

Evaluation of the Staphylococcus Protein A Absorption
Method for Detecting Anti-Hepatitis
A Virus Immuno-Globulin M

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OBJECTIVE : To evaluate the Staphylococcus Protein A absorption modification of the HAVAB (R) solid phase competition radioimmunoassay as a method for detecting anti-HAV IgM.

BACKGROUND : Establishing a laboratory diagnosis of acute infection with the hepatitis A virus is difficult. In most patients, viral excretion in the stool has ceased before the appearance of jaundice, and frequently the serum antibody titer has approached peak levels by the time the patient seeks medical care, so that demonstration of an unambiguous rise in titer may not be possible (1). Research efforts have therefore been directed towards developing assays to detect IgM antibodies with anti-HAV activity as presumptive evidence of acute infection.

At present the only widely available assay for anti-hepatitis A virus antibody is a commercial solid phase radioimmunoassay (SPRIA) based on the competition of the patient's antibody with labelled anti-HAV (competitive binding assay, CBA). Three basic methods have been proposed for pretreating sera to detect evidence of specific acute phase antibodies using the CBA method :

1. Absorb the sera with staphylococcus aureus protein A (SA absorption) to remove IgG antibodies and demonstrate residual non-absorbable activity in the absorbed sera (2).

2. Treat the whole serum with 2-mercaptoethanol (2-ME) to inactivate IgM and demonstrate a decrease in activity (3-4).

3. Fractionate the serum by sucrose density gradient ultra-centrifugation and assay the activity of the 19S and 7S fractions (5). In this study we compared the SA absorption method to the other two methods for detecting diagnostic acute phase anti-HAV antibodies in serum specimens from patients with acute hepatitis.

METHODS

Screening SPRIA on undiluted serum : Undiluted sera were screened using the HAVAB (R) as directed by the manufacturer without modification. Greater than 50% blocking of counts was taken as evidence for specific anti-HAV antibodies in undiluted sera.

Serum specimens : Sixteen acute phase serum specimens (obtained within six weeks of the onset of symptoms) were selected from an acute hepatitis study serum bank. All serum bank specimens had previously been assayed for HBsAg (AUSRIA-II (R) Abbott Laboratories) anti-HBs (AUSAB, (R), Abbott Laboratories) total anti-HAV activity, and anti-HAV activity before and after SA absorption.

Two sera lacking anti-HAV activity by screening CBA were selected as negative controls. Fourteen sera with anti-HAV in unabsorbed sera formed the study battery: in seven the anti-HAV could be absorbed out by SA absorption (Group A) while in seven others the anti-HAV activity was not removed by SA absorption (Group N). The age and sex of the patient, day of illness, HBsAg status, % blocking with undiluted sera, and % blocking before and after absorption of a 1:15 dilution of the specimen, are presented in Table 1.

Staphylococcal protein A absorption of sera : A strain of *S. aureus* was kindly provided by Dr. Savanat Tharavanij at the Faculty of Tropical Medicine, Mahidol University. The staphylococci were grown for 24 hours in 2 litre volumes of trypticase soy broth. While bacteria were prepared for absorption of sera by heat inactivation and formaldehyde treatment according to the method of Kessler (6). Before absorption, all sera were diluted 1:15 in PBS pH 7.4 regardless of the percent blocking activity in the screening CBA. Absorption of an aliquot of the 1:15 dilution of serum was performed according to the method of Bradley et al. (2), except that sera were absorbed twice. Unabsorbed and absorbed aliquots were tested for percent blocking activity by the CBA method.

Sucrose density gradient fractionation of sera : Thawed serum specimens were clarified by filtration through millipore 0.45 micro filters. A 0.1 ml specimen was layered on top of a 10-40% gradient of sucrose in a 5.0 ml ultracentrifuge tube and centrifuged for 18 hours in a SW50 rotor in a Beckman L-5 ultracentrifuge at 35,000 rpm. Five drop fractions were collected by piercing the bottom of the tube. Fractions were tested by immunodiffusion against anti-human immunoglobulin (Antibodies Incorporated). IgM was always confined to fractions 2, 3 and 4 and these were pooled to form the "19S" fraction. IgG and IgA were confined to fractions 6, 7, 8, and 9 in all tubes; these fractions were pooled to form the "7S" fraction. "19S" and "7S" fractions from each serum were dialyzed against PBS pH 7.5 for 24 hours, then brought to 1.5 ml total volume with PBS so that each immunoglobulin was at a 1:15 dilution compared to the original serum concentration.

