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Advances in Diagnostic Methods for Zika Virus Infection

The Zika virus (ZIKV) is one of the most infamous mosquito-borne flavivirus on recent memory due to its potential association with high mortality rates in fetuses, microcephaly and neurological impairments in neonates, and autoimmune disorders. The severity of the disease, as well as its fast spread over several continents, has urged the World Health Organization (WHO) to declare ZIKV a global health concern. In consequence, over the past couple of years, there has been a significant effort for the development of ZIKV diagnostic methods, vaccine development, and prevention strategies. This review focuses on the most recent aspects of ZIKV research which includes the outbreaks, genome structure, multiplication and propagation of the virus, and more importantly, the development of serological and molecular detection tools such as Zika IgM antibody capture enzyme-linked immunosorbent assay (Zika MAC-ELISA), plaque reduction neutralization test (PRNT), reverse transcription quantitative real-time polymerase chain reaction (qRT-PCR), reverse transcription-loop mediated isothermal amplification (RT-LAMP), localized surface plasmon resonance (LSPR) biosensors, nucleic acid sequence-based amplification (NASBA), and recombinase polymerase amplification (RPA). Additionally, we discuss the limitations of currently available diagnostic methods, the potential of newly developed sensing technologies, and also provide insight into future areas of research. [DOI: 10.1115/1.4041086]

Introduction

Zika virus (ZIKV) is a mosquito borne flavivirus that has been linked to a series of neurological malformations in recently born children, e.g., microcephaly [1]. Microcephaly is a birth defect in which the brains of some babies have not developed properly when compared to other babies of the same age and sex [2]. More specifically, the American Academy of Neurology (AAN) defines microcephaly on neonates as having an occipitofrontal circumference greater than 2 standard deviations below the mean, when comparing neonates of the same age and gender [3]. In experimental studies, ZIKV has shown abrogate neurogenesis by down-regulating genes involved in cell proliferation, while upregulating genes involved in apoptosis. This in turn reduces cell viability and growth [4]. In the cases of ZIKV infection during fetal development, preliminary studies seem to indicate that the risk of fetal

microcephaly is high during the first trimester of gestation. However, as the pregnancy process through the third trimester, the risk of microcephaly decreases, but there is an increase in other types of neurological impairments [5–7]. In adults, it appears that there is a correlation between ZIKV and the re-emergence of Guillain–Barre syndrome [8]. Guillain–Barre syndrome (GBS) is an autoimmune disorder characterized by having either a T cell mediated or a humoral autoimmune response against the peripheral nervous system causing the demyelination of nerve cells which leads to progressive muscle weakness, paralysis, and it can even lead to respiratory failure and autonomic dysfunction if not treated [9,10]. A significant number of GBS cases have been seen in recent ZIKV outbreaks in both the Americas, and in French Polynesia, which would indicate a possible association [11]. Fearing a pandemic, The World Health Organization (WHO) declared that ZIKV was a serious threat to wellbeing of the world's population and issued some restrictions to limit spread of the disease [12]. Although, it was previously known that ZIKV could infect humans, it was rarely investigated, or otherwise misdiagnosed as other closely related virus, e.g., dengue, which shows similar

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clinical presentations and serological cross-reactivity [1]. This could be one of the main reasons as to why there was so little information available on ZIKV (269 articles in PubMed) in the years that preceded the first outbreak, when compared to other viruses from the same family such as Dengue (9187), West Nile (5949), and Chikungunya (2183) [13]. ZIKV has been around for over 71 years, as it was first discovered in 1947, albeit it was first considered to be a zoonoses [14]. The first documented case of human transmission occurred in Uganda in 1962 [15], and from there, ZIKV has been slowly spreading throughout the African and Asian continents, although the infected were not even aware of it, as most did not show any severe symptoms from infection [8]. In 2007, ZIKV became severely pathogenic and began to hinder the quality of life for those who were infected. Figure 1 depicts the spread of ZIKV from 2007 to 2016. The outbreak started in 2007 on a small island in the western pacific known as Yap [16]. Six years later, in the south pacific, a larger outbreak occurred in French Polynesia which was then followed by smaller outbreaks on other pacific islands [17]. The virus was then introduced into Brazil between 2013 and 2015, and caused a large epidemic that reached its peak at the end of 2015, subsequently spreading rapidly throughout the rest of the Americas, while continuing to circulate in the Pacific islands in 2016–17 [18,19]. Current diagnostic methods of ZIKV infection include viral nucleotide detection in blood, urine, amniotic fluid, and saliva samples, or the use of antibody-based bioassays that detect viral antigen-specific antibodies (IgG or IgM) in the patient's serum. The conventional assays are time-consuming and not portable, hence shows limited applicability to detect ZIKV infection rapidly at the point-of-care (POC) settings. Further, conventional serological assays show high cross-reactivity between ZIKV and other flaviviruses. Herein, we discuss the current and emergent diagnostic techniques available for ZIKV detection. We describe the limitations of conventional diagnostics assays and discuss the development of improved assays that can potentially be applied at POC settings.

