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Mosquito-borne arboviruses in Norway: further isolations and detection of antibodies to California encephalitis viruses in human, sheep and wildlife sera

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SUMMARY

Seven virus strains antigenically related to the California encephalitis (CE) virus group were isolated from Norwegian *Aedes* spp. mosquitoes collected in 1976. So far CE viruses have been isolated from five different *Aedes* spp. in Norway. Furthermore, two virus strains related to the Bunyamwera group were isolated from *Anopheles claviger*.

Antibodies to CE viruses were demonstrated in 22% of 1014 military recruits tested. Among 91 soldiers who were monitored by monthly blood samples during the mosquito season, seroconversions were detected in 11 individuals. Specific IgM antibodies were found in seven of them. Disease symptoms in connexion with the CE virus infections were not seen. The prevalence of CE antibodies in patients with CNS or respiratory infections was not higher than in control groups. Seroconversions were not seen in any of the groups.

Screening of sheep sera from six different areas in northern Norway indicated significantly different degrees of CE virus activity. Passerine birds may be important CE virus hosts, while small rodents seem unimportant. Specific IgM antibodies were detected in the sera of one of three hares and one of two squirrels.

Of the methods used, single radial haemolysis (SRH) and immuno-electro-osmophoresis (IEOP) seemed to be well suited for serological screenings. However, an indirect immunofluorescence antibody test (IFAT) which was used may be an attractive alternative if high-quality anti-species conjugates are available. The haemagglutination-inhibition (HI) test used gave a high number of false positive results.

INTRODUCTION

The isolations of three virus strains related to the California encephalitis (CE) group from Norwegian *Aedes* spp. mosquitoes were reported in 1978 (Traavik, Mehl & Wiger, 1978). The mosquitoes were collected in 1975.

From mosquitoes collected in 1976, nine additional filterable agents, pathogenic for suckling mice, were isolated (Traavik, 1979*b*). Seven of these isolates were from *Aedes* spp., the last two were from *Anopheles claviger*. Results presented in this paper show that the agents are viruses, and that the isolates from *Aedes* spp. are all related to the CE group, while the viruses from *An. claviger* react serologically with a reference antibody preparation to the Bunyamwera group.

Some CE viruses from both North America and Europe can cause human disease, ranging from nonspecific fever to serious encephalitis, which may sometimes be followed by behavioural changes (Henderson & Coleman, 1971).

One of the earlier reported Norwegian CE strains caused a clinical CNS infection in the laboratory, probably by inhalation of infectious aerosol (Traavik, Mehl & Wiger, 1978).

The investigations described in this paper were aimed at giving a provisional distribution map for CE viruses in Norway, and providing some insight into the incidence of CE infections in man and his domestic animals. Finally, the sera of some selected human patients and military groups have been examined for antibodies to CE viruses. This was done in order to obtain preliminary data related to a hypothetical threat presented to human health by CE viruses.

MATERIALS AND METHODS

Mosquito collections

The collection sites were marked in Fig. 1, and relevant data in connection with the isolation materials are presented in Table 1. The collections are more thoroughly described elsewhere (Mehl, Traavik & Wiger, 1983). Briefly, mosquitoes attracted by the investigators and CO_2 baits were caught in fine-meshed nets, lightly anaesthetized in CO_2 containers, and aspirated into glass tubes by a special device (Mehl, 1983). The tubes were carefully closed with rubber stoppers, sealed with tape and immediately frozen on dry ice. During the intervals until isolation procedures were performed, all materials were kept at −70 °C.

Isolation procedures

Mosquitoes were removed from the −70 °C freezer and pooled according to species, sex, state of engorgement and collection site. The pools were triturated in PBS with bovine albumin and inoculated intracerebrally (ic) into mouse litters (Bom: NMRI, SPF), 1–3 days old. The procedures for harvest, filtration and passage of brain isolates were as earlier described (Traavik, Mehl & Wiger, 1978). As a precautionary measure, neither the 1975 Norwegian CE isolates nor the Tahyna prototype strain were handled in our laboratory during the period of isolation work.

Cell cultures

BHK 21/c13, KB and *Aedes albopictus* cells were originally purchased from Flow Laboratories, Irvine, Scotland. BHK cells were cultured in the Glasgow modification of Eagle's medium with new-born calf serum and KB cells in minimum essential medium (MEM) with foetal bovine serum (FBS) at 37 °C, while *A. albopictus* cells were cultured in Mitsuhashi and Maramorosch basal medium (Flow Labs) with

FBS at 28 °C. For haemagglutinating (HA) antigen production MEM was supplemented with bovine serum albumin instead of FBS.

