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Arbovirus Identification by an Agar-Gel Diffusion Technique

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A double diffusion-in-agar test was used to investigate precipitation reactions of 75 arboviruses. Specific reactions were regularly observed with members of arbovirus groups B, California, Simbu, Turlock, Hart Park, vesicular stomatitis, and several other arboviruses as well as with a member of the Tacaribe group and a herpesvirus. The results demonstrated the feasibility of applying this technique to the identification of arboviruses.

Since the beginning of 1965, this laboratory has identified nearly 1,300 viruses isolated from diagnostic or field-collected specimens that were submitted to or collected by the Arbovirus Unit, National Communicable Disease Center (NCDC), Atlanta, Ga. The viruses included members of arbovirus groups A, B, C, Bunyamwera, Guama, Patois, Simbu, California, Turlock, and several other arboviruses as well as a member of the Tacaribe group and *Herpesvirus hominis*. For identification, complement-fixation (CF), hemagglutination-inhibition (HI), and neutralization (NT) techniques were used singly and in combination.

This work load indicated the need for a simple screening test which could be used in conjunction with other tests for more rapid identification and, particularly, for differentiation of closely related strains. The work of Murphy and Coleman (11) with the California virus group and Clarke (6) with the group B tick-borne complex had indicated the feasibility of the Ouchterlony double diffusion-in-agar system for arbovirus differentiation.

The present paper comprises an evaluation of the precipitin-in-agar technique for differentiation and identification of a wide variety of arboviruses.

MATERIALS AND METHODS

Viruses. A list of the viruses used in the initial studies is given in Table 1. Arbovirus strains isolated from field-collected specimens were also used (Table 7). *H. hominis*, although not an arbovirus, was included in these studies because it is occasionally isolated from specimens of human brains. Tamiami, a Tacaribe group virus, was included in these studies because it is isolated under circumstances similar to those of arbovirus isolations.

Antisera and ascitic fluids. Immune sera (IS) and immune ascitic fluids (IAF) were produced as described elsewhere (2, 11). Four injections of antigen were usually required to elicit satisfactory precipitating antibody in Ha/ICR mice; from one to four injections were required for domestic rabbits (*Oryctolagus cuniculus*). The most suitable immune system for precipitin studies with a given antigen was selected by prescreening several immune preparations in the gel diffusion system. Selection for the prescreening was made on the basis of strength of homologous HI, CF, or NT reactions, availability, and lack of evidence of nonspecific cross-reactivity with normal suckling mouse brain.

Anti-mouse tissue precipitins are frequently found in sera from rabbits inoculated with mouse tissue. Nevertheless, the large number of immune preparations available at NCDC provided a variety from which a suitable serum could be selected; that is, either the serum did not contain sufficient anti-mouse precipitins to produce visible mouse–anti-mouse arcs or the system was in such total antigen excess that no precipitin lines were seen. Thus, no adsorption of rabbit sera with uninfected mouse tissue was done.

Antigens. Antigens were usually prepared by inoculating 2- to 4-day-old ICR mice with a dilution of seed virus (approximately 500 suckling mouse intracranial 50% lethal doses), collecting the mice when signs of illness appeared, and harvesting the infected suckling mouse brain (ISMB). Sucrose-acetone-extracted antigens were prepared by the method of Clarke and Casals (7).

Agar gel diffusion technique. The method of Murphy and Coleman (11) was used. Undiluted, uncentrifuged ISMB brei was the antigen in these tests. Antigens were expelled into the agar (1% Ionagar 2, Consolidated Laboratories Inc., Chicago, Ill.) wells through a 19-gauge needle attached to a 1.0-ml tuberculin syringe. In all tests, undiluted normal suckling mouse brain was included as a control to detect nonspecific reactivity of the immune preparation. Precipitin results were recorded on a 0 to 4+ scale, depending upon arc intensity. A value of 0 indicated no reac-

