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HCV associated glomerulopathy in Egyptian patients: Clinicopathological analysis

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Abstract

Background: Hepatitis C virus (HCV) infection in Egypt has reached an epidemic proportion and is associated with many extra hepatic manifestations; Glomerulonephritis (GN) is one of the most consequences of HCV infection often resulting in end stage renal disease in some cases. Detection of viral genome or particles within the kidney biopsies from HCV-infected patients has proven to be difficult. Histological characterization of renal lesions still represents a major challenge. The aim of our work was to describe the histological pattern of HCV-associated nephropathy.

Methods: Fifty Patients out of 233 presented to Mansoura Urology and Nephrology clinic with manifestations of glomerular disease were screened for HCV antibodies by a 3rd generation ELISA test. Those tested positive for HCV antibodies were confirmed by PCR for HCV-RNA and subjected to more detailed clinical, biochemical and histological study. Kidney biopsies and in appropriate cases liver biopsies were examined by LM and electron microscopy (EM).

Results: Histological study of renal biopsies revealed membranoproliferative (MPGN) type 1 to be the most common lesion encountered (54%), followed by focal segmental glomerulosclerosis (FSGS) (24%), mesangioproliferative GN (18%), membranous nephropathy (MN) (4%) in that order. EM examinations of renal biopsies were successful in identifying HCV like particles in frozen renal tissue.

Conclusion: HCV-associated glomerulopathy is a distinct category of glomerulonephritis. Results of LM showed some peculiar features. In addition, we were successful in location and detection of HCV particles in renal tissues by EM.

Keywords: Glomerulopathy; Glomerulosclerosis; Nephropathy

Introduction

In Egypt, HCV infection has reached epidemic proportions with up to 21.9% of the population affected. This has been partly attributed to the routine parenteral administration of anti-Shistosomal drugs during the 1960s (Frank et al., 2000). The incidence of HCV infection in Egypt is being studied both at a community level as well as by the Hepatology committee of the Ministry of Health. In the general population, anti-HCV has been reported in 12% of children in a rural primary school, in 18% of the general population in a rural area (increasing with age) and in 16% of children with endemic hepatomegaly. In multitransfused children and patients on maintenance hemodialysis, the frequency has been found to be 44.5% and 46.0%, respectively (Zakaria et al., 1994).

Extra-hepatic manifestations of HCV infection are diverse and appear during late middle age (Wilson, 1997). Among these, glomerulonephritis, arthritis, dermal vasculitis and sialadenitis are thought to develop as a result of the deposition of immune complexes. Membranoproliferative glomerulonephritis (MPGN) is thought to be the most common whether cryoglobulinemic or non-cryoglobulinemic (Johnson et al.,

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1994; Ohsawa et al., 1999). An association between HCV infection and FSGS (Altraif et al., 1995), membranous nephropathy (Stehman-Breen et al., 1995) has also been reported. Other forms of renal disease have been previously linked to chronic HCV infection including acute exudative and proliferative glomerulonephritis and IgM nephropathy (Kamatsoda et al., 1996). The seroprevalence of HCV is thought to be greater in individuals with chronic glomerulopathy compared with those suffering from other renal disease (Daghestani and Pomeroy, 1999).

While some studies conducted on a limited number of patients have failed to detect HCV protein in tissues from HCV-positive patients (Johnson et al., 1994; Stokes et al., 1997), others have reported successful detection of HCV proteins in renal tissues of HCV-infected patients (Okada et al., 1996; Sansonno et al., 1995, 1996).

A paucity of studies detected at a systematic analysis of HCV both liver and renal biopsy specimens and from patients with HCV-associated glomerulonephritis utilizing light and EM techniques prompted us to carry out such a study. Results of such a study constitute the basis of this report.

Materials and methods

Patient population

The study comprised the evaluation of a total of 233 patients that were screened at the Urology and Nephrology clinic located in Mansoura, Egypt. The study protocol was approved by our local human investigation committee (HIC) forum at the initiation of the study and appropriate uniform consent obtained from each patient in their native language.

All patients presented to our outpatient clinic complaining from symptoms suggestive of renal disease during a 2-year period. Each patient was screened for serum HCV antibodies using a 3rd generation ELISA technique (Abbott Diagnostics, Maidenhead, UK). Seropositive cases were confirmed to have HCV-RNA by polymerase chain reaction (Amplicor PCR diagnostics, Roche Diagnostic system, Lewes, UK).

