

## Development of a Modified Immunoelectroosmophoresis Method for Uukuniemi and Runde Virus Serology

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With 2 Figures

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### Summary

In search for a suitable method for sero-ecological screenings for arboviruses in Norway, efforts were undertaken to make the immunoelectroosmophoresis technique more sensitive than here to fare in detection of antibodies. The aim was to make it comparable to haemagglutination inhibition test in sensitivity, retaining the advantages in specificity, simplicity and capacity. This has been achieved by:

1. Using concentrated virus as antigen.
2. Performing electrophoresis in a gel consisting of an agar-agarose mixture in optimal concentrations.
3. "Sandwiching" the first specific electrophoretic run with an anti-species antiserum to the tested sample.

### Introduction

The isolation of Uukuniemi (UUK) group viruses and a new coronavirus-like agent, Runde virus, from Norwegian *Ixodes* ticks (19, 21, 22, 23), were intended to be followed up promptly by sero-ecological surveys by a standard haemagglutination inhibition test (HAI).

It was soon decided, however, that certain disadvantages of HAI, with regard to our specific needs, would make it worthwhile to search for another screening method.

After having solved the problem of making potent HA antigens for our weakly haemagglutinating viruses (24), the remaining disadvantages of HAI were linked to the question of unspecific serum inhibitors, and to its broad group reactivity. We intended to perform sero-ecological screenings comprising human beings, small mammals, passerine birds, sea birds, wild ruminants, domestic animals and

reptiles. However, little is known about the qualitative and quantitative contents of serum inhibitors in many of the groups above. Therefore it was desirable to use a serological screening method which eliminated the inhibitor problem but was comparable to HAI in sensitivity.

The use of the immunoelectroosmophoresis (IEOP) technique has attracted increasing interest in virus serology due to its speed, simplicity and modest requirements for reagents. But its use has been hampered by the lack of sensitivity, which is partly due to the anodal movement of most immunoglobulins under the conditions previously employed (12).

This paper reports how the adoption of relatively simple measures makes IEOP equal to HAI in sensitivity for detection of antibodies to Norwegian arbovirus strains. Since it eliminates the serum inhibitor problem and also has various practical advantages over HAI, it has become our standard screening method.

## Materials and Methods

### *Viruses*

The original isolations of the five virus strains used in these experiments are described elsewhere (22, 23). They were isolated from suspensions made from *Ixodes* ticks by intracerebral inoculations of 1—3 days old baby mice [Bom: NMRI (SPF)]. SF E 1 and By E 50 are Uukuniemi (UUK) group viruses from *I. ricinus* ticks. Ru E 82 is an UUK group virus from *I. uriae*, while Ru E 81 and Ru E 85 are coronavirus-like agents isolated from *I. uriae*. The two latter strains seem identical and we have tentatively termed them Runde virus. Ru E 82 differs antigenically from the two other UUK viruses.

Tahyna virus (strain 181) was kindly provided by Dr. V. Danielova, Institute of Parasitology, Czechoslovak Academy of Sciences, Prague as a 10 per cent lyophilized suckling mouse brain suspension from the 3rd passage. Tribec and Tickborne encephalitis (TBE) virus were kindly placed at our disposition by Dr. M. Gresikova, Institute of Virology, Slovak Academy of Sciences, Bratislava. Ten per cent lyophilized suckling mouse brain suspensions from the 13th and the 63rd passage, respectively, were used.

### *Precipitin Antigens*

The following kinds of antigenic preparations have been utilized in these experiments:

Crude suckling mouse brains (SMB antigen) are 20 per cent suspensions of infected suckling mouse brains in PBS pH 7.4 with 0.75 per cent bovine albumine (APBS).

Sucrose-aceton extracted suckling mouse brains (SA antigen) were prepared according to CLARKE and CASALS (5), but the final lyophilization step was omitted.

Cell culture antigens (BHK antigens) were prepared by growing the viruses in BHK 21/c 13 cells in Roux bottles. The infectious culture fluids are concentrated 100 to 300 times by precipitation with 6 per cent polyethylene glycol 6000/2.2 per cent NaCl at pH 7.6 and 4° C (14) overnight, followed by lowspeed centrifugation and resolution of precipitates in APBS. Finally sonication is performed. For TBE, HeLa Bristol cells were used instead of BHK.

It was found beneficial to include lipoprotein-absorption by the colloidal silicagel Aerosil (Degussa, Frankfurt a. Main) for all antigens (20, 24). This was done in order to avoid lipoprotein deposition around the electrophoresis wells. This may in some instances interfere with the interpretation of specific precipitation lines.

