REVIEW ARTICLE

A brief review on novel biomarkers identified and advanced biosensing technologies developed for rapid diagnosis of Japanese Encephalitis Virus

Akanksha Roberts[1,2] · Sonu Gandhi[1,2]

Abstract

Advanced biosensor technology research is imperative for the management of infectious disease outbreaks such as Japanese Encephalitis (JE), a zoonotic disease caused by the *flavivirus* JE virus (JEV) which is transmitted to humans (dead-end hosts) from the amplification host, pigs, via mosquitoes. To avoid future pandemic scenarios, proactive research rather than responsive research in the field of diagnostics is a requirement for development of rapid, sensitive and specific screening detection methods. In this mini-review, we have critically compared and evaluated the different types of biomarkers (antigen, antibody, nucleic acid) identified for JEV diagnostics and their specific roles in the manifestation of the infection which may be potentially used for therapeutics and drug development as no treatment is available for JE. Furthermore, different biosensors developed for the detection of JEV biomarkers have been discussed in detail to give an overview of the working principles (electrochemical, optical, etc.), fabrication components (signal amplifier, bioreceptor, etc.), detection limits and response times. This review provides a compact compiled base on available JEV diagnostic research work being currently carried out along with their limitations, future prospective, and major challenges faced. This will enable future development of rapid point-of-care diagnostic screening methods for JEV infection management, which may help reduce number of fatalities.

Introduction

The major reason for mosquito-borne haemorrhagic and encephalitic infectious diseases is the zoonotic *flavivirus* Japanese Encephalitis Virus (JEV) which belongs to the family *Flaviviridae*, consisting of more than 70 different viruses (Khristunova et al. 2020). The closest related viruses to JEV are other *flaviviruses* such as West Nile Virus (WNV), Dengue Virus (DENV), Yellow Fever Virus (YFV) and Zika Virus (ZIKV). JEV transmits via a zoonotic cycle which involves pigs as the amplification host, *Culex* mosquito as the transmission vector, and wading birds as the reservoir host. Human/cattle/horses act as dead-end or incidental host

as a result of low viraemia which prevents human to human transmission (Weaver and Barrett 2004). The first case was recorded in 1871 and the Nakayama strain was isolated for the first time in 1935, both in Japan (Solomon et al. 2003; Erlanger et al. 2009). JEV is a single stranded positive sense RNA virus of approximately a 11,000 bp genome. It exists as a single phenotype and five different genotypes (I, II, III, IV, V) based on the changes in the nucleotide homology of the Envelope (E) protein gene (Tsarev et al. 2000; Satchidanandam and Uchil 2001). It contains a polyprotein encoded in a single open reading frame (ORF) which consists of three structural proteins- envelope (E), capsid (C), pre-membrane protein (prM) and seven non-structural proteins numbered 1, 2 A, 2B, 3, 4 A, 4B, 5 (Sumiyoshi et al. 1992; Tiwari et al. 2012).

According to reports by the World Health Organisation (WHO), JEV endemic outbreaks occur every two to five years during the monsoon season, especially in rural regions where rice paddy fields and pig rearing are parallel occupations. JEV is often referred to as "a plague of the Orient" as outbreaks occur mostly in western pacific and south east

✉ Sonu Gandhi
gandhi@niab.org.in

1 DBT-National Institute of Animal Biotechnology (NIAB), Hyderabad, Telangana 500032, India

2 DBT-Regional Centre for Biotechnology (RCB), Faridabad, Haryana 121001, India

Asian countries where pig farming and rice cultivation are in abundance (Monath 1988) with 24 countries i.e. 3 billion people at risk of JEV transmission. In India, since 1955, major JE outbreaks occur periodically such as Bankura in 1973, Gorakhpur in 1978, and most recently Assam in 2019 (Tiwari et al. 2012; Datey et al. 2020). Approximately 68,000 JEV cases are reported worldwide annually with 13,600−20,400 fatalities (20–30%), most predominantly in children (Solomon 2009). JE is mostly asymptomatic but neuropsychiatric sequalae may occur in 30–50% cases and other clinical symptoms such as encephalitis, meningitis and febrile illness may also occur (Bandyopadhyay et al. 2013; Kakoti et al. 2013). Only palliative care can be given to JE patients as no specific therapy is available. Furthermore the vaccines have multiple limitations such as inherent risks (live-attenuated), short-term immunity (inactivated), allergic reactions (chimeric/mouse-brain derived) and expensive 3-dose requirement (Hegde and Gore 2017).

