Diagnostic 20 minute whole blood clotting test (WBCT20) in Russell's viper envenoming delays antivenom administration

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Running Title: Poor diagnostic utility of the WBCT.

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Abstract

Background: The 20 minute whole blood clotting test (WBCT20) is used widely for identification of coagulopathy in snake envenoming, but its performance in practice hasn't been evaluated.

Aim: We aimed to investigate the diagnostic utility of the WBCT20 for coagulopathy in Russell's viper envenoming.

Design: Prospective observational study.

Methods: Adult patients with snake envenoming were recruited. Age, sex, bite information, clinical effects, serial WBCT20 and antivenom treatment were recorded. Definite Russell's viper envenoming was confirmed with venom specific enzyme immunoassay. We assessed sensitivity of admission WBCT20 to coagulopathy (International normalised ratio,INR>1.5) in Russell's viper envenoming, the specificity of negative WBCT20 in non-envenomed patients and directly compared paired WBCT20 and INR.

Results: Admission WBCT20 were done in 140 Russell's viper bites with coagulopathy and was positive in 56/140 [sensitivity 40%(95%CI:32-49%)]. A negative WBCT20 led to delayed antivenom administration [WBCT20-ve tests; median delay 1.78h(IQR:0.83-3.7h) vs. WBCT20+ve tests: median delay 0.82h(IQR:0.58-1.48h);p=0.0007]. Delays to antivenom were largely a consequence of further WBCT20 being performed and more common if the first test was negative (41/84 vs. 12/56). Initial WBCT20 was negative in nine non-envenomed patients and 48 non-venomous snakebites [specificity:100%(95%CI:94-100%)]. In 221 paired tests with INR>1.5, the WBCT20 was positive in 91(41%). The proportion of positive WBCT20 only increased slightly with higher INR.

Conclusions: In clinical practice the WBCT20 has low sensitivity for detecting coagulopathy in snake envenoming and shouldn't over-ride clinical assessment based decisions about antivenom administration. There is an urgent need to develop a simple bedside test for coagulopathy in snake envenoming.

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Introduction

Snake envenoming is being increasingly recognised as a major medical problem in tropical and subtropical countries. One of the most important effects of snake envenoming is coagulopathy, most commonly a venom induced consumption coagulopathy (VICC).[1] The major treatment is antivenom administration which aims to neutralise the toxins in the venom.[2] However, antivenom is expensive, difficult to obtain in some parts of the world and associated with a significant risk of systemic hypersensitivity reactions.[3-5] It is therefore essential to be able to rapidly and accurately determine which patients have envenoming (and will require antivenom), and which are non-envenomed, or have been bitten by non-venomous snakes.

The 20 minute whole blood clotting test (WBCT20) has been used for decades in viper (and other snake) bites to determine if patients have a clinically significant coagulopathy.[6-8] The WBCT20 was not intended as a clotting test per se but as an indicator of envenoming (and need for antivenom) in patients bitten by snakes that cause coagulopathy. Despite the widespread reliance on this test and it being regarded as the standard of care for treatment of snake envenoming in resource poor settings,[9] there have been no studies that have determined the conditions under which the test can be performed accurately, validated it against standard tests, or demonstrated that it is accurate in the field.[6] There is little standardisation of the method for the WBCT20 and the original study describing it simply states that "a few ml of blood were placed in a clean dry glass test tube and left undisturbed for 20 minutes and then tipped to discover whether the blood had clotted".[7] Whether factors such as the size or type of tube (beyond that it should be glass), temperature and type of snake affect the test, has not been explored. Some studies even report a 30 minute whole blood clotting test, indicating even the time of the whole blood clotting test is not universally standardised.[10, 11] Coagulation

studies are notoriously difficult to standardise. For example, laboratory measurement of clotting function in plasma has required significant standardisation to make test results reproducible (e.g. the prothrombin time being standardised as the international normalised ratio [INR]).[12] It is therefore remarkable that no attempt has been made to standardise the WBCT20, or to demonstrate that this lack of standardisation does not affect performance.

