviviruses.\textsuperscript{96}

### 3.4 Multiplex microsphere immunoassays

Currently, serological assays of ZIKV mainly depend on IgM capture, which can cause some problems due to the cross-reactivity with other homologous flaviviruses. Nevertheless, the MIA could enhance the sensitivity and specificity of ZIKV detection by adding one or more antigens. Compared with other diagnostics tests, MIA shows advantages in that it captures the diagnostic ability of viral envelope proteins (EPs; which elicits robust but cross-reactive antibodies against other flaviviruses) and the contradistinctive differential ability of viral nonstructural proteins (e.g., NS1 and NS5, which can increase the specificity (78–100%) of the assay).\textsuperscript{224,255,356,370–372} In 2017, Wong et al. reported the multiplex MIA for serologic diagnosis of ZIKV that combined viral structural with nonstructural proteins for the first time. The developed MIA platform could achieve rapid diagnosis (<4 h) with an improved diagnostic accuracy, and higher sensitivity than IgM-capture ELISA in detecting the ZIKV infection. Moreover, only a small specimen volume (10 μl) was required for a single reaction. In addition, the obtained MIA platform could not only be used for the clinical diagnosis of ZIKV infection, but was also able to monitor immune responses in vaccine trials.\textsuperscript{373} In a recent study, a novel multiplexed flavivirus IgM MIA (flaviMIA) was successfully developed and validated by Taylor et al., which could be used for differentiating ZIKV-specific IgM from the other flavivirus infections of humans. The developed flaviMIA could combine 12 inactivated flavivirus antigens (which were from yellow fever virus (YFV) and ZIKV) to form the unequal anti-flavivirus antibody coupled beads for interrogating the sera of infected patients (who were suspected to have a ZIKV infection after traveling to relevant countries). The obtained flaviMIA protocol displayed broad concurrent flavivirus serodiagnostic ability, and was easily operated. Moreover, the flaviMIA protocol was suitable for high throughput testing, and could achieve a rapid diagnosis and could generate repeatable results.\textsuperscript{249}

### 3.5 Reporter virus neutralization test

Currently, the serologic assay of ZIKV infection mainly depends on the labor-intensive PRNT, which requires the long turnaround time (over 7 days) and results in a bottleneck for the ZIKV diagnosis of patients. To solve this problem, Shan et al. successfully developed a novel high-throughput assay (RVNT), which could be used for the diagnosis of ZIKV and DENV (Figure 5). The newly developed RVNT was homogeneous, and neutralizing antibody could be quantified using the luciferase viruses in a 96-well format. It was found that RVNT had higher dynamic range and could keep the relative specificity as the current PRNT assay by using 91 human specimens. Moreover, the RVNT could improve assay throughput and shorten the turnaround time (<2 days). Furthermore, the study demonstrated that the RVNT could be employed as a first-line diagnostic test for vaccine clinical trials.\textsuperscript{374} Shortly
afterward, the same group evaluated the performance characteristics of the RVNT with 258 clinical serum specimens by employing the current PRNT as a reference strategy. The result demonstrated that the RVNT as a screening assay exhibited an excellent diagnostic specificity, sensitivity, and accuracy. Based on the confirmatory assay, the developed RVNT titers were verified to show 93.1% agreement with the current PRNT titers. Moreover, RVNT as an accurate and reliable strategy could be used for the detection of neutralizing antibodies in serum specimens of patients. In addition, RVNT could significantly shorten the turnaround time and greatly improve the assay throughput to detect many clinical specimens in a single run owing to its homogeneous 96-well format. As such RVNT has great potential in clinical diagnosis, serologic surveillance, and monitoring antibody response in vaccine trials. Similarly, Garg et al. also developed a microneutralization assay (reporter virus particles [RVPs]) for ZIKV infection using a virus-like particle-based method. The RVP assay was shown to work in a 96-well plate format, and its GFP readout could be quantitated using a simple fluorescence microscope. Moreover, the assay exhibited high specificity and reproducibility in the detection of ZIKV antibodies as PRNT. Though RVNT exhibits many advantages compared with traditional PRNT, it still needs more well-characterized samples of infected patients to be tested in order further strengthen the validation of the novel RVNT.

FIGURE 5  Experimental scheme of reporter virus-based infection assay to measure neutralization titers of specimens. Reprinted with permission from Ref. [Color figure can be viewed at wileyonlinelibrary.com]
Other immunoassays are also employed for the detection of ZIKV infection. For example, Bosch et al. has investigated a rapid immunochromatography test for the detection of ZIKV infection. Moreover, a new rapid immunochromatography approach (Figure 6A) and reagents have been successfully developed by their group for the differential clinical diagnosis of acute ZIKV cases and could be used to develop a novel rapid diagnostics for detecting the antigen of emerging viruses. In addition, Hage has comprehensively summarized the applications of immunochromatographic assays for selectively detecting ZIKV in serum. Recently, based on the domain III of the envelope protein (EDIII) and recombinant forms of NS1, Cabral-Miranda et al. have successfully developed a new biosensor (Figure 6B). Using square wave voltammetry and electrochemical impedance spectroscopy (EIS), the developed biosensor exhibited high sensitivity and specificity for detecting the ZIKV antibodies in saliva and blood samples. The biosensor was used to quickly distinguish the specific antibodies of ZIKV from DENV. Moreover, because of the high sensitivity, the new biosensor could be applied to early infection detection when conventional ELISA approaches could not detect circulating antibodies whose levels were still very low. In addition, the biosensor showed the ability to detect the ZIKV antibodies in the saliva samples, which could solve the problem of collecting serum samples from volunteers or infected individuals. Furthermore, the saliva test could be used for rapid screening of people from sensitive areas during a ZIKV outbreak (e.g., the airports or border). With the advances in NPs and digital health systems, portable and sensitive detection technologies have been quickly developed for timely managing emerging viral infections. For example, a nanomotor-based bead-motion cellphone (NBC) system was successfully developed by Draz et al. for the specific and sensitive immunological detection of ZIKV infection, which was rationally designed with nanotechnology, cellphone, and microfluidics. Interestingly, the platinum (Pt)-nanomotors could be accumulated on the surface of beads and would move in H₂O₂ solution when the ZIKV was present in a test sample. The concentration of ZIKV was determined by correlation of changes in the beads motion. The obtained NBC system was able to detect the ZIKV in samples with a low concentration of 1 particle/μl. The newly developed NBC system displayed high specificity for detecting the ZIKV in the presence of 

**FIGURE 6** (A) Schematic illustration of a biosensor. (B) Biosensor using ZIKV-derived proteins as biorecognition element bound to carbon support material. (BII) Oxidation of carbon material; (BIII) activation of carboxylic acids; (BIII) amination of activated carboxylic surface with p-phenylenediamine; (IV1) incubation with ΔNS1 protein; (IV2) incubation with EDIII protein; and (V) binding of the sensing layer to immune antibodies (anti-ΔNS1 (V1) and anti-EDIII (V2)). Reprinted with permission from Ref. Copyright 2018, Elsevier. EDIII, domain III of the envelope protein. ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
closely related viruses and other neurotropic viruses (e.g., human cytomegalovirus and herpes simplex virus type 1). Furthermore, the developed NBC system allowed for simple and rapid testing of viral load, and could be potentially employed for immunoassays that need rapid and simple identification of viruses.378 Recently, Sola et al. developed a novel, straightforward, and robust strategy (Figure 7) to extend the range of immune-diagnostic probes by copresenting peptides which belong to the identical antigenic surface. The group focused on ZIKV NS1 protein antigen putative antigenic region, a diagnostic confidence was reached through the oriented and spatially controlled coimmobilization of the peptide sequences found adjacent within the protein fold, which cooperatively interacted to provide an enhanced immunoreactivity with respect to single linear epitopes. Based on the developed strategy, ZIKV infected individuals could be differentiated from healthy controls. The method could be included in high-throughput screening platforms of mixed and linear peptide libraries. Moreover, it may facilitate a fast identification of the conformational immunoreactive region.379

**FIGURE 7**  (A) The working principle for enhancing antibody serodiagnosis using a controlled peptide coimmobilization method. (B) Strategy to enhance immunoreactivity by spatially controlled copresentation of peptidic probes on microarray surfaces through "Click" reaction of yne-modified peptides on Copoly Azide. Reprinted with permission from Ref.379 Copyright 2018, American Chemical Society [Color figure can be viewed at wileyonlinelibrary.com]
4 | MOLECULAR ASSAYS

