Analytical methods for detection of Zika virus

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Abstract

Due to the recent outbreak of the Zika virus in several regions, rapid and accurate methods to diagnose Zika infection are in demand, particularly in regions that are on the frontline of a Zika virus outbreak. In this paper, three diagnostic methods for Zika virus are considered. Viral isolation is the gold standard for detection; this approach can involve incubation of cell cultures. Serological identification is based on the interactions between viral antigens and immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies; cross reactivity with other types of flaviviruses can cause reduced specificity with this approach. Molecular confirmation, such as reverse transcription polymerase chain reaction (RTPCR), involves reverse transcription of RNA and amplification of DNA. Quantitative analysis based on real time RTPCR can be undertaken by comparing fluorescence measurements against previously developed standards. A recently developed programmable paper-based detection approach can provide low-cost and rapid analysis. These viral identification and viral genetic analysis approaches play crucial roles in understanding the transmission of Zika virus.

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## Introduction

Zika virus (ZIKV) is an arthropod-borne virus in the genus Flavivirus and the family Flaviviridae. ZIKV is most closely related to the Spondweni virus and other mosquito-borne viruses, such as dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis, and chikungunya virus (CHIKV).[1] ZIKV was first isolated in a Rhesus monkey that was used as a sentinel animal for yellow fever research in the Zika forest of Uganda. The second time the virus was isolated took place in early 1948 and involved an Aedes africanus mosquito in the same forest[2]. The first case of ZIKV isolation in humans occurred in 1954 during an epidemic of jaundice in Nigeria.[3] According to the phylogenetic and geographical analysis, ZIKV can be categorized into three lineages, the East Africa lineage, the West Africa lineage, and the Asia lineage. [4] The introduction of ZIKV from Uganda to West Africa and Malaysia may have occurred in the 1940s.[5] Although Zika virus has been introduced across a wide geographical range, only sporadic cases of human infection have been reported in Africa (e.g., Senegal[6, 7], Sierra Leone[8], Gabon[9, 10], Central African Republic [11], Ivory coast[12] and Egypt[13] ) and Asia (e.g., Indonesia[14], Pakistan[15], Malaysia[16], India[17], Thailand, North Vietnam[18] and the Philippines[19]). For example, 6.1% of the residents in regions of Uganda were shown to possess specific anti-ZIKA antibodies.[20] 40 % of Nigerians exhibited the Zika virus neutralizing antibody; [21] in Senegal, 10.1% and 2.8% of humans had IgM antibodies against ZIKV in 1988 and 1990[22], respectively. Underreporting or asymptomatic infection may account for the large difference between the number of reported infections and the number of individuals with antibodies against ZIKV. In 2007, an outbreak was reported on Yap island in the Federated States of Micronesia.[23] Approximately 73% of the residents were infected; 18% of those infected exhibited clinical illness. This outbreak represents the first transmission of ZIKV outside Africa and Asia. In October 2013, a second outbreak of ZIKV was reported in French Polynesia[24], with 19,000 suspected cases. Large outbreaks in New Caledonia[25] (1,400 confirmed cases), the Cook Islands (over 900 cases) and Easter Island[26] have also been reported. In addition to autochthonous cases, cases associated with travel have been reported. Infections have been imported to Norway[27], Japan[28] and Italy[29] from viremic people that traveled from French Polynesia. Infections have been imported to Canada[30] and Germany[31] from viremic people who traveled from Thailand. ZIKV may have been imported to the Americas by recreational travelers[32] to Brazil[33, 34]; a plausible hypothesis for the arrival of ZIKV in Brazil involves travel associated with the 2014

World Cup. The lineage that emerged in the Pacific region and in Brazil were distinguished as the Asia lineage. The migration of humans and airline travel appear to facilitate the spread of ZIKV outside its typical geographical range.

ZIKV is a single-stranded RNA virus that exhibits positive sense. The RNA genome consists of 10,794 nucleotides[35], with a long open reading frame that is flanked by 5' and 3' noncoding regions. The open reading frame encodes three structural proteins (capsid, premembrane or membrane, and envelope) and five nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).[36] Most of the phylogenetic analysis on ZIKV has involved analysis of the envelope protein[1, 25, 37, 38], which is the major virion surface protein, or NS5[5, 9, 39-41], which is the largest segment and is a well-conserved protein. NS5 contains an N-terminal component, which participates in synthesis of the 5' cap and a C-terminal RNA-dependent RNA polymerase (RdRP).[36]

Many types of sylvatic and peri-domestic mosquitos of the Aedes genus have been reported as major vectors for ZIKV, including Aedes africanus[2], Aedes aegypti[16], Aedes polynesiensis[24], Aedes luteocephalus, Aedes furcifer, Aedes taylori[22], Aedes apicoargenteus[7], Aedes albopictus[42] and Aedes hensilli.[43] The enzootic cycle of ZIKV and the broad range of hosts may facilitate recombination of ZIKV strains. Aedes africanus was the first species that was described as a vector for ZIKV; it has a wide dispersion among many animal species.[5] The main vectors in Asia were reported to be Aedes albopictus and Aedes aegypti; these species are widespread in tropical and subtropical regions. In addition, these species have a large presence in urban areas. In Europe, Aedes albopictus presents a major threat to public health.[44] It is considered to be one of the most invasive mosquito species in the world; it is widely distributed in 20 European countries, particularly in the Mediterranean region. In Brazil, an where outbreak began in May 2015, both Aedes albopictus and Aedes aegypti are widely distributed.[45] Although Aedes hensilli and Aedes polynesiensis are the predominant species in the Federated States of Micronesia and French Polynesia, respectively, no direct evidence has been presented that they were associated with outbreaks; however, it is plausible that these species are vectors for ZIKV transmission.

