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THE STUDY OF AFRICAN HORSESICKNESS VIRUS BY THE AGAR DOUBLE-DIFFUSION PRECIPITATION TEST. 1–STANDARDIZATION OF THE TECHNIQUE

By :

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Introduction.

Since the introduction of agar double - diffusion test to the study of antigen-antibody reaction by Ouchterlony on 1948, [16] the technique has extensively been used in identification and immunological research studies on different viruses including those of Arbovirus group. [1,2,3,6,8,9,14,15]

The first application of gel diffusion to the study of African horsesickness virus, a member of the Arbovirus group, was reported by Huq and Ansari [7], who employed it as a laboratory tool for the diagnosis of the disease. Serum obtained from rabbits hyperimmunized by 5 weekly intravenous injections of 1 ml. of a 10% infected horse splenic tissue suspension in saline was mostly used in the test. Formalized saline suspension of spleen tissues of infected animals, and in a few experiments virulent blood in O.C.G. or buffered glycerine, were used as antigens. It was stated that two precipitin lines were formed when such antigens and antiserum, were diffused towards each other through an agar medium.

With the recent advancement in the study of horsesickness virus, especially after successful adaptation of the virus in tissue culture [13,17], it was thought useful to further investigate the gel diffusion test on the study of this virus using antigens prepared from different host systems and antisera obtained from different animals.

An extensive study is now in progress to characterize the horsesickness viral antigen properties, precipitating antibody response of animals to the virus, and so on. The findings will be considered in the subsequent papers. The present communication describes the specificity of precipitin reactions between horsesickness antigen-antibody systems and deals mostly with the standardization of the technique.

Materials and Methods

Horsesickness Virus

The 102nd mouse passage of the neurotropic attenuated type 9 strain S2 and the 7th passage of the same strain in monkey kidney stable (MS) cells were mainly used for preparation of antigens and antisera employed in almost all the experiments.

However, neurotropic strains: A501, OD, L, Vryheid, VH, 114, Karen, and 18/60, adapted to the MS cell cultures, were also used as representatives of type 1 to 8 of HS virus, respectively, in a preliminary experiment to study antigenic similarities of these virus strains.

Strain S2 was isolated in Iran and was attenuated through more than 100 intracerebral passages in adult mice. The other strains were obtained from the Onderstepoort Veterinary Laboratory, South Africa. [17]

Preparation of Antigens

Mouse brain antigen (MBA). Suckling mice, 2-to-5 days old, were inoculated intracerebrally with horsesickness neurotropic mouse adapted virus. The brain tissue materials of inoculated mice in extremis were harvested, pooled and kept at -20 C. until use. The mice were partially bled before harvesting.

To prepare the antigen, the stored brain materials was thawed, ground in a tissue grinder and then mixed with an equal volume of saline. The mixture was homogenized in the same tissue grinder and the homogenate, in a glass container held in an ice-bath, was then subjected to 4 minutes ultrasonification at 20 Kc/sec. in a Branson Sonifier Cell Disruptor tuned to 2.5 amps. This preparation was centrifuged at 3000 r.p.m. for 60 minutes in a refrigerated centrifuge. The supernatant was collected, kept without any preservative at -20 C. until use as mouse brain precipitating antigen (MBA).

Control antigen (MBCA) was prepared in a similar manner from noninfected normal mouse brain.

Tissue culture antigen (TCA). Monkey kidney stable (MS) cells were grown in Roux bottles as described previously [18]. The cells which had formed complete monolayers were infected with 5 ml./bottle of 7th MS passage material of the virus strain. After an adsorption period of 2 hours at room temperature, the inoculum was decanted, the cells was then rinsed with 50 ml. of warmed Earle's Balanced Salt Solution and 50 ml. of the same solution containing 0.005% yeast extract, 0.5% Lactalbumin hydrolysate, 0.0015% phenol red, and antibiotics, (YLE), was replaced. The bottles were reincubated at 37 C. At the time of complete virus-specific cell destruction, while most of the destructed cells were still fixed on the glass surface, the fluid and the infected cells were harvested by vigorous shaking of the bottles. After centrifugation at 2500 r.p.m. for 15 minutes, the sedimented cells were resuspended in the sufficient amount of the supernatant to have a final volume of 0.5 N ml., where N is the number of Roux bottles used for preparation of antigen.

The infected cells were then disrupted by ultrasonification of the preparation according to the procedure already described.

The antigen, so prepared, without any preservative, was kept at -20 C. after being partially clarified by centrifugation at 2500 r.p.m. for 20 minutes.

Control antigen (TCCA) was simoultaneously prepared from noninfected MS cell cultures in a similar fashion. The cells in this case were detached and harvested by means of sterile glass beeds.

Preparation of Immune Sera

Immune sera were obtained from horse, rabbits and chickens. The blood was collected in sterile centrifuge tubes and allowed to clot at room temperature. Soon after the clotting, the clot was loosened from the sides of the tube using a sterile pipette for each tube. The tubes then were left standing at 37 C. for maximum 60 minutes following by 60 minutes in a refrigerator. The tubes were centrifuged at 1800 r.p.m. for 15 minutes and clear supernatant serum was pipetted off and pooled.

The sera, without any preservative, were stored in 2-ml. amount at -20 C. and used unheated in the test. Serum samples were diluted in veronal buffer or saline whenever needed.

Preinoculation sera of the same animals were employed as negative control sera (NS).

Horse immune serum (HIS). A horse recovered from an experimentally infection with type 9 horsesickness virus was bled daily following the onset of clinical symptoms. The serum collected 4 weeks after infection, possessing a high precipitin antibody titer, was used in the study.