Antigen production

HA antigens were made from infected KB cells as earlier described for BHK cells (Traavik, Mehl & Wiger, 1978). Briefly, virus from infected KB cells was concentrated 100 times by PEG 6000/NaCl precipitation, low-speed centrifugation and resolution of the virus-pellet in borate saline with 0·4 % bovine serum albumin (BABS), pH 9·0. We have earlier used sonication and colloid silica treatment to improve HA preparations. Since the untreated concentrates from KB cells constantly gave HA titres of 160–640, we used them in these studies without further treatment. In addition to cell culture preparations, third passage 10 % infected suckling mouse brain (SMB) suspensions were used as antigens in complement fixation test (CFT) and immunoelectro-osmophoresis (IEOP).

Antigens for fluorescent antibody tests (FAT) were produced by a modification of the method used for Hantaan virus (French *et al.* 1981; Traavik *et al.* 1983). Infected KB cells were trypsinized approximately 18 h post-infection. The cells were then re-seeded at a density of about 5×10^5 cells/ml on sterile 10-spot slides and cultured an additional 18 h, at which time they approached confluency. Five spots (one row) on each slide received infected cells, the other five received uninfected cells and served as negative controls. The slides were fixed in acetone and kept at −70 °C until used.

Sera

Reference hyperimmune ascitic fluids to the CE and the Bunyamwera groups were obtained from NIAID, Bethesda, Maryland. Rabbit antisera and mouse ascitic fluids to Tahyna virus and Norwegian CE virus isolates S 586 and S 618 were produced by conventional procedures, as earlier described (Traavik, Mehl & Wiger, 1978). In 1972/73 an epidemiological study of hepatitis B in the Norwegian armed forces was performed (Smevik & Traavik, 1974). By this time 7270 soldiers were screened for HB_sAg on admission to the armed forces. The seropositive soldiers and their closest colleagues were followed up with serum samples taken each month during the following year. Sera from this collection have now been tested for antibodies to CE viruses. The locations of the relevant military camps are shown in Fig. 1.

Sera from patients with symptoms of viral CNS and respiratory infections were sent to the Virus Diagnostic Laboratory of the University Hospital in Tromsø for investigation. We selected a panel of paired sera from patients whose symptoms began during the months of May to September (corresponding to the mosquito season) in the years 1978–80. Three control groups were chosen from the files of the Virus Diagnostic Laboratory: (*a*) healthy individuals (samples taken for serological controls in connexion with pregnancy and/or vaccinations), (*b*) patients with CNS or respiratory symptoms which began during the months of November to March and (*c*) patients with other infections whose symptoms began during the arbovirus season.

Sheep sera were selected from the files of the National Institute of Veterinary Medicine, Oslo. The sera had been collected as part of a screening programme on

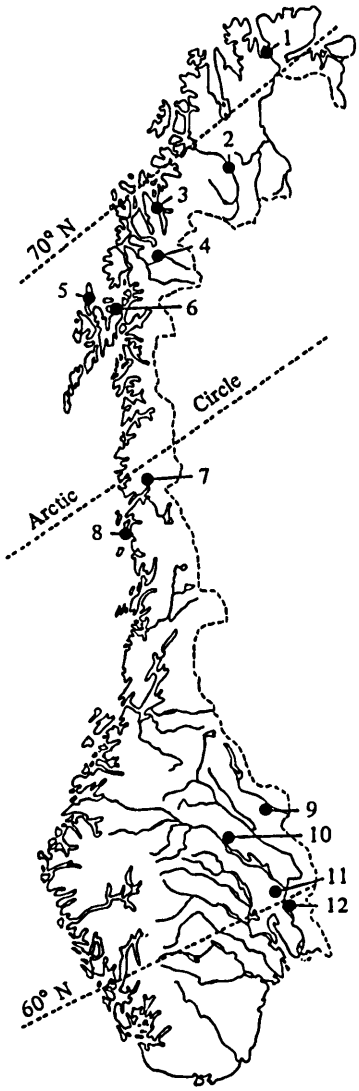

Fig. 1. Map of Norway showing localities for collections of sera and viruses. 1, Tana; 2, Masi; 3, Lyngseidet; 4, Setermoen; 5, Andøya; 6, Harstad; 7, Rana; 8, Tjøtta; 9, Trysil, 10, Sjusjøen; 11, Trandum, 12, Øyeren. Serum samples from sheep were collected in locations 1, 3, 5, 6, 7 and 8. Serum samples from soldiers were collected with monthly intervals in locations 4, 5 and 11. Viruses were isolated from mosquitoes collected in locations 9, 10 and 11. CE viruses were earlier isolated from mosquitoes in locations 2, 11 and 12 (Traavik, Mehl & Wiger, 1978).

