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Original Article

Study on Evaluation of Alanine Aminotransferase (ALT) as Surrogate Marker in Hepatitis Virus Test

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Nucleic acid amplification test (NAT), which was introduced by the Japanese Red Cross Society in October 1999, began to be performed for screening of blood transfusion formulations in Japan in August 2014. In this study, the precision of immunological screenings of hepatitis B (HBsAg, HBcAb, and HBsAb), hepatitis C (HCVAb), and human immunodeficiency (HIVAb) virus antigens in donated blood were evaluated. In addition, the sensitivity of the alanine aminotransferase (ALT) test for detection of the hepatitis B and C viruses was re-evaluated.

Immunological screenings showed high precision of detecting the viral antigens. In contrast, the ALT test showed much lower precision of detecting the presence of the hepatitis B and C viruses.

Results of the NAT and immunological screenings revealed that ALT levels in donors were more strongly correlated with their levels of gamma-glutamyltranspeptidase (γGTP) and body mass index (BMI), than with the results of NAT and immunological screening. Our study indicates that elevated level(s) of ALT, were more likely to be associated with lifestyles factors such as high intake of alcohol or obesity than with infection. Therefore, ALT may be excluded as surrogate markers of HBV, HCV, and HIV in donated blood.

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I. Introduction

It is anticipated that the availability of blood transfusion products derived from donated blood may decrease with the advent of serious declines in the birth rate and an aging population. In this situation, it is necessary to effectively utilize valuable donated blood, while trying to achieve maximum safety of transfusion. The nucleic acid amplification test (NAT) was introduced by the Japanese Red Cross Society in October 1999, with a focus on improving the safety of blood products by shortening the window period in diagnosis of hepatitis B (HBV) and C (HCV), and human immunodeficiency (HIV) viruses in donated blood. The test also took into consideration lessons learned from the HIV-tainted blood scandal of the 1980s in Japan (by interview with Laboratory Management Division of the Japan Red Cross). Since August 2014, NAT has been performed on every blood product in Japan[1]. Immediately after the introduction of individual NAT, more accurate evaluations of immunological and alanine aminotransferase (ALT) test became possible for the first time.

In this study, the precision of immunological screening for the detections of hepatitis B (HBsAg, HBcAb, and HBsAb), hepatitis C (HCVAb), and HIV (HIVAb) antigens in donated blood, we compared their results with those results of NAT. In addition, the sensitivity of ALT test for the detection of hepatitis viruses was re-evaluated.

II. Objectives

Since the safety of donated blood and its products has been markedly improved by introduction of individual screening with NAT, this study aims to contribute to the existing knowledge on the safety criteria for blood products, by collecting and analyzing basic data on the effectiveness of current methods of blood screening.

In this study, the sensitivity of immunological screening of HBsAg, HBcAb, HBsAb, HCVAb, and HIVAb in donated blood was assessed. In addition, the rationality and effectiveness of testing for ALT were analyzed, by comparison with the results of NAT for each donor included in the study.

III. Methods

For 839,376 blood donations (Table 1.) made across Japan between August and September 2014 (the first 2 months immediately after the introduction of individual NAT), data such as hepatitis virus antibody values, ALT levels, and results of other biochemical examinations were extracted from the blood donor data uniform system of the Japanese Red Cross Society. Factors responsible for elevation of the ALT level were evaluated using statistical techniques. Furthermore, to assess the suitability of the ALT and other immunological tests for HBV, HCV, and HIV the screening of donated blood for infectious diseases was evaluated by comparison with the results of NAT for the same diseases in those donors.

Correlations among the immunological indicators and between the NAT and ALT test were analyzed by using the Receiver Operating Characteristic (ROC) curves, and multiple regression analyses by the forced-entry method. Factors correlated with elevated level of ALT were analyzed by multiple regression analysis by the step-wise method. SPSS Statistics 22 (IBM, Armonk, NY, U.S.A.) was used for analysis of the data.