As a consequence of the cross-reactivity with other homologous flaviviruses (e.g., DENV), the serological assays of ZIKV are extremely challenging (due to an uncertainty of the results).\(^{225}\) Therefore, molecular assays have played an important role in confirming the detection of viruses. Molecular assays as an important tool for the detection of ZIKV can be performed using RT-PCR. Moreover, owing to its high sensitivity and high selectivity, the RT-PCR has been viewed as the gold standard for the molecular amplification and diagnostics of ZIKV.\(^{235,300,381,382}\) To date, the ZIKV RNA has been found in various biofluids (e.g., semen, blood, urine, saliva, breast milk, and amniotic fluid) and could be detected by RT-PCR.\(^{184,208,383-385}\) It has been confirmed that ZIKV is more frequently detected in saliva and urine samples than in blood using the RT-PCR tests. Saliva samples are particularly interesting since they have increased the prospects of ZIKV detection at the acute phase of infection and provides appropriate results when blood is hard to be collected particularly in neonates and children. Nevertheless, viral load has been discovered to be higher in urine samples than that in saliva samples. Moreover, the detection of ZIKV in patients’ urine more than 10 days after disease onset have indicated that the urine sample was of great importance for the diagnosis of ZIKV infections.\(^{19,222}\) In this review, RT-PCR methods have been categorized into traditional RT-PCR, real-time RT-PCR, pan flavivirus RT-PCR, and nested RT-PCR. Among these assays, the pan flavivirus RT-PCR and nested RT-PCR are rarely used. In addition, several other molecular assays have been illustrated, such as real-time PCR based neutralization assay, localized surface plasmon resonance (LSPR), and DNA sequencing.

4.1 | Traditional RT-PCR

RT-PCR with high sensitivity and selectivity has been considered as the gold standard for the molecular amplification and assay of virus. In the early phase of the acute period of ZIKV infection, it is feasible to determine the ZIKV RNA in serum by RT-PCR.\(^{225,355,386-388}\) Based on previous reports, the RT-PCR has also been employed for detecting the ZIKV in multiple sample matrices, such as semen, plasma, saliva, serum, urine, and conjunctival fluid.\(^{188,191,200,381,389-391}\) For example, Bingham et al. compared the result of ZIKV RNA detected by RT-PCR in saliva, serum, and urine specimens from infected people with a history of traveling to Florida. It was found that the urine samples could be used as the most suitable specimen to identify acute infections of ZIKV.\(^{392}\) Similarly, by studying small sample size or several cases using both the blood and urine samples, Lamb et al. found that the ZIKV RNA detected in urine possessed higher levels after the onset of ZIKV infection than that in blood. Based on the result, they recommend that serum and urine were collected for molecular assays and served as a standard for evaluating ZIKV infection of patients. Moreover, it was found that urine could serve as a good alternative sample when blood collection was difficult and ZIKV was hard to be detected in serum samples during early disease infection.\(^{393,394}\) Interestingly, Tan et al. evaluated strategies to maximize the detection and quantitation of ZIKV RNA in urine samples, and determined that urine samples required special consideration because pre-analytical factors (e.g., the freeze-thaw, length of storage, and storage temperature) could adversely affect the stability of the urine samples. Moreover, it was found that urine samples stored at 4°C for more than 48 h could influence the detection of ZIKV RNA by RT-PCR, and urine samples frozen at −80°C could lead to the significant loss of detectable ZIKV RNA. Furthermore, it was found that the detection of ZIKV could be achieved by adding a nucleic acid stabilizer (e.g., Shield or ATL) when delays in testing were anticipated.\(^{395}\) In another study, George et al. detected the envelope (E) protein and NS2B genes of ZIKV in serum and urine samples collected from infected patients (including newborns, pregnant women, travelers to ZIKV epidemic areas in New York, and suspected symptomatic GBS patients) using real-time RT-PCR. Given the short viremia time period of ZIKV infection, selecting appropriate samples for detection was a promising strategy, because the ZIKV RNA could not be detected in plasma or serum samples obtained 10 days after the onset of symptoms. However, the results indicated that urine samples were more dependable than serum samples due to their higher viral load. Moreover, compared with other serological assays, the test results of this strategy were more reliable.\(^{396}\)
To compare the detection of ZIKV RNA in multiple specimen types, Rossini et al. used the plasma, whole urine and blood as samples to detect ZIKV RNA obtained on the same day (3–28 days after the onset of symptoms) from 10 patients infected by ZIKV. The results indicated that ZIKV RNA was positive in urine samples at the 21st day after the onset of symptom while the ZIKV RNA could be detected up to 26 days after the onset of symptom. In addition, it was demonstrated that the simultaneous test of different specimen types could increase the probability of disease diagnosis. ZIKV RNA has been detected in breast milk of mothers. However, it has not been reported that ZIKV can be transported by breast milk. Another study reported by Murray et al. indicated that ZIKV in whole blood samples could persist for 81 days, while ZIKV in serum samples could persist for 73 days. Therefore, ZIKV RNA could be detected using the whole blood samples collected from asymptomatic patients. Soon afterward, Frankel et al. successfully developed a dual targeting RT-PCR assay for detecting the ZIKV RNA within urine, whole blood, plasma, and serum, which used an automated m2000 system to extract ZIKV RNA from samples. The assay was highly specific and sensitive with a limit of detection (LOD) of 120 copies/ml in whole blood, 40 copies/ml in urine and plasma, and 30 copies/ml in serum. While, Hancock et al. used electronic health records (RT-PCR) in a syndromic surveillance program to monitor asymptomatic pregnant women from American Samoa in order to establish a timeline to cease routine testing.

### 4.2 Real-time RT-PCR

Compared with conventional RT-PCR, real-time RT-PCR has some advantages, including low false positives, rapidity, higher specificity and sensitivity of quantitative analysis. In real-time RT-PCR, the RNA of a virus needs to be extracted from the infected samples, which is then added into a reaction mixture (made using the necessary factors, including the specific primers of ZIKV, reverse transcriptase, DNA polymerase and dNTPs, buffer solution, and the intercalating fluorescent dye used for quantification) for amplification. With the amplification of the target, the fluorescence in the samples enhances as the amount of product increases. Thus, the concentration of ZIKV in the samples can be determined by the fluorescence changes with high specificity and sensitivity. Owing to its high specificity, sensitivity, and ability for quantitative analysis, real-time RT-PCR has been viewed as the most powerful tool for molecular assays. Moreover, real-time RT-PCR was recommended by the CDC for detecting and quantifying ZIKV RNA during the early stages of ZIKV infection (Figure 8A). According to reports, at the early onset of ZIKV infection, the ZIKV RNA could be detected by real-time RT-PCR. Furthermore, the real-time RT-PCR could easily detect the ZIKV RNA in urine samples with the advantage of collecting samples easily and noninvasively, which was extremely useful for evaluating travelers during an epidemic. It was reported that ZIKV infection could be confirmed using the real-time RT-PCR. By using the real-time RT-PCR to detect the ZIKV in plasma and urine samples, Pessôa et al. confirmed that the viral loads were lower in urine than that in plasma samples. In another study, Corman et al. made a comparison between seven published real-time RT-PCR assays and two novel assays developed by themselves. By analyzing the sensitivity of each assay, they discovered that there were some detection methods unsuitable for the specific diagnosis of ZIKV because of the limited sensitivity and the potential incompatibility of some strains. Likewise, to search for the best real-time RT-PCR for detecting ZIKV infections, Moraes et al. compared and evaluated several different protocols using the probes and primers reported previously. The previously published primers and probes for the detection of ZIKV infection were evaluated using spiked samples and the serum detected using real-time RT-PCR. It was found that the previously published primers exhibited different sensitivity when the spiked samples were analyzed, but the results were quite similar when the urine and serum samples from the patients were tested. Moreover, the real-time RT-PCR designed for amplifying ZIKV NS1 exhibited the highest sensitivity for all samples. In another study, based on the conserved sequences in the envelope (E) gene of ZIKV, Yang et al. successfully developed a rapid and specific one-step quantitative real-time RT-PCR assay. The assay offered specificity and broad coverage for detecting all African and Asian lineages of ZIKV, and could alleviate the problem (could not be used to detect all strains of ZIKV) of earlier real-time PCR. In addition, the assay also achieved a low LOD for five RNA transcript copies. To improve the diagnosis of ZIKV RNA in the specimens of animal and humans, Chan et al. successfully developed a new, highly
specific and sensitive real-time RT-PCR assay by targeting the 5’-untranslated region (5’-UTR) of ZIKV. The newly developed real-time RT-PCR assay (ZIKV-5’-UTR assay) displayed the lowest in vitro LOD of 5–10 RNA copies/reaction and did not exhibit cross-reactivity with other members of the Flaviviridae family. The ZIKV-5’-UTR assay exhibited an enhanced sensitivity for the detection of ZIKV in most tissues of human and possessed sensitivity in...
clinical specimens collected from the epididymis/testis and kidney compared to the ZIKV-E gene targeting real-time PCR. Furthermore, the ZIKV-5'-UTR assay has no in vivo or in vitro cross-reactivity with YFV, DENV, Japanese encephalitis virus, hepatitis C virus, chikungunya virus (CHIKV), and West Nile virus.407

