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Mechanisms of Inactivation of Hepatitis A Virus by Chlorine

Jun Wen Li,* Zhong Tao Xin, Xin Wei Wang, Jin Lai Zheng, and Fu Huan Chao

Institute of Health and Environmental Medicine of Tianjin, Tianjin City 300050, People's Republic of China

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The study was intended to investigate the feasibility of reverse transcription-PCR (RT-PCR) for evaluation of the efficacy of inactivation of viruses in water and to elucidate the mechanisms of inactivation of hepatitis A virus (HAV) by chlorine. Cell culture, enzyme-linked immunosorbent assay, and long-overlap RT-PCR were used to detect the infectivity, antigenicity, and entire genome of HAV inactivated or destroyed by chlorine. The cell culture results revealed the complete inactivation of infectivity after 30 min of exposure to 10 or 20 mg of chlorine per liter and the highest level of sensitivity in the 5′ nontranslated regions (5′NTR), inactivation of which took as much time as the inactivation of infectivity of HAV by chlorine. However, antigenicity was not completely destroyed under these conditions. Some fractions in the coding region were resistant to chlorine. To determine the specific region of the 5′NTR lost, three segments of primers were redesigned to monitor the region from bp 1 to 1023 across the entire genome. It was shown that the sequence from bp 1 to 671 was the region most sensitive to chlorine. The results suggested that the inactivation of HAV by chlorine was due to the loss of the 5′NTR. It is believed that PCR can be used to assess the efficacy of disinfection of HAV by chlorine as well as to research the mechanisms of inactivation of viruses by disinfectants.

Hepatitis A virus (HAV) is a positive-strand RNA virus that belongs to the *Hepatovirus* group of the family *Picornaviridae*, which can bring about infectious hepatitis (hepatitis A). The infection rate of HAV is universally very high, being about 70% in China (18), and person-to-person spread of HAV is also very common. Disinfection with chlorine has been adopted worldwide to ensure the safety of drinking water, since HAV spreads mainly through drinking water and food. However, research on the mechanisms of chlorine inactivation of HAV has been inadequate. It is generally believed that chlorine inactivates HAV by damaging the nucleic acid of the virus (2, 3, 12). With the development of molecular biology methods, nucleic acid probes and PCR have been used to evaluate the effects of virus disinfection and to research disinfection mechanisms. However, the research problem most frequently encountered and difficult to solve is that, after disinfection, the virus nucleic acid can still be detected by PCR, although the infectivity of the virus actually no longer exists. Therefore, it is generally believed that molecular biology methods cannot be used to evaluate either the effects or the mechanisms of disinfection (2, 8, 9, 16).

The assumption that chlorine acts on the virus nucleic acid is likely to be measured by PCR. The main reason why no ideal result has ever been obtained in previous studies is that the length of the virus nucleic acid segment detected is limited and fails to reflect the overall status of viral nucleic acid. So in this study, in addition to using such techniques as cell culture and enzyme-linked immunosorbent assay (ELISA) to detect infectivity and antigenicity of virus, a large section of step-by-step shifting (long overlap) reverse transcription-PCR (RT-PCR) has been applied to make a full sequence. This technique thus made it possible to scan for HAV nucleic acids before and after disinfection in order to define the mode, area, and characteristics of chlorine's activity on HAV nucleic acid and thus lay a foundation for research on the effects and mechanisms of disinfection.

* Corresponding author. Mailing address: Mailing address: Institute of Health and Environmental Medicine of Tianjin, 1 Da Li Rd., Tianjin City 300050, People's Republic of China. Phone: 86-22-84655345. Fax: 86-22-23328809. E-mail: junwenli@eyou.com.

MATERIALS AND METHODS

Virus strains. The strain of HAV used in this study was Nj-3, isolated from the human liver cancer cell line PLC/PRF/5 (both were donated by the Institute of Military Medical Research of Nanjing).

