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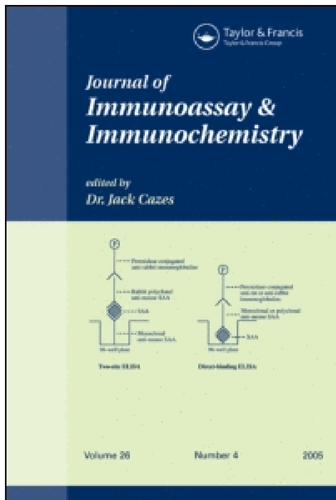

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Reverse Passive Hemagglutination Tests for Rapid Diagnosis of Snake Envenomation

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REVERSE PASSIVE HEMAGGLUTINATION TESTS FOR RAPID DIAGNOSIS OF SNAKE ENVENOMATION

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ABSTRACT

Reverse passive hemagglutination (RPHA) tests for the detection of six major poisonous snake venoms of Thailand were studied. Three different species of red blood cells i.e., sheep (SRBC), human (HRBC) and chicken (CRBC) were sensitized with protein A-affinity purified rabbit antivenom IgG using chromic chloride as a coupling reagent. The properties of these sensitized erythrocytes with regard to sensitivity, specificity, stability to venom enzymes and storage etc., were studied and compared. The sensitivities of the RPHA tests in venom detection were 2 to 635 ng/ml. Cross-reactions were observed with heterologous venoms at concentrations at least 62 times higher than those observed with homologous venoms. After treatment with glutaraldehyde, the coupled red blood cells showed reduced

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sensitivity but were stable at 4°C from 1 to 12 months depending upon the antibody and the species of erythrocytes. The entire test required 60 to 120 min. The RPHA using fresh SRBC correctly identified various venoms in 48 of 59 (81.3%) serum samples and 16 of 26 (61.5%) wound swabs. Venom mis-identifications were made in 2 sera (3.4%). In a comparison of 24 paired serum and wound swab samples, more positive identifications were made with serum than with swab samples but the difference was not statistically significant ($p > 0.05$).

(KEY WORDS: Immunodiagnosis, hemagglutination, snake venoms)

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INTRODUCTION

Therapeutic antivenoms against poisonous snakes consist of specific monovalent horse sera. These antivenoms are effective only against venoms used to produce them and cross-neutralization is minimal or absent (1). It is therefore essential that accurate species diagnosis is made so that the correct antivenom is administered to the snakebite victim. Capturing the culprit snake can be dangerous and is not always possible. Moreover, different snake venoms may produce similar clinical signs and symptoms, which may preclude clinical species diagnosis (2).

Various immunodiagnostic tests have been developed for the purpose of snake species identification to aid proper antivenom treatment. The most widely used methods are based on ELISA (3-5). A special version of ELISA using a glass capillary is very simple and convenient to use (6), but the cost is prohibitive for use in lesser developed countries. Some ELISA's were too slow to be useful for treatment purposes (7), or lacked the required

specificity (8, 9). A reverse latex agglutination test is simple and rapid and the reagents are stable to tropical climates, but the sensitivity of the test is relatively low, at about 0.16 to 1.2 ug venom/ml (10).

Reverse passive hemagglutination (RPHA) has been shown to be very sensitive for the detection of various antigens (11, 12). However, different species of red blood cells (RBC) have different rates of agglutination and sedimentation (13). They also show different degrees of stability to various enzymes present in snake venoms, and to different storage times and conditions. In an attempt to arrive at the most suitable RPHA, three species of RBC (sheep, chicken and human) were compared with respect to sensitivity, specificity, speed, stability and reproducibility in RPHA tests with various snake venoms. An RPHA with sheep RBC indicator cells was found to have the best overall suitability and it was used to test for venoms in body fluids of 59 snakebite victims.

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MATERIALS AND METHODS

Snake venoms and chemicals

Six major poisonous snake species were studied. For these snakes, horse monovalent antivenoms are available in Thailand. These snakes were _Naja naja siamensis_ (also called _Naja kaouthia_), _Bungarus fasciatus_, _Ophiophagus hannah_, _Calloselasma rhodostoma_, _Vipera russelli_ and _Trimeresurus albolabris_. The venoms were milked from 3-8 snakes of the same species and stored at $-20^{o}C$ until used. Extreme care was taken to avoid cross contamination of these venoms during milking and antisera production. Chemicals were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, unless otherwise indicated.