Table1. Classification of Donor

	Total	Male	Female
Number	839376	592940	246436
Age	41.21±12.61	42.11±12.18	39.03±13.33
Height (cm)	167.29±8.05	170.99±5.80	158.39±5.28
Weight (kg)	65.11±11.50	69.11±10.20	55.48±8.32
BMI	23.18±3.45	23.62±3.41	22.12±3.30

[Ethical considerations]

This study was approved by the Research Ethics Committee of Tokyo Medical and Dental University, Faculty of Medicine, and the ethical review board of Research for Blood Program of the Japanese Red Cross Society.

IV. Results

The cut-off value for each indicator conformed to the current standard values used by the Japanese Red Cross Society.

1. Evaluation of the sensitivity and specificity of each indicator (Table2.)

i. Immunological testing for HBV antigens as surrogate markers of hepatitis B

First, for HBV antigens (HBsAg, HBcAb, and HBsAb), a concentration of 1.0mIU/mL or more was defined as positive. The efficacy of immunological screening of these antigens for HBV detection was evaluated by comparison with that of NAT. For these three antigens, the sensitivities of immunological screening were 0.8384, 0.9091, and 0.3990, the specificities were 0.9998, 0.9712, and 0.8984, the positive predictive value were 0.4573, 0.007403, and 0.0009256, and the negative predictive value were 0.99996, 0.99998, and 0.99984, respectively.

Next, we re-evaluated the efficacy of immunological screening when HBV positivity was defined as either “HBsAg of 1.0mIU/mL or more” or “HBcAb of 1.0mIU/mL or more and less than HBsAb 200mIU/mL. Based on these definitions, the efficacy of combined immunological screening of HBsAg, HBcAb, and HBsAb in combination was evaluated. The sensitivity, specificity, positive predictive value, and negative predictive value were 0.9293, 0.9957, 0.05063, and 0.9998, respectively. The sensitivity, specificity, and negative predictive value were superior to those of the immunological screening of HBsAg alone.

In addition, we compared the ALT levels of HBV-NAT-positive/HBsAg-negative and HBV-NAT-negative/HBsAg-positive cases by performing the independent *t* test. P-value of Levene’s test is 0.384 (more than 0.05, significant level), and it is assumed that population variances are equal. P-value of t-test for Equality of Means is 0.19. This result revealed no significant differences between ALT levels in the two groups (Table 3).

ii. Immunological testing for HCVAb as a surrogate marker of hepatitis C

An HCVAb concentration of 1.0mIU/mL or more was defined as positive and the efficacy of immunological screening of this antigen was evaluated by comparison with that of NAT. The sensitivity, specificity, positive predictive value, and negative predictive value were 1.000, 0.9998, 0.320513, and 1.000, respectively. These results indicated that immunological screening of HCVAb was extremely effective and could be performed safely without additional testing for HCV infection. There were no HCV-NAT- negative/HCVAb-positive cases.

iii. Immunological testing for HIVAb as a surrogate marker of HIV

An HIVAb concentration of 1.0mIU/mL or more was defined as positive and the efficacy of immunological screening of this antigen was evaluated by comparison with that of NAT. The sensitivity, specificity, positive predictive value, and negative predictive value were 1.000, 0.9999, 0.01861, and 1.000, respectively. These results indicated that immunological screening for HIVAb was extremely effective and could be performed safely without additional testing for HIV infection. There were no HIV-NAT-negative/HIVAb-positive case.

iv. Elevated ALT levels as surrogate marker of hepatitis B and C

The Japanese Red Cross Society specifies that donated blood with an ALT level of 61 IU/L or more cannot be used for the formulation of blood transfusion products. Hence, in this study, an ALT level of 61 IU/L or more was defined as positive, and the efficacy of the ALT test for of HBV and HCV was evaluated by comparison with that of NAT for these viruses (HBV-NAT and HCV-NAT, respectively). For HBV, the sensitivity, specificity,

