REVIEW

Progress in the diagnosis of dengue virus infections and importance of point of care test: A Review

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Abstract: It is an urgent need of highly sensitive, specific and economical diagnostic tools for early and fast diagnosis of highly challenging dengue virus infections. Many laboratory methods including virus detection, genome detection, antigen detection and serological detection of such short-lived viremia were explored but promising outcomes for economical immunochromatographic tests have been reported in this review. With the trend of fast, easy operation, rapid diagnostic tests (RDT) based on immunochromatographic assays are of great importance due to point of care test (POCT) in the dengue endemic regions where it is short of laboratory equipments and cold storage conditions. Such kind of point of care diagnosis is more efficient, fast and user friendly. Moreover, the development of highly advance RDT is dependent on the use of anti-dengue monoclonal antibodies highly specific for particular analyte/antigen.

Keywords: Dengue, diagnosis, rapid diagnostic tests, antibodies.

INTRODUCTION

Dengue is emerging as an enduring global threat in the tropical and subtropical developing countries of the world. This arthropod-born viral disease causes millions (50-100 million) of infections each year. World Health Organization (WHO) recently estimated that the prevalence of dengue has increased 30-folds compared with the situation 50 years before, thus affecting both human health and the economy (WHO, 2012). The major factors contributing to the spreading dengue viruses include the expanding urban population and environment, an increase in mosquito vectors density and the ease and frequency of rapid air travel allowing the daily movement of viremic individuals (Chien et al., 2006; Gubler, 1999; Gubler and Clark, 1995). Dengue virus (Flaviviridae family) is transmitted to human by mosquitoes Aedes aegypti or Aedes albopictus. The incidence of all types of dengue viruses (serotypes 1-4) causes primary and secondary infections. Primary infection causes an acute febrile illness commonly identified as dengue fever, which is cleared in around seven days by a complex immune response while secondary infection causes dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF and DSS are more lethal and differentiated by high fever, bleeding, thrombocytopenia as well as haemoconcentration. Common symptoms of dengue patients after 3-4 days onset of fever are: rash, petechiae, epistaxis and gingival as well as gastrointestinal bleeding. Low pulse rate or hypotension, plasma leakage, cold and moist skin and disturbed mental conditions can also be observed in severe infection (Pan American Health Organization, 1994; Nimmannitya, 1987; Martinez, 1992; Halstead, 1992).

Dengue virus is positive-sense single-stranded RNA virus, consists of a single open reading frame (approximately 11 kb) encoding a polyprotein precursor, which is proteolytically cleaved into capsid (C), premembrane (prM) and envelope (E) proteins (structural proteins) while seven nonstructural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) are also observed. Replication and assembly of virus depend on the accurate cleavage of the viral protein (Rice et al., 1985; Cahour et al., 1992). There are several evidences that these proteins especially NS1 play an important part in different stages of the virus life cycle and contribute a vital role in RNA replication and various cellular functions (Welsch et al., 2009; Mackenzie et al., 1996; Lindenbach and Rice, 1997; Muylaert et al., 1997; Westaway et al., 1997). Dengue viremia is very short and within few hours after infection, thousands copies of viral molecules are produced from a single viral molecule, which causes cell damage as well as becomes lethal. RNA polymerases and other cellular factors are involved in catalyzing the infection cycle to dengue virus (Hidari and Suzuki, 2011; Filomatori et al., 2006).

This review is focused on the potential of commonly used different laboratories methods for diagnosis of dengue virus infections and importance of immunochromatographic assays with their future needs.

Diagnosis of dengue infection

With the increasing global incidence of dengue disease due to short viremia and the absence of effective vaccines
for its prevention, rapid, economical and accurate diagnosis of infections during the first few days of clinical symptoms is crucial to provide proper treatment for the patient and early public health control of dengue outbreaks (Andries et al., 2012; Tang and Ooi, 2012). Recently WHO stated that morbidity and mortality from dengue infections can be reduced to almost zero by the implementation of timely, appropriate clinical management, which involves early clinical or laboratory detection, proper staff training, intravenous rehydration and hospital reorganization (WHO, 2012). Diagnosis of dengue infection has always been a great challenge due to short life cycle of virus. Following laboratory methods are generally adopted for diagnosis of dengue infections.

