## Transfusion-transmitted Hepatitis C: a cluster of cases in transfusion-dependent thalassaemia patients in Sri Lanka

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**Abstract**

**Objectives**

To report the clinical and virologic epidemiology of a recent epidemic of Hepatitis C in thalassaemia patients in Sri Lanka.

**Background**

Transfusion-dependent thalassaemia patients remain at risk for Hepatitis C (HCV). Here we report a cluster of recent HCV infections in Sri Lankan thalassaemia patients and examine the phylogenetic relationship of viral sequences.

**Methods**

We conducted two prospective cross-sectional surveys of 513 patients in four Sri Lankan thalassaemia centres in 2014/2015 and re-surveyed one centre in 2016. We screened for anti-HCV antibodies using the CTK Biotech ELISA kits and confirmed active infection by RT-PCR for HCV-RNA. HCV genomes were sequenced by unbiased target enrichment.

**Results**

Anti-HCV antibodies were found in 116/513 (22.6%) of patients initially tested. Active Hepatitis C infection was found in 26 patients with no cases of active Hepatitis B infection. Of 26 patients with HCV, two were infected with genotype 1(a), and the rest had 3 (a). In a single centre (Ragama) 122 patients (120 new cases and two previously tested but negative) were retested for anti-HCV antibodies. 32/122 (26.2%) patients were seropositive. 23 (23/122; 18.8%) of these new cases were confirmed by HCV PCR (all genotype 3(a)).

**Conclusion**

There is a significant cluster of recent HCV cases in multiply transfused thalassaemia patients in several centres in Sri Lanka. Most of the viruses shared a close phylogenetic relationship. The results are consistent with recent continuing transfusion-transmitted HCV infection. Routine surveillance for HCV of chronically transfused patients is required irrespective of screening of blood products.

**Background**

Transfusion transmitted infection (TTI) by Hepatitis C virus (HCV) is a major health concern for patients with thalassaemia.1,2 There is no effective vaccine for Hepatitis C and so ensuring the safety of the transfused blood remains the single most important factor for prevention of HCV infection in regularly transfused thalassaemia patients.

Transfusion with HCV contaminated blood transmits the infection in more the 90% of cases.3 Transfusion-related transmission of HCV was substantially reduced following the introduction of antibody testing in 1990 and, with third-generation serological assays HCV antibodies, can be detected within 6-8 weeks of exposure with sensitivity of ~ 97%. HCV RNA can be detected within days of infection and transfusion-related transmission is exceptionally rare in parts of the world where screening by nucleotide acid amplification testing is routine.4

The global prevalence of hepatitis C virus is 1.0%.5,6,7 The seroprevalence of markers of HCV is variable in the regions in the world where thalassaemia is found. The seroprevalence for Hepatitis C is extremely high in Egypt and Pakistan (where >3% of adults are positive for anti-Hepatitis C IgG antibodies).6,7,8,9

A much lower prevalence of antibodies to HCV has been documented in Sri Lanka in multiple studies involving the general public, medical students, blood donors as well as high risk populations such as prison inmates.10,11,12,13,14 Indeed, data from South Asia published recently shows that Sri Lanka has the one of the lowest incidences of HCV, when comparing data from nine countries in the region. In Pakistan it is 6%–6.8%, the second highest in the world; in Bhutan 1.3%; in Myanmar 0.34%–2.03%; in Afghanistan 1.0%; in Nepal 0.6%; in Bangladesh 0.6%; in India 0.33%; and in Sri Lanka 0.16%-0.4%; the incidence in Maldives is not known.15

In previous surveys in Sri Lanka, in 2009 and 2010, anti-HCV antibodies were found among blood donors with a prevalence of 0.3% and 0.4% respectively.16 In a study conducted in 2001 in multiply transfused patients, 33% of haemophiliacs and 10% of thalassaemia patients were positive for HCV antibodies.17 This study however did not confirm its results with PCR testing.