The study widely cited as demonstrating the reliability of the WBCT20 compared it with fibrinogen concentrations, rather than to conventional laboratory tests, such as a thrombin time (TT), prothrombin time (PT), or international normalised ratio (INR). These are the usual tests used and are more representative of global clotting function. Evaluation of the WBCT20 against such standard tests in the clinical setting is necessary to determine if it is an appropriate diagnostic test in snake bite coagulopathy or VICC. We have been conducting a prospective randomised controlled trial (RCT) of antivenom infusion rates for snake bite in Sri Lanka. A major feature of envenoming is coagulopathy from Russell's viper bites. Doctors in these hospitals routinely do a WBCT20 to determine if antivenom is required (as outlined in the National guidelines for Sri Lanka[13]). The determination of WBCT20 was therefore routine practice and not part of our study, however, we measured formal coagulation studies within the RCT. We compared the results of the WBCT20 done by the treating clinicians with an INR done on immediately frozen samples for definite Russell's viper envenoming cases and non-envenomed patients.

Methods

This was a prospective observational study of patients with snake envenoming in central western Sri Lanka conducted in conjunction with a randomized controlled trial of different infusion rates of antivenom.[14] Our primary aim in this paper is to determine the sensitivity and specificity of the WBCT20 in clinical practice under the usual conditions of its use for detection of coagulopathy in Russell's viper bites. The study had approval from the Ethical Review Committee, Faculty of Medicine, University of Colombo. All patients gave written and informed consent for the collection of data and parents/guardians also gave consent for children.

Study Patients

Patients were recruited from a secondary referral hospital in Chilaw, Sri Lanka between 21st January 2007 and 31st July 2009. Every patient who was older than 13 years of age who presented with a snake bite was identified on arrival to hospital. All patients then had baseline clinical data collected, including demographic features (age, sex), bite information (type of snake, time of bite), clinical features of envenoming, complications and treatment. A WBCT20 was done routinely in all patients by the treating hospital team, including repeat testing in patients with envenoming. A few ml of blood were placed in a small glass test tube. After 20 minutes the tube was inverted to determine if a clot had formed. The test was negative if a clot formed and positive if no clot formed (incoagulable blood). We identified from review of INR and Russell's viper venom specific enzyme immunoassay results two groups: definite Russell's viper envenoming (venom concentration > 2.5ng/mL) plus VICC (INR>1.5) and suspected Russell's viper bites with no venom detected and no VICC (INR<1.3). An INR cut-off of 1.5 was chosen because this was above the normal range and

higher than any non-envenomed patients. An INR>1.5 was present in all but one patient with detectable RV venom.

During the study, patients who were deemed to require antivenom were recruited to a clinical trial comparing different rates of antivenom infusion which is reported elsewhere.[14] All other decisions about treatment were made by the treating clinicians.

Patients had a 10 mL sample of blood collected at the time of initial assessment and close to the time the WBCT20 was done. In patients given antivenom, 5 mL samples were also collected one, four and twelve hours later, and thence once daily until discharge. Blood was collected in serum tubes for venom specific enzyme immunoassays (EIAs) and citrated tubes for coagulation studies. All samples were immediately centrifuged, aliquoted and frozen at -20°C and then transferred to -80°C freezers within 2 weeks until the completion of the study.

Laboratory Assays

Frozen samples were transported to Australia. Frozen serum was then thawed and used to measure Russell's viper venom concentrations using a previously described EIA.[15] In brief, polyclonal antibodies (IgG) to Russell's viper (*Daboia russelli*) were raised in rabbits.[16] These were conjugated to biotin and then used in a sandwich EIA with the detecting agent streptavidin-horseradish peroxidase. The limit of detection for the assay was 2.5ng/ml.