Propagation and purification of virus. The methods used for proliferation and purification of virus were described previously by Peterson et al. (13) and Shieh et al. (15). All cell cultures were grown in Eagle's minimum essential medium (Difco Laboratories, Detroit, Mich.) containing 8% fetal bovine serum (FBS), 0.015 M HEPES buffer, and antibiotics (50 μg [each] of kanamycin and gentamicin per ml) and maintained in the same medium with 1.5% FBS. For virus propagation and isolation, cell cultures in 75-cm^2 flasks were drained of medium, inoculated with small volumes of stock virus, and inoculated for 1 h at 37°C with periodic rocking for viral adsorption. Cultures were then supplemented with maintenance medium and incubated at 35°C for viral propagation. Medium was replaced for 2 to 3 days of incubation. HAV was harvested by freezing and thawing, concentrated with 12% (wt/vol) polyethylene glycol, and further purified by dialysis with ultracentrifugation in CsCl equilibrium gradients. The details were described by Wang et al. (16).

Extraction of virus RNA. A virus RNA extraction kit made by Life Technologies for extraction of exceedingly pure viral RNA was utilized in our experiment to extract virus RNA, and all operations were strictly implemented in accordance with the reagent instruction manual.

Primer design for assay of HAV nucleic acid. Seven sets of primers were designed with Oligo 4.0 software on the basis of published reports on several strains of HAV sequences (1, 13). Each set of the primers contains three strips of primers: S and A are the first PCR primers, and SN is a seminested PCR primer. The first PCR products are around 1,000 bp, with adjacent primers on top of another, so that a full-sequence scanning of HAV nucleic acid could be carried out (Table 1). All primers were synthesized and purified by the Dalian Bao Biology Company.

Amplification of HAV nucleic acid by RT-PCR. The one-step single-tube RT-PCR reagent used in the experiment was purchased from the Bao Biology Company, and the specific operations were carried out in line with the reagent instruction manual. The RT-PCR parameters are listed as follows. RT was performed at 50°C for 30 min. Pre-denaturation was performed at 94°C for 5 min. This was followed by denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 1 min for 35 cycles and, finally, extension at 72°C for 10 min. Next, 1 μl of RT-PCR-expanded product, diluted at a ratio of 1:10, was taken for the seminested PCR to verify the particularity of the expanded

TABLE 1. Primer sequences and their locations in the entire genome of HAV

Primer name and properties[a]	Locations	Sequence	Product length (bp)
I			
S	1–~18	TTCAAGAAGGGTCTCCGG	
A	1002–~1023	GAGCATGTGTAGTAAGCCAATC	1,023
SN	652–~671	AAGGCCCCAAAGAACAGTCC	671
II			
S	997–~1020	CTGCAGATTGGCTTACTACACATG	
A	2033–~2056	TACTCACCTTTCTGTTGTGCTGAC	1,060
SN	1556–~1577	TGCTTTGGATCAGGAAGATTGG	521
III			
S	2045–~2067	GAAAGGTGAGTACACTGCCATTG	
A	3191–~3215	AGGAGGTGGAAGCACTTCATTTGA	1,171
SN	2713–~2734	GTTGGGTAGAGAAGGAGTCAGC	503
IV			
S	3040–~3059	AATTGCAGCTGGAGACTTGG	
A	4166–~4185	TTGTCTGCTTCTTCAATGGC	1,146
SN	3642–~3661	GGATTGTCTGGAGTTCAGGA	544
V			
S	4101–~4122	GTGAATTATGGCAAGAAGAAGG	
A	5329–~5348	CACCAACTCCAAACTGAACC	1,249
SN	4629–~4648	GTGTCAGGATGTCCAATGAG	620
VI			
S	5327–~5346	CTGGTTCAGTTTGGAGTTGG	
A	6494–~6513	AAAGGACAAGCATCAATGGC	1,187
SN	5976–~5995	GTTCAATGAATGTGGTCTCC	538
VII			
S	6496–~6515	CATTGATGCTTGTCCTTTGG	
A	7478–~poly(A)	TTTTTTTTTTTTTTT	985
SN	6696–~6715	CCAGTCTTAGTCCATTTATG	785

[a] S, upstream primer; A, downstream primer; SN, seminested primer.

product. The reaction conditions were the same as those for the one-step single-tube RT-PCR, but with 15 cycles.