The Sri Lankan National Transfusion Blood service introduced Hepatitis C screening in 2003 using an ELISA-based assay for Anti-HCV IgG and IgM antibodies (Innotest HCV Ab 2003-8; Eiagen HCV Ab 2008-12; Ortho HCV Ab 2012-13; Monolisa Hepatitis HCV Ab Version 2.0 2013-14; Monolisa Hepatitis HCV Antigen Antibody Version 2.0 2015-present). No routine HCV-RNA screening tests were carried out. The reported seroprevalence by screening HCV Antibody and confirmatory tests HCV Antigen tests was 109/38303 (0.28%) (Range 0.15-0.40%) in 4 blood collection centres in Sri Lanka. (personal communication)

Sri Lanka has approximately 2500 patients with severe thalassaemia across the island. Health care in Sri Lanka is free and blood transfusion as well as care for thalassaemia happen almost exclusively in the government managed hospitals. These patients are transfused with blood almost exclusively provided by using blood supplied by the National Blood Transfusion Service (NBTS).18 Collection of blood and screening and testing for TTIs in blood donors is done by the regional blood banks.

Testing for Hepatitis C or HIV status among the thalassaemia patients is not routinely done in most of the thalassaemia clinics in Sri Lanka. This practice perhaps stems from the belief that TTIs are not a major health concern among thalassaemia patients in Sri Lanka at least partly due to the strict screening for Hepatitis C and other potential transfusion transmitted infections by the NBTS.

We initially identified one patient with thalassaemia major from Anuradhapura in Sri Lanka who had anti-HCV antibodies and was PCR-positive for HCV RNA. This was an incidental finding during the work-up for bone marrow transplantation. We therefore decided to survey the prevalence of HBV and HCV infection in our transfusion-dependent thalassaemia cohorts across Sri Lanka to understand significance and origins of this Hepatitis C epidemic.

**Materials and Methods**

The first part of the study was conducted between 2014 January to 2015. A prospective cross-sectional study was carried out at Ragama, Anuradhapura, Badulla, and Chilaw Thalassaemia Units. The first three centres are among the top five centres in Sri Lanka in terms of patient numbers and all centres are situated in different districts in the country. Chilaw and Ragama centres are the closest to each other with a physical distance of about 70km. All the centres have their own individual blood banks supporting them with transfusions. All consenting patients, with transfusion-dependent thalassaemia (beta-thalassaemia major or haemoglobin E beta-thalassaemia) were included in the study.

Ethical approval for the study was obtained through the Ethics Review Committee of the Faculty of Medicine, University of Kelaniya (ERC No. P/86/05/2014). Details of participants were collected, using an interviewer administered structured questionnaire, by trained research assistants. This included demographic data, disease history including diagnosis, past transfusions, immunization status for HBV and other risk factors for HBV and HCV infection. Two 5ml samples of blood were obtained for screening and confirmation of HBV and HCV. The sample for screening was transported to the laboratory within one hour of collection and the sample for confirmation was stored at -80oC. Screening for HBV and HCV was performed using Hepatitis B surface antigen (HBs Ag) and Anti-HCV antibodies (anti-HCV) using CTK BIOTECH ELISA kits. Confirmation of active infection in those positive for screening was by HBV-DNA and HCV-RNA assays using quantitative reverse transcription-polymerase chain reaction (RT-PCR). The sensitivity of the RT-PCR assay was 92.0% with a lower limit of detection (LOD) of 300 copies/mL

In the Ragama thalassaemia centre, all patients who underwent initial testing in 2014 and 2015 were followed up and those who were negative for HCV initially were re- tested whenever unusually high ALT and AST levels (> 2 X upper limit of normal) were found during their regular monthly clinic visits or if their serum ferritin levels did not fall despite adequate doses of iron chelators.