Recently, Santiago et al. successfully developed a novel Trioplex real-time RT-PCR assay (Trioplex assay) for detecting ZIKV infections and differentiating ZIKV infections from CHIKV and DENV. The analytical performances of all Trioplex modalities were evaluated through the detection of these three viruses (i.e., ZIKV, CHIKV, and DENV) in whole blood and serum samples, and ZIKV in urine samples. The developed Trioplex assay had a LOD close to \(10^3\) GCE/ml for the three pathogens using different RNA extraction methods. Moreover, simultaneous detection of more than one sample from each patient could increase detection sensitivity by 6.4%. The results indicated that the developed Trioplex assay has a high sensitivity for confirming ZIKV infections.409 By combining the primers previously reported and a novel Taqman probe, Judice et al. developed a new ZIKV NS5 real-time RT-PCR assay. The developed ZIKV NS5 real-time RT-PCR assay was evaluated and compared with the previous assay (ZIKV 1107) using 42 urine and 51 blood samples collected from 54 suspected patients infected by ZIKV. Compared with ZIKV 1107, the ZIKV NS5 real-time RT-PCR assay displayed better sensitivity for the detection of ZIKV in both urine and blood samples. In general, the two assays had good overall agreement for blood (\(\kappa = 0.825\)) and urine (\(\kappa = 0.770\)) samples.410 In a very recent study, Mansuy et al. successfully developed a novel one-step real-time PCR for simultaneous detection of pathogens (including ZIKV, CHIKV, and DENV). Moreover, the one-step real-time PCR could be used to distinguish between ZIKV, CHIKV, and DENV, and is particularly suitable for whole blood samples. Moreover, there were no PCR inhibition and no cross-reactivity found for the clinical specimens.270 Real-time RT-PCR has been widely used, and has exhibited numerous advantages such as high sensitivity and specificity, fewer false positives, and quantitative analysis. However, there are some limitations. The main limitation is that the real-time RT-PCR depends on a good laboratory infrastructure with skilled operators. Moreover, it is time-consuming, and requires several hours to obtain a test results. Furthermore, the equipment for PCR is expensive, and requires thermocycling.

Droplet digital PCR (ddPCR) is a method for the quantitation of nucleic acid, where the PCR reaction mix is partitioned into approximately 20,000 nanodroplets. Then, when PCR is performed, an automated droplet flow cytometer is used for reading the droplets. The ddPCR method exhibits improved accuracy and higher sensitivity than that of real-time PCR. Moreover, ddPCR serves as an endpoint measurement and can achieve an absolute quantification of ZIKV RNA levels without the requirement of a calibration curve, which makes it a promising tool for the determination of brain viral load.411 Utilizing the micro-ddPCR and real-time RT-PCR, Hui et al. established a new protocol for the ZIKV detection based on the NS5 gene amplification. Compared with real-time RT-PCR, the established ddPCR method achieved a linear range from \(10^3\) to \(10^6\) copy/\(\mu\)l, and a LOD as low as \(1\) copy/\(\mu\)l. Therefore, the proposed ddPCR method illustrated great potential for early diagnosis, laboratory evaluations, and ZIKV monitoring.412

4.3 | Pan flavivirus RT-PCR and nested RT-PCR

The pan flavivirus RT-PCR may sometimes be employed using a degenerate primer to detect flavivirus. However, pan flavivirus RT-PCR can lead to a loss of sensitivity. Similarly, the nested RT-PCR is also rarely utilized.
The nested RT-PCR needs two successive PCRs and two primer sets (an inner primer set and an outer primer set). The primary primer or outer primer is targeted on a general area of the RNA. Subsequently, the nested primer or inner primer is targeted on a specific coding region. This method can reduce the amplification of an unforeseen binding site.\textsuperscript{189,226} Even though the RT-PCR has been reported to have many advantages, the probes and primers having high specificity when applied in RT-PCR may cause significant errors. Therefore, the problem of missing unanticipated sites should be noted, which may lead to failure in amplification.

4.4 Other molecular assays

According to previous reports, other molecular assays have been developed for detection of ZIKV.\textsuperscript{323} For example, by combining real-time PCR with serum neutralization tests, Wilson et al. developed a new real-time PCR based neutralization assay. The real-time PCR was used for measuring the neutralization endpoint rather than by counting plaques to measure the neutralization endpoint, but the test took 72 h. The assay could be used to confirm test results (the serum samples were positive) obtained from an IgG/IgM ELISA. Moreover, the real-time PCR based neutralization assay had a high sensitivity (100\%) for both DENV and ZIKV.\textsuperscript{413} In another study, a one-step reverse transcription-insulated isothermal PCR (RT-iiPCR) reagent set (Figure 8B) was developed for the detection of the ZIKV RNA in spiked samples. Comparison of the CDC and PAHO real-time RT-PCR assays for various spiked sample types including mosquitoes, the performance of ZIKV RT-iiPCR reagent set could be evaluated. Moreover, these assays displayed an exclusive specificity for the ZIKV (Asian and African lineages) with detection endpoints of 10 PFU/ml of infected tissue culture fluid, and the LOD ranged from 10 to 100 for in vitro transcribed RNA copies/\mu{l}. In addition, it was confirmed that there was 92\% agreement between the real-time RT-PCR assays and the ZIKV RT-iiPCR.\textsuperscript{408}

Surface plasmon resonance based biosensors as label-free technology have been widely applied for both bioassays and analytical chemistry.\textsuperscript{414-417} Moreover, LSPR biosensors are an effective strategy for the detection of ZIKV RNA. For example, based on LSPR-mediated fluorescence signals from a molecular beacon (MB), Adegoke et al. recently developed a novel LSPR-mediated quantum dot (Qdot)-MB biosensor probe for selectively detecting low concentrations of ZIKV RNA (Figure 8C). It was found that the NPs-Qdot bimetallic hybrids (e.g., AuAgNP-Qdot646-MB) could detect trace amounts of ZIKV RNA over a sample with a range of 6-673 copies/ml. Moreover, compared to single metal NPs, bimetallic NP-Qdot-mediated fluorescence signals were stronger for detecting ZIKV RNA. In this case, due to the differences between NP-Qdots, it was very important to choose the most appropriate one for the detection of ZIKV RNA.\textsuperscript{291}

DNA sequencing has been widely applied and used to confirm ZIKV infection, and the relationship between ZIKV strains could be demonstrated by sequencing of the NS3, NS5, and E genes.\textsuperscript{101,124,418-420} To solve the problem with the lack of ZIKV genomic data, Metsky et al. used multiple sequencing strategies to produce 110 ZIKV genomes from mosquito and clinical samples collected from 10 countries and territories. Viral genetic diversity from the ZIKV outbreak in America was greatly expanded. Then by comparing the generated 110 ZIKV genomes with another 64 published genomes from the NCBI GenBank, it was found that the ZIKV outbreak started in Brazil.\textsuperscript{421} Buechler et al. analyzed the ZIKV genome using deep sequencing. The results indicated that a comparison of the ZIKV genome and sequences from the data bank could be used to identify the nucleotide changes of ZIKV specific strains.\textsuperscript{422} Recently, Gu et al. has studied the first whole-genome sequence of ZIKV strain AFMC-U using next generation sequencing, which was amplified from the urine specimens collected from a Korean traveler returning from the Philippines. The study indicated that urine samples were very important for the detection of ZIKV infection.\textsuperscript{423}

5 ZIKV DETECTION BASED ON SENSING METHODS

Though serological and molecular assays play a key role on the detection of viruses, there are some disadvantages limiting their widespread applications (e.g., extensive sample preparation, false-positive and false-negative results, the requirement of expensive laboratory equipment, requiring skilled operators, and time consuming generation of...
results). Fortunately, opportunities exist for the development of sensing technologies toward ZIKV detection. In particular, sensing strategies that have been developed for use in multiple fields, including biochemical analysis, medical diagnostics, environmental monitoring, food quality control, and so forth. As such, numerous sensing strategies have been developed for the sensitive and selective detection of ZIKV infection, such as electrochemical, colorimetric, fluorescence, and surface-enhanced Raman spectroscopy (SERS) methods.

