Low prevalence of hepatitis C virus RNA in blood donors with anti-hepatitis C virus reactivity in Rwanda

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BACKGROUND: Hepatitis C virus (HCV) is the leading cause of severe liver disease worldwide and is highly endemic in Africa, where it often has nosocomial spread. Little is known on the HCV prevalence, risk for transfusion-transmitted HCV, and circulating genotypes in Rwanda. This study was performed to investigate the prevalence of anti-HCV among blood donors from all regions of the country and genetically characterize identified HCV strains.

STUDY DESIGN AND METHODS: Data on anti-HCV reactivity for all 45,061 Rwandan blood donations during 2014 were compiled. Samples from 720 blood donors were reanalyzed for anti-HCV in Sweden. Line immunoassay INNO-LIA HCV and detection of HCV RNA by polymerase chain reaction were used to confirm anti-HCV reactivity. The NS5B and core regions were sequenced and phylogenetic analysis was performed.

RESULTS: The anti-HCV prevalence among all first-time blood donors was 1.6%, with the highest occurrence in donors from the eastern region. On further analysis, only 25 of 120 primarily anti-HCV–reactive samples could be confirmed reactive and 15 samples had indeterminate results by INNO-LIA. Confirmed reactivity was more common among females than males (p = 0.03) with no regional difference. Phylogenetic analysis of the sequences showed a predominance of subtypes 4k, 4q, and 4r, with no geographical difference in their distribution.

CONCLUSION: The prevalence of anti-HCV among Rwandan blood donors has probably been overestimated previously due to the high rate of nonconfirmable anti-HCV reactivity. Further study of the involved mechanism is needed to avoid loss of blood products and distress for blood donors and other test recipients.

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus classified into the Flaviviridae family in the genus Hepacivirus. Its genome is approximately 9.6 kb and translated into a 3000-amino-acid-long polypeptide, which is posttranslationally cleaved into 10 different proteins, three structural and seven nonstructural.1,2 An error-prone replication by the viral polymerase is responsible for emergence of mutations, some of which may become fixed and thereby a high genetic diversity of the virus evolves with time. To date, HCV has been classified into seven different genotypes.

ABBREVIATIONS: CMIA = chemiluminescent microparticles immunoassay; CML = Clinical Microbiology Laboratory; NCBT = National Center for Blood and Transfusion; S/CO = signal to cutoff.

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genotypes, designated 1 to 7; each subdivided into subtypes, designated with letters, a to t. The genotypes and subtypes have different geographical distribution across the globe, exhibit differences in sensitivity to antiviral compounds, and may have differences in the rate of progression to severe forms of liver disease. Genotypes 1 and 3 are ubiquitously found but are more predominant in Western Europe, North America, and East Asia. Genotypes 2, 4, and 5 are mainly reported from African countries, with Genotype 2 being common in West Africa, Genotype 4 in North and Central Africa, and Genotype 5 in South African regions.

HCV infection is a major health problem worldwide. More than 170 million of the global population are chronically infected and are at different stages of HCV-induced liver disease including asymptomatic forms, liver cirrhosis, and hepatocellular carcinoma. There is a geographic disparity in global prevalence with East Asia and North Africa having the highest rates while most sub-Saharan African countries have moderate prevalence estimated at 3% to 4%.

The clinical pattern of HCV infection includes a usually asymptomatic acute phase from which only 25% to 40% of patients spontaneously clear the infection during the first 12 months. Thereafter, those remaining with the infection will progress to a chronic phase with a low rate of spontaneous clearance. These patients are at long-term risk to develop liver cirrhosis and hepatocellular carcinoma, if efficient treatment is not initiated. The therapeutic approach to chronic infection has until recently relied upon pegylated interferon and ribavirin with comparatively low antiviral effectiveness, a long duration of the treatment, and severe side effects. Nowadays, new direct-acting antivirals targeting one or more nonstructural proteins of HCV such as the protease, NS5A, and the RNA polymerase have been developed achieving higher sustained viral response rates (>90%) with a short duration of treatment and minimal side effects. These antiviral compounds are, however, very expensive and therefore not available to many patients in need of treatment. Current therapeutic protocols have high efficiency against Genotypes 1, 2, and 3, but studies are ongoing toward the development of a pan-genotypic treatment. Determination of the infecting HCV genotype is therefore still needed before the initiation of therapy and the determination of circulating HCV genotypes nationwide together with the prevalence of infection are paramount for health care planning and resource allocation.

The prevalence of HCV and the circulating genotypes are well known in almost all developed countries and, to a lesser extent, in some Asian and North African countries. However, this is not the case for several sub-Saharan countries where substantial epidemiologic data on HCV prevalence are still lacking. In Rwanda, in particular, efforts are being made to allow access to HCV antiviral compounds but the gaps in knowledge on the number of potential beneficiaries and epidemiology of circulating genotypes nationwide need to be addressed. The aims of this study were to investigate the prevalence of HCV among volunteer blood donors across all regions of Rwanda and to determine the circulating HCV genotypes and their geographical distribution.