Zika Virus Structure, Transmission, and Molecular Biology

Zika virus is a positive sense single-stranded RNA virus from the genus *Flavivirus* which also includes dengue, yellow fever, and chikungunya [20]. ZIKV has an icosahedral symmetry whose measurements range between 50 and 60 nm in size [21]. The length of its genome is about 10,794 kb, and it contains a single open reading frame (ORF) which codes for all of its structural and nonstructural components. As shown in Fig. 2, the encoded structural components contain the capsid and its associated membrane, proteins (C & M). The nonstructural portion of the ORF includes its envelope protein (E) and 7 other proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [20]. Phylogenetic analysis indicates that ZIKV has more than one lineage, in fact it has two, an African and an Asian lineage. The African lineage seems to be less invasive than its counterpart, as it has not disseminated outside of Africa, while the Asian lineage has made its way into previously unaffected regions of the world [22].

Mosquito bites from the *Aedes* spp. are the main routes of transmission. However, additional forms of transmission such as perinatal, sexual, as well as blood transfusions have also been identified [23–25]. Perinatal transmission is thought to occur as a result of transplacental transmission or during the process of childbirth [26]. ZIKV likely enters the host cell through receptor-mediated endocytosis; this process is initiated by the interaction of the virus envelope glycoprotein with cell surface receptors [27]. The virus then fuses with the endosomal membrane and releases its genome into the cytoplasm. This process is mediated by the low pH inside the endosome. Once in the cytoplasm, the viral RNA is translated into a polyprotein which is then cleaved into both structural and nonstructural ZIKV proteins [28]. The surface of the endoplasmic reticulum is the primary site for ZIKV replication where the genomic ssRNA (+) is made double stranded. The dsRNA genome is then transcribed and replicated thus increasing the amount of available viral mRNAs and new ssRNA (+). Viral assembly is finalized in the endoplasmic

Fig. 1 Geographical distribution of ZIKV outbreak from 2007 to 2017. This image illustrates the spread of ZIKV from its inception in the small island of Yap to its conclusion in the Americas [18] (Reprinted from *The Lancet* with permission from Elsevier © 2017).

Fig. 2 Zika virus external morphology and viral proteins. This illustration depicts the encoded structural components contain the capsid and membrane proteins [29] (Reprinted with permission from The American Society for Microbiology © 2017). The nonstructural portion of the ORF includes an envelope protein and 7 other proteins; NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 [30] (Reprinted with permission of Creative Commons Attribution BY 4.0).

reticulum, where virion buds are eventually be transported to the Golgi apparatus and released through exocytosis [29].