SA absorption of unfractionated serum and 19S and 7S fractions : Aliquots of a 1:15 dilution of whole serum in PBS and the 1:15 dilutions of 19S and 7S protein were absorbed twice with staphylococci. Absorbed and unabsorbed aliquots were tested for blocking activity in the CBA.

2-ME treatment of unfractionated serum and 19S and 7S fractions : To 0.2 ml aliquots of 1:15 dilutions of serum, 19S antibody and 7S antibody, 14 microliters of a 10% dilution of 2-mercaptoethanol in distilled water (wt/wt) were added, to give a final concentration of 0.1 molar 2-ME. After mixing and incubating for 1 hour, the treated and untreated aliquots were assayed for CBA blocking activity. No attempt was made to dialyze away excess 2-ME; we assumed that the 1:20 dilution of the specimen in the first step of the

HAVAB (R) assay would adequately dilute the 2-ME.

Diagrammatic summary of protocol : Figure 1 is a diagrammatic summary of the protocol.

Statistical analysis of data : P values were assigned to differences in sample means between untreated and treated serum and serum fraction groups using student's t test for paired observations (7).

RESULTS : Results are presented in tabular form in Table 2 and in graphic form in Figure 2.

In describing the staph absorption technique for detecting acute phase anti-HAV antibodies, Bradley et al. (2) expressed their results as the ratio of (raw CPM absorbed/raw CPM unabsorbed). With unfractionated sera they found a ratio <2.0 only in acute sera and not in convalescent specimens. However, to achieve consistent results each serum specimen required variable and unique dilution before absorption.

We believe that this variable dilution step was unnecessary. By calculating the results as :

$$\frac{(\% \text{ inhib absorbed})}{(\% \text{ inhib unabsorbed})} = \frac{\frac{(\text{CPMNC-CPM test absorbed})}{(\text{CPMNC-CPM PC})}}{\frac{(\text{CPMNC-CPM test unabsorbed})}{(\text{CPMNC-CPM PC})}}$$

We found that we could follow a set procedure, including a fixed pre-absorption dilution, and still clearly separate IgM positive and IgM negative sera.

Sera selected on the basis of non-absorbability of activity (Group N, inhibition ratio ≥ 0.5) fractionated into 7S and 19S fractions with $64 \pm 18\%$ and $62 \pm 18\%$ respectively, of the blocking activity of whole serum, while sera with absorbable activity (Group A, inhibition ratio < 0.5) fractionated into 7S and 19S fractions with $64 \pm 20\%$ and $17 \pm 17\%$ respectively. That the 19S activity in the Group N sera was due to IgM was proven by its sensitivity to treatment with 2-ME; 2-ME treatment had no effect on the 7S fractions.

In agreement with the results by Pastore et al. (3) and Girardet et al (4), treatment of whole serum with 2-ME produced a decrease in activity of specimens in group N but not of those in group A. However, the magnitude of the decrease in the activity of group N sera produced by 2-ME treatment ($27 \pm 10\%$) was less than that produced by staph absorption of group A sera ($84 \pm 12\%$).

We initially thought that most of the activity in the group N sera not inactivated by 2-ME was probably IgG; however, considerable activity remained in the 7S fraction of the group N sera even after SA absorption, while SA absorption of the 7S fraction of group A sera almost completely removed activity ($36 \pm 18\%$ removed vs $81 \pm 24\%$ removed). As determined by immunodiffusion; both IgG and IgA were present in our 7S fractions; the non-absorbable

7S activity may be due to either IgA or to a high concentrations of non-absorbable IgG (?IgG₃) anti-HAV in acute sera from patients with HAV virus infections.