TABLE 1. *Laboratory-adapted virus strains used for Immunodiffusion tests*

Group	Virus	Strain	Passage level
A	Eastern equine encephalomyelitis	NJO	6
	Western equine encephalomyelitis	Fleming	>100
	Sindbis	W-32309	2
B	St. Louis encephalitis	TBH-28	5
	Bussuquara	BeAn 4073	7
	West Nile	Ar-248	3
	Dengue type 1	Hawaii	13
	Dengue type 2	Tr1751	58
	Ilheus	331	28
	Japanese B encephalitis	G8924	8
	Murray Valley encephalitis	11A	14
	Yellow fever	Asibi	>200
	Modoc	Pero	9
	Cowbone Ridge	W-10986	7
	Rio Bravo	U.S. Bat salivary gland virus	9
C	Apeu	BeAn 848	14
	Caraparu	BeAn 3994	13
	Gumbo Limbo	FE3-71H	9
	Marituba	BeAn 15	4
	Oriboca	BeAn 17	12
	Murutucu	BeAn 974	2
	Nepuyo	Tr18462	4
	Itaqui	BeAn 12797	5
	Ossa	BT1820	4
	Madrid	BT4075	3
Bunyamwera	Bunyamwera	RI-1	40
	Tensaw	A9-171B	4
	Cache Valley	Bun-144	9
	Guaroa	352111	11
	Chittoor	Original	3
	Wyeomyia	Prototype	9
Guama	Guama	BeAn 277	9
	Mahogany Hammock	FE4-56F	4
	Catu	BeH 151	3
	Moju	BeAn 12590	2
	Bimiti	Tr8362	2
	Mirim	BeAn 7722	3
Patois	Patois	BT4971	7
	Zegla	BT5012	7
	Shark River	FE4-1R	15
	Pahayokee	FE3-52F	15
Simbu	Simbu	Ar-53	10
	Akabane	JaGAr 39	11
	Buttonwillow	7962	15
	Ingwavuma	An 4165	7
	Manzanilla	Tr3587	4
	Mermet	AV-782	3
	Oropouche	Tr9760	15
	Sathuperi	India	16
	Utinga	BeAn 84785	2
California	California encephalitis	BFS-283	16
	La Crosse	177	6
	Snowshoe hare	Burgdorfer	20
	San Angelo	20230	3
	Tahyna	92	198
	Lumbo	W-32369	6
	Jamestown Canyon	61v-2235	3
	Jerry Slough	BFS-4474	3
	Keystone	B64-5587.05	6
	Trivittatus	FE3-61BR	7
	Melao	Tr9375	4
		NJO-94F	11
Capim	Capim	BeAn 8582	3
	Guajara	An 10615	2
	Moriche	Tr57896	5
	Bush Bush	Tr26668	3
Turlock	Turlock	Malt 150	3
Hart Park	Hart Park	AR-70	17
	Flanders	W-18904	2
Vesicular stomatitis	Vesicular stomatitis	New Jersey	9
	Vesicular stomatitis	Indiana	3
	Cocal	Tr40233	4
Ungrouped	Colorado tick fever	Florio	38
	Kern Canyon	41117	4
	Silverwater	Canada	12
Tacaribe	Tamiami	W-10777	5
Herpesvirus	*hominis*	P-15096	5

tivity visible to the naked eye; 1+, a minimal reproducible specific reaction; 2+, a weak to moderate but readily visible arc; 3+, strong reactivity; 4+, maximum intensity and a narrow precipitin arc. Although the values were assigned arbitrarily and depended upon individual perception and the quality of reagents, they provided a scale for measuring the intensity of heterologous precipitation reactions.

RESULTS AND DISCUSSION

The procedure described was not intended to be analytical. Differences in the intensity of reactions between antigens and IS or IAF can be attributed to the degree of hyperimmunity of the preparation as well as to other factors such as stability and diffusibility of antigens, immunizing antigens, immunization schedule, host, and source of antibody.

Serological relationships between arboviruses can be detected in HI, CF, and NT tests by using immune preparations with low titers for viruses related closely or with high titers for less type-specific, more group-specific cross-reactions. Thus, the 0 to 4+ scale used in this study is

useful on a quantitative basis alone. Cross-reactivities shown here are intended only to reflect the capacity of a given antigen or antibody preparation to produce a visible precipitin arc with a homologous, or closely related, virus.

The present technique, as adapted, is useful only insofar as it is capable of rapidly, specifically, and simply detecting type- or group-specific antigens of certain arboviruses.