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Rabbit antisera

Antiserum against each of the six snake venoms was produced in rabbits and prepared as purified immunoglobulin G (IgG) by using Sepharose 4B-protein A affinity chromatography (14). This purified IgG was utilized in the sensitization of RBC.

Reverse passive hemagglutination (RPHA) test

Perparation of trypsin-treated red blood cells (TT-RBC)

Cells were washed 6 times in PBS, 15 mmol/L, pH 7.2 and a 10% suspension in PBS was mixed with an equal volume of 0.25% trypsin (Gibco grade, 1:250) at pH 7.0. The mixture was gently shaken in a 37 ^{o}C waterbath for 30 min. It was then centrifuged and the cells were washed twice with PBS. The cells were then resuspended at 10% (vol/vol) in PBS and mixed with an equal volume of 0.025% soybean trypsin inhibitor. The suspension was shaken at room temperture for 30 min. The cells were then washed twice with 0.9% NaCl, and packed (15). The efficiency of trypsin treatment was tested by examining the relative increase in agglutinability of a 1% TT-RBC suspension with rabbit anti-red cell serum by the hemagglutination assay (16).

Coupling of purified IgG to trypsin-treated red blood cells via chromic chloride

Chromic chloride ($CrCl_3.6H_2O$) was dissolved in 0.9% sodium chloride to a concentration of 1% (mass/vol). The pH of the solution was immediately adjusted to 5.0 by the dropwise addition of 1 M NaOH. The solution was then stored at 4 ^{o}C for 3 weeks, its pH being readjusted to 5.0 with 1M NaOH three times per week. After such treatment, the $CrCl_3$ was ready for use as a coupling reagent.

Purified IgG from Sepharose 4B-Protein A affinity chromatography was diluted with 0.9% NaCl to give a final protein

concentration of 2-3 mg/ml. A 1% stock solution of chromic chloride was diluted to various concentrations with 0.9% NaCl on the day of coupling. Ten microliters of IgG solution was then mixed with 10 ul of packed washed TT-RBC, and 20 ul of the chromic chloride solution was added slowly while vortexing. After mixing for 30 sec, SRBC tubes were rotated slowly at a 60° angle to the horizontal whereas HRBC and CRBC tubes were vigorously mixed with the vortex and then shaken. The coupling reaction was at room temperature for 60 min. Cells were then washed 3 times and resuspended at 1% (vol/vol) in PBS. The optimal dilution of $CrCl_3$ solution was the one at which the resulting sensitized cells detected the lowest concentration of homologous venom with no agglutination in negative controls. The preparations were checked for autoagglutination and stored at 4^oC. The coupled cells had to be prepared fresh every 1-2 weeks. Cells coupled to normal IgG were prepared in the same manner and were used as control.

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<u>Variation of antivenom IgG concentration in the sensitization of red blood cells</u>

Various concentrations of purified IgG were allowed to bind to TT-RBC as described above. The optimal concentration of IgG was the one at which the resulting sensitized cells detected the lowest concentration of homologous venom antigen with no agglutination in negative controls.

<u>Variation of the incubation time of antivenom IgG and TT-RBC during the coupling reaction</u>

Ten microliters of 2-3 mg/ml IgG was mixed with 10 ul of packed washed TT-RBC, and 20 ul of chromic chloride solution was then added slowly, mixed and incubated at room temperature for

varying periods of time (i.e., 0, 5, 15, 30, 60 and 120 min). After incubation, cells were washed 3 times in PBS and resuspended at 1% (vol/vol) in PBS. The preparations were assayed by RPHA in the detection of snake venoms.

Glutaraldehyde treatment of antivenom IgG-coupled RBC

The IgG-coupled TT-RBC were treated with glutaraldehyde and then prepared as a 1% suspension in PBS containing 0.1% sodium azide as described by Cranage et al., (17).