Detection of PCR product. To detect PCR products, an agarose gel electrophoresis method (11) was used. Amplification reaction mixtures (10 μl) were mixed with 2 μl of 6× gel loading buffer and loaded onto 2% agarose gels. After electrophoresis, gels were soaked in 5 μg of ethidium bromide per ml for 5 min and destained for 1 h. The bands were then visualized and photographed.

Preparation and analysis of halogen solutions. Chlorine demand-free water was prepared according to Peterson's methods (13). All reagents and buffers used in chlorination experiments were prepared with chlorine demand-free water. Chlorine solution was made by dissolving the chlorine gas, liberated by the action of concentrated HCl on potassium permanganate, in dilute NaOH. The stock chlorine solution was stored in amber-colored bottles at 4°C. The concentration was defined by iodometry before being used.

Chlorine inactivation experiments. For chlorine inactivation, the glassware was thoroughly cleaned and rinsed with chlorine demand-free water. The chlorine demand of the HAV solution was determined by the *N*,*N*, diethyl-*p*-phenyldiamine (DPD) colorimetric method to be 0.5 to 0.6 mg/liter. The bulk volume of the overall reaction system of the disinfection experiment was 10 ml, of which virus-containing original liquid and disinfectant occupied 1 ml, respectively. The rest of the volume was filled by sterilized phosphate-buffered saline. The treatment solution was prepared at pH 7.0 with the desired free residual chlorine at 5, 10, 20, and 50 mg/liter plus 0.6 mg/liter to satisfy the demand of the HAV solution, and the exposure times were 10, 30, and 60 min. The rest of the chlorine was neutralized by 1% $Na_2S_2O_3$.

Test of virus infectivity. After disinfection, samples at every time point were used to inoculate cells, and the titer of infectivity was determined (6, 11) in terms of the 50% tissue culture infective dose ($TCID_{50}$) per milliliter. The following equation was used to calculate the infectivity/inactivation ratio of virus.

$$\text{Rate of inactivation (\%)} = \frac{TCID_{50}\text{/ml of control group} - TCID_{50}\text{/ml of disinfection group}}{TCID_{50}\text{/ml of control group}} \times 100\%$$

Antigenicity measured by ELISA. In this study, we used anti-HAV antibodies containing a human polyclonal anti-HAV serum collected after natural infection and a rabbit monoclonal anti-HAV antibody. To quantify antigenicity, an ELISA method was used. A 96-well microtiter plate coated with polyclonal anti-HAV antibody was used for the assay. HAV was serially diluted, loaded, and incubated overnight at room temperature. After being washed five times, horseradish peroxidase-labeled anti-HAV antibody was added for 1 h at 37°C. Microtiter plates were washed, and the optical density at 450 nm (OD_{450}) was measured.

TABLE 2. Inactivation of infectivity of HAV at different chlorine dosages and exposure times[a]

Chlorine dosage (mg/liter)	Inactivation in $TCID_{50}$/ml (%) at exposure time:		
	10 min	30 min	60 min
5	$10^{5.63}$ (24.14)	$10^{5.33}$ (61.98)	$10^{4.78}$ (89.29)
10	$10^{4.50}$ (94.38)	0 (100)	0 (100)
20	$10^{3.78}$ (98.93)	0 (100)	0 (100)

[a] The initial concentration of HAV was $10^{5.75}$ $TCID_{50}$/ml. The inactivation rate of virus infectivity is shown in parentheses.

RESULTS

Inactivation of virus infectivity. When the chlorine concentration is 5 mg/liter, HAV infectivity still cannot be completely inactivated, despite exposure for 60 min. Only with a chlorine concentration of 10 or 20 mg/liter and after an exposure time of 30 min can HAV infectivity be inactivated completely (Table 2).

Destructibility of virus antigenicity. When the chlorine concentration is 5 or 10 mg/liter, HAV antigenicity still exists (OD_{450} for the experimental group/OD_{450} for the control group, >2.0), even after exposure for 60 min. The antigenicity was not destroyed completely by a chlorine dosage of 20 mg/liter. Only with a chlorine concentration of 20 mg/liter and after exposure for 60 min did HAV antigenicity become negative (Table 3).

