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Clinical Case Study

A Febrile Blood Donor

Julian W. Tang,[1*] Yvonne Ng,[2] Evelyn S. C. Koay,[1] Gek Har Leow,[1] Eng Soo Yap,[3] Douglas Chan,[1] Lip Kun Tan,[3] and Paul A. Tambyah[4]

CASE

A 4-day-old neonate born prematurely at 29 weeks gestation developed thrombocytopenia (platelet count 43×10^9/L) with associated severe pulmonary and intracranial hemorrhaging. Urgent transfusions with 10 mL/kg of packed red cells and 10 mL/kg of platelet concentrate were given. These were sourced from blood donated 2 days earlier in Singapore by a 21-year-old female university student who was clinically well at the time. The day after these blood products were given to the neonate, the blood donor contacted the blood transfusion service to inform them that she was now sick with a febrile flu-like illness. Her blood donation screening plasma sample was retrieved and tested by PCR for several viruses, including dengue, chikungunya, enterovirus, and Epstein-Barr virus.

Dengue RNA (serotype 2) was found to be present at a very low amount (i.e., at a real-time PCR cycle Ct number of 39–40) (Fig. 1A) from the initial RNA extract (extract A). To confirm the presence of this low amount of dengue 2 RNA, the same extract (extract A, which had been stored at −20 °C) was retested (Fig. 1B), and again found to give a low-positive result.

QUESTIONS TO CONSIDER

1) What steps should be taken next?
2) What can cause false-negative and false-positive PCR results?
3) How should low-positive PCR results affect patient management?

DISCUSSION

Recently, Wilder-Smith and colleagues highlighted the important issue of blood transfusion safety with regard to dengue infection in a dengue-endemic country, such as Singapore *(1)*. Although at the time of submission of their report Wilder-Smith et al. could not identify any dengue transmission events associated with blood transfusion, there has since been a report presenting convincing evidence for several such transmission events *(2)*. In addition, there has been at least 1 previous case of dengue transmission in Singapore, via organ transplantation *(3)*.

It was important to verify the status of this blood donation as accurately as possible, because this event had additional implications for the blood transfusion service. Three approaches were taken: retesting the original specimen, testing the neonate, and further testing of the donor.

Retesting the original specimen was done by going back to the original blood-screening sample (stored at 4 °C) and reextracting the RNA again (extract B). All RNA was extracted by using the Qiagen Biorobot EZ1 system (Qiagen). In addition, another RNA extract (extract C) that had been obtained earlier, at the same time as extract A, was retrieved from storage at −20 °C for further testing. Extracts A, B, and C were tested as replicates of 5 (i.e., $3 \times 5 = 15$ tests) in the same dengue RNA PCR assay. Surprisingly, all these tests were negative—even extract A, which had tested low positive twice previously. Additional, retrospective dengue NS1 antigen testing on the first serum sample (i.e., the original serum sample from which extracts A and B were obtained) was also negative.

The neonate was then urgently tested for dengue RNA by PCR on days 2, 5, and 8 posttransfusion and, fortunately, all dengue RNA test results were negative. Dengue NS1 antigen test results were also negative. At the time, the neonate's negative status was thought to be due to the low volume of blood products transfused (combined volume of 16 mL), which reduced the potential for dengue virus transmission.

In this case, it was fortunate that the donor agreed to return for convalescent antibody testing to determine whether dengue or some other viral infection was responsible for her fever and flu-like illness. She was serologically tested 15 days after her reported onset of illness for dengue and Epstein-Barr virus IgM and IgG, as well as influenza A and B, parainfluenza types 1–3, adenovirus, and respiratory syncytial virus. All serology test results were negative.

