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Phylogenetic analysis in the clinical risk management of an outbreak of hepatitis C virus infection among transfused thalassaemia patients in Italy

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SUMMARY

Background: Occurrence of hepatitis C virus (HCV) infection is reduced by effective risk management procedures, but patient-to-patient transmission continues to be reported in healthcare settings.

Aim: To report the use of phylogenetic analysis in the clinical risk management of an HCV outbreak among 128 thalassaemia outpatients followed at a thalassaemia centre of an Italian hospital.

Methods: Epidemiological investigation and root-cause analysis were performed. All patients with acute hepatitis and known chronic infection were tested for HCV RNA, HCV genotyping, and NS3, NS5A, and NS5B HCV genomic region sequencing. To identify transmission clusters, phylogenetic trees were built for each gene employing Bayesian methods.

Findings: All patients with acute hepatitis were infected with HCV genotype 1b. Root-cause analysis, including a lookback procedure, excluded blood donors as the source of HCV transmission. The phylogenetic analysis, conducted on seven patients with acute infection and eight patients with chronic infection, highlighted four transmission clusters including at least one patient with chronic and one patient with acute HCV infection. All patients in the same cluster received a blood transfusion during the same day. Two patients with acute hepatitis spontaneously cleared HCV within four weeks and nine patients received ledipasvir plus sofosbuvir for six weeks, all achieving a sustained virological response.

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Conclusion: Combined use of root-cause analysis and molecular epidemiology was effective in ascertaining the origin of the HCV outbreak. Antiviral therapy avoided the chronic progression of the infection and further spread in care units and in the family environment.

Introduction

The occurrence of hepatitis C virus (HCV) infection in healthcare settings has been markedly reduced by application of effective risk management procedures and use of highly sensitive screening tests to detect HCV infection among blood donors [1–3]. Although transfused patients, including thalassaemia major (TM) patients, are presently at very low risk of acquiring HCV infection by transfusion in high-income countries, HCV persists as a major cause of liver-related morbidity among these patients [4,5]. New infection, or reinfection with a new viral strain, has been detected in transfused TM patients and other high-risk groups, both in developed and developing countries [6–8].

Few studies have reported the use of HCV genotype and phylogenetic analyses combined to investigate HCV infection associated with nosocomial exposure [9–11].

Since more than 50% of thalassaemia patients with acute HCV infection develop chronic liver damage, it is important to test the effectiveness of direct-acting antiviral (DAA) therapies in preventing the spread of HCV and the progression of liver damage in the chronic phase of the infection [12]. Although DAA therapy is attractive due to its potential short duration, high efficacy, and favourable toxicity profile, none of the available DAAs has been licensed for acute hepatitis thus far. Recent studies have reported the results of DAA therapy in patients with acute HCV hepatitis, but only one study has evaluated DAA therapy in patients with genotype 1b without HIV coinfection [13–15].

This article describes an HCV outbreak in a group of transfused TM outpatients which started in May 2016 and ended in July 2017. Root-cause analysis was applied to investigate the causes of virus transmission, and HCV genotype and phylogenetic analyses were employed to support the epidemiological investigation and the clinical risk management.

Methods

Following an outbreak of HCV infection among 128 TM patients being treated at the Thalassaemia Centre at a hospital in Sicily, Italy, the transfused TM patients were screened annually as recommended by international guidelines [16]: all anti-HCV negative patients were tested for anti-HCV antibodies (HCV-IgG), while those known to be anti-HCV positive were tested for serum HCV RNA. Liver function tests (aspartate aminotransferase, alanine aminotransferase) were evaluated at the time of any blood transfusion.

Patients who tested negative for anti-HCV antibodies were considered free from HCV infection. Anti-HCV-positive patients with repeatedly positive serum HCV RNA were considered to have a chronic HCV infection, while anti-HCV positive patients with a negative serum HCV RNA test were considered to have recovered from infection, spontaneously or after antiviral therapy.

A new HCV infection was diagnosed if seroconversion (a positive anti-HCV test in previously anti-HCV negative patient) was detected and serum transaminase values were higher than the normal values (40 IU/mL), while an HCV reinfection was diagnosed when increased serum transaminase values and a positive serum HCV RNA test were detected in a patient who had previously been anti-HCV positive but persistently negative for serum HCV RNA.

