ated with the missing sequences, which now form a set by themselves, it provides for a 'duality'. This arises because for every *real event A*, there is a *virtual event A'*, opposite in character, since a one-to-one correlation exists between *A* and *A'*. Every sequence in the missing set arises from an element denied in the diagonal set.

Whether the wave and particle nature of matter which are both observable, are found in *A* or *A'*, depends on whether *events* belong to either (*A*)'s or (*A* ')'s respectively. It also depends on the validity of the Complementarity Principle of Bohr. If particle and wave behaviour are described as both belonging to *A* only, then the wave and particle property can be observed simultaneously which is contrary to that predicted by Bohr (see ref. 4). If one is from *event* set *A* and the other from set *A'* , their detection will be mutually exclusive.

Conclusion

This work is based on Buddhist Logic of Conditional Reality, Kshana and Nothingness[5]. In the matrix I, if the causes of events are dependent on each other it leads to what is known as 'conditioned reality'. While in classical physics, space and time are separate, in reality this is not so and it

leads to new physics. In quantum mechanics, the wave nature is merged with particle nature and leads to many paradoxes but this is all a part of 'conditioned reality'. 'Kshana' is the theory of time averages and 'nothingness' is the existence of virtual states. These are described in a forthcoming paper entitled 'Causality,[n]cardinality and conditioned reality' in the project of history of Indian science, philosophy and culture, Calcutta.

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Indian strains of hepatitis-C-virus: Prevalence and detection

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A simple polymerase chain reaction (PCR) method for detecting Indian strains of hepatitis-C virus (HCV) directly using serum samples is described. Owing to known genetic variability in HCV strains, commonly used primers for detecting US and Japanese strains were found unsuitable for Indian strains. We report the successful use of primers designed from minimum variable regions of the HCV genome for detecting Indian strains of the virus by PCR. The PCR products have been authenticated and one of them sequenced. We also show that the method we have developed can detect the presence of HCV in ELISA-negative (using commercially available kits) patients who had received blood transfusion.

THE existence of a parenteral non-A, non-B (NANB) hepatitis agent was first reported[1-3] in 1989. A number

of reports based on enzyme immunoassays indicate that this virus, termed hepatitis-C (HCV), is a predominant cause of post-transfusion NANB hepatitis around the world[2,4-8]. Anti-HCV is associated with most community-acquired NANB hepatitis cases in the United States[2,9] and Western Europe[5,10,11] and is a major cause of cryptogenic chronic liver disease in Italy[12]. In addition, anti-HCV is associated with most cases of hepatocellular carcinoma (HCC) in Japan[13], Italy[11] and Spain[15]. On the other hand, practically no information is available on either the prevalence or the nature of HCV in India.

More than 50% of the patients who acquire acute HCV infection develop chronic hepatitis[16]. Clinical manifestations of HCV infection include acute hepatitis which may be resolving or fulminant, chronic hepatitis, cirrhosis and hepatocellular carcinoma. Some of the

rare complications of hepatitis-C infection include myocarditis, pancreatitis, aplastic anaemia and transverse myelletis. Even though chronic hepatitis-C generally appears to be an indolent disease, it has been reported that at least 20% of the patients developed cirrhosis and as many as 25% of the patients developed liver failure[16]. The association between chronic HCV infection and HCC has been described, although the exact oncogenic mechanism is unknown[3].

The major mode of transmission of the virus is through blood transfusion[17]. Vertical transmission from infected mothers to their children[18] as well as sexual transmission of the virus have also been reported[19].

The significance of HCV infection therefore lies in the considerable diseases load both from morbidity and mortality points of view. HCV infection increases the risk of developing liver cancer and the development of chronic carrier state contributes to spread of hepatitis-C and jeopardizes the safety of blood and blood products.

The genome of hepatitis-C is a single positive stranded RNA of about 9.4 kb with a single open reading frame encoding for a precursor polyprotein of approximately 3000 amino acids[20]. The genomic structure possesses similarity to flaviviruses of humans, pestiviruses of animals and potyviruses of plants. There is considerable genomic heterogeneity amongst different isolates. Based on sequence analogy, at least six different strains of HCV have now been recognized in different parts of the world[21].

Generally, HCV is detected through commercially available enzyme immunoassay kits. The second generation enzyme immunoassay which includes the antigens C33C, C22 and C100-3 has replaced the less sensitive one-antigen capture assay. These immunoassays have shown increased sensitivity to anti-HCV in chronic hepatitis-C and have shortened the period between infection and detection of anti-HCV by four to five weeks when compared with the first generation assays[22,23]. Apart from enzyme immunoassay, other sensitive methods of detection of the virus include recombinant immunoblot assay as well as detection of the virus nucleic acid in the serum by reverse transcriptase polymerase chain reaction (RT-PCR) and the quantitative branched chain DNA signal amplification assay[24]. It should be added that in immunosuppressed patients who are often seronegative for anti-HCV in the presence of active infection, the RT-PCR method provides distinct advantages.