The conventional diagnostic techniques for JEV detection include virus isolation, plaque reduction neutralization test, molecular assays, haemagglutination test, complement fixation test, immunofluorescence assay, and enzyme-linked immunosorbent assay. These have various drawbacks such as time-consuming, laborious, expensive and require skilled technicians (Roberts and Gandhi 2020) and are often not feasible in rural areas were JE is prevalent. Furthermore, since the handling of the highly infectious clinical body fluid samples from JEV patients for certain conventional method diagnosis require Biological Safety Level-3 (BSL-3) facility with proper personal protective equipment, it poses a serious health hazard for the diagnostician. To overcome these diagnostic hurdles as well as non-availability of therapy and full-proof vaccines, advances are being made in the field of biosensor technology to meet the need for rapid portable point-of-care diagnostic screening methods for JEV, with a small amount of sample available/target biomarker present (Jones et al. 2008).

The upcoming diagnostic tool i.e. biosensors (Kaushik et al. 2021), especially for viruses (Banga et al. 2019; Nandi et al. 2020; Roberts et al. 2021a), function on various sensing methods such as optical (Damborský et al. 2016), electrochemical (Bakker 2004), mechanical (Arlett et al. 2011), thermal (Mosbach 1991), etc. or a combination of these. Based on the biomarker analyte being targeted e.g., antigen (Healy et al. 2007), antibody (Guerrero et al. 2020), enzymes (Chambers et al. 2009), nucleic acid (Adam and Hashim 2015), etc., a specific bioreceptor is selected. These are used as the immobilized biorecognition element (Roberts et al. 2021b, 2022b) on the fabricated sensor such as antibody (Holford et al. 2012), aptamer (Song et al. 2008), peptide (Liu et al. 2015), enzyme (Kim et al. 2011), etc. Different nanomaterial (Mishra et al. 2019) for example graphene (Shahdeo et al. 2020; Dey et al. 2022; Mahari et al. 2022),

graphitic carbon nitride (Chouhan et al. 2021), gold (Shahdeo et al. 2022) etc. may be incorporated as signal enhancers in the biosensing system.

In this review, we have critically compared various biomarkers identified which may be used as target analytes for development of JEV diagnostic and therapeutic purposes against which highly specific bioreceptors can be potentially generated. Furthermore, we have discussed different types of biosensors already developed for detection of JEV and its biomarkers along with the limitations and future research prospective. Although there isn't enough literature available as there is a dearth of JEV diagnostic research, this review will provide researchers with a base to proactively develop on-site rapid mass screening point-of-care kits for JEV. This will enable better management and monitoring of the infectious disease as well as reduce outbreak incidence by decentralizing clinical applications using 'alternative-site' (rural endemic areas) testing.

Biomolecules identified as potential target JEV biomarker candidates

It is not possible to accurately diagnose JE based on clinical symptoms alone and hence research has been extensively carried out to identify various biomarkers specific for JEV which could facilitate accurate prognosis, vaccine development and guided therapy. Biomolecules expressed by either the virus or the host organism against viral entry and cell invasion display biological characteristics which can be measured in the patient clinical samples (Desai et al. 1994; Shrivastva et al. 2008) and may be employed as biomarker candidates. Some of these may include JEV antigens, inflammatory reactive proteins, antibodies, gene mutations/polymorphisms, virus induced peptides, lipid metabolites, etc. (Kant Upadhyay 2013). While there exist various biomarkers used for imaging, anatomical, physiological, therapeutic and immunohistochemical purposes, we have listed those biomarkers that may be used as targets in biosensing applications.

