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SEROLOGICAL, EPIDEMIOLOGICAL AND MOLECULAR ASPECTS OF HEPATITIS C VIRUS INFECTION IN A POPULATION FROM LONDRINA, PR, BRAZIL, 2001-2002

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SUMMARY

Serological, epidemiological and molecular aspects of hepatitis C virus (HCV) infection were evaluated in 183 subjects from Londrina, Paraná, Brazil, and adjacent areas. Serum samples which tested anti-HCV positive by microparticle enzyme immunoassay (MEIA) obtained from eight patients with chronic hepatitis C, 48 blood donors, and 127 patients infected with the human immunodeficiency virus (HIV) were submitted to another enzyme immunoassay (ELISA) and to the polymerase chain reaction (PCR). About 78.7% of samples were also reactive by ELISA, with the greater proportion (70.8%) of discordant results verified among blood donors. A similar finding was observed for HCV-RNA detection by PCR, with 111/165 (67.3%) positive samples, with higher rates among HIV-positive subjects and patients with chronic hepatitis than among blood donors. Sixty-one PCR-positive samples were submitted to HCV genotyping, with 77.1, 21.3 and 1.6% of the samples identified as types 1, 3 and 2, respectively. Finally, analysis of some risk factors associated with HCV infection showed that intravenous drug use was the most common risk factor among HIV/HCV co-infected patients, while blood transfusion was the most important risk factor in the group without HIV infection. The present study contributed to the knowledge regarding risk factors associated with HCV infection and the distribution of HCV genotypes in the population evaluated.

KEYWORDS: Hepatitis C virus; Anti-HCV; Genotypes; Epidemiology.

INTRODUCTION

About 170 million people all over the world are infected with hepatitis C virus (HCV), although many ignore such fact[30]. In most cases, the infection develops as an asymptomatic clinical picture but with severe consequences, since about 80% of the infected subjects develop chronic hepatitis, cirrhosis or hepatocellular carcinoma[16,28].

Co-infection of HCV-infected subjects with the human immunodeficiency virus (HIV) has important implications for the prognosis and management of both diseases. These subjects show elevated alanine aminotransferase levels and are at a higher risk of developing fibrosis, cirrhosis, liver failure or hepatocellular carcinoma than subjects infected only with HCV[6]; in addition, they show a higher risk of developing hepatic toxicity related to the use of highly active antiretroviral therapy than patients infected only with HIV[5,26].

The use of the polymerase chain reaction (PCR) for the detection of HCV-RNA allows the identification of the presence of active HCV infection. However, RNA levels may show fluctuations with non-detectable periods even in patients not submitted to interferon therapy[7]. Patient counseling regarding the risk-benefit of treatment also requires analysis of a liver biopsy and the determination of the HCV genotype. Differences in the response to treatment depending on the HCV genotype have been observed, with type 1 being more resistant than types 2 and 3[19]. Genotyping based on the amplification of the 5' untranslated region (5'-UTR) has the advantage that it can be performed on PCR amplification products obtained from HCV-RNA detection tests[7,10].

HCV prevalence varies among different regions of the world or even among different groups studied. Prevalence is lower in populations highly selected by previous screening for risk factors associated with the infection, such as blood donors, and is higher among groups highly exposed to parenteral transmission of the virus, such as intravenous drug users or hemodialysis patients[2,13,21,29]. In Brazil, available data usually refer to large centers[8,12,22]. In Londrina, Paraná, data are also very scarce. PONTELLO *et al.* (1993) detected 1.2% positivity of anti-HCV antibodies in blood donors[23], and REICHE *et al.* (2000), studying 1,006 pregnant women assisted at a University Hospital during the period from 1996-1998, reported 0.8% positivity determined by enzyme immunoassay (ELISA), with no confirmation of antibody specificity or determination of the presence of HCV-RNA in seropositive cases[24].

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The aim of the present study was to evaluate the serological, epidemiological and molecular aspects of HCV infection in subjects living in the Londrina region, Paraná.