Uninfected mouse brains and BHK-cultures were treated in parallel and used as control antigens.

*Mouse Hyperimmune Sera and Ascitic Fluids*

Each mouse initially received between  $10^4$  and  $10^5$  BMLD<sub>50</sub> (baby mouse lethal doses) in 0.25 ml brain suspension mixed with an equal volume Freund's complete adjuvant (Difco) intraperitoneally. Thereafter 3 weekly injections without adjuvant were given. After another week, an injection identical to the first one was performed. Seven to 8 days later paracentesis and bleeding from the retroorbital sinus were done.

Reference mouse immune ascitic fluids to UUK (strain S23), TBE (RSSE) and Tribec had been obtained from the Yale Arbovirus Research Unit.

Rabbits received 3 injections of virus grown on BHK cells, concentrated 100 times by PEG/NaCl and sonicated. They first received 5 ml intraperitoneally, and then 2 ml intravenously 2 and 4 weeks later. The rabbits were bled by cardiac puncture before the immunization started, and 2 weeks after the last injection. The antisera were adsorbed with packed BHK cells. All sera were inactivated at 56° C and stored at -20° C.

*Antispecies Antisera*

The antispecies sera used in these experiments were Rabbit antimouse Ig (RAM/Ig) and Rabbit anti-human Ig (RAHu/Ig) from Nordic Immunol. Lab., Tilburg, the Netherlands.

*Human Sera*

The human sera were selected from the files of the Virus Laboratory, Haukeland hospital, Bergen. The sera had been remitted for all kinds of suspected virological diseases and controls. Our only criterion for inclusion was that the patient should live in or near by areas infested by *I. ricinus*.

*The Original IEOP Method*

The technique is a modification of the method of ALTER *et al.* (1), employing the commercial Hepascreen electrophoresis apparatus (Spectra Biologicals, Oxnard, California). For the electrode trays as well as for the gel a barbital buffer with an ionic strength of 0.050 and a pH of 8.2 (Spectra Biologicals) is employed. Gels are made on precoated lantern slides, 8 × 8 cm. Twelve ml molten 1 per cent agarose are poured on to each slide, giving an average gel thickness of 1.9 mm. Three double-rows of 10 wells are cut in the gel. The well diameters and interdistances are 3 mm. Two slides are run in the apparatus at the same time. Running time is 60—90 minutes. The reagents are applied by the aid of a 10  $\mu$ l Oxford automatic pipette. In screenings, this technique permits the simultaneous testing of 58 unknown samples.

*Experimental Conditions*

The following parameters have been tested:

- a) The optimal antigen preparations and concentrations.
- b) The composition of gels. Mixtures of Bacto agar (Difco) and agarose (l'Industrie Biologique Française) from 0.1—0.9 to 0.9—0.1 per cent have been used.
- c) The effects of antispecies antiserum. After the termination of the first electrophoresis run, the antiserum well was filled with antispecies (anti-mouse or anti-human Ig) and the electrophoresis was continued for 30—60 minutes. This sandwiching should theoretically trace immunocomplexes which are not big enough to be read directly as precipitates.
- d) The effects of varying well-diameters and -interdistances.

*Confirmation of IEOP Results*

When screening unknown samples, the specificity of IEOP precipitation lines was secured by the sensitive gel precipitation method (CHI) (closed hexagon immunodiffusion) (18). Antigens were allowed to diffuse for 8—12 hours before filling of antiserum wells. Repeated fillings of wells were necessary in some instances. When needed, sera were concentrated by polyacrylamide gel (Lyphogel, Gelman Instr. Co.) (2).

In all IEOP runs, positive and negative controls for antigens as well as antisera were included.

*Haemagglutination Inhibition Test (HAI)*

HAI was performed according to CLARKE and CASALS (5), using microtitration equipment (Cooke Engineering Co., Alexandria, VA). For UUK viruses optimum conditions were pH 5.6 at 37° C, for Runde virus pH 6.4 at 4° C (22, 23).

*Complement Fixation Test (CFT)*

CFT was performed by standard procedures (10) using 2 units of complement in the final test. The optimum antigen dilutions used for antibody detection was found by checker-board titrations.

**Results***The Effect of Antigen Source and Concentration*

In 1 per cent agarose gel, crosstitrations showed that all 3 types of antigens had to be diluted to obtain maximal sensitivity in antibody-detection. The cell culture antigens had the highest titers, and also effected a 2—4-fold increase in the titers of the antisera. Sandwiching the reactions with rabbit anti-mouse serum gave only a two-fold rise in antibody titers. These results are illustrated in Table 1.