**MATERIALS AND METHODS**

**Blood donor samples**

Data obtained from the National Center for Blood and Transfusion (NCBT) in Rwanda for anti-HCV analysis were aggregated for all 45,061 blood donations from five regions of Rwanda during 2014, 13,637 from first-time donors and 31,424 from repeat donors as previously described. Anti-HCV–positive reactivity was found in 323 of these blood donor samples (Table 1).

For this study saved samples containing more than 1 mL of serum from the 45,061 blood donations were selected. Only 512 samples were available: 79 anti-HCV reactive and 433 nonreactive. An additional 208 blood donor samples (35 with anti-HCV reactivity in Rwanda) were prospectively collected between January and February 2015. Thus, in total, 114 anti-HCV–reactive samples and 606 anti-HCV–nonreactive samples were available for further analyses at the Clinical Microbiology Laboratory (CML) of the University of Gothenburg (Fig. 1). Hepatitis B surface antigen (HBsAg) has previously been identified in six of the anti-HCV–reactive samples and in 134 of the nonreactive samples. At both laboratories, reactive samples were those with a signal-to-cutoff (S/CO) ratio of at least 1.0. At NCBT only anti-HCV reactivity or nonreactivity were manually recorded for each sample, and the S/CO values for the samples were not saved.

**Ethical considerations**

The Rwanda National Ethics Committee (RNEC) approved the whole research project (No. 243/RNEC/2014).

**Determination of anti-HCV**

All samples were assayed for anti-HCV by automated chemiluminescent microparticles immunoassay (CMIA) on an immunoassay analyzer (Architect i2000R, Abbott) during the first 24 hours after blood collection at NCBT. The 720 samples available for further analysis were transported on dry ice to CML in Sweden, where they were kept at −80°C until analyzed. At CML, the anti-HCV analysis was repeated on all samples using CMIA on Architect i4000R systems, and all reactive samples were reanalyzed with CMIA. The repeatedly reactive samples and those with discordant results from analyses at NCTB and CML were reassayed for anti-HCV using a third-generation enzyme-linked immunosorbent assay (ELISA; MUREX anti-HCV Version 4.0, DiaSorin) according to the manufacturer’s instructions.
All samples reactive for anti-HCV with both CMIA and MUREX anti-HCV ELISA and samples that were reactive with CMIA alone with values of at least 2.2 S/CO (Fig. S1, available as supporting information in the online version of this paper) and without detectable HCV RNA were further analyzed for anti-HCV by a line immunoassay (INNO-LIA HCV score; Innogenetics), with 3 hours of incubation procedure according to the manufacturer’s description.

Determination of total IgG

To investigate if the IgG concentration in the samples could influence the anti-HCV reactivity in the assays, the amount of total IgG was determined by ELISA (IgG human ELISA kit, Abcam) according to the manufacturer’s description. The amount of total IgG was investigated in all samples reactive for anti-HCV with CMIA, MUREX anti-HCV ELISA, and INNO-LIA HCV, together with all samples that were reactive with CMIA but nonreactive with ELISA and vice versa. In total, there were 83 samples analyzed, 11 with confirmed anti-HCV reactivity, 14 with indeterminate INNO-LIA reactivity, 20 with nonconfirmed anti-HCV reactivity, 28 samples with low S/CO values between 1 and 4.9 in CMIA, and 10 samples from anti-HCV–nonreactive blood donors with S/CO values below 0.05. The amount of IgG was also determined in samples from five Swedish anonymized blood donors negative for anti-HCV with CMIA.

Nucleic acid extraction

Nucleic acids were extracted from 200 μL of all serum samples reactive for anti-HCV with CMIA or with MUREX anti-HCV ELISA with NucliSENS EasyMAG (bioMérieux) and eluted in 100 μL as previously described.\(^1\)

Quantitative polymerase chain reaction for HCV RNA detection

The extracted nucleic acids were analyzed by a TaqMan quantitative polymerase chain reaction (PCR) assay using primers corresponding to the nucleotide position 180 to 354 of the 5’UTR of the HCV genome. Each 50-μL reaction mix contained 20 μL of RNeasy plate, 25 μL of 2× universal master mix/MgCl\(_2\), 40 U of recombinant ribonuclease inhibitor (RNaseOUT, Invitrogen), 200 U of reverse transcriptase Taq mix (SuperScript III, Invitrogen), 0.1 mmol/L HCVKOD sense primer (5’-CTAGCCGAGTAGYGGTTGGT-3’), 0.4 mmol/L HCVKOD antisense primer (5’-CATGTTGCACGGTTACGAG-3’), and 0.2 mmol/L probe HCVKDO-(FAM-5’-CTCGCAAGGCACCTATCGGACGATC-3’-BHQ1). The amplification was performed with an initial reverse transcription at 50°C for 45 minutes followed by an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