Group A arboviruses. Hawkes and Marshall reported precipitin reactions with five group A arboviruses from Australasia (9). In the present study, five recent isolates of eastern equine encephalomyelitis (EEE) virus and six of western equine encephalomyelitis (WEE) virus from birds and horses were compared with homologous systems prepared from standard strains. These isolates had been identified in CF and mouse NT tests by using laboratory-adapted strains of EEE (NJO strain) and WEE (Fleming strain) viruses as controls. The EEE (NJO) and WEE (Fleming) viruses were also tested in agar, as was an isolate of Sindbis virus from Israel (13). Strong precipitin reactions (4+) were obtained with the Sindbis homologous system but not with the Sindbis antigen and EEE or WEE antibody, EEE antigen and Sindbis antibody, or with WEE antigen and Sindbis antibody. Although the homologous system for EEE (NJO) was moderate (2+) and that of WEE (Fleming) was weak (1+), reactivity varied in intensity from test to test, indeed with the same reagents. None of the field-collected WEE virus strains precipitated with WEE (Fleming) antibody, even though seven immune preparations were tested. Five of five EEE virus preparations gave reactions with EEE (NJO) IS or IAF, although a given reacting immune preparation did not react with all five isolates. The immunodiffusion test as conducted here, therefore, does not appear to be useful for identifying the WEE strains tested.

Group B arboviruses. The utility of an immunodiffusion system for group B arbovirus identification has been demonstrated by Chan (5) and Ibrahim and Hammon (10) with dengue viruses, Clarke (6) with the tick-borne group, Hawkes and Marshall (9) with a variety of Australasian viruses, and Murphy and Coleman (11) with St. Louis encephalitis virus. In our experiments, the test satisfactorily differentiated the group B members studied (Table 2). Reactions between two or more systems reflect the antigenic relatedness of the viruses. A more quantitative method would undoubtedly give more analytical results. Although it is unfortunate that the Cowbone Ridge antigen did not titer sufficiently enough to

TABLE 2. *Reactivities of group B arboviruses by immunodiffusion*

Antibody	Antigen												
	St. Louis encephalitis	West Nile	Japanese B encephalitis	Yellow fever	Bussuquara	Ilheus	Modoc	Cowbone Ridge	Murray Valley encephalitis	Rio Bravo	Dengue-1	Dengue-2	Normal
St. Louis encephalitis (ASF)[a]	4[b]	2	2	1	1	0	3	0	0	0	0	0	0
West Nile (ASF)	2	3	1	0	0	0	2	0	3	0	0	0	0
Japanese B encephalitis (ASF)	0	0	2	0	0	0	0	0	0	0	0	0	0
Yellow fever (RIS)[c]	0	0	0	2	0	0	0	0	0	0	0	0	0
Bussuquara (ASF)	0	0	0	0	3	0	3	0	0	0	0	0	0
Ilheus (ASF)	0	0	0	0	0	3	0	0	0	0	0	0	0
Modoc (RIS)	0	0	0	0	0	0	2	0	0	0	0	0	0
Cowbone Ridge (ASF)	0	0	0	0	0	0	2	0	0	0	0	0	0
Murray Valley encephalitis (RIS)	0	0	0	0	0	0	0	0	3	0	0	0	0
Rio Bravo (RIS)	0	0	0	0	0	0	0	0	0	2	0	0	0
Dengue-1 (ASF)	0	0	0	0	0	0	0	0	0	0	4	0	0
Dengue-2 (ASF)	0	0	0	0	0	0	0	0	0	0	0	4	0
Normal (ASF)	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] ASF = mouse hyperimmune ascitic fluid.
[b] Precipitin results recorded on a 0 to 4+ scale.
[c] RIS = rabbit immune serum.

TABLE 3. *Cross-reactivities of California encephalitis group viruses by immunodiffusion*

Antibody	Antigen													
	California encephalitis	La Crosse	Snowshoe hare	San Angelo	Tahyna	Lumbo	Jamestown Canyon	Jerry Slough	Keystone	Waycross	Trivittatus	Melao	NJO	Normal
California encephalitis (RIS)[a]	4[b]	3	3	3	2	3	3	1	0	0	0	0	3	0
La Crosse (RIS)	3	4	3	1	2	3	3	3	0	0	0	0	3	0
Snowshoe hare (RIS)	3	3	3	3	2	3	3	3	0	0	0	0	3	0
San Angelo (ASF)[c]	3	2	3	3	0	0	0	2	0	0	0	0	2	0
Tahyna (ASF)	0	0	0	0	4	4	0	0	0	0	0	0	0	0
Lumbo (ASF)	0	1	0	0	4	4	0	0	0	0	0	0	0	0
Jamestown Canyon (ASF)	3	3	2	0	0	2	4	3	1	0	0	0	4	0
Jerry Slough (ASF)	2	2	2	2	0	0	1	3	0	0	0	0	1	0
Keystone (ASF)	0	0	0	0	0	0	2	2	4	4	0	0	2	0
Waycross (RIS)	0	0	0	0	0	0	2	3	4	4	0	0	2	0
Trivittatus (RIS)	0	0	0	0	0	0	0	0	0	0	4	0	0	0
Melao (RIS)	0	0	0	0	0	0	0	0	0	0	0	3	0	0
NJO-94F (RIS)	0	1	0	2	0	1	4	4	0	0	0	0	4	0
Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] RIS = Rabbit immune serum.
[b] Precipitin results recorded on a 0 to 4+ scale.
[c] ASF = Mouse hyperimmune ascitic fluid.