Zika Virus Diagnostic Methods

Zika virus diagnosis is performed through the detection of its viral components (RNA or viral proteins), or through serological assays which measure antibody concentration against viral proteins (host immune response) [31]. The previous studies from the outbreaks on French Polynesia and Brazil seem to indicate that ZIKV concentrations vary between sample matrices whether it is blood, urine, or saliva. When it comes to serum samples, the RNA concentration can range between 5 and 3.7×10^6 copies/mL in patients who were suspected to have an acute phase of infection [32]. Interestingly enough, the concentration of ZIKV does not necessarily correlate with onset of symptoms, as asymptomatic people can present a higher concentration of ZIKV without showing any signs of the disease (range 2.5×10^3 to 8.1×10^6 copies/mL) [32]. In the case of urine and saliva, the lowest viral RNA detected has been 10^2 copies/mL in urine and 40 copies/mL in saliva with their highest range being 2.68×10^3 copies/mL and 7.44×10^4 copies/mL, respectively [33]. Taking all of these factors into consideration, both urine and saliva would be the easiest way of collecting infected samples from the general population especially neonates and young children. However, it is important to note that the physiological pH values in both urine and saliva can be less than 7 (pH of Urine: 4.5–8/pH of Saliva: 6.2–7.6) [34,35]. A low pH value of less than 6.5 can cause an irreversible conformational change on the envelop protein of ZIKV leading to a decrease in the amount of ZIKV present on the recovered sample [36]. The best way to accurately diagnose a ZIKV infection would be to collect more than one fluid sample if possible and test them together. This approach would significantly reduce the number of false positives, and if identified early enough prevents the spread of the disease. Among all other diagnostic methods, RT-qPCR is most popular due to its sensitivity and specificity for ZIKV. However, this method is only useful at the beginning stages of infection, as the viremic phase of ZIKV infection usually lasts for about one week, making the molecular diagnostic window of ZIKV detection very narrow [37]. On the other hand, serological detection are better for long-term studies, as antibodies against ZIKV will remain in the body for a longer period of time [38]. In this paper, we discuss the most traditionally used diagnostic methods in ZIKV detection, as well some new potential candidates for diagnosis. Refer to Table 1 for currently FDA approved ZIKV diagnostic methods.

Conventional Molecular Diagnostics—Reverse Transcription Quantitative Polymerase Chain Reaction. At the early onset of infection, the Centers for Disease Control and Prevention (CDC)

recommends the use of reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) to detect and quantify ZIKV viral RNA [57]. In RT-qPCR, viral RNA extracted from an infected sample is placed into a reaction mix containing all of the necessary factors for amplification such as ZIKV specific primers (to bind to the RNA template), reverse transcriptase (to make cDNA from RNA), DNA polymerase and dNTPs (to amplify the DNA), a buffer solution (to maintain an optimal environment for the polymerase), and an intercalating fluorescent dye for quantification. As the target undergoes amplification, the fluorescence present in the sample increases as more product is being made. This fluorescence can then be quantified to determine the concentration of ZIKV present in the sample. This process typically last about two hours (Fig. 3(a)). The major benefit that this assay provides is high specificity and sensitivity. ZIKV can be detected from a multiple variety of sources such as whole blood, serum, saliva, urine, cerebrospinal fluid, and amniotic fluid [58]. The major limitation of PCR-based assays is that they rely on a well-developed laboratory infrastructure filled with people with a significant degree of expertise. Additionally, PCR assay is time-consuming and take several hours to get the results. The PCR equipment is costly and requires thermocycling [59,60]. In the real world scenario, e.g., during an outbreak, sourcing all of the necessary samples and delivering those samples to where they need to go (logistics) severely hinders the efficacy of this test, as contamination due to improper handling is always present [61].

Conventional Serological Testing

Zika MAC ELISA. In the later stages of infection, typically during the first week of symptoms, the body of an infected person starts making neutralizing antibodies (IgM) against ZIKV, which then can be used to detect the presence of the disease. In this instance, the CDC recommends targeting and quantifying the available IgM present in the infected blood samples. This can be done by using the Zika IgM antibody capture enzyme-linked immunosorbent assay (Zika MAC-ELISA) [57]. In this procedure, a patient's blood sample is incubated in a well plate containing antibodies against IgM. If IgM is present, then it will strongly bind to the antibodies in the plate. If not, then the patient's sample will be washed away and there would be no reaction when the secondary antibody is added in the final step. The final step involves the use of an antibody conjugated with horseradish peroxidase enzyme (HRP, chromogenic substrate) to specifically bind to the original antigen (Fig. 3(b)). The presence of ZIKV IgM is indicated by a detectable signal that can be read and measured using spectrophotometer to determine the concentration of IgM in the sample. When using this assay, one should take into account that there is always a possibility for false negative and false positive results. False negatives could arise from simply failing to follow assay procedures, or by collecting blood samples before IgM has

Table 1 Currently authorized ZIKV diagnostic tests under emergency use authorization