| TABLE 1. Anti-HCV reactivity among all blood donors in Rwanda from 2014 and in 720 samples collected for this study* |
|---|---|---|---|---|---|---|
| Region and types of donors | All blood donors from 2014 | Blood donor samples from 2014 and 2015 for this study anti-HCV reactive in Rwanda† | Males | Females | Total | Males | Females | Total |
| | anti-HCV reactive/total number analyzed | anti-HCV reactive in Rwanda†/total number analyzed | | | | | | |
| East (n = 4,338) | | | | | | | | |
| First-time | 55/961 (5.7) | 21/516 (4.1) | 76/1,477 (5.1) | 29/72 (40) | 3/11 (27) | 32/83 (39) |
| Repeat | 63/2,314 (2.7) | 11/547 (2) | 74/2,861 (2.6) | 9/88 (10) | 0/11 (0) | 9/99 (9) |
| Subtotal | 118/3,275 (3.6) | 32/1,063 (3) | 150/4,338 (3.5) | 38/160 (24) | 3/22 (14) | 41/182 (23) |
| Kigali (n = 19,479) | | | | | | | | |
| First-time | 32/2,326 (1.4) | 14/1,002 (1.4) | 46/3,328 (1.4) | 1/44 (2.3) | 0/9 (0%) | 1/53 (1.9) |
| Repeat | 7/13,424 (0.05) | 3/2,727 (0.1) | 10/16,151 (0.06) | 1/10 (10) | 1/4 (25) | 2/14 (14) |
| Subtotal | 39/15,750 (0.25) | 17/3,729 (0.5) | 56/19,479 (0.3) | 2/54 (3.7) | 1/13 (7.7) | 3/67 (4.5) |
| North (n = 7,083) | | | | | | | | |
| First-time | 17/1,618 (1.1) | 1/523 (0.2) | 17/2,141 (0.8) | 8/56 (14) | 2/13 (15) | 10/69 (14) |
| Repeat | 0/4,550 (0) | 0/392 (0) | 0/4,942 (0) | 1/10 (10) | 0/1 (0) | 1/11 (9) |
| Subtotal | 17/6,168 (0.3) | 1/915 (0.1) | 17/7,083 (0.2) | 9/130 (6.9) | 2/23 (8.7) | 11/153 (7.1) |
| South (n = 9,081) | | | | | | | | |
| First-time | 30/2,144 (1.4) | 24/1,865 (1.3) | 54/4,009 (1.3) | 19/79 (24) | 11/31 (35) | 30/110 (27) |
| Repeat | 13/3,319 (0.4) | 5/1,753 (0.3) | 18/5,072 (0.3) | 18/119 (15) | 5/29 (17) | 23/148 (15) |
| Subtotal | 43/5,463 (0.8) | 29/3,618 (0.8) | 72/9,081 (0.8) | 37/198 (19) | 16/60 (27) | 53/258 (21) |
| West (n = 5,080) | | | | | | | | |
| First-time | 21/1,577 (1.3) | 7/1,105 (0.6) | 28/2,682 (1) | 6/46 (13) | 3/6 (50) | 9/52 (17) |
| Repeat | 0/1,642 (0) | 0/756 (0) | 0/2,398 (0) | 3/8 (37) | 0/0 (0) | 3/8 (37) |
| Subtotal | 21/3,219 (0.6) | 7/1,861 (0.4) | 28/5,080 (0.6) | 9/54 (17) | 3/6 (50) | 12/60 (20) |
| Total (n = 45,061) | | | | | | | | |
| First-time | 155/8,626 (1.8) | 67/5,011 (1.3) | 222/13,637 (1.6) | 63/297 (21) | 19/70 (27) | 82/367 (22) |
| Repeat | 83/25,449 (0.3) | 19/6,175 (0.3) | 102/31,624 (0.3) | 32/299 (11) | 6/54 (11) | 38/353 (11) |
| Total | 238/33,875 (0.7) | 86/1,186 (0.8) | 323/45,061 (0.7) | 95/596 (20) | 25/124 (20) | 120/720 (17) |

*Data are reported as number (%) †Including six samples probably wrongly recorded in Rwanda as anti-HCV negative.
by denaturation at 95°C for 10 minutes; thereafter, the amplification was performed for 45 cycles with denaturation at 95°C for 30 seconds and elongation at 60°C for 60 seconds in a sequence detection system (ABI7300, Applied Biosystems). Three 10-step serial dilutions from 1 in 10 to 1 in 1000 of extracted RNA from a sample with $1.1 \times 10^5$ IU HCV RNA/mL was analyzed in duplicate and nuclease-free water was used as negative control in each assay. The amount of HCV RNA in this sample had been determined with the Cobas Taqman assay (Roche). Regression analysis based on the Ct values and dilutions of the sample with known amount of HCV RNA was used for estimation of the viral load of HCV RNA in the samples. The lower detection limit was at 10 IU HCV RNA/mL.