The primary aim in the present studies has been to develop a method for the molecular diagnosis of Indian strains of HCV using PCR and carrying out molecular cloning and expression of the envelope gene of HCV for both immunodiagnostic and vaccine studies. In this article, we report the successful development of a dependable and sensitive RT-PCR method for detecting Indian strains for HCV and its advantages in comparison with commercially available reagents.

Methods

Blood samples from patients with liver disorders from Osmania General Hospital were tested for the presence of HAV and HBV using ELISA kits (obtained from Abbott Laboratories, USA) according to manufacturer's instructions. From among the NANB patients, based on clinical symptoms of liver diseases, a subset of patients were selected and were tested for anti-HCV antibodies using the second generation Abbott ELISA kit. Polymerase chain reaction (PCR) was carried out in the presence of reverse transcriptase according to methods described elsewhere[25].

Two types of procedures were used for PCR. In the first method, after extracting RNA from 200 μl of blood sample the isolated nucleic acid was used for RT-PCR. Round-1 PCR was carried out with outer primers (see Figure 1) and round-2 PCR with inner primers. The amplifications were carried out for 35 cycles in each round with denaturation at 95°C for one minute, primer annealing at 50°C for 1.5 minutes and extension at 72°C for 3 minutes. Appropriate positive and negative controls were included in each set of experiments to preclude false positive reactions.

In the second method, PCR amplifications were carried out directly on serum samples. Serum samples (2 μl of serum diluted to 10 μl in PBS) were heat denatured at 95°C for 2 minutes and quick chilled in an ice bath. RNasin (40 ul) was added to inhibit the RNase activity. Reverse transcription (43°C for 30 minutes) and the first phase of PCR was carried out as a single tube reaction in a total volume of 100 μl. Aliquots (10 μl) were further reamplified in 50 μl reaction mixtures. The conditions for denaturation, annealing and extension steps were identical to those

Primers	Sequence (5'→3')	Position	Final PCR Product
External			
A1L*	CACTCCCCTGTGAGGAACTACTGT	29-52	
A2R	CCCTGTTGCATAGTTCACGCCGTC	810-833	
Internal			
A3L	ACTGCTAGCCGACTAGTGTTGGGT	237-260	
A4R	AGGTTGCGACCGCTCGGAAGTCTT	483 506	270 bp

* L and R indicates sense and antisense polarity respectively

Figure 1. Sequence of commercially available primers commonly used for the detection of Japanese and American strains of HCV.

used for PCR using RNA isolated from sera as described above. The amplified products were analysed on 2% agarose for checking their size. Southern hybridizations and restriction enzyme digestions were carried out using standard methods[26]. Cloning of one of the PCR products was carried out by ligating the blunt ended product at the Sma I site of pGEM-3 using T4 DNA ligase[26]. Nucleic acid sequencing was carried out using the dideoxy nucleotide chain termination method using the Sequenase kit version 1.0 (USB, USA) according to manufacturer's protocol. ^{32}P-end labelled probes were made as described by Sambrook et al.[26].

Results

Prevalence of HCV antibody in hepatic patients

Initially, 2000 patients with clinical features of liver diseases were tested for serological markers for HAV and HBV infection. After testing a group of 140 patients which included 122 NANB and 18 randomly selected hepatitis B antigen (HBsAg) positive cases, a total of 26 patients were found to be seropositive for anti-HCV antibodies. The distribution of patients with anti-HCV antibodies among the different types of hepatic patients is shown in Table 1. Among the non-specific cases we had tested, three individuals had multiple blood transfusion and the remaining had hepatomegaly. It may be noted that although only one of the post-transfusion individuals was positive for anti-HCV antibody by ELISA, all the three were positive by PCR (see following section). From among the 18 HBsAg positive patients (included in the acute hepatitis group in Table 1) three patients were found to be positive for anti-HCV antibodies also. It is of interest to note that the large majority of ELISA positive patients had cirrhosis of the liver.

The age-wise distribution of HCV-positive patients is shown in Table 2. It also shows that the incidence of hepatitis-C tends to increase with age. The number of

Table 1. Prevalence of antibodies to Hepatitis C virus in different liver diseases

Disease condition	No. of cases investigated	No. of anti-HCVAb positive	HCV infection (%)
Acute hepatitis	48	5	10.4
Chronic active hepatitis	36	10	27.7
Cirrhosis	38	9	23.6
Hepatocellular carcinoma	3	1	–
Non-specific*	15	1	6.6

*Includes post-transfusion and hepatomegaly cases, but without any clinical symptoms of hepatitis.