Once the mosquitos take a blood meal from an affected host, the virus is transmitted from the midgut to salivary gland. The infection rate, dissemination rate and transmission rate can be determined by the fraction of the mosquitos with infected midguts, by the number of infected salivary glands over the total number of mosquitos with infected midguts, and by the number of positive infected salivary glands over the number of infected salivary glands, respectively.[42] When mosquitos take a blood meal from a human, transmission through skin immune cells, including dermal fibroblasts, epidermal keratinocytes, and immature dendritic cells has been demonstrated.[46] Symptoms associated with Zika virus infection appear after an incubation period of a few days. The clinical symptoms associated with Zika virus infection include arthralgia, myalgia, headache, retro-orbital pain, conjunctivitis, cutaneous maculopapular rash, edema, and mild fever; severe fever has also been reported (>39° C) [23, 34, 47].[23, 34, 47] Some patients have also described symptoms such as malaise, anorexia, vomiting, diarrhea, and nausea.[48] Most patients recover after one or two weeks; however, arthralgia can last for one month.[47] Although all of the symptoms mentioned above have been reported, a great portion of cases of infection are asymptomatic, which can lead to underdiagnosis and underreporting. ZIKV infection can be complicated by a neurological disorder, Guillain-Barré syndrome (GBS); this phenomenon was reported in French Polynesia.[49] Also, leukopenia and thrombocytopenia may be noted in laboratory measurements.[50] In addition to the human infections, infections of non-human primates, water buffalo, elephants, goats, hippos, impala, kongoni, lions, sheep, wildebeest, zebras, and rodents have been reported[15, 51-53]. The symptoms associated with infection of various animals need further investigation.

Although the route of transmission is through mosquito bite, transmission of the Zika virus through perinatal transmission[54], sexual intercourse[47, 55] and blood transfusion[56] has been reported. The possible routes of perinatal transmission include transplacental, delivery, and breastfeeding routes. In the case of sexual intercourse, RRT-PCR results have indicated the presence of ZIKV in semen. Transfusion-transmitted infection is theoretically possible with WNV[57] and DENV[58] infections; the presence of CHIKV[59] in blood has also been suspected. Due to the concurrent circulation of ZIKV with other arbovirus in a given area, co-infection with ZIKV and other arboviruses is a possibility. For example, co-infection of DENV and ZIKV has been reported in New Caledonia[25]; however, the cross-reactivity and synergy

between viruses is unknown and requires additional study.

Due to the recent outbreak of the ZIKV in multiple areas and the absence of specific antiviral therapy, a rapid and accurate characterization method for ZIKV is needed, particularly for regions that are undergoing outbreaks; a map of affected regions is shown in Figure 1.[60] Novel materials may facilitate faster detection of ZIKV. For example, surface plasmon resonance and piezoelectric devices rely on advanced materials for sensitive detection of analytes. Novel materials for electrochemical sensing, lab on a chip-based sensing, lateral flow assay-based sensing, or optical sensing of Zika virus infection may facilitate more rapid detection and improved public health in affected regions. To date, detection of ZIKV has been based on viral isolation, serological identification, and molecular confirmation[61]. It is important to note that the similarity of ZIKV to other flaviviruses may hinder accurate virus identification (e.g., ZIKV may sometimes be misdiagnosed as DENV). In addition, the relationship between clinical symptoms and the presence of ZIKV can be more completely understood with the aid of more accurate detection methods.

## Viral isolation

Viral isolation in cell cultures has been recognized as the “gold standard” for viral detection and has been commonly performed since the 1960s.[62] Viral isolation can be undertaken via intracerebral inoculation of mice, C6/36 cells (derived from Aedes albopictus), Vero cells (derived from the kidney epithelial cells of the African green monkey), or the AP 61 cell line (derived from Aedes pseudoscutellaris) with\ appropriate growth media and supplements. At the current time, researchers use Vero cell, C6/36 cell and AP 61 cell lines more frequently than intracerebral inoculation. Briefly, the samples are added to cell culture for incubation under controlled temperature and for an appropriate duration. Series passages can be performed to study the infectious capability of the virus. Microscopic identification of the unstained cell culture is the standard approach for examining viral proliferation. The presence of the virus causes degeneration in the cells; these changes are collectively called the cytopathic effect (CPE). However, there are some disadvantages to the viral isolation approach. Although viral isolation is not costly, it requires a long incubation period[40, 62], which can be a few days; in addition, expertise may be needed to

evaluate the CPE. It should be noted that the viremic stage is fleeting; it can cease at the time of or soon after the onset of symptoms; the viral load in the serum is often at a low level. Viral isolation with cell culture can be unsuccessful and is not practical for most clinical situations. As such, viral isolation is mainly utilized for research purposes.

