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From the Department of Veterinary Virology and Immunology and Department of Pathology, Royal Veterinary and Agricultural University, Copenhagen, Denmark and Danish Fur Breeders Association, Glostrup, Denmark.

COUNTER CURRENT LINE ABSORPTION IMMUNOELECTROPHORESIS IS AN ALTERNATIVE DIAGNOSTIC SCREENING TEST TO COUNTER CURRENT IMMUNOELECTROPHORESIS IN ALEUTIAN DISEASE (AD) ERADICATION PROGRAMS

By

Bent Aasted, Søren Alexandersen, Anders Cohn and *Mogens Hansen*

AASTED, BENT, SØREN ALEXANDERSEN, ANDERS COHN and MOGENS HANSEN: *Counter current line absorption immunoelectrophoresis is an alternative diagnostic screening test to counter current immunoelectrophoresis in Aleutian disease (AD) eradication programs.* Acta vet. scand. 1986, 27, 410—420. — Counter current immunoelectrophoresis (CCIE) is the diagnostic method used in the ongoing Aleutian disease virus eradication program on Danish mink farms. There has been an increasing demand for an alternative diagnostic test especially to evaluate suspected false positive CCIE reactions. We compared test results of a number of negative and positive mink sera in indirect counter current immunoelectrophoresis (ICCIE), counter current line absorption immunoelectrophoresis (CCLAIE) and radio immuno assay (RIA) with test results from counter current immunoelectrophoresis and found that counter current line absorption immunoelectrophoresis is the best alternative diagnostic screening test to counter current immunoelectrophoresis for Aleutian disease eradication programs. Not only proved the CCLAIE test to be useful for evaluation of doubtfully positive CCIE reactions, but it was found to have a higher sensitivity than the CCIE test.

Aleutian disease virus; mink; parvovirus.

Mink plasmacytosis (Aleutian disease) is caused by a persistent parvovirus infection (for reviews see *Porter et al.* 1980, *Lodmell & Portis* 1981, *Aasted* 1985). The etiological agent is known as Aleutian disease virus (ADV). The disease is responsible for great losses in mink production. As a consequence of this, a Dan-

ish eradication program started in 1976. At that time all of 53 farms tested had ADV-infected animals and 65 % of 30,000 tested mink from the 53 farms had antibodies to ADV. In 1985, 2800 farms (65 % of all Danish mink farms) had joined the program, and 30 % of all mink farms were declared A farms. A farms are farms, in which no AD positive mink have been detected for the last 3 test periods (1½ year). In 1985, 50 % of the Danish mink farms were free from ADV infection as judged by one test only.

The diagnostic test used in this huge screening program is counter current immunoelectrophoresis (CCIE, *Cho & Ingram* 1972), which is a fast, easy and quite sensitive test with high specificity.

Occasionally a few mink sera from A farms are found to be positive in the CCIE test. There are several possible reasons for such findings: 1) An ADV infection has taken place at the A farm. 2) The mink may have harboured trace amounts of ADV for years without producing antibodies in sufficient concentration for the diagnostic method to record the serum as positive. Suddenly an activation of the infection takes place and the mink produces antibodies in high enough concentration to become positive. 3) A virus serologically crossreacting to ADV has infected the mink, causing a production of antibodies which makes the CCIE positive (no such virus is presently known). 4) Other false positive reactions in CCIE (for instance precipitation lines which are not caused by ADV-antigen-antibody reactions). Some of these reactions are known as zone phenomena, which are precipitations of unknown composition close to the serum containing well in CCIE. 5) Personal errors at various levels of the diagnostic procedure and handling of the files.

The purpose of this study was initially to find an alternative diagnostic screening test to CCIE to be used in situations where doubtful positive reactions were found in CCIE for instance in serum samples from A farms. We compared the reactions of a panel of negative and positive sera in CCIE as well as in other immuno assays. The alternative tests chosen were the indirect counter current immunoelectrophoresis (ICCIE, *Aasted & Cohn* 1982), counter current line absorption immunoelectrophoresis CCLAIE, *Alexandersen et al.* 1985) and the most sensitive of the assays for anti-ADV antibody detection, which is a direct binding radio immuno assay (RIA, *Aasted & Bloom* 1984).

MATERIALS AND METHODS

Counter current immunoelectrophoresis (CCIE)

Counter current immunoelectrophoresis was performed as described by *Cho & Ingram* (1972) except that 0.7 % agarose gels (HSA agarose, Litex, Glostrup, Denmark) were used instead of the 1 % described. The barbital buffer system was the one published by *Cho & Ingram* (1972). The antigen source was in vitro produced ADV-G (10 µl per well of titer 1 material, kindly donated by the Research Foundation of Danish Fur Breeders Association).