Root-cause analysis was employed to exclude the transmission of HCV infection from blood donors and to investigate possible errors in blood processing, storage, and transfusion. The Ishikawa diagram representing the root-cause analysis of the HCV outbreak is shown in Figure 1.

Whole blood and serum samples from each patient with acute or chronic HCV infection were collected and stored at −20°C and underwent testing for HCV RNA, HCV genotypes, viral genome sequencing, and phylogenetic analysis. As previously described, HCV RNA was extracted and then amplified by reverse transcriptase–polymerase chain reaction (RT–PCR) and, when appropriate, a second nested amplification was performed [17]. Sanger methodology and next generation sequencing were applied following validated protocols to sequence the NS3, NS5A, and NS5B HCV genomic regions [18,19].

To identify potential transmission clusters, a phylogenetic analysis was carried out using firstly the MEGA 6.0 software to build a phylogenetic tree for each gene with the maximum likelihood (ML) method. The ML tree was inferred with the general time-reversible nucleotide substitution model (GTR) with gamma-distribution among site rate heterogeneity, a proportion of invariable sites ($G + I + \Gamma_5$) and 1000 bootstrap replicates [20]. The tree was rooted using a midpoint rooting by FigTree software v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Finally, the Bayesian phylogenetic tree was reconstructed with MrBayes, using a GTR+ $I + \Gamma_5$. The Monte Carlo Markov Chain (MCMC) search was run for 5×10^6 generations with the trees sampled every 100th generation (with a burn-in of 10%) [21]. Statistical support was obtained by calculating the posterior probability of each monophyletic clade, and a posterior consensus tree was generated after 10% burn-in. Clades with a posterior probability of $\geq$0.90 were considered transmission clusters.

The data set also contained the specific reference sequence 1b 58355 plus twenty NS3/NS5A/NS5B sequences, randomly selected from routine clinical samples, as control. IL28B genotyping for *rs12979860* was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA) as previous described [12].

Patients experiencing new acute hepatitis or reinfection and all patients with chronic HCV hepatitis received a

