Original Article

Distribution of Hepatitis B virus genotypes among healthy blood donors in eastern part of North India

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Abstract:

Aim: We evaluated the distribution HBV genotypes among non-remunerated healthy blood donors in eastern North India. **Materials and Methods:** During screening of donated blood, 176 consecutive HBsAg positive, samples comprised the study. HBV-DNA was quantitative detected in 150 samples by PCR. HBV genotype was determined by identifying genotype-specific DNA band using nested PCR. **Results:** Majorities were of age group 31-40 yrs (65.3%). Males (92.7%) outnumbered females (7.3%) and were HbeAg-negative HBsAg carriers. Over all, genotype-A was the most prevalent (54%) followed by D (21.3%). We did not find genotype-G and H. Districts under study, divided into four zones: *Zone–I* genotype-A was most common (62.3%) followed by D (18.8%); *Zone–II* genotype–C (41.2%) was more frequent followed by D (20.6% and A (17.7%). *Zone–III* in adjoining Bihar state close to *Zone–I,* A was more prevalent (81.8%) followed by B and C (9.1%). In *Zone-IV* adjoining *Zone- II* had genotype-A (100%) only. Genotype–D had more sporadic distribution. Genotype-E and F were prevalent in Zone I and II (3/150, 2%). **Conclusions:** Among blood donors HBV genotype-A followed by D was the most prevalent in eastern North India. Genotype–A had pattern of distribution signifying common focus, while D was more sporadic and C had single large pocket (Zone-II) probably common focus but restricting to particular area. Evidences are suggestive of association of HBV genotype in liver dysfunction. An effective treatment and preventive strategies based of genotypes will reduce the disease burden and increase the blood safety.

Key words:

Hepatitis B virus, HBV in blood donor, HBV in India, Hepatitis B virus genotype, PCR genotyping

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Introduction

Chronic hepatitis B virus (HBV) infection remains a major public health problem worldwide with more than 300 million chronic carriers.[1] The course of HBV infection depends on several factors that can influence the immune system, including age at infection and host genetic factors, and genetic variability of the virus influencing the expression of the viral antigens. HBV has been classified into eight genotypes (A-H) based on inter-group divergence of 8% or more in the complete nucleotide sequence.[2-4] The distribution of HBV genotypes may vary with time and geographical region. It is becoming increasingly evident that the genotypes of HBV may have a role to play in predicting the response to various therapies and that this should be taken into account as a variable before initiating any treatment.[5,6] However, differences in host and environmental factors make it difficult to extrapolate findings from one geographical region to another. Moreover, the advent of new molecular antiviral makes it paramount that the genotypes are well-characterized, so that the drugs can be tailor-made to the virus prevalent in the different regions of the world.

Data on the clinical relevance of HBV genotypes are not available from many parts of the world including India. Majority of the available data are based on studies of patients with chronic HBV infection in Asia. Genotypes A and D have been reported from Western Europe and North America; in Mediterranean region, the middle East, and central Asia genotype- D is dominant. Limited data from India, however, suggest that genotypes A and D are most prevalent.[7-9]

India has one of the largest pools of hepatitis B-infected patients.[10,11] But the data regarding the regional distribution of hepatitis B genotypes is scarce.[12] We do not have data regarding prevalence of different genotype of the HBV and rate of progression to cirrhosis and liver cancer among patients with all known HBV genotypes. Such data is largely from thickly populated (500-600 people/km^2) middle part of the gigantic basin which comprised of eastern Uttar Pradesh and western Bihar state. Therefore, present study was planned to know the prevalence of different HBV genotype among the apparent healthy blood donors representing the eastern part of north India.

Materials and Methods

The present study was carried out on the HBV-positive donors coming to the Blood Bank of Sir Sundarlal Hospital, Institute of Medical Sciences,

Banaras Hindu University, Varanasi, India during the period of December 1, 2006-May 31, 2008. This is a 1000-bed hospital catering tertiary level health facility to the population of eastern part of Uttar Pradesh, western part of Bihar and occasionally adjoining part of Jharkhand, Chhattisgarh and Madhya Pradesh.