Table 2. Distribution of HCV infection in different age groups

Age group (years)	No. of cases investigated	No. of anti-HCVAb positive	HCV infection (%)
0–10	6	2	33.3
11–20	18	1	5.5
21–30	32	3	9.3
31–40	21	3	14.2
41–50	37	9	24.3
51–60	16	5	31.2
61–75	10	3	30.0

patients tested belonging to the age group 0–10 were only six and out of these, only two were HCV-positive. Because of the small number of patients tested in this group, it would not be correct to place undue emphasis on the percentage positivity value for this group.

HCV detection by PCR

As indicated earlier, there is considerable genomic heterogeneity among different HCV isolates from different parts of the world. There is as high as 30% variation in nucleotide sequences in these isolates. However, in the 5' non-coding region there is 95–98% conservation of the sequences. As a consequence, it becomes necessary to make use of sequence information in the 5' non-coding region for designing primers for PCR detection of HCV.

To begin with, we used the set of primers shown in Figure 1 for PCR. This sequence is commercially available and has been successfully used for detecting several strains of HCV of American or Japanese origin. Our serum samples which were found to be HCV-positive using the second generation ELISA kit, when tested with this set of primer did not show the expected amplified fragments in a large majority of samples. The first set of experiments were carried out using primers obtained commercially by the US collaborating laboratory. In order to rule out possible mistakes in sequences that could be present in commercially available samples, we synthesized primers corresponding to the sequences shown in Figure 1 making use of the oligonucleotide synthesis facility in CCMB. The new set of primers were also found unsatisfactory for faithful detection of HCV.

It may be noted that the primers shown in Figure 1 were designed on the basis of the nucleotide sequence of Japanese isolates. Although they were found satisfactory for detecting a number of American and Japanese strains of HCV, it so happened that the downstream outer primer shown in Figure 1 overlapped the 5' coding region of the HCV genome. We argued that there is the likelihood of sequence variation in this region in the Indian strains of HCV. Should this be the case there

could be failure in PCR owing to template primer mismatch. Consequently, we decided to design a new set of primers to amplify the 5' non-coding region. The sequences were selected from within this region which showed minimum variability among different strains of HCV. The sequences of the new sets of primers are shown in Figure 2. Using this set, the expected fragment size at the end of the first round of PCR is 272 bp. Nested PCR using internal primers is expected to amplify a fragment of 256 bp size. The new set of

Primers	Sequence (5'-3')	Position	Final PCR Product
External			
D1L*	ACTGTCTTCACGCAGAAAGCGTCTAGCCAT	40-69	
D2R	CGAGACCTCCCGGGGCACTCGCAAGCACCC	282-311	
Internal			
D3L	ACGCAGAAAGCGTCTAGCCATGGCGTTAGT	49-78	256 bp
D4R	TCCCGGGGCACTCGCAAGCACCCTATCAGG	275-304	

* L and R indicates sense and antisense polarity respectively

Figure 2. Sequence of primers confined to the 5' non-coding region of HCV used for the detection of Indian strains of HCV.

primers were found to be quite satisfactory for detecting Indian strains as can be seen from a number of samples tested positive as shown in Figure 3. It may be noted that lanes 2–7 in Figure 3 correspond to the products from the first round of PCR and lanes 8–13 correspond to the products of the second round of PCR. The concentration of the amplified products after the first round of PCR is low and the bands corresponding to this round are not clearly visible on the gels, unlike those from the second round of PCR