## Serological identification

Serological identification is based on the interaction between antibodies and antigens. Enzyme-linked immunosorbent assay (ELISA) with Plaque reduction neutralization test (PRNT) and immunofluorescence assay (IFA) are the most commonly used tests for ZIKV detection. An immunofluorescence assay for ZIKV infected fibroblasts is shown as in Fig. 2.[46] This type of assay was performed during the first outbreak on Yap Island. It should be noted that cross reactivity of antibodies between flaviviruses makes diagnosis using this approach challenging[1]. Data from patients at both the acute phase and the convalescent phase is shown in Table 1. Patients with antibodies to more than one flavivirus show higher serological reactivity; this phenomenon is referred to as the "original antigenic sin."[1] A complementary serological assay, PRNT, may needed for further confirmation of the ZIKV infection[30, 39, 49]. Plaques can be counted and a plaque forming unit (PFU) per unit volume can be calculated. Titers of neutralizing antibody to ZIKV and other flaviviruses with a cutoff value of 90% ($PRNT_{90}$) can be determined. For confirmation of ZIKV infection, a titer of ZIKV greater than four times of the titer of other flaviviruses is recommended.[23] Alternatively, ZIKV can be detected by indirect IFA using specific hyperimmune mouse ascitic fluid. A secondary antibody that binds to the primary antibody and causes an amplification of the signal can be used to provide enhanced sensitivity. It should be noted that low titers of IgG or IgM antibodies may be present during the early phase of infection.[63] IgM antibodies may be found three days after the disease onset; IgG antibodies may be present in serum during the convalescent phase[48]. In addition, PRNT and indirect IFA may also show cross reactivity associated with various flaviviruses[1].

## Molecular confirmation

Since its development, polymerase chain reaction (PCR) became a major tool for viral characterization. For ZIKV, reverse transcription PCR (RTPCR) can be performed to generate cDNA from RNA. RTPCR

can be categorized into traditional RTPCR, real time RTPCR (quantitative), pan flavivirus RTPCR, and nested RTPCR. Full length sequences of ZIKV MR766[35] and ZIKA EC 2007 were published in GenBank; these sequences were isolated from rhesus monkey in the Zika forest in 1947 and from an outbreak on Yap Island, respectively. The MR766 strain was most commonly used for RTPCR; it is commonly compared with other strains since it was first isolated strain of ZIKV. For reverse transcription, the envelope or NS5 region are used as the target for primers. The primer is designed to be the counterpart to the nucleotides of the target segment. RNA is extracted from ZIKV, most commonly via the QIAamp viral RNA kit by means of centrifugation or a vacuum procedure. Viral RNA is then separated out using a QIAamp silica membrane. The mixture of ZIKV, primers, reverse transcriptase, DNA polymerase and buffer solution is added to the test tube and subject to thermocycling. The reverse transcriptase and DNA polymerase provide reverse transcription and DNA amplification functionalities, respectively. The amplicon size is determined by the forward primer and reverse primer. Rapid amplification of cDNA ends (RACE) kits, with a reverse primer for 5'-RACE and forward primer for 3'-RACE, can be used for amplification. Deoxynucleotide and buffer can be added for tailing to form a poly-nucleotide primer.[35] The RTPCR products can be analyzed using agarose gel electrophoresis. The pore size and strength of agarose gel make it an appropriate medium for DNA analysis. After applying voltage to the agarose gel, the nucleic acid migrates, with the velocity of migration being inversely proportional to the number of base pairs. The result can be visualized using colored dyes. ZIKV identification with a 364bp amplicon is shown in Fig. 3[34]. Many groups utilize a one-step RTPCR approach, which is suitable for a rapid diagnosis and is associated with less contamination than the two-step RTPCR approach.

Real time RTPCR (or quantitative RTPCR) was considered to be the most powerful tool for molecular confirmation due to its high sensitivity and capability for quantitative analysis. During reverse transcription, a probe containing a reporter and a quencher is included with the primer. Emission of fluorescence from the probe occurs when the reporter is separated from the quencher by interaction with the primer; the optical density (OD) can subsequently be measured. In processing real time RTPCR, the product of each PCR cycle is be recorded. The threshold cycle (Ct) versus the fluorescence is obtained during the course of the process; quantitative analysis can be obtained from a comparison of the

threshold cycle and a previously established standard curve. It is important to note that the Ct value is inversely proportional to the amount of RNA in the sample. Real time RTPCR results of samples from ZIKV-positive patients, Yap State, Micronesia, 2007 are shown in Table 2.[1] In contrast, traditional RTPCR is an end-point study that does not provide information during the process.

A pan flavivirus RTPCR or nested RTPCR may sometimes be utilized. Pan flavivirus RTPCR is performed with a degenerate primer. This degenerate primer can detect a range of flaviviruses; the notable trade-off in this approach is the loss of sensitivity. Nested RTPCR requires an outer primer set, an inner primer set, and two successive PCRs. An outer primer or primary primer is targeted on a general area of the RNA, followed by the inner primer or nested primer that is targeted on a specific coding region. This approach can reduce the amplification of an unexpected binding site. Although RTPCR is widely utilized and has many advantages, the highly specific primers and probes used in RTPCR can pose a major drawback. It should be noted that there is a possibility of missing unanticipated sites, which may lead to failed amplification.