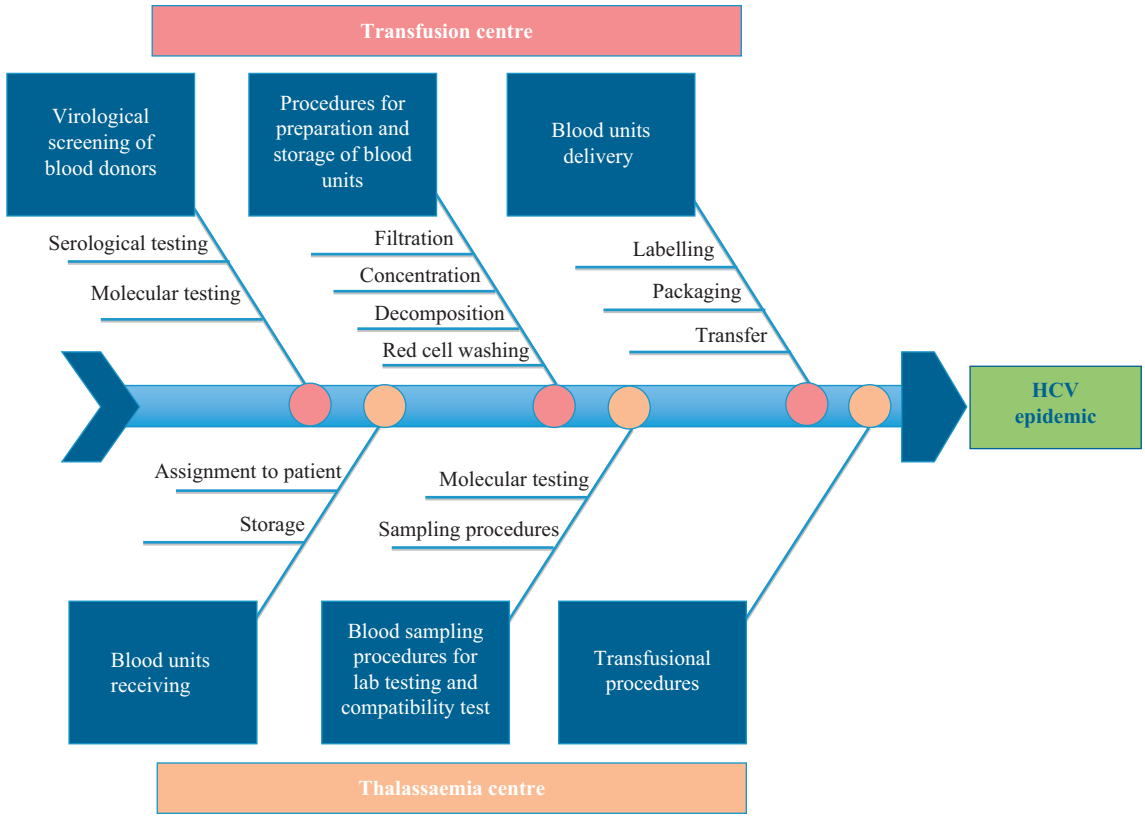

Figure 1. Ishikawa diagram representing the root-cause analysis of the hepatitis C virus (HCV) outbreak occurred among 128 thalassaemia major transfused patients.

description of the scheduled virological tests and all signed informed consent for testing and personal data management.

This study was conducted in agreement with the Helsinki Declaration. Since the available DAAs were not licensed for acute hepatitis, the regional health authorities and local ethics committee authorized DAA therapy in patients with diagnosis of acute hepatitis.

Guidelines for transparent reporting of outbreak reports of nosocomial infection (ORION statement) were applied (see Supplementary Appendix) [22].

Data were analysed after regional health authorities suspended the embargo on confidentiality of procedures and the root-cause analysis.

Results

Root-cause analysis

A multi-disciplinary team consisting of an epidemiologist, a haematologist and a hepatologist investigated the causes of the HCV outbreak and disposed any action needed to prevent any occurrence of new infections.

The first patient with acute HCV hepatitis was identified on October 5th, 2016, and eight more acute cases were identified through November 7th, 2016. After a retrospective evaluation of all TM patients followed in 2016 in the Thalassaemia Centre, three additional cases of acute HCV hepatitis were identified between May and September 2016.

A complete list of the patients who were admitted to the centre from January to December 2016 was consulted together with the results of their serum HCV RNA and anti-HCV antibodies, the diary reporting date of blood transfusion, pre-transfusion blood tests, and the results of biochemical tests performed on patients with HCV acute infection. In March 2016 all thalassaemic patients followed in the Centre performed the annual HCV screening. Twelve patients with previously known chronic HCV hepatitis who had not been treated with DAA were found to be anti-HCV positive and HCV RNA positive. Seven of the eleven patients who subsequently showed acute hepatitis were anti-HCV negative, while four were anti-HCV positive and HCV RNA negative (Tables I and II).

A lookback of all blood donors from whom TM patients experiencing acute hepatitis had received blood was organized: no blood donors reviewed were positive for serum HCV RNA and anti-HCV antibody testing. The procedures adopted for blood filtration, concentration, decomposition and red cell washing, storage and delivery of the blood units were all judged as safe. The interviewed nurses reported that the intravenous cannulas of TM patients who received blood transfusion on the same day could have been washed with the same saline solution package using the same syringe (Figure 1). Thus, the main deficiency highlighted during the on-site visit in the Thalassaemia Centre was a procedural error in the management of peripheral venous access for blood transfusion. The investigative team prescribed the use of two different peripheral cannulas for pre-transfusion and blood transfusion procedures and these measures were effective in containing

Table I
Screening of patients with HCV acute hepatitis before the outbreak

Patient ID	Sex	Age (years)	Date HCV screening	Anti-HCV	HCV RNA
1	Female	35	Mar 2nd, 2016	Neg	
2	Male	33	Mar 14th, 2016	Neg	
3	Male	61	Mar 2nd, 2016	Neg	
4	Female	42	Mar 3rd, 2016	Pos	Neg
5	Female	30	Mar 14th, 2016	Neg	
6	Female	30	Mar 31st, 2016	Neg	
7	Male	23	Mar 3rd, 2016	Neg	
8	Female	40	Mar 11th, 2016	Pos	Neg
9	Female	45	Mar 17th, 2016	Pos	Neg
10	Male	34	Mar 14th, 2016	Neg	
11	Female	30	Mar 1st, 2016	Pos	Neg

HCV, hepatitis C virus; Neg, negative; Pos, positive.