5.1 Electrochemical sensing

In recent years, electrochemical sensing has been widely applied in biochemical analysis, food quality control, and environmental monitoring. To develop an electrochemical sensing methodology for the detection of targeted disease, many advanced technologies have been utilized, such as nanostructured immobilizing materials, transduction techniques, molecular recognition, and novel sensing arrays. Moreover, electrochemical sensing methods have made great advances for the detection of viruses. For example, Kaushik et al. proposed that a nano-enabled electrochemical immunoassaying technology could be used for the detection of ZIKV infection in biofluid specimens collected from the infected patients, and particularly the pregnant women and newborn babies (Figure 9A). The proposed miniaturized electrochemical nano-sensing systems could be used for point-of-care (POC) ZIKV detection, and could provide better disease management and treatment by promoting high-speed personalized health-care for patients. The proposed electrochemical nano-sensing systems could be fabricated using nano-structured sensing materials, miniaturized sensing transducers, nano-/micro-electrodes and microelectronics for selectively monitoring, screening and detecting ZIKV infections. Recently, by using an interdigitated micro-electrode by functionalization of a gold (IDE-Au) array, a novel electrochemical immunosensor for selectively and sensitively detecting the protein of ZIKV was developed. In the electrochemical immunosensor, the IDE-Au immunoassaying chip was constructed by immobilizing the specific envelop protein antibody (Zev-Abs) of ZIKV onto the functionalized IDE-Au with an electrode width/gap of 10 µm. The electrical response of the IDE-Au immunoassaying chip as a function of ZIKV protein concentrations could be measured using EIS, and it was verified that the sensing chip could selectively detect ZIKV protein. Furthermore, the electrochemical immunosensor displayed a high sensitivity of 12 kΩM⁻¹ with a low LOD of 10 pM, and the ZIKV-protein could be detected from 10 pM to 1 nM. In addition, by integrating with a miniaturized potentiostat (MP) which was interfaced with a smartphone (Figure 9B), the as-prepared IDE-Au immunoassaying chip could be used to rapidly detect ZIKV infections during the early stages in a POC application. In a very recent study, a novel electrochemical biosensor based on nanocarbon materials (graphene oxide) and imprinted polymers (SIPs) composites was successfully developed for the detection of ZIKV in both serum specimens and buffer (Figure 9C). The electrochemical biosensor could be employed for the detection of ZIKV using variation of electrical signal with a change of ZIKV concentrations in both serum specimens and buffers. The electrochemical biosensor could detect ZIKV with a low concentration of 2 × 10⁻⁴ PFU/ml (1 RNA copy/ml) in PBS. Moreover, the LOD for ZIKV was close to the LOD of the real-time RT-PCR approach.

5.2 Colorimetric sensing

Owing to the ease of read-out and fast visual detection by the naked eye/low-cost portable instruments, colorimetric sensing technologies have been widely applied in bioanalysis, clinic diagnostics, environmental monitoring, food safety, public safety control, and biological technology. In recent years, colorimetric sensing has been employed for the detection of ZIKV infection. For example, Pardee et al. successfully developed a novel portable, low-cost sensing platform (the cell-free paper-based biosensor; Figure 10A) for the colorimetric detection
of ZIKV. The paper-based biosensor could be used to detect ZIKV RNA genome using a CRISPR/Cas9-based module. The biosensor displayed high selectivity because of the hybridization between the ZIKV RNA and the CRISPR/Cas9 system (which was specifically designed). Moreover, the study indicated that ZIKV could be detected at femtomolar (fM) concentrations. By using the sequences of DENV as a negative control, the selectivity of the prepared biosensor could be confirmed. In addition, the results indicated that the developed paper-based biosensor exhibited high specificity and selectivity and the capability of discriminating viral strains with single-base resolution by using CRISPR/Cas9.277 Using the color change of leuco crystal violet dye, Song et al. developed a POC ZIKV sensing system (Figure 10B) using the RT-LAMP with high sensitivity, which only used a chemically heated cup to adjust to the sensing temperature of the system without a requirement for electrical power. Moreover, the sensor was considered as an instrument-free POC detection device, because the color variation could be examined by the naked eye without any instrumentation. In addition, it was shown that the sensor could detect ZIKV from saliva, blood, semen, and urine samples with a high sensitivity of 5 PFU in a short time (<40 min).278 Shortly afterward, Müllner et al. developed a high-throughput colorimetric assay for the detection of ZIKV infection. The colorimetric assay could be used to quantify ZIKV infections with a broad range of viral dilutions in both human and monkey cells. On this basis, the neutralizing antibody titers in ZIKV antisera could be defined or the inhibitory activities of antivirals that block ZIKV could be tested using the colorimetric assay. More importantly, the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT)-based colorimetric assay could be evaluated by using the naked eye and analysed using computational tools (Graphpad Prism), achieving a good linearity over a broad range and did not require costly reagents or equipment. Therefore, the colorimetric assay as a cheap, simple, and fast strategy for the testing of antiviral compounds and the quantification of ZIKV neutralizing antibodies is a promising substitute for antibody-based detection, especially with large specimen numbers in resource-poor settings.485 To meet the requirement of rapid and noninvasive ZIKV diagnostic screening assays for the prenatal care of women living in the areas where the ZIKV may outbreak, Calvert et al. developed a novel RT-LAMP assay for the colorimetric detection of ZIKV RNA in serum and urine specimens with high specificity and sensitivity using a simple visual detection method. The results of the RT-LAMP assay indicated a LOD 10-fold higher than real-time RT-PCR. The developed RT-LAMP assay could detect ZIKV RNA collected from a panel of 178 diagnostic samples with a low LOD of 1.2 RNA copies/μl. Moreover, it was shown that the RT-LAMP assay exhibited a high specificity for the ZIKV RNA when the ZIKV was examined with diagnostic samples positive for the CHIKV and DENV. In addition, the developed assay could be potentially used as a fast, sensitive, specific, and reliable assay for detecting ZIKV in serum or urine specimens under the conditions of clinical/field setting with minimal technological expertise and equipment.279 Smartphones have attracted widespread interest due to their potential application in pathogen detection and POC usage.486 In a very recent study, Priye et al. reported that the smartphone CMOS sensor could be used for reliably and reproducibly quantifying the colorimetric sensing of nucleic acid specimens with no vision biases (Figure 11). Using luminescence-based analysis, positive or negative discrimination was higher by about an order of magnitude compared with traditional RGB analysis. Moreover, the chromaticity part of the analysis could achieve a reliable multiplexed detection for different targets that were labeled using spectrally separated fluorophores. Using the end point RT-LAMP, a

**Figure 9** (a) An interdigitated microelectrode is modified with different nanostructures for high loading of ZIKV-specific antibodies for the detection of ZIKV proteins at picomolar concentrations using an appropriate electrochemical transduction technique. Reprinted with permission from Ref.230 Copyright 2016, Elsevier Ltd. (b-A) Illustration of IDE-Au based electrochemical ZIKV immunosensor. (b-B) Roadmap for the development of an electrochemical ZIKV immunosensor at POC. Reprinted with permission from Ref.273 (c) The preparation of SIPS-graphene oxide composites on an Au surface to generate a ZIKV biosensor. Reprinted with permission from Ref.274 Copyright 2019, American Chemical Society. IDE-Au, interdigitated micro-electrode by functionalization of a gold; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 10  (a) Workflow of a paper-based ZIKV colorimetric biosensor using CRISPR/Cas9 technology. (A) CRISPR/Cas9-based paper sensor for detecting the ZIKV RNA genome. (B) Colorimetric detection of ZIKV or DENV. Reprinted with permission from Ref.277 Copyright 2016, Elsevier Ltd. (b) Instrument-free ZIKV detection. A microfluidic chip is established to achieve colorimetric ZIKV detection based on the RT-LAMP strategy. Reprinted with permission from Ref.278 Copyright 2016, American Chemical Society. DENV, dengue virus; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
chromaticity-luminance formulation could simultaneously detect ZIKV and CHIKV RNA. In addition, the chromaticity-luminance analysis was suitable for other types of multiplexed fluorescence detection using a smartphone camera.⁴⁸⁷