Indirect counter current immunoelectrophoresis (ICCIE)

Indirect counter current immunoelectrophoresis was performed as described by *Aasted & Cohn* (1982). In this technique 5 µl of the serum and 5 µl of ADV-G antigen (titer 2) is mixed together and incubated for 5 min before electrophoresis against a known positive serum. If the antigen is not bound to antibodies in the well, the antigen will migrate into the agarose gel and give a precipitation line with the applied antibody. The agarose and buffer conditions were the same as for CCIE.

Counter current line absorption immunoelectrophoresis (CCLAIE)

Counter current line absorption immunoelectrophoresis was performed as described by *Alexandersen et al.* (1985) for the thin layer modification of this technique, but with some modifications to adapt the test for mass screening. A mould was constructed for making gels of 1.5 mm thickness containing 96 wells (hemispherically shaped) each with a volume of 7 µl. This mould is shown in Fig. 1. The agarose and buffer constitutions were the same as for the CCIE and ICCIE, except that NaCl was added to a final concentration of 0.15 mol/l. In one of the troughs 200 µl of ADV-G (titer 2) was pipetted and in the other trough 200 µl of a known positive serum (diluted 1:10) was added. Fig. 2 shows an example of the precipitation patterns of some negative and positive sera.

Radio immuno assay

The radio immuno assay (RIA) was carried out according to *Aasted & Bloom* (1984). The method consists of a two step direct

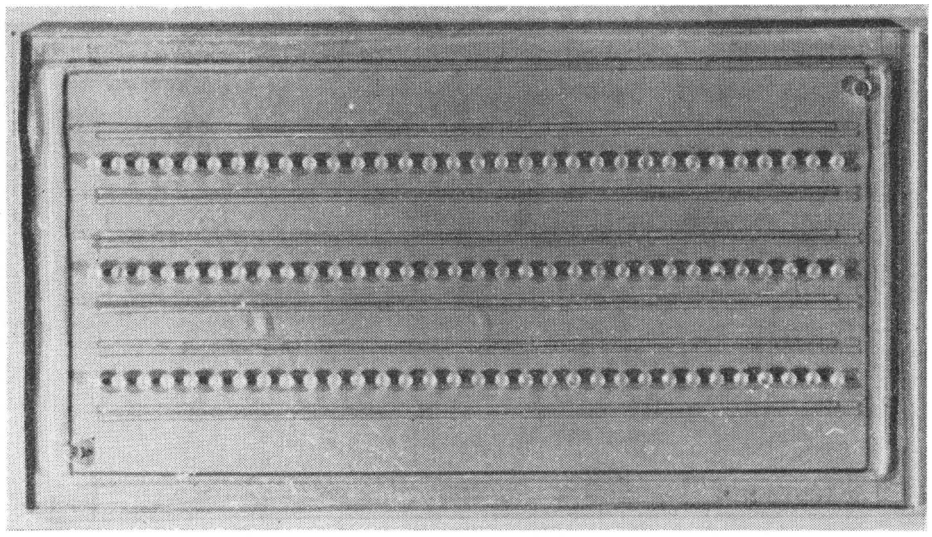

Figure 1. Plastic mould for preparation of 1.5 mm agarose gels containing 96 wells and 6 troughs to be used for the counter current line absorption inmmunoelectrophoresis technique.

binding RIA using antigen (a highly virulent DK strain of ADV) coated plastic beads incubated with serum for 2 h at 37°C followed by 3 times rinsing in a phosphate buffered saline solution and incubation with 50,000 cpm of 125 iodine labelled protein A (New England Nuclear, Mass., USA) for ½ h at 37°C followed by 3 times rinsing. The beads were then counted in a gamma-counter.

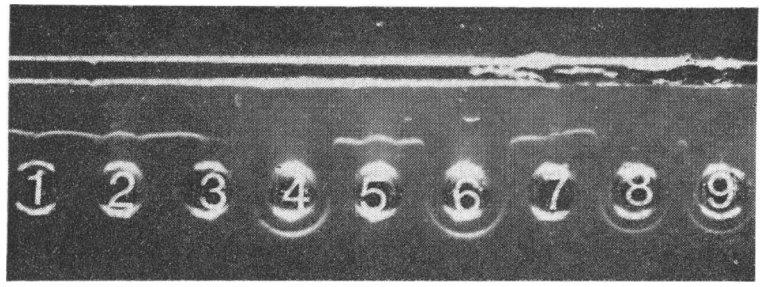

Figure 2. Positive and negative sera analysed by the counter current line absorption immunoelectrophoresis technique. Serum no. 4, 6, 8 and 9 are positive. The others are negative.

Test conditions

Sera from different mink farms were blind tested. Most of the recordings were done on unstained agarose plates, but in some instances recordings were made on stained plates. The staining procedure was as described by *Svendsen et al.* (1983).

Serum samples

We collected 3321 sera from different sources: 272 sera were obtained from a farm with no positive animals as judged by testing in CCIE for 1½ year (A farm). Fifty sera were from experimentally infected mink, and 2999 sera were collected from 15 farms with different levels of ADV infection (from beavily positive to almost negative farms).