(A) Study Population: A total of 176 HbsAg-positive HbeAg-negative blood donors were included in the study. HBV-DNA was positive in 150. The geographical areas were divided in four zones depending on their location which is depicted in Figure 1. *Zone–I* comprised of districts around Varanasi (Varanasi+Bhadohi+Mirzapur+Jaunpur); Zone-*II* (Azamgarh+Mau+Ghazipur+Chandauli). While *Zone-III* consists of districts of Bihar located at eastern boarder of Uttar Pradesh (Buxar+Kaimur+Rohtas+Aurangabad) and *Zone-IV* districts of Bihar located on north-eastern boarder of Uttar Pradesh (Gopalganj+Bettiah).

(B) Genotyping of HBV

(i) Collection of specimen: Five milliliters of blood was collected and allowed to clot for 30 min. The serum was separated and stored at –40˚C till further use.

(ii) Detection of HBV:

(a) For HBs Ag: All 150 HBV-positive serum found during primary screening for HBV (Bio-Rad Lab.) were further tested to reconfirm the positivity for virus following the method using commercially available EIA kit (Abbott Laboratories., Chicago, USA)

(b) For HBe Ag: Method using commercially available EIA kit (Abbott Laboratories).

(iii) Extraction of DNA from serum (Annexure-A):
The DNA was extracted by Phenol chloroform technique following the method of Maniatis *et al.*[13]

Table 1: Primer sequences used for HBV genotyping by nested PCR

Primer	Sequence[a] (position, specificity, and polarity)	Annealing Temp.
First PCR		
P1*b*	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt 2823–2845, universal, sense)	
S1-2	5'-CGA ACC ACT GAA CAA ATG GC-3' (nt 685–704, universal, antisense)	55˚C
Second PCR		
Mix A		
B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67–86, types A to E specific, sense)	
BA1R	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113–134, type A specific, antisense)	
BB1R	5'-CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324–345, type B specific, antisense)	
BC1R	5'-GGT CCT AGG AAT CCT GAT GTT G-3' (nt 165–186, type C specific, antisense)	
Mix B		58˚C* and 60˚C
BD1	5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979–2996, type D specific, sense)	
BE1	5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (nt 2955–2978, type E specific, sense)	
BF1	5'-GYT ACG GTC CAG GGT TCA CA-3' (nt 3032–3051, type F specific, sense)	
B2R	5'-GGA GGC GGA TYT GCT GGC AA-3' (nt 3078–3097, types D to F specific, antisense	
BD1	5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979–2996, type D specific, sense)	

[a]An "**M**" represents a nucleotide that could be either an A or a C; a "**Y**" represents a nucleotide that could be either a **C** or a **T**. nt: Nucleotide.
[b]The sequence for primer P1 was determined by Lindh *et al.*[11]
*First cycle only