Table II
Screening of patients with HCV chronic hepatitis before the outbreak

Patient ID	Sex	Age (years)	Date HCV screening	Anti-HCV	HCV RNA	HCV genotype
12	Male	38	Mar 4th, 2016	Pos	Pos	1b
13	Male	55	Mar 21st, 2016	Pos	Pos	1b
14	Female	55	Mar 16th, 2016	Pos	Pos	1b
15	Male	45	Mar 14th, 2016	Pos	Pos	1b
16	Male	35	Mar 14th, 2016	Pos	Pos	1b
17	Male	47	Mar 11th, 2016	Pos	Pos	1b
18	Female	51	Mar 11th, 2016	Pos	Pos	1b
19	Female	50	Mar 7th, 2016	Pos	Pos	1b
20	Male	35	Mar 11th, 2016	Pos	Pos	2c
21	Male	70	Mar 29th, 2016	Pos	Pos	1b
22	Female	41	Mar 23rd, 2016	Pos	Pos	1b
23	Male	41	Mar 15th, 2016	Pos	Pos	2c

HCV, hepatitis C virus; Pos, positive.

the HCV outbreak. From December 2016 to July 2017 no new cases of acute hepatitis were observed.

Molecular epidemiology investigation

The molecular epidemiology investigation identified 11 patients with acute HCV infection (seven with new infection and four with reinfection). Of these, two showed spontaneous clearance of serum HCV RNA, and two patients had a very low viral load that did not enable definition of the phylogenetic profile. Twelve patients with chronic HCV infection managed in the Thalassaemia Centre gave consent for the phylogenetic analysis. HCV sequencing was successful for the NS5B gene in 19 patients, for NS3 gene in 18 patients, and for NS5A gene in 17 patients. Seventeen available patients had a genotype 1b infection and two patients with HCV chronic infection had a genotype 2c.

The phylogenetic trees based on HCV genotype 1b NS3, NS5A, and NS5B nucleotide sequences from seven patients with acute infection and 12 patients with chronic infection, who had at least two amplified genetic sequences, highlighted five transmission clusters (Figure 2). Four clusters included at least one patient with chronic and one patient with acute HCV infection. The fifth cluster included three patients with chronic hepatitis who had received the same virus strain in their previous transfusions when screening for HCV infection was not yet available. The remaining five chronically HCV-infected patients had no viral similarities with other patients.

Patients of each cluster including patients with chronic infection and patients with new infection or reinfection were together at the Thalassaemia Centre during the same day to receive a blood transfusion, and the mean time between the day identified as the moment of the HCV infection and the diagnosis of acute hepatitis was 9 weeks (range: 3–24). In addition to the phylogenetic similarity of the HCV strains, the incubation time and the clinical diagnosis of the acute hepatitis cases were consistent with HCV infection acquired on a specific day, and from a patient with chronic infection (Table III).

All clusters had a statistically significant bootstrap ML $\geq$90% and a Bayesian posterior probability $\geq$0.99, providing convincing evidence that the virus of each cluster had a common origin.

Antiviral therapy

Two of the 11 patients with acute hepatitis showed a spontaneous clearance of HCV within four weeks following the clinical and virological diagnosis of acute hepatitis. The two patients with spontaneous clearance had a new infection and had a CC gene of the IL28B polymorphism, while among the