Rabbit hyperimmune serum (RIS). Rabbits were hyperimmunized using mouse or cell culture adapted virus strain S2 as antigens. The animals were given 10 intramuscular injections of 5 ml. antigen at weekly intervals. The rabbits were bled 10 days after the last inoculation and the sera were separated and pooled.

Freshly prepared 10% infected suckling mouse brain suspension in normal saline was used as mouse adapted virus antigen. The suspension was centrifuged at 3000 r.p.m. for 60 minutes before inoculation.

The tissue culture adapted virus antigen was prepared as described under tissue culture antigen preparation. The fluid containing virus harvested at the time of complete destruction of infected cells, was lightly centrifuged and the supernatant was used as antigen.

Chicken hyperimmune serum (CHIS). The serum was obtained from adult Brown Leghorn chickens hyperimmunized in a similar fashion as the rabbits. The serum was collected and stored under the same conditions as above.

The sera obained from rabbits and chickens hyperimmunized by (MBA) and (TCA) antigens, hereafter, will be referred as (MB) and (TC) antisera, respectively.

Diffusion Media

The agar gel was used as the diffusion medium throughout the experiments. Difco purified agar or Difco special agar-Nobel was used. The medium was prepared in double distilled water with and without electrolytes, or in various buffer solutions, as described in the text. Sodium Azide, phenol or merthiolate was added to the medium when the inhibition of contaminating micro organisms' growth in or on the medium was needed or when the effect of these chemicals on precipitation reactions were being studied.

To prepare the medium, the agar and other ingredients were added to the required amount of warmed double distilled water or buffer solution in

a sterile container. The mixture was then autoclaved at a presure of 10 pounds for 10 minutes. The final pH of the medium was adjusted to the desired pH using N/10 NaOH.

Some of the prepared test media needed to be filtered to improve their clarity. This was performed by passing the hot melted medium through a Buchner funnel containing filter paper pulp.

Immediately after preparation the medium was distributed into flat bottomed pettri dishes of 90 mm. diameter, which had been placed open on level surfaces.

The agar were allowed to solidify at room temperature before cutting. Wells were cut in solidified medium with the aid of a metal empty tube possessing a uniform sharp edge, and the cut portions were removed cleanly with the help of a sterile bent needle.

The wells were punched to a predetermined pattern and specified equal distance from each other.

The plates so prepared were generally kept inverted at room temperature overnight, and then in a 4° C. refrigerator, for period of not longer than 2 weeks, before use.

Procedure of the Gel Diffusion Test

The punched wells in the diffusion medium were filled to capacity with sera or antigens. The reagents were allowed to diffuse towards each other through the medium at desired temperatures and examined for precipitin lines at specified time intervals.

The plates were examined in a darkened room over an indirectt light from a microscope lamp reflected by a concave mirror.

The degree of precipitin reactions were recorded as follows: O, no precipipitin; Tr., Trace but recognisable; D, diffuse; 1, faint; 2, faintly delineated; 3, distinct and sharp; 4, quite distinct and well defined dense precipitin lines.

Experiments and Results

Formation of Precipitin Reactions

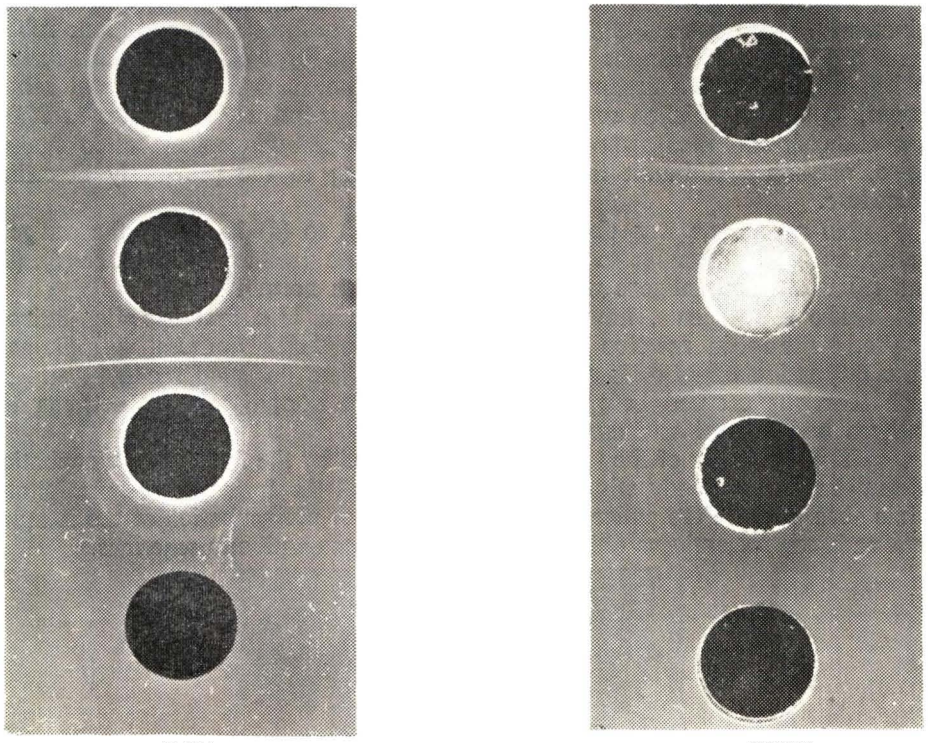
Convalescent horse serum, sera from rabbits and chickens hyperimmunized by mouse adapted S2 antigen were subjected to gel diffusion precipitation test against homologous tissue culture antigen. Similarly, sera obtained from rabbits and chickens hyperimmunized by MS adapted S2 strain were tested against mouse brain antigen.

