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High prevalence of infection with a single hepatitis C virus genotype in a small rural community of Argentina

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Abstract: *Background:* During the years preceding this study, we noticed a relatively unusual high number of individuals with elevated alanine aminotransferase (ALT) levels in O'Brien, a small rural town in Argentina. Moreover, four individuals from this town underwent liver transplantation owing to hepatitis C virus (HCV)-induced liver cirrhosis. These findings prompted us to conduct a large population-based survey to evaluate the prevalence of HCV in this community. *Methods and Results:* A total of 1637 individuals were studied. The overall HCV-seroprevalence was 5.7% (93/1637), being slightly higher in men (45/769; 5.9%) than in women (48/868; 5.5%). HCV seroprevalence increased with age, reaching a peak rate of 23.9% among individuals between 61 and 70 years of age. HCV RNA was present in 82.7% of all HCV seropositive individuals identified and 100% of them were infected with genotype 1b. ALT elevations were detected in 44% of HCV+ patients and were only observed among viremic individuals. Hepatitis B virus infection was also prevalent (52%) among HCV-seropositive patients. The most common risk factor associated with HCV transmission identified was the apparent use of inadequately sterilized glass syringes by a health care provider serving the community; however, other risk factors may have also played a role in the dissemination of HCV. *Conclusions:* Our findings provide an explanation for the relative high number of individuals with elevated ALT levels observed in this community and form the basis of future prospective studies on the natural history of genotype 1b infection.

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Hepatitis C virus (HCV) infection is a major health problem with more than 170 million infected individuals worldwide (1). There is considerable geographic variation in the incidence and prevalence of this infection. Owing to the limited availability of population-based epidemiological studies, prevalence estimates have been mainly derived from studies in selected populations such as blood donors. Although these studies provide valuable information toward understanding the epidemiology of HCV infection, they probably underestimate the real magnitude of dissemination of this virus in the general population. For example, significantly different HCV prevalence rates have been reported in the United States between blood donors

(0.6%) and the general population (1.8%) (2). These observations suggest that additional epidemiological studies involving general population are needed to determine, more accurately, the extent of dissemination of HCV.

O'Brien is a rural town of approximately 2300 inhabitants in the County of Bragado, Province of Buenos Aires. During the years preceding this study, one of the authors (N. M.) responsible for routine clinical testing in O'Brien noticed a relative high proportion of individuals with elevated alanine aminotransferase (ALT) levels, some with confirmed HCV infection. Moreover, four individuals from this town underwent liver transplantation because of HCV-related liver cirrhosis.

These findings prompted us to conduct a large epidemiological study to investigate the prevalence of HCV infection in this rural community.

Patients and methods

Patient enrollment and sample collection

The population of O'Brien was offered to participate in a study designed to investigate the prevalence of HCV infection stressing the fact that results would be maintained strictly confidential and that counseling and additional testing would be provided if necessary at no charge. Written consent was obtained from each participant enrolled or from the parents if the participant was under 18 years of age. The study was approved and sponsored by the Health Department of Bragado County. A total of 1637 individuals were enrolled into the study between August and September of 1999 and a similar number of serum samples were collected.

Serology

The presence of anti-HCV antibodies was investigated with a third generation enzyme immunoassay (EIA), HCV 3.0 ELISA with enhanced SAve (Ortho Clinical Diagnostics, Raritan, NJ). Repeatedly reactive samples were assayed by Chiron recombinant immunoblot assay (RIBA) HCV 3.0 SIA (Ortho Clinical Diagnostics) according to the manufacturer's instructions. The presence of hepatitis B core antigen (anti-HBc) antibodies was investigated in all HCV-seropositive samples using a sandwich-based EIA (Ortho HBc ELISA System, Ortho Clinical Diagnostics). Anti-HBc-positive samples were further analyzed for the presence of hepatitis B surface antigen (HBsAg) and anti-HBs by EIA (Hepanostika HBsAg Uniform II and Hepanostika anti-HBsAg, respectively, Organon Teknika, Boxtel, the Netherlands) according to the manufacturer's recommendations.