Stability of the glutaraldehyde - treated antivenom IgG-sensitized RBC.

Glutaraldehyde-treated antivenom IgG-sensitized RBC were prepared as 1% (vol/vol) suspension in PBS containing 0.1% sodium azide. The suspension was stored at 4 ^{o}C, -20 ^{o}C or snap frozen in dry ice-ethanol and stored at -20 ^{o}C for varying periods of time. The stability of the cells was checked weekly and monthly. The sensitized cell suspension was also tested for its ability to withstand cycles of freezing-thawing and lyophilization. Freezing was made by placing the cell suspension at -20 ^{o}C and thawing was done at 25 ^{o}C. The agglutination properties of the sensitized RBC were studied in positive and negative serum controls.

Detection of snake venoms by the RPHA test

The RPHA test was performed on a "U" bottom microtiter plate. To 0.05 ml of serial 2-fold dilutions of venom in diluent (PBS containing 0.5% BSA and 0.1% sodium azide), was added 0.05 ml of a 1% suspension of antivenom IgG-coupled TT-RBC, with or without glutaraldehyde treatment. The mixture was gently shaken and left at room temperature for 30 min (CRBC), 60 min (HRBC) or 90 min (SRBC) before the result was read.

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Diluent controls consisted of 0.05 ml of the diluent mixed with 0. 05 ml of antivenom IgG-sensitized TT-RBC. Sample controls consisted of 0.05 ml of venom or sample mixed with 0.05 ml of normal rabbit IgG-coupled TT-RBC. These negative controls had to show no agglutination. Judgement of the result was made by observing the agglutination of test materials in comparison with the agglutination of the negative control. The reciprocal of the highest dilution of sample showing complete agglutination (4+) was taken as the titer of the sample. Reactions of samples which, at all concentration tested, did not reach 4+ were considered to be incomplete agglutinations.

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Clinical specimens

Serum samples and wound swabs were collected from snakebite victims who arrived at hospitals for treatment regardless of whether systemic poisoning had occured. All the patients brought the culprit snakes for identification. A total of 59 sera were obtained from snakebite victims including *N. n. siamensis* (4 samples), *V. russelli* (19 samples), *T. albolabris* (5 samples) and *C. rhodostoma* (31 samples). Twenty six wound swab samples were gathered from victims of *C. rhodostoma* and 24 of these were accompanied by paired serum samples. The clinical specimens (59 sera amd 26 wound swabs) were kept frozen at -20 ^{o}C until used. The dried wound swabs were soaked with 1 ml of 0.85% normal saline solution and the cotton swabs were removed prior testing.

Control human plasma, serum, and urine were collected and similarly prepared. These samples were assayed for interference by the RPHA method.

Determination of snake venoms in clinical specimens by the RPHA test

Three hundred microlitres of each sample were adsorbed with 150 ul of packed TT-SRBC and 150 ul of a 50% suspension of normal rabbit IgG-coupled TT-SRBC in the presence of 15 ul of EDTA 200 mmol/L. The mixture was rotated at room temperature for 30 min and centrifuged at 500 xg for 5 min. The pellet was removed. To 0.05 ml of serial 2-fold dilutions of adsorbed sample starting with a 1:2 dilution, was added 0.05 ml of a 1% suspension of each of the six kinds of antivenom IgG-sensitized fresh TT-SRBC. After shaking, the mixture was left at room temperature for 90 min, at which time the result was read and expressed as the titer.

Statistical analysis

In comparing the effect of sample source on the detection of snake venoms by the RPHA test, the Chi square test (χ^2) was used.

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RESULTS

Optimization of parameters for the RPHA test

Variation of chromic chloride and IgG concentrations in RBC sensitization

The optimal concentration of chromic chloride varied with the species of RBC (Table 1). Human RBC required less chromic chloride than SRBC and CRBC. In addition, anti-*N. n. siamensis* and anti-*B. fasciatus* IgG needed less chromic chloride for coupling than did the other four antivenom IgGs. The range of chromic chloride concentrations for successful coupling was narrow, as excess antibody inactivated chromic

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TABLE 1 Optimum concentrations of antivenom IgG and optimum dilutions of chromic chloride solution in the coupling to TT-RBC.