5.3 | Fluorescence sensing

In recent years, fluorescence sensing has been employed for the detection of ZIKV infections.⁴⁸⁸ For example, a new ultrasensitive electrogenerated chemiluminescence (ECL)-based immunoassay (Figure 12A) was developed by Acharya’s group for specifically and sensitively detecting the ZIKV using human biological fluids. The anti-ZIKV-PSBs were prepared by loading polystyrene beads (PSBs) with an ECL label, and then conjugating them with anti-ZIKV monoclonal antibodies. The ZIKV in solution could be efficiently captured by the as-prepared anti-ZIKV-PSBs to generate ZIKV-anti-ZIKV-PSB complexes, and the ECL intensity of anti-ZIKV-PSBs measured after being separated by magnetic beads. The results indicated that the anti-ZIKV-PSBs could capture the ZIKV at as little as 1 PFU in 100 μl of human urine sample, human plasma specimens, or saline.²⁸⁰ Interestingly, Priye et al. developed a novel smartphone-based diagnostic platform (Figure 12B) for the rapid, highly specific and sensitive detection of ZIKV, DENV, and chikungunya using RT-LAMP with the quenching of unincorporated amplification signal reporters
FIGURE 12  (A) ECL-based immunoassay for ZIKV detection. Copyright 2016 nature. Reprinted with permission from Ref.280 (B) Smartphone-based ZIKV assay. (B-A) Scheme of RT-LAMP detection setup depicting the isothermal heater with Bluetooth microcontroller (Arduino Uno), LED excitation source, and reaction tubes. (B-B) A 3 watt RGB LED coupling a RGB multi band pass filter can ensure a narrow excitation source for detection reagents. (B-C) The isothermal heater can supply a uniform surface temperature distribution of 1°C. The heaters are loaded with either (B-D) off the shelf real-time PCR polypropylene tubes or (B-E) custom made laser-cut reaction wells. (B-F) Thermal management and heat ramp rates have a great improvement by using custom laser-cut wells. (B-G) The smartphone app can wirelessly actuate the RGB LED excitation source and isothermal heater to achieve real-time monitoring and change of the heater temperature along with illumination of the samples using an appropriate excitation light source. The illuminated reagent can be captured through a smartphone camera with an interchangeable emission filter and the image is able to be subsequently analyzed. Reprinted with permission from Ref.281 ECL, electrogenerated chemiluminescence; RT-LAMP, reverse transcription-loop-mediated isothermal amplification [Color figure can be viewed at wileyonlinelibrary.com]
(QUASR) technique. A simple, portable, and cheap “LAMP box” using a smartphone (consumer class) could be used to conduct the reactions, which could be powered using a 5 V USB source (e.g., solar panel or USB power bank). Compared with the detection using the naked eye or the traditional RGB intensity sensors, the use of a smartphone could increase fivefold the discrimination of positive or negative signals, because the smartphone exploited chromaticity to analyze fluorescence signals using an algorithm. The developed diagnostic device could directly detect ZIKV collected from saliva, blood, and urine specimens, which demonstrated that the device was suitable for clinical deployment.\textsuperscript{281} Based on the multimode interference wave guide-based optofluidic-chip platform, Parks et al. developed a highly specific and sensitive multiplexed fluorescence assay for dual detection of ZIKV protein and nucleic acid. The device could be used to detect different types of molecular targets such as ZIKV protein and nucleic acid complexes with high specificity, sensitivity, and reliability.\textsuperscript{489} Recently, Ochmann et al. for the first time proposed a target-specific FQH-based DNA detection method using a novel physical concept (DNA origami optical antennas enhanced the fluorescence of molecules placed in the plasmonic hotspot; Figure 13), and used it to detect specific artificial DNA and RNA of ZIKV. Using DNA origami-based optical antennas (the height of which was about 125 nm), the hotspot could be created by metallic NPs and was then employed for enhancing the fluorescence signal through plasmonic effects. Equipping the hotspot with a MB-like structure resulted in the combination of plasmonic signal enhancement with specific signal generation, producing an enhanced and easily detected signal for specific target nucleic acids. The applicability of the approach was verified by using it to detect the specific artificial

\begin{figure}
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\caption{
FQH and DNA origami design: (A) DNA-based FQH is used for the detection of Zika-specific DNA. (B) The DNA origami pillar with a total height of 125 nm is immobilized via biotin modifications on to a BSA-biotin/neutravidin surface at low concentration to ensure single molecule detection. Reprinted with permission from Ref.\textsuperscript{282} Copyright 2017, American Chemical Society. FQH, fluorescence-quenching hairpin [Color figure can be viewed at wileyonlinelibrary.com]
\end{figure}
DNA and RNA of ZIKV (in heat-inactivated human blood serum and in buffer). In addition, it was shown that the modularity provided by DNA nanotechnology could enable multiplexing through combining orthogonal fluorescent labels for simultaneous detection of different sequences. In another study, Xie et al. successfully fabricated a novel stable and water soluble 3D Cu-based zwitterionic metal-organic framework (MOF) for the simultaneous detection of the RNA sequences of ZIKV and DENV. The as-prepared MOFs could be used for forming the electrostatic, hydrogen bonding interactions and/or π stacking with the fluorophore-labeled DNA probes to construct two different sensing systems. The sensing systems displayed high sensitivity and selectivity, and could simultaneously detect the RNA sequences of ZIKV and DENV by using the single and synchronous fluorescence analysis. The single detection strategy could simultaneously detect the RNA sequences of ZIKV and DENV with a LOD of 192 and 332 pM, respectively. For the synchronous fluorescence detection strategy, the LODs of 121 and 184 pM were respectively obtained. Moreover, the two assays with high specificity had no interference with other mismatched RNA sequences, including single-base mismatched RNA sequences. In addition, two DNA probes could be used for synchronous ZIKV detection without cross-reaction. In a recent study, Zhang et al. reported a novel Janus emulsion agglutination assay (Figure 14) that could be used to detect interfacial protein-protein interactions. Janus emulsion droplets could be functionalized using rcSso7d-ZNS1 (an antigen binding the hyperthermophilic rcSso7d protein) with thermal stability, which was used to detect ZIKV NS1 protein. In hydrocarbon and fluorocarbon phases, the emulsion droplets containing fluorescent dyes could be used to intensify the intrinsic optical signal, which could be determined through a simple optical fiber. In addition, two novel optical transduction strategies were successfully developed, which could be used for the analyte quantification. Through the incorporation of dyes in emulsion droplets and detection of the emitted light or backscatter, or the multiple emissions modulated by an inner filter effect (IFE), robust ratiometric signals were successfully used for the detection of agglutination. The proposed assay based on two optical schemes could be employed for the detection of ZIKV NS1 protein with a LOD of 100 nM.

5.4 SERS sensing

Due to their merits of unique LSPR properties, excellent multiplexing ability, large dynamic range, and high sensitivity, SERS has been used in multiple fields, such as bioanalysis, disease diagnosis, immunoassays, environmental surveillance, food safety supervision, and biomedicine. SERS has been widely investigated and applied for the detection of viruses. Due to its high sensitivity in a multiplexed assay, SERS has recently been used to detect ZIKV infections. For example, Sánchez-Purrà et al. successfully developed the new SERS-based sandwich immunoassay (SERS-lateral flow assays [LFAs] platform; Figure 15A) for the multiplexed detection of ZIKV and DENV with lower LODs than those achieved using the colorimetric readout of LFAs. Interestingly, SERS encoded-Au nanostars could be conjugated to specific antibodies to distinguish the NS1 biomarkers between ZIKV and DENV using 4-mercaptobenzoic acid and 1,2-bis (4-pyridyl) ethylene (BPE) as Raman reporter molecules. By combining SERS with LFAs (the simplicity of a LFA was combined with the high sensitivity of SERS), the low sensitivity of LFAs for detecting low concentrations of biomarkers could be greatly improved, and the combined SERS-LFA platform exhibited high sensitivity and simplicity. Moreover, compared with the colorimetric LFAs, the SERS-LFA platform led to 7.2-fold LOD decrease for DENV NS1 and 15-fold for ZIKV NS1, respectively, resulting in LODs down to 7.67 ng/ml for DENV NS1 and 0.72 ng/ml for ZIKV NS1, respectively. In addition, the SERS-LFA platform could be connected wirelessly to the cloud or a PC, which could analyze and report the data of new disease cases in real-time during an outbreak. In a recent study, to solve the problems of the high LOD and the cross-reactivity between DENV and ZIKV, Camacho et al. designed a novel immune-specific assay based on SERS nanoprobes (Figure 15B). The gold shell-isolated nanoparticles (Au-SHINs) were fabricated using Au NPs (100 nm) and silica shell (4 nm) coated with Nile Blue (NB; Raman reporter). Following that, NB molecules were modified on the outer silica layer. After wrapping in a final silica shell, the SERS nanoprobes were functionalized with the...
FIGURE 14  (a) Functionalization of the droplets with a polymer surfactant. (a-a) Preparation of maleimide-functionalized surfactant P1-MA using a polystyrene-b-poly polymer. (a-b) Bioconjugation of rcSso7d to the droplet H/W interface using maleimide-thiol chemistry. The streptavidin was added into the rcSso7d-functionalized droplets assay and leads to agglutination. The hydrocarbon phase is dark gray for illustrative purposes. The scheme is illustrative and is not to scale. (b) Optical detection using backscattering. (b-a) Experimental setup with both excitation and detection source from the top of the emulsion layer. (b-b) Backscattering of light by naturally oriented Janus droplets and agglutinated droplets. (c-a) Experimental setup with the optical fiber at the bottom of the emulsion layer. (c-b) Mechanism for the attenuated emission of the perylene dye, is dependent on the orientation of the droplets. Reprinted with permission from Ref.285 Copyright 2019, American Chemical Society [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 15  (a) SERS-LFAs platform for detecting and distinguishing the NS1 biomarkers of ZIKV and DENV. Reprinted with permission from Ref.286 Copyright 2017, American Chemical Society. (b-A) ZIKV-mAb-SERS nanoprobe assembly: gold-SHIN (about 100 nm gold core + 4 nm silica shell thickness); gold-SHIN + NB Raman reporter layer; gold-SHIN + NB Raman reporter layer + final about 10 nm silica shell (SERS nanoprobe); conjugation onto ZIKV-NS1 monoclonal antibodies (ZIKV-mAb). (b-B) SERS immunoassay platform for the detection of various concentrations of ZIKV NS1. The platform can be irradiated using a 633 nm laser line and the SERRS signal produced from NB molecules, located a close to the AuNPs (about 4 nm), is recorded using area mappings. Brighter spots indicate higher intensity of the NB band at 593 cm$^{-1}$. Reprinted with permission from Ref.287 Copyright 2018, American Chemical Society. DENV, dengue virus; LFA, lateral flow assay; NS1, nonstructural protein 1; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
monoclonal antibodies against ZIKV NS1. Owing to the specificity toward ZIKV antigens provided by the monoclonal anti-ZIKV NS1 antibodies functionalized SERS nanoprobes, the assay system possessed the capability of decreasing the cross-reactivity between ZIKV antigens and DENV antigens to avoid false positives. Moreover, it was found that the immune-specific assay based on the SERS nanoprobes could be used for detecting ZIKV antigens with very low concentration of 10 ng/ml, and no cross-reactivity with DENV NS1 antigens.