JEV protein biomarkers

The 11 kb JEV genome encodes three structural proteins-envelope (E), capsid (C), pre-membrane protein (prM) and seven non-structural (NS) proteins- NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. The C and prM proteins contain glycosylation sites which may be targeted for mutation to reduce the entry and neurovirulence of the virus (Chen et al. 1996). Similarly, the E protein contains target neutralization epitopes which facilitate attachment of the virus, fusion of the membrane and viral entry into the host cell. The virulence phenotype is determined by the NS154

putative glycosylation site of the E protein and its receptor binding domain induces an immunological response in the host organism (Kawano et al. 1993). Beside the E protein, NS3 also causes a major immune response along with neurovirulence, processing the polyprotein and genome replication (Rice et al. 1986). NS1 glycoprotein exists both as a cell-membrane associated as well as a secretory protein assisting in neuronal invasion, cell profusion, pathogenesis and elicits an immunological response from the host (Muller and Young 2013; Rastogi et al. 2016).

Blood serum biomarkers

Serum marker profiles are analysed as biomarkers and generated using techniques which include a combination of chip microarrays, proteomics (ProteoMiner technology) (Li et al. 2017) and surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) (Dattelbaum and Iyer 2006; Poon 2014). The most common and highly reproducible targeted blood serum biomarkers are JEV specific serum antibodies such as IgM-Immunoglobulin M (4–5 days after infection) or IgG-Immunoglobulin G (7–10 days after infection). Complement fixation and ELISA tests are based on detection of JEV serum antibodies. Also, the secretory JEV NS1 Ag is also detectable in clinical serum samples since Day 1 of infection. Furthermore, complete blood count (CBC) also acts as a biomarker for thrombocytopenia, anaemia, leucocytosis, and leukopenia that may occur in JE patients (Kantake 2011). Fluctuations in serum chemokine (monocyte chemoattractant protein-1) and cytokine (inflammation) levels (Misra et al. 2010) along with glucose levels and mononuclear white blood cell counts may also be employed as potential serum biomarkers for JEV. The major advantage of using serum samples for diagnosis is the reduced risk of infection for the diagnostician as the virus is no longer present in the serum as compared to other types of samples such as cerebrospinal fluid (CSF) and a BSL-3 facility is no longer required to handle JEV patient serum samples.

Cerebrospinal fluid biomarkers

Since JEV is a neurological virus, cerebrospinal fluid (CSF) is the major sample collected for diagnostic purposes once the virus crosses the blood-brain barrier. One of the most important biomarkers targeted are the virus neutralising antibody titres (both IgM and IgG) which are often detected by the conventional techniques, especially ELISA (Shrivastva et al. 2008). Viral secretory NS1 and E protein enveloping the virus can also be detected in CSF samples using biosensors using specific monoclonal antibodies as bioreceptors (Kimura-Kuroda et al. 1993). The limitation of handling CSF samples is the need for a BSL-3 facility with increased

risk of infection and a more invasive procedure than blood collection.

Plasma biomarkers

Infected cells have their own antibody-dependent cell mediated cyto-toxicity (ADCC) mechanism to destroy all infected cells by inducing different classical pathways. Complement formation (C3 and C4b) leads to proinflammatory peptide synthesis as well as proteolytic fragmentation which can be potential biomarker candidates. Other inflammatory response cytokines such as IL-1, IL-6, IL-8, TNF-α, IL-12, chemokines, monocyte chemo-attractant protein (MCP-1), macrophage inflammatory protein (MIP) 1a and 1b were higher in JE infected patient samples (Stein and Nombela-Arrieta 2005). Serum albumin, vitamin-D-binding protein, fibrinogen gamma and beta chains, actin cytoplasmic-1, lysozymes, immunoglobulins, enzymes and a large protein properdin can all be targeted in plasma whose main purpose is to destroy invading microorganisms (Sengupta et al. 2015). Changes in the chemical balance of ion channels in the plasma are also an indicator of infection.