Name of the Kit	Organization	Sample Type	Volume	Sensitivity	Specificity	Process Time	Point of Care
Nucleic assay based assays:							
Aptima Zika Virus Assay [39]	Hologic, Inc.	Serum, Plasma and Urine	Serum and Plasma: 1200 μ L Urine: 2000 μ L	Serum and Plasma: 100% Urine: 100%	Serum and Plasma: 97.2% Urine: 100%	3.5 h	No
Gene-RADAR Zika virus test [40]	Nanobiosym Diagnostics, Inc.	Serum	Serum: 50 μ L	100%	100%	1 h	No
RealStar® Zika virus RT-PCR Kit 1.1 [41]	Altona Diagnostics	Serum and Urine	Serum and Urine: 140 μ L	100%	96.6%	2 h	No
Realtime Zika [42]	Abbott Molecular, Inc.	Whole Blood, Serum, EDTA Plasma and Urine	All Samples: 500 μ L	Whole Blood: 96.6% Serum: 92.6% Plasma: 93.4% Urine: 87.3%	Whole Blood: 100% Serum: 97.0% Plasma: 100% Urine: 95.5%	6 h	No
Sentosa SA ZIKV RT-PCR test [43]	Vela Diagnostics, Inc.	Serum, EDTA Plasma and Urine	All Samples: 250 μ L	Serum: 94.2% Plasma: 94.9% Urine: 100%	Serum: 100% Plasma: 100% Urine: 100%	3 h	No
TaqPath Zika virus kit [44]	Thermo Fisher Scientific	Serum and Urine	All samples: 25 μ L	Serum: 94.9% Urine: 88.6%	Serum: 100% Urine: 100%	12 h	No
Triplex rRT-PCR [45]	CDC	Whole Blood, Serum, Urine, Cerebrospinal and Amniotic Fluid	All samples: 200 μ L	Whole Blood: 96.1% Serum: 100% Cerebrospinal and Amniotic Fluid: 100%	Whole Blood: 100% Serum: 98.2% Cerebrospinal and Amniotic Fluid: 98.2%	Auto	No
VERSANT Zika RNA 1.0 assay kit [46]	Siemens Diagnostics, Inc.	Serum, EDTA Plasma, and Urine	All samples: 140 μ L	Serum: 90.6% Plasma: 100% Urine: 86.7%	Serum: 85.2% Plasma: 88.5% Urine: 84.5%	N/A	No
xMAP Multi-FLEX Zika RNA assay [47]	Luminex Corporation	Serum, Plasma, and Urine	All samples: 200 μ L	Serum: 95.8% Plasma and Urine: 97.1%	Serum: 98.1% Plasma and Urine: 100%	3 h	No
Zika virus detection by RT-PCR [48]	ARUP Laboratories	Serum, EDTA Plasma and Urine	All samples: 200 μ L	Serum: 98.0% Plasma: 90.67% Urine: 91.1%	Serum: 100% Plasma: 93.3% Urine: 95.7%	2 h	No
Zika virus real-time RT-PCR test [49]	Viracor Eurofins	Serum, Plasma and Urine	All samples: 500 μ L	Serum: 100% Plasma: 92% Urine: 100%	Serum: 96.4% Plasma: 87.5% Urine: 93.9%	8–12 h	No
Zika Virus RNA Q real-time RT-PCR [50]	Quest Diagnostics, Inc.	Serum and Urine	All samples: 500 μ L	Serum: 94.6% Urine: 100%	Serum: 100% Urine: 100%	N/A	No
Zika ELITe MGB Kit [51]	ELITechGroup, Inc.	Serum and EDTA Plasma	All samples: 200 μ L	Serum: N/A Plasma: 94.7%	Serum: N/A Plasma: 90.9%	3 h	Yes
Serological assays:							
ADVIA Centaur Zika Test [52]	Siemens Diagnostics	Serum and Plasma	All samples 15 μ L	Serum and Plasma: 91.23%	Serum and Plasma: 95.91%	1.5 h	Yes
DPP Zika IgM assay system [53]	ChemBio Diagnostic, Inc.	Whole Blood, Serum, EDTA Serum and Plasma	All samples: 10 μ L	Whole Blood: 96.1% Serum: 95.1% Plasma: 98.1%	All samples: 100%	1 h	Yes
LIAISON XL Zika capture IgM assay [54]	DiaSorin, Inc.	Serum	25 μ L	100%	99.6%	N/A	Yes
Zika MAC-ELISA [55]	CDC	Serum and Cerebrospinal Fluid	50 μ L	93.98%	100%	3 Days	No
ZIKV detect IgM capture ELISA [56]	InBios International	Serum	4 μ L	100%	92.5%	2 h	Yes