A diffusion medium consisting of 1.25 per cent Difco purified agar in distilled water was used for testing of horse and rabbit sera. The same medium but containing 4% sodium chloride was employed for testing the chicken sera. The wells were 7 mm. in diameter and were spaced at 5 mm. apart. The plates were incubated at 37 C. for 3 days and observed for precipitates.

Two precipitin lines were usually observed. The first line which was more sharp and defined appeared as early as 6 to 8 hours after incubation and was either a straight line, (RIS), or slightly bent towards the serum well, (CHIS), and

mostly located midway between antigen-antibody wells. The second line appeared close to the first and was usually slightly curved away from antigen well. This line formed 12 to 24 hours later than the formation of the first line. (Fig. 1)

In some cases these two lines, being fused to each other, formed a single line which was in fact consisting of two overlapping lines. In these instances if different dilutions of antiserum was tested against different dilutions of the antigen, at least in one system, where the optimal concentration of reagents were diffused towards each other, the separation of the overlapped lines could be observed.



(RIS) (CHIS)

Fig. 1. Precipitin reactions between tissue culture Type 9 antigen and homologous MB hyperimmune sera from rabbit and chicken. Top row shows wells filled with undiluted serum, second row with antigen, third row with 1/2 diluted serum, and forth row with tissue culture control antigen.

Specificity of the Reactions

The convalescent horse serum, MS hyperimmune rabbit and chicken sera were mixed with an equal volume of 1) homologous tissue culture antigen (TCA), 2) tissue culture control antigen (TCCA), and 3) normal saline. The mixtures were incubated either at 37 C. for a period of 90 minutes or at 4 C. overnight. During the storage period the containers were occasionally shaken. The absorbed antisera were recovered by centrifugation at 2500 r.p.m. for 30 minutes and then

were tested against (TCA), (TCCA) antigens according to the procedure already described.

Normal sera were also similarly treated and served as controls.

Precipitin lines were formed when sera in system (3) were diffused towards the homologous antigen. Identical reactions were obtained when preparation in system (2) were tested against the same antigen. In no instance did normal or normal absorbed sera produced any visible lines in the similar tests. (Fig. 2).

No precipitin formation was observed in the system (1) in which the antisera were treated with tissue culture antigen.

In another experiment tissue culture and mouse brain type 9 antigens were mixed with equal volume of 1) immune horse serum, 2) MB hyperimmune rabbit serum, 3) MB hyperimmune chicken serum, 4) MS hyperimmune rabbit serum, 5) MS hyperimmune chicken serum, 6) Normal horse serum, 7) Normal rabbit serum, 8) normal chicken serum. An absorption period of 90 minutes at 37 C. was allowed, and then all the preparations were simoultaneously tested against horse, chicken and rabbit immune sera according to the previously described procedure.

No precipitate was observed in systems (1) to (6) in which the antigens were absorbed by immune sera. Precipitin lines were produced in systems (6), (7), and (8) where the antigens were preincubated with normal sera.

The experiments, however, indicated that a true and specific antigen-antibody precipitin reaction existed when horsesickness antisera and antigens were diffused towards each other in an approperiate diffusion medium, and no such reactions occured between the antigens and normal sera or between the antisera and normal antigens.

The specificity of the test was also supported by the fact that preincubation of antigen and antibody with each other inhibited the formation of precipitates while absorption of the antigens with normal sera or preincubation of the immune sera with control antigens did not reduce the antigen-antibody reactions.

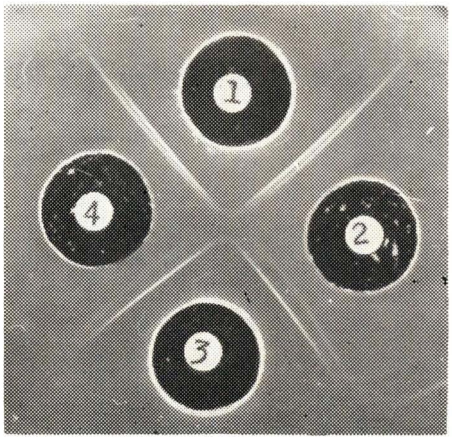


Fig. 2. Identical precipitin reactions formed by diffusing: 2) MB type 9 hyperimmune rabbit serum absorbed with control antigen, 4) MB type 9 hyperimmune rabbit serum treated similarly with equal volume of saline, against 1) undiluted, and 3) diluted homologous tissue culture antigen.

Selection of Diffusion Medium.

In order to find out the most suitable diffusion medium for horsesickness antigen-antibody precipitation reactions, 9 different media of 1.25% Difco purified agar in double distilled water with and without varying concentration of sodium chloride or in different buffer solutions, as shown in Table 1, were prepared The pH of each medium was adjusted to 7.2.

Immune horse serum (HIS), MB hyperimmune rabbit serum (RIS), and MB hyperimmune chicken serum (CHIS) were simoultaneously titrated against an optimal dilution of (TCA) antigen in these media. The plates were observed for precipitin lines every 12 hours after being incubated at 37 C. The results read after 24 hours were shown in Table 2.

The results, however, indicated that buffering the medium with buffer salts was not absolutely necessary for the precipitin reactions. The lines, under the conditions of the test, were in fact more clear & sharp and better defined in the non buffered media. Medium No. 9 having the disadvantage of not being as clear as the others needed to be filtered for its clarity.

The concentration of sodium chloride had an obvious effect on the precipitation reactions. Rabbits and horse sera gave a higher titer and sharper lines in medium with sodium chloride concentration of less than 4%, the concentrations of 0 to 1% being optimal.

Chicken sera, on the other hand, reacted better in media with higher concentration of sodium chloride. In this case, although good lines could be obtained in media containing varying concentration of sodium chloride from 2 to 10 per cent, preference should still be given to the media with 3 to 6% of the salt.