A molecular-based diagnostics method was recently proposed by Pardee et al., which offers a programmable sensor approach and paper-based detection (Figure 4).[64] The detection approach, which is anticipated to take three hours, includes sample collection, RNA extraction, RNA amplification, and toehold reaction steps prior to colorimetric detection. A PCR approach was used to ligate the sensor to a LacZ reporter element. The sensor was embedded in paper, which was subsequently freeze dried. A reaction would occur in the presence of ZIKV trigger RNA, activating the LacZ gene for a colorimetric result. The result was associated with a change in the color of the paper from yellow (from chlorophenol red-β-D-galactopyranoside) to purple (from chlorophenol red). An electronic reader, which contained a 570 nm light emitting diode source, was used to perform twenty-nine readings from each sample; it provided low noise measurements of the color change. Boiling of the virus sample allowed for destruction of the Zika virus capsid and enabled a sufficient amount of RNA to be generated; this approach can be used in low resource envrionments and provides a straightforward alternative to traditional virus extraction. Use of nucleic acid sequence-based amplification (NASBA) with the paper-based system

enabled Zika RNA to be distinguished from dengue RNA. NASBA-CRISPR cleavage (NASBACC), exploiting the Cas9 endonuclease enzyme, cleaves DNA if a specific protospacer adjacent motif (PAM) is present (Figure 5) [64]. The clade (i.e., the branch of the viral family tree) that the Zika virus belongs to can be readily distinguished from the colorimetric results that are provided by the NASBACC approach. More recently, Chan et al. described use of magnetic particle (MP) and a consumer-grade 3D printer for Zika virus RNA extraction and purification from Zika virus-spiked urine samples (Figure 6).[65] Their device was shown to perform reverse transcription recombinase polymerase amplification and post-amplification detection (e.g., illumination by a light emitting diode and analysis using a smartphone camera) of 8-12 urine samples in under 15 minutes. An absence of cross-reactivity with  cross-reactivity with chikungunya and dengue was demonstrated using reverse transcription recombinase polymerase amplification assay and RTPCR from the 3D printer-processed urine.

## Discussion and Conclusions

In comparing the three types of characterization methods, molecular confirmation is the most widely used method. Although viral isolation is the gold standard for detection, it is neither a time saving nor a labor saving approach. Serological identification can be hampered because of the cross reactivity of flaviviruses. RTPCR is a powerful tool for diagnosis of Zika virus infection and for genome sequencing analysis. The full sequence can be obtained using the genome walking approach. By Basic Local Alignment Search Tool (BLAST) analysis, a Zika virus strain can be compared with other flaviviruses or other ZIKV strains. A phylogenetic tree can be prepared, showing the clades of viruses; a potentially new strain of ZIKV can be identified as Asia lineage or Africa lineage. Real time RTPCR can provide quantitative analysis; it is less prone to contamination and error than agarose gel electrophoresis. However, the specificity of primer and probe should be taken into consideration. A finite mismatch between ZIKV MR766 strain and ZIKA EC 2007 strain led to a failed amplification of Yap island virus during the initial analysis.[1] In addition to patient blood samples, saliva[66] and urine[50] samples were subjected to RTPCR. ZIKV in both samples were detectable with higher loads; use of saliva samples did not increase the duration of detection window. ZIKV can be detected over twenty days after infection from

collected urine samples. These two body fluids are useful if blood samples are difficult to collect (e.g., for potentially infected infants around the time of birth). In areas that lack medical facilities, non-invasive detection methods can are beneficial since they allow for collection of samples by patients. However, the onset of infection is sometimes difficult to define. A such, diagnosis with three kinds of samples is recommended.

In regions with concurrent circulation of ZIKV and other flaviviruses, multiple RTPCR can be expensive and time consuming. Due to the low availability of RTPCR equipment and high cost of RTPCR, Pardee et al.[64] proposed a novel technology for molecular detection that enables rapid and low-cost diagnosis. This programmable and paper-based sensor does not require interpretation using laboratory equipment. With NASBA, a result with high sensitivity can be provided even in low resource regions. NASBACC can provide genetic information on the virus. As noted by Meagher et al., reverse transcription recombinase polymerase amplification and reverse transcription loop-mediated isothermal amplification can be used for highly sensitive detection of RNA with crude (minimally-processed) samples such as heat-treated blood.[67] A combination of a liposome-based lateral flow assay and nucleic acid sequence-based amplification or a liposome-based immunoassay may also provide another avenue for rapid detection of Zika virus in clinical samples.[68]

Due to the ongoing ZIKV outbreak and the absence of either a vaccine or a curative treatment, a rapid mechanism for diagnosis of Zika virus infection is urgently needed. An inaccurate diagnosis may misguide individuals and may either underestimate or overestimate the incidence of infection in a given region. The cost and availability of various diagnostic methods should also be considered. Viral isolation is time consuming and serological identification may be hampered by cross reactivity with other viruses. Compared with viral isolation and serological identification, RTPCR provides a rapid and sensitive approach to diagnose Zika virus infection; however, specific primers and probes may fail to provide appropriate amplification. A recently developed programmable and paper-based detection provides a low-cost and rapid detection method. A major challenge with current diagnostic approaches is that infection with another flavivirus (e.g., DENV) may be misdiagnosed as Zika virus infection.

More sensitive and accurate diagnosis methods are needed for faster treatment of affected individuals. With the development of novel biosensor materials, better detection accuracy, miniaturization of devices, increased stability, and a decrease in cost can be realized.[68, 69] The key features of a biosensor (e.g., low power consumption, short assay time, selectivity, sensitivity, stability, and reproducibility) are primarily determined by the component materials.[68,69] Use of nanomaterials may enable detection of Zika virus infection with smaller samples. As the analyte and the detector become closer in size, a reduction in non-specific binding and single molecule detection become possible.[68] Nanoplasmonic sensors may enable group-specific antibodies in rapidly evolving viruses to be detected.[69] It should be noted that conversion of fluids obtained from a blood draw or a urine collection into biosensor samples may complicate use of new biosensor technologies.[69] Developing portable biosensors that are stable for at least six months and stable at high temperatures remains a significant challenge.

