REVIEW ARTICLE

AN ATTEMPT TOWARDS LAB AND CLINICAL COMBINE APPRAISED, INCLUDING FUTURE CONCERNS REGARDING DENGUE INFECTION

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Background: Dengue infection is a deadly global pandemic due to its fatal nature, being emerged from mild stage to turn into more severe stages and consequently causing casualties. it upsurges various phases, namely subclinical infection, undifferentiated febrile fever, Dengue fever (DF) and devastating states which often ends to life, they are Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Dengue infection is a mosquito born disease which has engulfed many regions in general and tropical zone in particular, causing many casualties and has posed a threat to humanity, demanding strategies to resolve the global issue. It is reported that 50-200 million people fall prey to it globally by dint of its causative agents and imperative to mention that over proportional are the minor among the victims. Because of awful joint pain dengue fever is also named break bone fever. The common indicator in infected individuals is thrombocytopenia, coagulopathy and vasculopathy. Apart from supportive therapy, no aphoristic therapy has been introduced so far, however care may prove rescuer. Timely prognosis thwarts to enter it in deteriorating phase. **Methods:** In the list of laboratory diagnosis virus serology and detection of Ribonucleic acid are primed. In general, there's no specific decisive diagnostic biomarker present through which accurate and prompt prognosis can possible during the entire patient presentation time, particularly in case of secondary dengue infection. Although, through the advancement and commercialization of point-care combined tests, capable of tracking disease markers present during various phases of infection (viral non-structural protein 1 and immunoglobulin M), such evaluation massively improved the treatment through lab-based. **Conclusion:** Despite such improvements, major hurdles persist in the clinical management of patients with dengue infection, particularly lack of dependable biomarkers that have an efficacious prognostic gauge to predict steady progress to severe disease. In the described review both clinical and laboratory diagnosis of dengue infection has been highlighted, including concern regarding future accessibility.

Keywords: Dengue shock syndrome; Dengue haemorrhagic fever; thrombocytopenia; Dengue fever

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INTRODUCTION

Dengue virus infection can lead to spectrum of illness, initially moderate febrile illness to gradually riskier folds like haemorrhagic disorders; Dengue haemorrhagic fever (DHF) and Dengue shock syndrome(DSS).[1] Majorly it is self-limiting whereas in minor cases it develops serious complications in the form of DHF; having got its roots from south east, claiming the lives of number of peoples, the aforementioned clinical phase is based on increased vascular permeability and haemoconcentration, leading to DSS;[2–4] most delicate phase of dengue infection posing to hypovolumic shock clinically fluctuation in plasma level and RBCs count, which is alarming. Extensive studies on DHF show that emergence of two dengue serotypes in an area at the same time can peril DHF. Serologic research reveals that inheritance of secondary antibodies can worsen DHF phase.

Presently no specific antiviral therapy is available, however severe complications can be prevented through instant detection and suitable management.[5–9] It is broadly reported these days, that dengue complications threatening the whole world community both tropically and sub-tropically, according to World health organization (WHO) dengue virus is awfully turning saddening issues due to fertility, environmental factors, inappropriate prevention of vector, lack of health care facilities and lethargy of concern authorities, so for 30 folds increases has been witnessed from 1960–2010.[10–12] Four genetically distinct serotypes are identified up to date, i.e., Den-1, Den-2, Den-3, and Den 4, all of them have the ability to cause severe illness (DHF/DSS). In Asian region Den-2 and Den-3 are more frequently found and these are associated with severe complications.[13,14] Infection with one serotype may lead to produce immunity against that particular serotype. Infection with another serotype in future may lead to severe complications, i.e., thrombocytopenia and increased vascular permeability, which can result in haemorrhagic and shock complications. The latest review put stress upon clinical, diagnostic and management aspects of Dengue infection transparently.