Whole genomes were sequenced using the Illumina veSEQ protocol specifically developed for unbiased target-enrichment of HCV.19 In brief, 30 μl of total nucleic acid was extracted from 500 μl of plasma using the Nuclisense EasyMag system (Biomerieux) and 8 μl was used to synthesise total RNAseq Illumina libraries using the Ultra Directional Sequencing Kit (New England Biolabs), with 15 cycles of PCR amplification using customised indexed primers. Libraries were purified with Ampure XP silica beads and equal masses pooled for target-enrichment using the SeqCap ® EZ probe hybridization system (Roche) with M-270 streptavidin Dynabeads (Life Technologies) and a custom panel of 120mer biotinylated-oligonucleotide HCV probes (IDT, Iowa, US), previously shown to capture the full pan-subtype diversity of HCV. Probes specific to all major pandemic subtypes are included in the panel, and each probe tolerates up to 20% divergence from the reference sequence used in their design; the panel has been cross-validated against multiple subtypes for genotypes G1, G2, G3 and G4.19 The enriched pool of libraries was PCR amplified directly from the streptavidin-beads with a further 12 cycles of PCR amplification then sequenced on an Illumina MiSeq Instrument with v3 chemistry producing 300bp paired-end reads.

Short read data was processed to produce HCV whole genomes using a previously described custom pipeline that incorporates the *de novo* assembler, VICUNA20, with genome annotation and frame-shift correction using VFAT (<https://www.broadinstitute.org/viral-genomics/v-fat>). Near full length genomes (> 80 % coverage at a minimum deduplicated read depth of 5) were aligned using MAFT and manually curated in Genious software. Maximum likelihood trees were constructed with IQ-TREE using a GTR nucleotide substitution model and 1000 “ultrafast” bootstrap replicates ([http://www.cibiv.at/software/iqtree).21](http://www.cibiv.at/software/iqtree%29.21) A panel of G1 and G3 reference sequences derived with the same sequencing pipeline, and previously published in GenBank, were included in the phylogenetic analyses as a comparator for subtype diversity. G1 sequences were obtained from an Oxford study of first-generation direct-acting antiviral treatments and the G3 sequences were obtained from the BOSON clinical trial, which sampled from five different countries (Australia, Canada, New Zealand, United Kingdom and United States).22,23 Consensus sequences obtained for the subjects selected for this study are available on GenBank {Accession numbers to be provided}.

**Results**

***Prevalence of chronic Hepatitis C infection***

A total of 513 patients were tested in the initial study. This included 210 patients from Anuradhapura, 184 patients from Ragama, 70 patients from Badulla and 49 patients from Chilaw (Table 1). Anti-HCV antibodies were positive in 116/513 (22.6%) [97/210 (46.2%) patients from Anuradhapura, 14/184 (7.6%) from Ragama, 5/70 (7.1%) from Badulla and 0/49 patients from Chilaw. Active HCV infection was confirmed by detection of HCV-RNA in 21/210 (10.0%), 4/184 (2.2%), 1/70 (1.4%) from Anuradhapura, Ragama, Badulla, Chilaw respectively. Of the PCR positive patients 57.7% were females and 92% were Thalassaemia major (Table 1). There were no cases of HBV infection.

PCR positive patients’ age ranged from 6-27 years (mean 13.0; SD 5.4). Total transfusions ranged from 66-312 (mean 150.6; SD 61.7). A single patient out of 26 had tattooing as a traditional risk factor for acquiring HCV in addition to multiple blood transfusions. Two out of four HCV infected patients from Ragama and the patient from Badulla had blood transfusions from Anuradhapura prior to changing care to present centre. The mean number of blood transfusions per PCR positive patients in the two units were significantly different at 133.0 units in Anuradhapura, 228.0 units in Ragama.(p<0.05). Badulla had only one PCR positive patient.

***Hepatitis C genotypes***

Viraemic samples, positive by RT-PCR, were sequenced using the veSEQ bait-capture protocol, which has been shown to produce whole genome sequences at viral loads of 104 IU per ml and partial genomes at viral loads below this threshold.24

Of the 26 patients with whole, or near-whole genome sequences, all but two had infection with HCV genotype 3(a) (G3a). The two remaining HCV-positive patients, from Anuradhapura and from the Ragama centres, both had HCV genotype 1(a) (G1a). The patient from Ragama had received blood from both Anuradhapura and Ragama centres.

