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The performance of RT-PCR compared with a rapid serological assay for acute dengue fever in a diagnostic laboratory

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Summary The laboratory diagnosis of dengue has largely relied on serological assays, although many different RT-PCR protocols have been reported. Owing to its limited use, the value of RT-PCR in the clinical laboratory has not been fully evaluated. During the outbreak of severe acute respiratory syndrome (SARS) in Singapore in 2003, RT-PCR to detect dengue viral RNA was used as a rapid diagnostic tool to differentiate dengue from SARS among patients who presented to a hospital designated to manage and quarantine SARS cases. A total of 343 results for RT-PCR and 439 results for serology were analysed and compared with the final discharge diagnosis. Our experience indicates that RT-PCR for dengue can be set up rapidly in a clinical laboratory, with very sensitive and specific results for the diagnosis of dengue, particularly in the first 5 days from onset of symptoms.

1. Introduction

Dengue virus, a flavivirus, is an RNA virus that causes dengue fever and dengue haemorrhagic fever. It is a major cause of febrile illness in the tropics, particularly in cities where favourable habitats for *Aedes aegypti* breeding are abundant (Gubler, 1997, 2004). Detection of thrombocytopenia with a clinical picture of fever, rash and bone

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pain, including orbital pain, provides a presumptive diagnosis of dengue fever (George and Lum, 1997), whilst dengue haemorrhagic fever has other important signs, particularly haemoconcentration due to plasma leakage (WHO, 1997). However, laboratory confirmation is necessary, as clinical case definitions show low positive predictive values, ranging from 22% to 62% for several different combinations of symptoms (Dietz et al., 1990). Many laboratories rely on serology as the primary diagnostic method, but antibodies may take several days to appear so initial serology is often negative (Guzman and Kouri, 2004). Several RT-PCR protocols for dengue have been described (Chow et al., 1993; Deubel et al., 1990; Henchal et al., 1991; Lanciotti et al., 1992; Morita et al., 1991) but these are not widely used in the clinical laboratory (Guzman and Kouri, 2004).

During the outbreak of severe acute respiratory syndrome (SARS) in 2003 in Singapore, all patients with fever were directed to Tan Tock Seng Hospital. As SARS and dengue fever had similar initial presenting symptoms (Peiris et al., 2003), RT-PCR was introduced to detect dengue viral RNA as a routine test, with the results reported on the same day. The test proved invaluable because a rapid and early positive diagnosis of dengue made a diagnosis of SARS much less likely, although co-infection with the SARS coronavirus was theoretically possible. This relieved the whole health infrastructure of the need to isolate these patients and to trace their contacts, a strategy used by Singapore to control the SARS outbreak (Gopalakrishna et al., 2004). As isolation rooms were at a premium, a confirmed diagnosis of dengue fever was helpful. Final decisions to remove patients from isolation required interpretation of all clinical and laboratory evidence.

This paper reports the results of a routine audit that describes the performance of the dengue RT-PCR that was rapidly set up as well as the current serological method. The aim of the audit was to assess the use of these dengue tests for diagnostic purposes in a hospital setting. The results were circulated to hospital staff to encourage an evidence-based approach to test selection and results interpretation.

2. Methods

2.1. Subjects

RT-PCR results, serology results and sample collection dates were taken from the Laboratory Information System for patients tested in May and June 2003. The date of onset of fever and the discharge diagnosis were taken from the electronic discharge records. The 'day of illness' was calculated as the number of days between the date of collection of the sample and the date of onset of fever. Patients were excluded if the diagnosis or the date of onset of fever were not available. Some patients had multiple samples sent, which were all included. This was a routine retrospective audit, therefore extra samples were not collected.