Global burden

Throughout the 19th century, dengue was considered a sporadic disease, causing epidemic at long intervals, but now dramatic changes occur in this hearsay, presently it is considered an important mosquito born disease. In the past 50 years, Dengue virus hit a depicting ratio of the world population and its prevalence is dramatically scaling up with enormous outbreaks.[15–17] Recently WHO claim that Approximately 3.6 billion of the world populations are residing in tropical and subtropical region, are at stake. Aedes mosquitoes are scattered in these regions. Presently more than 125 countries are effected by dengue virus, including: south East Asia, United States of America, western pacific and in African countries.[18] Each year approximately 50-200 million new cases emerge of DF, out of it 500,000 cases turn threatening (DHF/DSS), consequently near to 20,000 people die due to the complication of the virus, amongst the victims 90% are innocent children having age less than 15 years.[19,20] DHF was 1st investigated in Manila in 1954, now it is spread approximately all over the world. major epidemics of the dengue virus were happened in 1980s-90s, the predominant serotype at that time was Den-2, over the last few years it has changed to Den-3 serotype.[21–23] 1st dengue pandemic occurred in 1998, in which 1.2 million cases from 56 different countries were reported of DF and DHF. Recently a new sub genotype is originated from Indian sub-continent and later spread to other countries, this sub genotype was also a major cause of pandemic.[24]

Characteristics of Dengue virus

Dengue virus is a single stranded RNA virus belongs to genus flavivirus from family Flaviviridae, also known as arbovirus.[25] The virus genome is approximately 11 KB in length. Mature virion has three structural protein (core ′C′, precursor membrane ′prM′ and envelope ′E′) and seven non-structural proteins (NS1, NS2, NS2a, NS2b, NS3, NS4a, NS4b and NS5) Figure-1. The envelope protein has important functions, it binds to a specific receptor on host cell and allows the virus to be transported through it, it is also related to hemagglutination of erythrocytes, induction of neutralizing antibodies, and protects immune responses.[26] It has also an important role in RNA replication and production of infectious virus particles.[27] Dengue virus share their antigenic epitopes with other flaviviruses, the sharing leads to production of cross reactive antibodies and hence, interfere serological analysis. Antibodies directed to the prM protein are species specific and are beneficial for sero-epidemiological research on dengue.[28]

Vector of dengue infection

Dengue is a mosquito born disease, *Aedesaegypti, Aedesalbopictus* and *Aedespolynesiensis* play a central role in transmission of dengue virus. Aedes Aegypti is the primary and most critical vector and the rest two depend on geographical region.[29] In some countries like Singapore, Thailand, India, and Mexico, Aedes albopictus has been found in the transmission of dengue while Aedes aegypti can transmit virus in both tropical and sub-tropical region. It is a day biting mosquito and rests mostly in cool and dark places or indoors particularly in residing rooms, bed rooms and areas where small collection of water along with flower pots are found.[30] It is difficult to control it, because it breeds in polluted water, plant saucers, flower vases, uncover water tanks, barrels, water coolers and other places where rain water collect and stored, it lays eggs in water bins around the homes, which take 10 days to be hatched.[31,32] Damp condition offers suitable breeding opportunity to mosquito larval that′s why the epidemic prevails over proportionally in rainy seasons, however rise in temperature shorten their life span.[30,33]

Figure-2 shows the transmission of Dengue virus infection, the infected mosquito take longer to bite as compare to uninfected one. This increased time corresponds dengue virus infection of organs known to control or influence the activities associated with feeding.[34] Several research endorse the presence of transovarial dengue virus transmission in infected female Aedes Aegypti, allowing the virus to spread their progeny, such process act as a reservoir for virus to support during interepidemic period.[35]