5.5 Other sensing methods

There are some other sensing methods reported for the detection of ZIKV infection. For example, using an immobilized monoclonal antibody with high specificity, Afsahi et al. developed a new portable and cost-effective graphene-enabled biosensor for the detection of ZIKV (Figure 16A). By covalently linking the anti-ZIKV NS1 to the graphene, the biosensor could be used for the quantitative, real-time detection of ZIKV antigens through the incorporation of field effect biosensing technology. Moreover, the biosensor functionalized with anti-ZIKV NS1 could detect ZIKV antigens (ZIKV NS1) in buffer at concentrations as low as 0.45 nM. It was found that the biosensor could serve as a potential diagnostic tool for measuring ZIKV NS1 in simulated human serum. The selectivity of the biosensor was verified by using Japanese encephalitis NS1 (a homologous viral antigen with potential cross-reaction), no measurable cross-reactivity was observed. In addition, a graphene-enabled ZIKV biosensor with the merits of high speed, sensitivity, and selectivity was considered as an ideal candidate, which could be further developed as a medical diagnostic test. Recently, Liao et al. successfully constructed a new ZIKV liquid biopsy system by integrating a dendritic Ru(bpy)32+-polymer-amplified ECL approach as a valid signal output mode (Figure 16B). The system could be used to achieve amplification-free analysis of ZIKV using a drop of blood, with superior specificity and sensitivity. By adopting the humoral biomarker as a detection indicator to simplify the analysis process, a novel nondestructive detection mode was successfully established. In addition, clinical ZIKV samples were evaluated, the results of which demonstrated that the Ru(bpy)32+-polymer-amplified ECL approach displayed a reliable response toward ZIKV in urine, blood, and saliva specimens. The system could meet the strict clinical demands for the liquid biopsy of ZIKV, and may possess the potential of serving as a novel paradigm for the detection of ZIKV infection and biomedical analysis. Xiong et al. successfully developed a new dual-modality readout immunoassay platform (Figure 17) using an aggregation-induced emission luminogen (AIEgen), which was used to detect the viruses with high sensitivity. The constructed platform achieved robust naked-eye discernible dual signal plasmonic colorimetry and turn-on fluorescence for the detection of viruses.

6 OTHER STRATEGIES FOR DETECTION OF ZIKV

6.1 Reverse transcription loop mediated isothermal amplification

The RT-LAMP as an isothermal amplification technology could be used for nucleic acid amplification without requiring thermocycling. Compared with the traditional real-time PCR and quantitative real-time RT-PCR, the RT-LAMP assay displayed excellent specificity and sensitivity for the detection of ZIKV. Due to its high specificity and sensitivity, RT-LAMP has been widely applied for the detection of ZIKV. For example, Wang et al. have developed a one-step reverse RT-LAMP platform with high specificity and sensitivity for the detection of ZIKV. Moreover, RT-LAMP was more convenient than previous approaches and could be easily implemented and distributed. The RT-LAMP assay with superior specificity and sensitivity could be used for detecting ZIKV RNA with a LOD of 20 copies/test, and a LOD of 0.02 PFU/test for the simulated ZIKV clinical samples. The results indicated that the LOD was equal to the real-time PCR but an order of magnitude above the RT-PCR. To distinguish the ZIKV of African and Asian lineages, Chotiwan et al. developed a rapid LAMP assay...
with high sensitivity and specificity. The LAMP assay could directly detect ZIKV RNA in mosquitoes, cultured infected cells, and the body fluids of infected patient (e.g., semen, blood, urine, saliva, and plasma) and in plasma, semen, and serum specimens of infected patients without the requirement of ZIKV RNA isolation/reverse transcription. By incorporating the optimized RT-LAMP and a LFA (Figure 18A), a novel, user-friendly, simple strategy was developed by Lee’s group for the highly sensitive detection of ZIKV. The method could be used to identify ZIKV RNA in the human whole blood and pure water. When the RT-LAMP reaction was optimized by adjusting the concentrations of dNTP and Mg$^{2+}$; this resulted in the complete elimination of nontarget

**FIGURE 16** (a-A) Diagram of the sensor element of the graphene biosensor chip. Antibodies are immobilized on pristine graphene using a zero-length linker. The graphene surface is blocked and passivated to reduce nonspecific interactions. PEG has been demonstrated as an effective block against nonspecific interactions in general and specifically when covalently attached to graphene devices. Antibodies (i.e., anti-ZIKV NS1) and PEG form the dielectric in a graphene based liquid gated transistor. (a-B) Illustration of the entire sensor chip system, incorporating the sensor chip, reader electronics and digital control, and PC running control and data presentation software. Reprinted with permission from Ref.511 (b) Workflow of ZIKV liquid biopsy system for detection of ZIKV. Reprinted with permission from Ref.290 Copyright 2018, American Chemical Society. PEG, polyethylene glycol; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
amplification (which was caused through nonspecific primer dimers amplification). Moreover, ZIKV RNA obtained from human whole blood with no requirements for special equipment (e.g., thermal cycler and ultracentrifuge) could be directly amplified through a direct RT-LAMP reaction. In addition, ZIKV RNA could be detected down to the single copy level within 35 min using a combination of the optimized RT-LAMP and the LFA.294 Yaren et al. have developed a LAMP assay to detect ZIKV using a dTTP-dUTP mix and a thermolabile uracil DNA glycosylase (Figure 18B). The assay could be used for the detection of viral RNA in unprocessed urine specimens and other biological specimens, and could distinguish ZIKV, DENV, and chikungunya in urine specimens and in infected mosquitoes. Squares of Q-paper impregnated with crushed infected mosquitoes or plasma and urine samples were directly added into RT-LAMP mixtures, and the result from the assay could be read in 20–40 min by visualizing three-color coded fluorescence signals using the naked eye. The tests resulted in detection levels of ~38 copies of chikungunya viral RNA, ~0.71 PFU equivalent viral RNAs for ZIKV, and ~1.22 PFU equivalent viral RNAs for DENV.283 In another study, Sabalza et al. employed a generic strategy to detect the RNA and/or DNA of a pathogen based on the LAMP and reverse dot-blot (RDB) approach, which was automatically operated in a microfluidic device. They described how to convert a microfluidic assay for the detection of HIV RNA into an instrument for the detection of ZIKV RNA. The assay could be implemented using a microfluidic device, and the developed method was used to simultaneously analyze 24 specimens and could automatically detect specimens through RDB. In addition, preliminary data obtained using saliva specimens spiked with ZIKV indicated that the diagnostic system could detect ZIKV RNA in saliva samples.517 To overcome the limitations of conventional molecular diagnostics, Song et al. rationally designed and successfully developed a novel simple, hand-held, inexpensive, smartphone-based mobile assay platform ("smart-connected cup" [SCC]) (Figure 19). The detection platform used the bioluminescent assay in real-time loop-mediated isothermal amplification technology with