Using the staph A absorption method with a fixed dilution of serum before absorption, we have now examined samples of anti-HAV positive blood from over 200 patients with acute hepatitis. A histogram of the distribution of patients according to the ratio (% inhibition absorbed/% inhibition unabsorbed) is presented in Figure 3. The distribution is clearly bimodal adding further proof that the SA absorption modification of the CBA does crisply differentiate between sera with acute versus chronic anti-HAV activity.

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Table 1. Anti-HAV Antibody Content of Group N and Group A Sera.

Serum#	pt. age	Sex	Day of illness serum obtained	HBsAg	% blocking undiluted	1:15 serum dilution:% blocking		Abs/unabs activity ratio
						Unabsorbed	Absorbed	
63064	5	F	6	+	98%	74%	66%	.89
64037	6	M	43	-	96%	82%	70%	.86
63960	10	F	"Acute"	-	93%	73%	60%	.82
64024	3	M	2	-	90%	66%	62%	.94
63078	17	F	5	-	98%	79%	63%	.80
62959	10	F	2	-	90%	64%	50%	.78
64036	21	M	4	-	ND	73%	67%	.91
64053	15	F	31	-	97%	75%	-03%	-04
63062	6	F	2	-	87%	63%	07%	.11
63114	18	M	21	-	99%	75%	32%	.43
63080	23	F	30	-	98%	73%	00%	.00
63162	27	M	3	-	ND	97%	07%	.10
63233	21	M	2	-	ND	72%	04%	.06
64004	21	M	11	+	98%	77%	08%	.11

Table 2. Percent Inhibition by Serum and Serum Fractions in HAVAB (R)

$$(\% \text{ inhibition} = \frac{\bar{XNC} - \text{Test}}{\bar{XNC} - \bar{XPC}})^a$$

	Serum #	Unfractionated Serum			7S Fraction			19S Fraction		
		UN	SA	ME	UN	SA	ME	UN	SA	ME
Group N	63064	61	52	48	39	36	34	58	41	32
	64037	80	71	65	66	40	54	36	42	18
	63960	73	57	43	44	31	37	50	37	21
	64024	64	57	41	31	21	26	46	38	14
	62959	68	58	45	25	12	28	28	24	24
	64036	79	68	58	53	37	40	48	39	12
Group A	64053	77	16	73	68	08	73	00	6	24
	63062	57	06	35	22	00	32	14	21	34
	63114	84	31	82	62	13	62	04	06	11
	63080	59	05	62	32	-11	39	15	08	20
	63162	35	06	43	28	-21	25	16	02	06
	63233	46	12	50	18	07	11	07	04	18
	64004	52	-09	56	37	23	39	-04	00	05
Controls	63247	-02	05	22	17	12	04	-02	03	21
	63285	12	29	10	16	03	16	08	10	23

^a \bar{XNC} = mean negative control counts per minute.

\bar{XPC} = mean positive control counts per minute.

Test = mean counts per minute in test using specified serum fraction.