Pathogenesis

The average incubation period of Dengue virus is 7–10 days as soon as the infected mosquito bites, showing its symptoms regarding to the victim′s age and immune status, the symptoms (intense fever, constitutional signs) are observed for a week, having entered (virus) host circulation simultaneously grip the body through white blood cells. Dendritic cells, mast cells and endothelial cells are usually infected by the virus through endless series of replication with in B-cell, macrophages and in monocytes[36], probably (Dengue virus) effect spleen, bone marrow, leukocytes, lymph node, liver heart, stomach, thymus, kidney plus also disrupt blood brain barrier[37].There is possibility of both early recovery and severity in preliminary stage[38]. Occurance of overstimulation of immune system during infection tenure the virus dwindled away whereas the process leads to over production of cytokines, which badly paralyze the monocytes, hepatocytes and endothelial cells, as they have TNF-α, IFN-α and various chemical mediators. Both infected and non-infected dendritic cells are activated by IFN-α and TNF-α.[39,40]. Enormous number of various mediators and cytokines ascend vascular permeability, shock, leakage of plasma, haemostatic abnormality and hypovolemia, and it witnesses that endothelial cells go through apoptosis which disrupting endothelial cell barrier and causes generalized vascular leak syndrome.[41]

Dengue infection is caused by any of the dengue viral serotype. Generally, infection with one

serotype conferring to produce lifelong immunity against that particular serotype but no other, however new serotype infection jeopardize life, because antibodies produce during 1st infection were unable to resist, during this condition large number of antibodies were produce which lead to severe infection. This scenario is called antibodies dependent enhancement. Pre-existing antibodies cross-reacts with a new stereotype of secondary infection and cause extreme attack. Antibodies Coated virions can take up quickly by macrophages, monocytes and dendritic cells. This condition lead to high viral load, and dendritic cell present large number of antigen presentation to the T-cells which result in huge amount of T-cell activation and proliferation of memory T-cells, this condition lead to low expression of IFN-γ.[42] Common pathological findings of this infection include peritoneal effusions, petechial haemorrhages, pulmonary oedema, and serous pleural. There is no such data available which show the severity of infection belongs to which particular serotype or which serotype has significant outcome.

Clinical diagnosis

Clinical progression of the infection is venture and unambiguous in order to predict the severity of the infection, which is important for accurate prognosis. Across the globe, variety of pathogens and manifestation of same appearance and signs, may be observe in other infectious diseases, i.e., In the initial phase of dengue infection, patient present moderate undifferentiated "flu-like" fever and certain other symptoms, but such symptoms may also appear in measles, influenza, Zika, yellow fever, malaria, and chikungunya.[11] Timely and precisely identification of this viral pathogen is of particularly vital, as it leads to subsequent shock presentation. Early grabbing of the pathogen ultimately helpful in the treatment approach for the dengue-acquired shock, which is also arising from sepsis condition conventionally needs different tactics.[11] Though, paradigm-shifting observation that DENV infection triggers similar immune response close to that of sepsis, in the form of innate immune pathways as those induced in sepsis, usually targets for treatment.[43] As Dengue clinical symptoms are ambiguous, precise and rapid diagnosis is a challenge, Physicians should follow the clinical manifestation along with laboratory investigations in order to treat infection precisely.

Clinical presentation

In 2009, a team was appointed by World Health Organization (WHO), they established a set of guidelines for clinical management of dengue infection.[11] They set an organize classification for dengue disease; dengue fever, DHF, and DSS with or without warning signs and severe dengue (Figure-1). The objective of this modification is to create uniform and standard criteria that can recognize internationally for classification of this disease. Furthermore, infection with dengue virus can occur either symptomatically or asymptomatically.[44] Approximately 20% of all infections are supposed to be Symptomatic with signs of illness and experiencing a disease state having clinical spectrum of mild serious to severe clinical manifestations.[45]