Table 1. — Composition of 9 different diffusion media used in the experiments.

	M 1	M2	M3	M4	M5	M6	M7	M8	M9
Agar	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
ClNa	0	1	2	4	6	8	10	0	1
VB^+	0	0	0	0	0	0	0	100	0
NaH2PO4 2H2O	0	0	0	0	0	0	0	0	0.156
Na2HPO4 2H2O	0	0	0	0	0	0	0	0	0.178
DDW	100	100	100	100	100	100	100	0	100
pH adjusted to	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2

The number indicated "ml." for DDW and VB, and "Gramme" for the rest of the ingredients.
VB = Veronal buffer pH 7.2
M9 : was clarified before use.
DDW: Glass double distilled water

Medium	Rabbit serum dilution				chicken serum dilution			Horse serum dilution			
	1/1	1/2	1/4	1/8	1/1	1/2	1/4	1/1	1/2	1/4	1/8
M1	4	4	3	1	TR	TR	0	4	4	4	4
M2	4	4	3	1	2	2	0	4	4	4	4
M3	4	3	1	TR	4	2	TR	4	3	3	2
M4	4	4	1	TR	4	3	3	3	2	2	1
M5	3	3	2	1	4	3	3	2	2	1	1
M6	2	1	1	1	4	3	TR	2	TR	TR	1
M7	D	D	D	D	4	3	1	D	D	D	D
M8	4	4	2	TR	4	2	1	4	4	4	2
M9	2	3	2	0	3	1	0	3	3	2	1

Table 2. — Titration of horse immune serum, MB hyperimmune rabbit and chicken sera against optimal dilution of tissue culture precipitating antigen in different diffusion media. Results recorded 24 hours after incubation the plates.

Effect of Agar Concentration

MB hyperimmune rabbit serum and horse convalescent serum were simoultaneously titrated against (TCA) antigen in diffusion media containing 1, 1.25, 1.5, 2, and 2.5 % of Difco purfied agar.

The titer of sera did not vary significantly in different media, but there were some differences as far as the time of first appearance and separation of the two precipitin lines were concerned. The lines were visible earlier and separated better in media of 1, 1.25, and 1.5% agar than in th others. In the media with 1% agar some difficulty was encountered with cutting the wells, and therefore, from various concentration of agar being tried, 1.25 and 1.5% were found optimal.

Effect of Distance between Antigen and Antibody Wells

In a preliminary test, investigating the effect of agar thickness on precipitin formation, it was found that the reactions were not significantly different in plates containing 15,20, and 25ml. of the diffusion medium, although in plates with 15 ml. agar precipitin lines appeared somewhat sooner than in the others.

To determine the effect of distance between antigen and antibody wells on the reactions in agar test, reagents were allowed to diffuse towards each other at 37 C. through an approperiate medium from wells spaced at 3,5,7,10,12,16,18, and 20 mm distances.

The results of such an investigation testing horse serum and MB hyperimmune rabbit serum against (TCA) antigen, are shown in Table 3. It can be seen that the precipitin reactions occured earlier between wells with narower distances. The sharpness and strength of the reactions were also dependent on the distance

between antigen and antibody wells. With the distance of 3 and 5 mm. the lines were more sharp and solid, but as the distances increased the sharpness of the lines decreased, accordingly. However, a distance of 3 to 5 mm. was found optimal under the condition of the test. At these distances the lines were sharp, well defined and better separated.

Effect of the Temperature

The antigens and antibodies were allowed to react through similarly prepared media at 4C, 22C., 30 C and 37 C. The plates were observed at varying intervals for 3 days.

The precipitin lines were sharper and better defined in plates incubated at 37 C. and 30 C. Moreover, at this temperature, due to a probably better and more rapid diffusion of the reagents, the precipitin lines appeared several hours sooner than in plates incubated at the lower temperatures. (Table 4).

Effect of pH on Precipiiin Reactions.

The horsesickness immune sera were simoultaneously tested against homologous antigens in diffusion media with pH values of 5.5, 6.5, 7.5, and 8.5. The plates were incubated at 37 C. for 3 days and observed daily for precipitin reactions. No obvious difference was noticed on the time of appearance or the density of precipitin lines at the above pH variations.

Table 3. — Precipitin reactions between HS antigen and antibody diffused from wells at varying distances.

Time	Serum	Distance between antigen-antibody wells (mm.)							
		3	5	7	10	12	16	18	20
6 h.	RIS	TR	0	0	0	0	0	0	0
8 h.		2	1	0	0	0	0	0	0
16 h.		3	3	2	0	0	0	0	0
24 h.		4	4	3	0	0	0	0	0
48 h.		4	4	4	2	1	TR	0	0
72 h.		4.	4.	4	3	2	TR	TR	0
96 h.		4.	4.	4	4	3	2	1	0
6 h.	HIS	0	0	0	0	0	0	0	0
8 h.		2	TR	0	0	0	0	0	0
16 h.		3	2	TR	0	0	0	0	0
24 h.		3	3	TR	0	0	0	0	0
48 h.		4	4	3	TR	TR	0	0	0
72 h.		4	4	2.	TR	TR	0	0	0
96 h.		4	4	2.	TR	TR	TR	0	0

h. = hours

. = two line were visible.

Table 4. — Appearance of precipitin lines when HS antigens and antibodies were allowed to react at different temperature.