In contrast, if an unbalanced system is encountered, i.e. the antigen is too strong to attain optimal sensitivity, the effect of antispecies serum is much more striking, on occasions it has increased the titer of antiserum by a factor of 8.

*Varying Concentrations of Agar-Agarose*

A remarkable higher sensitivity was demonstrated by mixing increasing amounts of agar into the electrophoresis gel. The concentration of antigen needed for optimal conditions had to be elevated in parallel to the relative agar-concentration. By using a gel composed of 0.4 per cent agar and 0.6 per cent agarose, and concentrated BHK antigens, results comparable to the HAI titers were obtained for the reference sera with all the viruses tested. This is demonstrated in Table 2.

The reasons for the improvement was clearly seen by immunoelectrophoresis as demonstrated in Figure 1. With increasing agar concentration in the gel, more of the antibodies move cathodically, but at the same time the anodal movement of the antigens are hampered, so that more concentrated antigens are needed.

*Anti-Species Antiserum*

Under balanced conditions, i.e. near to equivalency, the effect of "sandwiching" is not very pronounced, raising end-point titers by 1—2 dilution steps

Table 1. *IEOP in 1 per cent agarose gel. Different antigen preparations titrated against a mouse antiserum with HAI titer<sup>a</sup> 640*

Antigen preparation	Dilution of ag <sup>b</sup> giving max. ab titer	Corresp. ab titer	Ab titer after RAM <sup>c</sup> for 1 hour
By E 50 SA	1/16	32—64	64—128
By E 50 SMB	1/16	32—64	64—128
By E 50 BHK	1/48	64—128	128—256

<sup>a</sup> Antibody titers are given as reciprocal titers

<sup>b</sup> Ag—antigen, ab—antibody

<sup>c</sup> Rabbit antimouse Ig

(Tables 1, 2). However, when unbalanced systems are met with, "sandwiching" is improving the results considerably (Table 2).

*Prozone*

The ability to detect antibodies and antigen increased to some extent with dilution of antigen and antibody as demonstrated by checker-board titrations.

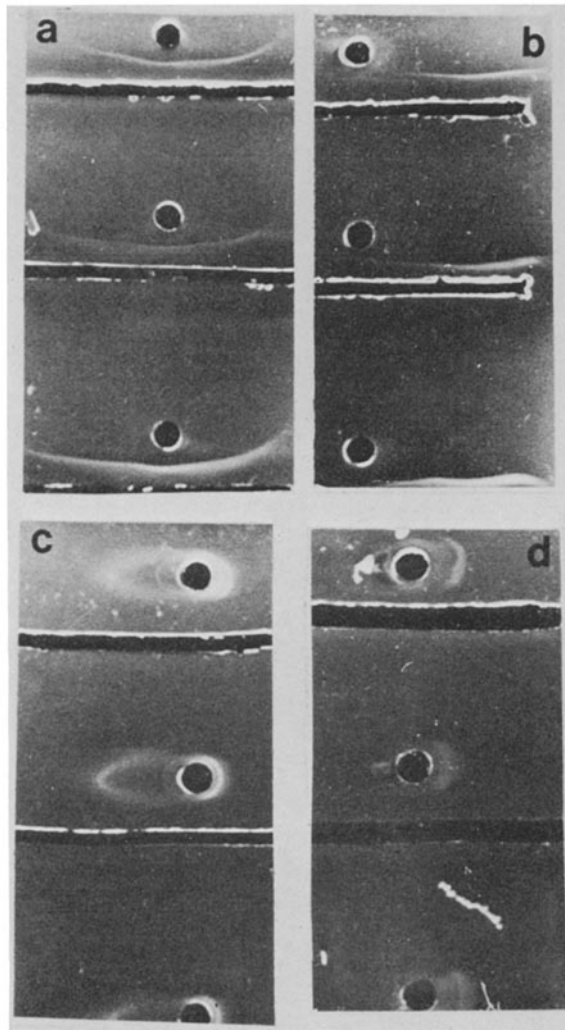


Fig. 1. Immunoelectrophoresis with Norwegian arboviruses, demonstrating the migration of antibodies and antigens in 1 per cent agarose gel (Figs. 1 a and 1 c) and in gel composed of 0.6 per cent Bacto agar and 0.4 per cent agarose (Figs. 1 b and 1 d). In Figures 1 a and 1 b mouse hyperimmune sera were applied in the wells, and electrophoresis was performed for 90 minutes. The corresponding viruses, SA antigens, were applied in the troughs, and diffusion took place overnight. In Figures 1 c and 1 d antigens were electrophoresed and the corresponding antisera applied in the troughs. The viruses used were from above: By E50, Ru E82 and Ru E81

A marked prozone was seen for all viruses. The prozone effect could be restricted to some degree by "sandwiching", but the effectiveness of this varied from one experiment to another, and could not be relied upon. Consequently, the optimal dilution of antigen is considered the one giving precipitation lines for all antibody-dilutions, discriminating the question of absolute end-point titer. These features are illustrated in Figure 2.