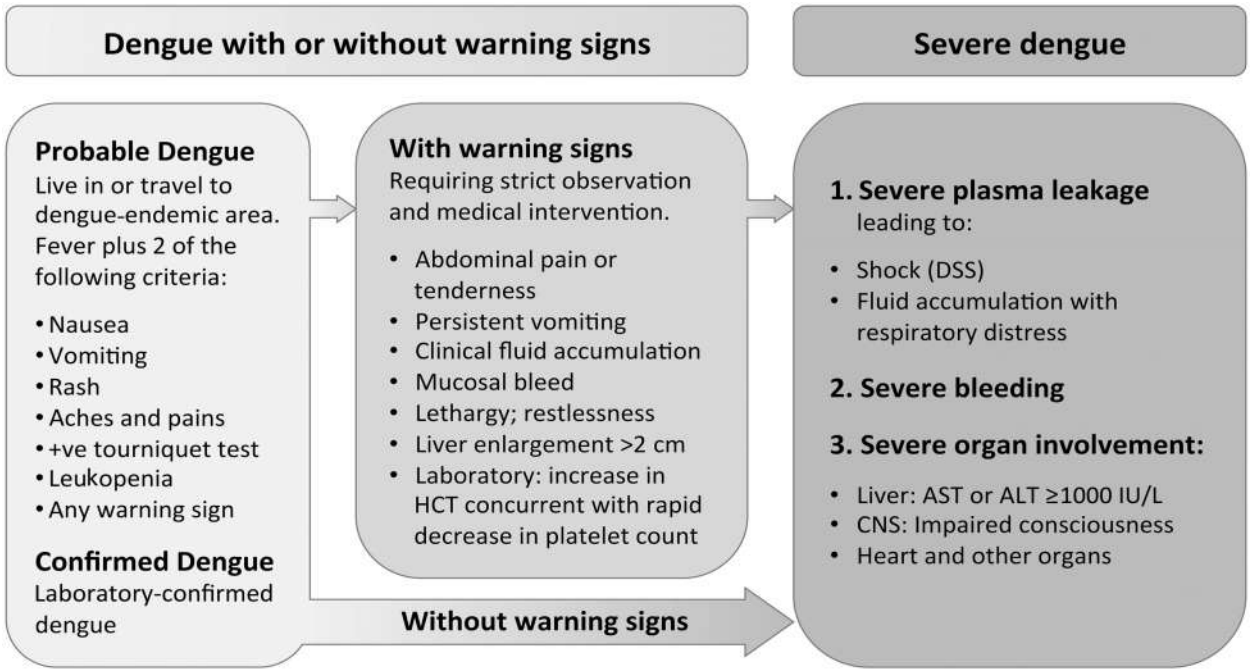

Figure-1: Development phases of dengue fever.

The WHO recommended guidelines of dengue outbreak are listed both with and without warning signs. Abbreviations: alanine aminotransferase; AST, +ve, positive, CNS, central nervous system, AST aspartate aminotransferase.[11]

Dengue induced disease has three commonly recognized phases i.e. febrile, critical, and recovery phases.[11] Proper viral diagnosis and appraisal of alarming signs of disease progression to severe stage of disease are vital for good management of patient. The febrile is the primary phase as mentioned earlier, rapid onset, with high-grade fever[44], and usually loss within 2–7 days. Patients in the febrile phase being identified by a facial flushing skin erythema, generalized body ache, arthralgia, retro-orbital eye pain, myalgia, rubeli form exanthema, photophobia, and headache[46], as well as sore throat, nausea, anorexia, and vomiting.[20] This phase is distinguished from other diseases by possessing similar symptoms, positive tourniquet test, provide demarcation.[47] Haemorrhagic signs are sometime also observe i.e., petechiae to spontaneous bleeding from the gastrointestinal tract, gums, nose, and other mucosal sites[11], along with positive tourniquet testing primary phase. Symptoms develop during this phase do not predict the degree of severity of the infection, therefore patient should be closely and regularly observed for the appearance of early warning signs of critical phase.[11]