Table2. Sensitivity and specificity of the immunological screenings

HBsAg	HBV-NAT		
	positive	negative	total
positive	166	197	363
negative	32	838845	838877
total	198	839042	839240

HCVAb	HCV-NAT		
	positive	negative	Total
positive	75	159	234
negative	0	839006	839006
total	75	839165	839240

HIVAb	HIV-NAT		
	positive	negative	total
positive	8	422	430
negative	0	838810	838810
total	8	839232	839240

HBsAg	Sensitivity	0.8384
	Specificity	0.9998
	Positive likelihood ratio	0.4573
	Negative likelihood ratio	1.0000

HCVAb	Sensitivity	1.0000
	Specificity	0.9998
	Positive likelihood ratio	0.3205
	Negative likelihood ratio	1.0000

HIVAb	Sensitivity	1.0000
	Specificity	0.9995
	Positive likelihood ratio	0.0186
	Negative likelihood ratio	1.0000

Table3. Independent Samples (Comparison with the ALT levels of Test HBV-NAT-positive/HBsAg-negative and HBV-NAT-negative/HBsAg-positive)

	Frequency	Mean	Standard Deviation	Standard Error
HBV-NAT-positive/HBsAg-negative group	31	24.87	14.532	2.61
HBV-NAT-negative/HBsAg-positive group	197	21.42	13.639	0.972

One sample of HBV-NAT-positive/HBsAg-negative group has been excluded because of the decifit of ALT value.

	Levene's Test for Equality of Variances		*t*-test for Equality of Means				
	F value (test statistic)	Significance Probability	t value (test statistic)	Degrees of freedom	Significance Probability (2-tailed)	Mean Difference	Standard Error Difference
Equal variances assumed	0.760	**0.384**	1.299	226.000	**0.195**	3.455	2.659
Equal variances not assumed	—	—	1.240	38.779	0.222	3.455	2.785

positive predictive value, and negative predictive value of the ALT test were 0.02052, 0.977962, 0.032667, and 0.964948, respectively. For HCV, these values were 0.095368, 0.978031, 0.001821, and 0.999597, respectively. The sensitivity and positive predictive value of the ALT test were extremely low, which implied highly probability of overlooking an infected person (i.e., a false-negative result).

2. Evaluation with Receiver Operating Characteristic (ROC) curves (Figure 1.)

i. Evaluation of the immunological tests with ROC curves

In this analysis, the Areas Under the Curves (AUCs) of HBsAg, HBcAb, and HBsAb for HBV were 0.927, 0.972, and 0.677, respectively. These results showed that the tests for HBsAg and HBcAb were high-precision, while that for HBsAb was low-precision. The AUC of HCVAb for HCV was 1.000, while that of HIVAb for HIV was 1.000, which showed that both tests were extremely high-precision.

ii. Evaluation of the ALT test with ROC curves

In this analysis, the AUCs of ALT for HBV and HCV (as assessed by comparison with the results of NAT) were 0.629 and 0.864, respectively. These results showed that the ALT test was low-precision for the detection of both HBV and HCV.

3. Evaluation with multiple regression analysis

A multiple regression analysis with the forced entry method was conducted for the results of NAT for HBV, HCV, and HIV (HIV-NAT) as dependent variables (the value for NAT-positive was set to 1 and that for NAT-negative was set to 0). Correlations among the immunological indicators and between the NAT and ALT test were analyzed.

i. Multiple regression analysis with HBV-NAT as a dependent variable (Table 4.)

The adjusted R^2 value (adjusted coefficient of determination) was 0.663, and the standardized coefficients for determination of HBsAg, HBcAb, HBsAb, and ALT were 0.806, 0.046, –0.013, and 0.000, respectively. These results indicated that elevation of HBsAg levels was strongly correlated with HBV-NAT-positivity. In contrast, elevation of ALT levels showed almost no correlation with HBV-NAT positivity.