Inactivation of virus nucleic acid. (i) Amplification of HAV nucleic acid by PCR before disinfection. Positive results can be tested through large-section, step-by-step shifting RT-PCR for the virus nucleic acid in a positive control group (Fig. 1), and its specificity was confirmed by the seminested PCR (data not shown).

(ii) Detection of virus nucleic acid after chlorine disinfection. (a) Full-sequence scanning results for virus nucleic acid. When the chlorine concentration was 10 mg/liter with exposure for 30 min, the RT-PCR product of the I primers was negative. It is shown that chlorine acts on HAV nucleic acid best within the range of amplification area of the I primers, near the 5′ nontranslated region (5′NTR) of the virus nucleic acid (Fig. 2). This result is consistent with that from inactivation of virus infectivity. Besides, the 3′NTR of the nucleic virus was also negative when the chlorine concentration was 10 mg/liter with

TABLE 3. Destructibility of virus antigenicity at different chlorine dosages and exposure times

Chlorine dosage (mg/liter)	Destructibility (P/N ratio) at exposure time[a]:		
	10 min	30 min	60 min
5	5.17	5.09	4.85
10	4.87	3.21	2.01
20	3.49	2.98	1.57

[a] The initial P/N value of HAV was 5.23; a P/N value below 2.0 means that the antigenicity was destroyed completely. P is the OD_{450} for the experimental group, and N is the OD_{450} for the control group.

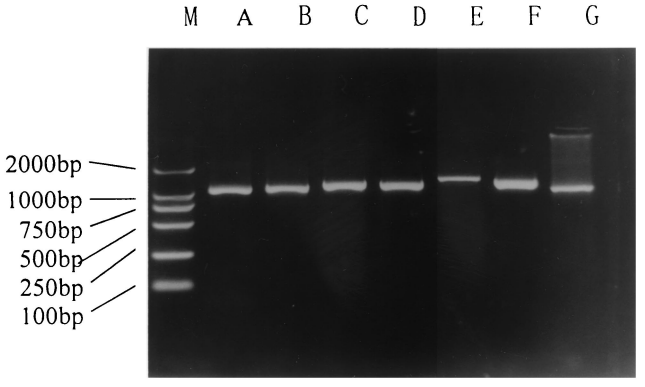

FIG. 1. RT-PCR results for the scanned entire genome of HAV. Lanes: M, DNA marker; A to G, amplification with the outer primers I to VII, respectively.

exposure for 60 min, while the coding area of the virus nucleic acid revealed stronger resistance to chlorine (Table 4).

(b) Amplification results for the sensitive area of virus nucleic acid. The structure of the 5′NTR in HAV nucleic acid is very complicated. The amplification area (bp 1 to ~1023) of the I primer pair is further divided into three jointed sections according to the composition characteristics of HAV genome in order to find the specific position of chlorine action. The primer sequence and amplification areas are listed in Table 5.

Take exposure to 10 mg of chlorine per liter for 30 min as an example. The amplification was made segment by segment for the 5′NTR, and a strict control procedure was set up. The result showed that two segments (bp 1 to ~174 and 155 to ~671) tested negative with contact with the disinfectant for 30 min; the segment from bp 669 to ~1023 was positive, and the three segments of the positive control groups were all positive (Fig. 3).

DISCUSSION

Inactivation of infectivity is the most important and direct index with which to evaluate the effects of viral disinfection. Previously, different experimental designs had led to different results in studies of chlorine inactivation of HAV. For example, with 30 min of exposure, the effective chlorine concentrations required by different researchers were between 1 and 25 mg/liter (14, 17). Our study found that HAV could be inactivated when the chlorine concentration exceeds 10 mg/liter with 30 min of exposure time.

Because it is fast, sensitive, specific, and simple to operate, ELISA has often been adopted to evaluate the effects of HAV inactivation. Our study suggests that the antigenicity of virus can be inactivated when the chlorine concentration is 10 mg/liter with an exposure time of 60 min, which means that the disappearance of antigenicity comes after the inactivation of infectivity. This result agrees with the study by Scarpino et al. (14). It is suggested that the original target of chlorine inactivation in HAV may not be in the capsid protein of virus, but rather in the nucleic acid.