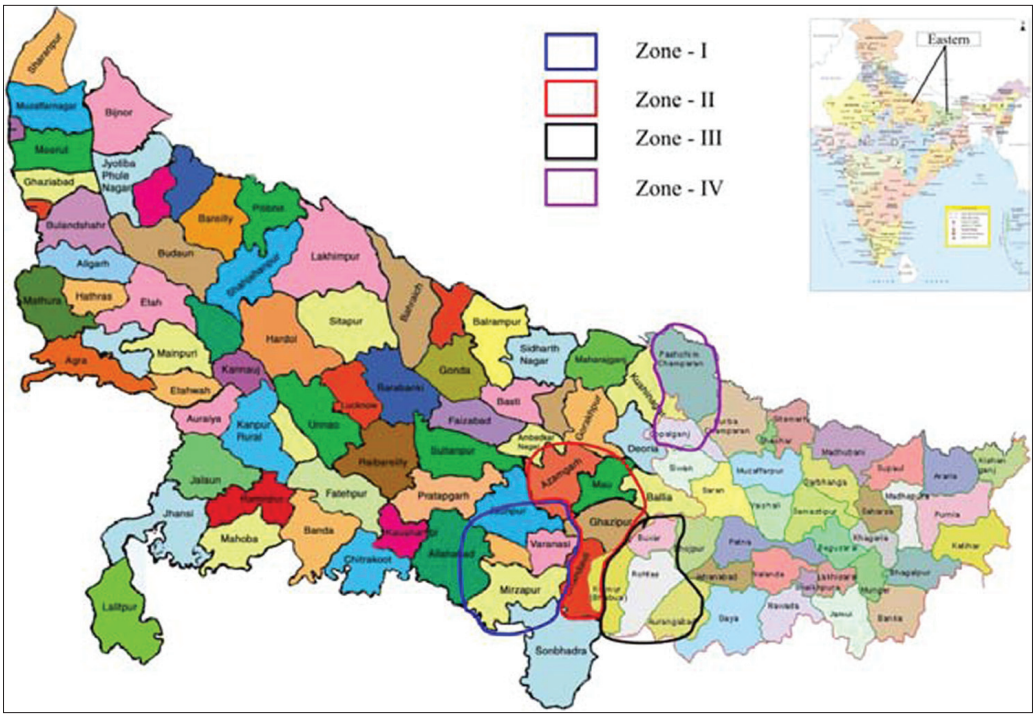

Figure 1: Geographical distribution of HBV genotypes

Table 2: Age and HBV genotypes (*n* = 150)

Genotype	Age group (yrs)						Total	
	21-30		31-40		41-50			
	No.	%	No.	%	No.	%	No.	%
A	11	42.3	63	64.3	7	26.9	81	54.0
B	7	26.9	3	3.1	0	0	10	6.7
C	5	19.2	4	4.1	6	23.1	15	10.0
D	0	0	20	20.4	12	46.2	32	21.3
E	3	11.5	6	6.1	0	0	9	6.0
F	0	0	2	2.0	1	3.8	3	2.0
Total	26	100	98	100	26	100	150	100

In Brief: Three milliliters of serum was mixed with 100 µL of lysozyme and incubated at 37°C for 1 h. To inactivate the enzyme and break the virus particle 1 mL of 0.1% Triton X 100 and 5 µL of proteinase K was added and incubated at 65˚C for 2 h to precipitate the debris, mixture of chloroform and isoamyl alcohol (IAA) was added in the proportion 24:1 and vortexed for 15 s and centrifugation was done at 2000 g for 10 min. Phenol, chloroform and IAA (25: 24:1) mixture was added to it in 140 µL volume and vortexed again for 15 s following which again centrifugation done for 10 min. Aqueous phase was collected. The process was repeated again. The supernatant was treated with 2 µL of RNAse (20 mg/mL) at 37°C for 30 min. To inactivate RNAse incubation was done at 65°C in water bath for 30 min. To precipitate the DNA equal volume of isopropanolol was added and kept at room temperature for 5 min. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded and pellet was washed twice with 200 µL of 70% ethanol. The pellet was dried at 37°C and dissolved in 20 µL of Tris-EDTA (TE) buffer (pH 8.0) and preserved till further use at 4°C.

(iv) Amplification of target sequence by PCR:
For genotyping of HBV, the primers were used targeting S gene for amplification. Sequences were taken as already reported by Naito *et al*.[14] The primers and their annealing temperatures have been shown in Table 1.
In brief: 10 ρ moles each of the primers, 200 µ moles of each dNTPs, 1 U T*aq* polymerase and 1 × PCR buffer containing 1.5 mmoles $MgCl_2$ were added in a reaction volume of 50 µL. The template DNA was added in concentration 100 ng per reaction. The PCR was done for 32-40 cycles with temperature of 92°C for 20 s, corresponding annealing temperature 55˚C for 20 s and extension at 72°C for 1 min [Table 1]. Both, primary and nested PCR amplification was done as described above.

(v) Visualization of amplicon:
After amplification, 3 µL of gel loading buffer (0.1% bromophenol blue; 50% glycerol) was mixed with each amplified product, and 15 µL of the mixture was loaded onto a 1% agarose gel containing 0.5 µg ethidium bromide/µL in 1 x TBE buffer [40 mM tris-borate,1 mM EDTA (pH 8.0)] DNA ladders 100 bp and 1 kb (MBI, Fermentes) was used as a size marker. The gels were photographed under UV light, after electrophoresis, to record results and visually compare isolates to test for diversity within each isolate [Figure 2].

Statistical methods

Statistical analysis was done using Fisher's exact probability test.