maedi in Norway. A total of 238 sera from sheep from northern Norway were tested for antibodies to CE viruses. The locations included are shown in Fig. 1.

Sera from small rodents, passerine birds and other wildlife were collected during arbovirus (Traavik, 1979*a*, *b*) and other investigation programmes during the 1960s and 1970s.

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Fractionation of sera

To evaluate the specificity of HI results, serum antibodies and non-specific inhibitors (lipoproteins) were separated by density centrifugation in NaBr as described by Blom & Haukenes (1974). Due to interference of NaBr with erythrocyte settling patterns, the bottom (immunoglobulin) fractions were dialysed overnight against three changes of borate saline buffer before being tested for HI activity. Separation of 7S and 19S (IgG and IgM) antibodies were performed by a conventional density-gradient centrifugation test (DGCT) in sucrose (Rawls & Chernesky, 1976). Identification of human 7S and 19S antibodies and of 7S antibodies from sheep was done by a modified Ouchterlony gel precipitation technique (Traavik, Siebke & Kjeldsberg, 1972).

Methods for virus identification and antibody detection

The procedures for HI, CFT and IEOP have been described in detail previously (Traavik, 1977; Traavik, Mehl & Wiger, 1978). Single radial haemolysis (SRH) was performed exactly as described by Duca *et al.* (1979). Preliminary results with CE reference sera indicated a good correlation between SRH diameters and HI titres.

The indirect fluorescent antibody test (IFAT) was used to compare CE virus isolates antigenically. The technique was also employed to detect human IgG or IgM antibodies and sheep and small rodent IgG antibodies to CE viruses. We have given a detailed account of IFAT as it was used on spot slides to detect Nephropathia epidemica antibodies (Traavik *et al.* 1983). Anti-human, -sheep and -mouse FITC conjugates of high quality were purchased from Litton Bionetics, Kensington, Maryland. Preliminary experiments demonstrated that the anti-mouse IgG antiserum reacted with 7S antibodies from wood mice (*Apodemus sylvaticus*) as well as from bank voles (*Clethrionomys glareolus*) and field voles (*Microtus agrestis*).

RESULTS

Viruses isolated from Aedes *spp. mosquitoes*

Seven filterable agents were isolated by intracerebral inoculations of suckling mice with suspensions of *Aedes* spp. mosquitoes (Table 1). The three different collection localities are mapped in Fig. 1. Five strains were from *A. communis* collected at Trandum, one was from *A. punctor* collected at Sjusjoen, and the last was from a pool of *Aedes* mosquitoes collected in Trysil. The individuals in the latter pool could not be determined as to species because they lacked parts which were vital for classification.

The exact compositions of the virus-yielding *Aedes* spp. pools are shown in Table 2. After the original isolations by suckling mouse brain inoculations, it was possible to pass all seven agents in suckling mice, and also in KB and BHK cells. The four isolates which were tested were shown to multiply also in *A. albopictus* cells (Table 2).

All the seven filterable agents were shown to belong to the CE virus group (Table 3). They reacted with antibodies to the CE group, Tahyna (European prototype) virus and the Norwegian S 586 CE virus isolate (Traavik, Mehl & Wiger,

Table 1. *Composition of the mosquito collections from the three virus-yielding localities*

Mosquito species	Locality and date: Trandum 11–18 June 1976 Mosquitoes	Pools	Isolates	Sjusjøen 11 Aug. 1976 Mosquitoes	Pools	Isolates	Trysil 8 Aug. 1976 Mosquitoes	Pools	Isolates
A. communis	1710	21	5	—	—	—	—	—	—
A. diantaeus	57	3	0	—	—	—	—	—	—
A. punctor	50	2	0	234	4	1	38	1	0
A. excrucians	7	1	0	—	—	—	—	—	—
Aedes spp.	80	1	0	—	—	—	370	3	1
An. claviger	502	7	2	—	—	—	—	—	—
Total	2405	35	7	234	4	1	408	4	1