**cDNA synthesis**

cDNA synthesis was performed on extracted RNA from all samples with detectable HCV RNA by the TaqMan quantitative PCR assay. The reaction mixture contained 5 μL of extracted nucleic acids, 4 μg of random primers, 0.5 mmol/L deoxynucleotide triphosphates (dNTP), 1× first-strand buffer (Invitrogen), 5 mmol/L of dithiothreitol (Invitrogen), 40 U of recombinant ribonuclease inhibitor (RNaseOUT, Invitrogen), and 200 U of reverse transcriptase (Superscript III, Invitrogen) in a final volume of 25 μL. The mixture was incubated at 25°C for 10 minutes.

Fig. 1. Flow chart showing the selection and analysis results of the samples obtained for this study. *Probably wrongly recorded as anti-HCV negative in Rwanda.
followed by 50°C for 90 minutes. The products were stored at −80°C until further analyses.

**PCR amplification of the NS5B and core regions**

PCR and seminested PCR were performed on cDNA from the HCV RNA–positive samples to amplify 364 nucleotides of partial NS5B and 411 nucleotides of partial core regions. The PCR mixture contained 5 µL of the synthesized cDNA, 1× Taq buffer (Applied Biosystems), 0.2 mmol/L dNTP, 0.3 mmol/L of each primer, cs1/cs1 (ACTGGCCTAGGTTGGCTC/ATGTTACCCCATGAAGTCG) for the core region, and ns5bs/ns5bas (TATGAACTGCCTGA/GAGGAGCAAGATGTTATCAGCTC) for the NS5B region, 1 U of Taq DNA polymerase, MgCl2 in a final volume of 50 µL. The PCR procedure was performed with initial denaturation at 95°C for 3 minutes followed by 40 cycles with 94°C for 30 seconds, 61°C (core region) or 59°C (NS5B region) for 45 seconds, and 72°C for 75 seconds and one cycle at 72°C for 10 minutes. Three microliters of the first-round product was used as template in the second amplification round with primers cs2 (AGGTTCTTGACGTGA/GAGGAGCAAGATGTTATCAGCTC) for the core region and ns5bs/ns5bas2 (GAACCTGTATAGGCTTGC/ATGTTACCCCATGAAGTCG) for the NS5B region. The PCR procedure was performed as for the first PCR for the core region and for the NS5B region with an annealing temperature of 60°C.

**Sequencing of the NS5B and core regions**

The PCR products obtained were purified and extracted with a PCR purification kit (QIAquick, Qiagen) as previously described18 and sequenced in both sense and antisense directions using the same primers as in the PCR with a cycle sequencing kit (BigDye Terminator 3.1, Applied Biosystems) on a genetic analyzer (3130xl, Applied Biosystems) as previously described.18 All sequences obtained are deposited in GenBank with Accession Numbers MF000430-MF000456.

**Phylogenetic analysis**

The sequences obtained were analyzed in the SeqMan Pro 13 program in the DNAStar Program package version 13.0.2 (DNAStar, Inc.). The sequences were aligned with the corresponding region of 115 NS5B and 234 core sequences of HCV obtained from GenBank. Most sequences were of Genotype 4 and originated from Africa, Middle East, Western Europe, and Northern America. Evolutionary distances were calculated using the Hasegawa-Kishino-Yano (HKY) algorithm in the DNADIST program in the PHYLIP package version 3.6519 with a transition/transversion ratio of 4.02 for NS5B and 2.86 for the core region with gamma correction with alpha 0.36 and 0.34 for the NS5B and core region, respectively. Phylogenetic trees were constructed using the unweight pair-group method using arithmetic averages (UPGMA) and the neighbor-joining method in the NEIGHBOR program of the PHYLIP package. The trees were visualized with the program Tree View, Version 1.6.6.20

**Statistical analysis**

Categorical data are presented by percentages and comparisons between groups using chi-square test and odds ratio (OR) with 95% confidence interval (CI). Linear regression was performed for the association between the total IgG and the concentration of anti-HCV R², the slope (k), and 95% CI are given. All statistical analyses were performed by using a computer program (SPSS 24.0, IBM Corp.), and a p value of less than 0.05 was considered significant.

**RESULTS**

**Anti-HCV reactivity in Rwanda for all blood donors in 2014**

From all of the 45,061 blood donations collected in 2014, a total of 323 (0.7%) were recorded as anti-HCV positive at NCBT in Rwanda, 221 (1.6%) from first-time donors and 102 (0.3%) from repeat donors (Table 1). The donors from the Eastern region were significantly more often recorded as anti-HCV positive than those from the other regions of the country (OR, 8.4 [95% CI, 6.7-10.5]; p < 0.0001). There was no difference between the sex with 0.7% of the males and 0.8% of the females being reactive for anti-HCV.