So far, we have completed PCR analysis of 17 of the HCV antibody positive samples. All of them were PCR-positive with the possible exception of a single borderline ELISA-positive sample. What is of greater interest is that a large percentage of ELISA-negative samples was PCR-positive. We have tested ten ELISA-negative samples including five post-transfusion patients who had received multiple blood transfusion. The post transfusion patients included one with acute myelocytic leukaemia, and another with β-thalassaemia. Two others had received transfusion, one post operatively and another after an abortion (a patient who was HBsAg positive earlier). A fifth post-transfusion patient had hepatoma. All these cases were found to be PCR-positive for HCV. The three other PCR-positive but ELISA-negative patients had acute hepatitis. Two of
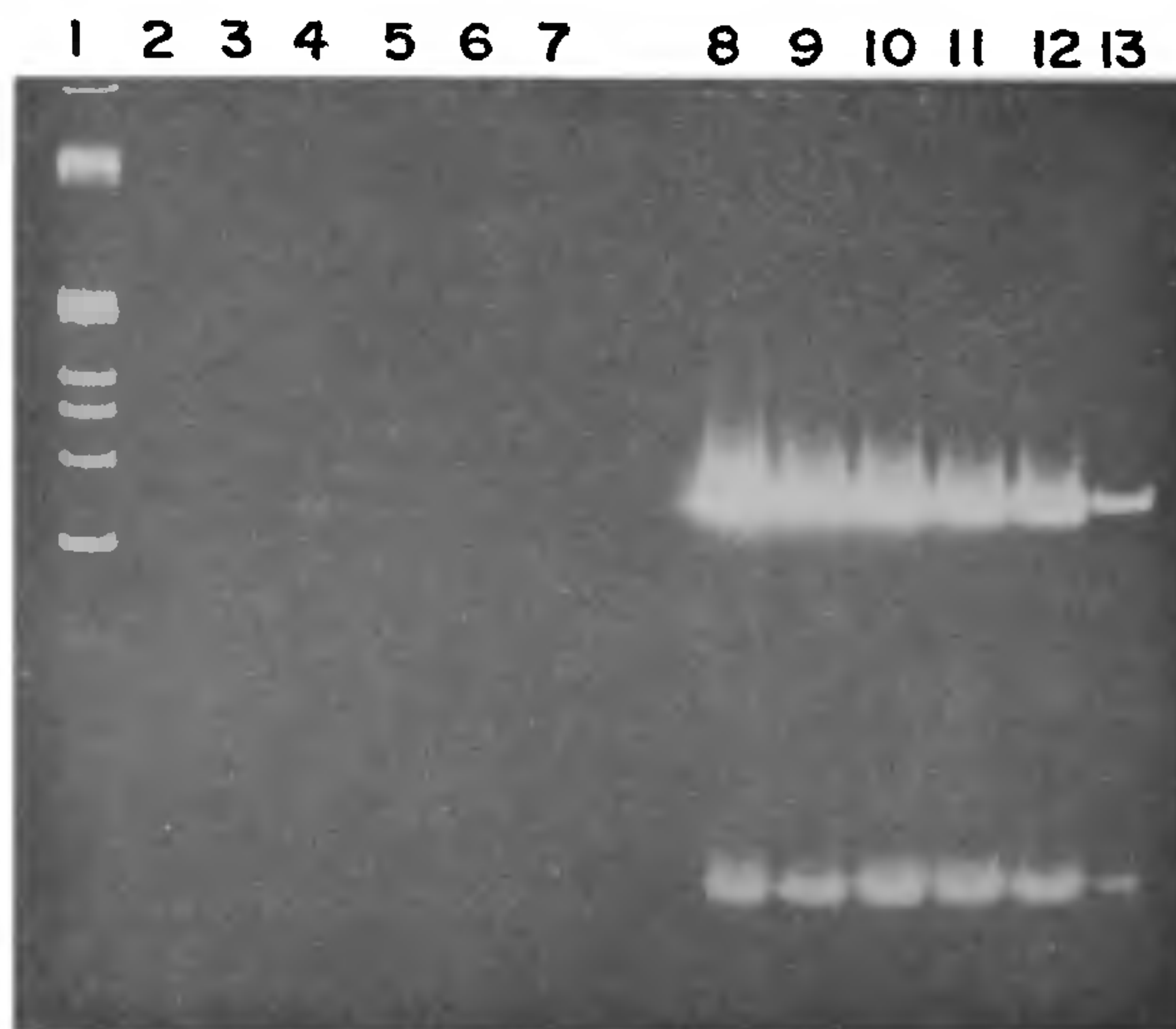