Fig. 3 CDC recommended tests for ZIKV detection. (a) In RT-qPCR, viral RNA extracted from an infected patient's sample is placed into a reaction mix containing all of the necessary factors for amplification such as ZIKV specific primers (to bind to the RNA template), reverse transcriptase (to make cDNA from RNA), DNA polymerase and dNTPs (to amplify the DNA), a buffer solution (to maintain an optimal environment for the polymerase), and an intercalating fluorescent dye for quantification. Amplification takes place through thermal cycling, and its product is later identified and quantified based on fluorescence. (b) In MAC ELISA, the activity of IgM antibodies is measured in response to ZIKV infection. The process starts when a patient's blood sample is incubated in a well plate containing antibodies against IgM. If IgM is present, then it will strongly bind to the antibodies in the plate. If not, then the patient's sample will be washed away and there would be no reaction when the secondary HRP antibody is added to the plate. (c) PRNT tests are carried out to confirm serological results. In PRNT, a patient's serum undergoes a series of dilutions that are then added to a ZIKV viral suspension, mixed, and incubated alongside cell cultures. If antibodies against ZIKV are present, then there will be a reduction in the number of observable ZIKV plaques [64] (Reprinted with permission of Creative Commons Attribution BY 4.0).

reached detectable levels, typically 4 days after onset of symptoms [62]. On the other hand, false positives could arise due to cross-reactivity with closely related flaviviruses [63]. If the obtained results are inconclusive, the CDC recommends using the plaque reduction neutralization test (PRNT) to validate or discard previously made assumptions that arose as a result of an inconclusive MAC-ELISA [8,62].

Plaque Reduction Neutralization Test. The first step in plaque reduction neutralization tests involves a series of dilutions from serum samples collected from the infected patients. These dilutions are then added to a ZIKV viral suspensions, mixed, and incubated alongside cell cultures. If antibodies against ZIKV are present in serum, there will be a decrease in the amount of plaque forming units present in those cell cultures (Fig. 3(c)). Unlike MAC-ELISA, this assay provides a greater sensitivity and specificity for ZIKV detection [64]. PRNT assay is more labor intensive than that of ZIKV

MAC-ELISA, and it has higher throughput as you can analyze 12 patient's specimens in 2 six well plates, while a MAC ELISA only has the capacity to analyze eight specimens per 96 well microtiter plates. PRNT has the added advantage of detecting Zika antibodies beyond the normal IgM window, which is 12 weeks post onset of symptoms, when IgM concentration is at its lowest [65].

Recent Development in Zika Virus Testing and Diagnostics

Reporter Virus Neutralization Test. Recently, a new serological ZIKV-based assay similar to MAC ELISA and less labor intensive than PRNT has shown significant improvements in turnaround times. The reporter virus neutralization test (RVNT) uses a luciferase-tag to directly mark the neutralizing antibodies found in the blood of an infected patient. This in turn reduces the processing time of this assay to around 24h, which is significantly better than

Fig. 4 Localized surface plasmon resonance biosensor schematics and ZIKV sensitivity. (a) Qdot bimetallic nanohybrids are made by coupling plasmonic nanoparticles such as gold to quantum dots. These hybrids are then infused alongside ZIKV specific DNA loops to the molecular beacon of the biosensor. (b) LSPR relies on the resonance that occurs through the hybridization of viral ZIKV RNA with the complementary DNA loop sequence found in the molecular beacon. This in turn induces a signal that can be picked up, amplified and quantified by nearby quantum dot nanocrystals [77]. (Reprinted with permission of Elsevier © 2017).

the typical processing times of conventional PRNT assay which can range from 2 to 7 days. RVNT assay uses a 96 well plate format that facilitates the testing of multiple samples in a single run, without sacrificing specificity, accuracy, or sensitivity [66]. RVNT fixed plates can also be stored for up to six months, if later quantification is needed without any loss in the GFP signal [67].