Mostly dengue patients are diagnosed in the febrile phase, and after successful treatment the patients are unable to develop the vital dangerous state. Patients progress to advance stages may experience increase capillary permeability and eventually to vascular plasma leakage. Typically, patients get worse at the time of deffervescence (from illness day 4) when patient`s temperature tumbles to 37.5–38°C[48], and during such critical phase vascular/plasma leakage may be seen. Leukopenia, followed by a sudden drop in platelet counts in dengue fever, sometimes leads to plasma leakage[46]. Simultaneous dropping of platelet counts become a reason of in rising hematocrit level. Plasma leakage state last in 24–48 hours while in this duration, haematocrit levels needs to be carefully monitored as a predictor in term of intravenous fluid adjustment.[46,49] Alternative approaches can be used, i.e., Ultrasound for the purpose to detect free fluid in the thoracic region, if profound leakage is suspected clinically.[50] Warning signs as shown in (Figure-1) are present in patient throughout the episode before the shock appear.[50] Significant amount of plasma leakage leads to shock. The tissue perfusion occurs during intense/prolonged hypovolemic shock leads to metabolic acidosis may further progress to progressive organ disturbances and eventually intravascular coagulation.[51]

Once patient pass through this critical phase of 24–48 hour, the patient mitigates from the disease rapidly. Re-absorption of extra vascular fluids in the general benefit of the patients occur, yields appetite, and terminates all the symptoms.[51] Patients may show up “recovery rash” with patches of normal skin resembling to “isles of white in a sea of red” that appear on the trunk and leading to the head and extremities of the patients.[52] Through this recovery phase, the patient`s blood quantity stabilize and return to normal. Disease severity is characterized by the degree of plasma leakage that certainly leads to shock with fluid accumulated condition along with respiratory distress in which there is severe bleeding and eventually may result in severe organ impairment (Figure-1).[11] Noteworthy as previously mentioned, dengue-acquired shock occurs at defervescence and at a phase when viral load are dropped (Figure-2), suggesting likely immune-mediated pathology.[38] The hypovolemic shock (HVS) that appears after prolonged vascular permeability causing plasma leakage.[53] DSS patients, due to asymptomatic capillary leakage in the instance trap leading to compensated shock to deadly hypotensive shock, and eventually progress to cardiac arrest.[54,55] Dengue shock patients need to be monitored closely and regularly, the duration between alarming signs and the onset of compensated shock, hypotensive shock, and ultimate cardiac arrest can be differentiated in a short time.[53] In term of in-depth review regarding clinical presentation of severe dengue disease and its management find WHO reference book for clinical management of dengue fever.[11]

Predictive Algorithms

There is a potential algorithm has been used by different researchers for the purpose of evaluating variable feature of the DENV infection in order to predict the progression of dengue to fever to severe disease.[45,51,54] In this regard the scholars have noticed/looked the both clinical manifestation and markers (viral and host derived), which were used previously to distinguish substantially dengue fever and severe patients of dengue.[56–59] Talking about the virological markers, pin pointily virus itself or viral genome and NS1 protein in company with host factors were credited. Also, virus-specific immune responses, liver enzymes AST and ALT[60], and some haematological factors (platelet counts and haematocrit counts) have been entertained[60]. Moreover, by theses scholars some have claimed, a comprehensive multicentre clinical study which is yet to be appraised and conducted.[61] Although, the maturing of a word wide predictive algorithm in term of progression to severe disease shows challenges

due to significant variables found statistic gave by local virus evolution. Including the virus host delicate interaction also geographic or major spread of disease, and host genetics with ethnic background. However, according to the WHO are currently instructed and help clinicians while providing an unambiguous set of clinical alarming signs to assess and predict episode of severe disease and his onset. In spite of that, not all the warning or alarming signs manifest early in disease. So, when properly implemented in the respective clinic, accompany with reliable laboratory diagnosis, there is a strong framework is given by WHO guidelines in order to provide an effective appraising of severe disease.