Figure 3. Detection of HCV RNA in seropositive sera by RT-PCR. Lanes: 1, × 174 DNA, HaeIII digest. Product of PCR phase I (lanes 2–7) and phase II (8–13). The low molecular weight bands are arising from primer-dimers.
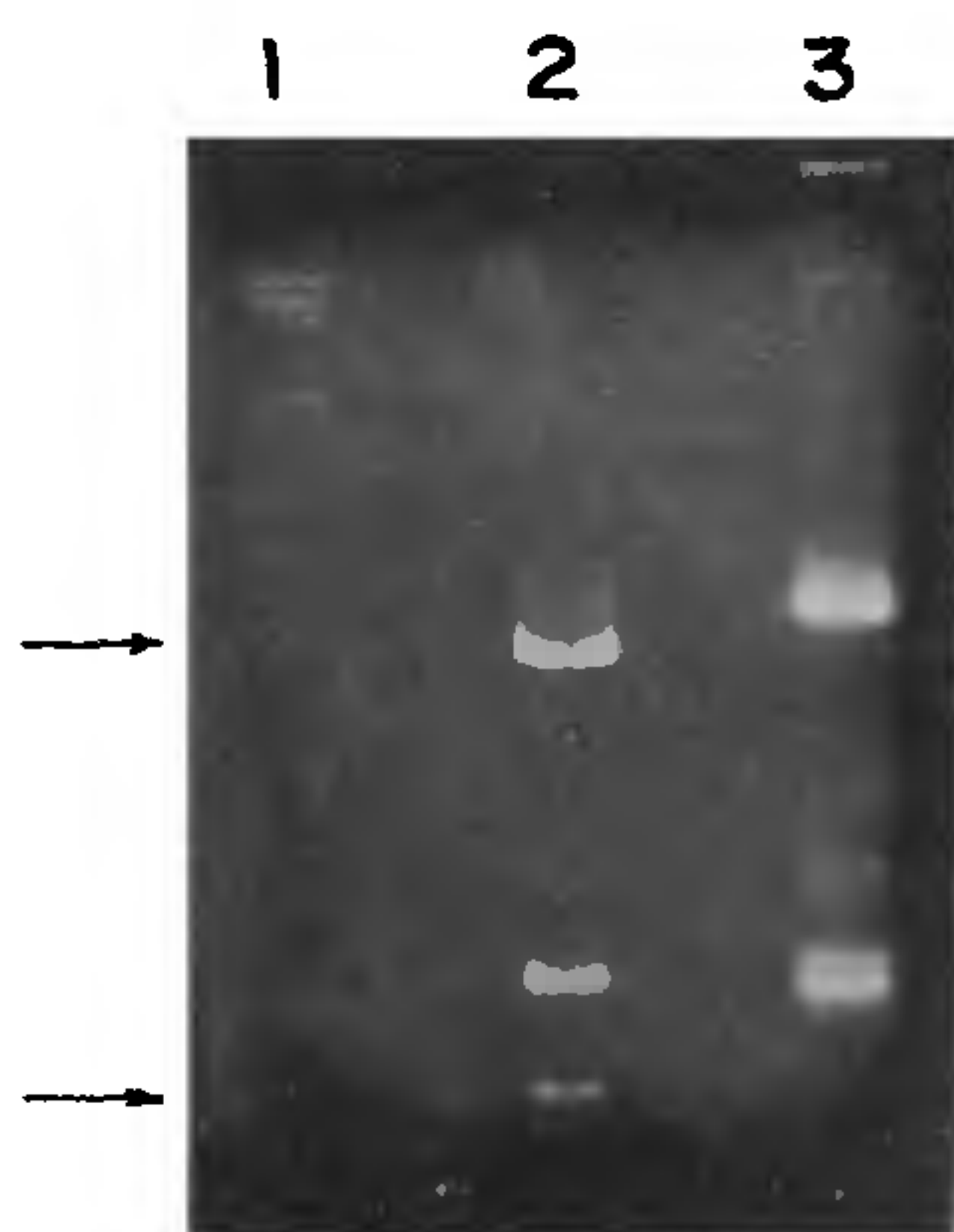
the clinically asymptomatic patients who tested negative for anti-HCV antibody were found to be negative by PCR also.

Authentication of the PCR product

From the known sequence of the conserved region at the 5' end of the HCV genome, the expected amplified product in our PCR reactions is expected to have one *Hae*III restriction site. Digestion of the product with *Hae*III should therefore generate two fragments of 213 bp and 43 bp. Likewise, the amplified sequence has three *Bst*N1 restriction sites and treatment with *Bst*N1 should result in 4 fragments of sizes 41 bp, 50 bp, 63 bp and 102 bp. Figure 4 shows the *Hae*III restriction pattern of the amplified product and Figure 5 shows the *Bst*N1 restriction pattern of the same product. They clearly show the expected pattern confirming the authenticity of the amplified product. It may be mentioned at this point that at least one Japanese isolate is known to be a variant lacking the *Hae*III restriction site. Our isolates are similar to the major group of Japanese isolates with the preserved *Hae*III site.

The amplified 256 bp sequence contains the region spanning from the 237th to 260th base pairs (from the 5'-end) of the HCV genome. This region, in turn, corresponds to the A3L primer shown in Figure 1. In order to further confirm the authenticity of the amplified product, this primer was endlabelled with ^{32}P and used as a probe for hybridization. The autoradiographic profile of the hybridization experiment is shown


Figure 5. Restriction enzyme digestion of 256 bp purified PCR product. Digestion was carried out with *Bst*N1 for 2 h at 60°C and electrophoresed on 7% polyacrylamide gel. Lanes: 1, DNA marker; 2, undigested PCR product, 3, digested with *Bst*N1.

in Figure 6. It clearly shows excellent hybridization in all the HCV RNA positive samples tested.