Multiplex Microsphere Immunoassays. Current serological diagnostics of ZIKV rely on IgM capture, which can encounter problems with cross-reactivity among different flaviviruses. Multiplex microsphere immunoassay (MIA) on the other hand allows the addition of one or more antigens to augment the sensitivity and specificity of ZIKV detection. The advantage of using MIA over other diagnostics tests is that it allows combining the diagnostic power of viral envelope proteins (which elicit the higher sensitivity response, but shows cross-reactivity) with the contrastive differential power of viral nonstructural proteins such as NS1 and NS5 (which increases the specificity (78%–100%) of the assay, when compared to IgM ELISA (67% specificity)). Compared with IgM ELISA, MIA also provides a rapid turn-around time (less than 4 h) and only requires a small specimen volume (10 μ L) for a single reaction [68].

Microfluidic Paper-Based Analytical Devices for Zika Detection. Microfluidic Paper-based Analytical Devices (μ PAD) are being developed to detect various infectious diseases at the POC settings [69]. These μ PADs provide several advantages including low-cost, flexible, light-weight, and availability around the world [70–72]. These μ PAD platforms are easy to use and disposable, hence can be discarded after single use. Papers used in these devices have microporous structures that allow efficient absorption and sample flow without any peripheral pumps. Therefore, paper-based devices provide attractive alternative to traditional lab-based assays and allows affordable and rapid disease testing [70]. Recently, paper-based microfluidic devices are being investigated for ZIKV detection [73,74]. At one instance, paper-based device consists of a large area at the top for sample loading (blood or plasma) followed by several areas coated with specific antibodies against ZIKV's NS1 protein [73]. All of the areas are separated lines of yellow wax that facilitates the antibody-antigen reactions by reducing the fluid flow rate. Using the device, up to 10 ng/mL of NS1 protein can be detected rapidly. The major advantages of paper-based device over others are quick processing time (less than 8 min), and cost [75]. However, it should be noted that the developed paper-based device is only a proof of concept, and it requires thorough testing with clinical samples.

Localized Surface Plasmon Resonance for Zika Virus Detection. The surface plasmon resonance based biosensor is a label-free technology that is being applied to both analytical chemistry

Fig. 5 CRISPR/CAS9 biomolecular sensors. (a) Primers and toehold sensors are selectively designed to match specific ZIKV strains. These sensors, alongside their primers are then infused and freeze-dried into paper effectively completing the biosensor. (b) Adding a patient's sample (fluid) to the paper will cause it to become moisturized and active. If a specific ZIKV strain is present, then a color change from yellow to purple will take place on the surface of the paper, which would be indicative of a positive reaction. This sensor is specific enough to differentiate between closely related flaviviruses and even different ZIKV lineages within a single base resolution, when coupled to a CRISPR/Cas9-based module [78] (Reprinted with permission of Elsevier © 2016).

Fig. 6 Isothermal amplification technologies; RT-LAMP and RPA amplification. (a) Patients' samples are collected and lysed to extract viral nucleotides. (b) The lysed samples are then filtered through a porous silica membrane inside the microfluidic cassette to isolate ZIKV nucleic acids. Once the nucleotides have been isolated, a colorless leuco crystal violet indicator is pumped into the reaction chamber (where the filter membrane is located) to serve as a marker for amplification. (c) The inside of the device consists of a thermos that has been modified to hold a microfluidic cassette, a heat sink, an isothermal heat source (Mg-Fe alloy), and a heat sink to dissipate heat, while the outside (d) consists of a 3D printed lid for sample collection, and a water port to trigger the isothermal reaction. (e) Photographs depicting the isothermal amplification reactor prior and after use. If ZIKV is found, then the colorless crystal violet indicator reacts and becomes violet in color [81] (Reprinted with permission of ACS Publications: <https://pubs.acs.org/doi/10.1021/acs.analchem.6b01632>). (f) This modified 3D printer is able to perform high throughput nucleotide extraction, and isothermal amplification all within the same enclosure. Due to its configuration, you can run up to twelve patient samples per run, as this printer holds 96 well plates. The heated bed underneath the plate maintains the temperature at a constant rate, as to not affect the amplification process. (g) RPA amplification can also be performed within a thermos like structure as seen in RT-LAMP. However, this process is not as efficient, as you cannot run as many samples in it [85] (Reprinted with permission of Elsevier © 2018).