Table 2. *IEOP in varying concentrations of agar-agarose. (E50 mouse antiserum: HAI titer<sup>a</sup> 640)*

Antigen	Titers of E50 mouse antiserum									
	0—1% <sup>b</sup>		0.2—0.8%		0.4—0.6%		0.5—0.5%		0.6—0.4%	
	RAM <sup>c</sup>		RAM		RAM		RAM		RAM	
By E50 BHK	— <sup>d</sup>	+	—	+	—	+	—	+	—	+
dilut. 1/8	32	128	128	512	512	1024	256	512	8	64

- <sup>a</sup> Antibody titers given as reciprocal values
- <sup>b</sup> Concentration of agar mentioned first
- <sup>c</sup> Rabbit antimouse Ig in a second electrophoresis run
- <sup>d</sup> Minuses and pluses refer to titers before and after addition of antimouse serum

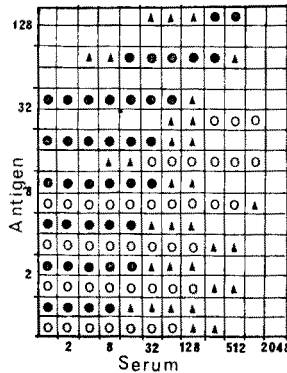


Fig. 2. Checkerboard titrations in IEOP of Ru E81 against its mouse antiserum Antigen: Virus grown in BHK 21/c 13 cells, concentrated 300 times by PEG 6000/NaCl. Antiserum: Reciprocal HAI titer 640. Filled circles: Reciprocal IEOP titers in 1 per cent agarose gel. Open circles: Reciprocal titers in 0.4—0.6 per cent agar-agarose. Triangles: Reciprocal titers after "sandwiching" with anti-mouse Ig

*Varying Well Diameters and Interdistances*

Increasing the well diameter to 4 mm and varying the interdistances between 3 and 8 mm gave no benefit in antibody detection. Increased interdistances effected the optimal running time adversely.

*Titration of Human Sera*

Some sera which had been found sero-positive to UUK virus in HAI were titrated in parallel in HAI, the original IEOP and IEOP with 0.4 per cent agar to 0.6 per cent agarose. The results are shown in Table 3. There was no correlation

between actual end point titers in HAI and IEOP. But it was clearly demonstrated that IEOP has been made comparable to HAI by the modifications used.

Table 3. *IEOP with human patient sera for detection of antibodies to the By E50 UUK strain. Antigen: E50 BHK antigen diluted 1/8*

Human serum	HAI titer	IEOP titers <sup>a</sup> in	
		0—1% <sup>b</sup>	0.4—0.6%, RAH <sup>c</sup> for 1 hour
103	40	4	64
120	80	2	32
779	40	8	128
1047	40	1	32
1121	20	2	32

<sup>a</sup> Antibody titers given as reciprocal values

<sup>b</sup> Concentration of agar mentioned first

<sup>c</sup> Rabbit antihuman Ig in a second electrophoresis run

Table 4. *Comparisons of the optimal results obtained in HAI, CFT and IEOP with selected viruses*

Antibody preparation	Optimal antigen in IEOP	Maximum titers <sup>a</sup> in		
		HAI	CFT	IEOP <sup>b</sup>
anti-Tahyna, rabbit <sup>c</sup>	BHK-antigen <sup>d</sup>	80—160	32—64	32—64
anti-Tahyna, mouse	diluted 1/4	640—1280	256—512	256—512
anti-Tribec, rabbit	BHK-antigen	—	64—128	64—128
anti-Tribec, mouse	diluted 1/2		256—512	256—512
anti-TBE, rabbit	Hela Bristol antigen	160—320	63—128	4—8
anti-TBE, mouse	Concentrated	1280—2560	256—512	8—16
anti-By E50 UUK, rabbit	BHK-antigen	80—160	32—64	64—128
anti-By E50 UUK, mouse	diluted 1/8	640—1280	256—512	512—1024
anti-Runde, rabbit	BHK-antigen	80—160	32—64	64—128
anti-Runde, mouse	diluted 1/4	320—640	128—256	512—1024