Time	Temp. (C)	(CHI) dilutions 1/1	1/2	1/4	(RIS) dilutions 1/1	1/2	1/4	(HIS) dilutions 1/1	1/2	1/4	1/8
6h.											
	4	0	0	0	0	0	0	0	0	0	0
	22	0	0	0	0	0	0	0	0	0	0
	30	TR	TR	0	TR	TR	0	0	0	0	0
	37	1	TR	TR	1	TR	TR	TR	TR	0	0
8h.											
	4	0	0	0	0	0	0	0	0	0	0
	22	TR	TR	0	TR	0	0	TR	0	0	0
	30	2	TR	0	1	TR	0	TR	TR	0	0
	37	3	1	1	3	1	1	2	TR	TR	0
12h.											
	4	TR	0	0	TR	0	0	0	0	0	0
	22	2	TR	TR	TR	TR	0	2	2	TR	0
	30	3	1	TR	2	1	0	2	2	1	0
	37	3	3	1	3	2	2	3	2	TR	TR
24th.											
	4	1	TR	0	2	0	0	2	0	0	0
	22	3	2	TR	3	2	0	3	3	3	TR
	30	4	2	TR	3.	2.	0	4	3	3	TR
	37	4	4	2	4.	3.	2.	4	4	3	2
48h.											
	4	3	1	TR	3	2.	TR	3	2	2	TR
	22	4	3	TR	4.	3.	TR	4	4	3	TR
	30	4	3	TR	4.	3.	TR	4	4	4	2
	37	4.	4	2	4.	4.	2.	4	4	4	2
72h.											
	4	4	3	TR	4	2.	TR	3	3	2	TR
	22	4.	3	TR	4.	3.	TR	4	4	4	2
	37	4.	4	TR	4.	4.	TR	4	4	4	2
	30	4.	4	2	4.	4.	2.	4	4	4	2

. = Two lines were visible.

Effect of Preservatives

The horsesickness precipitating antisera produced in horse, rabbits and chickens were allowed to react against homologous antigens in media containing: 1) 0.5% phenol, 2) 0.25% phenol, 3) 0.5% sodium azide, 4) 0.25% sodium azide,

5) 1/10,000 merthiolate, 6) 1/5,000 merthiolate, and 7) no preservative.

Identical results were obtained by using media Nos. 1, 2, 3, 4, 5, and 7, but lines in medium No. 6 appeared slightly weaker in comparison to the others. Moreover in this medium usually one single line was only formed.

Effect of Protamine Sulphate

To investigate the possibility of improving the precipitin reactions when horsesickness antigen antibody were tested by the gel diffusion technique, protamine sulphate was incorporated into the diffusion medium at the final concentrations of 0.5 mg., 1.0 mg,. and 2 mg./ml.

(RIS), (CHIS), and (HIS) were simoultaneously tested against homologous antigen using the approperiate standard medium and media containing the above concentration of protamine sulphate. The procedure of the test was as already described. The comparative results indicated that the two precipitin lines formed sooner and better separated in protamine added media. The concentrations of 0.5 mg. and 1.0 mg./ml seemed to be optimal. At the higher concentration (2 mg./ml.) the medium became cloudy although the lines appeared slightly better separated.

Recruiting Effect

The object of this experiment was to determine if testing the horsesickness antigens or antibodies in conjunction with known positive control reagents made any difference on the precipitine formation.

Precipitating antiserum was diluted in a 0.2 $\log._{10}$ fold manner with veronal buffer. Then each dilution was allowed to react on its own and in conjunction with undiluted serum against the homologous antigen as shown in Fig 3.

The results sugegsted that the precipitin reactions, in each case, was more readily demonstrated when the diluted antiserum was placed into wells next to the undiluted antiserum well. This was specially noticeable when the dilution of antiserum just beyond the serum titer was tested. Such a serum dilution formed a precipitin line when placed at an angle $< 90^{\circ}$ to the antigen-antibody system and did not react when tested on its own against antigen. Fig. 3.

The same results were observed when the dilution of antigen were similarly tested against an optimal dilution of antiserum.

This phenomenon, termed the "recruiting effect" in gel diffusion test by Klontz [8], influences the results of the test whenver the estimation of horsesickness antibody in sera or detection of HS antigen are concerned, and therefore it should be taken into consideration.

Antigenic similarity of HS Virus Types

In order to find out the antigenic relationship of the 9 distinct immunological types of horsesickness virus in gel diffusion test, type antigens were prepared in MS cell cultures by using neurotropic MS adapted virus strain of each type.

The antigens were then tested against the type 9 specific MB (CHIS), and MB (RIS) sera. The test was so designed that the heterologous antigens were

tested next to the homologous one, as shown in Fig 4. The sera reacted identically to homologous type 9 and heterologous types 1 to 8 antigens. In absorption experiments all the different types absorbed out type 9 antibody, and did not react in precipitin test when they were preincubated with type 9 hyperimmune sera.

These findings indicated that all the type antigens shared at least two common precipitating antigenic components and that, under the conditoins of the test, gel diffusion technique could not be employed to identify the immunological types of horsesickness virus.