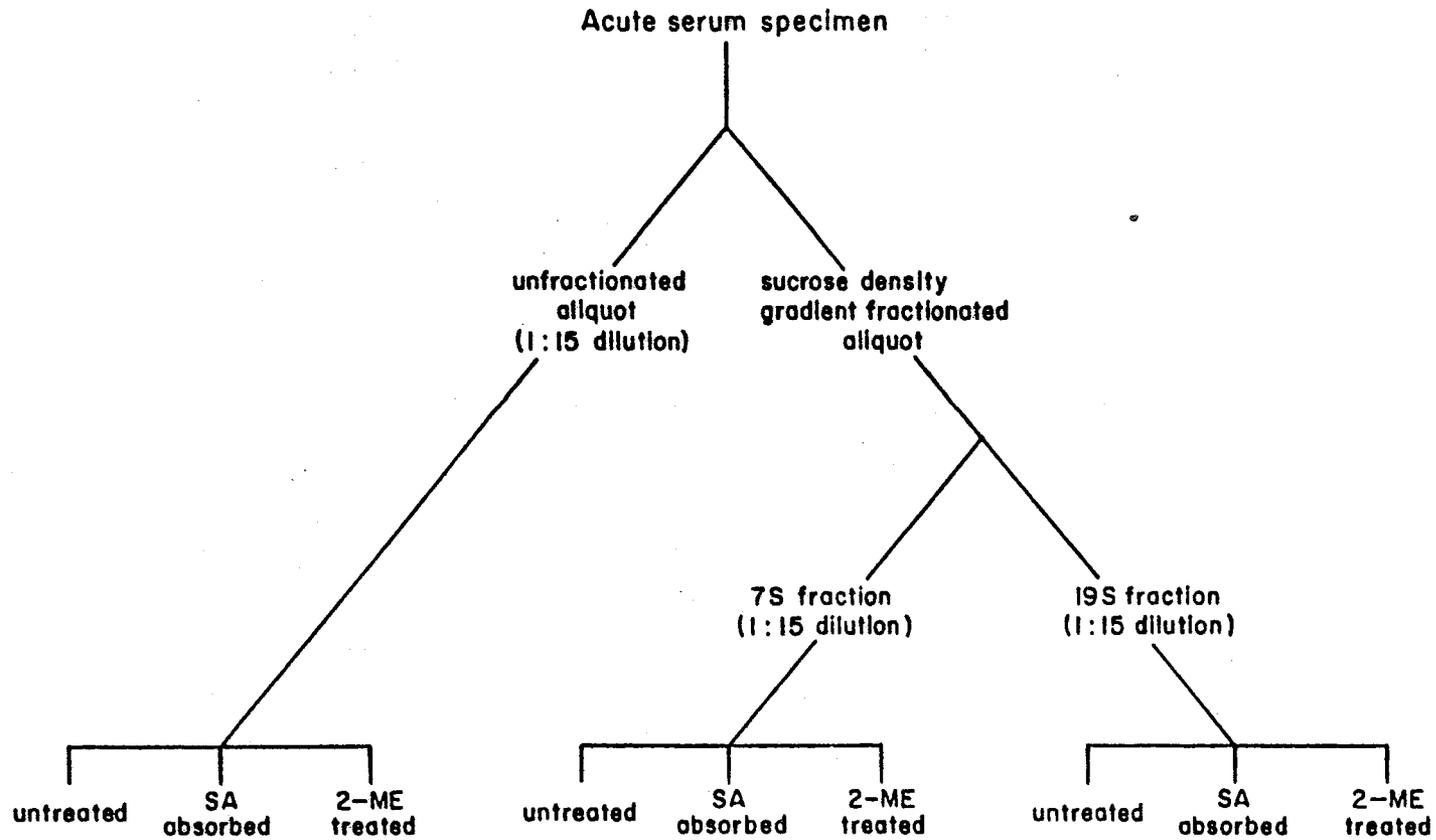


Figure 1. Diagrammatic summary of serum fractionation and treatment of serum fractions. SA absorbed = staphylococcus aureus absorbed; 2-ME treated = 2-mercaptoethanol treated. See text for details.