Frozen citrate specimens were thawed to measure the international normalised ratio (INR). The INR was performed using a standard coagulometric method provided by the manufacturer on a Behring Coagulation System. An INR of greater than 1.5 was considered to be representative of coagulopathy in snake bite. The use of frozen citrate specimens to measure the INR in blood collected at a distant hospital, centrifuged, frozen and stored has

previously been used in an Australian study of VICC.[17] INR was also measured in plasma from nine non-envenomed snake bite patients and the INR ≤ 1.4.

Data Collection

Demographic information (sex, age), bite site, clinical features of snake envenoming, WBCT20 results and treatments (antivenom) were all recorded on datasheets which were then entered into a relational database (Microsoft Access). The results of all WBCT20 and the time of each WBCT20, all INR results and the time they were tested, and the time of antivenom administration were extracted from the datasheets.

Data Analysis

We assessed the sensitivity of the admission WBCT20 in detection of the presence of VICC in Russell's viper bites defined as a peak or maximum INR > 1.5 on frozen citrate samples collected anytime during the admission. We also examined the proportion of negative WBCT20 in non-envenomed patients and made a direct comparison of paired WBCT20 and INR. The WBCT20 and the research INR were not collected at exactly the same time so a paired comparison of a WBCT20 and INR was done for samples where the WBCT20 and INR were collected within 1 hour of each other. We also examined how the time to antivenom was influenced by the result of the first WBCT20.

Statistical Methods

In addition to visual analysis, sensitivity and specificity analyses was performed.[12] Sensitivity was defined as the proportion of envenomed cases with VICC where the WBCT20 was positive. Specificity was defined as the proportion of non-envenomed patients with a negative WBCT20. Ninety five percent confidence intervals (95% CI) were calculated using a normal approximation. Continuous variables are summarised as medians and interquartile ranges

(IQR) and proportions are presented with 95% confidence intervals (CIs). All analyses and graphs were done with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Of 1004 patients presenting with suspected snakebites during the study period, there were 145 definite Russell's viper bites that developed coagulopathy based on the detection of Russell's viper venom in patient serum and an INR greater than 1.5. Thirty one patients suspected to be Russell's viper bites were excluded because of a negative EIA for Russell's viper venom – 21 were probable Russell's viper bites but no pre-antivenom blood was available, six were unknown and three were more likely to be hump-nosed viper **(***Hypnale hypnale***)** bite. Only one patient with detectable Russell's viper venom did not develop an INR >1.5. The remaining 828 patients were bitten by other venomous snakes including hump-nosed vipers, krait and cobras, unidentified venomous snakes or non-venomous snakes.

The 145 patients with Russell's viper envenoming had a median age of 39 years (IQR: 29 to 49yr; Range: 16 to 82yr) and 109 (75%) were male. The majority of bites occurred in people walking outdoors, working in the paddy fields or in the garden (Table 1). Only one patient was bitten while indoors. The bite site was most commonly the lower limb. The median peak venom concentration was 199 ng/ml (IQR: 70 to 480 ng/ml; Range: 4.5 to 1952 ng/ml). The median maximum INR was 6.8 (IQR: 3.7 to >12; Range: 1.7 to >12) and Figure 1 shows the association between pre-antivenom venom concentrations and the maximum INR for 123 patients who had pre-antivenom blood samples available. No patient had an initially normal INR that then became abnormal although the INR increased in a proportion of patients on the second test.

WBCT20 Outcomes

On admission a WBCT20 was done in 140 of the 145 Russell's viper bites with VICC. It was positive in 56/140 patients [sensitivity 40% (95% CI: 32 to 49%] (Figure 1). A negative test result led to a delay in administration of antivenom [for WBCT20– tests: median delay 1.78

hours (IQR: 0.83 to 3.7) vs. WBCT20+ tests: median delay 0.82 hours (IQR: 0.58 to 1.48); p=0.0007] (Figure 3). Delays in both groups were often caused by further WBCT20 tests being performed (Figure 2). Even 12 of 56 positive WBCT20 tests had one or two further WBCT20 performed before antivenom (versus 41 of 84 in WBCT20 negative tested patients).