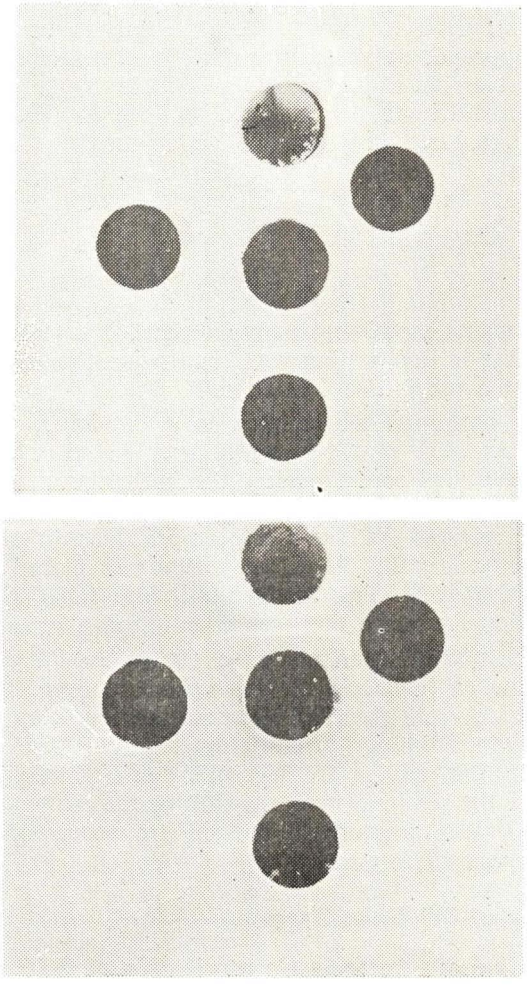


Fig. 3. Recruiting effect; Wells in the Centre filled with tissue culture antigen, on the top with undiluted MB hyperimmune rabbit serum, the other wells in (A) 1/8 dilution and in (B) 1/16 dilution of the serum.

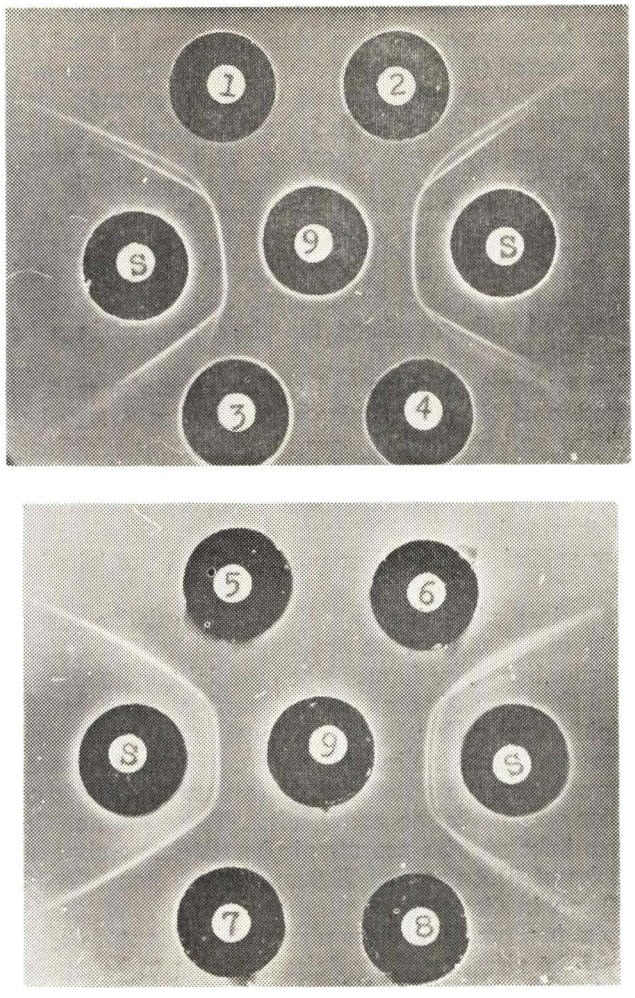


Fig. 4. Identical precipitin reactions between MB type 9 hyperimmune rabbit serum and homologous type 9 & heterologous types 1 to 8 HS viral antigens.

Discussion

Monkey kidney stable (MS) cell cultures and suckling mice were used for propagation of horsesickness virus in preparation of both precipitating antigen and antibody. The satisfactory results of using either host systems, made it possible to test the antisera obtained from animals immunized by the virus propagated in one host system against the antigens prepared from the other host system. In such a case no non-viral precipitin line, due to the mouse brain or tissue culture materials were observed in the agar diffusion tests.

Precipitating antigens were prepared from ultrasonically disrupted cells of

suckling mouse brain or monkey kidney tissue culture system infected with the virus. The mouse brain materials were obtained from infected suckling mice in extremis, and the infected (MS) cells were harvested at the time of complete virus-specific cell destruction. Antigens prepared under these conditions gave a titer of 1/4 to 1/6. No attempt was made to find the optimal time for harvesting the infected materials in order to prepare an antigen wtih a higher titer.

In two cases the antigens produced from ultrasonically disrupted cells of either systems, were compared with those obtained by freezing, the same infected materials, in an alcohol solid CO2 mixture and thawing in cold running water, for 5 times. The results, not presented in this communication, indicated that although good antigens could be prepared by alternate freezing and thawing, but ultrasonification of infected cells containing the virus, resulted in a better release of the precipitating antigens.

In addition to horse, chickens and rabbits were also used with satisfactory results in preparation of HS precipitating sera. Hawkes [6], stated that, in production of precipitating antisera against several arboviruses, chickens were superior to mice, rabbits, and guinea pigs. In the present study, chickens, while showing some individual differences, were also found a good producer of precipitating serum to horsesickness virus. Rabbits reacted more regularly and were found to be the laboratory animal of choice for producing HS precipitating hyperimmune serum.

Mice and guinea pigs, due to their full or relative susceptibility to the neurotropic strains of HS virus, were not employed in this study.

Two precipitin lines were formed when precipitating antigens and antibodies were allowed to diffuse towards each other through an approperiate diffusion medium. The precipitin lines differed in the time of first appearance as well as their density and curvature. These indicated that mouse brain tissues and cell cultures infected with horsesickness virus contained at least two antigenic components with different molecular weight. Polson and Madsen [19], by ultracentrifugation studies, showed that tissue infected with neurotropic horsesickness virus strains contained 2 infective particles of 31.2 mμ and 50.8 mμ, and a non infective but antigenic component of 12 mμ in diameter.