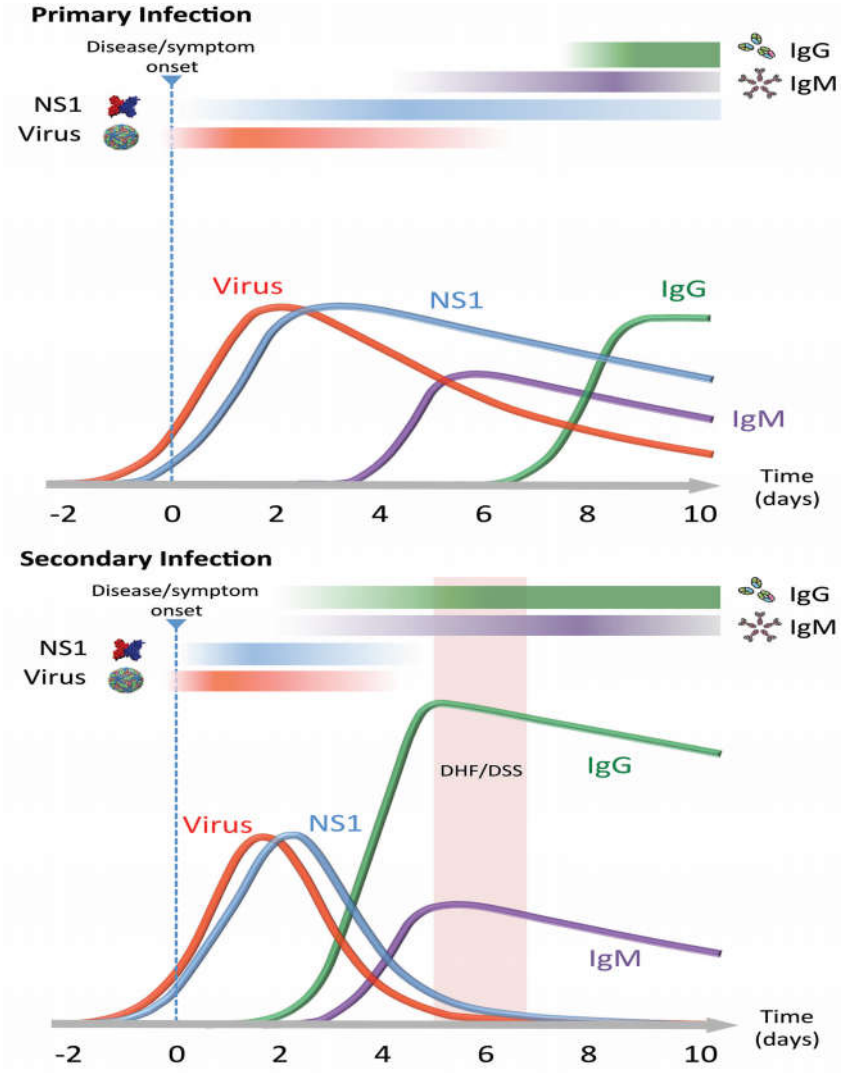

Figure-2: Time frame of the appearance of dengue screening tools in patients with secondary and primary infections

In acute infection, non-structural protein 1 (NS1), viremia, immunoglobulin M (IgM) appearing about days 3 of illness, and immunoglobulin G (IgG) appear till the end of the acute period. Secondary infections (bottom panel) are characterized by the presence of IgG early in the acute phase of disease and a shorter duration of NS1 and virus detection. Note onset of severe dengue (dengue haemorrhagic fever [DHF]/dengue shock syndrome [DSS]), primarily in secondary infections and at a time when virus and NS1 levels are falling.[73]

LABORATORY DIAGNOSIS

Biomarkers by mean dengue infection is detected are; the virus antigen itself (virus isolation in culture or mosquitoes or the direct detection of viral genomic RNA), viral products (detection of NS1 protein

which is secreted during the infection), or the host immune response to virus infection (via detection of virus-specific antibodies IgM and IgG).The time duration of these biomarkers in both acute and secondary dengue infection is shown in Figure 2. Both old and new approaches are briefly discussed below.