Fifty of the 233 patients – among 90– who tested positive for HCV RNA – were selected for the present study. They were adults and all have manifestations of glomerulopathy (proteinuria, hematuria). None of these patients was either HIV1 or 2 positive (Murex immunoassay) or HBV-positive. Secondary causes of glomerulonephritis were excluded by testing for fasting and 2 hour post-prandial levels of blood glucose, autoimmune antibodies (Serum autoantibodies) and Schistosmal circulating cathodic antigen (double antibody sandwich ELISA technique). All included patients were subjected to thorough laboratory investigations including urinalysis, serum creatinine, electrolytes, liver function test, and prothrombin time estimation.

Histological work-up

Light microscopy

While renal biopsies were carried out for all selected patients, liver biopsies were performed in only 10/50 (20%) with high liver enzymes. Biopsy tissue specimens were fixed in formalin and embedded in paraffin. 4 um sections were cut and stained with hematoxylin and eosin.

Polymerase chain reaction for formalin-fixed paraffin embedded renal and liver sections

RNA Isolation. 1 ml Xyelene was added to 30 um of the paraffin sections in autoclaved Eppendorf tubes and the tubes incubated for 10 min at room temperature. The fluid was removed following centrifugation for 2 min and then 1 ml of 100% ethanol added to the tubes which were incubated for 10 min at room temperature. This step was followed by two alternative RNA extraction procedures. The first one termed the Guanidinium Isothiocyanate method (200 UL volume of Guanidinium Isothiocyanate was added, 4 different choices were tried 15 min incubation at room temperature, 15 min incubation at 4 C, Overnight incubation at room temperature and overnight incubation at 4 C). The second extraction procedure termed the Proteinase K digestion method: using Proteinase K buffer preparation(100 mM NaCl +10 mM Tris pH 7.4 + 25 mM EDTA pH 8(Autoclaved for 24 h) + 5% Sodium dodecyl sulphate. 10 ml of the buffer was prepared by adding 1 ml from each to 6 ml PCR water. The digestion was performed in a 200-UL volume utilizing different concentrations (0.5 μg/ml–5 mg/ml).

Complementary DNA (cDNA) synthesis. The protocol utilized the first strand cDNA kit-Pharmacia 27-9261-01. 4 UL RNA was first heated to 65 C (on heat block) for 10 min then chilled on ice. A master Mix was prepared which constitutes of (2.5 μl bulk 1st strand reaction mix + 0.5 μl DTT + 0.5 μl primer (Pd (N) 6)+ 4 μl of the heat denatured RNA is added to 3.5 aliquots, mixed by pipetting, incubated at 37 C for 1 h.

First round PCR. Master Mix was Prepared by adding (2.5 UL 10 × buffer (Boehringer Mannheim 306527) + 0.25 UL dNTPs 10 μM (Boehringer Mannheim 158295) + O.3 UL Tag DNA Polymerase (Boehringer Mannheim 306528) + 1 UL outer primer (sense) + 1 UL outer primer (antisense) + 20.1 UL PCR water + Outer primer sequences: chosen from the highly conserved untranslated 5' region (Life Technologies Ltd., UK)-Sense (AGC GTC TAG CCA TGG CGT), Antisense (GCA CGG CTC ACG AGA CCT), (Outer product runs from 74 to 338). 1 UL of the cDNA was added to 24 UL of the master mix, overlaid with mineral Oil to prevent evaporation. A thermal cycler was used (PTC-100 programmable thermal controller) set for 94 C for 45 s (Denaturing), 50 C for 45 s (Annealing) and 72 C for 120 s (Extension) for 35 cycles.

Second round PCR. Master Mix was prepared (1 UL inner primer-GTG GTC TGC GGA ACC GG (sense), 1 UL inner primer-GGG CAC TCG CAA GCA CCC (antisense) (Life Technologies Ltd., UK)(Inner product runs from 143 to 316), 1 UL of the first round product was added to 24 UL of the master mix, overlaid with mineral oil. The same thermal cycle was used (PTC-100 programmable thermal controller MJ-Research INC) set up 94 C for 45 s, 50 C for 45 s and 72 C for 120 s for 35 cycles. The PCR product was visualized under ultraviolet illumination, captured by Polaroid Ca-5 camera on a 2% agarose gel.

Polymerase chain reaction for renal frozen sections

Frozen renal tissue was first homogenized using a glass homogenizer and plunger in 500 UL of solution D. The rest of the steps for RNA extraction were the same as utilized for formalin fixed paraffin embedded sections.