produce a detectable arc with its homologous antibody, this antibody reacted moderately with the broadly reactive Modoc antigen. By other tests, Cowbone Ridge and Modoc are closely related (2).

California virus group. The antigenic relatedness of the California group members suggested by others (11, 14) was confirmed and extended to include some other types (Table 3). The Keystone virus strain used in the cross-test was isolated from a pool of *Aedes atlanticus-tormenter* mosquitoes caught in Waycross, Ga., in 1963 (4). Although the isolate had previously been shown by CF and NT tests to be a unique member of the California group, its identity with Keystone virus had not been determined. The present studies confirm this relationship. Prototype Keystone antigen (Keystone virus kindly supplied by Gladys Sather, University of Pittsburgh School of Public Health) precipitated equally with IS to the Waycross strain and IAF prepared to Keystone virus, and the reciprocal cross also reacted identically. Moreover, these antigens and immune fluids reacted indistinguishably with reagents of other group members; significant cross-reactions were seen between the Jerry Slough, Jamestown Canyon, and New Jersey antigens and Keystone or Waycross IS or IAF; Keystone and Waycross antigens, on the other hand, reacted only with IS or IAF to each other, and, in addition, Keystone antigen reacted very weakly with Jamestown Canyon IS.

On the basis of differences in cross reactivities with other viruses of the group, the Jamestown Canyon, Jerry Slough, and New Jersey types appear to constitute one subgroup, and California encephalitis, La Crosse, and snowshoe hare constitute another subgroup. San Angelo, closest to the latter subgroup, appears distinct from it because of its reaction with the New Jersey type and its lack of reaction with Jamestown Canyon.

As Sather and Hammon (14) have shown by other methods, Lumbo and Tahyna viruses are closely related. One minor difference we found was that Lumbo antigen, but not Tahyna antigen, precipitated with Jamestown Canyon and New Jersey IAF. Melao and Trivittatus are the only members of the California group with no relationship to any other member by the precipitin-in-gel method.

By the immunodiffusion system, a number of recently received isolates did not appear to be identical to known types and thus may represent still more members of the California complex. There appear to be a plethora of California complex types. It is possible that laboratory passage level, type, availability of vertebrate host,

species of vector, or geographical distribution, or all of these, may contribute to the antigenic variations. Perhaps minor degrees of variation should be ignored unless evidence indicates some special significance of strain differences.

The La Crosse strain is associated with human illness, particularly meningoencephalitis. Paired human sera, kindly supplied by Howard Stegmiller (Ohio Department of Health), were tested in the immunodiffusion system against La Crosse, California encephalitis, snowshoe hare, Keystone, and Trivittatus viruses. The precipitin results, together with HI, CF, and NT titers supplied by Howard Stegmiller, are presented in Table 4. Sera reacted in the precipitin-in-agar system only with La Crosse or, weakly, with snowshoe hare. The reactivity with snowshoe hare antigen probably was due to heterologous cross-reactivity. Precipitin arcs with La Crosse but not with snowshoe hare antigen were present after adsorption of the sera with snowshoe hare ISMB, whereas no arcs were present after adsorption with La Crosse ISMB.

TABLE 4. *Comparison of neutralization (NT), hemagglutination-inhibition (HI), complement-fixation (CF), and immunodiffusion testing of paired human sera from Ohio*

Patient	Date drawn	Titers given as reciprocals			Immuno-diffusion (La Crosse antigen)
		NT	HI	CF	
VP	8/18/66	40	40	<8	0
	9/03/66	80	40	16	0
DC	8/29/66	40	20	<8	0
	9/19/66	≥160	80	16	4+
KB	7/21/66	ND[a]	20	8	0
	8/18/66	≥160	320	16	4+
JLT	Unknown	ND	20	<8	0
	Unknown	≥160	80	32	4+
SSr	8/22/66	40	80	16	1+
	9/22/66	160	160	256	4+
TC	Unknown	20	160	8	1+
	Unknown	160	80	16	4+
JMC	9/28/66	20	20	<8	0
	10/19/66	≥160	80	32	4+
SSt	9/14/66	ND	<20	<8	0
	9/29/66	≥160	80	8	4+
JM	9/28/66	ND	20	<8	0
	10/19/66	80	80	32	4+

[a] Not done.

