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Hepatitis C virus surveillance and identification of human pegivirus 2 in a large Cameroonian cohort

Mary A. Rodgers[1] | Vera Holzmayer[1] | Ana Vallari[1] | Ana Olivo[1] | Kenn Forberg[1] | Jill Fuhrman[1] | Kelly E. Coller[1] | Bih Awazi[2] | Jules Bertrand Kenmegne Sidje[2] | Matthew B. Frankel[1] | Michael G. Berg[1] | Dora Mbanya[2,3] | Nicaise Ndembi[4] | Gavin A. Cloherty[1]

[1]Abbott Laboratories, Abbott Park, Illinois, USA

[2]Université de Yaoundé I, Yaoundé, Cameroon

[3]University of Bamenda, Bamenda, Cameroon

[4]Institute of Human Virology, Abuja, Nigeria

Correspondence
Mary A. Rodgers, Abbott Laboratories, Abbott Park, IL.
Email: mary.rodgers@abbott.com

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Summary

The prevalence of chronic hepatitis C virus (HCV) and the presence of human pegivirus 2 (HPgV-2) have not been examined in Cameroon, although HCV has been associated with HPgV-2 infections previously. Herein we aimed to characterize the burden and genetic diversity of HCV and the presence of HPgV-2 in Cameroon. Retrospective plasma specimens collected from N = 12 369 consenting subjects in South Cameroon from 2013 to 2016 were included in the study. The majority (97.1%) of participants were patients seeking health care. All specimens were screened for HCV using the Abbott RealTime HCV viral load assay and positive specimens with remaining volume were also screened for HPgV-2 antibodies on the Abbott ARCHITECT instrument, followed by molecular characterization. Overall, HCV RNA was detected in 305 (2.47%; 95% CI: 2.21%-2.75%) specimens. Notably, the prevalence of HCV RNA was 9.09% amongst participants over age 40 and 3.81% amongst males. Phylogenetic classification of N = 103 HCV sequences identified genotypes 1 (19.4%), 2 (15.5%) and 4 (65.1%) within the study cohort. Amongst HCV RNA-positive specimens, N = 28 (10.6%; 95% CI: 7.44%-14.90%) specimens also had detectable HPgV-2 antibodies. Of these, N = 2 viremic HPgV-2 infections were confirmed by sequencing and shared 93-94 median % identity with strains found on other continents. This is the first study to determine the prevalence of chronic HCV in Cameroon, and the discovery of HPgV-2 in this study cohort expands the geography of HPgV-2 to the African continent, indicating a widespread distribution exists.

KEYWORDS
Cameroon, chronic HCV, human pegivirus 2 (HPgV-2), surveillance, viral diversity

1 | INTRODUCTION

Although chronic hepatitis C virus (HCV) infection affects an estimated 71 million people worldwide, HCV is now a curable disease that can be treated by potent new direct-acting antiviral (DAA) therapies.[1,2] Evidence-based policymaking to ensure that DAAs are delivered to the appropriate recipients requires identification of infected patients through diagnostic testing and prevalence

estimates to predict the cost of eradication efforts. HCV is endemic to Cameroon, with meta-analysis prevalence estimates varying from 3.6% in low-risk populations to 12.2% in high-risk populations.[3,4] A large study cohort is key to determining the actual burden of chronic HCV. The Cameroon 2011 national health survey of dried blood spots from N = 14 150 residents reported the prevalence of anti-HCV-positive individuals to be 1.1%.[5] However, the prevalence of viremic HCV infections in Cameroon remains unknown.

To date, estimates of HCV prevalence in Cameroon have relied entirely on the detection of anti-HCV antibodies.[3,4,6] Seroprevalence rates do not directly reflect the rates of chronic HCV infection since they include patients who have spontaneously cleared their infection, and antibody-negative acute infections can also be missed. In contrast, detection of viral components, such as HCV RNA or core antigen, provides a direct measurement of the prevalence of viremic HCV infections that can be cured by DAA treatment. Although a direct measurement has not been made in Cameroon, a prediction model estimates the prevalence of HCV RNA in Cameroon to be 0.7%,[7] which is similar to the rate of 0.9% (95% CI: 0.3%-1.6%) recently observed in the neighbouring Democratic Republic of the Congo.[8]