Cloning and sequencing of part of an Indian HCV isolate

One of the samples that was PCR positive using both the sets of primers (Figures 1 and 2) was used for

Figure 4. *Hae*III digestion of PCR amplified fragments. The 213 and 43 bp fragments (lane 2) are marked with arrows. Lane 3 shows unpurified PCR product.
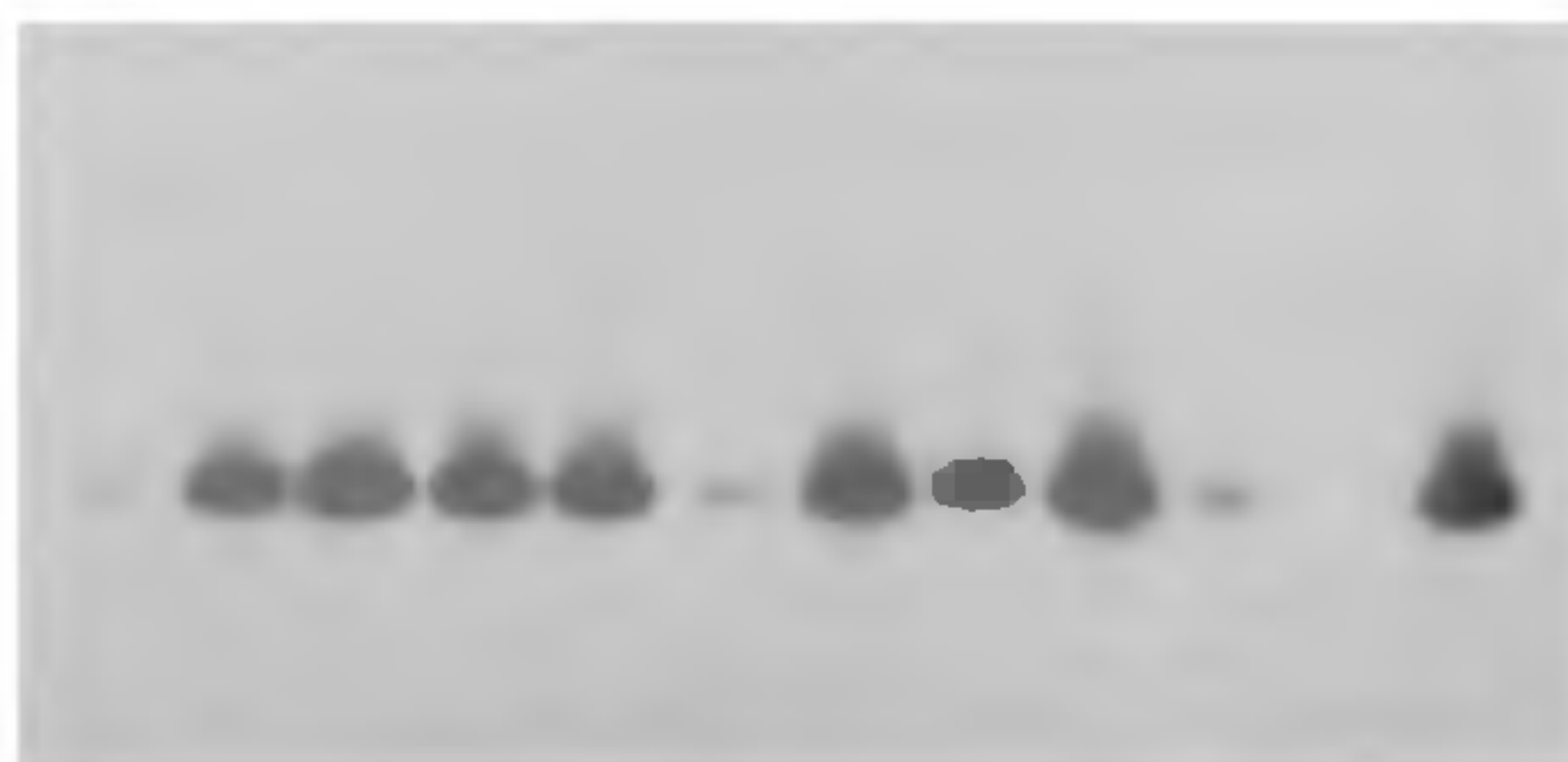
Figure 6. Southern blot hybridization of PCR products with ^{32}P end labelled A3L primer.

cloning and sequencing the amplified fragment. The PCR product obtained using primers shown in Figure 1 (270 bp in length) was cloned in pGEM-3 and was sequenced. The sequence is shown in Figure 7. This sequence shows 98 to 99% homology with Japanese isolates and 96% homology with American isolates. It should be pointed out that the homology shown by comparing sequences of the 270 bp fragment in the 5′ region would overestimate the homology for the simple reason that comparison is being made using a region of minimum variability.