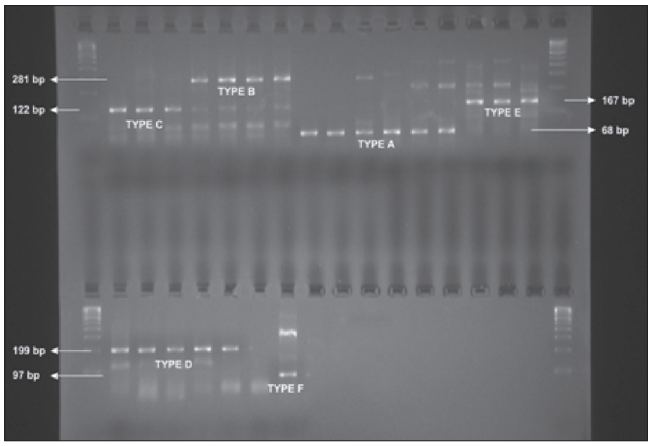

Figure 2: Electrophoresis patterns of PCR products from different HBV genotypes as determined by PCR genotyping system

Results

Of the 176 HBsAg-positive samples, HBV-DNA could be detected in 150. In the present study genotype-A was most prevalent (54%, 81/150) followed by genotype D, C, B, E and F with corresponding prevalence percentage 21.3, 10.0, 6.7, 6.0 and 2.0, respectively [Table 2]. All 176 HBsAg-positive samples had AST/ALT in normal range.

HBV genotype and Age

Among the 26 HBsAg-positive specimens [Table 2] belonging to the age group 21-30 yrs, majority were of genotype-A (42.3%, 11/26) followed by B (26.9%, 7/26). In an age group 31-40 yrs also genotype-A was most prevalent (64.3%, 63/98) followed by D (20.4%, 20/98). However, in age group 41-50 yrs, genotype-D was detected at the highest rate (46.2%, 12/26) followed by genotype-A (26.9%, 7/26).

Table 3 shows that age group 31-40 yrs of blood donors was found to have highest prevalence of genotype-A, D, E and F 78%, 63%, 67% and 67%, respectively. Genotype-B with positivity of 70% (7/10) was most prevalent in age group 21-30 yrs while with 40% (6/15) prevalence of genotype-C dominated age group 41-50 yrs.

HBV genotype and gender

Although numbers of females in the study were only 11 [Table 4], it was genotype-A which was most prevalent in males 57.6% (80/139) while in females genotype-B was most prevalent 63.6% (7/11).

HBV genotype and geographical distribution

In the present study based on type-specific primer (TSP) PSR, six of the eight genotypes were found in this part of country. Among them irrespective of gender and zonal distribution, overall, HBV genotype-A was commonest (54%) followed by genotype-D (21.3%) and C (10%); HBV genotype-B and E were least common. We did not find HBV genotype-G and F [Table 5].

Within Zone –I, genotype-A was most common (62.3%) followed by genotype-D (18.8%). Similarly, in Zone –II genotype–C (41.2%) were more frequent followed by genotype-A (17.7%)

Table 3: Age with distribution of HBV genotype (*n* = 150)

Age group	A *n* (%)	B *n* (%)	C *n* (%)	D *n* (%)	E *n* (%)	F *n* (%)
21-30 yrs	11 (13)	7 (70)	5 (33)	0	3 (33)	0
31-40 yrs	63 (78)	3 (30)	4 (27)	20 (62)	6 (67)	2 (67)
41-50 yrs	7 (9)	0	6 (40)	12 (38)	0	1 (33)
Total (150)	81	10	15	32	9	3

Table 4: Sex and HBV genotypes (*n* = 150)

Genotype	Male		Female	
	No.	%	No.	%
A	80	57.55	1	9.09
B	3	2.16	7	63.64
C	14	10.07	1	9.09
D	30	21.58	2	18.18
E	9	6.47	0	0
F	3	2.16	0	0
Total	139	100.00	11	100.00

Table 5: Geographical distribution of genotypes of HBV (n=150)