**Virus detection**

The dengue virus was isolated for the first time from the blood samples of patient in Japan in 1943 by Kimura and Hotta (1943). Four isolation methods for dengue virus detection have been employed: mosquito inoculation (Aedes aegypti or Aedes albopictus) (Rosen and Gubler, 1974; Jarman et al., 2011), mosquito cell cultures using C6/36 lines or AP61 (Chua et al., 2011; Oliveira et al., 2003; Tesh, 1979; Kuberski and Rosen, 1977), mammalian cell culture using vero cells or LLC-MK2 cells (Matsumura et al., 1971; Yuill et al., 1968) and intracerebral inoculation of suckling mice (Meiklejohn et al., 1952; Lee et al., 2005). Although virus isolation methods provide high sensitivity and specificity (table 1), but there are few limitations, which include long time (about 7 days), acute samples (0-5 days post onset), experienced staff, appropriate facilities and high cost. Consequently, this approach is not commonly used in routine diagnostic laboratories.

**Genome detection**

As cell culture based detection methods usually require seven days, reverse transcriptase polymerase chain reactions (RT-PCR) were developed for rapid genome detection and typing of dengue viruses (Lanciotti et al., 1992). Usually, RT-PCR need one day for operation and has been confirmed as a valuable research tool in pathogenesis and molecular studies. RT-PCR is also commonly used for dengue virus (DENV) RNA detection in infected cells, serum, plasma, infected mosquito larvae, fresh and paraffin-embedded tissues and formalin-fixed tissue. Sensitivity and specificity of the conventional RT-PCR in different detections ranges from 48.4-100% and 100%, respectively (Lanciotti et al., 1992; Raengsakulrach et al., 2002; Chua et al., 2011). Later, real time RT-PCR for diagnosis and quantification of dengue virus was developed and its sensitivity ranged from 58.9-100% and specificity was 100% (Shu et al., 2003; Lai et al., 2007; Gurukumar et al., 2009; Pok et al., 2010; Hue et al., 2011; Waggoner et al., 2013). The dengue virus isolation rate was also improved by nested PCR and mostly it was up to 79.4% (Jarman et al., 2011). Nucleic acid sequence based amplification assay (NASBA) is another method to detect the dengue viral RNA from the whole blood or serum from infected patients in the acute phase (Wu et al., 2001) and it showed high sensitivity (98.5%) and specificity (100%). Moreover, RNA extraction from whole blood was found more sensitive (90%) as compared to plasma/serum (62.0%) in the same pool of sample (Klungthong et al., 2007). RNA detection methods can confirm the infections within 24-48 hours with high sensitivity and specificity. Identification of serotype and genotype can also be performed. But these methods require acute phase samples, expensive laboratory equipments and trained technicians. False positive results are also frequently observed due to sample contamination (Peeling et al., 2010).

**Antigen detection**

Among all virus antigens, NS1 was found at the concentration of several micrograms per milliliters in the serum of infected patients from the start of fever up to the ten days (Young et al., 2000; Alcon et al., 2002; Alcon-Le Poder et al., 2006). The quantity of NS1 circulating in the blood of DHF infected patients was found to be significantly higher than dengue fever patients (Libraty et al., 2002). The maximum diagnosis of NS1 antigen can be obtained between days 3 and 5 in both kinds of primary and secondary infections. All these findings indicate that NS1 is an attractive marker for the diagnosis of infection (Alcon et al., 2002).