Human pegivirus 2 (HPgV-2) is a newly identified virus that is most closely related to rodent and bat pegiviruses, sharing <32% amino acid identity with these relatives,[9,10] HPgV-2 infections have been identified in plasma specimens from the USA, UK, Germany, Iran and China,[9-16] indicating that this virus is already present on at least 3 continents. The expansion of HPgV-2 surveillance to additional parts of the world will determine the prevalence and extent of genetic diversity of HPgV-2. Although the effects of HPgV-2 on human health have not been determined, an association between the detection of HPgV-2 RNA and HCV co-infection has been observed.[10-13,15]

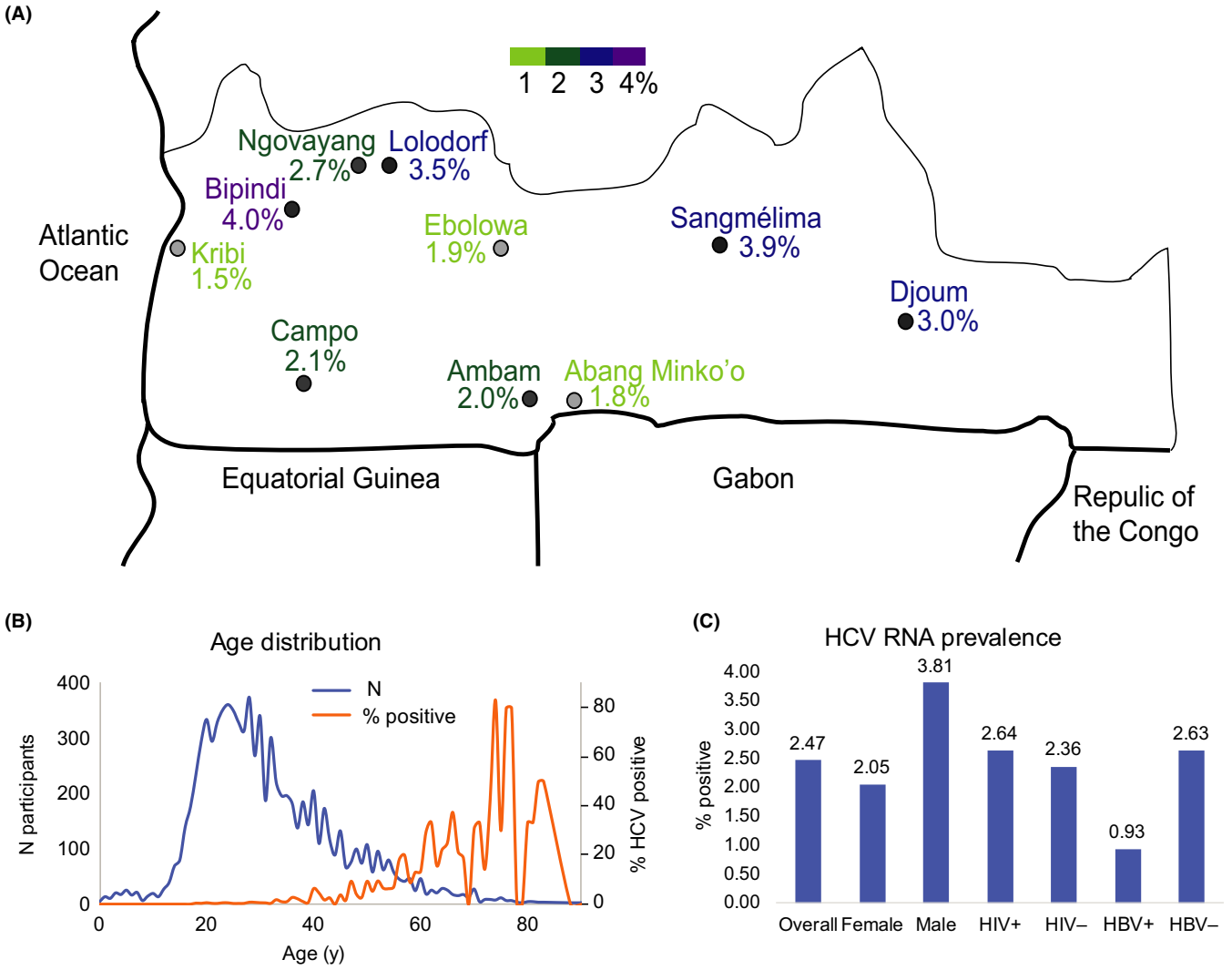

FIGURE 1 Study sites and demographics. A, The 10 study sites with at least 100 participants are shown by dots coloured to represent HCV prevalence as indicated by colour key on a map of the South Region of Cameroon with the prevalence of HCV RNA at each site indicated as a percentage of the total samples at each site. B, The distribution of ages of all study participants is shown in orange and the prevalence of HCV RNA for each age is shown in blue as a percentage of total samples for each age. C, The prevalence of HCV RNA in each cohort is indicated as a percentage