WBCT20 Testing

There were 232 paired WBCT20 and INR tests done within one hour of each other during the admission for the 145 patients with Russell's viper envenoming. In 221 of the paired tests the INR was greater than 1.5 and the WBCT20 was only positive in 91 (41%) of these. Figure 4 shows the number of tests that were positive or negative based on the paired INR result. The proportion of WBCT20 that were positive only increased slightly with higher INR values (those indicating more severe coagulopathy). Figure 5 compares the INR values for negative versus positive WBCT20. The median INR for WBCT20+ was 4.2 (IQR: 2.4 to 8.5; Range: 1 to >12) compared to 3.0 (IQR: 2.1 to 6.3; Range: 1.3 to >12; p=0.004) for WBCT-.

There were nine non-envenomed patients with paired INR and WBCT20 tests available where the INR $\leq$ 1.4. All WBCT20 were negative in these patients. A further 48 patients bitten by non-venomous snakes (identified as cat or rat snakes) had a negative WBCT20. This indicates the WBCT20 had good specificity on admission in this setting [100% (95% CI: 94 to 100%)].

Discussion

This study has shown that at the bedside in Sri Lanka, in a busy unit treating large numbers of snakebites for years with this method, the WBCT20 had an unacceptably high false negative rate for the detection of coagulopathy in Russell's viper envenoming with a sensitivity of only 40%. The test performed only marginally better for those with severe coagulopathy and cannot be used to safely rule out coagulopathy. In contrast, the false positive rate in non-envenomed patients was zero suggesting a very high specificity of 100% (95% CI: 94 to 100%) in this setting. However, in patients with Russell's viper coagulopathy with normalising coagulation, there were some false positive WBCT20 (Figure 4). Despite the poor sensitivity of the WBCT20 to Russell's viper envenoming, all patients received antivenom, although just over one third had delayed administration.

The WBCT20 was done by the clinical staff on the ward without any supervision or training by the investigators. A protocol was used but there was not standardisation of the type of tube or whether tubes were re-used. This study assessed the WBCT20 in the clinical setting. Thus, the low sensitivity of the WBCT20 may have reflected the manner in which the test was conducted in these wards. However, this hospital has used WBCT20 in clinical practice and research for decades, including studies by the developer of the method.[18] Further studies using a standardised approach to the whole blood clotting test (i.e. use of one size of tube, the same volume of blood collection and trained operators undertaking the test), are needed to identify whether the WBCT20 can perform much better in this setting and whether it is simply poor standardisation of clinical use of the test that is the problem.

The idea behind the development of the whole blood clotting test was inspired by an obvious clinical need in the developing world and a simple logic. However, the WBCT20 has moved into dozens of guidelines around the world without any translational research; there has been

no systematic attempt at further refinement, standardisation or evaluation of its strengths and limitations. Sri Lankan doctors are using the test because they have no alternative. However, it is clear that using the test can delay appropriate treatment (Figure 3) and that the doctors treat despite a negative result (Figure 2). In 43 patients antivenom was given after one negative WBCT20 test on admission. A further 15 patients were given antivenom after a second negative WBCT20 and 9 after three consecutive negative WBCT20 (Figure 2). Therefore antivenom was given in almost half of the envenomed patients without a prior positive WBCT20.

Although numerous studies have reported whole blood clotting test results,[7, 8, 10, 11, 18-24] they provide little information on the timing of the WBCT20 and its use as a diagnostic test of coagulopathy associated with envenoming – VICC. Many clinical trials have used abnormal WBCT20 as an inclusion criteria,[23] but provide no information on the diagnostic value of the test. One previous study in Russell's viper bites in Burma included 54 cases with systemic envenoming.[19] Only 32 patients (60%) had an abnormal WBCT20 on admission, and 16 patients had a delay of 30 minutes to 15 hours until the WBCT20 was abnormal and presumably antivenom was given.[19] The proportion of abnormal WBCT20 on admission and the delay in abnormal WBCT20 is similar to our study. Another study of *E. carinatus* in Sri Lanka doesn't report the admission WBCT20 but does find a similar delay in abnormal WBCT20 of 40 minutes to 18 hours.[21] One study of *E. ocellatus* and another of *Bothrops* species report a clotting test where 30 minutes is the cut-off for incoagulable blood.[10, 11] Thus these studies, while not designed to directly assess this issue, support the issues we highlight of low sensitivity in diagnosis of coagulopathy and envenoming, and lack of standardisation.