**Anti-HCV reactivity in samples obtained for this study**

A total of 114 of the 720 blood donor samples available for further analysis in this study were reactive for anti-HCV with CMA in Rwanda. On reanalysis of these samples, only 76 (67%) remained repeatedly reactive with CMA in Sweden (Fig. 1). An additional six samples that were reported as negative for anti-HCV in Rwanda were reactive for this marker in Sweden. Five of these six samples had probably been wrongly recorded at NCBT as HbsAg instead of anti-HCV positive due to the manual recording of the results. The sixth sample was only recorded as HbsAg positive at NCBT. These samples were included in a study on HbsAg prevalence among blood donors in Rwanda.18

A total of 120 of the 720 samples were thus anti-HCV reactive in Rwanda, 82 of them in Sweden (Fig. 1). The majority of these anti-HCV-reactive samples in Sweden (64; 78%) had low anti-HCV reactivity with S/CO values between 1 and 4.9 (Table 2). This low anti-HCV reactivity was found in samples from donors originating from all parts of the country with no regional difference (p = 0.772; OR, 1.29 [95% CI, 0.38-4.39]; Table 2).

Only 43 of the 106 samples reactive for anti-HCV with CMA in Rwanda and without detectable HCV RNA were reactive also with ELISA (Fig. 1 and Fig. S1). Concordant
| Variable          | Number | CMIA in Sweden S/CO reactive (%) | 0-0.99 | 1-4.99 | > 5 | CMIA assay in Sweden, N reactive (%) | HCV RNA positive | Indeterminate | Positive | Number of first-time blood donors/anti-HCV reactive in Rwanda (%) | Number of repeat blood donors of anti-HCV reactive in Rwanda (%) | Total number of blood donors of anti-HCV reactive in Rwanda (%) |
|------------------|--------|---------------------------------|--------|--------|----|-------------------------------------|-----------------|--------------|----------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Region           |        |                                 |        |        |    |                                     |                 |              |          |                                                              |                                                              |                                                               |
| East             | 182    | 41 (22)                         | 153    | 24     | 5  | 29 (16)                            | 3                | 6            | 6        | 12/32 (38)                                                   | 3/9 (33)                                                      | 15 (37)                                                        |
| Kigali           | 67     | 3 (4.5)                         | 65     | 1      | 1  | 2 (3)                              | 0                | 0            | 0        | 0/1                                                          | 0/2                                                           | 0 (0)                                                          |
| North            | 153    | 11 (7.1)                        | 143    | 7      | 3  | 10 (6.5)                           | 3                | 1            | 0        | 4/10 (40)                                                    | 0/1                                                           | 4 (36)                                                         |
| South            | 258    | 53 (20)                         | 228    | 26     | 6  | 32 (12)                            | 5                | 6            | 4        | 13/30 (43)                                                   | 2/23 (9)                                                      | 15 (28)                                                        |
| West             | 60     | 12 (20)                         | 51     | 6      | 3  | 9 (15)                             | 3                | 2            | 1        | 5/9 (56)                                                     | 1/3 (33)                                                      | 6 (50)                                                         |
| Age (years)      |        |                                 |        |        |    |                                     |                 |              |          |                                                              |                                                              |                                                               |
| <29              | 380    | 72 (19)                         | 336    | 34     | 10 | 44 (12)                            | 6                | 12           | 8        | 23/56 (41)                                                   | 3/16 (19)                                                     | 26 (36)                                                        |
| 30-39            | 253    | 37 (15)                         | 224    | 25     | 4  | 29 (11)                            | 4                | 3            | 2        | 7/20 (35)                                                    | 2/17 (12)                                                     | 9 (24)                                                         |
| 40-60            | 87     | 11 (13)                         | 78     | 5      | 4  | 9 (10)                             | 4                | 0            | 1        | 4/6 (67)                                                     | 1/5 (20)                                                      | 5 (45)                                                         |
| Sex              |        |                                 |        |        |    |                                     |                 |              |          |                                                              |                                                              |                                                               |
| Male             | 596    | 96 (16)                         | 534    | 50     | 12 | 62 (10)                            | 10               | 9            | 8        | 22/63 (35)                                                   | 5/32 (16)                                                     | 27 (28)                                                        |
| Female           | 124    | 25 (20)                         | 104    | 14     | 6  | 20 (16)                            | 4                | 6            | 3        | 12/19 (63)                                                   | 1/6 (17)                                                      | 13 (52)                                                        |
| Type of donor    |        |                                 |        |        |    |                                     |                 |              |          |                                                              |                                                              |                                                               |
| First time       | 367    | 82 (22)                         | 308    | 43     | 16 | 59 (16)                            | 13               | 11           | 10       | 34 (41)                                                      |                                                              | 34 (41)                                                        |
| Repeat           | 353    | 38 (11)                         | 330    | 21     | 2  | 23 (6.5)                           | 1                | 4            | 1        | 6 (16)                                                       |                                                              | 6 (16)                                                         |
| Total            | 720    | 120 (17)                        | 638    | 64     | 18 | 82 (11)                            | 14               | 15           | 11       | 34 (41)                                                      | 6 (16)                                                        | 40 (33)                                                        |

*All samples with S/CO > 1.0 were confirmed for anti-HCV reactivity by INNO-LIA HCV or HCV RNA.
results for anti-HCV reactivity with all three assays were obtained for 33 of the 106 samples (31%); eight of the anti-HCV–reactive samples with ELISA were nonreactive with CMIA in Sweden (Fig. 1).