Molecular biomarkers

The disease marker genes and mutation analysis expression profiles can assist in diagnosis, status and morbidity of the infection. Ranging from RT-PCR to electron microarrays, targeted changes in the genome are detected for accurate prognosis of JEV in clinical samples. Phylogenetic analysis can help pin-point the genotype while molecular substitutions in the genome may help reduce neurovirulence. Inhibition of the replication of JEV mediated by DNAzyme has been established as an imperative biomarker to detect neurotropic viral titre within the host organism (Appaiahgari and Vrati 2007). Systemic mRNA profiling and circulating viral microRNAs have also been reported as excellent markers for neuroinvasive JEV diagnostics (Zhang et al. 2010; Baluni et al. 2022).

Biosensors for JEV diagnostics

JEV outbreaks mostly occur in rural areas where there is no access to extensive laboratory set-ups, skilled diagnosticians and expensive detection methods. Hence, biosensor technology is the need of the hour to prevent periodic JE endemic breakouts, better management and guided therapy. While no extensive research has been carried out in developing point-of-care biosensors for rapid detection of JEV, there have been a few advances in biosensor technology development that have been briefly compiled. Initially, JEV biosensors were developed for analysis of vaccine assays

such as gold nanoparticles (AuNPs) and Cobalt(III)tris(2,2'-bipyridine) (Co(bpy)$_3^{3+}$) films assembled as layers on gold electrodes modified with L-cysteine. Japanese B encephalitis antisera was immobilised as the bioreceptor to determine Japanese B encephalitis vaccine with a limit of detection (LOD) 3.5×10^{-8} log pfu/ml in the linear range 8.1×10^{-8} to 3×10^{-6} log pfu/ml (Zhang et al. 2006). Similarly, amperometric (Yuan et al. 2005) and potentiometric (Zhang et al. 2004) sensor were fabricated by immobilising Japanese B encephalitis antisera. One on bi-layered film of AuNPs and the other on polymerized o-phenylenediamine deposited on platinum electrodes with a detection limit of 6×10^{-9} log pfu/ml in the range 1.1×10^{-8} to 2×10^{-6} log pfu/ml.

Exploiting the surface plasmon resonance (SPR) property of silver nanoparticles (AgNPs), an optical based sensing probe was fabricated with synthesised AgNPs drop casted onto (3-Aminopropyl)triethoxysilane (APTES) amine functionalized glass immobilised with JEV antibodies. Changes in the absorbance of light was detected upon binding of target JEV antigen (Ag) and analysed which resulted in a detection limit of 12.8 ng/ml (Lim et al. 2017). Similarly, an optical Lateral Flow Assay (LFA) was developed for detecting JEV antibodies (Ab) in swine serum for sero-surveillance and mass screening using the SPR properties of AuNPs. The fabricated LFA consisted of AuNPs-Ag on the conjugate pad with staphylococcal protein A coated on test line and anti-antigen IgG as control line. The LFA was able to successfully detect JEV Ab in 500 clinical pig serum real samples with 100% relative diagnostic specificity as well as sensitivity in monsoon and post-monsoon (Dhanze et al. 2019). Another recently developed LFA using AuNPs conjugated with JEV NS1 Ab as immunochromatic probes was fabricated for successful point of care detection of JEV NS1 Ag in clinical pig serum samples with a visual LOD of 10 pg/ml (Roberts et al. 2022d).

Synthesised polyaniline (PANI) nanowires on inter-digitated platinum microelectrodes imbibed with anti-JEV polyclonal IgG antibody Ab were fabricated using 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimde (NHS). Electrochemical impedance spectroscopy (EIS) method was used to detect JEV at an LOD < 10 ng/ml in the range 10–500 ng/ml (Tuan et al. 2013). Another PANI electrochemical sensor with anti-JEV polyclonal IgG Ab immobilised on multi-walled carbon nanotubes upon nanocomposite microelectrodes was fabricated. The detection limit using EIS was determined as 2 ng/ml in the range 2 to 250 ng/ml for JEV detection (Hien et al. 2016). A label-free electrochemical non-Faradic impedance based interdigitated sensor, composed of protein A and glutaraldehyde along with anti-JEV sera for detection was developed. This sensor displayed an LOD of 0.75 µg/ml in the concentration range 1 to 10 µg/ml of JEV Ag (Huy et al. 2011).