Comparison of the G3a genotype sequences with 491 G3a viruses showed, with high confidence (>99%, bootstrap support), they were highly similar (Figure 1). The mean nucleotide similarity amongst the G3a sequences, considering all pair-wise comparisons, was 95.7%, with a range of 94.8% - 99.1%. Of note, this nucleotide similarity falls within the range reported following seven mother-to-child transmission events in a previously published case series (94%-99%).25 We therefore concluded that the G3 infections observed in this study arouse from donors sharing very similar strains or possibly from a single or few source(s). On the other hand, the two G1a viruses were quite different with 100% bootstrap support to showing they sit within two separate clades of forty G1a viruses (Figure 2).

***Re-testing thalassemia patients for Hepatitis C***

A total of 122 patients with thalassaemia in the Ragama centre were retested for anti-HCV antibodies following raised ALT or AST levels. All but two had been included in the previous screening and had been found to be negative. 32/122 (26.2%) patients were seropositive and out of these 32 seropositive patients, 23 (23/122; 18.8%) new cases of HCV were identified by PCR. Of these 23 patients, 9 were diagnosed in 2016, 13 in 2017 and one already diagnosed within the first three months of 2018. They were all of genotype 3(a). They included one patient with haemoglobin E-beta thalassaemia and 22 patients with beta thalassaemia major. The two previously untested patients had recently transferred care for the Eastern province of the country. None of the new patients had a link to the Anuradhapura hospital. The age of the new study group ranged 14- 39 years (mean 24.2 years; SD 6.2). The total transfusion load in this group ranged from 120-400 (mean 271.8; SD 74.9). All infected cases are now received treatment with directly active anti-viral drugs.

**Discussion**

In this study we have described evidence of a seroprevalence of Hepatitis C antibodies in transfusion dependent thalassaemia patients in 4 centres in Sri Lanka. We have also shown a substantial number of patients have active infection with Hepatitis C with evidence of on-going transmission of the virus. Infections with Hepatitis C occurred in 4 regional centres for the care of thalassemia. The infections would be consistent with failure of screening of blood donors to detect and exclude donors carrying Hepatitis C or direct spread from patient to patient. This is the first extensive survey of the incidence of Hepatitis C in chronically transfused patients in Sri Lanka since screening for Hepatitis C was introduced and suggests a review of screening of blood donors and hospital practices is urgently required to prevent further infection.

In the initial survey in 2015, anti-HCV antibodies were positive in 22.2% (116/513) of patients and HCV was confirmed in 5% (26/513) of patients in this cohort of transfusion-dependent thalassaemia patients. There were no cases of HBV infection in this cohort. In the absence of the other traditional risk factors, 97.3% of HCV positive cases were very likely to have acquired HCV from blood transfusions. There is no practice of reusing syringes or using glass syringes in the thalassaemia units and all procedures are done using disposable plastic syringes. The number of centres involved would be consistent with infection by blood transfusion rather that isolated problems with procedures at one centre. Screening for Hepatitis C is carried out by the regional transfusion services and so suggests a technical failure of screening procedures and/or kits.

This is the first study to report such high prevalence of HCV infection among a high-risk group in Sri Lanka. The only other documented study from thalassaemia patients was done in 2001 merely using an ELISA test for anti-HCV antibodies and where the positivity rate was 10%. The 5% HCV PCR positivity in the thalassaemia patients contrasts quite significantly with data from other high-risk populations in Sri Lanka, for example a 0.5% HCV prevalence was found in intravenous drug abusers.14 The prevalence of anti-HCV and HCV RNA positivity was higher in the Anuradhapura Centre when compared with other three Centres. The reason for the observation remains unclear as the transfusion screening policies at all four centres were identical and there have been no reports of increased HCV community prevalence from the Anuradhapura districts compared to other districts.