Coupling agents	Anti-NS	Anti-BF	Anti-OH	Anti-CR	Anti-VR	Anti-TA
Antivenom IgG concentration (mg/ml)	2.50	2.38	3.22	5.95	2.80	2.75
Dilution of chromic chloride (10 mg/ml)						
Sheep red blood cells	1:80	1:60	1:45	1:45	1:45	1:45
Human red blood cells	1:100	1:60	1:80	1:60	1:60	1:80
Chicken red blood cells	1:80	1:60	1:60	1:45	1:45	1:45

chloride while deficient antibody or excess chromic chloride led to spontaneous agglutination.

The optimal concentration of IgG for sensitization was 2.38 to 3.22 mg/ml, with the exception of the IgG against C. rhodostoma which required 5.95 mg/ml (Table 1). Higher IgG concentrations caused prozone effects, whereas lower IgG concentrations resulted in spontaneous agglutination. The acceptable range of IgG concentrations was very narrow.

Variation of incubation time for RBC sensitization.

Anti-V. russelli IgG was coupled to TT-SRBC, HRBC or CRBC using chromic chloride as the coupling reagent. The reaction mixture was incubated for varying periods of time. It was found that the optimal incubation time with any species of RBC was 15 min, after which the RPHA titers did not change (data not shown). Non-specific agglutination took place when the reaction mixture was stopped immediately without incubation.

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Snake venom detection by the RPHA test

The results of sensitivity and specificity in venom detection by the RPHA method are shown in Table 2. It is apparent that the lower agglutination limits of the IgG-sensitized RBC by the respective homologous venoms at various concentrations were dependent almost entirely upon the particular venom/antivenom IgG, i.e., they were minimally influenced by the species of erythrocytes. Except for the determination of O. hannah and C. rhodostoma venoms, the sensitivity of the RPHA was 2 to 7 ng/ml. The detection limits for O.hannah and C.rhodostoma venoms were not improved when para-methyl sulfonyl fluoride (PMSF) 10 mmol/L was included in the RPHA reaction

TABLE 2 Summary of the sensitivity and cross-reactions of antivenom IgG-sensitized RBC of three species for detection of six snake venoms by the RPHA test.

Antivenom	Species of RBC sensitized with antivenom IgG	Cross-reacting venom* (ug/ml)					
		NS	BF	OH	CR	VR	TA
Anti-NS	Sheep	0.004	2,300	1,862	1,050	812	308
	Human	0.002	575	3,700	66	1,625	10
	Chicken	0.002	2,300	3,700	131	1,625	154
Anti-BF	Sheep	225	0.007	3,700	525	1,625	615
	Human	56	0.004	1,862	66	203	10
	Chicken	112	0.004	116	131	812	308
Anti-OH	Sheep	450	1,150	0.317	525	812	615
	Human	112	575	0.317	262	812	38
	Chicken	225	1,150	0.635	262	812	154
Anti-CR	Sheep	450	575	3,700	0.61	406	308
	Human	450	1,150	3,700	0.61	812	38
	Chicken	450	2,300	1,862	0.61	203	154
Anti-VR	Sheep	450	575	1,862	131	0.01	615
	Human	900	575	3,700	33	0.01	38
	Chicken	112	2,300	3,700	525	0.02	308
Anti-TA	Sheep	225	288	3,700	262	812	0.003
	Human	225	288	3,700	33	812	0.002
	Chicken	450	575	1,862	262	812	0.002

* Cross-reaction was observed when the amount of heterologous venom was higher than the indicated number.

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(data not shown). The agglutination results could be read within 90 min for SRBC, 60 min for HRBC and 30 min for CRBC. Cross-reactions in the hemagglutination were recorded at concentrations of heterologous venoms which were a minimum of 665 fold, 62 fold and 242 fold higher than the detection limits of the homologous antigens for SRBC, HRBC and CRBC, respectively. In most cases, cross-reactions were detected at very high concentrations (> 500 ug/ml) of heterologous venoms. Only certain venoms showed obvious cross-reactions and only in conjunction with RBC of a particular species,e.g.,heterologous T. albolabris venom with antivenom IgG-sensitized TT-HRBC (Table 2).