In the present study, we aimed to determine the prevalence of HCV viremia and presence of HPgV-2 in South Cameroon by screening a retrospective cohort of 12 369 plasma specimens for HCV RNA, followed by HPgV-2 antibody screening of the positives. We report the viral sequences and classifications for N = 103 HCV and N = 2 HPgV-2 strains identified in the study population, adding to the known diversity of both viruses.

2 | MATERIALS AND METHODS

2.1 | Study population

This study was approved by the Ministry of Health, the Cameroon National Ethical Review Board, and the Faculty of Medicine and Biomedical Science IRB in Cameroon. Informed consent was obtained from all participants and plasma specimens were collected anonymously in 21 towns and villages in South Cameroon (Figure 1 and Table S1). All specimens were initially collected and screened for HIV, HBV and HTLV as described previously.[17] From the larger initial study population, a retrospective cohort of N = 12 369 specimens collected from 2013 to 2016 with sufficient remaining volume were included in the present study. Collection sites and categories have been previously described, with the majority of participants reporting illness of unknown aetiology and a prescreening bias to include approximately 40% HIV-positive specimens in the study.[17] A flow chart describing the testing algorithm can be found in Figure 2.

2.2 | HCV screening

Specimens were tested for HCV RNA in pools of 5 in an off-label use of the m2000 HCV RealTime (RT) viral load assay (04J86; Abbott Molecular Diagnostics, Des Plaines, IL, USA). Positive pools were dissected by retesting individual samples at a 1:5 dilution in recalcified normal human plasma by the RT assay. Final viral load results have been corrected for the dilution factor.

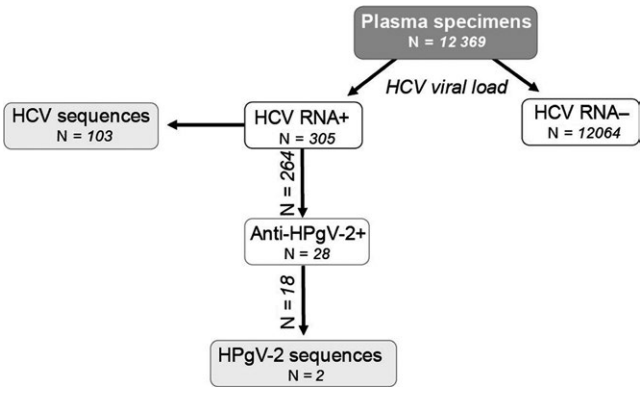

FIGURE 2 HCV and HPgV-2 testing algorithm. The number of specimens tested for each assay is shown in black adjacent to the appropriate arrow and the number of positives is indicated in the boxes for each test. Samples were tested for HCV viral load in pools of 5 in an off-label use of the Abbott RealTime assay, followed by individual testing for specimens in positive pools. Anti-HPgV-2-positive specimens include both grayzone and reactive test results

2.3 | HPgV-2 antibody screening

HCV RNA-positive specimens with sufficient volume were screened by prototype serology assays on the ARCHITECT instrument to detect IgG antibodies to HPgV-2 NS4AB and E2 proteins as previously described.[15] Results of 0.80-0.99 S/CO were considered grayzone while samples with S/CO of at least 1.0 were positive.

2.4 | HCV sequence characterization

Total nucleic acid was extracted from 0.2 ml of plasma using automated Abbott open mode TNA-200-50 protocol (Abbott Molecular Diagnostics) or alternatively, leftover RNA from the viral load dissection testing was used for RT-PCR to amplify 5′UTR-core (749 bp, H77 nucleotides 127-875) and/or NS5B (383 bp, H77 nucleotides 8250-8638) regions. For the 5′UTR-core region, one-step RT-PCR was performed with 15 μl of RNA, forward primer HCV-127F (5′ TCCCGGGAGAGCCATAGT), reverse primers HCV-852Rb (5′ AGGAAGATAGAGAAAGAGCAACC) and HCV-852Rc (5′ AGGAAGATAGAAAAGGAGCAACC) using QIAGEN One-Step RT-PCR reagents (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Cycling conditions were 50°C for 30 minutes, 95°C for 15 minutes, 50 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 1 minute 30 seconds, and final extension at 72°C for 10 minutes.