F

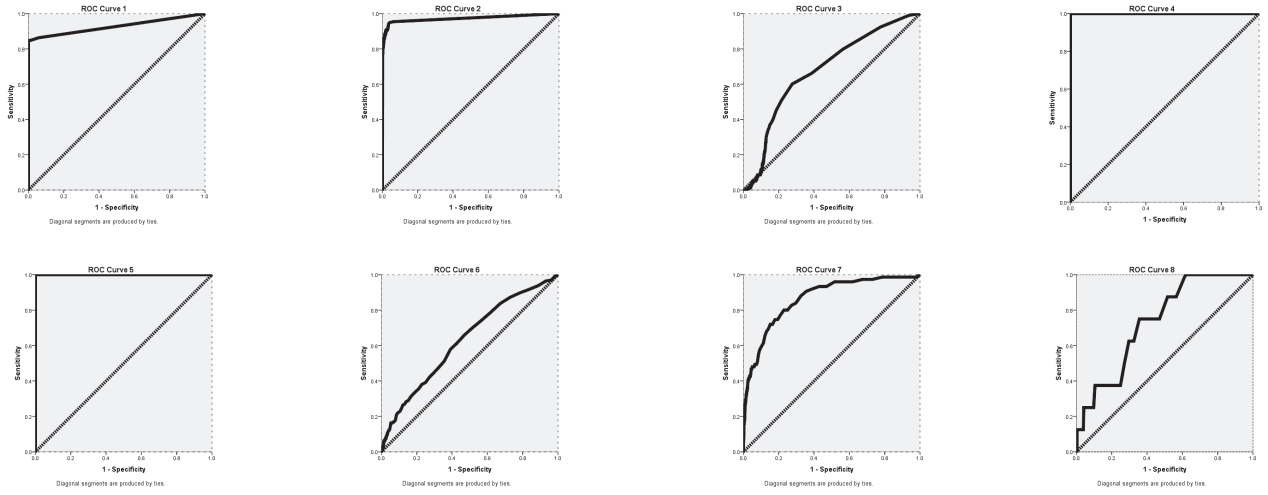

Figure 1. ROC Curves

Curve 1.	Result Variable: HBsAg	State Variable: HBV-NAT	(AUC=0.927)
Curve 2.	Result Variable: HBcAb	State Variable: HBV-NAT	(AUC=0.972)
Curve 3.	Result Variable: HBsAb	State Variable: HBV-NAT	(AUC=0.677)
Curve 4.	Result Variable: HCVAb	State Variable: HCV-NAT	(AUC=1.000)
Curve 5.	Result Variable: HIVAb	State Variable: HIV-NAT	(AUC=1.000)
Curve 6.	Result Variable: ALT	State Variable: HBV-NAT	(AUC=0.629)
Curve 7.	Result Variable: ALT	State Variable: HCV-NAT	(AUC=0.864)
Curve 8.	Result Variable: ALT	State Variable: HIV-NAT	(AUC=0.735)

ii. Multiple regression analysis with HCV-NAT as a dependent variable (Table 4.)

The adjusted R^2 value was 0.663, and the standardized coefficients for determination of HCVAb and ALT were 0.920 and 0.002, respectively. These results indicates that elevation of HCVAb levels was strongly correlated with HCV-NAT-positivity. On the other hand, elevated level of ALT showed almost no correlation with HCV-NAT-positivity.

iii. Multiple regression analysis with HIV-NAT as a dependent variable (Table 4.)

The adjusted R^2 value was 0.175, and the standardized coefficients for determination of HIVAb and ALT were 0.418 and 0.000, respectively. It was found that elevation of HIVAb levels was strongly correlated with HIV-NAT-positivity. On the other hand, elevated levels of ALT showed almost no correlation with HIV-NAT positivity.