and bioassay development [76]. The use of localized surface plasmon resonance (LSPR) biosensors has shown to be an effective method for detecting low concentration of ZIKV nucleic acids. NP-Qdot bimetallic hybrids such as AuAgNP-Qdot646-MB are able to detect trace amounts of ZIKV in a sample ranging from 6 to 673 copies/mL [77]. LSPR relies on the resonance that occurs through the hybridization of viral ZIKV RNA with the complementary DNA loop sequence found in the molecular beacon (MB). This in turn induces a plasmon signal that can be picked up nearby by quantum dot nanocrystals which are also infused within the MB of the biosensor probe (Figs. 4(a) and 4(b)). This test relies on successful RNA extraction from patients' serum samples, although in this case, the samples were obtained from cultured supernatant derived from ZIKV infected Vero cells. As per limitations, there are differences between NP-Qdots, so selecting the most appropriate one is important, as bimetallic NPs produce a stronger LSPR-mediated signal than its single metallic counterpart. Its production cost also needs to be brought down to be an accessible POC device in remote areas of the world [77].

Nucleic Acid Sequence-Based Amplification RNA Amplification Followed by CRISPR/CAS9 Based Detection. A newly developed cell-free based biosensor has been proven to be effective at detecting the presence of ZIKV RNA, and it is specific enough to differentiate between closely related flaviviruses and even different ZIKV lineages within a single base resolution, when coupled to a CRISPR/Cas9-based module [78]. This diagnostic test relies on the isothermal amplification (does not require thermo cycling as in the case of PCR) of viral RNA by nucleic acid sequence-based amplification (NASBA). The mixture produced as a result of amplification is then used to moisturize and activate the previously freeze-dried paper sensors. If ZIKV is present, then a color change from yellow to purple will take place on the surface of the paper, which would be indicative of a positive reaction (Figs. 5(a) and 5(b)). This test used RNA extracted from cultured Vero cells infected with ZIKV, which was then spiked into serum and water to mimic clinical samples. The RNA samples were easily extracted by simply boiling viral capsid for one minute and amplified by NASBA. The benefits of using this test include specificity for ZIKV over other flaviviruses, sterility and stability of biomolecular components, low-cost, easy storage and distribution, and field readiness, as it does not need a stable power supply for use [78]. However, the developed assay takes at least 3 h for ZIKV detection, hence not suitable for rapid and POC settings [78].

Reverse Transcription Loop Mediated Isothermal Amplification for Zika Virus Detection. Reverse transcription-loop mediated isothermal amplification (RT-LAMP) is an isothermal amplification technology that does not require thermocycling for nucleic acid amplification [79]. LAMP-based assays have been developed for ZIKV detection [80,81]. The advantage of using the developed LAMP-based devices such as microfluidic cassettes is that they can carry out a series of processes ranging from nucleic acid isolation and purification to amplification and detection of viral RNA, all within an enclosed chamber. As amplification takes place, one of the reagents inside the cassette (colorless leuco crystal violet) undergoes a redox reaction that causes it to turn violet in the presence of the amplified product (Figs. 6(a)–6(e)). This LAMP assay can be carried out with a variety of samples, e.g., saliva, blood, urine, and semen. It can also be used in resource-poor settings, as there is no need for an expensive thermocycler or even electricity [80,81]. Another advantage of using RT-LAMP assay is its ability to differentiate between both African and Asian ZIKV strains [82], showing similar specificity and sensitivity as in the case of RT-qPCR [83]. However, the RT-LAMP based assay is prone to contamination, and a closed tube setup is recommended [84].