AuNPs modified screen-printed electrode (SPCE) impedimetric biosensor with JEV Ab bioreceptor and horseradish peroxidase (HRP)-labelled secondary Ab for signal development was fabricated. Using EIS, the LOD showed a great linear response in the range 500 - 5×10^5 pfu/ml with an LOD 167 pfu/ml for JEV detection (Geng et al. 2016). Another SPCE functionalised with APTES, was fabricated using JEV antibody modified carbon nanoparticles (CNPs) synthesised from starch nanoparticles. Electrochemical sensing was carried out using cyclic voltammetry (CV) and EIS of JEV E Ag with a detection limit of 2 ng/ml in 20 min within the linear range 5–20 ng/ml (Chin et al. 2017). Similarly, another SPCE was modified with amine functionalised CNPs synthesised from preformed chitosan nanoparticles with JEV Ab immobilised as the bioreceptor as shown in Fig. 1. Using CV and EIS, the LOD of the sensor was found to be 0.36 ng/ml in the range 1–20 ng/ml of JEV E Ag with a rapid response time of 10 min (Lai et al. 2017).

Recently, a graphene-based field effect transistor (GraFET) sensor was developed using JEV monoclonal NS1 Ab as the biosensing element as shown in Fig. 2. The FET chip was fabricated using E-beam lithography and thermal evaporation followed by EDC-NHS surface activation and immobilisation of JEV NS1 Ab. A lock-in amplifier was employed for readout with LOD of 1 fM in the range 1 fM to 1 µM for the detection of secretory JEV NS1 Ag (Roberts et al. 2020).

Reduced graphene oxide (rGO)-based Fluorine Doped Tin Oxide (FTO) sensor was fabricated using in-house generated JEV NS1 Ab for detection of JEV NS1 Ag as shown
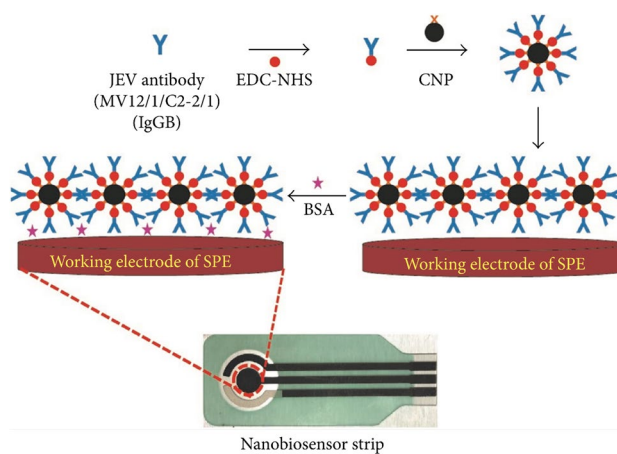