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Interference by various body fluids

Non-specific agglutinations of control human plasma and sera with antivenom IgG-sensitized SRBC and CRBC were registered in the absence of venom, and some samples caused hemolysis. This interference was eliminated by adsorption with TT-RBC and normal rabbit IgG-coupled TT-RBC in the presence of EDTA, 10 mmol/L, at room temperature for at least 30 min (data not shown). This procedure added 30 min to the total test time when SRBC or CRBC were used. The adsorbed samples were 2-fold serially diluted before assay since prozone-like effects occurred at higher concentrations of sera. When HRBC were used, plasma (10/24 cases) showed a variable pattern of non-specific agglutination in diluent controls (data not shown). This non-specific agglutination was eliminated by including normal rabbit serum in the samples or by adsorption with normal rabbit IgG-sensitized TT-HRBC. In contrast, only 1/25 serum samples showed a strong non-specific reaction. At high concentrations of sera or plasma samples regardless of venom concentration,

prozone-like effects with sensitized HRBC were observed, unless they were diluted 8 to 64 fold where positive hemagglutination appeared.

Effect of glutaraldehyde treatment.

The IgG-sensitized RBC could be stored at 4 ^{o}C for 7-14 days. In order to keep them stable for longer periods, they were treated with 0.1% glutaraldehyde at room temperature for 1 hr. This was successful in all cases with SRBC but not with CRBC (Table 3). With HRBC, the glutaraldehyde treatment (0.1 % for 1 hr) resulted in incomplete hemagglutination (3 + or less) at all venom concentrations tested, and thus, it reduced sensitivity of the test. When the glutaraldehyde solution was two-fold serially diluted before reaction with RBC, better patterns of agglutination were observed. However, at these concentrations, the treated HRBC, kept at 4 ^{o}C, tended to lyse. When IgG-coated TT-HRBC were treated with glutaraldehyde at a concentration of 0.1% for 5 min, only a slight decrease in sensitivity (reaction +3 in the RPHA) was obtained.

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Table 3 shows a comparison of fresh and glutaraldehyde treated antivenom IgG-sensitized red cells in the detection of the six poisonous snake venoms by the RPHA tests. Only the anti-*N. n. siamensis* IgG-sensitized cells revealed no sensitivity reduction after glutaraldehyde treatment with any of the 3 species of erythrocytes. With the other antivenom IgG's, glutaraldehyde treatment resulted in variable end point titer reductions of 2 to 4 fold, depending upon the species of RBC.

Stability of glutaraldehyde-treated, sensitized RBC

IgG-linked RBC treated with 0.1% glutaraldehyde kept at 4 ^{o}C in PBS containing 0.1% sodium azide were stable for 3 to 12 months for SRBC, 1 to 6 months for HRBC or 1 to 5 months

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TABLE 3 Comparison between fresh and glutaraldehyde-stabilized antivenom IgG-linked RBC for the detection of six snake venoms by the RPHA test

Treatment	Recipocal RPHA Titer+					
	NS:Anti-NS	BF:Anti-BF	OH:Anti-OH	CR:Anti-CR	VR:Anti-VR	TA:Anti-TA
SRBC						
Fresh	1024	128	32	16	64	256
GA	1024	32	8	16	32	128
HRBC						
Fresh	2048	256	32	16	64	512
GA	2048	128	16	4	32	128
CRBC						
Fresh	2048	256	16	16	32	64
GA	2048	256	*	*	32	32

+ the starting antigen concentrations in the 'undiluted' wells were as follows : NS, 3.516 ug/ml; BF, 0.903 ug/ml; OH, 10.156 ug/ml; CR, 4.883 ug/ml; VR, 0.635 ug/ml and TA, 3.075 ug/ml.

* Treatment with 0.1% glutaraldehyde resulted in autoagglutination

for CRBC, depending on the antivenom IgG used (data not shown). Glutaraldehyde-treated red cells were not stable at $-20^{o}C$ or at $-20^{o}C$ after snap freezing. They were not stable to freezing and thawing or to lyophilization. These treatments caused autoagglutination in diluent controls.