Detection of HCV RNA and HCV genotyping

HCV RNA was extracted from 200 µl of serum using TRIZOL reagent (Gibco BRL, Grand Island, NY), precipitated, and subsequently resuspended in 40 µl of RNase free water. HCV cDNA was synthesized by reverse transcription from 10 µl of HCV RNA in the presence of M-MLV RT (Gibco BRL) and the antisense primer 209 (3). The resulting cDNA was subjected to two rounds of polymerase chain reaction (PCR) amplification (35 cycles each) in the presence of AmpliTaq Gold polymerase (Perkin

Elmer, Foster City, CA) and the following primers from the 5'NC region: 209, 939, 211, 949 (3). PCR products were separated by electrophoresis in acrylamide gels and visualized by ethidium bromide staining. A sample containing approximately 100 IU/ml of HCV RNA genotype 1a was prepared by diluting a sample of known copy number in HCV RNA-negative plasma. The sample was subsequently aliquoted and included as a positive control. This control sample was consistently detected in all PCR reactions performed. Genotypes were determined by restriction fragment length polymorphism (RFLP) analysis of the amplified 5'NC region product as described previously (4).

Statistical analysis

The χ^2 test was used for the evaluation of differences in proportions between groups; a *P*-value <0.05 was considered significant.

Results

The prevalence of infection was studied in a total of 1637 individuals, which represented approximately 71% of O'Brien's population (Fig. 1). The overall seroprevalence rate for HCV was 5.7% (93/1637), being slightly higher in men (45/769; 5.9%) than in women (48/868; 5.5%) (*P*>0.05). HCV seroprevalence increased with age, reaching a peak rate of 23.9% among individuals 61–70 years old (Fig. 2a). HCV EIA-positive samples (*n* = 93) were further analyzed with the RIBA 3.0 assay. The results showed that 90.4% (84/93) of the samples resulted RIBA-positive (Table 1). Only 9/93 samples (9.6%) showed reactivity against a single antigen (Table 1) and were consequently classified as RIBA-indeterminate. The presence of HCV RNA was investigated in all HCV EIA-reactive samples; results were positive in 77/93 (82.8%) cases (Table 1). HCV viremia correlated well with RIBA reactivity, being detected in 76/84 (90.5%) RIBA-positive samples and only in 1/9 (11.1%) RIBA-indeterminate samples (*P*<0.001). Although it did not achieve statistical significance, HCV RNA was detected less frequently in women (36/48; 75%) than in men (41/45; 91%) (*P*>0.05). The absence of viremia in 7/12 (85.7%) women was associated with isolated and weak reactivity against the c22 peptide.

ALT levels were measured on one opportunity in 45% of the samples, and in two, three, or more opportunities in the rest. ALT levels were considered abnormal if all available measurements were above the upper normal range (40 U/l). We

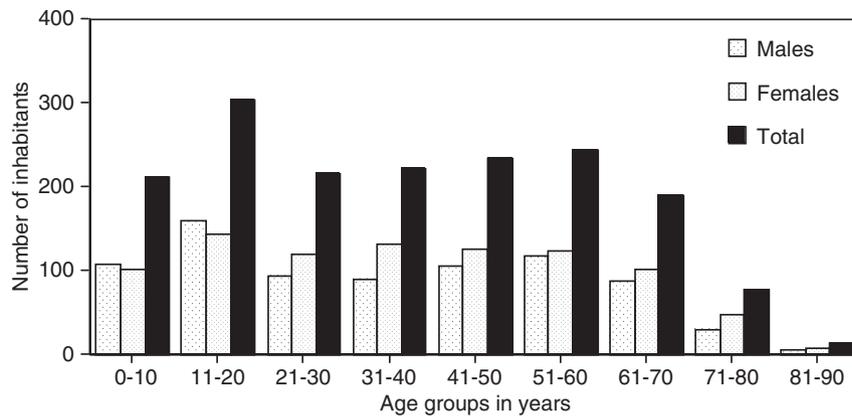


Fig. 1. Distribution of the population studied according to age and gender. A total of 1637/2300 (71.2%) subjects living in O'Brien, Argentina, were studied.