Table 2. *Virus isolations from mosquitoes collected in* 1976

Virus isolate	Collection site	Pool composition: Mosquito	Number	Sex	Passage in: SMB*	KB	BHK	A.alb.†	Identification
S 16/17	Trysil	*Aedes* spp.	170	F	+	+	+	+	CE group
S 20/76	Sjusjøen	*A. punctor*	13	F	+	+	+	+	CE group
S 43/76	Trandum	*A. communis*	100	F	+	+	+	+	CE group
S 45/76	Trandum	*An. claviger*	9	M	+	−	−	−	Bunyamwera
S 46/76	Trandum	*A. communis*	100	F	+	+	+	NT‡	CE group
S 51/76	Trandum	*A. communis*	100	F	+	+	+	NT	CE group
S 54/76	Trandum	*A. communis*	100	F	+	+	+	+	CE group
S 60/76	Trandum	*An. claviger*	100	F	+	−	−	−	Bunyamwera
S 65/76	Trandum	*A. communis*	100	F	+	+	+	NT	CE group

* Suckling mouse brain.
† *Aedes albopictus* cells
‡ Not tested.

1978) by all the serological methods employed. No significant difference was found between the newly isolated strains, nor were there any antigenic differences between the new strains and Tahyna or S 586.

According to our data (Table 1) the minimum field infection rate for CE virus in *A. communis* was 1 in 342 at Trandum at the time of collection.

Virus isolates from An. claviger

The two filterable agents from *An. claviger*, one from 100 females, the other from nine males, had been shown previously to be unrelated to Tahyna, WEE, EEE, UUK, Kemorovo or TBE viruses (unpublished results). In these studies the two virus strains (Table 1 and 2) did not show any CFT activity against CE group, Tahyna or S 586 antibody preparations. However, both virus strains reacted specifically with the Bunyamwera hyperimmune ascitic fluid. The CFT titres were low (10–20), but reproducible. The activity was shown by fractionation to be due to the 7S immunoglobulins of the ascitic fluid. We could not demonstrate infectivity, haemagglutinins or FAT antigens in any of the cell cultures used.

Table 3. *Serological identification of the virus isolates from* Aedes *spp. mosquitoes*

Virus strain	Antibody titres using stated antisera											
		CE group			Tahyna				S 586			
	HI	CFT	IFAT	IEOP	HI	CFT	IFAT	IEOP	HI	CFT	IFAT	IEOP
Tahyna	80	40	160	32	640	320	640	256	320	80	320	128
S 586/75	40	20	40	16	160	80	320	64	640	160	640	256
S 618/75	80	40	80	32	640	160	640	256	320	80	320	128
S 16/76	80	40	80	32	320	80	320	128	320	80	160	128
S 20/76	80	40	40	8	160	80	320	128	320	80	640	128
S 43/76	40	20	80	16	320	160	160	256	320	80	640	128
S 46/76	40	20	40	16	160	160	320	128	160	160	320	128
S 51/76	40	40	80	8	320	320	320	256	160	160	160	128
S 54/76	40	20	80	16	320	160	320	128	160	80	160	128
S 65/76	80	40	80	32	160	80	320	128	320	80	160	128

CE antibody screening of human sera

The 1014 serum samples from the soldiers were selected according to their place of residence so that most parts of the country were included. All sera were screened by HI for antibodies to the Norwegian CE strains S 586 and S 618 (Traavik, Mehl & Wiger, 1978). HI activity was found in 243 sera. By using SRH, 219 out of these 243 sera gave positive reactions. The 24 SRH-negative sera had HI titres of 10–40, and six of them had shown HI activity to only one of the two viruses. The 24 SRH-negative, HI-positive sera and 20 selected sera which were both SRH and HI positive were fractionated on NaBr gradients. HI activity in the bottom (immunoglobulin) fractions was demonstrated for all 20 SRH-positive, but for none of the 24 SRH-negative, HI-positive sera.

The prevalence of CE virus antibodies in the tested population determined by SRH was 22 % (219/1014). By IFAT 206/219 (94 %) SRH-positive sera were shown to contain IgG antibodies to CE viruses, and by IEOP 184/219 sera (84 %) demonstrated precipitation lines.