Whether the two precipitin lines observed in this study could be related to the components of the virus stated by Polson, remain to be studied.

No precipitin line was formed between antigens and normal sera, and between control antigens and precipitating or normal sera, indicating that a true and virus specific antigen-antibody reaction has occured. This was also supported by the fact that in absorption experiments horsesickness antigens absorbed out the antibody in the precipitating sera and that the reactions were blocked when antigens had been treated by a positive serum before being used in the test.

No definite difference in precipitin reactions was observed, in a preliminary test, when undiluted hyperimmune type 9 rabbits and chickens sera were reacted against 9 immunologically different types of the horsesickness viral antigens. Further investigations, using immune sera obtained at varying intervals after immunization of horses and rabbits, are being followed to find out whether the agar diffusion test could be used to detect the antigenic differences among different types of horsesickness virus.

Hyperimmune rabbit serum and convalescent horse serum gave higher titer

and better defined lines in diffusion medium with 0 to 1 per cent sodium chloride. Hyperimmune chicken sera, on the other hand, reacted better in media with higher concentration of sodium chloride.

The necessity of a high concentration of sodium chloride in measurement of Precipitating antibody in chicken antisera, in liquid and semisolid media, has been previously recommended [4,5,20]. In the agar gel diffusion studies of Hawkes et al [6], using antiserum obtained from chickens, a 4 per cent sodium chloride in the medium was found to be optimal, and that below this concentration the intensity of the precipitin lines diminished, although the number of visible lines did not alter.

In the present studies a level of 4 to 6 percent of sodium chloride in the diffusion medium was found optimal for testing hyperimmune chicken sera, while at concentrations higher than this the two precipitin lines were often fused to each other.

Agar concentration of the diffusion medium, and the temperature in which the reagents were allowed to react, were found to affect the precipitin formation. pH variations of the medium between 5.5 to 8.5, on the other hand, did not make any obvious difference on the time of first appearance or the density of the precipitin lines.

Mansi [10,11], showed that, in agar double diffusion test, the thickness of the diffusion medium and the distance between wells were important factors in formation of precipitin lines. He stated that in agar plate a distance of 4 mm. was optimum for the antigen-antibody system in myxoma, fibroma, swine fever, canine distemper, Rubarth's disease and lymphocytic choriomeningitis.

In our experiments, with wells of 7 mm. diameter, cut in plates prepared by 20 ml. of a diffusion medium containing 1.25% agar, distances of 3 to 5 mm. between antigen and antibody wells gave the best results.

The addition of protamine sulphate to diffusion medium, as a factor improving the precipitin reactions between antigen and antibody, was first reported by Conant and Barron [3B], in the study of entrovirus antigens. In the present studeis it was found that the two precipitin lines formed sooner and better separated when protamine sulphate was incorporated into the diffusion medium at the optimal concentrations of 0.5 to 1 mg./ml.

Hopkins et al [6B] showed that plaque size of horsesickness virus were increased 2 to 6 fold in the cultures overlaid with medium containing 0.5 mg./ml. of protamine sulphate. It seems that agar gel containing protamine sulphate in both studies improved the diffusion of HS virus and viral antigen through the medium resulting to the increase of plaque size and precipitin reactions of the virus.

Klontz et al [8], studying the precipitating antibody directed to blue tongue virus, showed that "an antigen -antibody system containing one reagent in low concentration precipitated to a precipitating control system but not if placed by itself or on an angle $> 90°$ to the control system". This phenomenon termed "recruiting effect" was also observed in the present studies and should, therefore, be taken into account in the study of horsesickness by agar double diffusion test. In detecting of horsesickness antigen in tissues, testing the materials in conjunc-

tion to known positive antigen, as recommended by Mansi and King [12] in the study of swine fever, increased the sensitivity of the test and was, therefore, recommended.

Summary

Precipitating horsesickness viral antigens were produced from ultrasonically disrupted cells of suckling mouse brain or monkey kindney tissue culture system infected with the virus.

Horsesickness convalescent serum from a recovered horse, as well as, horsesickness hyperimmune sera obtained from rabbits and chickens, reacted specifically and formed two distinct precipitin lines when diffused towards the homologous and heterologous precipitating antigens in Ouchterlony agar gel diffusion test.

In studying factors affecting the sensitivity of the test, it was found that the composition of the diffusion medium, among the other factors, was of more importance when testing sera of different sources were concerned. Rabbits and horse sera gave higher titer and better defined lines in medium with 0 to 1 per cent sodium chloride, while a relatively higher concentration (4-6 per cent) of this chemical was needed for the better detection of precipitating antibody of chickens sera.

The comparative tests using media with pH values of 5.5 to 8.5 revealed no obvious difference in the number or the intensity of precipitin lines.

Performing the test at the temperatures 4, 22, 30, and 37 C., the precipitin lines appeared and were completed within a shorter time at 37 C. This, however, depends on the percentage of the agar, the depth of the medium in the plate, the diameter of the wells and the distance between antigen-antibody wells. With wells of 7 mm. in diameter, cut in a plate prepared by 20 ml. of a diffusing medium consisting of 1.25% agar, a distance of 3 to 5 mm. between antigen and antibody wells were found optimal.

The effect of preservatives, such as phenol, merthiolate, and sodium azide, and the addition of enhancing factors, such as protamine sulphate to the media, were also studied on the reactions in agar precipitation test.

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RESUME

Les antigènes précipitants du virus de la Peste équine ont été produits sur des cellules cérébrales de souriceaux non sévrés, ou bien sur les cellules rénales de singe infecté par le virus et détruit par un appareil ultra-sonique.