MATERIAL AND METHODS

Specimens: We studied 183 samples sent to the Clinical Laboratory of the University Hospital, Londrina, PR, between December 2001 and September 2002. The samples were selected from patients attending infectious diseases ambulatories and detected anti-HCV reactive at routine laboratorial screening. These subjects (48 blood donors and 127 HIV-patients with serological evidence of co-infection with HCV) were undergoing their first clinical evaluation for hepatitis C after the anti-HCV reactive result, and their status about HCV infection was unknown. Other eight patients selected had a confirmed diagnosis of chronic hepatitis C, and they were previously known to be HCV-RNA positive, but HCV genotype was not still determined. No patient was under interferon therapy. The study was approved by the Research Ethics Committee of the institution and all patients signed a written informed consent.

Anti-HCV screening: Anti-HCV antibodies were initially detected by an automated microparticle enzyme immunoassay (MEIA; AXSYM® HCV version 3.0, Abbott Laboratories, USA), the method routinely used in that laboratory, and confirmed by a second ELISA assay (Murex® anti-HCV version 4.0, Murex Biotech-Abbott, South Africa). Results were expressed as the optical density of the sample (S) divided by the cut-off value (CO). Samples were considered to be strongly reactive in one assay when showing a S/CO ratio ≥ 3.0 or ≥ 5.0 in ELISA or MEIA, respectively. Samples were classified as weakly reactive when the S/CO ratio was between 1.0 and 2.9 in ELISA or between 1.0 and 4.9 in MEIA[14,15]. Samples with a S/CO ratio < 1.0 in ELISA or MEIA were considered to be non-reactive.

Detection of HCV-RNA: Samples were stored at -20 ºC and later submitted to nested-PCR using primers for the amplification of the 5' untranslated region (5'-UTR) of HCV. The assay was performed on 165 samples; other 18 samples were stored at 4 ºC for several days, and were not tested by PCR because of possible RNA degradation. Viral RNA was extracted from 100 µL serum with 300 µL Trizol® (Gibco-BRL, USA), separated in an aqueous phase by chloroform addition and precipitated with isopropanol. The pellet was washed in 70% ethanol and dissolved in 12 µL of a solution containing diethyl pyrocarbonate-treated water and 300 ng of random primers (Pharmacia Biotech, Sweden)[4]. After a denaturation step at 70 ºC for 10 min, cDNA was synthesized from RNA by the addition of a solution containing 100 U reverse transcriptase (SuperScript™ II RNase H-Reverse Transcriptase, Gibco-BRL), 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 µM DTT, 5 U RNase inhibitor, and 0.5 mM deoxynucleoside triphosphate mix (dNTP), in a final reaction volume of 20 µL. The mixture was incubated at 42 ºC for 90 min and then heated to 70 ºC for 10 min. A two-round PCR was run in a final reaction volume of 50 µL using a protocol including an initial cycle at 94 ºC for one min and 40 cycles at 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, followed by a final extension cycle at 72 ºC for five min. NCR1 (5'-GTATCTCGAGGCGACACTCCACCATAG-3') and NCR2 (5'-ATACTCGAGGTGCACGGTCTACGAGAC-3') were used as outer primers, and NCR3 (5'-CCACCATAGATCTCTCCCCTGT-3') and NCR4 (5'-CACTCTCGAGCACCCTATCAGGCAGT-3') as inner primers. For the first amplification, 5 µL of cDNA was mixed with 45 µL of a reaction mixture containing 20 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.15 mM dNTP mix, 1 U Taq DNA polymerase (Gibco-BRL), and 12.5 pmol of each outer primer. The second amplification was performed using 3 µL of the first amplification product and a mixture of the same composition as described above, but using the inner primers, 0.2 mM dNTP mix and 1.5 mM $MgCl_2$. The second PCR amplification product was separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light[9].

HCV genotyping: Nucleotide sequencing of HCV 5'-UTR was carried out for 61 PCR-positive samples (seven blood donors, eight chronic patients and 46 HIV-seropositive subjects). The PCR product was purified (QIAquick®, QIAGEN, Germany) and submitted to sequencing (ABI PRISM® Big Dye Terminator Cycle Sequencing Ready Reaction Kit, PE Applied Biosystems, USA) in an automatic GeneAmp 9600 thermal cycler using 25 cycles at 96 ºC for 10 s, 50 ºC for five seconds and 60 ºC for four min. The product was precipitated with sodium acetate and ethanol, and later dissolved in loading buffer and submitted to electrophoresis in the ABI PRISM 377 DNA Sequencer (PE Applied Biosystems). The sequences obtained were edited with the SEQUENCHER program, aligned using the BIOEDIT program, and compared with published sequences available at GenBank: 1a (M62321 and D10749), 1b (D90208), 1c (D14853), 2a (D00944), 2b (D10998), 3a (D28917), 3b (D49374), 3c (D16612), 4a (Y11604), 5a (Y13184), and 6a (Y12083).