The results of RT-PCR and serology were analysed by day of illness. Performance data were calculated using the clinical discharge diagnosis as the gold standard. Clinically defined cases of dengue with samples negative by RT-PCR in the first 6 days of illness and cases with samples negative by serology after day 6 of illness were examined in further detail, including a *Rickettsia* screen (Multi-test, DIP-S-Ticks; PanBio, Brisbane, Australia). Day 6 was chosen as suggested by our past unpublished data, and we advised the medical staff that RT-PCR is the test of choice until day 7 when serology becomes more sensitive.

2.2. Serology

All samples had been processed in the routine diagnostic microbiology laboratory at Tan Tock Seng Hospital. Dengue serology was performed daily with the Dengue Duo IgM and IgG Rapid Strip Test (PanBio, Brisbane, Australia). The IgG test is designed with a high cut-off so that it is positive in secondary but not primary dengue. For the purpose of comparing serology with RT-PCR results and the clinical diagnosis, any samples positive for IgM and/or IgG were considered 'positive'.

2.3. RT-PCR

For RT-PCR, RNA was extracted from serum with the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Heiden, Germany) and 2 μl was used immediately to set up the RT-PCR on a Px2 thermal cycler (Thermo Electron Corporation, Needham Heights, MA, USA). The Superscript One—Step RT-PCR with Platinum *Taq* System (Invitrogen, Carlsbad, CA, USA) was used to perform reverse transcription and PCR in one tube according to the manufacturer's instructions with 1 μl of each primer. The sequence of the forward primer (CDC10418) was 5′-TTGAGTAAACYRTGCTGCCTGTAGCTC-3′ and the reverse primer (CDC10590) was 5′-GGGTCTCC TCTAACCTCTAGTCCT-3′. These primer sequences targeted the 3′ end of the dengue viral genome. They were developed by G.J. Chang (Division of Vector-borne Diseases, NCID, Fort Collins, CO, USA)

and L.J. Chien (Center for Disease Control, Taiwan) and shared with the participants of the Asia Pacific Economic Congress (APEC) Workshop on molecular epidemiology of dengue virus held in Taipei in December 2002, the objective of which was to achieve a standardised method for molecular surveillance of dengue viruses in the Asia Pacific. RT-PCR conditions were: 1 cycle of 60 °C for 30 min and 95 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 65 °C for 45 s and 72 °C for 10 min and finally 1 cycle of 72 °C for 10 min. Then, 10 μl of RT-PCR product with 2 μl of loading dye were run in a 2% agarose gel with 30 μg/ml ethidium bromide. Bands were visualised with UV light, captured by Polaroid and interpreted visually. Three controls were included on each run: a negative control using water, a positive patient control and a control from heat-inactivated cultured dengue virus serotype-2 that was isolated in Singapore. Test samples were run in duplicate. Inhibition was not assessed.

2.4. Virus isolation

Virus isolation was carried out using C6/36 *A. albopictus* cell line (ATCC CRL-1660). Briefly, 1 ml of serum was inoculated onto a 90% confluent monolayer of cells in a cell culture tube. Liebovitz (L-15) medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 5% fetal calf serum was added after adsorbing the serum to the cells at 32 °C for 1 h. The culture was incubated at 32 °C and observed daily for cytopathic effects (CPE). After 7 days of culture, the culture supernatant was harvested if CPEs were observed in 75% or more of the monolayer. If no CPEs were observed, the supernatant was passaged onto a fresh monolayer of C6/36 cells. This was repeated twice. Upon harvesting the supernatant, the cell monolayer was scraped off with a rubber policeman, washed with PBS solution and spotted onto a Teflon-coated slide. Flavivirus, dengue complex and dengue serotype-specific monoclonal antibodies, derived from the hybridoma (ATCC HB-112, HB-114, HB-46, HB-47, HB-48, HB-49) culture supernatant, were then used to confirm the isolation of dengue virus via indirect immunofluorescence.

2.5. Statistical analysis

The mean 'day of illness' and the 95% CI were calculated. Statistical analysis was carried out using Student's *t*-test.