To amplify the NS5b region, a first round of RT-PCR was performed with EF101F[18] and a mix of two reverse primers HCV-NS5b-R1e (5′ GAGTACCTGGTCATAGCCTCCGTGAA) and HCV-NS5b-R2e (5′ GAGTACCTCGTCATAGCYTCCGTGAA). Cycling conditions were 50°C for 30 minutes, 95°C for 15 minutes, 5 cycles of 94°C for 15 seconds, 50°C for 30 seconds, 72°C for 1 minute; 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 1 minute; followed by final extension 72°C for 10 minutes.

Nested PCR was performed with NS5B internal primer pair HCVNS5F2p/HCVNS5Rnb[18] using AmpliTaq DNA Polymerase reagents (Applied Biosystems, Carlsbad, CA, USA) and 2 μL of first-round product as template. Cycling conditions were 95°C for 1 minute, 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 1 minute followed by final extension at 72°C for 10 minutes. Amplified products were sequenced as previously described.[17]

Sequence data were edited and assembled using Sequencher software version 5.4.6 (Genecodes Corp, Ann Arbor, MI, USA). Sequences were aligned to N = 117 reference strains including subgenotypes of HCV genotypes 1-7 by MUSCLE in Sequencher. Gaps were manually removed in BioEdit version 7.0.4.1[19] and Neighbour-Joining phylogenetic inference was performed using the PHYLIP 3.5c (J Felsenstein, University of Washington, Seattle, WA, USA) software package as previously described.[17]

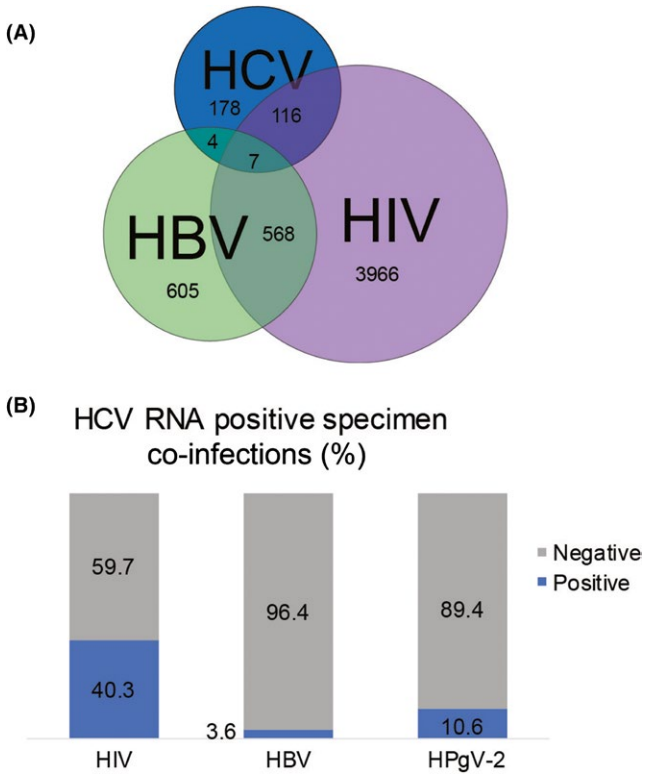

FIGURE 3 Summary of co-infections. A, The number of single, dual and triple infections identified in the study cohort is indicated by a Venn diagram. B, The percentage of all HCV RNA-positive specimens with HIV, HBV or HPgV-2 co-infections are indicated in the blue section of each corresponding bar; each portion is labelled with the percentage value plotted

2.5 | HPgV-2 sequence characterization

Leftover nucleic acid from viral load testing or benzonase-treated plasma extractions were templates for RT-PCR amplification of the 5′UTR region using QIAGEN One-Step RT-PCR reagents (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Amplified products were sequenced as previously described.[17] Sequences were aligned to all global N = 26 HPgV-2 strains and rodent pegivirus by MUSCLE in Sequencher version 5.4.6 (Genecodes Corp), and sequence identity to reference sequences was determined using BioEdit version 7.0.4.1.[19]

2.6 | HPgV-2 and HCV next generation sequencing (NGS)

Benzonase pre-treatment of plasma,[20] total nucleic acid extraction on the m2000 instrument, metagenomic library preparation, sequencing on the MiSeq instrument and analysis are described in detail elsewhere.[10] All partial HCV genomes obtained from NGS data sets had different coverage and gap positions so phylogenetic inference was applied as described above for Sanger sequences for each individual partial genome except that alignments were gap-stripped to the regions where specimen sequence was available. NGS coverage and average read depth are shown in Tables S2 (HCV) and S3 (HPgV-2). Alignment and phylogenetic inference were completed as described above.