Twenty selected sera with HI titres > 160 and significant SRH reactions were fractionated by DGCT. None of these sera had HI activity in the 19S IgM fraction, while activity in the 7S fraction was demonstrated for all sera. The same 20 sera were tested for specific IgM antibodies by IFAT and all were negative.

Human seroconversions

Forty-one soldiers from Trandum, 34 from Andøya and 16 from Setermoen (Fig. 1) were screened each month from April to December 1972. In April 14 of 41 soldiers at Trandum, 3 of 16 at Setermoen and 6 of 34 at Andøya had antibodies to CE virus. Seroconversions occurred at Trandum (6 of 27; 22 %) and Setermoen (5 of 13; 40 %). All seroconversions took place between June and September 1972. Clinical signs were not reported for any of the seroconverting soldiers. Antibodies to CE viruses in 6 of 11 of these soldiers were in the 19S IgM class. The sera from these six and the serum from one additional seroconverting soldier were positive also by the IFAT IgM test.

Selected patient groups

We found no evidence that linked CNS or respiratory disease to CE viruses in northern Norway. No seroconversion was demonstrated in the paired sera. No serum with high antibody content according to HI (titre > 160) and SRH demonstrated specific IgM antibodies.

The CE antibody prevalence rates for patients showing symptoms during the mosquito season were 17 % (9 of 52) for CNS, also 17 % (11 of 66) for respiratory and 21 % (18 of 84) for other infections.

During the part of the year when mosquitoes are not active, 19 % (18 of 93) of the patients showing CNS or respiratory infections had antibodies to CE viruses, and the same rate (19 %, 23 of 120) was found among healthy control subjects.

Antibodies to CE viruses in sheep sera

The variation in prevalence of seropositive animals between the different areas (Fig. 1) was considerable, ranging from 75 % in Lyngseidet to only 7 % at Andøya

Table 4. *Prevalence of CE virus antibodies in sheep*

Location	Number of sheep tested	Number seropositive by: HI	SRH	IEOP	IFAT	Positives* (%)
Tana	58	28	23	19	21	40
Lyngseidet	20	18	15	11	14	75
Harstad	49	23	20	18	20	41
Andøya	46	4	3	2	3	7
Rana	25	13	11	8	10	44
Tjøtta	40	12	8	8	8	20
Grand total	238	98	80	66	76	34

* Based on results obtained by SRH.

(Table 4). Eighteen sera which were positive by HI and negative by SRH were fractionated on NaBr gradients. No HI activity was found in the immunoglobulin fractions of these sera. For 20 sera positive by all four serological methods used, specific immunoglobulins were found after fractionation. After DGCT separation we could not detect HI activity in the 19S region for any of 20 high-titred (> 160) sera.

Wildlife sera

A total of 322 small rodent sera were tested by HI, SRH and IFAT. HI activity to CE viruses was detected for 12 animals, and in 9 of these antibodies were also detected by SRH. This comprised 1 of 41 field voles (*Microtus agrestis*), 2 of 56 wood mice (*Apodemus sylvaticus*) and 6 of 225 bank voles (*Clethrionomys glareolus*). These wood mice and bank voles were also found positive by IFAT, while the field vole serum was negative by this method. The limited serum volumes prohibited fractionation and determination of specific IgM antibodies.

Twenty-six passerine bird sera were examined by HI and SRH. Eighteen of the sera were positive by HI, while none reacted in SRH. After NaBr fractionation, HI activity was detected in the immunoglobulin fraction for 14 of these sera, (54 %).

We have tested sera from three hares (*Lepus timidus*) and two squirrels (*Sciurus vulgaris*). Two of the hare sera gave positive HI reactions. One had a titre of 80, the other of 1280. Both sera were positive also by SRH, and after NaBr fractionation HI activities were detected in the immunoglobulin fraction. HI reactions were detected in both the 7S and 19S fractions for the serum with titre 1280, but only in the 7S fraction for the serum with titre 80.

One of two squirrel sera tested had an HI titre of 2560, while the other was negative. The former serum was positive also by SRH, and HI activity was found in the immunoglobulin fraction after NaBr separation. HI activity was demonstrated in both the 7S and 19S fractions.

DISCUSSION

The results of these investigations demonstrate that CE viruses are widespread in Norway, and thus confirm and extend findings that we have reported earlier (Traavik, Mehl & Wiger, 1978; Traavik, 1979*a*, *b*).