The convalescent serum of patient SSr, with a CF titer of 1:256, was titrated with twofold dilutions of La Crosse antigen. The 1:16 serum dilution (with an equivalent CF titer of 1:16) demonstrated a 4+ reaction, but the 1:32 dilution (with an equivalent CF titer of 1:8) demonstrated only a 1+ reaction.

Bunyamwera group. Although a large number of IS and IAF, including single-dose and hyperimmune preparations, were tested, no differences could be detected between the six members of the Bunyamwera group listed in Table 1. Whether the group- and type-specific antigenic determinants are present on the same moiety remains to be determined for viruses of this and other groups. It is possible that, with inclusion of more members of the group in future tests, subgroup separations may be realized.

Guaroa virus, which is a member of the Bunyamwera group by CF (17), is related to the California group by both HI and NT. In cross-precipitin tests with Guaroa, no lines of precipitation were seen with the battery of California group reagents, but weak (2+) reactions were

TABLE 5. *Reactivities of Simbu group arboviruses by immunodiffusion*

Antibody[a]	Antigen									
	Simbu	Sathuperi	Akabane	Oropouche	Utinga	Button-willow	Mermet	Ingwa-vuma	Manzanilla	Normal
Simbu.....	2[b]	3	3	0	0	0	0	0	0	0
Sathuperi.....	0	4	2	0	0	0	0	0	0	0
Akabane.....	0	1	4	0	0	0	0	0	0	0
Oropouche....	0	0	0	4	0	0	2	0	0	0
Utinga.....	0	0	0	0	4	0	4	0	0	0
Buttonwillow..	0	0	0	0	0	4	2	0	0	0
Mermet.....	0	0	0	0	0	0	4	4	4	0
Ingwavuma....	0	0	0	0	0	0	4	4	4	0
Manzanilla....	0	0	0	0	0	0	4	4	4	0
Normal.....	0	0	0	0	0	0	0	0	0	0

[a] Mouse hyperimmune ascitic fluid.
[b] Precipitin results recorded on a 0 to 4+ scale.

obtained with Bunyamwera prototype, with Tensaw, and with the homologous reagent.

Group C, Patois, Guama, and Capim groups. IAF and IS were tested against both sucrose-acetone-extracted and crude ISMB antigens. Although one or more of the antigens and immune systems titered as high as 1:256 by CF, lines of precipitation were not seen with all homologous systems. In addition, no correlation was noticed between CF titer and precipitin line production or between sucrose-acetone-extracted and crude ISMB antigens with either homologous or heterologous systems. Whether sucrose-acetone extraction removes an accessory antigen remains to be shown.

Simbu group. The most intense arcs of all of the arbovirus antigens studied were seen with members of the Simbu group. Not only were the precipitins distinct by their specificities (Table 5), but the arcs were as long as 8 mm, and most were easily visible without the usual aid of a dark background. Cross-reactivities of Ingwavuma, Manzanilla, and Mermet viruses correspond with CF and NT test results (3).

Because the relationship between Buttonwillow and the California group has been shown by HI and NT (W. C. Reeves, *unpublished data*), cross-precipitin tests were carried out with both Buttonwillow ISMB and IAF and the battery of California group reagents. Crossing would have been expected if the antigen responsible for arc formation had been the CF moiety, but no crossing was noted.

"Bullet" viruses. Results presented in Table 6, which provides a summary of cross-testing with bullet-shaped viruses, support the CF and NT test results of Murphy and Fields (12). Although these viruses (and rabies virus) are morphologically similar, the gel method revealed no previously undetected serological crossing at the group or strain level. The relationship between vesicular stomatitis virus (NJ), vesicular stomatitis virus (Indiana), and Cocal viruses has been documented (12). Multiple arcs, demonstrated previously (1), were also seen in the present study. The single precipitin line obtained with Kern Canyon homologous system was remarkable in intensity and length.