2.7 | Statistical analysis

Confidence intervals were determined by the Wilson Score method[21] in Microsoft Excel 2016. Standard deviations were calculated in Microsoft Excel 2016.

2.8 | Data availability

HCV sequences can be found in Genbank under accession numbers MH477292-MH477414 (Sanger) and MH447417-MH477426 (NGS). HPgV-2 sequences can be found in Genbank under accession numbers MH477415 (D2931) and MH477416 (K4583).

3 | RESULTS

3.1 | Demographics

A convenience cohort of 12 369 residual plasma specimens collected in 2013-2016 as part of an HIV diversity study was selected for HCV screening based on available volume for additional testing.[17] Age and gender data were available for the majority of participants, indicating that 70.5% of the study population was female and 29.5% were male. The participant ages ranged from 6 months to 92 years old, with an average age of 31.5 years (standard deviation 12.8 years) (Figure 1). Specimens were collected mainly from ill patients at hospitals and clinics (97.1%) in 21 sites in South Cameroon.[17] Prescreening of specimens indicated that 37.7% (N = 4657) of the specimens were seropositive for HIV and 9.6% (N = 1184) were positive for HBV surface antigen (HBsAg), including 575 (4.6%) specimens from HIV-HBV co-infected participants.

3.2 | HCV prevalence

HCV RNA was detected in 305 (2.47%, 95% CI: 2.21%-2.75%) specimens (Figures 1 and 2). Of these viremic patients, 123 were also HIV positive, including 7 HIV-HBV-HCV triple infections (Figure 3). The resulting prevalence of HCV RNA within the HIV-positive group (2.6%, 95% CI: 2.2%-3.14%) was similar to the overall prevalence, while the rate amongst HBV-positive participants (0.93%, 95% CI: 0.52%-1.66%) was lower than that of HBV-negative participants or the overall prevalence (Figure 1), consistent with previous reports.[22,23] Notably, the prevalence of HCV RNA was nearly twice as high amongst males (3.81%, N = 99/2599) compared to females (2.05%, N = 127/6199, Figure 1). The mean age of the HCV RNA-positive participants was 52.5 years old while the mean age of the HCV RNA-negative participants was 31. Accordingly, the prevalence of HCV was higher amongst participants over age 40 (9.09%, 95% CI: 7.86%-10.49%) compared to the overall study prevalence (2.47%,

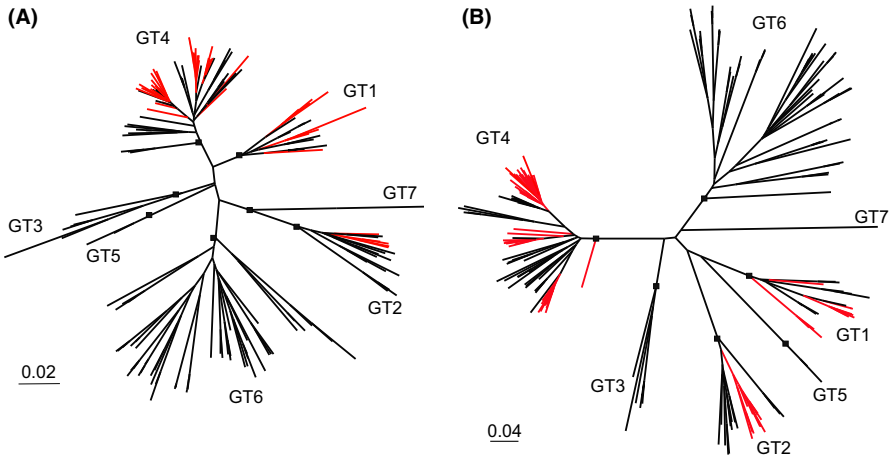

FIGURE 4 HCV phylogenetic trees. Neighbour-joining phylogenetic trees for (A) 5′UTR-core and (B) NS5B are shown with specimen sequences in red and references in black. Bootstrap values >90 are indicated by a black box