The anti-HCV reactivity was also analyzed by INNO-LIA HCV in the 33 samples reactive with all assays and for additional 14 HCV RNA negative samples. Nine of these 14 samples were reactive for anti-HCV with CMIA both in Sweden and in Rwanda; they were, however, nonreactive with ELISA. Five of the 14 samples were those probably wrongly recorded as anti-HCV negative in Rwanda (Fig. 1). Ten of the 33 samples reactive with ELISA and CMIA in Sweden and CMIA in Rwanda were confirmed anti-HCV positive with INNO-LIA, an additional eight of these samples were indeterminate reactive with INNO-LIA, and the remaining 15 triple-reactive samples could not be confirmed anti-HCV reactive by INNO-LIA (Fig. 1). Two samples only reactive for anti-HCV with ELISA and CMIA in Sweden could not be confirmed reactive with INNO-LIA. Among the 12 samples reactive with CMIA in both Sweden and Rwanda but nonreactive with ELISA, one was confirmed reactive, seven were indeterminate, and four were nonreactive with INNO-LIA.

Anti-HCV reactivity by CMIA and ELISA could thus be confirmed with INNO-LIA or HCV RNA for 25 samples and was indeterminate by INNO-LIA for 15 of the initially 120 reactive samples (Table 2). None of the samples with indeterminate anti-HCV reactivity had detectable HCV RNA. If indeterminate reactivity would be considered as a marker of past infection there were thus only 40 of the 120 (33%) initially reactive blood donors that could be confirmed anti-HCV reactive. There was no age difference of the donors with or without confirmed anti-HCV reactivity (<30 years vs. 30 years and more; p = 0.553, chi-square test; Table 2). There was, however, a difference in sex, with females more often having confirmed anti-HCV reactivity (p = 0.03, chi-square test; Table 2).

### Determination of total serum IgG concentration

A high amount of total IgG in serum has been suggested for unspecific reactivity for anti-HCV and was analyzed in 83 samples. The amount of total IgG in all Rwandan samples ranged between 6.43 and 39.4 mg/mL (mean, 25 mg/mL) and was somewhat higher than in the five Swedish donors (12.1-20.5 mg total IgG/mL; mean, 16.7 mg/mL) and the internationally recognized normal range of 10 to 17 mg/mL (Fig. S2, available as supporting information in the online version of this paper). There was no difference between total IgG concentration in samples with S/CO below 1 in CMIA (mean, 28.9 mg/mL; range, 12.2-39.4 mg/mL) compared to those samples with S/CO between 1 and 4.9 (mean, 24.9 mg/mL; range, 6.43-36.5 mg/mL). Nor was there any difference in total IgG concentration between samples with indeterminate or negative anti-HCV reactivity with INNO-LIA (mean, 27 mg/mL vs. 24.5 mg/mL) or between sex (26.5 mg/mL for males vs. 23.2 mg/mL for females).

### HCV RNA detection and phylogenetic analysis

HCV RNA was detected in 14 samples, all with S/CO values of more than 10.0 with CMIA. Partial NS5B was sequenced for these samples and the core region could be sequenced for 13 samples. All but one belonged to Genotype 4. The non-Genotype 4 sample was of Genotype 3, 3h, based on the NS5B sequence, and could not be amplified in the core region. This strain was isolated from a donor from the Western province (Table 3). On phylogenetic analysis it was revealed that 10 strains belonged to subtypes 4k (six samples), 4q (two samples), and 4r (two samples; Fig. 2). Two samples (RW-236 and RW-151) could not be classified into any known subtype and were found on separate branches in both phylogenetic trees formed by sequences from the NS5B and the core region (Fig. 2). Another sample (RW-93) was found in the clade formed...

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**TABLE 3. Age, sex, and origin of 14 blood donors with HCV RNA in relation to subtype and viral load of the infecting HCV strain**