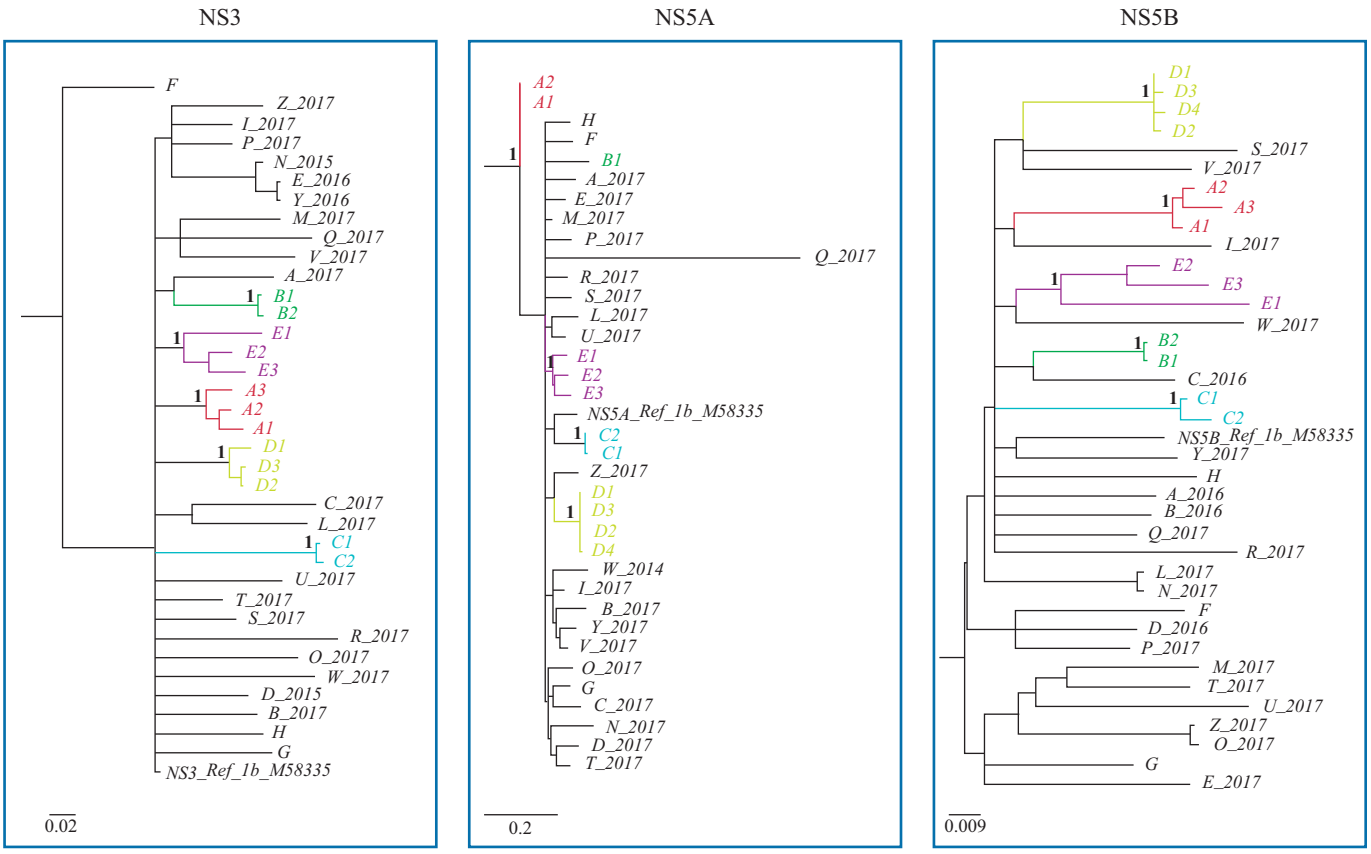

Figure 2. Neighbour-joining phylogenetic tree based on hepatitis C virus (HCV) genotype 1b NS3, NS5A, and NS5B nucleotide sequences. Clusters are highlighted in different colours.

nine patients who did not have a spontaneous clearance of HCV infection patients 4, 3, and 2 had a CC, CT and TT genotype, respectively. None of the four reinfected patients showed spontaneous HCV clearance.

Between December 19th, 2016 and January 27th, 2017, nine patients with acute hepatitis started antiviral treatment (Table IV). The mean time between the diagnosis of acute hepatitis C and initiation of antiviral therapy was 10 weeks (range: 4–26).

Patients received ledipasvir plus sofosbuvir as a fixed dose combination of 90 mg ledipasvir and 400 mg sofosbuvir once daily for six weeks. The serum HCV RNA was measured at baseline, week 2, week 4, and week 6 of antiviral treatment and 12 weeks after the end of therapy. Mean HCV RNA viral load at baseline was 11.35 IU/mL (range: 212.0–45.3).