Risk factors: Subjects with detectable HCV-RNA were asked regarding possible risk factors, such as the use of intravenous drugs, blood transfusion and sexual relations with multiple partners (defined as more than 10 lifetime sexual partners)[3].

Statistical analysis: Results were expressed as absolute and relative frequencies. The kappa coefficient (κ) was used for comparison of the performance of the different assays[17]. Proportions were compared by the chi-square test (χ^2). The odds ratio (OR) and 95% confidence interval (CI) were calculated to determine the association between variables. The statistical tests were performed using the EPI-INFO program version 6.04d (CDC, Atlanta, USA), and statistical significance was assessed at the 0.05 probability level in all analyses ($p < 0.05$).

RESULTS

Anti-HCV screening: Of 183 anti-HCV-positive samples by MEIA, 59 (32.2%) were weakly reactive (S/CO ratio between 1.0 and 4.9) and 124 (67.8%) were strongly reactive (S/CO ≥ 5.0). This reactivity was confirmed in 144 (78.7%) samples by a second screening assay (ELISA). Positive results by the two screening assays were observed in 22/59 (37.3%) and 122/124 (98.4%) samples from the weakly reactive and the strongly reactive groups, respectively. Only 29.2% (14/48) of the blood donor samples tested positive by the two assays used, in contrast to the 96.1% (122/127) and 100% (8/8) agreement between these two methods obtained for HIV-positive and HCV chronic patients, respectively ($\chi^2 = 95.22$; $p < 0.001$).

PCR: HCV-RNA was detected in 111/165 (67.3%) samples

evaluated. PCR positivity was higher in patients with chronic hepatitis C (100%; 8/8 samples) and HIV-positive patients (75.6%; 96/127 samples) than in blood donors (23.3%; 7/30 samples) ($\chi^2 = 34.19$; $p < 0.001$). All PCR-positive samples were reactive in both MEIA and ELISA. A comparison between the MEIA, ELISA and PCR results is shown in Table 1.

Genotyping: HCV genotype was determined in 61/111 PCR-positive samples. Genotypes found were type 1 (77.1%), followed by type 3 (21.3%) and type 2 (1.6%). The most common subtypes were 1a (42.6%), 1b (24.6%) and 3a (19.7%). Differentiation between subtypes 1a and 1b was not possible in 8.2% of the samples due to the small variability in the 5'-UTR region. This was observed for the sample identified as type 2, in which subtypes 2a and 2b could also not be differentiated. Both subtypes 1c and 3c were also identified in only one sample (1.6%).

Risk factors: The 111 subjects with detectable HCV-RNA were asked regarding the possible route of infection. Among the subjects co-infected with HCV/HIV, 52.1% (50/96) were intravenous drug users, 14.6% (14/96) had received blood transfusion and 59.4% (57/96) had sexual relations with multiple partners. Among the subjects infected with HCV alone, only 6.7% (1/15) were intravenous drug users, 33.3% (5/15) had received blood transfusion and 20.0% (3/15) had multiple sexual partners. No risk factors associated with HCV could be identified in 53.3% and 5.2% of subjects infected with HCV only or co-infected with HCV/HIV, respectively (Table 2). Subtype 1a was very common among drug users (53.8%; 14/26 samples) and subjects with multiple sexual partners (48.1%; 13/27 samples), and subtype 1b was common among recipients of blood transfusion (45.5%; 5/11 samples).

Table 1
Comparison between the results obtained by microparticle enzyme immunoassay (MEIA), enzyme immunoassay (ELISA), and polymerase chain reaction (PCR) for 183 serum samples initially reactive in MEIA

MEIA	ELISA	PCR			Total
		Positive	Negative	Not done	
$1 \leq S/CO < 5^b$ (n = 59)	$1 < S/CO^a$	0	23	14	37
	$1 \leq S/CO < 3^b$	1	8	0	9
	$S/CO \geq 3^c$	5	7	1	13
$S/CO \geq 5^c$ (n = 124)	$1 < S/CO^a$	0	1	1	2
	$1 \leq S/CO < 3^b$	0	0	0	0
	$S/CO \geq 3^c$	105	15	2	122
Total		111	54	18	183