Norwegian CE virus have now been isolated from five *Aedes* spp. mosquitoes collected in five different locations in both southern and subarctic areas. Yet we still do not know whether any of these species are true biological vectors (Henderson & Coleman, 1971; LeDuc, 1979; Parkin, Hammon & Sather, 1972).

Two virus strains isolated from *An. claviger* were shown to be related to the Bunyamwera group of the *Bunyaviridae* (Porterfield *et al.* 1974). The methods employed did not allow a more precise identification of these virus strains. In Finland an epizootic of Batai virus, a member of the Bunyamwera group, seems to have occurred in the early 1960s (Brummer-Korvenkontio, 1974; Brummer-Korvenkontio & Saikku, 1975). The vector of this virus is assumed to be *An maculipennis*. Batai virus seems to occur epidemically in Finland. It has been hypothesized that the virus is brought in by insect migrations from south-eastern Europe (Brummer-Korvenkontio & Saikku, 1975). Whether the Norwegian Bunyamwera viruses are endemic or regularly imported is not known. *An. claviger* is, however, a constant part of the fauna in the area where the virus-carrying mosquitoes were. Also, one of the isolates was from male mosquitoes, indicating the possibility of transovarial virus transmission (Watts *et al.* 1973).

The present investigations have shown that CE virus infection of man is not uncommon in Norway. The mean antibody prevalence (approximately 20%) detected among military recruits and selected patient groups is similar to the results from antibody screening of Finnish populations (Brummer-Korvenkontio & Saikku, 1975).

In Finland there is no indication that CE viruses cause human disease. In Czechoslovakia, however, the CE virus type Tahyna has been connected with both influenza-like disease and symptoms of CNS infection (Simkova & Sluka, 1973; Bardos *et al.* 1975). The Norwegian CE virus strain S 586 caused clinical CNS infection following a laboratory accident (Traavik, Mehl & Wiger, 1978). During the present investigations we detected 11 cases of recent CE virus infection among Norwegian soldiers. Clinical symptoms were not seen in any of the infected individuals, but the cases have not been followed up by neurological examinations. North America CE virus strains have been shown beyond doubt to cause clinical CNS infections, and suggestions have been made that such infections may result in lasting mental disability (Henderson & Coleman, 1971; LeDuc, 1979; Parkin, Hammon & Sather, 1972).

In Norwegian patients with signs of CNS and respiratory infections during the arbovirus season, the prevalence of CE antibodies was not different from that found in healthy subjects and other control groups. No seroconversion or significant rise in antibody titre was detected in paired sera. IgM antibodies, as a sign of recent infection, were not detected in any of these patients. So far our investigations do not indicate that Norwegian CE viruses represent a serious threat to human health.

The antibody screenings in sheep demonstrated that the CE virus activity varies

considerably between different locations in northern Norway. IgM antibodies were not detected in any of the sheep. The reason for this is probably that the serum samples were taken in the spring, at a time when *Aedes* mosquitoes are not active in those parts of the country.

Antibody screening of wildlife sera indicated that small rodents are not important as CE virus hosts in Norway, whereas passerine birds may be important in the biotopes included in the investigations. Studies in Finland seemed to exclude both small rodents and passerine birds as CE virus hosts (Brummer-Korvenkontio, 1974; Brummer-Korvenkontio & Saikku, 1975). Lack of SRH-reactivity for passerine birds may be due to the inability of avian antibodies to fix complement.

Hares and squirrels are the main hosts for various CE virus strains in other parts of the world (Henderson & Coleman, 1971; Leduc, 1979; Parkin, Hammon & Sather, 1972). Although the number of animals tested is very limited, our results indicate that these animals may play a role in CE virus ecology in Norway.

Various serological methods were employed in these investigations. HI following kaolin absorption seemed to be an unreliable method for CE virus diagnostics and sero-ecological screenings. A considerable number of positive HI reactions were due to residual non-specific inhibitors in the sera. Both SRH and IEOP seem to be methods of practical importance, being specific, easy to perform and consuming minimal volumes of sera.

In the versions we have used, SRH was more sensitive for antibody detection than IEOP. The modified IFAT employed in these studies may be an attractive alternative provided that satisfactory anti-species FITV-conjugated antibodies are available. For diagnosis of human CE virus infections a somewhat different IFAT method has recently been shown to be useful (Beaty *et al.*, 1982).

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