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Figures

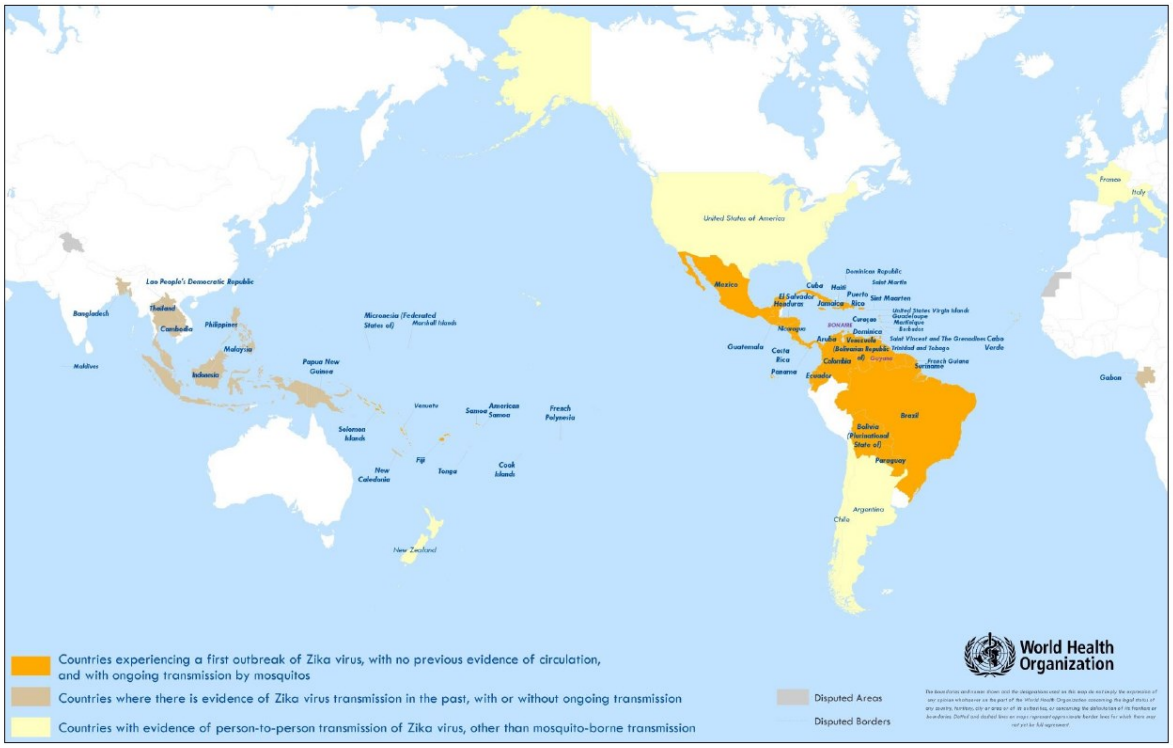


FIG. 1. Countries reporting Zika virus infection/outbreak, 2007-2016. Reprinted with Ref. 60 : World Health Organization, Zika Virus Microcephaly and Guillain-Barre Syndrome Situation Report, 31st March 2016, Copyright (2016).

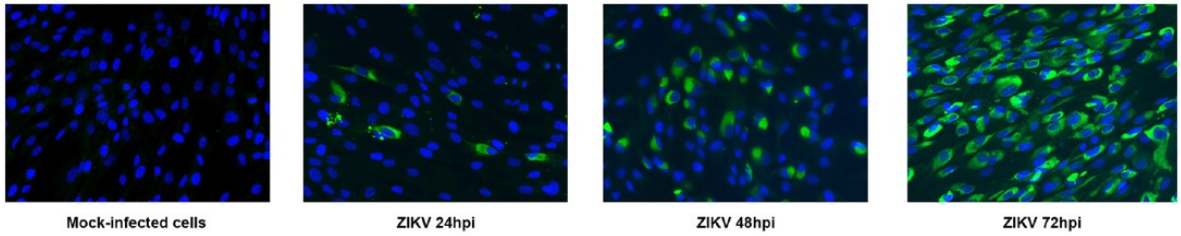


FIG. 2. Primary human fibroblasts are susceptible to ZIKV. Primary fibroblasts infected with ZIKV (MOI =1) and mock-infected cells were analyzed at different times post-infection for the presence of the viral envelope protein by immunofluorescence with 4G2 MAb and FITC-conjugated anti-mouse IgG. Reprinted with Ref. 46 : *J. Virol.* **89**, 8880 (2015).