95% CI: 2.21%-2.75%, Figure 1). In contrast, HCV RNA was not detected in any of the N = 695 juvenile participants of ages 6 months to 17 years old (Figure 1). Furthermore, HCV RNA prevalence varied considerably by study collection site, with the highest prevalence rate amongst sites with at least 100 participants in Bipindi (4.0%) and the lowest rate in Kribi (1.5%, Figure 1, Table S1). The HCV viral load of positive specimens ranged from detectable (<1.48 log IU/mL at a 1:5 dilution) to 6.42 log IU/mL, with the majority of specimens having 4 log IU/mL or higher viral load (N = 218, 71.5%).

3.3 | HCV diversity

HCV genotypes were classified from sequences in the NS5B (N = 71) and/or 5′UTR-Core regions (N = 53). In parallel, partial and complete HCV genomes were obtained from the NGS data sets of 10 specimens during metagenomic sequencing of HPgV-2-positive specimens described in the following sections, with HCV coverage ranging from 36% to 100% (Table S2). The predominant classification was genotype 4 (65.1%), with genotypes 1 (19.4%) and 2 (15.5%) also detected (Figure 4, Table 1, Figure S2). As expected, all specimens sequenced in two regions had concordant classifications. A variety of subgenotypes were found in the study population, including 1a, 1c, 1e, 1g, 1h, 1l, 2q, 4f, 4p and 4t (Figure 4, Table 1, Figure S2). The diverse set of HCV subgenotypes detected here is consistent with previous reports from Cameroon.[24-27]

3.4 | HPgV-2 prevalence

Since HPgV-2 infections have predominantly been identified in viremic HCV co-infected individuals,[10,12-15] a subset of N = 264 HCV RNA-positive specimens were screened for antibodies to HPgV-2 NS4AB and E2 proteins (Figure 2). Overall, N = 28 specimens were reactive in at least one antibody assay and N = 11 additional specimens had at least one grayzone result, giving a HPgV-2 seroprevalence of 10.6% amongst HCV RNA-positive specimens (Figure 3). Higher HPgV-2 seroprevalence rates were found amongst HIV-HCV co-infected participants (13.8%, N = 15/109) compared to HCV mono-infected (8.39%, N = 13/155) participants, consistent with

TABLE 1 HCV classifications. The total number (N) of samples with each HCV classification is listed along with the percentage (%) of all sequenced HCV samples

Classification	N	%
1c	1	0.97
1e	10	9.71
1g	2	1.94
1h	6	5.83
1l	1	0.97
2	14	13.59
2a	1	0.97
2q	1	0.97
4	5	4.85
4f	37	35.92
4p	11	10.68
4t	14	13.59
Total	103	

previous reports.[12,28] Similarly, a higher HPgV-2 seroprevalence rate was observed amongst females (11.0%, N = 12/109) compared to males (5.13%, N = 4/78). However, HBV-HCV-HPgV-2 co-infections were not identified in the study cohort. Out of the N = 24 HPgV-2 seropositive participants with demographic data, the ages ranged from 24 to 77, with a mean age of 51 (standard deviation 13.6) years old. HPgV-2 seropositive specimens had HCV viral loads ranging from 1.81 to 5.98 log IU/mL and were identified in specimens from 9 study sites (Table S1, Figure S1). Amongst N = 13 HPgV-2 seropositive specimens with HCV sequences, genotypes 1e (N = 2, 15.4%), 2 (N = 2, 15.4%), 2a (N = 1, 7.7%), 4f (N = 7, 53.8%) and 4t (N = 1, 7.7%) were identified.

To identify viremic HPgV-2 infections, RT-PCR of the 5′UTR region and metagenomic next generation sequencing (NGS) methods were applied to RNA extracted from dilutions of all the remaining volume from N = 18 HPgV-2 seropositive specimens. The presence of HPgV-2 RNA was confirmed by two sequencing methods for N = 2 specimens (11.1%), both of which were co-infected with HIV