MEIA: microparticle enzyme immunoassay (AXSYM®, Abbott, USA); ELISA: enzyme immunoassay (Murex®, Murex Biotech-Abbott, South Africa); PCR: polymerase chain reaction; S/CO: ratio of sample optical density divided by the reaction cut-off (interpreted as non-reactive[a], weakly reactive[b] or strongly reactive[c]); n: number of samples; MEIA (weak/strong) x PCR (+/-): $\kappa = 0.682$ ($p < 0.001$); MEIA (weak/strong) x ELISA (+/-): $\kappa = 0.670$ ($p < 0.001$); MEIA (weak/strong) x ELISA (weak/strong): $\kappa = 0.539$ ($p < 0.001$)

Table 2
Frequency of risk factors associated with human immunodeficiency virus (HIV) infection among subjects with hepatitis C virus (HCV)-RNA detected by the polymerase chain reaction (PCR)

Risk factor	HIV (+) (n = 96) N[2] (%)	HIV (-)[1] (n = 15) N[2] (%)	OR	95% CI	*p**
Parenteral					
Intravenous drug user	50 (52.1)	1 (6.7)	15.22	1.92-120.35	< 0.001
Blood transfusion	14 (14.6)	5 (33.3)	0.34	0.10-1.15	0.131
Sexual					
Multiple partners [3]	57 (59.4)	3 (20.0)	5.85	1.55-22.09	0.004
Male homosexual	11 (11.5)	0 (0.0)	-	-	-
Drug user partner [4]	6 (6.3)	0 (0.0)	-	-	-
Not identified	5 (5.2)	8 (53.3)	0.05	0.01-0.19	< 0.001

[1] blood donors (7) + patients with chronic hepatitis C (8); [2] number of subjects who presented the risk factor; [3] more than 10 lifetime sexual partners; [4] subject who is not a drug user; OR: odds ratio; CI: confidence interval; *$p < 0.05$

DISCUSSION

Retesting MEIA reactive samples by a second ELISA showed that the agreement between the two methods was higher in the chronic hepatitis C group and HIV-positive subjects than in blood donors. Since all MEIA and ELISA reactive samples submitted to PCR showed a positive result, one may suppose that a reactive result by two different screening tests indicates that the detected antibodies are anti-HCV specific. The use of a second ELISA for confirmation of anti-HCV-positive results has first been proposed as an applicable strategy to reduce the use of supplemental tests in up to 85% of cases found in a clinical laboratory[14].

The level of reactivity in the anti-HCV antibody screening test is related to the positivity in PCR. A PCR-positive result was observed in 86.8% of samples with a strong reactivity by MEIA, in contrast to only 13.6% of samples with weak reactivity by MEIA that presented detectable HCV-RNA. In a similar study, 71% of samples with a S/CO ratio higher than 3.0 in ELISA and 50% of the samples with a S/CO ratio between 1.0 and 3.0 tested positive by PCR[15]. Another study reported 91.5% positivity in PCR when the S/CO ratio in ELISA was higher than 3.0 and only 17.6% when the S/CO ratio was between 1.0 and 3.0[11].

The procedure employed for the determination of the genotype of the samples evaluated was nucleotide sequencing of 5'-UTR. Although this is the most conserved region of the HCV genome, it is polymorphic enough for genotype differentiation of clinical specimens and has the advantage of using the same PCR amplification product generated in viremia detection tests, thus reducing laboratory costs. Difficulties in the differentiation between subtypes 1a and 1b and subtypes 2a and 2b were observed for some samples. A study comparing sequencing of the 5'-UTR and NS5 regions showed that 3.4% of strains classified as 1a by 5'-UTR sequencing were 1b when the NS5 region was sequenced, and 25% of those classified as 1b by 5'-UTR analysis resulted in 1a when sequencing the NS5 region[10]. Nevertheless, this fact does not invalidate the method since for therapeutic purposes determination of the main types is sufficient[1].

In the present study, most samples were identified as HCV genotype 1, with a predominance of subtype 1a followed by subtype 1b. These results are in agreement with the literature, with genotype 1 being the most common HCV genotype in North and South America, Europe and Asia[27]. In a previous study carried out in a Brazilian population consisting of different HCV exposure categories, genotype 1 was detected in 72% of the subjects, type 2 in 2%, type 3 in 25.3%, and type 4 in 0.7%[21]. Differences in the distribution profile of the HCV subtypes in the different geographical regions of Brazil have been reported, with subtype 1b being the most prevalent in the southeast area followed by subtype 1a, subtypes 1b and 3a being the most frequent in the northeast area, and subtype 1a being the most common in the center-west region, followed by subtype 3a[18].