These studies also supported the evidence (12) that Hart Park and Flanders viruses are antigenically related. Single-injection immune rabbit sera prepared against arboviruses reacted more specifically in precipitation reactions than antisera produced by multiple injections of a single virus. The latter sera showed a broadened antibody spectrum because of augmented response to group antigen.

Other viruses. Satisfactory specific homologous reactions ($\geq 3+$) were obtained with Colorado Tick Fever, Silverwater, and Turlock viruses. In addition, the system was suitable for detection of *H. hominis*, as previously reported for a similar system (16).

Three strains of Tamiami virus were tested against a Tacaribe horse IS, a Tacaribe group IAF, and an IAF prepared against one of the Tamiami virus strains. Multiple arcs were seen with the horse IS and the Tamiami IAF, but no lines were observed with the IAF with which Tamiami was originally identified by a CF test. This inability to see arcs with the IAF indicates the necessity for a high-titering positive control for visualization of precipitin reactions.

Attempts to identify isolates from field-collected specimens. The agar diffusion system was used to test 248 isolates from field-collected material.

TABLE 6. *Reactivities of "bullet" viruses by immunodiffusion*

Antibody[a]	Antigen						
	Vesicular stomatitis (NJ)	Vesicular stomatitis (Indiana)	Cocal	Kern Canyon	Flanders	Hart Park	Normal
Vesicular stomatitis (NJ)	3[b]	0	0	0	0	0	0
Vesicular stomatitis (Indiana)	0	3	3	0	0	0	0
Cocal	0	0	2	0	0	0	0
Kern Canyon	0	0	0	4	0	0	0
Flanders	0	0	0	0	4	4	0
Hart Park	0	0	0	0	4	4	0
Normal	0	0	0	0	0	0	0

[a] Mouse hyperimmune ascitic fluid.
[b] Precipitin results recorded on a 0 to 4+ scale.

TABLE 7. *Results of testing field-collected specimens*[a]

Virus	No. identified/ no. tested
Eastern equine, encephalomyelitis.	5/5
Western equine, encephalomyelitis	0/6
Sindbis	1/1
St. Louis encephalitis	7/7
Dengue type 2	3/3
Tensaw	9/10
California	185/185[b]
Turlock	6/7
Flanders	17/19
Tamiami	5/5

[a] Identity confirmed by CF or NT test, or by both tests.

[b] Includes five isolates identified as to group but not type.

These strains had been isolated from mosquitoes, mammals, or birds by various members of the Arbovirus Unit, had undergone two or three SM passages, had never been lyophilized, and had previously been identified by CF tests. Results of the identification tests with double diffusion are presented in Table 7. As mentioned previously, five of five EEE but none of six WEE were identified, as was one isolate of Sindbis, the other group A arbovirus tested. Of 236 other virus strains tested, 232 were identified; this included 185 strains of California virus.

One of the Flanders isolates identified by immunodiffusion had been submitted to this laboratory as a possible isolate of St. Louis encephalitis virus, one of the Tamiami strains had not reacted by CF with a Tacaribe group IAF in another laboratory and was received at NCDC for identification, and the three dengue type 2 isolates were obtained from human sera collected during the 1968 to 1969 Caribbean dengue fever epidemic. In all five instances, rapid identification of the virus was important. Immunodiffusion tests provided rapid and satisfactory identifications for all of these isolates.

It was suggested by Hawkes and Marshall (9) that their technique of precipitin-in-agar might have been limited by the need for a high concentration of antigen; the present technique probably provides sufficient reactants for attaining the limited goals established here. Should material having undergone two or more SM passages be used, satisfactory adaptation of most viruses probably would obviate any difficulties which might be brought about by host modifications. The alternate reagents problem can be accommodated by the production of more potent and specific antibody.

The feasibility of a technique also depends upon its cost, simplicity, and rapidity. The agar-gel diffusion method presented does not require extraction of antigen and uses only about one-third the volume of reactants required for a micro-CF test. The time lapse between initiation and reading is about the same for the agar-gel test and the micro-CF test, but actual working hours are nearly one-fourth. Protein stains, such as Thiazine red R, may be used to prepare the slides for photography or reading at a later date; results may then be filed in the form of the stained reactions themselves.

Although the present system works well enough for many applications, additional work will undoubtedly lead to many improvements. The substitution of a more purified agar preparation could eliminate certain nonspecific inhibitors (8, 15). Such an inhibitor might well have been responsible for low or nonreactive antigens in the present gel diffusion system when the same antigen preparations titered substantially by CF and infectivity tests.

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