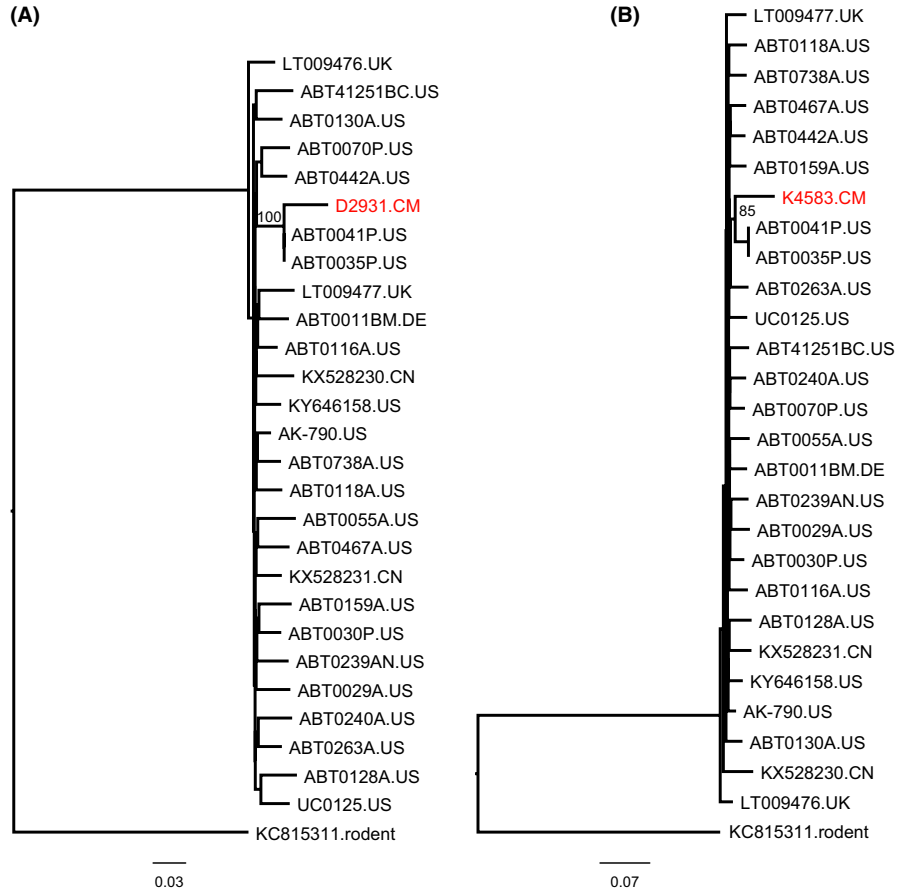

FIGURE 5 HPgV-2 phylogenetic trees. HPgV-2 phylogenetic trees. Neighbour-joining phylogenetic trees generated from alignments gap-stripped to the regions NGS coverage for a) D2931 and b) K4583 partial genome sequences are shown with specimen sequences in red and references in black. Bootstrap values are indicated for branches containing specimen sequence

(Figure 2). Each of the viremic HPgV-2 specimens was collected from 52-year-old females seeking health care; one in Ebolowa in 2013 and the other in Kribi in 2015. Metagenomic NGS data confirmed that each viremic HPgV-2 patient carried a different strain of HCV (2, D2931; 4f, K4583) and HIV (CRF02, D2931; F2, K4583). The merged Sanger and NGS consensus HPgV-2 sequences from each of the two viremic specimens resulted in 19.3% (1901 bases, D2931) and 38.3% (3778 bases, K4583) HPgV-2 genome coverage, respectively. Between the two sequences, gaps in coverage occurred in different regions of the genome, with only 281 nucleotide positions covered for both samples (Figure S3). Separate phylogenetic analyses of each Cameroonian HPgV-2 sequence indicated that the strains identified in South Cameroon branched closely with other strains (Figure 5), sharing 91.3-96% nucleotide identity with closest relative strains (ABT0035P/ABT0041P) and a median 93-94% identity with all other HPgV-2 strains.