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Miscellaneous observations on sensitization and sensitivity

After treatment with trypsin, a gel-like solution developed from the mixture of CRBC, trypsin and PBS. This was hardly observable in the case of SRBC and HRBC. Trypsin-treated SRBC were less stable at $4^{o}C$ than TT-CRBC and TT-HRBC. The degree of lysis was in the order: TT-SRBC>TT-CRBC>TT-HRBC. Coupling of TT-HRBC and TT-CRBC to IgG was rather difficult; there were several occasions when couplings were not successful. In contrast, TT-SRBC could be easily coupled to IgG in the presence of chromic chloride and the result of the RPHA was highly reproducible. The propensity for cross-reactions was in the order of TT-HRBC>TT-CRBC>TT-SRBC. Adsorption of heterophile antibodies was required with SRBC and CRBC but not with HRBC. The total test times therefore were 120, 60 and 60 min for SRBC, HRBC and CRBC, respectively.

Species diagnosis in clinical specimens

The 59 serum and 26 wound swab samples were assayed by an RPHA test for species identification using GA-treated SRBC. Figure 1 shows the RPHA titers of serum samples with positive identifications for N. n. siamensis (4/4), C. rhodostoma (26/31), V.russelli (17/19) and T. albolabris bites (1/5). Of the 26 swab samples tested, 16 cases were positive, all for C. rhodostoma venom.

Of the total 59 sera, 48 gave correct positive identifications, 2 samples were mis-identified due to strong

FIGURE 1 Venom titers from the RPHA test using either sera (●) or wound swabs (▲) as the source of antigen from snakebite victims. NS, N.n.siamensis; CR, C.rhodostoma VR, V.russelli and TA, T.albolabris.

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cross-reactions and 9 were false negatives. Slight cross-reactions were observed in 16 cases, but the reactions were weaker than those of the homologous systems and thus they could easily be differentiated. The 29 sera containing C. rhodostoma venom from a Songkla provincial hospital gave prozone-like effects. However, after the sera were inactivatied at 56 ^{o}C for 30 min followed by adsorption and dilution in PBS, 150 mmol/L, pH 7.2, the prozone effect was eliminated. When the wound swabs were treated in the same manner, 16/26 cases showed positive hemagglutination and there were no mis-identification.

Comparison of serum and wound swab samples

When the 24 paired serum and wound swab samples from snake bite victims were assayed by the RPHA test, there were 12 dual positives, 8 serum-only positives and 2 swab-only positives

TABLE 4 Comparison of 24 paired serum and swab samples from snakebite victims of C. rhodostoma assayed by the RPHA test.

Serum	Swab		
	Positive	Negative	Total
Positive	12	8	20
Negative	2	2	4
Total	14	10	24

p-value > 0.05 by X^2 Test

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(Table 4). Although a higher number of venom identifications were obtained with serum than with swab samples, there was no statistically significant difference between results from the two sample sources by the chi square test (p-value > 0.05).

DISCUSSION

Chromic chloride was used as a coupling reagent between IgG and RBC since antibody activity was maintained after the reaction (18). Studies were made to assess many variables which influenced the efficiency of the coupling process. These variables included aging of chromic chloride; a solution of chromic chloride aged for 3 weeks was more efficient in coupling than a freshly prepared solution (19, 20). Optimizations of concentrations of antibody and chromic chloride were essential. The reactants had to be added in precise order with chromic

chloride being added in the last step, dropwise with constant mixing, because it was rapidly inactivated by protein. The reagents must have no phosphate ions as they precipitate the chromic ions (19, 20). It should also be mentioned that coupling of antivenom antibodies to erythrocytes at pH 4.0 in acetate buffer (21) was not successful.

Of the three species of erythrocytes tested, SRBC were the most suitable for venom detection by RPHA. Coupling of antibodies to SRBC could be performed easily and reproducibly. Of importance is the observation that the IgG coupled SRBC could readily be stabilized by glutaraldehyde; the glutaraldehyde-stabilized IgG-coated SRBC were stable at $4^{o}C$ for 3 to 12 months. The major disadvantage of the SRBC is the relatively slow rate at which they agglutinate, and thus the longer test time required.