3. Results

During the 2-month period, 475 patients had tests performed; 411 of these were eligible for study, with 439 results for serology and 343 for RT-PCR. The results for RT-PCR and serology are tabulated by day of illness in Tables 1 and 2, respectively.

3.1. RT-PCR

3.1.1. Previous experience with the RT-PCR protocol

The primers used in this study had been validated prior to their distribution to the participants of the APEC Workshop (G.J. Chang and L.J. Chien, personal communication). Separately, we tested the RT-PCR protocol against human serum spiked with culture-derived dengue (all four serotypes), Japanese encephalitis (Nakayama), yellow fever (YF17D) and West Nile (Sarafend) viruses in a separate study (data not shown). The RT-PCR was able to detect 1 plaque-forming unit (PFU)/ml for dengue serotype-1, -3 and -4, and 10 PFU/ml for dengue

Table 1 Performance data (number of samples) for RT-PCR by day of illness

Day of fever	Final diagnosis: dengue		Final diagnosis: not dengue		Sensitivity (%)	Specificity (%)
	RT-PCR positive	RT-PCR negative	RT-PCR positive	RT-PCR negative		
1	1	0	0	10	100	100
2	12	0	0	11	100	100
3	20	3	0	15	87	100
4	37	5	0	17	88	100
5	30	7	0	23	81	100
6	37	13	0	11	74	100
7	15	14	0	11	52	100
8	2	10	0	9	17	100
>8	1	7	0	22	13	100
Total	155	59	0	129	72	100

Table 2 Performance data (number of samples) for serology by day of illness

Day of fever	Final diagnosis: dengue		Final diagnosis: not dengue		Sensitivity (%)	Specificity (%)
	Serology positive	Serology negative	Serology positive	Serology negative		
1	0	2	0	10	0	100
2	0	9	0	10	0	100
3	1	21	1	20	5	95
4	4	26	0	15	13	100
5	12	34	1	17	26	94
6	21	34	0	13	38	100
7	23	16	0	11	59	100
8	23	6	0	17	79	100
9	14	4	0	8	78	100
10	13	5	0	6	72	100
11	4	1	0	7	80	100
>11	6	2	0	22	75	100
Total	121	160	2	156	67	99

serotype-2, with no cross reaction with the other flaviviruses (data not shown). In addition, we had also used this RT-PCR protocol in conjunction with virus isolation using C6/36 *A. albopictus* cell line in a sentinel physician-based epidemiological surveillance of dengue. Of 307 serum samples, 130 were positive by RT-PCR; 108 samples were positive by virus isolation and all of these were similarly positive by RT-PCR. None were detected by virus isolation without being RT-PCR positive (Ooi et al., unpublished data).

3.1.2. RT-PCR in the present clinical study

Of the 165 samples tested from patients in the first 6 days of illness and with a final discharge of dengue, 137 (83.0%) were positive by RT-PCR. The 28 RT-PCR negative samples were taken from 22 patients. Of these 22 patients, 17 had another sample positive for dengue. Four had a positive RT-PCR on a sample collected just 1 day earlier and 13 had positive serology. Of the remaining five patients, one had a test positive for *Rickettsia typhi* (his minimum platelet count was 139×10^9/l) and negative dengue serology at day 12; two had no other diagnostic test and two had negative dengue serology on days 5 and 6 of illness. These last four had thrombocytopenias of 96×10^9/l, 21×10^9/l, 30×10^9/l and 39×10^9/l, which are all suggestive of dengue in clinically compatible cases, but otherwise did not have any laboratory evidence of dengue. The samples in these four cases were drawn on days 5 or 6 of illness.

The positive predictive values are 100% for all days of illness. The negative predictive values are 100% in the first 2 days of illness and then decrease with increasing number of days from onset of illness, to give an overall figure of 69%.