FIGURE 17  Fluorescence and plasmonic colorimetric dual-modality virus detection based on a multifunctional AlEgen. Reprinted with permission from Ref.512 Copyright 2018, American Chemical Society [Color figure can be viewed at wileyonlinelibrary.com]
smartphone-based assay for a connected, quantitative, and rapid molecular diagnostics without the need of optical filters and excitation source (which are essential for fluorescence detection). Moreover, they developed a custom smartphone Android App for monitoring and analyzing bioluminescence signals, quantitative analysis of targets, data sharing, and diseases' spatiotemporal mapping. It was shown that the SCC could be used to quantitatively detect ZIKV in saliva and urine samples and HIV in blood within 45 min. In addition, it was found that SCC was suitable for nucleic acid amplification, and could carry out target quantification with a high sensitivity of 5 PFU per saliva/urine sample. Recently, based on RT-LAMP, Lamb et al. developed a new robust diagnostic test for the rapid detection of ZIKV in urine and infected mosquito specimens (<30 min). The study specifically detected ZIKV

FIGURE 18  (a) LFA procedures. (a-A) The LFA. (a-B) Operation procedures for LFA: (i) 1 μl RT-LAMP product can be loaded onto the conjugate pad, (ii) 45 μl of buffer is added to buffer loading pad, and (iii) AuNPs are transferred from the conjugate pad to the test and control line through the capillary flow. Reprinted with permission from Ref.294 Copyright 2016, American Chemical Society. (b-A) ZIKV assay of mosquito specimens using Q-paper technology. The bodies of mosquitoes were first crushed on Q-paper and treated with 1 M aqueous NH₃ solution (pH 12), and subsequently the Q-paper was washed with 50% EtOH and water. Q-papers with mosquito sample were then dipped into the RT-LAMP mixture and incubated at 65°C for 30 min and visualized using a LED blue light (470 nm) passed through an orange glass filter. The images were recorded using a cell phone camera. (b-B) Real-time RT-LAMP for the detection of ZIKV. Fluorescence emission on irradiation using a blue LED (470 nm) observed using an orange glass filter after being incubated at 65°C for 30 min. Reprinted with permission from Ref.283 LFA, lateral flow assay; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 19  IoMT system and SCC platform for the mobile molecular detection based on a BART-LAMP assay. (A) IoMT design. (B) SCC platform containing a smartphone, 3D-printed holder, and a thermos cup body. (C) Photograph of the SCC platform. (D) Photograph of MIAR chip suitable for on-chip NA extraction and BART-LAMP assay. (E) Images of bioluminescence emissions using smartphone camera from the isothermal amplification reactors (right: positive control; left: negative control) at 1 and 16 min after starting incubation. Positive and negative control specimens contained 0 and 500 PFU of ZIKV. Reprinted with permission from Ref.298 Copyright 2018, American Chemical Society. BART-LAMP, bioluminescent assay in real-time loop-mediated isothermal amplification; IoMT, internet of medical things; SCC, smart-connected cup [Color figure can be viewed at wileyonlinelibrary.com]
in serum, urine, and mosquito specimens without the requirement of RNA extraction from the urine and mosquito samples.\textsuperscript{518} Zhao et al. developed a LAMP assay using the EP coding regions and the NS5 protein (nonstructural protein) coding region as the target sequences, as a fast, specific, and sensitive strategy to detect ZIKV. Based on the calcein/Mn\textsuperscript{2+} complex chromogenic approach (the color variation of the calcein/Mn\textsuperscript{2+} mixture), resulted in a visual strategy for detecting ZIKV. The assay could detect ZIKV with LODs of $1.12 \times 10^{-11}$ pmol/µl DNA for E coding region and $0.5 \times 10^{-9}$ pmol/µl DNA for NS5 protein coding region, respectively. In addition, compared with real-time PCR and RT-PCR, the assay displayed a 100-fold increase in sensitivity.\textsuperscript{8}

6.2 | TMA technology

TMA technology requires two enzymes, including RNA polymerase and reverse transcriptase, which can produce the RNA amplicons and lead to a higher amplitude amplification in 15–60 min. The Aptima ZIKV assay was evaluated by Ren’s group using specimens spiked with ZIKV (three different lineages, $n = 10$) and clinical urine and serum samples ($n = 124$) collected from two different patient populations. Compared with RT-PCR as a reference approach, the TMA technology-based Aptima Zika virus assay showed a sensitivity of 94.7%, a specificity of 94.8%, and 94.8% diagnostic accuracy.\textsuperscript{299}

6.3 | Reverse transcription strand invasion based amplification

RT-SIBA performed with portable battery-operated instrumentation for the detection of ZIKV was reported by Eboigbodin et al. The RT-SIBA depended on a recombinase-coated single-stranded invasion oligonucleotide for the separation of complementary target duplex. The DNA polymerase could extend the single-stranded target template under a constant and low temperature. With RT-SIBA a fluorescence signal could not be produced if the target template was absent. Moreover, The RT-SIBA exhibited a high sensitivity and rapid detection of ZIKV, indicating that the developed strategy may be used as a portable and powerful molecular diagnostic tool to detect the ZIKV.\textsuperscript{300}

6.4 | Bioplasmonic paper-based device

BPD has been applied to the diagnosis of ZIKV through the detection of IgM and IgG antibodies to NS1 protein of ZIKV.\textsuperscript{519} Jiang et al. developed a novel and adaptable BPD to detect ZIKV infection by determining the anti-ZIKV-NS1 IgG and IgM in serum samples (Figure 20). The NS1 protein of ZIKV served as a capture element and the gold nanorods acted as plasmonic nano-transducers. The BPD displayed excellent selectivity and sensitivity for both IgM and IgG antibodies to NS1 protein of ZIKV in human serum. Furthermore, the BPD also exhibited excellent stability at room temperature and elevated temperatures for 30 days, which was achieved using a MOF dependent biopreservation strategy.\textsuperscript{520}

6.5 | Reverse transcription isothermal recombinase polymerase amplification

Recombinase polymerase amplification (RPA) as a promising tool exhibits a great many merits, such as simple primer design, speed, power saving (runs at 37°C), robustness and no initial heating step for biological substances. Compared with real-time PCR and other isothermal amplification strategies (e.g., LAMP, rolling circle amplification [RCA], nucleic acid sequence-based amplification, helicase-dependent amplification, and strand-displacement
amplification) RPA could play an extremely critical role by saving time in screening infected patients and managing the treatment process. Recently, it was reported that the reverse transcription (RT) isothermal recombinase polymerase amplification assay (RT-RPA) was employed for the detection of ZIKV. For example, Wahed et al. developed a portable and sensitive RT-RPA assay for the detection of ZIKV. The RT-RPA assay targeted the NS2A region and indicated 100% specificity and 92% sensitivity compared with real-time RT-PCR. Moreover, there was no cross-reactivity with other flaviviruses, arboviruses, and alphaviruses. The RT-RPA assay could rapidly detect 21 RNA molecules within 3–15 min. By combining RPA with a highly specific LAMP (RAMP), Song et al. proposed a sensitive two-stage, hybrid isothermal enzymatic amplification strategy for the detection of concurrent multiplex molecules (Figure 21). The developed RAMP could increase the sensitivity of the amplification process and displayed the merits of isothermal amplification (e.g., low power consumption and simple instrumentation). The RAMP was used to test specimens with similar targets like the various strains of ZIKV and the HPV, resulting in no false negatives or false positives. Compared with the traditional multiplexing methods (e.g., isoPCR, mPCR and nmPCR), RAMP was faster and more inhibitor-tolerant, and took less time (<40 min), even when using low-abundance specimens. Interestingly, Chan et al. described a repurposed 3D printer and RPA for the rapid and low-cost POC molecular diagnostics. In the device, a 3D printer was used to rapidly perform the nucleotide extraction with high throughput. The nucleotide extraction and isothermal amplification was performed within the same enclosure. With the amplification of RNA, the assay could produce a fluorescence signal that could be detected using a smartphone camera or portable detector. Moreover, the ability of this device was verified using ZIKV and foodborne pathogens. The device could extract and purify up to 12 specimens from infected patients within <15 min per run. Unfortunately, compared with the conventional spin column, the device displayed a slightly lower RNA extraction, which may cause a decrease in the detection sensitivity when specimens with lower viral load are used.
Recently, a number of emerging strategies have appeared for the detection of ZIKV. For example, Koish et al. developed a new high-throughput image-based fluorescent neutralization assay for detection of ZIKV infection. Compared with conventional PRNT, the method has a higher throughput. Moreover, the method exhibited higher specificity and 50.53% less cross-reaction than MAC-ELISA when ZIKV was detected with DENV specimens. Moreover, the method could simultaneously detect many specimens and different viruses. However, it could not discriminate between the antibody classes, and could only be applied in selected laboratories with expensive equipment. NIRS as a rapid, cost-effective and reagent-free tool has been used for the noninvasive detection of ZIKV in thoraces and heads of intact mosquitoes (A. aegypti mosquitoes). NIRS could be used to detect ZIKV using a beam of light targeted on the mosquitoes resulting in a diagnostic spectrum. Surprisingly, a 94.2–99.3% accuracy relative to real-time RT-PCR was obtained. Moreover, compared to real-time RT-PCR, the NIRS was 110 times cheaper and 18 times faster. In another study, a novel NP-enhanced viral lysate electrical sensing assay was developed for the specific and sensitive detection of ZIKV on cellulose paper microchips using screen-printed electrodes (Figure 22). ZIKV could be isolated from biological specimens using antibodies, and was able to be labeled by platinum NPs to enhance the electrical signal. Then, the captured ZIKV-platinum NP complexes were lysed employing a detergent to release the charged molecules associated with the intact virus and the platinum NPs on the
captured viruses. The electrical conductivity of the solution could be changed by the platinum NPs and the released charged molecules, could be gauged using the paper microchips with printed electrodes. The strategy resulted in a high specificity for the detection of ZIKV in the presence of other closely related flaviviruses (e.g., DENV-1 and DENV-2) with a LOD of $10^7$ virus particles/μl. Moreover, the strategy displayed a higher sensitivity (>10 times) compared with direct lysate virus sensing, and could improve the