Sensitivity of PCR detection

In our experiments we used two approaches. In the first one, HCV RNA was isolated from the serum of sero-positive individuals and then used for RT-PCR. In the second method, we directly made use of serum samples. This was with a view to simplifying the procedure and to develop a methodology for routine diagnostic purposes. In principle, this should avoid complications arising from RNA degradation. A number of samples which were found PCR-positive using the RNA extraction method were also independently tested using serum samples directly (without extracting RNA). Without exception, all the samples tested by either procedure were found to be positive (data not shown).

It may be noted that cDNA PCR is thought to be less sensitive than DNA-PCR. It has been estimated that at least 10^3 to 10^4 molecules of RNA are needed to be present for successful amplification by cDNA-PCR, as opposed to DNA-PCR which can, in principle, amplify even a single molecule of DNA. Consequently, among the NANB samples which are negative for PCR,

ACTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTAC

TGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACC

GTGCACCATGAGCACGAATCCTAAACCTCAAAGAAAAACCA

AACGTAACACCAACCGCCGCCCACAGGACGTCGAGTTCCCGGG

CGGTGGTCAGATCGTTGGTGGAGTTTACCTGTTGCCGCGCAGGGG

CCCCAGGTTGGGTGTGCGCGCGCCCAGGAAGACTTCCGAGCGGTCGCAACCT

Per Cent homology with other HCV sequences.

1. HPCHMUR   =   99 7 (Japanese)

2. HPCJCO   =   97 7 (Japanese)

3. HPCPLYPRE =   96 0 (North American)

Figure 7. Sequence of HCV isolate from Indian sample. The 270 base pair PCR product was cloned in pGME-3 and sequenced in both directions using SP6 and T7 promotor primers. Per cent homology was evaluated after alignment with reported HCV sequences

there could still be subsets such as those where viraemia is absent and the infection has been resolved, where virus titres could be below the detection limit, where virus could be sequestered in the liver and not circulating, where RNA in the serum samples could be degraded or where a virus may be having major sequence variations. In those instances where the virus titre is low, it is possible to use larger amount of serum for RNA extraction which could then be used for PCR.

Failure to detect HCV antibodies using ELISA in samples which were PCR-positive could arise from different reasons. One of them could be the known long incubation period required for the generation of HCV antibodies. Another possibility is genetic variability in the virus strains in the immunodominant regions responsible for recognition by commercially available reagents.

Conclusions

It has been clearly demonstrated that using primers designed from regions of the HCV genome showing minimum variability (from the 5′ region of the genome), HCV infection can be detected in Indian patients with a very high degree of confidence. All the ELISA-positive samples we have tested so far were found to be PCR-positive. What is of greater interest is that we were able to detect the presence of HCV in ELISA-negative samples using the primers shown in Figure 2. This observation is especially important because at least five of the ELISA-negative patients are likely to have been infected by blood transfusion and they can be detected at the present time only by the PCR method we have developed. This finding underscores also the importance of testing blood samples for the presence of HCV, before clearing the samples for transfusion. Another point that emerges from our studies is the fact that regional variations of virus strains have to be taken into account for developing successful diagnostic procedures. Characterization of the structural proteins of the virus, especially the envelope proteins of Indian strains of HCV would also be important in the development of effective vaccines also. It may also be added that these considerations hold good not only for HCV but also for other viruses, such as HIV strains which are likely to show equal or greater genetic variability and they underscore the necessity for understanding the molecular biology and strain differences of viruses present in different geographical regions.

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RESEARCH COMMUNICATIONS

A simple white-light interferometer operating on the Pancharatnam phase

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We describe a very stable interferometer that can be set-up with readily available optical components. This interferometer can be used with a white-light source and provides an effective demonstration of the Pancharatnam (Berry) phase.

It is well known that the wave function of a quantum system may undergo a phase shift (the geometric phase, or Berry phase) when the parameters of the system are varied in a cyclic manner before being brought back to their original values[1]. The optical analogue of the Berry phase is the Pancharatnam phase, which is generated by a cyclic change in the polarization state of a beam of light[2-5].

Several interferometric systems have been used for measurements of the Pancharatnam phase[6-9], but all of them require fairly sophisticated laboratory facilities. This paper describes a very simple interferometer that can be set up in any university laboratory. This interferometer will work even with a white-light source, and provides an effective demonstration of the Pancharatnam (Berry) phase.