Antigen detection is considered to be more important and highly sensitive method compared with others at present. For dengue viral antigens detection, immunofluorescence assay (IFA) and radioimmunooassay (RIA) can not be used for routine diagnosis tests because of low sensitivity (Monath et al., 1986). Dengue antigen detection by immunohistochemical techniques (using alkaline phosphatase labels or horseradish peroxidase) (Pelegino et al., 1997) is also used but not effective. Young and co-workers (2000) firstly successfully standardized a capture NS1 ELISA for NS1 detection in serum during the acute phase of secondary infection. The availability of commercial kits in the markets for dengue NS1 antigen diagnosis in acute serum provides an alternative to the existing methods such as virus isolation, PCR and serology. Some commercial kits based on ELISA format are also available in markets. Panbio (Australia), Biorad (France), and Standard Diagnostics (SD) Bioline (South Korea) are the major companies for manufacturing the commercial rapid diagnostic tests (RDT) for NS1 antigen tests based on ELISA assays. Many studies were performed to evaluate these commercial kits (Kumarasamy et al., 2007; Bessoff et al., 2008; Chaterji et al., 2011; Puttkhun et al., 2011; Blacksell et al., 2012), which showed in table 1. The sensitivities observed in these studies ranged from 94.7-98.3% for...
primary infections and 67.1-77.3% for secondary infections while specificities were 100%. However, the NS1 detection was less sensitive in secondary infections as compared to primary infections. The lower NS1-antigen detection in dengue secondary infection can be attributed to NS1 antigen complexing with anti-NS1 antibodies (Young et al., 2000; Libraty et al., 2002). These ELISA format rapid diagnostic tests employ serotype-specific anti-NS1 monoclonal antibodies to capture and detect soluble NS1 antigen in serum, plasma, or blood. Antigen detection assays based on ELISA can confirm the infection easily and are less expensive. But it is not as sensitive as virus isolation or DNA detection methods.

Serological detection
Serological testing is the widely adopted method in the hospitals for the diagnosis of dengue infections. Serology is a valuable support in the diagnosis of dengue infections (Lam, 1993; Lam, 1995). Traditionally, hemagglutination inhibition (HAI) assays have been used for the diagnosis of dengue (Vaughn et al., 2000). Complement fixation (CF) and neutralization test (NT) are also used for diagnosis but not very common (WHO, 1986; Clarke and Casals, 1958).

For proper and timely treatment of dengue-infected patients, it is compulsory to diagnose the primary and secondary infections accurately. Mostly, hospital laboratories and commercial kits manufacturers defined acute primary dengue virus infection as an IgM positive and IgG negative and acute secondary dengue virus infection as IgM positive and IgG positive or IgM negative and IgG positive. But authenticity of such claims was only confirmed by several studies (Blacksell et al., 2006; 2007; 2011).

Innis et al. (1989) demonstrated that stronger and specific IgM antibody isotype was the first immunoglobulin to emerge in the primary infections while anti-dengue IgG appears at the end of the first week of clinical symptoms in a low titer and then increased slowly. On the other hand, antibody titers increase rapidly during a secondary infection and generally antibody reacts with other flaviviruses. In the acute phase of infection, IgG is detectable with high levels following by the considerable rise during the next two weeks. False negative reactions of anti-dengue IgM can also be observed in secondary infections. Enzyme-linked immunosorbent assays (ELISA) especially MAC-ELISA corresponds to the most important advances for IgM detection for routine dengue diagnosis. Other formats like dot-ELISA, capture ELISA, AuBioDOT IgM capture and capture ultramicro-ELISA, GAC-ELISA for IgG are also developed (Innis et al., 1989; Nawa et al., 2001; Vázquez et al., 1998). ELISA based kits for anti-dengue IgM and IgG detection are also commercially available prepared by different companies (Dengue virus IgM ELISA and IgG ELISA by Standard Diagnostics, South Korea; Panbio dengue virus IgM capture ELISA, Panbio dengue virus IgG capture ELISA by Alere, Australia etc.). Several studies used these commercial kits for the evaluation of these commercial kits (Blacksell et al., 2012; Berde et al., 2012; Hunsperger et al., 2009; Chua et al., 2011; Groen et al., 2000). The ranges of sensitivities for IgM and IgG ELISA kits were observed from 20.22-99% and 7.8-88.9%, respectively (table 1) while specificities ranged from 52-100% for IgM and 63.5-100% for IgG. Due to the cross-reactivity of IgM with other flaviviruses such as yellow fever and Japanese encephalitis, dengue serotype determination is not reliable (Vázquez et al., 2003). Presently, anti-dengue IgM and IgG detection based on ELISA is generally used to differentiate between the primary and secondary infection. Because IgM and IgG show the cross action with other flavivirus diseases, this method is not very successful and false positive results are obtained.