The RPHA test using SRBC showed a sensitivity in the detection of venoms of 2-20 ng/ml except with O. hannah and C. rhodostoma venoms which were detected at 610 ng/ml. Judging from previously reported venom concentrations of 200 ng/ml in wound aspirates and 100 ng/ml in serum (28), the sensitivities of venom detection should be adequate except for O.hannah and C. rhodostoma, where improvement is needed. The lower sensitivity in the detection of O. hannah and C. rhodostoma venoms most likely was caused by the venoms themselves. However, the possibility that serine proteases in these venoms (22) were responsible was excluded since inclusion of PMSF did not increase the sensitivity of the RPHA.

Correct venom detections were made in 48 out of 59 serum samples (81.3%) and 16 out of 26 (61.5%) wound swabs. Out of 59 clinical samples, there were 2 venom mis-identifications (3.4%).

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Cross-reactions were observed in some samples but could be differentiated by the stronger positive hemagglutination of the homologous antigen-antibody system. Most of the cross-reactions were among venoms of different families and should not present any problems in the identification of snake species.

The negative venom identifications observed with RPHA were most likely due to insufficient venom concentration in the serum/swab samples. This is possible since the amount of venom delivered by a snake depends on its size, the amount of venom remaining in the venom gland, and the directness of the bite. Thus, less than half of snakebite cases result in systemic poisoning (23). Our serum/swab samples were collected from victims regardless of the severity of the bite, and we obtained 81.3% and 61.5% correct venom identifications from serum and swab samples, respectively. This compared favorably with the ELISA results of 39% for acute bites by N.n.siamensis and B.fasciatus (7), but somewhat less favorably with ELISA results of 97% for systemic poisoning cases by C.rhodostoma (24).

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Prozone effects were associated with serum samples obtained from Songkla Province. Although the sera were inactivated at 56 °C for 30 min to destroy serum complement components thought to produce the prozone effect, (25, 26), the positive samples still exhibited weak hemagglutination. When albumin was not included in the phosphate-buffered saline used as a diluent in the RPHA test, strong positive hemagglutination appeared. Non-specific interference with the antigen-antibody interaction by serum components, e.g. albumin, was also found in the detection of venom by competitive radioimmunoassay (27). In this latter study, sera were shown to give positive results only after being

diluted 1:10. Difficulty in using serum for snakebite diagnosis probably favors the use of wound swabs.

According to various reports (7, 9, 24), the ELISA technique established in Thailand requires overnight incubation to complete the procedure for the detection of snake venoms. It has been shown to have considerable value in retrospective diagnosis for epidemiological studies and in the measurement of serum venom levels in systemic envenomation to relate with the clinical symptoms, antivenom treatment and the distribution of venom in the human body or in experimental animals. The RPHA test is simple and shows a comparable sensitivity to the ELISA's, but is considerably faster. The test is inexpensive and requires no special apparatus. However, interference from serum samples, even though it could be eliminated by various samples treatments, may still be a drawback. This together with the variable stability of the sensitized red blood cells are the major disadvantages of the RPHA test.

Thus, the RPHA test described here exhibits certain advantages and disadvantages when compared to ELISA. It may be useful to produce the RPHA test for venom detection at certain health centers so that it can be evaluated for accuracy and acceptability. Meanwhile, efforts should be made to improve the stability of the RPHA reagents and to remove interference from clinical samples more quickly, thus shortening the test time.

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ABBREVIATIONS

BF, Bungarus fasciatus; BSA, bovine serum albumin; CR, Calloselasma rhodostoma; CRBC, chicken red blood cell; ELISA, enzyme-linked immunosorbent assay; HRBC, human red blood cell; GA, glutaraldehyde; NS, Naja naja siamensis; OH, Ophiophagus hannah; PBS, phosphate buffered saline; PMSF, para methyl sulfonyl fluoride; RBC, red blood cell; RPHA, reverse passive hemagglutination; SRBC, sheep red blood cell; TA, Trimeresurus albolabris; TT-RBC, trypsin treated red blood cell and VR, Vipera russelli.

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