Virus Isolation

In this traditional approach, virus was directly isolated for diagnosing DENV infection. this technique has been replaced by molecular technique; reverse-transcription polymerase chain reaction (RT-PCR), and in latest time by more develop, rapid, and time saving protocol i.e. invading NS1 antigen through enzyme linked immunosorbent assays (ELISAs).[62] In isolating virus, patient sample are collected and cultured in different cell lines of either mosquito (AP-61, AP64, C6/36,Tra-284, and CLA-1 cells) or mammalian(Vero, LLCMK2, and BHK-21 cells) origin or in live mosquitoes.[26] For successful result, Blood sample must collect from infected patients facing about 5 days febrile illness after the onset of infection. Virus isolation in the patients having secondary infection is unfavourable due to rapid anamnestic production of cross-reactive antibodies during the acute phase of infection that form immune-complexes with circulating virus.[26] Though, virus isolation method is definitive for diagnosing DENV, but it is not in practical as isolation can take few days to weeks.[63]

Reverse transcription polymerase chain reaction (RT-PCR)

For effective diagnosis of DENV infection, molecular protocols such as RT-PCR and nucleic acid hybridization has made a significant contribution, PCR-based methods provide the same or next day diagnosis report of DENV. Initially, Lanciotti *et al*[63] reported a highly sensitive 2-step heminested RT-PCR assay, later approach was then updated to a single-step RT-PCR multiplex real-time assay that was implemented worldwide.

A significant benefit of PCR-based techniques is that it is possible to capture viral RNA from the onset of disease and is therefore more sensitive, accurate, fast, fewer complex and affordable than methods of viral isolation.[64] As PCR-based methods are rapid and accurate, need specialized equipment and experience trained staff to perform the protocol accurately and carefully. In resource-poor remote settings where dengue is endemic, in such areas these methods are not always an option. In regards, as can be seen by the availability of commercial kits, large proportion of documented RT-PCR methodologies are established in-house and there is no centre-to-centre standardization.[65]

NS1 Antigen Capture

The viral protein NS1 is an excellent therapeutic tool as it is secreted by infected cells, can be observed in the blood of infected people at a higher-concentrations, and thus can be detectable from onset of symptoms until 9 days or longer till the onset of infection, particularly in primary infections. NS1 can be identified concurrently with viral RNA in primary infections before an antibody response is elevated. NS1 concentration can be seen as a viremia substitute marker, to quantify viral titer.[27] Detection of NS1 was first outlined in 2000 via using an approach antigen-capture ELISA.[27] By using quantitative capture ELISA, it was observed that NS1 is secreted at high concentrations, varying from minimum nanograms per millilitre to micrograms per millilitre, in other infected people circulation up to 50 μg / mL. Later studies evaluate the kinetics of NS1 in secondary infections find NS1 amounts being a reliable indicator of progression to a more severe illness within the first 72 hours of disease.[38]

Such preliminary reports have resulted in the redevelopment of NS1 capturing ELISAs and rapid strip testing.[66] The redevelopment of NS1 as a biomarker has innovated dengue diagnosis as it is simple and inexpensive-tech assays and has offered high sensitivity and specificity. Such assay is now the default method for dengue prognosis[67], facilitating early detection as well as faster patient management. Regarding the potential predictive value of NS1 as a predictor of disease progression, the appropriate quantity remains the province of academic research, other commercial testing mostly presenting qualitative positive / negative results. The steady anamnestic increase in NS1 cross-reacting antibodies during acute phase has been a limiting factor for NS1 detection in patients with a secondary infection. In immune complexes, such antibodies sequester NS1 which could not be easily and efficiently identified in capture assays. Therefore, during secondary infections, the kinetics of identification of NS1 during course of disease are lower than for primary infections (Figure-2).

