Molecular Diagnostics in Dengue

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Diagnostic Approach for Dengue

- Viral antigen or viral nucleic acid
- Viral Isolation
- Serology
Diagnostics
Diagnostic markers – What, When and Why

OPPORTUNITY

DIRECT METHODS
Antigen Detection (NS1)  Genome detection  Virus Isolation

INDIRECT METHODS
Serology IgM  Serology IgG

CONFIDENCE

1 2 3 4 5 6 7 8 9 14 days 3 months Year

Isolation of virus
Onset of symptoms
Detection of IgM
Detection of IgG
TIME COURSE OF DEVELOPMENT OF IMMUNE RESPONSE TO DENGUE

**Antibody titre**

- D1
- D3
- D5
- D10-18
- D30-90
- D3
- D10-18

**Antigen titre**

- Viral RNA
- NS1
- IgM
- IgG
Molecular Techniques - History

- 1971- First description of an enzymatic reaction to replicate DNA (Kleppe; Khorana)
- 1976- Discovery of Taq polymerase
- 1983- PCR was invented (Mullis)
- 1986- First PCR machine was invented
- 2003- Real time chemistry was introduced
- Post 2005-now- Revolutionised diagnostics- able to detect and quantify, ability to multiplex, availability of lyophilised reaction mixs.
Principle behind PCR (eg of molecular technique)

DNA – 1 copy

Known sequence  Sequence of interest  Known sequence

PCR

Lots of copies
Molecular Detection of Dengue

- Utilising a platform to Detect Nucleic Acid component of Dengue virus

- Highly specific and sensitive due to selection of primers/probes or other platform for genomic detection.

- Enzymatic reaction, amplifying target to levels that can be detectable

- Many platforms available for this approach
Diagnostics
Dengue Viral RNA Detection by rRT-PCR

- Dengue Viral RNA Detection- Detection of dengue viral nucleic acid by Multiplex Real Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR)

- Useful in early onset (during viraemia), up to Day 5/6 of illness

- Able to detect and serotype simultaneously- useful for surveillance as information about circulating dengue serotype useful as a predictor for dengue outbreak.

- Also useful as one of the diagnostic tool in fatal dengue cases (samples include serum, liver biopsy and CSF)
Sensitivity of rRT-PCR can be hindered by inhibitors such as Rnase enzyme and antibodies, which are also present in patients.

Sensitivity best (first 5/6 days) vs NS1 antigen (Up to first 9 days) but highly specific due to use of probe.

Requires special temperature for storage and transportation.

Available in Virology Unit, IMR, National Public Health Laboratory, Public Health Laboratories.
The cost of the test is estimated to be about RM160 (four-plex PCR)

But currently cost is only RM100 per test as price revision has not been approved.

Charges are only for requests from private laboratories/hospitals.
Findings:

Den 1 and Den 2 co-pre dominate for the past 2 years

Starting from mid 2016, D1 pre dominate, followed by Den 3

As a warning system in the event of change in dengue serotypes
Dengue- Multiplex Real Time RT-PCR

- Single assay that can detect as well as serotype dengue viruses simultaneously
- Up to 5 targets including internal control
- Utilises fluorescents probes for each target
- Highly sensitive (can detect up to 2 copy number) and specific (primers and probe specific for each dengue serotype)
- Rapid- results in 60 minutes
Dengue- Multiplex Real Time RT-PCR

- Utilises individuals probe (colour) for each target
- Each colour is indicative for each dengue serotype
- Assay takes about 60 minutes to complete

Suitable samples: Serum, plasma, CSF, tissue samples

Test Controls: Positive Control, NTC, Negative Control

Threshold Cut-Off: 10-40, Sigmoidal curve
Dengue- Multiplex Real Time RT-PCR

Den 1

Den 2

Den 3

Den 4

Multiplex
Advantages of Multiplexing

- Safe on costs - more targets with optimised reaction
- Shorter TAT - able to detect more targets simultaneously
- Provide better algorithm of testing for “targeted group” for testing. Eg Flavivirus - Dengue & JE & Zika
  Or Arboviruses in Malaysia - Dengue & Chikungunya & Zika
- Challenges - optimisation to ensure probes and primers are able to “work together” without interferences or inhibiting each other or priming non intended targets
Dengue- Quantitative Real Time RT-PCR

- Able to quantify dengue viral load by including known copy number of dengue virus as standards

- Useful in dengue pathogenesis study, helpful in close monitoring of patient due to prolonged viraemia
Designated PCR Working Areas

- **Pre-PCR**
  - RNA/DNA extraction
  - Mastermix preparation

- **Post-PCR**
  - Equipment/Gel electrophoresis
Advances in Reagents for PCR

- Availability of newer taq polymerase- highly efficient and better thermo stability.