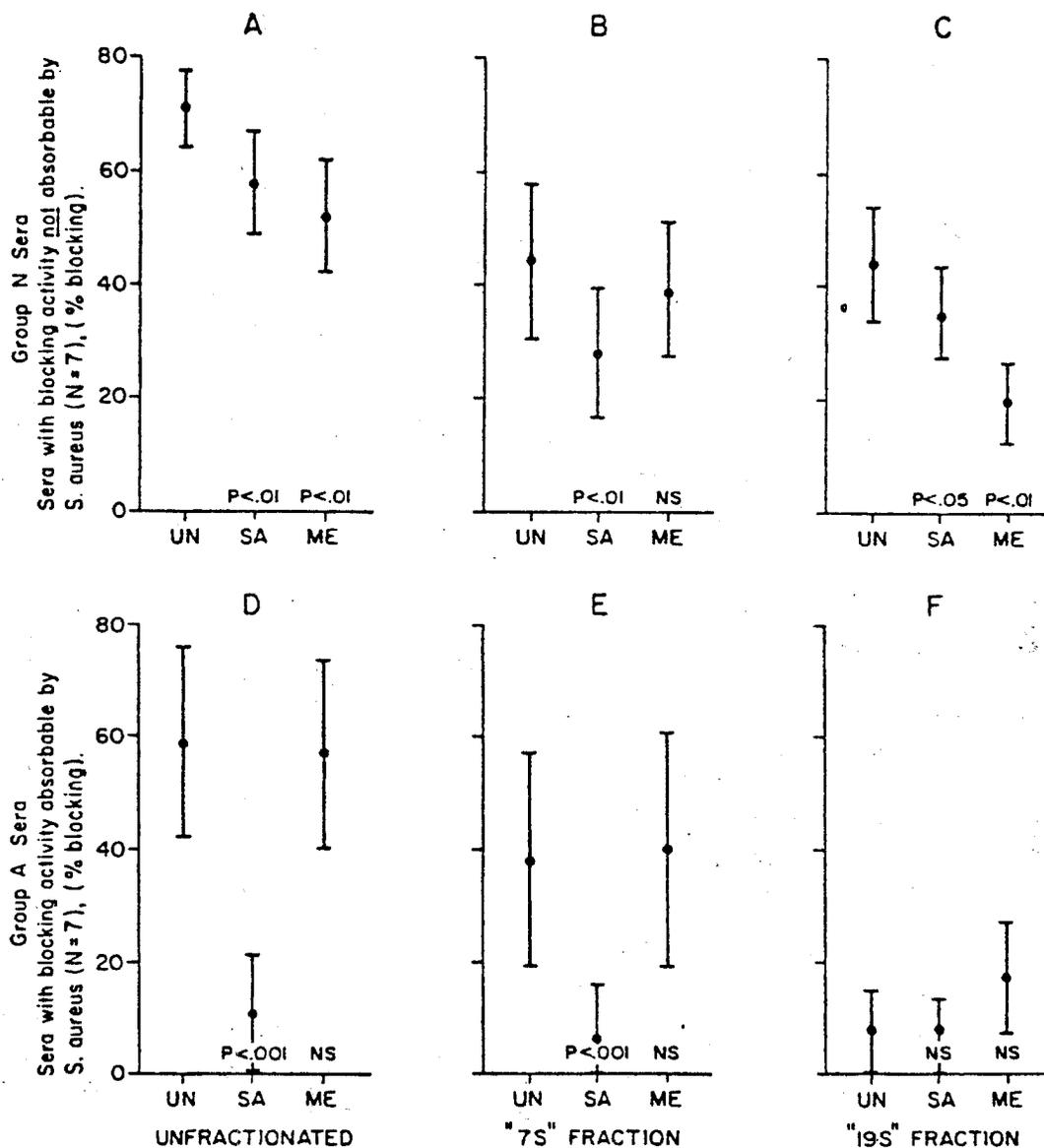


Figure 2. Graph of activity (% blocking) of unfractionated sera, 7S fractions, and 19S fractions from sera with SA absorbable activity (Group A) versus sera with activity not removed by SA absorption (Group N) A. Group N, unfractionated sera. B. Group N, 7S fraction. C. Group N, 19S fraction. D. Group A, unfractionated sera. E. Group A, 7S fraction. F. Group A, 19S fraction. UN = untreated; SA = absorbed with staphylococcus aureus; ME = treated with 2-mercaptoethanol. Mean +/- one standard deviation graphed. P values calculated for difference between treated specimens (either SA or ME treated) and untreated specimens.