Electron microscopy

Freshly cut biopsies were transferred immediately to a dish of modified Millonig's phosphate buffer (18.76 g of sodium dihydrogen phosphate ($2H_2O$) + 4.28g of Sodium Hydroxide + 1 l of distilled water + 3% phosphate-buffered glutaraldehyde + 12 ml of concentrated (25%) glutaraldehyde).

Half micron sections were cut and mounted on glass slides. The sections were heated at 150 °C for at least 5 min, stained with 1% aqueous toluidine blue in 1% sodium tetraborate for 15 s on same hot plate at 150°, allowed to cool and then excess stain was rinsed in xylene and coverslipped. The sections were examined under light microscope for assessable glomeruli. Additional sections were cut using a diamond knife utilizing Leica Ultracut E Ultramicrotome at interferance color gold (Approximately 95 nm thick). The section was picked up on a 200-mesh high transmission hexagonal copper grid left for half an hour at least to dry into grid at 37 °C, or overnight at room temperature. The sections were then stained and examined using a Philips 400 T TEM. Relevant areas were photographed, film developed and printed.

Statistical analysis

Results are presented as median and confidence intervals. The Fisher exact probability test and Mann–Whitney (Non parametric rank sum tests) were applied as appropriate. A P value of ≤ 0.05 was considered significant.

Results

Prevalence of HCV

Ninety of 233 patients (38%) were positive for HCV Ab by 3rd generation ELISA. Fifty of these patients fulfilled our inclusion criteria were subjected to more detailed clinical, biochemical and histological analysis.

Histological study of renal biopsies

Histological evaluation of renal biopsies from the 233 patients revealed MPGN and focal segmental glomerulosclerosis as the most common lesions observed accounting for 39% and 30.4%, respectively, among 233 patients presented with glomerulopathy (Table 1).

Clinical characteristics of HCV-positive patients

Male preponderance was observed among HCV-positive patients (70%). A past history of hospital admission (27 patients); blood transfusion (21 patients) and operative procedures (25 patients) were considered as risk factors for HCV acquisition. Twenty-three (56%) gave history of previous Schistosomal infection and its treatment, none tested positive for Schistosomal circulating cathodic antigen.

Biochemical characteristics

Among the 50 HCV-positive patients 27 were cryoglobulinemic, 18 patients were positive for rheumatoid factor, mean serum creatinine was 1.2 mg/dl, 24 h protein excretion was 3.7 g, and their levels of C 3 were normal but low for C4 (Table 2).

There was statistically no significant difference regarding demographic and clinical characteristics between cryoglobulinemic and non-cryoglobulinemic patients. The only difference between both groups is that the former received more blood transfusion. In addition, the cryoglobulinemic patients had statistically higher levels of serum creatinine and lower but not statistically significant levels of C3 (Table 3).

Table 1
Results of renal biopsy in 233 Egyptian patients with glomerulopathy

Histological classes	Frequency	Percent
Membranoproliferative GN	91	39
Focal segmental glomerulosclerosis	71	30.4
Diffuse Mesangioproliferative GN	30	12.8
Membranous nephropathy	10	4.29
Focal mesangial proliferative GN	8	3.4
Crescentic glomerulonephritis	5	2.14
Minimal change Nephropathy	4	1.71
Amyloidosis	4	1.71
IgA nephropathy	3	1.71
Normal by light microscopy	3	1.2
Tubulo interstitial fibrosis	2	0.8
Diabetic glomerulosclerosis	1	0.4
End stage renal disease	1	0.4
Total	233	100

* GN = glomerulonephritis.

Table 2
Results of laboratory tests on blood samples from 50 HCV-positive patients presented with glomerular disease (median and confidence interval)

Parameters	
Serum creatinine (mg/dl)	1.2 (1.15–1.47)
Proteinuria 24 h (g)	3.7 (3.66–6.00)
Serum cholesterol (mg/dl)	220.00 (226.08–310.04)
Hemoglobin (gm/dl)	12 (10.83–12.73)
Platelet count mm^3	217 (195.76–280.41)
White cell count (mm^3)	6.75 (6.43–8.56)
Serum bilirubin (mg/dl)	0.50 (0.45–0.94)
Total protein (gm/dl)	6.05 (5.61–6.27)

Results of radioimmunodiffusion for the cryoprecipitates of 27 patients revealed mixed cryoglobulinemia (Both IgM and IgG) in 25 of these cases.