All patients completed the scheduled course of DAA treatment. At week 2 of antiviral treatment, HCV RNA viral load was negative in eight patients and <15 IU/mL in one patient. At week 4 and at the end of therapy (treatment week 6), all nine patients had undetectable HCV RNA. All of the nine patients had undetectable HCV RNA 12 weeks after completion of treatment: thus, the proportion of patients with a sustained virological response 12 weeks after treatment was 100%. All patients achieved normalization of serum transaminases during the treatment and none of them experienced severe adverse events related to DAA. In July 2017, the HCV outbreak was considered closed and all patients with acute hepatitis were cured of the infection.

Discussion

This outbreak investigation identified seven new HCV infections and four HCV reinfections among 128 TM outpatients managed at a hospital in western Sicily, Italy. This observation confirms previously published data demonstrating the possibility of reinfection both in patients who have recovered spontaneously from HCV infection and in patients who have achieved a sustained virological response after antiviral therapy [6,23,24]. In fact, some defects in adaptive immune cell populations in patients with HCV infection can restrict themselves in obtaining only a limited functional memory and allowing the reinfection of cured individuals [25].

Patient-to-patient transmission of HCV infection in healthcare settings has been documented by molecular investigations sometimes assisted by HCV genotypic and phylogenetic analysis [9–11].

As previously reported, the molecular methodologies based on viral gene sequencing and phylogenetic analysis is the best approach to identify viral transmission, by assessing genetic viral similarities within the individuals involved [17].

Our study, through HCV sequencing, investigated the HCV quasi-species that were involved in the outbreak of HCV infection in TM patients. Among the 17 patients whose HCV sequences could be analysed, four patients with chronic HCV infection and seven with acute HCV infection were involved in transmission clusters. NGS analysis confirmed that all individuals were infected with HCV GT1b subtype, without cases of

Table III
Reconstruction of events related to the HCV outbreak: timing of the contacts between the source cases and the index/incident cases within each single infection cluster

HCV infection cluster	Patient ID	Cluster code	Type of case	Status of HCV infection	Day of transfusion procedures	Day of acute hepatitis diagnosis	AST (UI/mL)	ALT (UI/mL)	Time of incubation (weeks)	HCV genotype
A	12	A1	Source	Chronic infection	Aug 30th, 2016					1b
	4	A2	Index	Reinfection		Nov 11th, 2016	495	732	10	1b
	8	A3	Index	New infection		Oct 3rd, 2016	271	416	5	1b
B	16	B1	Source	Chronic infection	Aug 10th, 2016					1b
	6	B2	Index	Reinfection		Nov 14th, 2016	81	211	13	1b
C	19	C1	Source	Chronic infection	Oct 28th, 2016					1b
	7	C2	Index	Reinfection		Nov 28th, 2016	90	386	4	1b
D	23	D1	Source	Chronic infection	Apr 12th, 2016					1b
	10	D2	Index	Reinfection		May 1st, 2016	879	1550	3	1b
	9	D3	Index	New infection		May 3rd, 2016	599	713	3	1b
	11	D4	Index	New infection		Sep 27th, 2016	77	185	24	1b

HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

minority variants of other genotypes and/or HCV subtypes, excluding cases of coinfection [17]. The phylogenetic analysis on NS5A, NS5B, and NS3 genes confirmed four clusters considered epidemiologically monophyletic clades, with a posterior probability $\geq$99.

Each cluster included at least one patient with chronic infection and at least one patient with acute hepatitis, who received a blood transfusion on the same day. The Bayesian phylogenetic tree analysis performed on HCV sequences confirmed that the virus identified in every cluster had a high degree of similarity consistent with a common origin.

Phylogenetic HCV tree analysis, in combination with the analysis of transfusion diaries, allowed for accurate clustering of acute and chronic cases, clarifying that the outbreak was due to patient-to-patient transmission through incorrect procedures in the management of peripheral venous access for blood transfusion. Root-cause analysis excluded the possibility of transmission from blood donors or contamination of the blood supply. The corrective actions prescribed by the investigative team were effective in containing the HCV outbreak.

Whereas current blood policies provide safe access to blood and blood derivates, parenteral transmission still remains top of the list in HCV infection, particularly in outpatient settings. Transmission from hospital personnel or patient-to-patient transmission through the use of common instruments such as peripheral venous ports or subcutaneous infusion pumps, has been reported before [26,27]. Implementation of clinical risk management procedures, infection control strategies and practice, including aseptic techniques, is important in modern medicine [28].