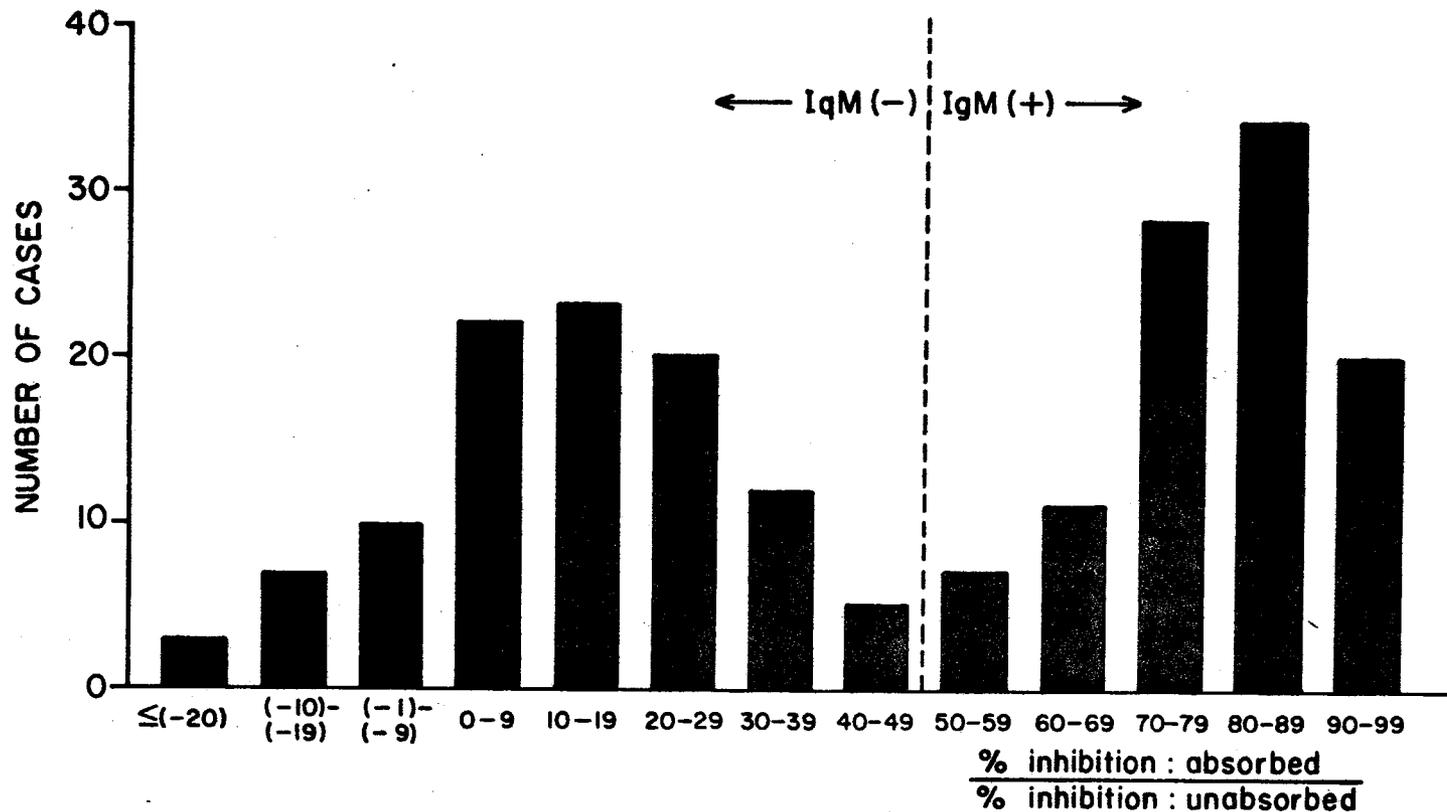


Figure 3. Histogram of distribution of acute sera according to the proportion of blocking activity remaining after SA absorption. Negative ratios denote sera in which the CPM in absorbed sera were greater than in negative control sera.

$$\frac{\% \text{ inhibition: absorbed}}{\% \text{ inhibition: unabsorbed}} = \frac{\frac{(\text{CPM:NC} - \text{CPM: test absorbed})}{(\text{CPM:NC} - \text{CPM: PC})}}{\frac{(\text{CPM:NC} - \text{CPM: test unabsorbed})}{(\text{CPM:NC} - \text{CPM: PC})}}$$

Where CPM = counts per minute; CPM:NC = CPM with negative control sera;
 CPM:PC = CPM with positive control sera.