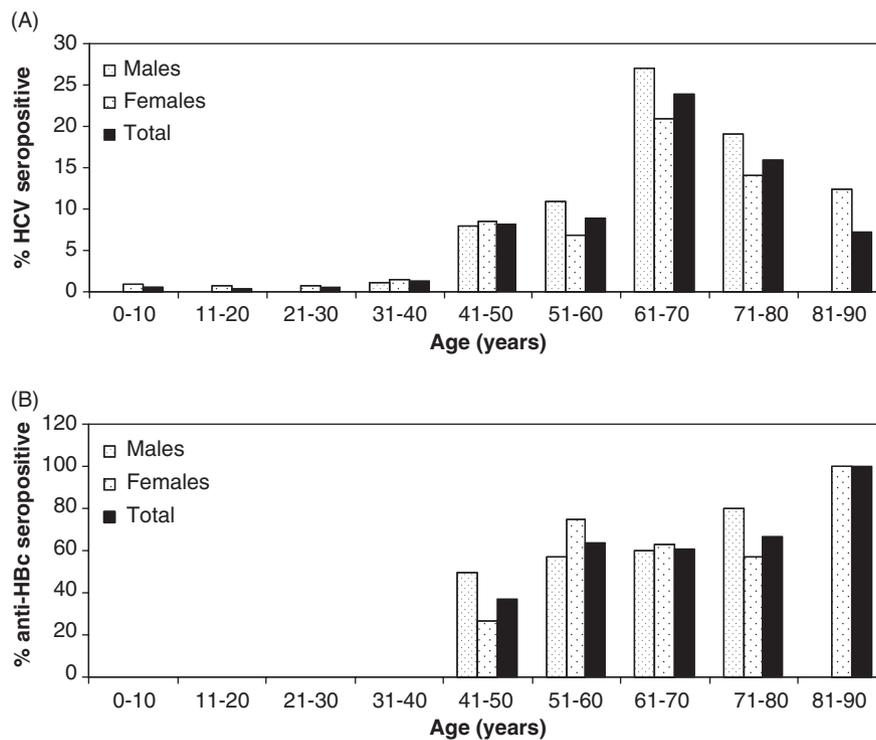


Fig. 2. Hepatitis C virus (HCV) and hepatitis B virus seroprevalence distributions according to age and gender. (A) HCV seroprevalence rates according to age and gender among 1637 subjects. The presence of HCV antibodies was investigated with a third generation enzyme immunoassay. (B) Hepatitis B core antigen seroprevalence rates according to age and gender among 93 HCV-seropositive individuals.

observed elevated ALT levels in 41/93 (44%) HCV-seropositive individuals. ALT elevation was more frequent in HCV RNA-positive than in HCV RNA-negative patients (53% vs. 0%, $P = 0.0002$).

HCV genotypes were determined by RFLP analysis in all RNA-positive samples. All samples were classified as genotype 1b and only one sample presented evidence of dual infection with genotypes 1b and 1a. A single RFLP pattern, consistent with pattern 'b' according to McOmish et al. (5), was observed in all samples analyzed

with the combination of restriction enzymes *RsaI* + *HaeIII*.

The prevalence of hepatitis B virus (HBV) infection was studied in all HCV-seropositive individuals. Anti-HBc antibodies were detected in 52% (48/93) of the cases. Anti-HBc seroprevalence also increased with age (Fig. 2b). None of the anti-HBc-reactive individuals was found to be positive for HBsAg and only 29/48 (60%) had detectable anti-HBs. The presence of HCV viremia was equally frequent among individuals positive (85.4%) or negative (80%) for anti-HBc

Table 1. Relationship between RIBA 3.0 results and HCV viremia among 93 HCV-seropositive individuals living in O'Brien

	RIBA 3.0 positive	RIBA 3.0 indeterminate	Total
HCV RNA positive	76 (81.7%)	1* (1.1%)	77 (82.8%)
HCV RNA negative	8 (8.7%)	8† (8.6%)	16 (17.2%)
Total	84 (90.4%)	9 (9.6%)	93 (100%)

*c22 band positive only. †Seven samples c22 band positive only, and 1 sample c33 band positive only. HCV, hepatitis C virus; RIBA, recombinant immunoblot assay.

antibodies ($P > 0.05$). However, it was slightly more frequent among those with isolated anti-HBc reactivity (94.7%) compared with those with resolved HBV infection (79.3%) ($P > 0.05$).