<sup>a</sup> Reciprocal of highest dilution giving positive reaction

<sup>b</sup> For TBE, gels consisted of 0.2 per cent agar—0.8 per cent agarose, all others 0.4 to 0.6 per cent was used

<sup>c</sup> Sandwiching with anti-rabbit antiserum has not been used

<sup>d</sup> Virus grown in BHK 21/c 13 cells, 300× concentrated by PEG 6000/NaCl

#### *The Specificity of the Method*

We consider the IEOP method described virus specific on the following grounds:

a) When one of the viruses used (UUK, Runde, TBE, Tribec, Tahyna) were run against antisera or ascitic fluids against all the others. no positive reactions were recorded. Checker-board titrations precluded that this was due to prozone-effect.

b) The precipitating antibodies of the antisera and mouse ascitic fluids could be adsorbed by using homologous virus-antigens, but not by using any heterologous antigen.

c) When anti-human antiserum was used to sandwich reactions with mouse antisera or immune ascitic fluids, and anti-mouse antiserum was used to sandwich human sera, no increase in titers was seen. Neither anti-human nor anti-mouse antisera could improve the results obtained by rabbit sera.

d) The UUK group viruses By E50 and Ru E82 had earlier displayed a one-way serological relationship in CHI (22). Antiserum to By E50 reacted with both viruses, while antiserum to Ru E82 reacted with the homologous virus phenomenon was seen also in IEOP.

#### *Comparison of Antibody Titers Obtained With Different Serological Tests*

Table 4 compares the titers obtained under optimal conditions in HAI, CFT and IEOP for Tahyna, Tribec, TBE, UUK and Runde viruses. It was demonstrated that, with an exception for TBE virus, the results obtained in IEOP can be favourably compared to HAI and CFT.

### **Discussion**

During the past few years, the IEOP method has found its main application in the detection of hepatitis B surface antigen (HB<sub>s</sub>Ag) (1, 7, 16, 20). It also has been used to some extent in the diagnosis of certain bacterial (6) and fungal infections (8). In virology, the IEOP method has been employed in studies of myxoviruses (11), poxvirus (13), herpes simplex (25) and some plant viruses (17). Recently, Tsotsos (25) examined the possibilities of using IEOP for typing and subtyping of various viruses belonging to major groups. However, very few researchers have investigated the possibility of using IEOP as a method for detection of viral antibodies. The main advantages of IEOP over other serological methods would be its simplicity, speed, the possibility of testing simultaneously many specimens, and its modest requirements for reagents and equipment. Obviously, however, the assumption has been that this could not compensate for the insensitivity of the method in detection of antibodies. Even so, IEOP modifications have been elaborated for screening of antibodies to influenza virus (4), California encephalitis virus (3), rubella virus (9) and measles virus (15). But in all these cases, the sensitivity in antibody detection seemed inferior to the methods routinely used for these viruses.

Even in the future, sero-ecological screenings will be an integrated part of efforts to evaluate the extent and significance of arboviruses. The most commonly used technique for this purpose has been HAI. In addition to the unreliability connected with unspecific inhibitors, HAI has certain other major disadvantages (apart from the fact that haemagglutination is not common to all arboviruses). The most serious objection to HAI as a sero-ecological tool is its very broad reactivity. Cross reactions occur between viruses which are not even transmitted by the same vector, but which might nevertheless circulate within the same areas. We have shown for the Norwegian arbovirus strains, as has already been demonstrated for other virus groups (25), that IEOP is virus type and even subtype



specific, and thus offers advantages over the HAI test in this regard for sero-ecological screenings. The low consumption of serum is another very important factor. In many instances very limited amounts of blood can be drawn from each individual of animal species that are to be investigated, and one often wishes to perform tests against more than one antigen.

The modifications which have been described in this paper to achieve a very sensitive IEOP test for antibody detection, are all simple and inexpensive. Some of the same effects have been obtained for hepatitis B diagnostics by employing discontinuous buffersystems (26). As pointed out by KĒLKAR and NĪPHADKAR (12) it is, however, a much simpler and cheaper solution to establish the optimal agar-agarose mixture for a given antigen/antibody system. High-grade concentration of sera is a laborious and timeconsuming task, and often is prohibited by the small volumes of blood available.

Provided the opportunity to concentrate the antigen, the modifications used in this work to obtain a sensitive IEOP for antibody detection might prove valuable also with other viruses. The relative failure experienced with TBE virus in these investigations may illustrate the problem of concentration. TBE has a considerably smaller molecular mass than the other viruses used, and according to TSOTSOS (25), this needs to be compensated for by the use of a more concentrated antigen.

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