| ID    | Age (years) | Sex   | First-time donor | Origin | Anti-HCV (S/CO) | Viral load (log IU/mL) | Subtype in the NS5B region | Subtype in the core region |
|-------|-------------|-------|-----------------|--------|-----------------|------------------------|---------------------------|---------------------------|
| RW-019| 19          | Female| Yes             | East   | 13.10           | 3.06                   | 4k                        | 4k                        |
| RW-127| 44          | Male  | Yes             | East   | 10.88           | 6.06                   | 4k                        | 4k                        |
| RW-185| 41          | Male  | Yes             | East   | 13.13           | 7.06                   | 4k                        | 4k                        |
| RW-062| 38          | Male  | Yes             | North  | 12.01           | 5.81                   | 4k                        | 4k                        |
| RW-080| 31          | Male  | Yes             | North  | 14.67           | 4.31                   | 4q                        | 4q                        |
| RW-146| 26          | Male  | Yes             | North  | 13.00           | 4.31                   | 4r                        | 4r                        |
| RW-133| 25          | Male  | Yes             | South  | 10.92           | 5.31                   | 4k                        | 4k                        |
| RW-092*| 32         | Female| Yes             | South  | 12.10           | 4.06                   | 4r                        | 4q                        |
| RW-151| 21          | Female| Yes             | South  | 13.03           | 4.31                   | 4r                        | 4r                        |
| RW-199| 35          | Male  | Yes             | South  | 12.06           | 5.39                   | 4k                        | 4k                        |
| RW-236| 40          | Male  | Yes             | South  | 13.51           | 6.06                   | 4                          | 4                         |
| RW-170| 22          | Female| Yes             | West   | 11.95           | 3.73                   | -                         | 3h                        |
| RW-195| 57          | Male  | No              | West   | 13.36           | 4.73                   | 4r                        | 4r                        |
| RW-213| 27          | Male  | Yes             | West   | 15.48           | 5.39                   | 4q                        | 4q                        |

*Discrepancy of subtype between the core and the NS5B regions.
Fig. 2. (A) Branch of a phylogenetic tree based on 436 nucleotides of the NS5B region in 13 strains sequenced in this study and 115 sequences obtained from GenBank. The origin and accession number of the strains are given at the nodes. The strains sequenced in this study are shown in red. (B) Branch of a phylogenetic tree based on 234 nucleotides of the core region of 13 strains sequenced in this study analyzed with 236 partial core gene Genotype 4 obtained from GenBank. The origin and accession number of the strains are given at the nodes in each phylogenetic tree. The strains sequenced in this study are indicated in red. The sample with different subtypes obtained in NS5B and core regions is marked with an arrow.
Fig. 2. (Continued).
by 4q strains when the core region was analyzed and in the 4r clade in the phylogenetic tree based on the NS5B region (Fig. 2). All Genotype 4 strains were intermixed in the phylogenetic trees with strains from other Central African countries and from mainly African immigrants in Western countries.

**DISCUSSION**

Relatively low anti-HCV reactivity among Rwandan first-time blood donors was found in this study, 1.6% compared to 3.1% to 4.9% reported from Rwandan HIV patients and pregnant women or from estimates based on the anti-HCV prevalence in neighboring countries.22-27 These previous reports may have overestimated the anti-HCV prevalence in the general population, since the prevalence among blood donors usually underestimates that by 24% to 44% owing to different factors such as predonation screening, higher socioeconomic status, and younger age of blood donors. However, even the relatively low prevalence of anti-HCV found in this study may also be an overestimation, since one-third of the reactive samples in Rwanda could not be confirmed reactive with the same assay in Sweden. The reason for this may be differences in the calibration of the assay and the manual recording, which indicates that training of technical staff and direct instead of manual recording of the results may be needed. In addition, near 50% of the blood donor sera with anti-HCV reactivity in both laboratories were shown to be nonreactive against HCV by confirmatory assays.

High rates of nonspecific IgG reactivity against HCV have been shown related to low concentration of anti-HCV as measured by low S/CO values, especially in African settings,28 and this has led to recommendations to change the cutoff values for the different serologic assays. For the CMIA assay used in this study, the Centers for Disease Control and Prevention in Atlanta, Georgia, has recommended an increase of the border value for anti-HCV reactivity from the recommended S/CO of 1 to 5 or more.10,29 If this value for reactivity had been used in this study, eight of 11 blood donors (73%) with confirmed anti-HCV reactivity would have been considered nonreactive. All these donors lacked detectable HCV RNA in serum, and their anti-HCV reactivity may indicate cleared HCV infection, or they may have a viral load below the detection limit, which then could lead to transfusion-transmitted HCV. In Rwanda at this time, change of the border value for anti-HCV reactivity should therefore not be recommended, and further research is required.

In this study, anti-HCV reactivity could not be confirmed by INNO-LIA for 46% of the samples with anti-HCV reactivity by CMIA and third-generation ELISA. This high nonconfirmed anti-HCV reactivity in blood donors has implications for destruction of apparently noninfected blood products, and for voluntary blood donors, who are informed that they are HCV infected, without further investigations. Therefore, an algorithm for onsite counseling and follow-up of the donors, as well as alternative assays for anti-HCV reactivity need to be introduced at the centers for blood transfusion to minimize the financial burden inherent to further medical visits and anxiety for the blood donors. It has been suggested that the high rate of unspecific anti-HCV reactivity is attributable to high concentration of human immunoglobulins in response to endemic parasitic diseases including but not limited to schistosomiasis in sub-Saharan African countries.30,31 In this study there was a higher concentration of total IgG in sera with both nonspecific and specific reactivity for HCV compared to Swedish blood donors. However, there was no support or refutation for the assumption that hyperglobulinemia may influence the anti-HCV reactivity.32 The nonverified anti-HCV reactivity may thus be due to the presence of specific IgG antibodies directed against non-HCV determinants with epitopes similar to those used in anti-HCV assays. Nearly half of all blood donors with anti-HCV reactivity during 2014 originated from the eastern part of Rwanda. There were, however, no regional differences in confirmed anti-HCV reactivity, indicating that there may be a higher prevalence of anti-HCV–infected persons in this part of the country. This is in concordance with our previous finding of higher HBsAg prevalence among blood donors from this region.18 One reason for the higher prevalence of blood-borne infections in the eastern regions may be due to the more common use of unsafe injections and traditional healing practices in this region compared to the rest of the country.