Departments of [1] Laboratory Medicine, [2] Neonatology, [3] Haematology and [4] Medicine, National University Hospital, Singapore.
* Address correspondence to this author at: Department of Laboratory Medicine, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074, Singapore. Fax +65-67771613; e-mail jwtang49@hotmail.com.
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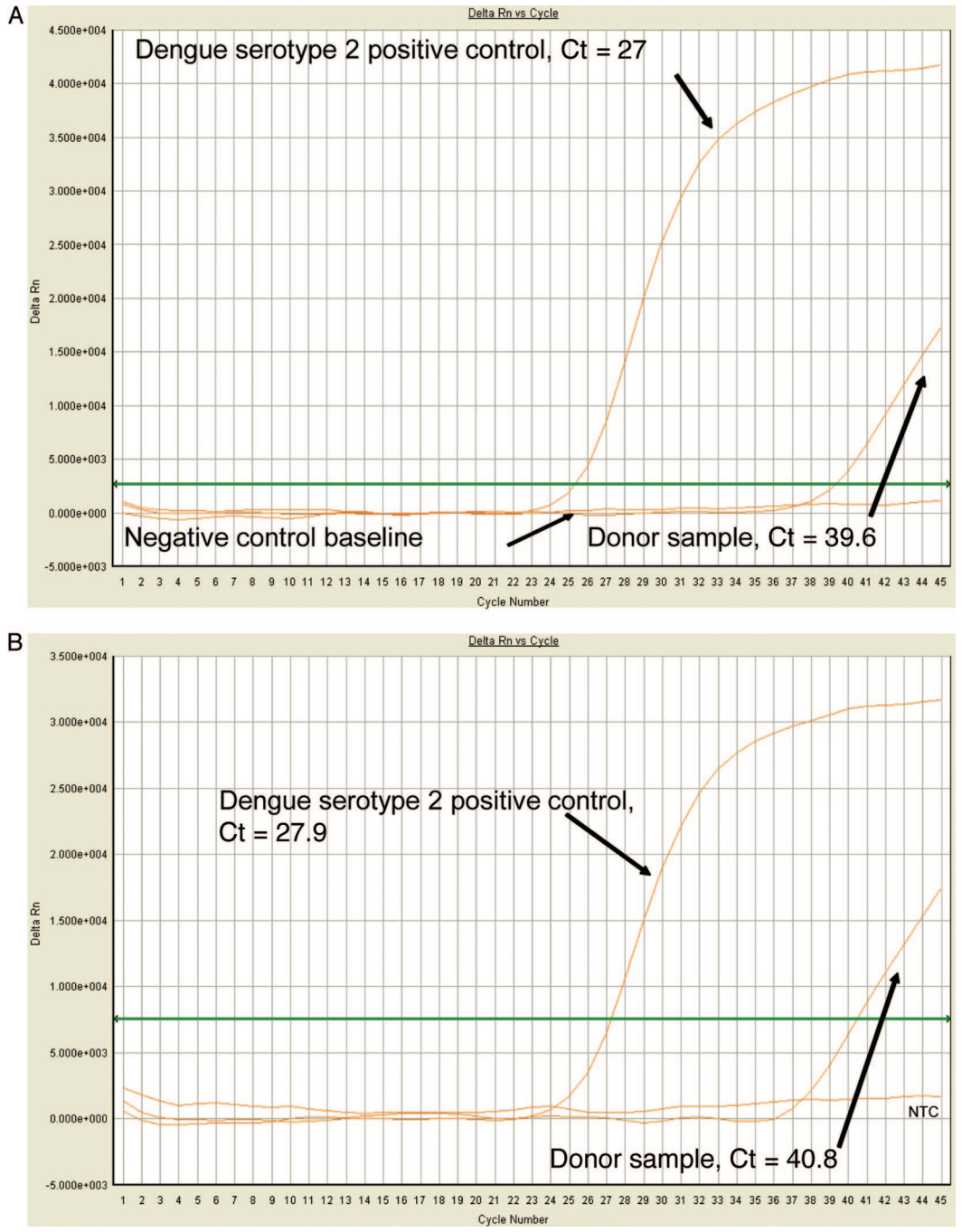

Fig. 1. Real-time dengue RNA PCR outputs for the donor plasma sample.

(A), Real-time PCR results on the RNA extract (extract A, first test) obtained from the first donor sample showing a Ct of 39.6, a low-level positive signal for dengue serotype 2 RNA. (B), Repeat testing on extract A after 1 cycle of freeze-thawing (from −20 °C), showing a higher Ct of 40.8, indicating an even lower amount of RNA present. The normal real-time PCR output is seen as a sigmoidal plot. The Ct value is where the signal sigmoid curve (indicating accumulation of the specific dengue PCR product) crosses the threshold (green horizontal line) indicating a positive result. The threshold for individual curves may be different and can be automatically set by the assay software, but can also be adjusted according to local dengue virus epidemiology and the expected Ct values for positive results in this specific patient population. NTC, nontemplate control.

When screening blood products, it is important for clinicians to be able to distinguish a possible low-level viral load in a transfused blood product from a low-level laboratory cross-contamination event. The case we report initially suggested the presence of low-level dengue RNA in a blood product, which was brought to our attention only when the donor called the hospital 2 days after the blood donation to report a febrile illness.