- Smaller volume reaction mix- reduced cost
  From reaction- 50 ul……..then 25 ul…..now 10 ul

- Enables higher throughput testing- can be used as screening assay test, resulting in shorter TAT

- Enables testing of pooled samples- re test pooled samples that had genomic material- saves costs
Advances in Reagents for PCR

- Availability of lyophilised pre mix for PCR reactions - reduce contamination risks due to many pipetting steps

- Availability of custom-made lyophilised pre mix reactions - shorter TAT eg can include primers as well, need to reconstitute with nuclease acid free water & probe

- Availability of wider probe chemistry selection and less cross reaction - enable multiplexing - reduce costs
Advances in PCR Equipment

- Availability of newer technology- open system, faster and more accurate

- Availability of smaller, “mobile” machine at reduced cost

- Availability of new technology eg “Prism” - full spectrum optics, better detection and selection

- High performance Peltier and Silver Blocks- Advantage in speed and thermal uniformity- faster heating and cooling – shorter TAT
Molecular Detection- its Role for Dengue Diagnosis

- Able to serotype and thus helpful in epidemiology surveillance

- Important diagnostic tool in fatal cases (liver biopsies/tissue samples) – Other tests may not be conclusive

- Able to perform multiplexing- can be used to determine co-infection with other dengue serotype as well as other arbovirus (eg zika or chikungunya or JE)

- Able to quantify dengue viral load
Example of Commercial Real Time RT-PCR Kits available
Commercial RT-PCR Kits

Dengue Virus subtypes 1, 2, 3 and 4

3’ Untranslated Region (3’UTR)
genesis® Standard Kit
Flavivirus Endemic Countries-Testing Approach

- Molecular detection is more suitable as serology may pose interpretation problem due to cross-reacting antibodies

- Perform Dengue RT-PCR or Chikungunya RT-PCR or Zika RT-PCR

CDC Triplex Real-Time RT-PCR Assay
Detection of RNA from Zika, Chikungunya, and/or Dengue Viruses
Diagnostic Test Application Form
Preliminary Findings of Identification of Proteomics and Genomic Markers in Den Patients With Multi Organ Failure

- Analysis completed for 60 patients (60 out of 100).

- RT-PCR serotyping (RNA detected among 49/60 pts (81.7%)

- Den 1 – 25 (51%)
- Den 2 – 20 (40.8%)
- Den 3 – 4 (8.2%)
- Den 4 – 0 (0%)

- NS1 ( Detected among 57/60 patients (95%)
NS1 (by ELISA) vs RNA (rRT-PCR)

- Detection rate of 95% (NS1) vs 81.7% (RNA)

- Inhibitors include Rnase (RNA) and protease (NS1)

- NS1 is the most important protein released in early dengue infection and important marker for pathogenesis/severity as well

- NS1 tend to stay longer, up to 8-10 days of onset
Viral Load for Den 1 by Real time RT-PCR

Highest load- $7.6 \times 10^{11}$ at admission and cleared by Day 7
On average takes about 4-6 days to clear ~depends on initial viral load
Viral Load for Den 2 by Real time RT-PCR

Highest load - $2.19 \times 10^8$ at admission and cleared by Day 4
On average takes about 2-4 days to clear ~depends on initial viral load
Findings

- Many patients demonstrated high viral load in the febrile stage, during admission and in the critical stage.

- Prolonged viraemia were seen 37/60 as virus was detected in all three stages of collection (admission, critical and discharge).

- Most of them had at least 1 organ involvement (mostly liver).

- On going tests- primary or secondary dengue infections

- Testing on the utility of severe dengue biomarkers
Conclusions

- Dengue diagnosis is important for both management of patient as well as epidemiological surveillance in Malaysia.
- Molecular diagnosis of dengue complements other existing assays (antigen detection or serology).
- Useful for serotyping, measure viral load or diagnosis of fatal dengue cases.
- Increased demand for molecular testing for dengue.
- Potential to be point of care test for patient management or to be used in field study.
- With threat from zika, a multiplex real time for dengue, zika and chikungunya will be an important diagnostic tool.