Histological characterization

Light microscopy

Results of light microscopy studies showed that 27/50 (54%) of the HCV -positive patients showed evidence of MPGN type 1. Out of these 27 patients 16 patients were cryoglobulinemic and 11 patients were non cryoglobulinemic. 24% had evidence of FSGS, 4% were MN and 18% showed evidence of Mesangial proliferative glomerulonephritis (Table 4). Of the 10 patients who had high liver enzyme values, each showed evidence of mild hepatitis with Knoddel scoring ranging from 2 to 9 in their liver biopsies (Table 5).

Electron microscopy

Immune complexes (IC) were detected in biopsy sections from 11 patients out of 20 samples examined. The IC appears to be located in the paramesangial and subendothelial spaces (Fig. 1).

With higher magnification (Fig. 2) viral-like particles were localized within these immune complexes in 5 sections. These findings were specific since similar analysis of biopsies from HCV-negative patients proved negative both for immune complexes and viral-like particles.

Table 3
Demographic and clinical characteristics for cryoglobulinemic and non-cryoglobulinemic patients (median and confidence interval)

Parameters	Cryoglobulinemic (n = 27)	Non-Cryoglobulinemic (n = 23)	P value
Age	40 (35.97–43.95)	45 (38.69–46.60)	0.283
Gender	Male, 17	18	0.239
Anasarca	16	14	0.908
Jaundice	3	3	0.834
Hypertension	15	13	0.945
Past history for blood transfusion	13	7	0.203
Serum creatinine	1.43 ± .49	1.21±	0.16
Serum complement C3	90.40 ± 46.18	116.24 ± 51.88	0.07
Serum complement C4	30.95 ± 11.6	33.1 ± 11.95	0.89

Table 4
Histological diagnosis of HCV-positive patients

Histological diagnosis	(n = 50)
Membranoproliferative glomerulonephritis type 1	27
Monocyte infiltration	17
Intraluminal hyaline thrombi	6
Accentuated lobular architecture	5
Focal segmental glomerulosclerosis	12
Membranous nephropathy	2
Mesangioproliferative glomerulonephritis	9

Polymerase chain reaction

Forty-two frozen renal biopsy samples were tested for HCV RNA by a nested PCR technique as described in the methods section. Of these 42 specimens 9 samples were positive, 4 from patients with cryoglobulinemic and 5 who were non-cryoglobulinemic (Figs. 3 and 4). HCV RNA was detected in liver biopsies from six of the 10 patients. HCV was also detected in 17 samples out of the 25 cryoprecipitate from HCV-positive cryoglobulinemic patients.

Discussion

Hepatitis C is a global health problem affecting a significant portion of the world's population. The World Health Organization estimated that in 1999, 170 million hepatitis C virus (HCV) carriers were present worldwide, with 3 to 4 million new cases per year. Based on data in blood donors from varying regions of the world, the infectivity rates range from 0.3% to 14.5%. The prevalence of HCV antibodies is relatively low in the United States, northern Europe, and Australia, ranging from 0.3% to 1.2% of the population (Robert and Paul, 2003).

Table 5
Clinical and histological criteria of patients with high liver enzymes

Parameter	Range (minimum–maximum)
Serum creatinine	1.5 (0.7–2.2)
24 h urinary protein	5.8 (0.5–6.3)
Cholesterol	332 (108–440)
Total protein	2.7 (5.2–7.9)
Serum albumin	2.4 (1.3–3.7)
ALT	63 (8–71)
AST	46 (12–56)
Serum complement 3	171 (60–231)
Serum complement 4	21.6 (10.2–31.8)
Knoddel scoring for liver biopsy	
Portal fibrosis	3 (0–3)
Portal inflammation	2 (1–3)
Piece meal necrosis	3 (0–3)
Spotty necrosis	2 (1–3)
Total scoring	7 (2–9)

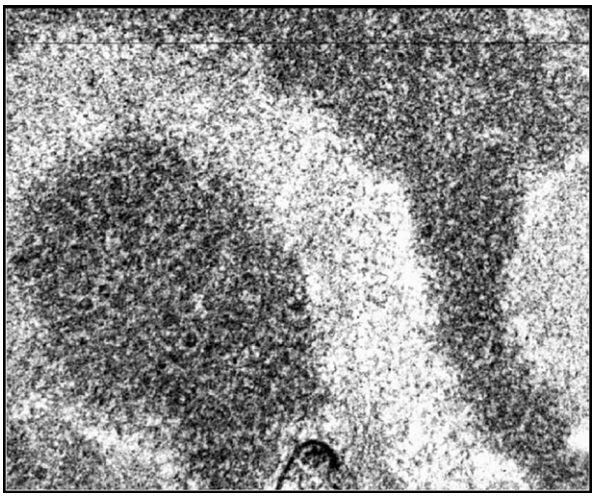

Fig. 1. Electron microscopy for HCV-positive patient with viral like particles in the electron dense deposits (56 nm) ×248418.