Figure 4 shows that the WBCT20 was still not sensitive enough in patients with the most severe coagulopathy. The proportion of positive WBCT20 only increased slightly with

increasingly severe coagulopathy based on the INR (Figure 4). Thus the sensitivity of the WBCT20 to detect severe coagulopathy is not much better that its sensitivity to detect envenoming. In practice, the test could not even apparently be relied on to rule out an INR>12. This therefore raises major concerns about the use of the WBCT20 for monitoring of ongoing coagulopathy (as used by some clinicians and recommended in some guidelines.

A limitation of our study was the use of frozen samples for the INR testing. However, this has been done previously[17] and meant that all of the tests were done during a short period of time. In addition, all samples were immediately centrifuged after collection and then aliquoted and frozen by trained clinical research assistants. Unfortunately the WBCT20 was not always collected at the same time as the citrate sample for the INR because the treating team collected blood for the WBCT20 while clinical research assistants collected the research bloods. Potential inaccuracies resulting from delayed testing may have implications for the quantitative INR –WBCT20 correlations (Figure 4 and 5), but this was only a secondary analysis in the study. An abnormal INR had over 99% agreement with the diagnosis of Russell's viper envenoming based on detectable venom concentrations, indicating that the INR was highly sensitive. In contrast the initial WBCT20 had a 40% sensitivity for patients with Russell's viper envenoming. So any potential inaccuracies in the paired comparison do not affect our conclusions about the lack of sensitivity of the WBCT20. Future studies need to compare an INR done on-site with the WBCT20, but currently INR testing is not readily available in Sri Lankan hospitals.

It is clear that an inexpensive bedside whole blood clotting test for use in remote areas and developing countries could fill an important role. There also should be more attention given to standardising the conditions, timing, use and interpretation of the current WBCT20. It is reassuring that clinicians eventually gave antivenom in all envenomed patients with a

negative WBCT20 based on clinical features, indicating they recognise it has limited sensitivity. Further work is required to develop such a test that performs to an acceptable standard in the field, and delays to antivenom are minimised. To do this, it may be worth exploring a range of methodologies; an ideal test would be able to provide a result more rapidly than after 20 minutes.

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Figure Legends

***Figure 1*:** Relationship between pre-antivenom venom concentrations and the maximum INR in 123 of the 145 patients where a pre-antivenom blood samples was available.

Figure 2: Flow diagram of all 140 patients with a definite Russell's viper bite and coagulopathy who had a WBCT20 on admission, showing all WBCT20 and when antivenom was administered.

Figure 3: Time to antivenom in patients with Russell's viper coagulopathy comparing those with an admission WBCT20 positive and those with a negative WBCT20.

Figure 4: Number of WBCT20 tests that were positive or negative based on the international normalised ratio (INR) done at the same time.

***Figure 5*:** INR values for all positive WBCT20 compared to those for all negative WBCT20

Table 1: Demographic features and clinical effects for patients in the study.

	Number	**%**
Sex (male)	107	75%
Age (median, IQR); years	38 (29 to 48)	
Activity		
Working in paddy fields	38	26%
Walking outside/road	70	48%
Working outdoors or in the garden	32	22%
Other	4	3%
Indoors	1	1%
Bite Site		
Foot or Ankle	127	88%
Lower Leg	15	10%
Forearm	1	1%
Hand	2	1%
INR		
1.5 to 5	51	35%
5 to 12	42	29%
> 12	52	36%
Neurotoxicity	68	47%
Systemic Symptoms	71	49%

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