Place	Zone (no.)	Genotype					
		A No. (%)	B No. (%)	C No. (%)	D No. (%)	E No. (%)	F No. (%)
Varanasi Bhadohi* Mirzapur Jaunpur	I (69)	43 (62.3)	7 (10.1)	1 (1.5)	13 (18.8)	4 (5.8)	1 (1.5)
Azamgarh Mau Ghazipur Chandauli	II (34)	6 (17.7)	0	14 (41.2)	7 (20.6)	5 (14.7)	2 (5.9)
Buxar Kaimur Rohtas Aurangabad	III (33)	27 (81.8)	3 (9.1)	0	3 (9.1)	0	0
Gopalganj Bettiah†	IV (4)	4 (100)	0	0	0	0	0
Ballia Fatehpur Faizabad	Sporadic-A (7)	0	0	0	7 (100)	0	0
Gaya Bhabhua	Sporadic-B (3)	1	0	0	2	0	0
Total		81 (54.0)	10 (6.7)	15 (10.0)	32 (21.3)	9 (6.0)	3 (2.0)

*Bhadohi (Sant Ravidas Nagar); †Bettiah (Paschim Champaran); Statistical analysis: Genotype –A: Zone-I vs Zone-II (*P*: < 0.0001); Zone-I vs Zone-III (*P*: <0.07); Zone-I vs Sporadic-A (*P*: <0.0000001); Genotype-B: Zone-I vs Zone-II (*P*: < 0.59); Genotype-D: Zone-I vs Zone-II (*P*: < 0.51); Zone-I vs Zone-III (*P*: <0.17); Genotype-E: Zone-I vs Zone-II (*P*: < 0.13); Genotype-F: Zone-I vs Zone-II (*P*: < 0.26)

and genotype-E (14.7%). Zone –III in adjoining Bihar state close to Zone –I, genotype-A was more prevalent (81.8%) followed by B and C (9.1%). In Zone-IV, neighboring Zone- II, though small in number, had genotype-A (100%) only. Genotype –D had more sporadic distribution. Low prevalence of genotype-E and F were seen in Zone I and II (3/150, 2% overall).

Discussion

More than half of the HBV-positive specimens were of genotype-A while about a quarter (21.3%) were of genotype-D. The genotype-C was found to be only in one-tenth (10%) of the study population. Our finding are in good agreement with other studies reported from Western India.[15] In a study reported from North India[7] genotype-D was most common in patient suffering from chronic liver disease (CLD). In contrast to this, a study conducted in Arunanchal Pradesh, Eastern state of India, reported C as the most prevalent genotype.[16] Genotype-C has been reported to be commonest type (89.3%) followed by genotype-B (7.4%) from northern Thailand.[17] The above data indicates that distribution of various genotypes depends on host characteristics and possible human genetic relatedness which explains as to why genotype-C is more prevalent in eastern India and neighboring countries.

There are number of factors which are associated with disease progression in HBV, such as route of infection, age of acquiring the infection,[18] immune competence of the host and influence of environmental factors. Therefore, it is imperative to exercise due care when interpreting the role of HBV genotypes in disease progression. The majority of studies showing association of genotypes in disease progression are from South-east Asia where HBV infection itself is hyperendemic and there is preponderance of genotype-B and C. In these studies genotype-C is associated more with liver dysfunctions.[19-21] Thus lower prevalence of genotype-C is better for this region. Thakur *et al.*[7] prospectively studied the prevalence and clinical significance of HBV genotypes-A and D in north India; histologically proven patients with chronic HBV showed that genotype-D is associated more with severe liver disease and may predict the occurrence of HCC in younger patients. But since genotype-D is more prevalent in study region, and influence of other confounding factors cannot be ignored, caution must be exercised to label the specific genotype in causation of CLD. In another study by Sanchez-Tapias *et al.*[22] observed that the rate of HBsAg clearance was higher in genotype-A compared with genotype-D. This has great implications in blood screening for HBsAg. However carriers with this genotype along with others should be followed up to assess the real association with hepatic and nonhepatic complications. In the current study, followup period is too small and long-term followup of the patient might throw light on association of genotype in disease progression.

When different age groups were analyzed, the commonest HBV genotype in 21-30 yrs was genotype-A followed by B. The genotype-A was the commonest in age group 31-40 yrs also but second commonest was genotype-D. In contrast to above two, 41-50 yrs age group had commonest genotype-D followed by A. In a study from India,[7] genotype-D was associated with more severe liver disease and with hepatocellular carcinoma (HCC) in younger age group while another study[15] did not find any association. Although, this age distribution among HBV genotypes needs further exploration, probably genotype-related protective immunity might be the reason to contain certain genotype in a particular age group. Further, certain genotypes of HBV might be causing more genetic instability leading to development of HCC.