Improvement of the diagnostic tests
All above-mentioned protocols represent many advances in dengue diagnosis and are performed in the laboratories with different equipments and tools for many years. However, these methods are time consuming, costly, requires specific laboratory equipment and experienced technicians. The global prevalence of dengue infections is remarkably increasing especially in the developing countries where it is in short of laboratories facilities. In such dengue endemic condition, development of more sensitive, specific, cheap and easy tests are still necessary for early dengue diagnosis.

Importance of the immunochromatographic assays
Presently, more advanced biosensors are developed and used extensively in diverse fields like forensic, medical or environmental areas (Nakamura and Karube, 2003). Among all those biosensors or assays, improvements of immunochromatographic assays using monoclonal antibodies as ligands, are of significance. These assays can be used easily in dengue endemic regions especially in the developing countries with limited or no laboratory resources and no cold storage facilities. The immunochromatographic assay consists of a membrane strip acts as the immunosorbet. This strip is very useful for point of care tests (POCT) with fast, cheap and one step analysis. The essential features of this assay include user-friendly operation and short time for analysis of results (Vaughn et al., 1998; Sang et al., 1998). For immunochromatographic assay, tests can be performed without using any reagents like ELISA and person without any expertise can perform it. This assay is also known as one-step assay, which speed up the analytical procedure. Due to all above-mentioned properties, this one step assay can be now broadly used as a suitable system for the detection of dengue infections especially in developing countries.
### Table 1: Description of different types of commonly used laboratory diagnostic tests for dengue infections

<table>
<thead>
<tr>
<th>Type</th>
<th>Methods</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Virus Detection</strong></td>
<td>Virus isolation by mosquitoes</td>
<td>71.5-84.2</td>
<td>100</td>
<td>Jarman et al., 2011; Kuberski and Rosen 1977</td>
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<td></td>
<td>Virus isolation by cell culture (C6/36)</td>
<td>40.5</td>
<td>100</td>
<td>Chua et al., 2011</td>
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<tr>
<td><strong>Genome Detection</strong></td>
<td>RT-PCR (RNA detection)</td>
<td>48.4-100</td>
<td>100</td>
<td>Lanciotti et al., 1992; Raengsakulrach et al., 2002; Chua et al., 2011</td>
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<td></td>
<td>Real time RT-PCR (RNA detection)</td>
<td>58.9-100</td>
<td>100</td>
<td>Waggoner et al., 2013; Hue et al., 2011; Pok et al., 2010; Lai et al., 2007; Chien et al., 2006</td>
</tr>
<tr>
<td><strong>Antigen Detection</strong></td>
<td>ELISA Kits (NS1 detection)</td>
<td>94.7-98.3 for primary infections and 67.1-77.3 for secondary infections</td>
<td>100</td>
<td>Kumarasamy et al., 2007; Bessoff et al., 2008; Chaterji et al., 2011; Puttikhunt et al., 2011; Blacksell et al., 2012</td>
</tr>
<tr>
<td><strong>Serological Detection</strong></td>
<td>ELISA for IgM detection</td>
<td>20.22–99.0</td>
<td>52-100</td>
<td>Hunsperger et al., 2009; Groen et al., 2000; Chua et al., 2011; Blacksell et al., 2012; Berde et al., 2012</td>
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<tr>
<td></td>
<td>ELISA for IgG detection</td>
<td>7.8-88.9</td>
<td>63.5-100</td>
<td>Groen et al., 2000; Hunsperger et al., 2009; Chua et al., 2011; Blacksell et al., 2012; Berde et al., 2012</td>
</tr>
<tr>
<td><strong>Immunochromatographic</strong></td>
<td>SD Bioline Dengue Duo kits for IgM detection</td>
<td>53.5-79.2</td>
<td>89.4-100</td>
<td>Wang and Sekaran, 2010; Blacksell et al., 2011; Tontulawat et al., 2011</td>
</tr>
<tr>
<td>Assays based detection with famous commercial kits</td>
<td>SD Bioline Dengue Duo kit for NS1 detection</td>
<td>44.4-70.6</td>
<td>73.4-100</td>
<td>Ferraz et al., 2013; Andries et al., 2012; Tontulawat et al., 2011; Blacksell et al., 2011; Osorio et al., 2010; Wang and Sekaran, 2010; Tricou et al., 2010</td>
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<td></td>
<td>SD Bioline Dengue Duo kit of NS1, IgM and IgG</td>
<td>75.5-92.9 for NS1/IgM; 80.7-85.7 for NS1/IgM/IgG</td>
<td>88.8-100 for NS1/IgM; 83.9-97.9 for NS1/IgM/IgG</td>
<td>Andries et al., 2012; Blacksell et al., 2011; Osorio et al., 2010; Wang and Sekaran, 2010; Tricou et al., 2010</td>
</tr>
<tr>
<td>combination detection</td>
<td>Panbio Dengue Duo cassette for IgM or IgG detection</td>
<td>54.5-81.8 for IgM; 62.1-87.5 for IgG</td>
<td>75.0-97.6 for IgM; 66.6-94.4 for IgG</td>
<td>Blacksell et al., 2006; Nga et al., 2007; Moorby et al., 2009; Blacksell et al., 2011; Pan-ngum et al., 2013</td>
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<td>Panbio Early Rapid NS1</td>
<td>45.9-88.06</td>
<td>92.5-100</td>
<td>Fry et al., 2011; Blacksell et al., 2011; Ferraz et al., 2013; Pan-ngum et al., 2013</td>
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<td></td>
<td>Panbio Early Rapid NS1 and Duo assay kit for detection of NS1, IgM, and IgG combination</td>
<td>89.0-89.9 for NS1/IgM; 87.0-93.0 for NS1/IgM/IgG</td>
<td>75.0 for NS1/IgM; 82.8 for NS1/IgM/IgG</td>
<td>Fry et al., 2011; Blacksell et al., 2011; Pan-ngum et al., 2013</td>
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The activity of immunochromatographic assay is dependent on the ligands. These ligands are highly specific monoclonal antibodies (MAbs) against dengue virus antigen. When blood sample of dengue infected patient containing antigen (analyte) is allowed to run, the analytical signals come out within 15-20 min in the form of red colored lines after the particular interaction of ligand (antibody) and analyte (antigen). The capillary action mechanism is involved for this kind of interaction occurred on the membrane. Such interaction of antigen to antibody can be monitored fastly, effortlessly and with high sensitivity (Vaughn et al., 1998; Sang et al., 1998).