A summary of the risk factors reported by all HCV-seropositive individuals is presented in Fig. 3. None of them reported a history of intravenous drug use (IVDU) or tattooing, while 21/93 (22.5%) had a prior history of blood transfusion or having received plasma-derived products mainly during surgical procedures. Although 14% of all HCV-seropositive patients reported having suffered from Argentine Hemorrhagic Fever during the 1950s and 1960s, only two were treated with immune plasma for this reason. The majority of patients (18/21, 86%) were transfused with blood or blood components before the implementation of routine HCV screening. Surgical procedures were very frequent among HCV-seropositive individuals being reported by 69/93 (74.2%) patients. Eighty-six percent of all HCV-seropositive individuals were treated by the same dentist. The HCV serological status of the dentist was not determined. Some anecdotal reports suggested that the dentist may have used inadequate sterilization procedures in the past but this could not be further confirmed. Five point four percent of the patients were treated with acupuncture. The most frequently reported risk factor appeared to be the use of unsafe injection practices. With exception of one 7-year-old patient, all HCV+ individuals received since the mid-1940s and until the mid-1980s injections which were all administered by the same professional. These at-home procedures, eventually involved the use of inadequately sterilized glass syringes among family and community members. The poor nature of the sterilization procedure used by the nurse, and the fact that needles and syringes were reused among family members during a home visit, was established based on anecdotal reports provided by HCV+ individuals during interviews but could not be confirmed by other means. The nurse declined to be studied and his HCV status remained unknown. Vertical transmission was only suspected in two females of 7 and 18 years of age, whose mothers were also found to be HCV+.

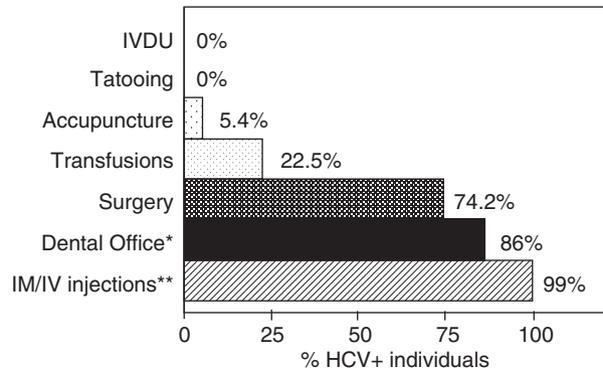


Fig. 3. Distribution and frequency of risk factors reported by hepatitis C virus (HCV)-seropositive individuals living in O'Brien, Argentina. *86% of all HCV-seropositive subjects were treated by the same dentist. **99% of all HCV-seropositive patients received intramuscular and/or intravenous injections with inadequately sterilized glass syringes that in all cases were administered by the same professional.

Similarly, sexual transmission seemed to have played a minor role, if any at all, in the dissemination of HCV in this community. We identified only three couples in whose members were found to be HCV+ and in all cases these individuals presented other risk factors associated with HCV transmission. Eighty-eight percent of all HCV+ individuals identified were born and lived exclusively in O'Brien. The remaining 11 lived in O'Brien for a median of 35 years (range 4–59).

Discussion

We determined that the prevalence of HCV infection in O'Brien (5.7%) is higher than previously reported in the general (1.9%) (O. Fay and J. Gonzalez, report during the Argentine HCV Consensus 2000 Meeting), and blood donor (0.5%) populations from Argentina (6). Our findings also provide an explanation for the relative high number of individuals with ALT elevations observed in O'Brien over the years preceding this study and the relative high frequency of individuals needing a liver transplant because of HCV-related cirrhosis. An additional finding was the high overall prevalence (>50%) of past HBV infection observed among HCV+ individuals. This finding was not entirely surprising considering that HBV shares similar

transmission routes with, and is more easily transmitted than HCV.