Fig. 1 Fabrication of SPCE sensor: EDC-NHS activated amine-functionalised CNPs were conjugated with JEV Ab on the working electrode of the SPCE followed by blocking with BSA for electrochemical detection of JEV E Ag. (Image reprinted with permission from (Lai et al. 2017) This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited)
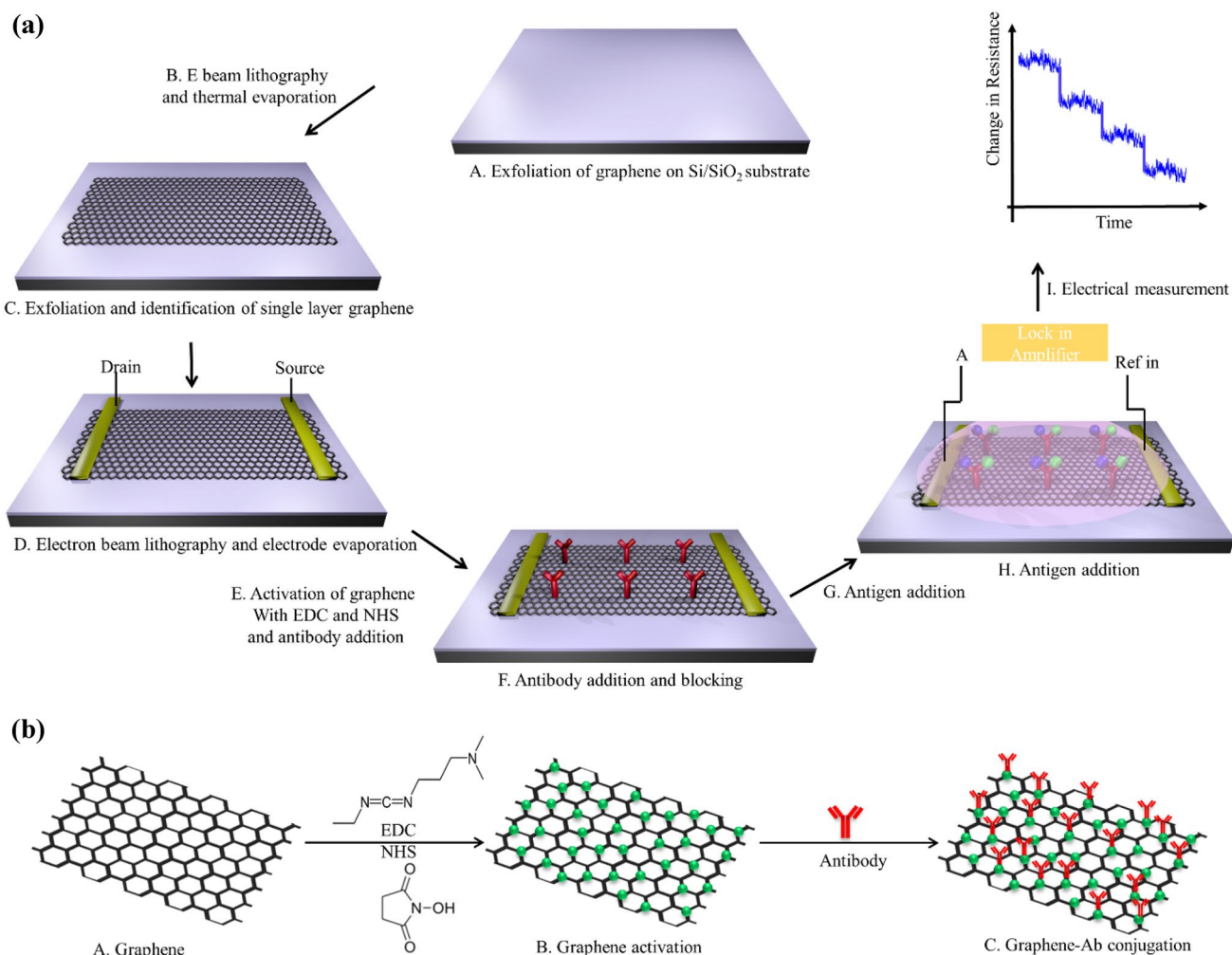
(a)

B. E beam lithography and thermal evaporation

A. Exfoliation of graphene on Si/SiO₂ substrate

C. Exfoliation and identification of single layer graphene

Drain | Source

D. Electron beam lithography and electrode evaporation

E. Activation of graphene With EDC and NHS and antibody addition

F. Antibody addition and blocking

G. Antigen addition

H. Antigen addition

I. Electrical measurement

(b)

A. Graphene

B. Graphene activation

C. Graphene-Ab conjugation

Fig. 2 a Fabrication of GraFET sensor: (A, B) Graphene exfoliated on Si/SiO2 base; (C, D) FET fabricated using e-beam lithography and thermal evaporation; (E, F) EDC-NHS activation of graphene followed by Ab immobilisation; (G, H) Target Ag captured on the sensor surface (I) Readings measured using a lock-in amplifier; **b** Binding of graphene-Ab conjugate: (A) Pre-activated graphene; (B) EDC-NHS graphene activation; (C) Immobilisation of Ab on activated graphene. (Image reprinted with permission from (Roberts et al. 2020). This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited)

in Fig. 3. Both the Ag and Ab were produced in-house and using cyclic and differential pulse voltammetry, the sensor was able to successfully detect JEV NS1 Ag at an LOD of 1.3 fM within 5 s in spiked serum samples (Roberts et al. 2022a). The same lab fabricated a similar electrochemical sensor using gold nanorods instead as the signal enhancer and detected JEV NS1 Ag at an LOD of 0.53 fM (Roberts et al. 2022c).