Evolution of dengue rapid diagnostic test based on immunochromatographic assay

Few rapid diagnostic kits based on immunochromatographic assays for dengue NS1 antigen, IgG, IgM and IgA antibodies detection are developed by some companies with higher specificities and sensitivities as compared to ELISA based RDTs. Immunochromatographic tests (ICT) for IgM, IgG or IgA antibodies or NS1 antigen detection have been existed in different forms for more than one decade (Standard Diagnostics BIOLINE Dengue Duo NS1antigen and IgG and IgM Combo Device for NS1 Ag IgM/IgG analytes, Alere Panbio Dengue Early Rapid Kit for NS1 Ag analyte, Alere Panbio Dengue Duo cassette for IgM/IgG analyte, Biorad STRIP for NS1 Ag analyte, MP Diagnostics ASSURE for IgA analyte).

Dengue RDT/ICT based on IgG detection has been forbidden due to the enduring persistence of dengue IgG antibodies in human blood, which gives false positive results. So, the first generation of kits for IgM, IgG or IgA antibodies detection developed by the above mentioned commercial companies had lower sensitivity and specificities, which is also mentioned by Blacksell et al. (2012). Therefore, we compared three kinds of kits developed by Standard Diagnostics BIOLINE and Panbio commercial companies with latest references (table 1).