4 | DISCUSSION

This is the largest HCV surveillance study to date in Cameroon, spanning 4 years (2013-2016) and including N = 12 369 participants from N = 21 South region sites. Furthermore, this study is the first to determine the prevalence of HCV RNA in a cohort from Cameroon, providing new insight towards developing appropriate diagnostic and treatment plans in the region. Notably, the prevalence of HCV RNA in our study (2.47%) was lower than the reported seroprevalence in Cameroon, which ranges from 3.6% in healthy cohorts to 12.2% in people who are ill,[3,4] indicating that seroprevalence measurements may not predict the number of viremic infections. Nonetheless, the HCV RNA prevalence we observed (2.47%) is consistent with the 0.7% prevalence calculated by a recent prediction model[7] and the measured 0.9% prevalence in the neighbouring Democratic Republic of Congo national survey[8] when the two major biases of our study are taken into account. First, the majority of the study population (97.1%) identified as ill patients seeking health care[17] and second, the prevalence of HIV was much higher amongst study participants (37.7%, Figure 3) than the overall prevalence of 4.3% in the general population as determined by a 2011 national survey.[17,29] Yet within the HIV-positive cohort of our study, the prevalence of HCV RNA (2.64%) was similar to the prevalence within the HIV-negative cohort (2.36%, Figure 1), indicating that the higher proportion of HIV-positive individuals in our study had a minor effect on the overall prevalence rate for viremic HCV infections. This leaves the

overrepresentation of patients seeking health care in our study population as a probable cause for a slightly higher prevalence of HCV RNA than predicted, suggesting that patients seeking health care in South Cameroon have a higher likelihood of chronic HCV infection than the general population.

Our study utilized a high throughput screening algorithm in which a primary HCV RNA screen of pools of 5 specimens was followed by dissection of positive pools and subsequent serology testing of positive specimens (Figure 2). While this method allowed us to determine the prevalence of viremic infections, the resulting prevalence could be an underestimate as the limit of detection (LOD) by our minipooling screening algorithm was 150 IU/mL (assay LOD × 5). However, the median viral load in a cohort of HCV-positive specimens from Cameroon was 6 log IU/mL in a recent study,[22] indicating that very few specimens are expected to have viral load below 150 IU/mL. Our study is consistent with previous reports of higher HCV prevalence amongst older adults in Cameroon.[5,24,26,30,31] In particular, adults over the age of 50 are at a higher risk for chronic HCV infection in Cameroon due to a spike in HCV transmission that has been estimated by population genetics to have occurred between 1920 and 1960, with prophylactic and therapeutic injections performed on a large scale during this time period as potential iatrogenic transmission routes of infection.[24,32-36] In this context, our observation that individuals over 40 years old had an elevated prevalence of viremic HCV infections (9.09%, Figure 1) further highlights that this cohort should be targeted for HCV diagnostic testing and DAA therapy towards HCV eradication.

This is the first study to report HPgV-2 infections on the African continent, with a seroprevalence of 10.6% amongst HCV RNA-positive individuals in Cameroon, which is higher than the rates observed in the USA (2.6%-3.31%)[10,15] and China (1.23%).[12] Amongst HPgV-2 seropositive individuals that we were able to test, 2/18 (11.1%) viremic infections were confirmed by two independent sequencing strategies (Figures 3 and 5), although this may be an underestimation of HPgV-2 viremic cases and an overestimation of clearance due to specimen dilution prior to molecular characterization. Notably, both of the viremic HPgV-2 infections identified in Cameroon were co-infected with HCV and HIV, consistent with previous reports of higher rates of HPgV-2 viremia within HCV/HIV-positive cohorts (3.5%, China; 10.9% USA).[12,14] The strains identified in Cameroon are most closely related to a US strain with two serial bleeds from a single individual (ABT0035P/ABT0041P).[10] The sequence identities of the Cameroonian HPgV-2 strains compared to this US strain (91.3%, K4583; 96%, D2931) and to others overall (93%, K8543; 94%, D2931) are consistent with previous reports.[9-12,14,16] HPgV-2 infections were not restricted to a single site in our study; rather, a total of 9 collection sites had specimens that were seropositive for HPgV-2, suggesting that the prevalence reported here is not due to a location-specific outbreak (Figure S1). Since HPgV-2 screening was limited to HCV RNA-positive specimens, the prevalence of HPgV-2 in HCV-negative populations of Cameroon is unknown. While previous studies have primarily identified viremic HPgV-2 infections in HCV co-infected individuals,[10-15] the role of HCV co-infection in the persistence of HPgV-2 viremia remains unknown. Likewise, additional studies are necessary to determine whether HPgV-2 impacts human health, and screening in additional geographical locations may identify previously underappreciated diversity amongst HPgV-2 strains.

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CONFLICT OF INTEREST

MAR, VH, AV, AO, KF, KEC, MBF, MGB and GAC are shareholders in Abbott Laboratories (Abbott Park, IL, USA).

ORCID

Mary A. Rodgers http://orcid.org/0000-0001-8815-8651

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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