Nucleic acid probes and PCR have been used to evaluate viral disinfection and to study the disinfection mechanisms. For

TABLE 4. Detection of infectivity and nucleic acid of HAV disinfected with chlorine

Group	Infectivity[a]	RT-PCR results of the entire genome						
		I	II	III	IV	V	VI	VII
Control	+	+	+	+	+	+	+	+
5 mg/liter								
30 min	+	N[b]	N	N	N	N	N	N
60 min	+	+	+	+	+	+	+	+
10 mg/liter								
30 min	−	−	+	+	+	+	+	+
60 min	−	−	+	+	+	+	+	−
20 mg/liter								
30 min	−	−	+	−	+	+	+	−
60 min	−	−	−	−	+	+	−	−
50 mg/liter								
60 min	−	−	−	−	−	+	−	−

[a] +, positive; −, negative.
[b] N, not detected.

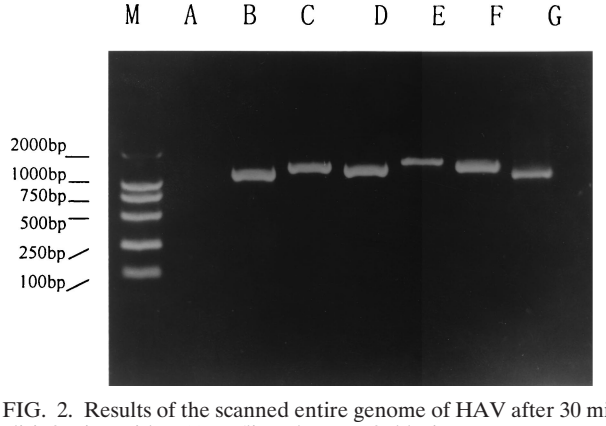

FIG. 2. Results of the scanned entire genome of HAV after 30 min of disinfection with a 10-mg/liter dosage of chlorine. Lanes: M, DNA marker; A to G, amplification with the outer primers I to VII, respectively.

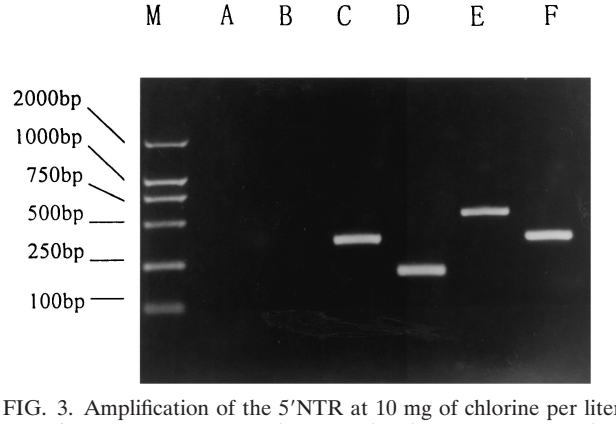

FIG. 3. Amplification of the 5′NTR at 10 mg of chlorine per liter for 30 min. Lanes: M, DNA marker; A and D, bp 1 to ~174; B and E, bp 155 to ~671; C and F, bp 669 to ~1023. Lanes A, B, and C represent the tested groups, and lanes D, E, and F represent the control group.

TABLE 5. Primers designed to amplify the near 5′NTR of HAV

Primer	Location	Sequence	Length (bp)	Amplified region
S	1–~18	TTCAAGAAGGGTCTCCGG		
A[a]	155–~174	AGAACCCAGAACCTGCAGGA	174	Before IRES
S[a]	155–~173	TCTGCAGGTTCTGGGTTCT		
A	652–~671	AAGGCCCCAAAGAACAGTCC	517	IRES
S[a]	669–~688	CCTTATGTGGTGTTTGCCTC		
A	1002–~1023	GAGCATGTGTAGTAAGCCAATC	355	Structure region

[a] Newly redesigned primer.