In the recent years, the most important development for the diagnosis of dengue infections was the advent of the specific detection of NS1 antigen in the blood or serum samples of dengue-infected patients. As NS1 was found to be an interesting target due to its presence from the first day of infection, next generation of ICTs for NS1 antigen detection showed better efficiency compared with the first generation of kits (table 1). The ICTs developed by SD Bioline for NS1 detection showed sensitivity ranged from 44.4-70.6% and specificity ranged from 73.4 to 100% while sensitivity and specificity for ICTs developed by Panbio was ranged from 45.9-88.06% and 92.5-100%, respectively (Andries et al., 2012; Blacksell et al., 2011; Tricou et al., 2010; Wang and Sekaran, 2010; Osorio et al., 2010; Tontulawat et al., 2011; Ferraz et al., 2013; Fry et al., 2011; Pan-ngum et al., 2013). Generally, the better results of strips for NS1 detection were specific to primary infections (Nga et al., 2007; Moorthy et al., 2009; Tricou et al., 2010; Hang et al., 2009). But several scientists also mentioned better diagnostic accuracy for dengue NS1 antigen during the secondary infections (Najioullah et al., 2011). NS1 antigen detection is very important for point of care diagnosis in the dengue endemic regions and it is found to be more useful for early phase of infection. Therefore, combination of NS1 antigen and IgM/IgG antibodies ICTs developed by different commercial companies appear to be more valuable for attaining the high sensitivity and specificity results. Recently, commercial kits for analyte combination of NS1 antigen and IgM/IgG antibodies developed by same companies are also evaluated by different researchers as mentioned in table 1. The overall sensitivities observed for ICTs for combination of analytes ranged from 80-93%. This new generation of ICTs is more efficient and showed higher sensitivities and specificities as compared to ELISA format tests (Lima et al., 2010).
Urgent need for highly specific antibodies

The requirement for point of care diagnostic tests has directed the proliferation of antibody based RDTs for tropical diseases (dengue, malaria and leptospirosis) using the immunochromatographic test format (Blacksell, 2012). Highly specific MAbs can be produced in unlimited quantities against any target antigen. MAbs recognize unique antigenic epitopes, thus allowing the accurate diagnosis of infection (Siddiqui, 2010). The efficiency of RDTs/ICTs becomes higher with the use of more serotype specific monoclonal antibodies during the preparation of rapid diagnostic kits. The production of monoclonal antibodies for dengue virus NS1 antigen detection or combination of NS1/IgM/IgG detection was found to be significant, which led to the improvement of rapid diagnostic tests for dengue infections with higher sensitivity and specificity. The immunodiagnostic assays with high sensitivity for NS1 detection can also be developed by the use of bi-specific monoclonal antibodies which are engineered bi-functional proteins with dual binding properties within a single entity. The inexpensive and sensitive NS1 detection based immunohosting utilizing bi-specific antibodies for the diagnosis of dengue is recently developed by Ganguly et al. (2013). Bi-specific antibodies which are composed of fragments from two different monoclonal antibodies, have ability to increase the sensitivity of existing diagnostic assays due to the monovalency for antigen as well as direct binding to the enzyme (Malabadi et al., 2012).

In this review, promising outcomes for economical and rapid immunochromatographic tests have been reported as compared to the existing laboratory techniques (table 1) but still a lot of scientific work has to be accomplished to take the technology to the next level. It is an urgent need to overcome the shortcomings for the improvement of existing technologies and instantaneous persuasion of new technologies for the proper control of dengue infections.

CONCLUSION

Dengue disease is spreading at alarming rate in almost every country located in the tropics. There is no suitable vaccine available in market until now. So, improved diagnostics is the only way for early and proper treatment of dengue infected patients. Although laboratory techniques like virus isolation in mosquito cell lines, PCR, IgM or NS1 capture ELISA have major advantages in dengue diagnosis. However, all these techniques are expensive and require experienced technicians. In addition, most of dengue endemic developing countries do not have laboratory or cold storage facilities. These techniques cannot be implemented in the developing countries. Consequently, immunochromatographic assays based rapid diagnostic strips were found to be successful and efficient tools in such developing countries for point of care test. These strips are user friendly, cheap, and good sensitivity and specificity results can be obtained even at high temperature. The high efficiency of rapid diagnostic kits based on ELISA or immunochromatographic assays is depended on the use of highly specific antibodies during the preparation of kits. Accordingly, it is need of the hour to emphasize on the development of highly specific and more advance antibodies that can be more useful for the development of highly advance diagnostic assays.

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