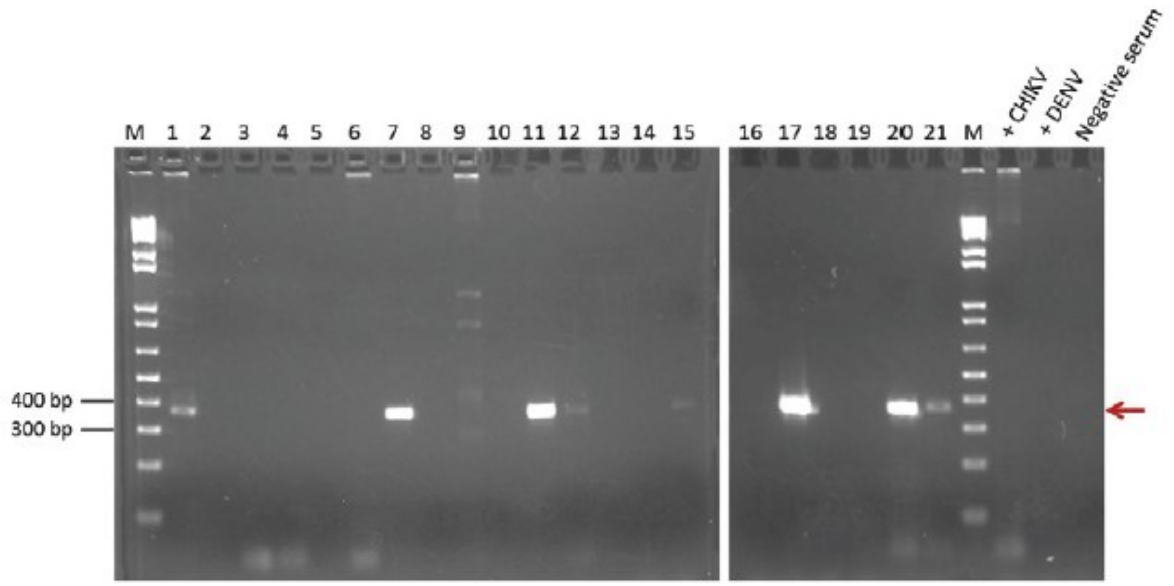


FIG. 3. Agarose gel electrophoresis of RTPCR products for ZIKV detection. Reprinted with Ref. 34 : *Mem. Inst. Oswaldo Cruz* **110**, 569 (2015).

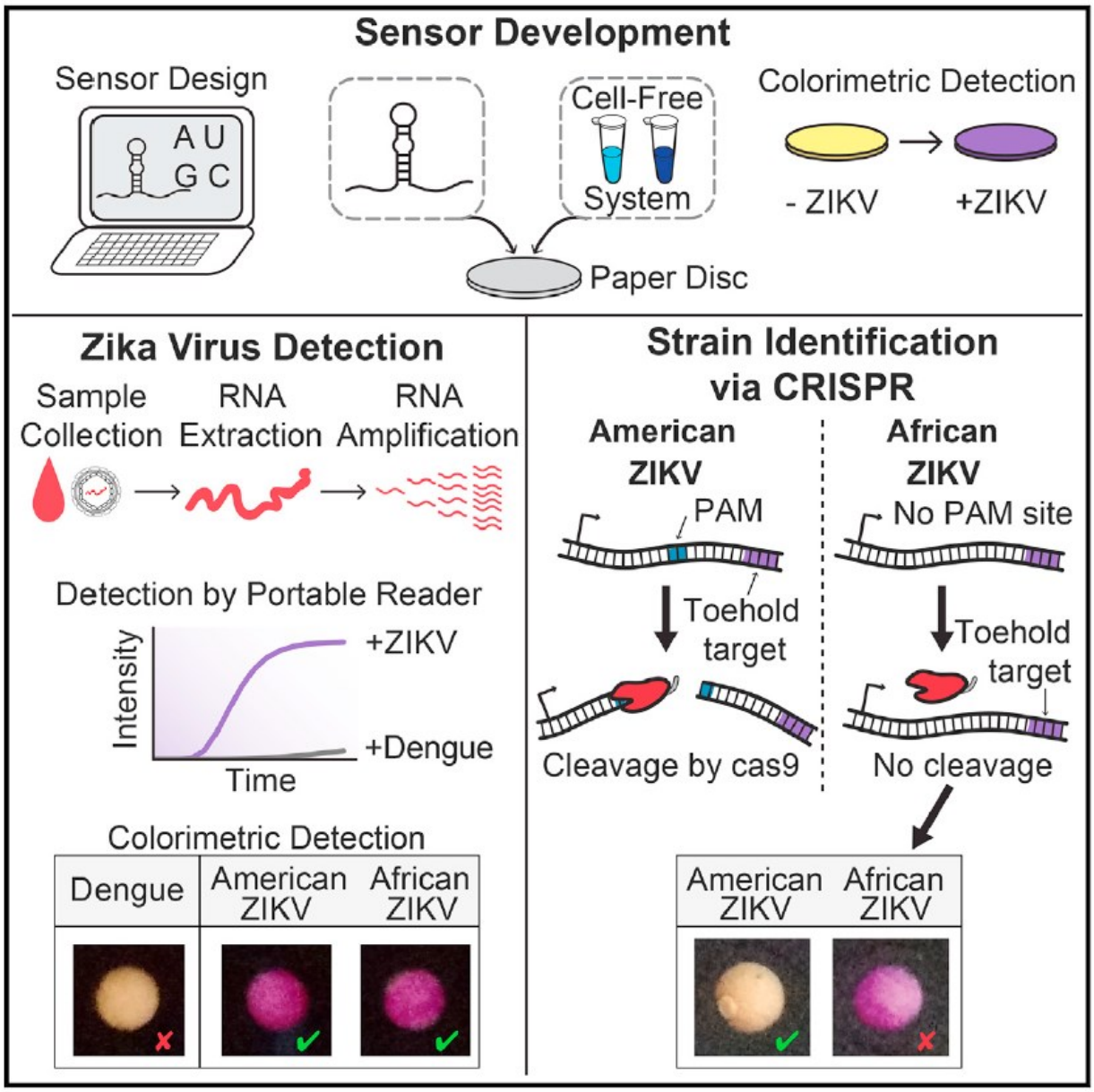


FIG. 4. A schematic of sensor development, Zika virus detection, and strain identification via CRISPR. Reprinted with Ref. 64 : *Cell* **165**, 1255 (2016), Copyright (2016), with permission from Elsevier.