Consequent to the overall low rate of specific HCV antibodies in our study, there were also few samples with detectable HCV RNA. Only 14 of 25 blood donors with confirmed anti-HCV reactivity (56%) were viremic, and this rate decreased to 35% when those with indeterminate reactivity were included. Such low rate has also been shown from blood donors in Kenya and HIV-infected pregnant women in Malawi, where only 10% of the anti-HCV–positive donors and none of the females were viremic.30,33 This may indicate that there is a relatively high rate of spontaneous clearance of HCV among the blood donors in Rwanda, since they are relatively young, and infection in younger age has been shown favorable for clearance of acute HCV infections,13 or that some donors may be viremic with HCV RNA concentrations below the detection limit of the assay used.

On phylogenetic analysis of the sequenced HCV strains, a predominance of Genotype 4 across Rwanda was revealed. This finding confirmed previous studies showing that Genotypes 1, 2, and 4 are prevalent in the Central African region, with Genotype 4 being predominant in patients treated for hepatitis C in Rwanda and in

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**REGIONAL DIFFERENCES OF HCV REACTIVITY IN RWANDA**

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patients from bordering countries like Burundi, Uganda, Gabon, and the Democratic Republic of Congo.6,25,26,34-36 This Central African region together with the Middle East and Mediterranean regions have been shown to account for the highest number of HCV Genotype 4 infections in the world.35,37,38 There are more than 22 reported subtypes of Genotype 4 and several unassigned lineages.39 The different subtypes are found in most Central African countries, with somewhat regional differences as predominance of 4h in Burundi and of 4e in Gabon.25,35 In this study three Genotype 4 subtypes were identified, with 4k being predominant. This subtype has previously been isolated from immigrants from Rwanda in France.40

One of the blood donors in this study was infected by a strain with discrepancies in subtype determinations between the NSSB and core regions, which may indicate a possible recombination between Subtypes 4q and 4r. However, a dual infection cannot be excluded since we were unable to sequence the whole genome of this strain. Reports of intersubtype recombination within Genotype 4 are relatively scanty in general, and from the Central African region in particular, where the only recombinant so far reported is an inter-Genotype 4/1 recombinant from Cameroon.41,42

Unlike the previous reports from Rwanda and other countries in the region, we did not find any Genotype 1–infected blood donor. This genotype seems to be relatively common in the region and has been reported together with Genotype 2 in liver disease patients after treatment in one hospital in Kigali and in Kenya, Uganda, and Burundi.25,26,30,43 The reason for the prevalence of Genotype 4 among the blood donors and the absence of the other genotypes found in antiviral-treated patients may indicate differences in disease progression and rate of spontaneous clearance between the genotypes. All this leaves room for a possibility of a more diverse presence of different genotypes and subtypes of HCV in Rwanda within the highly prevalent Genotype 4 and beyond. A study with a larger sample size including liver disease patients might provide more extensive results in that regard.

In conclusion, due to a high prevalence of nonspecific reactivity for anti-HCV among blood donors, the prevalence of HCV infection may be overestimated among this group and in the general population. Genotype 4, dominated by Subtype 4k, was the most prevalent genotype infecting blood donors from all regions of Rwanda. There is a high probability for the presence of other genotypes and/or subtypes of Genotype 4 given the geographical situation of Rwanda. A larger study with a particular focus on the actualization of the screening methods to refine the diagnostic strategies is crucial, as is a study on identification of epidemiologic aspects of transmission among adults in Rwanda.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s website. Fig. S1. The distribution of S/CO obtained for anti-HCV with CMA in Sweden for all 720 blood donor samples. The anti-HCV reactivity obtained for anti-HCV with Anti-HCV ELISA MUREX for the 68 samples with S/CO > 1.0 with CMA in Sweden and negative for HCV RNA are indicated.
Fig. S2. Scatter plots representations of CMIA anti-HCV concentration (S/CO) versus total IgG concentration (mg/ml) and regression lines of this association and regression statistics provided in the corresponding figures. A. Scatter plot of IgG vs. anti-HCV for all samples by category of INNO-LIA results. B. Scatter plot and regression line for INNO-LIA positive and indeterminate combined (true reactivity). C. Scatter plot of those anti-HCV positive but INNO-LIA negative (false reactivity). D. Scatter plot for samples that were anti-HCV negative.