3.2. Virus isolation

One hundred of these RT-PCR positive serum samples were also randomly selected for virus isolation. Of these, 68 were similarly positive by virus isolation. The mean day of illness for those samples that were positive by virus isolation was 3.85 days (95% CI 3.4—4.3) and for those negative by virus isolation it was 4.72 days (95% CI 4.1—5.3). The difference in the mean day of illness between the virus isolation positive and negative groups is 0.87 days, which is statistically significant ($t = 2.06$; $P < 0.05$).

3.3. Serology

Of the 117 samples tested from dengue patients after the sixth day of illness and onwards, 83 (71%) were serologically positive. Two patients were IgM positive (on days 3 and 5) but did not have dengue recorded as the discharge diagnosis. One was diagnosed with acute myeloid leukaemia (AML), the other with heart failure. Both serum samples, which had been stored at $-30\,^{\circ}C$, were re-tested; the patient with heart failure was still positive and the patient with AML was negative.

Two patients had a positive serology report followed by a later negative one. On re-testing the second serum samples, one was positive and one was negative. The patients' platelet counts were 32×10^9/l and 10×10^9/l, respectively, and both were RT-PCR positive.

Serology was negative in 29 patients tested after the sixth day of illness. Nine had had positive

RT-PCR reports, six others were found to be RT-PCR positive when the original serum sample was tested, seven others had positive serology in the later course of the illness, and seven had no other positive dengue test. One of these latter seven had a test positive for *R. typhi* (the same patient mentioned above); the remaining six only had one sample sent, between days 4 and 8, and they were all RT-PCR negative, with minimum recorded platelet counts of 88×10^9/l, 90×10^9/l, 88×10^9/l, 5×10^9/l, 48×10^9/l and 111×10^9/l.

The positive predictive value of serology after the sixth day of illness is 100% and the negative predictive value increases from 41% on the seventh day to 92% after the 11th day of illness.

4. Discussion

The literature is bursting with reports of applications for nucleic acid testing in diagnostic microbiology. However, laboratories may find it difficult to apply these techniques owing to lack of experience, training and clear evidence of an improved cost:benefit ratio. A high proportion of laboratories probably do not offer any nucleic acid tests (Guzman and Kouri, 2004). The clinical and public health need for a fast, sensitive test to help distinguish cases of dengue from SARS provided the impetus for us to introduce RT-PCR to our diagnostic laboratory. Equipment, reagents, primers and protocols were borrowed or obtained to set up this RT-PCR for dengue in concert with RT-PCR for SARS coronavirus. A small group of staff was chosen and taught some basic molecular biology and the principles of the test. Careful pipetting, avoiding contamination and proper storage of materials were emphasised throughout this period. Chemical (e.g. ethidium bromide) and biological hazards were discussed at length and samples were processed at biosafety level 2 with level 3 practices. A low threshold for consultation was encouraged so that staff was well supported.

Whilst many RT-PCR protocols are available that detect and distinguish between the four serotypes, we chose to use a generic RT-PCR for all four serotypes as this reduces the cost of the test. More importantly, whilst the information on serotype is useful for understanding dengue pathogenesis and epidemiological surveillance, it is not necessary for patient management.

The RT-PCR and dengue IgM ELISA results were compared with the diagnosis at discharge. Although this may not be completely accurate, as suggested in one patient who was positive for *R. typhi*, our results show that 17 of 22 clinically defined patients who had negative RT-PCR in the first 6 days of illness did have laboratory-confirmed dengue. The accuracy of the epidemiological data used to calculate the day of illness on which the sample was collected cannot be verified. However, the data were collected and recorded during the SARS epidemic when all staff and the general public were 'on alert' and the timing of onset of fevers was paramount. It would be difficult to reproduce this degree of care in history-taking without a prospective study.