Three different geographic patterns of HCV infection have been identified worldwide (7) based on a limited number of age-specific prevalence studies performed in the general population. The HCV prevalence pattern observed in O'Brien resembles the one found in Italy (8, 9) and Japan (10, 11), with low infection rates among children and younger adults (range 0.47–1.35%) and a sharp increase among individuals over 40 years of age (range 8.2–23.9%) (Fig. 2a). A striking finding of our study was that 100% of all viremic individuals ($n = 77$) harbored a common HCV genotype. All of the genotype 1b viruses examined exhibited a similar RFLP pattern when analyzed with restriction enzymes *RsaI* + *HaeIII*. Analysis of NS5b sequences further confirmed the initial RFLP findings (manuscript in preparation). In addition, phylogenetic analysis of these sequences showed that the genotype 1b isolates found in O'Brien are genetically homogeneous and form a clearly distinct cluster when compared against genotype 1b sequences from other geographic areas in Argentina (manuscript in preparation). The presence of a single genetically related infecting genotype suggests the existence of a common source of HCV infection in O'Brien, probably disseminated over a period of decades. The epidemiological data gathered based on patient interviews and anecdotal reports suggest that the use of inadequately sterilized glass syringes by the local nurse may have contributed to this process. However, this could not be definitively concluded as we were unable to confirm this finding using other approaches. This practice eventually stopped in the mid-1980s. In addition, we could not determine if the dental procedures to which most HCV+ patients were exposed also contributed to the dissemination of HCV. The serological status of both the local nurse and dentist also remained unknown. It is less likely however, that transfusions with HCV contaminated blood or surgical procedures played a role in creating the high prevalence of HCV infection found in O'Brien. Despite the fact that Saavedra et al. (12) reported a prevalence of HCV infection of 7.7% among donors of immune plasma used to treat Argentine Hemorrhagic Fever donating at a regional Hemorrhagic Fever Center, only two patients from O'Brien received plasma infusions for this reason and in total 20% reported having received transfusions, most of them in other geographic areas of the country. In addition, if transfusion had played a role, we would have expected to find a more diverse representation of genotypes including 1a, 2, 3,

and 4 among the HCV+ individuals identified. These genotypes have been extensively documented among Argentinean HCV+ patients (13–15) but were absent in HCV+ patients from O'Brien.

We estimate that most transmissions occurred 30–40 years ago. In agreement with other studies (16, 17), we found that 82.7% of all HCV+ individuals identified were chronically infected. HCV RNA could not be detected in the remaining HCV-seropositive individuals. Although we cannot totally exclude the presence of virus below the detection limit of our assay, it is possible that some of these individuals have successfully cleared the infection. Viral clearance has been associated with a progressive decrease in titer and eventual disappearance of anti-HCV antibodies (18). In agreement with these observations, we found that 50% and 18.7% of patients with undetectable HCV RNA exhibited weak antibody responses against one (c22) or two (c22, c33) HCV antigens, respectively. It seems unlikely however, that viral clearance in this subset of patients occurred early after primary infection as it has been described previously (19). The time elapsed between infection and initiation of this study was probably sufficiently long (>30 years) to allow for a complete disappearance of specific antibodies rendering them HCV-seronegative (20). Thus, it is interesting to speculate that some of these patients were capable of mounting a successful immune response leading to viral eradication after several years of chronic infection and not necessarily soon after primary infection. It is also possible, that a number of individuals within this community became infected and cleared HCV early on, showing no signs of a humoral response at the time of this study. In this regard, Takakai et al. (21) were able to detect CD4-specific lymphoproliferative responses in women who were exposed to HCV more than 20 years ago and exhibited no serological evidence of HCV infection at the time of the analysis. In O'Brien, several HCV-seronegative individuals between 50 and 80 years of age reported having similar risk factors as the ones reported by HCV+ individuals (data not shown). Thus, it is possible that an even larger number of individuals than those identified by this study were infected with HCV at some point in time.

We detected anti-HBc antibodies in 52% of HCV+ individuals. However, only 60% of them had detectable anti-HBs, a marker that denotes resolution of HBV infection. Grob et al. (22) have recently reviewed the significance of an 'isolated' antibody-positive test result against the core antigen of HBV. In our patients, this finding may reflect a stage of late HBV immunity where

anti-HBs concentrations have fallen below the detection limit or an unresolved HBV infection which has been more frequently observed in the context of HCV co-infection (23). Administration of one dose of HBV vaccine and detection of HBV DNA in these patients will be necessary to help solve this issue.

In summary, we have identified a small rural community with high prevalence of HCV genotype 1b infection in Argentina. Similar HCV epidemiological profiles to the one observed in O'Brien have been previously described in small communities in Italy (8, 9), and Japan (10, 11); however, none of them associated with a single infecting genotype.

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