Serology

Multiple approaches are exist in order to serological diagnosis of DENV, namely Western blotting, fixation complement testing, hemagglutination inhibition (HI) assays, indirect immunofluorescent antibody tests, dot-blot assays, plaque reduction neutralization tests and also some antibody capturing IgM and IgG ELISAs.[67] HI assays is demonstrated to have the most valuable serological diagnostic methods for routine DENV detection along with IgM and IgG antibody-capture ELISAs. For many years, the HI test has been used for dengue diagnosis, with most laboratories developing in-house

methodologies, although there are also commercial kits offered.[47] As in all antibody detection-based biomarkers, early acute disease period usually appears a negative window detection due to the need to provoke the substantial antibody response. IgM may appear in primary infection as early as day 3–5 and peak several weeks after recovery and persist elevated for the next few months.[68] IgG may not usually appear during primary disease acute phase. Furthermore, with secondary infecting there may be a steady anamnestic response of IgG to shared epitopes on multiple viral proteins, IgG making an appearance after 03 days onset of disease.[68]

IgM and IgG identification can thus provide a predictive indicator for primary or secondary infection depending on the ratio of IgM and IgG, during acute disease period[69] when practiced in parallel. Serological detection of DENV is complex in parts of the world where > 1 flavivirus circulates (e.g., yellow fever, Japanese encephalitis, and even more lately, Zika virus), due to shared cross-reactive epitopes of the E protein flavivirus, and thus cross-reactivity of the response of the antibody. It is hugely problematic in the latest prevailing epidemic of the Zika virus in Brazil, which occurs in the specific instance of complicating DENV infection and serology. In DENV serology screening tests, antibodies interact directly against certain flaviviruses, and turn results in misleading-positive results. IgM and IgG serology should be combined with NS1 antigen capture in order to minimize such erroneous-positive results. Commercial dengue NS1 antigen-capture ELISA and immunochromatographic (Rapid) strip testing were programmed to be highly specific, with little or no observable cross-reactivity with other NS1 species of flavivirus.[70] Though, the specificity of such assays need to be re-evaluated[71] with the probable cross-reactivity observed in an earlier patient infected with Zika virus.

Combined Approaches

In terms of diagnosis, each biomarker has different kinetics, no specific assay can we rely in order to assess the DENV in patients having different stage of infection. NS1 antigen detection could be the most vigorous among all DENV diagnostic assays, with a relatively long window detection primarily in primary infection. As mentioned earlier, the reliability of NS1 detection in secondary infection can be obscured by the production of antigen-antibody complexes (Figure-2). Although in such cases the investigation of NS1 along with antibodies IgG/IgM enhance the detection significantly.[72] Presently many diagnostic kits (SD Bioline Dengue Duo (NS1 Ag + Ab Kit) are available that use this approach in rapid point-of-care devices

Future Approaches

Currently researchers are working on several techniques, in order to develop rapid and effective diagnosis to the infection, which comprises micro/paper fluidics, in vivo micro-patches, electrochemical and isothermal PCR and piezoelectric detection.[73]

These technologies are at preliminary stage, need improvement in order to make them practical. In the latest review, it is suggested that utmost approach acquire to the infection can through an approach that can distinguish both primary and secondary infection by invading antibodies (IgM and IgG), along with investigating quantitative serotype-specific NS1.

CONCLUSION

It is pertinent to arrange accurate and appropriate patient`s care in order to diagnose and manage efficiently. Yet it is alarming and challenging for current global researchers as it doesn't have any reliable technique, to predict the state that progress towards severe phase. Some studies in early time have indicated that NS1 and viremia may have prognostic importance, but due to lack of availability of quantitative NS1 assay, less study has been conducted for its validation purpose. Host responses have significance role in terms of predicting disease state, after the importance of biomarkers assessment. Several proteins have diagnostic importance, however yet it is necessary to test the predictive value to validate their practice. A massive multicentre study is presently being progressed in different parts of the world in order to identify a reliable predictive marker to prevent infection progress from advance stages. Presently dual approach is used to diagnose the infection, including detecting virus or viral particle and by serological assay, for appropriate management clinician should also observe the clinical manifestation to ensure patient state of being progress to severe dengue disease.

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