A gold-coated magnetic beads electrochemical immunoassay was developed with JEV E Ab immobilised on the bead surface, multi-walled carbon nanotubes as signal enhancers and horseradish peroxidase as an enzymatic tracer. The decrease in electrical conductivity upon capture of the target E protein was analysed and the detection limit for E protein was found to be 0.56 ng/ml in the

range 0.84 to 11,200 ng/ml whereas for the whole virus was found to be 2.0×10^3 pfu/ml in the range 2×10^3 to 5×10^5 pfu/ml (Li et al. 2011). Another advanced sensing technology involves virus-molecular imprinted polymers (virus-MIPs). In one such study, MIPs were anchored onto fluorescence dye (pyrene-1-carboxaldehyde) modified silica microspheres. The change in fluorescence intensity on sensing of JEV virus was analysed using fluorescence resonance energy transfer (FRET) principle. The detection limit of the optical sensor was established at 9.6 pM within the range 24 to 960 pM (Liang et al. 2016). Similarly, another JEV-MIPs based optical sensor was fabricated by the same lab using dansyl chloride on silica microspheres, JEV template, APTES monomer and tetraethyl orthosilicate (TEOS) cross-linker. The sensor
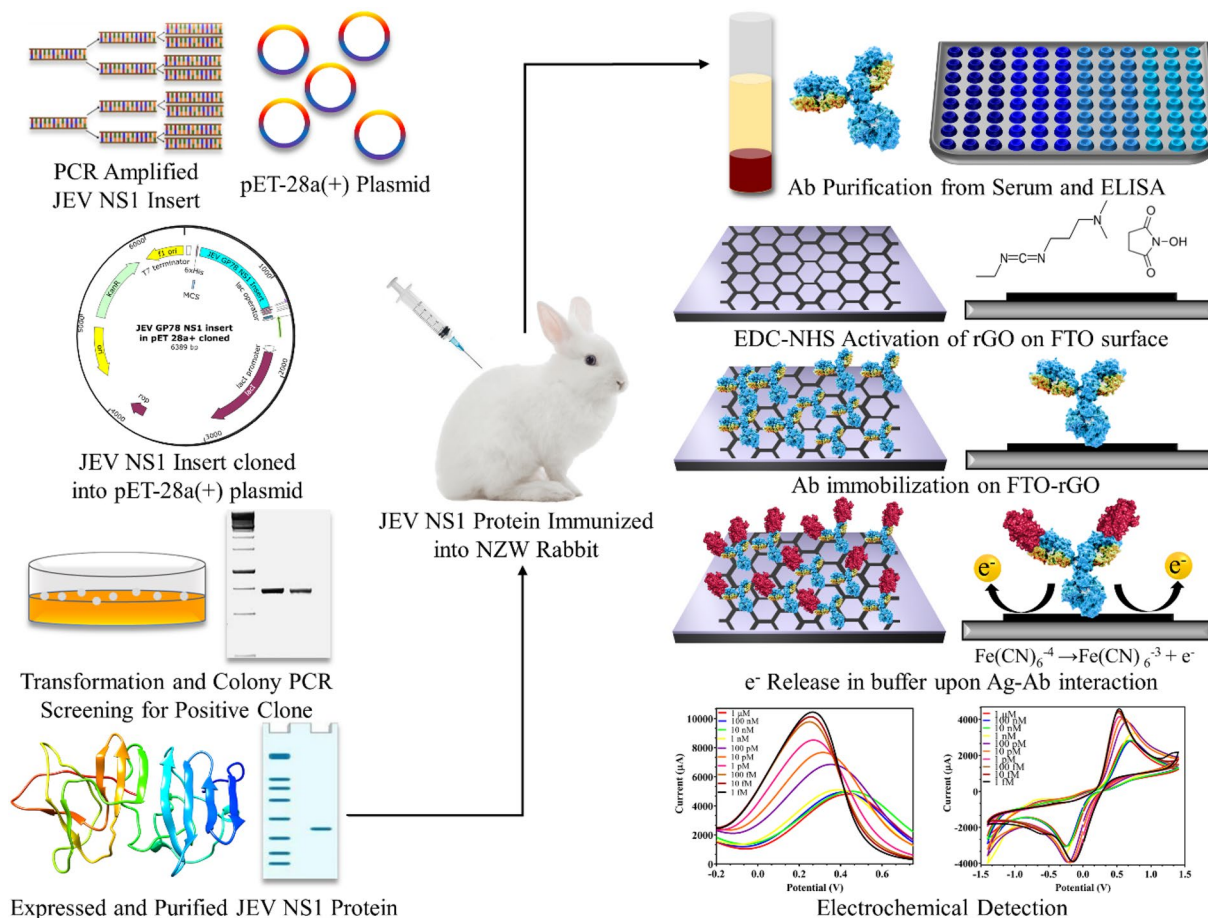
Fig. 3 JEV NS1 Ag was cloned, expressed and purified followed by immunisation in rabbits to generate JEV NS1 Ab. Both Ag and Ab were thoroughly characterised. EDC-NHS activated rGO was drop casted on FTO conductive surface followed by JEV NS1 Ab immobi-lisation. Changes in CV and DPV were measured upon interaction of sensor with target analyte spiked in serum. (reprinted from (Roberts et al. 2022a) with copyright permission for figure obtained from Else-vier)