There are several possible reasons for discrepant PCR results. The most obvious possibility is that there must have been 2 occurrences of low-level dengue RNA contamination of the PCR assays when extract A was tested the first 2 times. Such contamination is always possible in a molecular laboratory and may be difficult to exclude. Two different operators performed these first 2 dengue RNA PCR tests for extract A.

An alternative explanation is the low number of copies of the dengue RNA suggested by the PCR signal [cycle threshold (Ct) number of 39–40], a copy number that is virtually at our defined limit of clinical sensitivity for this in-house assay. With such a low RNA copy number in the original sample, stochastic effects become important, and it is possible to miss picking up with the pipette the target RNA to be amplified. From a plasma sample of 500–1000 μL, only 200 μL is used in the RNA extraction process. From this sample volume is obtained 50 μL containing RNA, and from this only 1 μL is used in the PCR reaction. It is possible that when there are very low copy numbers of RNA in the original sample, even after concentrating the RNA into the 50-μL elution volume, inserting the pipette into this volume to remove just 1 μL may fail to take up any RNA into the pipette tip. This type of potential "miss" is a stochastic phenomenon that has been well described previously *(4)*.

The process of freezing and thawing is also known to decrease the amounts of intact RNA in samples *(5)*. Extract A was freeze-thawed twice (from −20 °C), extract B was freeze-thawed once (from −20 °C), and extract C was obtained from the original plasma that had been freeze-thawed once (from −80 °C).

All the convalescent serology test results for various viruses proved negative in the donor. It is possible that her reported symptoms were due to the more common rhinoviruses or coronaviruses, for which PCR testing was not performed during the stage of acute illness and for which serological testing was not available to determine recent past infection with these pathogens. Given the negative results of this convalescent testing in the donor, it is therefore likely that the twice-positive dengue RNA PCR results (shown in Fig. 1) were probably due to a cross-contamination event rather than a low amount of dengue RNA in this sample. Further evidence for the lack of dengue transmission to the neonate was provided by negative dengue IgM and IgG results in convalescent serum taken from the neonate 4 weeks after the transfusion event.

We suspect that the source of the cross-contamination may have come from the dengue 2 positive control, because very few other samples were positive (there was 1 each of dengue 1, 2, and 3) during this week. Although we receive around 10–20 samples per week for dengue PCR testing, only about 10%–15% may be positive in any 1 week, and these samples are usually a mixture of dengue 1 and 2, although dengue 3 and 4 are occasionally seen. We do not routinely quantify the dengue in these positive samples, but the Ct can vary from 25–35, on average (with an arbitrary clinical cutoff set at a Ct of approximately 39–40, based on our

POINTS TO REMEMBER

PCR testing is a very sensitive technique that allows small amounts of RNA or DNA to be amplified more than a million-fold. Thus any low level contamination (e.g., from a positive control) will also be amplified.

Contamination should be monitored by placing several negative controls on every PCR run. If any of these negative controls gives a positive signal, the entire run must be considered invalid.

Reextraction from the original sample (which should be kept until all testing has been satisfactorily completed) is mandatory if a false-positive result is suspected.

Not all low-positive results are necessarily false positives, and there may indeed be a low amount of RNA or DNA in the sample. For acute (as opposed to persistent or chronic) viral infections, taking a second sample from the patient for retesting may not resolve the issue because most viral loads will decrease progressively from the date of onset of illness owing to immune-mediated viral clearance.

Repeat testing on the original extract (or on a new extract from the original sample) of a true low-positive sample may subsequently give a negative result owing to RNA or DNA degradation after freezing and thawing.

In situations in which an unexpected illness develops in either the recipient or the donor of a blood product, when infection is suspected, paired acute and convalescent (taken at least 10–14 days later) samples should be taken for parallel serology, PCR, and/or culture.

During these additional laboratory investigations, management of the patient must be closely coordinated with the diagnostic laboratory to avoid unnecessary use of potent antiviral drugs or missing the optimum window for necessary treatment.

experience of testing local clinical dengue cases, most of which have been confirmed on parallel serological testing).

In this case, the initial contact from the donor when she became symptomatic plus her willingness to cooperate with follow-up testing allowed us to resolve this case satisfactorily. Measures have now been put in place to prevent the recurrence of this situation, including a revision of and a careful review of the standard operating protocols with the staff members who routinely perform this particular dengue PCR test.