Recombinase Polymerase Amplification for Zika Virus Detection. Alternatively, recombinase polymerase amplification (RPA), another isothermal amplification technology, can also be used for nucleic acid amplification, without the need for active temperature

control in between runs. A simple 3D printer can be repurposed to perform high throughput nucleotide extraction and isothermal amplification all within the same enclosure. This assay produces a fluorescent signal as ZIKV RNA is being amplified; the amplification can then be detected by any run-of-the-mill smartphone camera, as long as it is able to capture images (some modifications to the camera software are required). The resulting images can then be later quantified with the help of its associated software (Figs. 6(f) and 6(g)). The main advantage of using this device is that it can extract and purify up to 12 patient samples in less than 15 min per run. Further, it does not require expensive components that would make it unsuitable for resource poor settings/real world conditions [85]. However, this device has a slightly lower RNA extraction performance than the conventional spin column, which may affect the assay sensitivity when detecting samples with lower viral load [85,86].

Microfluidic Devices Integrated With Impedance Sensing. Microfluidic devices provide a precise control on fluid flow and are widely investigated for various applications including disease detection [87–91], sample analysis [88], and biosensing [92–95]. The previous studies have shown that microfluidic devices can be used for the quantification of various viruses [69,74] and proteins through impedance sensing [96,97]. Recently, newly designed biochips integrating with electrical detection electrodes have been optimized by the addition of pillar structures, which serve to facilitate the functionalization of probes inside the chip, thus increasing the hit rate of target proteins [98]. For this device to work, plasma must be isolated from blood through centrifugation. The plasma is then incubated alongside beads that have been conjugated with antibodies specific for the target protein. After washing, a secondary antibody specific for the first is then introduced to bind to it and forms an immunoassay complex. The solution containing the complex is then introduced into the microfluidic device, and as it passes through the entrance counter, a change in impedance occurs which can be measured and recorded (first counting). The complex then makes its way into the capture chamber, where it is then captured by streptavidin if the target protein is present, if not, the unbound beads get washed away, and enter the exit counter where they get counted (second counting). A final analysis is then made by comparing the first counting and second counting results to elucidate and quantify the data. The advantage of using such a device over a conventional ELISA is that it requires very little sample (10 μ L of plasma), however, the entire detection process still requires about 4 h and involves multiple processing steps [98].

Recently, impedance sensing has also been utilized to detect various viruses including HIV and ZIKV viruses [69,74]. In this method, whole viruses are captured using magnetic beads functionalized with specific antibodies. Once the viruses of interest are isolated, they are lysed using detergent. The virus lysis step releases ions and biomolecules, which change the impedance of the sample. This impedance change is rapidly detected using paper-based flexible microfluidic devices viruses [69,74]. These impedance-based sensing methods provide rapid detection of disease (<30 min); however, quantification of viruses especially at low concentration is challenging and requires further investigation.

Conclusions and Future Perspectives

Even though the existence of ZIKV has been known for over 70 years, it is only until recently that it has made such a worldwide impact on our population's health. ZIKV has gone from being a noninnocuous pathogen capable of only causing mild cases malaise to one that can cause severe side effects such as microcephaly in neonates and Guillain–Barre in adults. The spread of the virus has also added more cause of concern, as it can cross countries and continents with the greatest of ease as seen over the last decade. This fast spread may be attributed to the “human factor” as

ZIKV is no longer spread by mosquito bites alone, it can also be spread through sexual contact with infected individuals or by blood transfusions. Due to these reasons, researchers across the world are concentrating their efforts in developing rapid confirmatory diagnostics and efficient drugs to prevent and/or control the spread of the disease. The gold standard for disease diagnosis, as the CDC puts it, involves the use of molecular and serological assays such as RT-qPCR and Zika MAC-ELISA to detect the presence of ZIKV at the early and late stages of the infection. However as previously mentioned, there needs to be a proper laboratory infrastructure set in place to run all of these tests, and that is a commodity that may not be available in all regions of the world. However, nowadays, we are moving in a new direction that involves the use of microfluidic-based devices that can run the multiple tests with high specificity and sensitivity, without the need of expensive equipment or human expertise. The cost associated with these devices is sure to be brought down through the use of alternative technologies such as paper-based devices and amplification techniques such as isothermal amplification, and through their mass production. The next step for all of these diagnostics tests/devices is to test them in realistic field conditions in ZIKV endemic countries. Understanding the molecular mechanism of the virus and its interactions with its host could help in the development of new and novel strategies to detect and counter future ZIKV infections.

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