4. Multiple regression analysis for factors correlated with elevated levels of ALT (Table 5.)

The correlations between ALT levels and the following 17 factors were analyzed: height, body weight, body mass index (BMI), gamma-glutamyltranspeptidase (γGTP), total protein, albumin, albumin-globulin ratio, total cholesterol, glycoalbumin, HBsAg, HBcAb, HBsAb, HCVAb, HIVAb, HBV-NAT, HCV-NAT, and HIV-NAT. Based on multiple regression analysis by the step-wise method, 12 independent variables revealing high collinearity were excluded. The correlation of ALT levels with the

Table4. Standardized Coefficients (multiple regression analyses with the forced entry method)

		Unstandardized Coefficients		Standardized Coefficients	test statistic Probability	Significance
		B	Standard Error	Beta		
Dependent Variable: HBV-NAT	(Constant)	0	0	—	–8.73	0
	HBsAg	0.001	0	0.806	1252.723	0
	HBcAb	0	0	0.046	68.554	0
	HBsAb	0	0	–0.013	–19.09	0
	ALT	0	0	0	0.465	0.642
Dependent Variable: HCV-NAT	(Constant)	–0.002	0	—	–247.447	0
	HCVAb	0.014	0	0.92	2151.055	0
	ALT	0	0	0.002	4.069	0
Dependent Variable: HIV-NAT	(Constant)	–0.001	0	—	–249.974	0
	HIVAb	0.012	0	0.418	421.787	0
	ALT	0	0	0	0.007	0.994

Table5. Standardized Coefficients (Multiple regression analysis by the step-wise method, Dependent Variable: ALT)

	Unstandardized Coefficients		Standardized Coefficients	test statistic	Significance Probability	95.0% Confidence Interval for B		Collinearity Statistics	
	B	Standard Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
(Constant)	–11.112	0.121	—	–91.729	0	–11.35	–10.875	—	—
γ-GTP	0.15	0	0.35	352.934	0	0.149	0.151	0.967	1.034
BMI	1.189	0.005	0.225	226.785	0	1.179	1.2	0.967	1.034
HCVAb	0.525	0.029	0.018	18.387	0	0.469	0.581	1	1
HBsAg	0.006	0.001	0.008	8.413	0	0.005	0.008	0.973	1.028
HBcAb	–0.039	0.006	–0.006	–6.297	0	–0.051	–0.027	0.973	1.028

remaining five factors was examined. The standardized coefficients of γGTP, BMI, HCVAb, HBsAg, and HBcAb were 0.350, 0.225, 0.018, 0.018, 0.008, and −0.006, respectively. These results indicated that the correlation of ALT levels with γGTP and BMI was relatively strong among the 17 factors assessed in this study.

V. Discussion

In this study, we evaluated the precision of immunological screenings of hepatitis B (HBsAg, HBcAb, and HBsAb), hepatitis C (HCVAb), and human immunodeficiency (HIVAb) virus antigens in donated blood. In addition, we also re-evaluated the sensitivity of the alanine aminotransferase (ALT) test for detection of the hepatitis B and C viruses.

According to previous reports from countries other than Japan, elevated levels of ALT were not observed in some patients despite being diagnosed with hepatitis B and/or C[2, 3, 4, 5, 6]. Our results support these previous reports. Further in our study, the ALT levels were more strongly correlated with the γGTP levels and BMI than with the indicators of viral infection. Similar findings were demonstrated in previous studies in other countries[7, 8, 9]. The investigation in 2013 revealed that economic losses in 2012 in Japan reached 3 billion yen because transfusion formulations could not be prepared due to high levels of ALT in donor blood[10]. The above mentioned findings suggest that it is desirable to exclude ALT from the indicators of screening for donated blood.

For the future, it is recommended that the current immunological tests be continued as basic screening and that beneficiaries be protected by introducing higher-precision laboratory procedures, securing the safety of donated blood by pathogen reduction technologies, and ensuring the delivery of a no-fault compensation system. These points are discussed below.

1. Introduction of laboratory procedures with higher precision

The "window period" from the establishment of infection until the viral antigens reach a concentration sufficient to be detected in blood still exists, though it has been shortened by the introduction of NAT in October 1999[11]. Besides, the risk of infection due to other unscreened viruses, protozoa, parasitic worms, and bacteria also remains[12, 13, 14].