With real-time PCR testing becoming more and more routine in hospitals, it is critical to distinguish in clinical samples true low amounts of pathogens from laboratory cross-contamination. During any such investigations and until the issue is resolved, the possibility of such potentially false-positive results must considered in the process of ongoing patient management. Such results may yet turn out to be true positives, and being attentive to them ensures that any opportunity for optimum intervention will not have been missed.

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Commentary

Lynne Uhl

In the wake of the recognition of transfusion-transmitted HIV, the blood-banking community introduced more extensive donor screening, improved donor testing, and postdonation product modification to improve blood supply safety and reduce the risk for transfusion transmitted infection (TTI). In addition to multiple predonation screening questions, in developed countries collected units are subjected to extensive laboratory screening, including nucleic acid testing for HIV/hepatitis C virus, and more recently hepatitis B virus and West Nile virus. Consequently, the risk for TTI has been reduced to levels ranging from 1/200 000 to 1/1–2 million transfusion episodes.

Despite these decreases in risk, ongoing surveillance for emerging infectious diseases potentially transmissible through blood transfusion is necessary *(1)*. As noted by Tang et al., the arbovirus agent responsible for dengue fever is widely endemic in equatorial regions and there is accumulating evidence for the risk of transfusion transmitted dengue infection. Currently, however, no good screening questions have been formulated to identify donors at high risk for dengue-associated TTI, nor is there an acceptable donor-screening test that can be practically applied *(1)*. Consequently, postdonation communication by a blood donor of a febrile illness within 7 days of donation is critical, as evidenced by this case report. Once the blood collection facility is presented with this information, it is incumbent on them to determine the risk for TTI, despite the low specificity of fever for a potential TTI. In most cases, blood collection facilities follow the precautionary principle and attempt to interdict release of blood components by initiating a market recall or withdrawal, often via a telephone call to the transfusion service that received the blood components in question *(2)*. In response, the transfusion service screens its inventory to determine the disposition of the implicated blood product.

Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, MA.
Address correspondence to the author at: Beth Israel Deaconess Hospital YA-309, 330 Brookline Ave, Boston, MA, 02215. Fax 617-667-4533; e-mail luhl@bidmc.harvard.edu.
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If still in inventory, the blood product is immediately quarantined until further information from the collection facility is available regarding the safety for distribution. If the product has been released for transfusion, it is the responsibility of the transfusion service to alert the recipient's physician regarding the risk for TTI and work with the physician to develop an action plan for further assessment and patient management. This case report illustrates the importance of coordinated teamwork between the blood collection facility, the department of laboratory medicine, and the clinical service in assessing the risk of TTI associated with a blood component for which concern was raised.

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Commentary

Roger Y. Dodd

Unfortunately, increased clinical diagnostic sensitivity comes at the expense of clinical diagnostic specificity. In addition, tests with high analytical sensitivity are susceptible to laboratory contamination. Thus, it is clear that diagnostically and analytically sensitive tests are subject to false-positive results. In this case, concern about the circumstances surrounding an apparently repeatably (but weakly) positive PCR result for dengue 2 RNA led to extensive and appropriate further studies that strongly support the final conclusion that the initial results were indeed due to contamination. A second and third extract from the original sample were tested multiple times and found nonreactive, and evaluation of subsequent samples for evidence of seroconversion provided supportive evidence that the donor (and the neonate recipient of her blood donation) were not infected with dengue virus. The laboratory cannot be faulted for these careful determinations, but would they have taken place in a less critical environment? Herein lies the lesson. However, a broader question that might be asked is why the testing was done in the first place and how were the analytes chosen? Given the recent finding of a dengue transfusion transmission in Singapore, and the involvement of some of the same authors in the case, it is understandable that dengue and even chikungunya viruses were of interest, but was there any helpful clinical information to provide a basis for the testing? What is the likelihood of accurately identifying the etiologic agent of a flu-like febrile illness by this approach? Could it not have been a bacterial or even parasitic infection, either of which would have had very different implications for the blood recipient? The case further illustrates that, in the absence of appropriate caution, the properties and potential failure modes of sensitive tests could lead an apparent finding of that which is expected, rather than what is really there.

Research and Development, American Red Cross, Rockville, MD.
Address correspondence to the author at: Research and Development, American Red Cross, Holland Laboratory, 15601, Crabbs Branch Way, Rockville, MD 20878. E-mail dodd@usa.redcross.org.
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