In Egypt, HCV infection has reached an epidemic proportion with up to 21.9% of the population affected (Darwish et al., 1993).

In addition to liver disease, HCV infection has been associated with a wide variety of extrahepatic manifestations including mixed cryoglobulinemia and renal involvement (Wilson, 1997). The most common form of renal disease is cryoglobulinemic membranoproliferative Glomerulonephritis, MPGN type 1 (D'Amico et al., 1989). This finding is consistent with a previous report that postulated that HCV is a significant cause of MPGN, especially in countries where HCV is highly prevalent (Bandi, 2003).

Results of our study also confirmed this observation since MPGN represents 39% of the population studied herein. It is important to note that the prevalence of HCV antibodies among patients with glomerulopathy (38%) was higher than among healthy blood donors (16%) which could be attributed to the association of HCV with renal disease.

Despite of the high prevalence of HCV among patients with chronic glomerulopathy, the histological characteristics and the prevalence of HCV-associated glomerulopathy remains to be determined.

The typical renal histological evaluation by light microscopy showed changes of MPGN and evidence of immune complex deposits in the glomeruli. Glomerular capillaries may have marked inflammatory cellular infiltrates with both mononuclear cells and polymorphonuclear leucocytes with accentuation of the glomerular lobulation and the tuft architecture and may have a combination of increased mesangial cellularity and matrix, capillary endothelial swelling, splitting of capillary basement membrane and intracapillary global accumulation of esinophilic material representing precipitated immune complexes or cryoglobulins (Johnson et al., 1993).

In our study marked inflammatory cellular infiltrates – mainly monocyte – was evident in biopsies from 63% of the patients, intra-capillary hyaline thrombosis was found in biopsies from 23% of patients and accentuation of glomerular lobulation in biopsies from 63% of patients.

Symptoms suggestive of cryoglobulinemia, such as purpura, arthritis or neuropathy, were present in only one half of HCV-infected patients with MPGN. In addition, only 20% of the patients had physical signs of liver disease, although the majority (60–70%) has mildly elevated serum transaminases.

Results of our study revealed that while 26% of the HCV-positive patients with cryoglobulinemia had elevated transaminase levels, only 10% had hepatomegaly, 6% had acral necrolytic erythema. These values are consistent with those reported by Wilson (1997). However, these values are not in agreement with Johnson et al. (1993, 1994) where two thirds of patients had high liver enzymes. The lack of extra-renal manifestations – particularly hepatic – in the majority of our patients may be explained by the fact that even those with high transaminase levels were biopsy proven to have mild hepatitis with Knoddel scoring.

It has been previously reported that the most reliable method for the diagnosis of HCV infection is the detection of viral RNA sequences by RT-PCR in serum and tissues (Akyol et al., 1992). While the non replicating form of HCV was successfully detected in paraffin-embedded renal biopsies, the viral replication could not be detected (Agnello et al., 1996) in these studies.

We tried many methods for RNA extraction from both formalin fixed and frozen liver and kidney tissues and also from the cryoprecipitates isolated from cryoglobulinemic patients. However we failed to extract HCV RNA from formalin-fixed, paraffin-embedded renal sections. These findings could be explained by the fact that HCV being a RNA virus is more labile than DNA viruses and susceptible to damage by the process of fixation and prolonged paraffin embedding (Shieh et al., 1991). This explanation is confirmed by our success in the isolation of HCV RNA from 22% of the frozen renal sections studied, four of them were from cryoglobulinemic MPGN patients, 2 MN, 2 FSGS and one mesangioproliferative glomerulonephritis. RNA was also

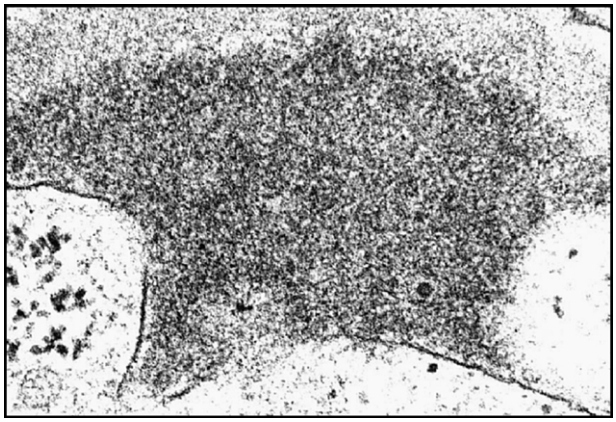

Fig. 2. Electron microscopy for HCV-positive patient with viral like particles in the electron dense deposits (56 nm) ×248418.