In particular, hepatitis E virus (HEV) is a transfusion-transmitted infectious disease, which is currently being addressed in Japan and the Western countries[15, 16, 17]. There are several reports on elevated levels of ALT in some patients who were infected with HEV. However, a correlation between HEV infection and elevated levels of ALT has never been definitively shown[18, 19, 20, 21]. Currently, the diagnosis of HEV is based on immunological tests and NAT[22].

The introduction of a test that is more economical and has a wider capture range is considered necessary in the future.

2. Securing the safety of donated blood by pathogen reduction technologies

Pathogen reduction technology can be used to enhance the protection of transfusion recipients against infection via blood donated during the window period for HBV, HCV, and HIV and against unscreened infections such as other viruses, bacteria, and protozoa[23]. This technology involves the addition of special compounds, which are activated by ultraviolet rays and inhibit replication of nucleic acids (DNA and RNA), to blood products for transfusion. Through this method, pathogens containing nucleic acids, such as virus, bacteria, and protozoa, can be inactivated simultaneously[24, 25, 26]. In addition, since these compounds inhibits the function of nuclei-containing white blood cells, they are effective in preventing post-transfusion graft-versus-host disease (GVHD)[27, 28].

However, there are limitations to pathogen-reduction technologies. For some pathogens, inactivation is either in effective or poorly responsive. These pathogens include prions, hepatitis A virus, parvovirus B19, and transfusion-transmitted virus. Screening for these pathogens via interview with the donors should be performed routinely.

Further, we cannot ignore the loss in quality and functional decline of blood products associated with pathogen-reduction technologies. Since pathogen-reduction technologies were first introduced, the possibility of side effects, which seem to be due to impaired activity of the congealing fibrinogenolysis system, has been noted. Similarly, in recent studies, low activity of various coagulation factors and impaired platelet function have been reported[29, 30, 31]. It is necessary to establish standard values to define the clinically permitted range for activity of the factors associated with hemostasis, coagulation, and fibrinolysis.

3. Protection of beneficiaries by no-fault compensation system

It is necessary to provide a safety net to protect transfusion recipients who contract transfusion-related illnesses despite the safety system provided by improved

precision of test methods and pathogen-reduction technologies.

A typical example of such a safety net is the Accident Compensation Corporation (ACC), which was founded in New Zealand for the first time in the world in 1972. The ACC provides insurance coverage for injuries caused by accidents in New Zealand. The coverage is guaranteed regardless of the cause of the accident, currently it is an independent system for personal injury by medical misadventure[32].

In the medical care system in Japan, a no-fault compensation system has been established in the field of obstetrics. Following the suggestion of founding a no-fault security system for children with cerebral palsy in 2004 by the Fukuoka Medical Association, the "obstetric medical compensation system" was established in January 2009, and has since achieved constant results for beneficiary protection[33, 34].

VI. Conclusion

This study revealed that immunological screening for HBsAg, HBcAb, HBsAb, HCVAb, and HIVAb has high sensitivity for the detection of infections with HBV, HBC, and HIV. It also revealed that degree of precision of screening for these viruses by the ALT test was extremely small. In addition, elevated levels of ALT showed a relatively stronger correlation with γGTP levels and BMI, than with the results of the immunological tests. According to our analysis, metabolic disorders associated with lifestyle factors, such as obesity or excessive alcohol consumption, rather than infection, are more likely to contribute to elevated level(s) of ALT. ALT may be excluded as surrogate markers of HBV, HCV, and HIV. On the other hand, metabolic abnormalities indicated by the elevation of ALT levels might be continued to be screened, and the harmful influences on blood products should be surveyed.

Finally, an ideal future scenario for blood projects in Japan to secure the benefit and safety of transfusion product recipients would be the continued NAT and immunological screening while also utilizing pathogen reduction technologies and establishing a no-fault compensation system.

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