**FIGURE 22** Preparation of Pt-nanoprobes and NPs-enhanced electrical ZIKV sensing using a paper microchip. (A) Synthesis of Pt-nanoprobes. Oxidized anti-ZIKV mAb were conjugated with the PtNP surface using 3-(2-pyridyldithio) propionyl hydrazide. 3-(2-Pyridyldithio) propionyl hydrazide possess a thiol group that can bind to the surface of PtNPs and a hydrazide group that can bind to the free aldehyde group of the FC region on the oxidized antibodies. The digital images confirmed the stability of the as-prepared Pt-nanoprobes. (B) TEM micrograph and the particle size distribution histogram of the synthesized PtNPs. (C) Virus particles were captured using magnetic beads modified with anti-ZIKV mAb followed by labeling with a specific Pt-probe to form Pt-virus complexes on the magnetic bead surface. (D) Representative impedance magnitude measurements toward ZIKV-spiked (red line) and virus-free control (black line) specimens. Reprinted with permission from Ref.526 NP, nanoparticle; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
reliability and specificity of the assay by limiting the number of false positive outcomes. Recently, a novel, simple, portable, and inexpensive smartphone-based fluorescent lateral flow immunoassay (LFIA) platform (Figure 23) was developed by Rong et al., for the detection of ZIKV NS1 with high sensitivity. In this platform, the external optical components (including an external lens, a high-power UV LED, optical filters, power unit) were integrated with a smartphone camera through 3D-printed attachment for image-based signal readout of the fluorescent LFIA strips. The QD microspheres with bright fluorescence signal were employed as probes, which could be excited using UV light and the fluorescence signal could be read out by the camera of the smartphone when the sandwich immune complexes formed on the test line of the fluorescent LFIA strip due to the existence of target ZIKV NS1 in the specimens. The developed platform could be used for the quantitative detection of ZIKV NS1 in buffer/serum specimens within 20 min. Moreover, LODs of 0.045 ng/ml in buffer and 0.15 ng/ml in serum samples were obtained. In addition, reduced cross-reactivity with a high-level DENV NS1 was observed. Saraf et al. have developed a novel polydimethylsiloxane-based microfluidic device (Figure 24) for detecting the viral EP (e.g., ZIKV and CHIKV). The channel was integrated with the microsized pillars that increased the surface area allowing more aptamers to attach to incoming E protein molecules. Therefore, the overall sensitivity of the system was increased. The developed device relied on the construction of protein-mediated sandwich morphology which was obtained by using aptamers and gold nanoparticles (Au NPs) functionalized with aptamers. The colorimetric signal was produced upon the addition of silver reagents into the channel, which were selectively deposited on the surface of Au NP, forming a gray contrast in the testing zone. The obtained microfluidic channel approach resulted in a high specificity for the detection of ZIKV and CHIKV E proteins, with a detection limit of 10 pM in calf blood and 1 pM of viral EP targets in PBS.

**FIGURE 23** Design and application of a smartphone-based fluorescent LFIA platform. (A) 3D schematic of the smartphone-based imaging device, showing the internal structure. (B) Photograph of the developed fluorescent LFIA reader. (C) Fluorescent LFIA for the detection of ZIKV NS1. (D) Images of the test strips in the presence (left) and absence (right) of ZIKV NS1. Reprinted with permission from Ref. Copyright 2019, Elsevier. LFIA, lateral flow immunoassay; NS1, nonstructural protein 1; ZIKV, Zika virus [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 24  (See caption on next page)
While ZIKV has been known since 1947, it was not a huge global threat for human health until recently. It was reported that ZIKV could cause congenital abnormalities in the fetuses of infected mothers, such as microcephaly, fetal demise, and other brain damage in infants. Moreover, ZIKV can lead to GBS in adults. With the spread of ZIKV between different continents/countries over the last decade, it has resulted in a cause of concern for human health. The rapid spread of ZIKV may be caused by multiple routes of transmission (including vector transmission, sexual transmission, maternal fetal transmission, and body fluid transmission) rather than just as a result of mosquito bites. Unfortunately, the ZIKV has recently resurged in India with the potential for devastating consequences. Therefore, rapid detection strategies and efficient medicines for the appropriate prevention and efficient control of ZIKV need to be urgently developed. Furthermore, the exhaustive molecular mechanism of ZIKV and host-pathogen interactions still need to extensively evaluated to understand the enhanced pathogenicity of ZIKV infection, to develop novel effective detection strategies and drugs to prevent and control ZIKV infections.

Currently, numerous strategies have been developed for the detection of ZIKV. Among these strategies, the MAC-ELISA/PRNT and real-time RT-PCR are gold standards and have been recommended by the CDC for the detection of ZIKV during infection. However, serological assays and molecular assays both suffer from some limitations. For the serological assays, the cross-reactivity between ZIKV and other homologous flaviviruses (e.g., DENV) is a problem. For the molecular assays, the missing unanticipated sites may cause failed amplification. Thus, the serological assays and molecular assays need to be further improved to enhance the detection stability and accuracy. In addition, serological assays, and molecular assays both require a laboratory environment and expensive equipment that requires human expertise in order to perform these tests. Such equipment and expertise may be not available for all regions of the world. Therefore, affordable, rapid, reliable, and portable sensing methods and devices still need to be developed for the detection of ZIKV infection. To date, some sensing methods have been put forward for the detection of ZIKV. The performance of these sensing methods and devices should be further evaluated and verified before they are used for the clinical diagnosis of ZIKV. In addition, ZIKV pathogenesis and genetics need be better understood to facilitate the design of new diagnostic methods and effective drugs and vaccines to counter ZIKV.

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FIGURE 24  (A) Polydimethylsiloxane-based microfluidic device for the multiplexed assay of viral E proteins (e.g., ZIKV and chikungunya) on a single platform using an aptamer-analyte interaction. (B) Illustration of the procedures established The surface was firstly conjugated with a linker molecule (i.e., PMPI) that attaches to the thiolated end of the aptamers (1); the control site was blocked by BSA. Specimens containing viral E proteins were introduced in to the channel where specific aptamers toward the proteins bind to the targets (2). AuNPs conjugated with protein-specific aptamers were subsequently added and to free epitopes on the previously captured E proteins for the formation of the sandwich morphology (3); if no arboviral E proteins are present, AuNPs are washed away. Finally, Ag reagents are introduced and deposited onto the bound AuNP surface, thus producing a colorimetric signal indicating existence of arboviral E proteins (4). Reprinted with permission from Ref.309 NP, nanoparticle; ZIKV, Zika virus
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