example, Leveque et al. have discovered that when HAV in seawater is irradiated by UV radiation for 60 min, HAV exhibits no infectivity, but its nucleic acid can still be detected by RT-PCR (8). Han et al. reached a similar conclusion (4). In addition, different disinfection methods for inactivation of poliovirus have been studied. Reports by Moore and Margolin (11) showed that neither a nucleic acid probe nor RT-PCR could determine the inactivation of poliovirus when used to detect virus nucleic acid after the virus had been treated with chlorine, chlorine dioxide, ozone, and UV radiation. Ma et al., Maier et al., and Moore and Margolin believe that although the infectivity of poliovirus cannot be tested by cell culture, its nucleic acid can still be revealed by RT-PCR when the virus is treated with UV radiation, free chlorine, HCl, and NaOH (9–11). They all agreed that evaluations of the effect or mechanism of disinfection cannot be carried out accurately with RT-PCR or a nucleic acid probe.

However, as is known to all, PCR is a kind of reaction that has strong particularity and strict requirements for template DNA. Any chemical change in the link of the template DNA chain, including a chain rupture, and any chemical change on base groups, such as an alkali base or pentose, can result in unmatched template and primers or failure to extend and replicate after mating, and hence no amplification. Therefore, the effects of disinfectant on the DNA can be fully reflected by the PCR results. The results mentioned above occur because of the adoption of inappropriate PCR methods where the length of target nucleic acid segment was only around 100 to ~400 bp, while the length of nucleic acid of HAV or poliovirus is about 7,500 bp. It follows that the changes in the nucleic acid segment of several tenths alone cannot achieve an objective evaluation of the effect of disinfectant for the whole length of the virus nucleic acid.

Large-fragment step-by-step shift RT-PCR was used in our study to make a full sequence scanning on HAV nucleic acid before and after disinfection. It has been found that different positions of the virus nucleic acid showed different levels of resistance to chlorine—the 5′NTR and 3′NTR are more sensitive to chlorine, whereas the coding area shows more resistance to chlorine. The 5′NTR segment could not be detected by PCR when the infectivity of virus was inactivated completely, meaning that the inactivation of virus infectivity is consistent with its damage, but some fragments of the coding area can still be detected, which is what researchers have neglected in their previous studies and made mistakes with.

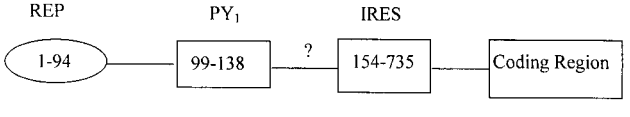

FIG. 4. Diagram of sequences in the HAV 5′NTR proposed for different functions (7). The numbers indicate the nucleotide sequences of pHAV/7. REP, replication-related sequence.

The functions of the 5′NTR in HAV nucleic acid are complex and can be classified as related sequence of virus replication (REP), an area rich in pyrimidine (pY1), an internal ribosome entry site (IRES), and an area between pY1 and the IRES (5, 7) (Fig. 4). To more precisely locate the specific sites of chlorine action on HAV nucleic acid, the sections of 5′NTR have been further shortened. It has been found that virus nucleic acid can still be detected in the region of bp 669 to ~1023, while the positions at bp 1 to ~174 and 155 to ~671 are negative according to many tests after virus infectivity is inactivated. It is thus evident that the segment running from bp 1 to ~671 of the 5′NTR plays an important role in the inactivation of virus infectivity. For the tiny RNA virus family, this area has a stable stem-loop structure close to the 5′ end (near the 10th nucleotide). The protein signal identification needed by virus replication depends on the space structure of the sequence in this area, and the identification of the signal will be affected by inactivation of the nucleic acid of this area. The IRES contains the *cis*-form action elements, which are required by external translation, and HAV protein translation requires the combination of various translation original genes with IRES. The expression of virus protein will be much affected by the failure to carry out the combination effectively (7). Therefore, the 5′NTR is closely associated with replication of virus RNA, the origination of translation, and the composition of the virus particles. Chlorine inactivation of HAV is just the result of inactivation of the virus nucleic acid 5′NTR. It is believed that the inactivation effect of disinfectant on virus can be evaluated completely by PCR in terms of the mechanism of inactivation of HAV nucleic acid by chlorine.

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