Further we tried to analyze the distribution of different genotype in various age groups. More than 75% of the genotype-A-positive HBV carriers belonged to age group 31-40 yrs. On the other hand genotype-B-positive blood donors mostly belonged to younger age group 21-30 yrs. Interestingly, genotype-C was found to be distributed, statistically compared, less in all three age groups. i.e., 21-30 yrs, 31-40 yrs and 41-50 yrs with positivity rate of 33%, 27% and 40% respectively. This distribution has indicated that

genotype-C behaves, probably in different manner in comparison to A and B genotype. It is really surprising to see that genotype-D, E and F were most prevalent in age group 31-40 yrs as we have observed with genotype-A. The reason and significance of such distribution is not known and needs to be studied further. Similarly higher prevalence of genotype-B in females and genotype-A in males is difficult to explain.

The exception of higher prevalence of genotype-C in zone-II as compared to other zones viz. I, III and IV is really extremely exciting. The cause and implications of this finding is very important and this will make us to understand the epidemiology of different HBV genotype in the community. People belonging to zone-II are apparently having genetic relatedness to other adjoining zones. But what other factors which are making genotype-C more prevalent in east and south East Asian countries needs to be explored. The pattern of genotype-A and D is almost same as present in west Asia and many European countries.

As 15% of isolates could not be genotyped by the method used, the prevalence of genotypes-G and F or their combinations cannot be ruled out. Further, TSP may miss out atypical, nonspecific forms. We did not find any coinfection of distinct genotype in any individual which was observed in another study.[23]

Conclusion

There is mounting evidence that HBV genotype may influence the natural history of the disease and predicting the response to various therapies. However, difference in host and environmental factors make it difficult to extrapolate the findings of one geographical region to another. Mutations, HBV and HIV coinfection has surfaced as challenge. In-depth studies on genotypic behavior of HBV are necessary to provide some solution. Most of the studies so far are cross-sectional, more longitudinal prospective studies could provide more information on the relationship of HBV genotypes to the severity of liver dysfunction and therefore net clinical outcome.

Screening the blood and donor tissue is one way to prevent the spread; another is adequate and effective vaccination program against HBV. Adequate screening of blood remains a problem in high endemic regions with available conventional ELISA method. Nucleic acid testing (NAT) are effective but current cost is prohibitive. Genotyping and NAT screening for HBV will definitely improve our understating to plane the appropriate medical strategies to treat, eradicate or confine HBV infection to low level.

Annexure-A

Extraction of DNA from blood samples

Serum sample (3 ml)
↓
Incubate with 1 mg (100 µl) of lysozyme at 37˚C for 60 min.
↓
1 ml 0.1% Triton-X 100 and 5 µl proteinase-K added and incubated at 65˚C for 120 min.
↓
to this equal volume of chloroform: Isoamyl alcohol (IAA) 24:1 dilution was added and mixed by vortexing for 15 s.
↓
Centrifuge at 10,000 rpm for 10 min.
↓
Aqueous phase was collected.
↓
140 µl of phenol: Choloroform: IAA (25:24:1)/ml of aqueous was added, vortexed for 15 s.
↓
Centrifuge at 10,000 rpm for 10 min.
↓
The aqueous phase was collected.
↓
Equal volume of phenol: Chloroform: IAA (25:24:1) was added and vortexed for 15 s.
↓
Centrifuge at 10,000 rpm for 10 min.
↓
Aqueous phase was collected.
↓
2 ml of RNAse (30µl/ml final concentration) was added.
↓
Incubated for 30 min at 37˚C
↓
Equal volume of isopropanol was added
↓
The solution was kept at room temperature for 5 min.
↓
The above was centrifuge at 10,000 rpm for 10 min and supernatant decanted
↓
The pellet (colorless, usually not evident visually) washed by 200 µl 70% ethanol.
↓
The above solution was centrifuge at 10,000 rpm for 10 min.
↓
Pellets were dried at 37°C (in inverted condition), for 30 min.
↓
The pellet was redissolved in 20 µl TE (pH 8)

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