The RT-PCR performed well, with high sensitivity and specificity in early disease. The high specificity of 100% reflects the robust nature of the assay and the technical care taken to avoid contamination despite the lack of experience. The performance data assume the clinical discharge diagnosis to be the gold standard. However, this diagnosis itself may have been influenced by the dengue RT-PCR result, so these encouraging specificity results should be interpreted with a little caution. The fall off in the sensitivity from 100% in early disease to 74% by day 6 is disappointing but expected because the viraemia disappears around days 5 to 7 (Innis et al., 1989; Summers et al., 1984; Vaughn et al., 1997). We did, however, find positives in a few samples on the eighth day of illness. More sensitive RNA extraction protocols or a nested RT-PCR would increase the sensitivity of the test and may thus extend this diagnostic window.

Our virus isolation rate from the RT-PCR positive samples was lower than that observed for those obtained during the epidemiological surveillance of dengue. This is probably due to the degradation of limited viable viral particles in the serum samples. This explanation is supported by the finding of a statistically significant difference in mean duration from onset of illness between the virus isolation positive and negative samples. Those RT-PCR positive but virus isolation negative samples were taken, on average, a day later than those that were positive by both methods. This could have resulted in a lower viraemia level in the sera that were negative by virus isolation. Whilst it was intended that samples were separated quickly and refrigerated or frozen at $-80\,^{\circ}$C while processing was awaited, the realities of a routine diagnostic laboratory dictate that samples may be left lying around pending attention. This was particularly so during the SARS outbreak as microbiological investigation was routine for all patients admitted to our hospital. In addition, the large workload delayed the transfer of samples for virus isolation, some by almost up to a year. Together, these factors could have contributed to the low virus isolation rate.

The RT-PCR protocol did not control for the presence of PCR inhibitors, which might explain some of

the loss of sensitivity. Inhibition controls are desirable but are not actually used in routine testing by other methods in microbiology: laboratories do not test for and report the presence of antibiotics when they report negative bacterial cultures. It is possible but expensive, so we applied the same standards to this RT-PCR system as we do to other test methods and did not do it. All 13 dengue cases tested on days 1 and 2 of illness were RT-PCR positive, so inhibition did not appear to be a problem in these cases.

The sensitivity of the serology test was very low in early disease, as expected (Vorndam and Kuno, 1997), but rose to 79% by day 8 of illness. It is disappointing that serology was negative in 29 patients tested after the sixth day of illness; 22 of these had laboratory-proven dengue. The product insert claims sensitivities at admission and discharge, respectively, of 88% and 94% for secondary dengue and 76% and 100% for primary dengue, although the day of illness is not given. These data are from studies in Thailand where patients may present later in disease. Two samples yielded false positive results according to the discharge diagnosis: one of them, drawn on day 2 of illness, was still IgM positive when the same sample was re-tested, although it was negative by PCR. The patient had a thrombocytopenia of 97×10^9/l. Two other patients with positive serology samples were reported negative on second samples, although both patients were RT-PCR positive. Re-testing the second serology sample showed one to be positive and the other negative. There is no definite reason but explanations include pre-analytical errors, technical errors with labelling and aliquoting, and the fact that the test is subject to intraobserver and interobserver variation. Advantages of this test include the rapid format with on-board controls so that tests can be run and reported throughout the day.

Individual RT-PCR tests cost more than the rapid serology test. However, because many patients have negative serology on admission, the serology is often repeated and other investigations ordered to investigate the cause of fever. Patients with RT-PCR taken early in admission are likely to be positive and there is no need for further dengue or other diagnostic tests. This has not been audited, but for patients caught in early disease we expect the RT-PCR to be cost effective. Samples received before 10:30 hours are reported the same afternoon. When we move the test to a real-time RT-PCR platform we expect more samples to be reported on the day of collection, as we will be able to delay the start of processing.

This analysis is restricted because this is a report of a laboratory audit and we did not have the luxury of samples collected according to a prospective protocol. Analysis of serial samples from individual patients would give better data and we plan to do this.

Our experience and the audit findings indicate that RT-PCR for dengue can be set up in a diagnostic laboratory in a matter of days with reliable results. The specificity was high both for RT-PCR and serology and, although RT-PCR outperforms serology in early disease, serology becomes the preferable test after the sixth day of illness.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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