High anti-HCV positivity of 22.2% of patients with only 5 % of patients with confirmation (HCV-RNA positive) may be a combination of false positivity, spontaneous clearance of HCV in the recipient or passive transfer of HCV antibodies from the donor. Such false positivity also arises in the presence of previous infections with other RNA viruses and is a common phenomenon in low prevalence countries. Only patients with anti-HCV positive were tested for HCV RNA, rather than the whole population. Therefore, we did not test for HCV RNA in anti-HCV negative patients. In doing so we may have missed some cases due to the window period of HCV infection. Despite this limitation we were able to demonstrate high active HCV infection rate in this population. Furthermore, due to logistical restraints we were unable to perform a second HCV antibody assay approved by FDA on the initial anti-HCV positive samples to confirm true anti-HCV positive cases.

The follow up study in the Ragama centre in which PCR positive HCV patients increased from 4/184 in 2015 to 27/122 in January 2018 suggest a high rate of new infection. There are several areas of concern in this situation; clearly it is very likely that the HCV was transmitted through blood products in Sri Lanka and it seems to be a relatively recent and an ongoing phenomenon. If it was not the case, earlier studies are likely to have identified this high prevalence. The rapid rate at which new HCV infected patients are being diagnosed at the only thalassaemia unit where HCV is being continuously monitored is of concern and suggests the need for urgent screening for all chronically transfused patients.

As donor-testing for HCV using ELISA was started in 2003 in Sri Lanka, it is a concern that there is a significant incidence of new hepatitis C that appear to be caused by blood transfusion. In a study in 2012, researchers had argued against introducing NAT testing in the NBTS for HCV testing in Sri Lanka citing the relatively low prevalence. This new data however may suggest the need to revise the decision.

The study also highlights a weakness in thalassaemia care in Sri Lanka. As mentioned before surveillance for TTI among thalassaemia patients is low. This clearly needs to change in the light of this study.

Further study of the seroprevalence of anti-Hepatitis C antibodies, their decline over time and the presence of HCV RNA in blood donors may help to explain the apparent failure of screening due to low antibody titres in donors with active infection or indeed transmission from seronegative donors. Such studies could inform future policy regarding screening for carriers of Hepatitis C and improvement of current antibody screening tests and/or implementation of NAT screening. While there are international guidelines for the care of thalassaemia patients that suggest annual Hepatitis C screening, these have not been translated into national guidance in Sri Lanka. This case series represents an opportunity to review Hepatitis C screening in blood donors and in chronic transfused patients. Furthermore, detection and reporting of transfusion transmitted infection and implementation of remedial measures if needed would be greatly helped by a national haemovigilance scheme. If there are systemic problems in screening donors for Hepatitis, it is also possible similar problems may exist in other jurisdictions.

This is the first report of high HCV prevalence in a specific group to be reported from Sri Lanka. The high prevalence among thalassaemia patients should not be seen as a problem restricted to thalassaemia patients but more so as a possible failure of haemovigilance, thus a much broader investigation is necessary to prevent widespread infection. These cases may also prompt action in other countries to review the effectiveness of Hepatitis C screening in blood donors.

**Declarations**

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AP., S.P. and M.N, devised the study, S.P., P.C.A., U.B.N., D.R. and I.D.S. collected samples and provided clinical data, S.P., D.B. and M.D.C. analysed clinical samples A.P., D.B., S.P., M.N. and A.A. analysed data, A.P., M.N. and D.J.R. wrote the drafts, all authors reviewed the final manuscript.

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**Conflict of interest**

The authors have no competing interests.

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Figure 1.

Phylogenetic tree estimated by maximum likelihood of 23 whole-genome consensus sequences identified as genotype 3 alongside 381 genotype 3a reference sequences publicly available on NCBI ([https://www.ncbi.nlm.nih.gov](https://www.ncbi.nlm.nih.gov/)). All Sri Lanka sequences are contained within a single clade (shown in red) with 100% bootstrap support, and with relatively short terminal branch lengths suggesting a relatively recent common ancestor or a single source.

Figure 2.

Phylogenetic tree of the two whole genomes identified as genotype 1a alongside 40 genotype 1a references. Sequences (shown in red) were contained within distinct clades in all of 1000 bootstrap resampled trees.