Le sérum d'animaux convalescents antipestiques a été obtenu sur des chevaux guéris de maladie, ainsi que les sérums hyperimmuns contre la maladie ont

été préparés sur des lapins et des poussins formant des lignes précipitantes bien distinctes vis-à-vis des antigènes homologue et hétérologue dans le test de diffusion sur gélose.

Considérant les facteurs qui affectent la sensibilité du test, nous avons trouvé que la constitution du milieu joue un grand rôle par rapport aux autres facteurs.

Les sérums provenant des lapins et des chevaux nous ont donné le meilleur test et les meilleures lignes avec le milieu constitué de 0 à 1% de clNa, tandisque la concentration de 4 à 6% de ce sel est nécessaire pour bien détecter les anticorps précipitants des sérums des poussins.

Les tests comparés utilisant les milieux avec de pH variant de 5,5 à 8,5 nous ont montré aucune différence dans le nombre des lignes de précipitation. Les tests des températures 4'C, 22'C, 30'C et 37'C ont montré que les lignes apparaissent très vite à 30'C, ce phénomène dépend du pourcentage d'agar, de la profondeur du milieu, du diamètre des cupules et enfin des distances entre les cupules antigéniques et les anticorps.

Les cupules de 7mm de diamètre préparées dans un milieu de 20cc constituée par 1,25% d'agar ont donné de bons résultats, quand la diamètre entre les cupules antigéniques et des anticorps est de 3 à 5mm.

Influence des préservateurs comme le phénole, le merthiolate, le sodium azide et l'additionnement des facteurs enrichissant comme les sulfates protamines ont été aussi étudiés dans les réactions de précipitation.

REFERENCES

1. Chan, Y.C.: Rapid typing of dengue viruses by the micro-precipitin agar gel diffusion technique. Nature, **206**, (1965): 116-117.

2. Clarke, D.H.: Antigenic relationship among viruses of the tick-borne encephalitis complex as studied by antibody absorption and agar gel precipitin techniques. In Biology of the tick-borne encephalitis complex. Edited by H. Libikova, New York and London, Academic Press, (1962): 67-75.

3. Clarke, D.H.: Further studies on antigenic relationships among the viruses of the Group B tick-borne complex. Bull. W.H.O., **31**, (1964): 45-56.

3B Conant, R.M. and Barron, A.L.: Enhanced diffusion of Enterovirus antigens in agar gel in the presence of protamine. Virology, **33**, (1967): 547-549.

4. Goodman, M., Wolfe, H.R., and Norton, S.: Precipitin production in chickens. VI. The effect of varying concentrations of NaCl on precipitate formation. J. Immunol., **66**, (1951): 225-236.

5. Grabar, P.: Immunoelectrophoretic analysis. in Meth. biochem. Anal., vol. **7**, pp. 1-38 (Interscience Publisher, New York, London 1959).

6. Hawkes, R.A., and Marshal, I.D.: Studies of arboviruses by agar gel diffusion. Am. J. Epidem., **86**, (1967): 28-44.

6B Hopkins, I.G. Hazrati, A., and Ozawa, Y.: Development of plaque technique for titration and neutralization tests with African horse-sickness virus. Am. J. Vet. Res. **27**, (1966): 96-105.

7. Huq, M.M., and Ansari, M.Y.: Gel-precipitin test for the diagnosis of South African horsesickness. Bull. off. int. Epizoot. **58**, (1962): 691-698.

8. Klontz, G.W., Svehage, S.E., and Gorham, J.R.: A study by the agar diffu-

sion technique of precipitating antibody directed against Blue Tongue virus and its relation to homotypic neutralizing antibody. Arch. Ges. Virusforch., **12**, (1962): 259-268.

9. Levitt, J., and Polson, A.: Physical and serological investigation of Rift Valley fever antigens. J. Hyg. (Camb.), **62**, (1964): 239-256.

10. Mansi, W. The study of some viruses by the plate gel diffusion precipitin test. J. Comp. Path. Ther., **67**, (1957): 297-303.

11. Mansi, W.: Slide gel diffusion precipitin test. Nature, **181**, (1958): 1289-1290.

12. Mansi, W., and King. A.A.: The study of swine fever by the gel diffusion precipitin test. Vet. Rec., **75**, (1963): 933-938.

13. Mirchamsy, H., and Taslimi, H.: Adaptation of horse sickness virus to tissue culture. Nature, **198**, (1963): 704-706.

14. Murphy, F.A., and Coleman, P.H.: California group arboviruses: Immunodiffusion studies. J. Immunol. **99**, (1967): 276-284.

15. Olinik, M.K., Dudnikov, A.I., and Malyarets, P.V.: Nauch. Trud. Ukrain. Ins. Teksp. Vet. **27**, 1, (1961): 39-45.

16. Ouchterlony, O.: Antigen-antibody reactions in gels. Arkiv. Kemi. Mineral. Geol. **26B**, (1948): 1-9.

17. Ozawa, Y., and Hazrati, A.: Growth of African horsesickness virus in monkey kidney cell cultures. Am. J. Vet. Res., **25**, (1964): 505-511.

18. Ozawa, Y., Hazrati, A., and Erol, N.: African horse-sickness live-virus tissue culture vaccine. Am. J. Vet. Res., **26**, (1965): 154-168.

19. Polson, A., and Madsen, T.: Particle size distribution of African horsesickness virus. Biochem. biophys. Acta, **14**, (1954): 366-373.

20. Weiner, L.M., Macho, I., Poulik, E., and Goodman, M.: Salt requirement for precipitation of chicken antisera in agar immunoelectrophoresis. J. Immunol. **93**, (1964): 228-231.