was able to detect the whole virus at a detection limit of 0.11 pM in the linear concentration range 2.4 to 24 pM (Feng et al. 2018). A magnetic based MIPs sensor was developed using Fe_3O_4 microspheres as the imprinting substrate, functional APTES monomers with JEV tem-plate and TEOS for polymerisation. The resonance light scattering intensity changes were analysed upon capture of target JEV virus within a response time of 20 min and the LOD was calculated as 1.3 pM (Luo et al. 2019). A very recent 2022 work involved a one-step point-of-care RApid VIsual Clustered regularly interspaced short palindromic repeats (RAVI-CRISPR) assay based on reverse transcription loop-mediated isothermal amplifi-cation (RT-LAMP) and CRISPR/Cas12a targeting. The developed assay could rapidly detect JEV with an LOD of 8.97 copies of the C gene sequence of JEV RNA within approximately 60 min (Xu et al. 2022).

Conclusion, limitations and future prospective

Biosensing technology is a cost effective, rapid, sensitive, and specific alternative to conventional techniques, and is crucial for rapid JEV diagnosis. It can help in better man-agement, monitoring and guided treatment of the infec-tious disease which may prevent fatalities and endemic outbreaks in rural regions. In this review, we have criti-cally compared and listed out different potential biomarker candidates that may be targeted for development of JEV biosensors. Furthermore, we have discussed the currently existing sensors developed for JEV along with their sens-ing components and detection limits. However, a large amount of research is still required in the field of JEV to bring it at par with other infectious disease diagnostics

such as the recent wave of SARS-CoV-2. The main challenge is the cross-reactivity with other flavivirus and how to increase the specificity for JEV detection alone. Another shortcoming lies in the miniaturisation of the biosensors without any compromise in accuracy and reliability, to make them portable point-of-care diagnostic tools for better monitoring, mass screening, guided therapy and management. Other future research scope includes reduction of response time and repeatability/safe disposal of used sensors. Diagnostic research can be carried out using advanced technologies such as clustered regularly interspaced short palindromic repeats (CRISPR) based sensing which is still in the nascent stage with respect to JEV (Bonini et al. 2021). Similarly, multiplex biosensor arrays for simultaneous detection of multiple biomarkers on a single microarray chip incorporating microfluidics, biomarker pattern software and artificial intelligence algorithms can also be explored. Ultimately, we need to develop Internet of Things (IoT) and machine learning biosensing systems which will result in automated sensors with maximum diagnostic accuracy. Hence, combined advances in cutting-edge IoT technology of biosensors could revolutionise all forms of infectious disease management and therapy.

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Declarations

Conflict of interest The authors have no conflict of interest to disclose.

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