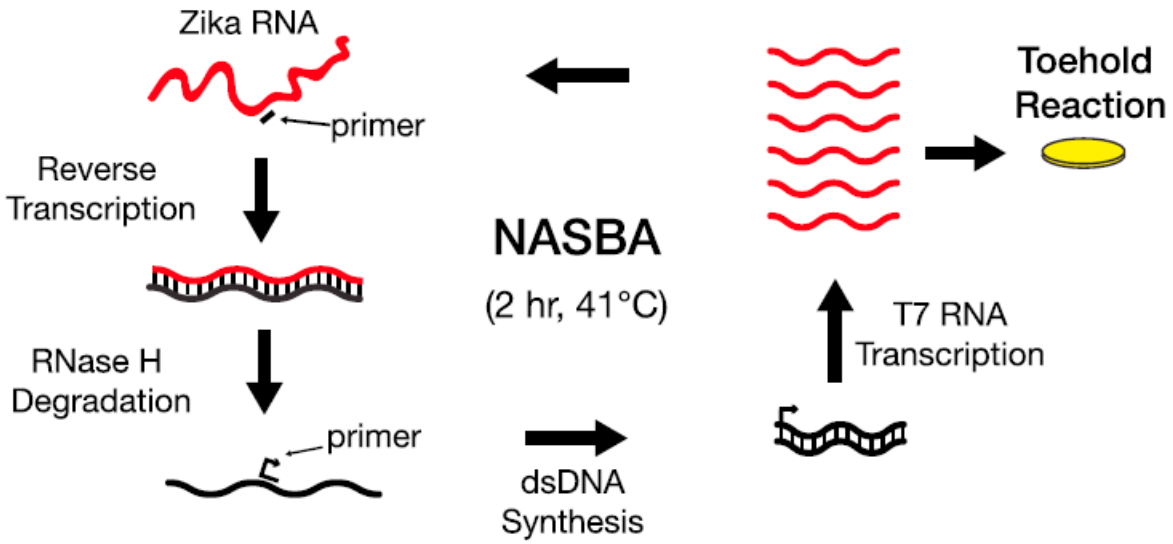


FIG. 5. A schematic of NASBA (nucleic acid sequence based amplification)-mediated RNA amplification. Reprinted with Ref. 64 : *Cell* **165**, 1255 (2016), Copyright (2016), with permission from Elsevier.

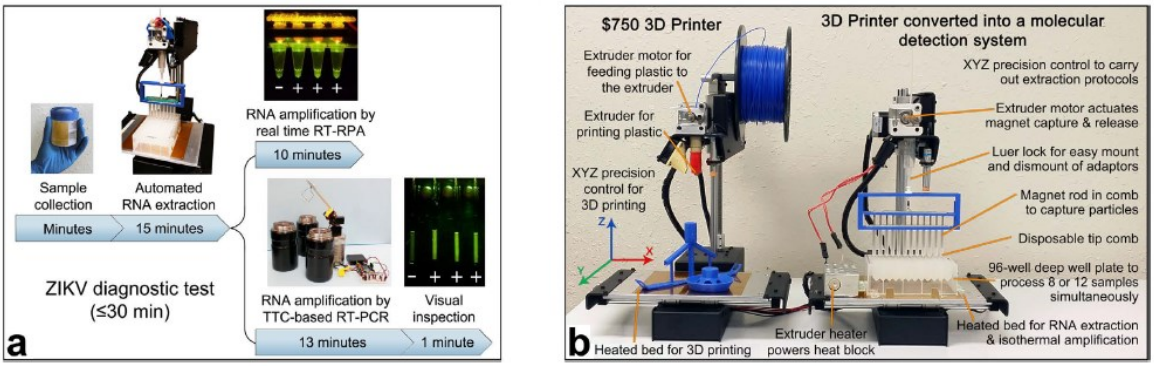


FIG. 6. (a) Rapid and low-cost diagnostics for ZIKV using a modified entry-level 3D printer. (b) Converting a low-cost 3D printer to perform rapid and automated nucleic acid isolation and real-time RPA amplification. Reprinted with Ref. 65 : *Sci. Rep.* **6**, 38223 (2016).