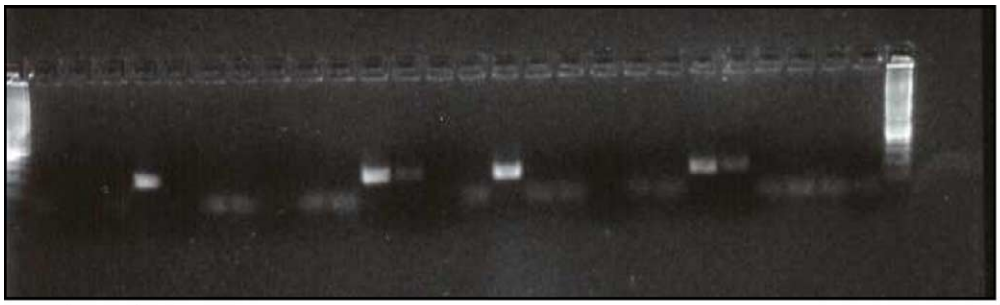

Fig. 3. PCR for HCV RNA in frozen renal tissues for cryoglobulinemic patients. *Positive samples are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extracted from 4 paraffin embedded liver sections. The success of RNA extraction from liver sections but not in renal sections might be explained in part by the fact that the liver is the major site for HCV replication and it may contain relatively higher viral load than renal tissue.

Detection of HCV by EM has been reported in a few studies and case reports which described the detection of aggregated or single viral like particles in the cytoplasm of hepatocytes in sections from HCV-infected patients (Bosman et al., 1998). To our knowledge, only one case in renal tissue was reported (Horikoshi et al., 1993). On the contrary, herein virus-like particles, mostly HCV-related, of about 30–45 nm in diameter, located in Electron-dense deposits in the paramesangial areas in 5 out of 20 sections examined in cryoglobulinemic MPGN patients.

In conclusion, the prevalence of HCV infection is higher among Egyptian patients with chronic glomerulonephritis when compared to the general population. The high

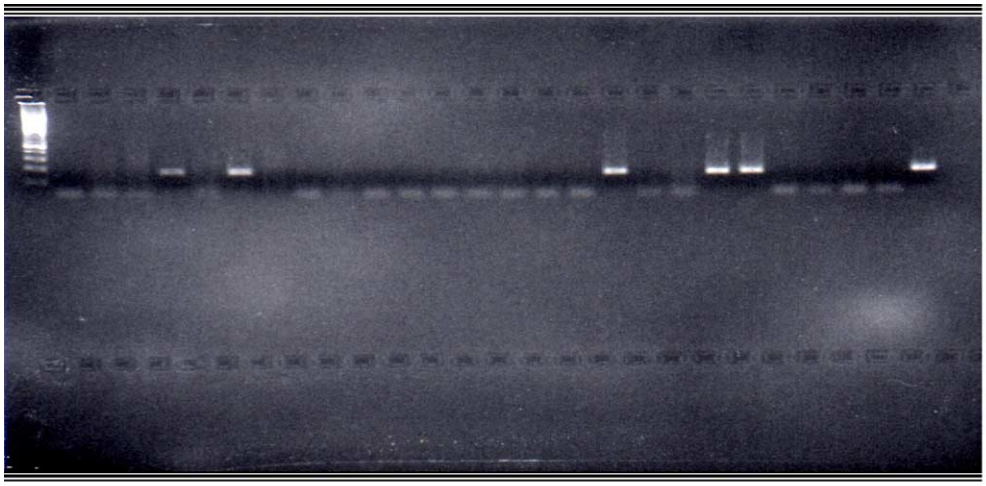

Fig. 4. PCR for HCV RNA in frozen renal tissues for non cryoglobulinemic patients. *Positive samples are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prevalence of MPGN in areas endemic for HCV could be attributed to such infection. MPGN-associated with HCV has peculiar histological features.

To our knowledge, results of the studies reported herein are the first that documents the detection of HCV particles in renal tissues in such number of patients. Our recommendation is to screen all patients with MPGN in endemic areas for HCV infection. Finally, combination of EM and PCR could help to establish diagnosis.

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