Figure 1 is a schematic of the optical system, which is a modification of that used earlier in an interferometer operating purely on the geometric phase[10]. The light source is a 1-mm pinhole S, illuminated by an automobile head lamp bulb and a condensing lens. This pinhole is placed at the focus of a +2 diopter spectacle lens L_1, which is used as a collimating lens. Alternatively, a low power He–Ne laser can be used as the source. A polarizer P and a $\lambda/4$ plate QWP produce a beam of circularly polarized light, which is divided at the 50:50 beam splitter BS into two beams that traverse the same rectangular optical path in opposite
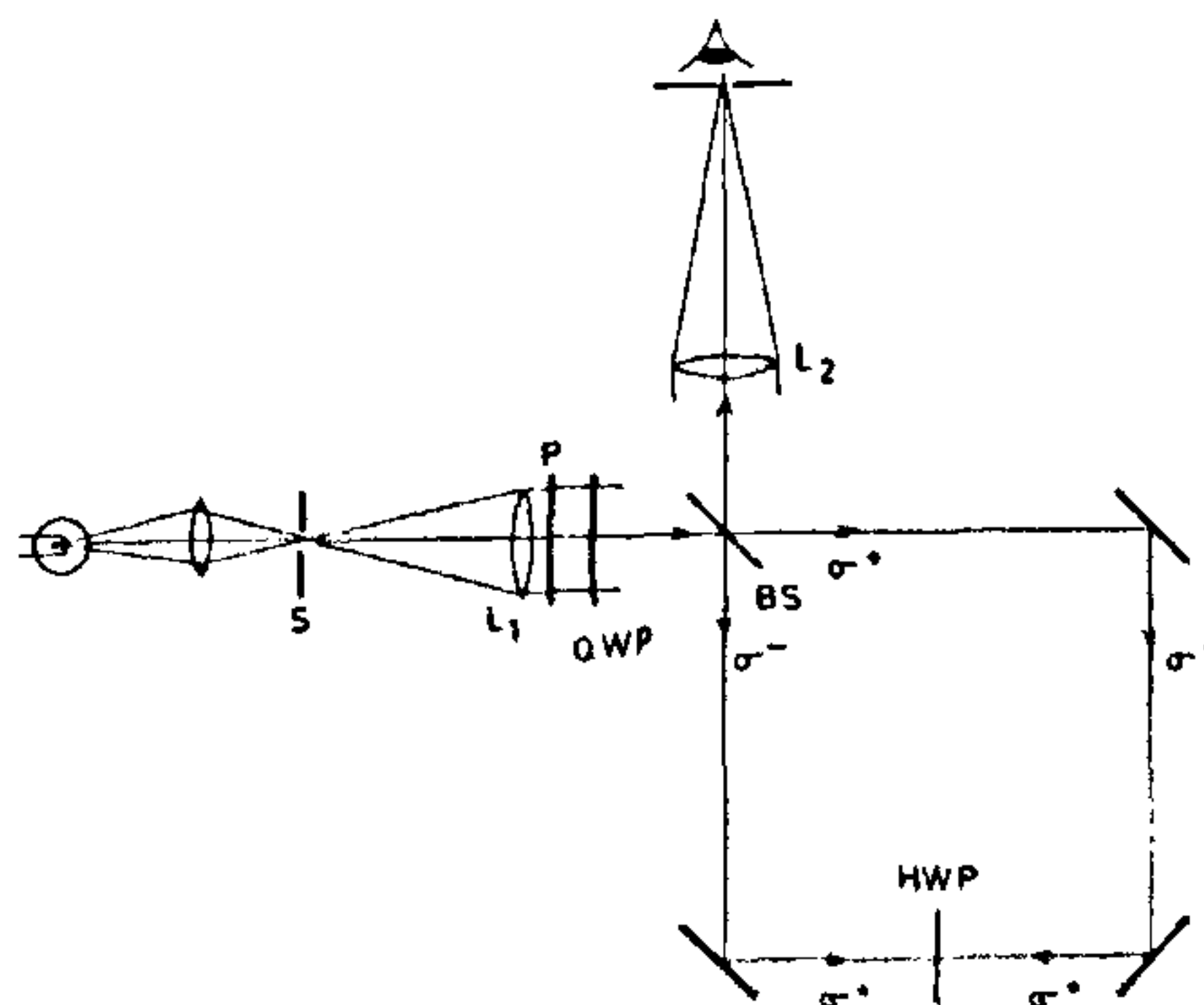

Figure 1. Schematic of the optical system of the interferometer