A definite conclusion about the route of HCV spread in small clusters of infection is often very difficult to reach, and only a few studies have used genome sequencing in support of epidemiological investigations in healthcare settings [29,30]. This study demonstrates the usefulness of viral phylogenetic analysis as a complement to more widely used virologic tests in epidemiological investigations. The detection of multiple clusters of HCV transmission pointing to patient-to-patient transmission, combined with the lookback of blood donor records, clarified the cause of HCV outbreak and led to effective action to improve the safety of healthcare for this group of high-risk, frequently transfused TM patients.

Regarding antiviral treatment, our results confirm that a short course of six weeks with ledipasvir and sofosbuvir regimen resulted in a sustained virological response in all patients with acute HCV genotype 1b infection.

Antiviral treatment was planned only after the discovery of the hepatitis C outbreak in the thalassaemia centre. Thus, the mean time between first diagnosis of acute hepatitis C and start of antiviral therapy was longer than that reported in clinical trials of patients with simple HCV infection or with HCV–HIV coinfection [13–15].

All patients were observed for at least four weeks before starting therapy and this allowed us to evaluate the rate of spontaneous clearance and the role of IL28B polymorphism. The nine patients treated with DAA had a persistence of HCV RNA for over four weeks and this delay before starting treatment does not appear to have compromised the outcome of antiviral therapy. Within the first two weeks of therapy eight of the nine patients had attained negative HCV RNA serum and by the fourth week of treatment all patients had negative HCV RNA serum. The observation after 12 weeks after the end of

Table IV
Characteristics, treatment management, and virological data of the nine patients with acute hepatitis that started antiviral treatment

Patient ID	Sex	Age (years)	Date of diagnosis of acute HCV	IL28B	Start of antiviral treatment	Baseline HCV RNA (IU/mL)	HCV RNA (IU/mL)			
							Week 2	Week 4	Week 6	Week 12
3	Male	61	Oct 19th, 2016	CC	Jan 27th, 2017	28.9	Neg	Neg	Neg	Neg
4	Female	42	Nov 11th, 2016	CT	Feb 21st, 2016	13.9	Neg	Neg	Neg	Neg
5	Female	30	Sep 27th, 2016	TT	Dec 19th, 2016	45.6	Neg	Neg	Neg	Neg
6	Female	30	Nov 7th, 2016	CC	Dec 22nd, 2016	212.0	<15	Neg	Neg	Neg
7	Male	23	Nov 14th, 2016	CC	Dec 26th, 2016	3.6	Neg	Neg	Neg	Neg
8	Female	40	Nov 28th, 2016	CC	Dec 23rd, 2016	5.9	Neg	Neg	Neg	Neg
9	Female	45	Nov 7th, 2016	CT	Jan 12th, 2017	3.1	Neg	Neg	Neg	Neg
10	Male	34	Oct 3rd, 2016	CT	Dec 23rd, 2016	416.0	Neg	Neg	Neg	Neg
11	Female	30	May 1st, 2016	CC	Dec 23rd, 2016	1.2	Neg	Neg	Neg	Neg

HCV, hepatitis C virus; Neg, negative.

antiviral therapy confirmed that all patients achieved a sustained virological response.

Whereas this study is limited by its observational design and by the small number of patients studied, it adds evidence that patients with previous HCV infection do not develop protective immunity against subsequent infections, even with viruses of the same genotype, and that treatment regimens based on DAAs, even if administered for a short time, can effectively induce viral clearance.

In conclusion, the combined use of root-cause analysis and molecular epidemiology was effective in ascertaining the origin of the HCV outbreak, but also in leading to improved clinical risk management of the infected patients and in containing the outbreak. The use of effective antiviral therapy avoided the chronic progression of the infection and further spread in care units and in the family environment.

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Author contributions

W.M. and V.D.M. conceived the analysis and wrote the manuscript. F.B. and V.D.M. enrolled patients and collected clinical data. R.P., F.G., and D.F. performed virological and genetical tests; M.A., L.F., V.C.D.M., and F.C.-S. performed the HCV Sanger sequencing, bioinformatics, and phylogenetic analyses. C.M., M.M., F.V., F.D.R., and F.C.-S. reviewed the manuscript. W.M. and V.D.M. have accessed and verified the data. All authors approved the manuscript.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2021.06.007.

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