The frequency of risk factors was evaluated in patients with active infection, evidenced by a PCR-positive result (n = 111). These data did not differ significantly from that found in all anti-HCV-reactive subjects (n = 183; data not shown). Nevertheless, the use of HCV-RNA-positive subjects might allow a better analysis of the results, with the exclusion of false-positive cases which are very common among blood donors.

A large number of subjects who admitted to have a sexual risk behavior or who were intravenous drug users were observed in the group co-infected with HCV and HIV. This finding agrees with previous observations made in HIV-seropositive patients from Rio de Janeiro, Brazil, where at least 70% of the subjects have been demonstrated to be engaged in high risk sexual activity and 70% of intravenous drug users were infected with HCV[22]. In HIV/HCV co-infected patients from São Paulo, Brazil, intravenous drug use was also the main risk factor detected[20].

The number of subjects in whom the infection source was associated with blood transfusion was small when compared to those with intravenous drug use as risk factor. Data reported by the Centers for Disease Control and Prevention (CDC, USA) indicate blood transfusion as the main cause of infections acquired more than 10 years ago. The number of cases associated with intravenous drug use is increasing, and currently accounts for 60% of HCV transmission in the United States[3].

The number of subjects with unknown source of infection was high, especially among blood donors and patients with chronic hepatitis C. In a community-based survey carried out in the State of Mato Grosso, Brazil, 78.9% of anti-HCV-reactive subjects did not present any evident risk factor for exposure to the virus, which was attributed to the fact that the group consisted of healthy subjects who probably could not remember such an important fact as a blood transfusion during childhood[25]. Risk factors related to HCV infection could not be detected in 5.2% of HIV/HCV co-infected patients, a rate similar to that observed in São Paulo, where 5.4% of HIV/HCV patients also denied any possible route of infection[20].

Although the serological, epidemiological and molecular aspects of HCV infection here obtained are related to a small population, this study was justified by the lack of information about HCV infection in the Londrina region, Paraná. Thus, it demonstrated the importance to continue this study in order to establish routine procedures that can be applied to the screening and confirmation of the diagnosis, as well as to provide an applied methodology for the epidemiological investigation of the virologic profile and therapeutic monitoring of HCV-infected patients.

RESUMO

Aspectos sorológicos, epidemiológicos e moleculares da infecção pelo vírus da hepatite C na população da região de Londrina, Paraná, Brasil, 2001-2002

Aspectos sorológicos, epidemiológicos e moleculares da infecção pelo vírus da hepatite C (HCV) foram avaliados em 183 indivíduos da região de Londrina, Paraná. Amostras soropositivas para anti-HCV pelo enzimaimunoensaio de micropartículas (MEIA), provenientes de oito pacientes com hepatite C crônica, 48 doadores de sangue e 127 indivíduos infectados pelo vírus da imunodeficiência humana (HIV), foram submetidas ao enzimaimunoensaio (ELISA) e a reação em cadeia da polimerase (PCR). Em 78,7% das amostras, verificou-se resultado

reagente no ELISA, ocorrendo maior proporção de resultados discordantes entre doadores de sangue (70,8%). O mesmo ocorreu com a pesquisa do RNA viral, na qual 111/165 (67,3%) amostras foram positivas com PCR, verificando-se maior positividade entre indivíduos HIV soropositivos e pacientes com hepatite crônica do que em doadores de sangue. Em 61 amostras com viremia detectável, realizou-se a genotipagem do HCV, encontrando-se os genótipos 1 (77,1%), 3 (21,3%) e 2 (1,6%). Por fim, foram avaliados os fatores epidemiológicos em indivíduos com infecção ativa, nos quais o uso de drogas injetáveis foi o principal fator de risco encontrado em indivíduos co-infectados pelo HIV/HCV e a transfusão sangüínea foi o mais comum em indivíduos sem infecção pelo HIV. O presente estudo contribuiu para o conhecimento do perfil da infecção pelo HCV em indivíduos da nossa população e da distribuição dos genótipos do HCV nesta região.

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