Table Captions

Table 1. Serological testing for heterologous flaviviruses of samples from ZIKV-positive patients, Yap State, Micronesia, 2007. Reprinted with Ref. 1 :*Emerg. Infect. Dis.* **14**, 1232 (2008).

| | | IgG | IgM | | | | | |
|---|---|---|---|---|---|---|---|---|
| Patient | Days after onset | ZIKV | ZIKV | DENV | YFV | JEV | MVEV | WNV |
| Primary flavivirus ZIKV | | | | | | | | |
| 822a | 5 | 1.5 | 23.2 | 1.3 | 1.4 | 1.7 | 1.1 | – |
| 822b | 10 | 1.2 | 39.5 | 1.2 | 1.0 | 2.4 | 1.2 | – |
| 822c | 24 | 3.3 | 13.1 | 2.7 | 0.63 | 1.8 | 1.3 | – |
| 830a | 2 | 1.1 | 1.3 | 4.4 | 0.48 | 4.4 | 2.9 | – |
| 830b | 21 | 1.8 | 16.3 | 1.9 | 0.63 | 1.3 | 1.6 | – |
| 849a | 3 | 1.5 | 4.5 | 0.92 | 0.95 | 1.2 | 0.66 | – |
| 849b | 18 | 3.0 | 18.2 | 2.2 | 1.0 | 2.7 | 1.5 | – |
| 862a | 6 | 1.9 | 25.4 | 1.7 | 1.1 | 1.8 | 1.0 | – |
| 862b | 20 | 2.6 | 15.4 | 2 | 1.1 | 2.3 | 1.1 | Eq |
| Secondary flavivirus ZIKV (probable) | | | | | | | | |
| 817a | 1 | 5.9 | 1.4 | 1.7 | 0.8 | 1.7 | 0.7 | – |
| 817b | 19 | 5.7 | 8.1 | 5.1 | 2.1 | 1.7 | 1.0 | – |
| 833a | 1 | 3.4 | 1.7 | 3.7 | 1.0 | 2.8 | 1.3 | – |
| 833b | 19 | 8.2 | 3.1 | 2.3 | 0.9 | 2.5 | 1.3 | – |
| 844a | 2 | 3.8 | 3.8 | 6.8 | 2.0 | 21.5 | 0.7 | – |
| 844b | 16 | 8.5 | 12.7 | 14.9 | 7.0 | 42.9 | 1.6 | – |
| 955a | 1 | 5.0 | 1.8 | 3.7 | 1.0 | 3.4 | 2.4 | Eq |
| 955b | 14 | 26.6 | 10.9 | 3.4 | 0.8 | 1.7 | 4.0 | Eq |
| 968a | 1 | 4.0 | 1.7 | 1.3 | 0.6 | 1.2 | 1.2 | – |
| 968b | 3 | 12.3 | 20.4 | 2.9 | 0.8 | 0.9 | 2.0 | – |
| 839a | 3 | 1 | 0.92 | 3.4 | 0.7 | 2.7 | 2.1 | – |
| 839b | 20 | 4.9 | 17.2 | 2.2 | 2.1 | 1.9 | 1.8 | – |
| 847a | 5 | 0.9 | 0.94 | 4.1 | 4.1 | 2.3 | 1.3 | – |
| 847b | 8 | 14.1 | 21.5 | 1.4 | 3.3 | 1.1 | 2.6 | – |

*Ig, immunoglobulin; ZIKV, Zika virus; DENV, dengue virus type 1–4 mixture; YFV, yellow fever virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; WNV, West Nile virus; –, negative. Eq, result in equivocal range of the assay. IgG and IgM testing was conducted by ELISA except for WNV, which was tested by microsphere assay; ELISA values are patient optical densities divided by negative control optical densities; <2, negative; 2–3 equivocal; >3 positive.

Table 2. Threshold cycles and estimated copies/mL using real time RTPCR of samples from ZIKV-positive patients, Yap State, Micronesia, 2007. Reprinted with Ref. 1 :*Emerg. Infect. Dis.* **14**, 1232 (2008).

| | Days after | ZIKV real-time RT-PCR | | | |
|---|---|---|---|---|---|
| Patient | onset | Ct-860† | Ct-1107† | Result | Estimated copies/mL‡ |
| 824 | 1 | 34.3 | 34.7 | + | 11,647 |
| 939 | 2 | 32.0 | 32.4 | + | 67,817 |
| 947 | 2 | 34.3 | 33.9 | + | 21,495 |
| 949 | 2 | 35.1 | 35.1 | + | 8,573 |
| 969 | 1 | 29.4 | 29.3 | + | 728,800 |
| 037 | 1 | 32.1 | 32.5 | + | 62,816 |
| 830a | 2 | 30.7 | 30.0 | + | 426,325 |
| 847a | 5 | 34.8 | 34.7 | + | 11,647 |
| 950a | 0 | 32.2 | 32.7 | + | 53,894 |
| 943 | 3 | 37.6 | 35.6 | + | 5,845 |
| 952 | 1 | 29.3 | 29.5 | + | 625,280 |
| 958 | 11 | 29.9 | 30.3 | + | 338,797 |
| 970 | 1 | 35.5 | 34.8 | + | 10,788 |
| 42 | 0 | 32.9 | 33.6 | + | 27,048 |
| 941 | 3 | 31.1 | 38.0 | + | 930 |
| 964 | 0 | 38.3 | 37.6 | + | 1,263 |
| 063a | 2 | 37.5 | 38.0 | + | 930 |

*RT-PCR, reverse transcription–PCR; ZIKV, Zika virus; Ct, crossing threshold; +, positive.
†Ct values with primer set 835/911c/860-FAM or 1086/1162c/1107-FAM. Values <38.5 are positive.
‡Estimated by testing quantitated dilutions of ZIKV RNA transcripts and standard curve calculation generated by the iCycler instrument (Bio-Rad, Hercules, CA, USA; see Methods).