RESULTS

Analyses of mink sera in CCIE, ICCIE and CCLAIE

Five µl of undiluted samples from each serum were analyzed in the different electrophoretic methods (the RIA findings will be presented later in this section). Table 1 shows the results. One serum out of the 272 sera from the farm with no known positive animals was diagnosed as positive. Retesting of this serum and several sera taken from the same animal later also gave a positive reaction in the CCLAIE test.

The 50 sera from experimentally ADV infected mink all showed positive reactions by the 3 methods.

Regarding the sera from the 15 different ADV infected farms the general pattern was that the CCLAIE test was considerably more sensitive than the CCIE. Altogether the CCLAIE scored 275 more sera as positive than the CCIE, or 9 % more of the 2999 sera from the infected mink farms. The ICCIE was shown to be more sensitive than the CCIE.

Of the 3321 sera tested in total, 11 sera were recorded to give positive reactions in CCIE but negative reactions in CCLAIE. With the higher sensitivity of the CCLAIE, these sera most likely should be regarded as giving false positive reactions in CCIE, a wellknown phenomenon discussed in an earlier paper by *Alexandersen et al.* (1985).

Table 1. The reactivities of 3321 sera in counter current immunoelectrophoresis (CCIE), indirect counter current immunoelectrophoresis (ICCIE) and counter current line absorption immunoelectrophoresis (CCLAIE)[1].

	Number of sera	CCIE		ICCIE		CCLAIE		% positive as judged by CCLAIE
		neg.	pos.	neg.	pos.	neg.	pos.	
Negative farm	272	272	0	271	1[2]	271	1[2]	0.4
Exp. infected mink	50	0	50	0	50	0	50	100
ADV infected farm no 1	238	13	225	6	232	5	233	98
ADV infected farm no 2	36	19	17	ND	ND	8	28	78
ADV infected farm no 3	408	216	192	ND	ND	106	302	74
ADV infected farm no 4	583	237	346	ND	ND	236	347	60
ADV infected farm no 5	68	31	37	ND	ND	30	38	56
ADV infected farm no 6	34	23	11	ND	ND	18	16	47
ADV infected farm no 7	162	130	32	ND	ND	88	74	46
ADV infected farm no 8	124	90	34	ND	ND	72	52	42
ADV infected farm no 9	144	119	25	ND	ND	92	52	36
ADV infected farm no 10	208	171	37	ND	ND	155	53	25
ADV infected farm no 11	191	158	33	ND	ND	156	35	18
ADV infected farm no 12	257	237	20	ND	ND	207	50	19
ADV infected farm no 13	103	97	6	ND	ND	96	7	7
ADV infected farm no 14	248	241	7	ND	ND	238	10	4
ADV infected farm no 15	195	193	2	ND	ND	193	2	1
Total number of sera	3321							

[1] The reactions were recorded on the unstained plates.
[2] One serum gave a weak positive reaction in both ICCIE and CCLAIE, but was negative in CCIE.
ND: Not Done.

Comparison of the sensitivities of CCIE, ICCIE and CCLAIE

Ten positive sera of various strenghts were titrated using two fold dilution steps (starting from 1:10 serum dilutions) and analyzed in the 3 tests. Fig. 3 shows an example of the precipitation patterns in the different techniques and Table 2 summarizes the average titers and the standard deviations (SD) of the titers.

Radio immuno assay (RIA)

The RIA setup chosen for the present comparative study is a very sensitive two-step direct binding test (*Aasted & Bloom* 1984). Strongly positive sera have been shown to react with significantly positive binding values (when compared to buffer

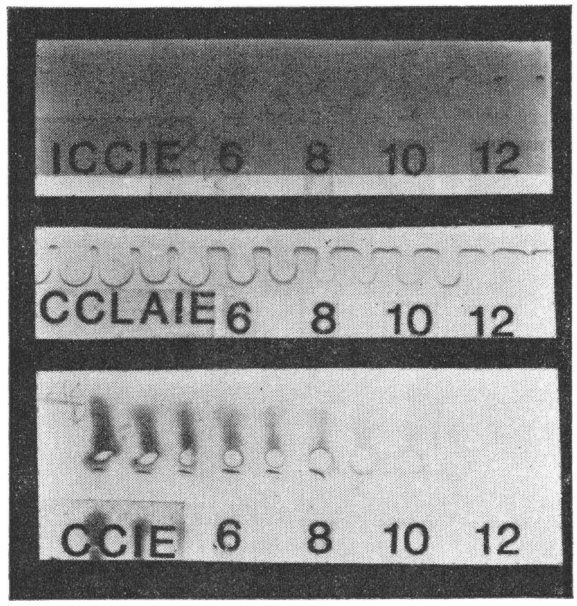

Figure 3. Comparative titrations of a single serum in indirect counter current immunoelectrophoresis (ICCIE), counter current line absorption immunoelectrophoresis (CCLAIE) and counter current immunoelectrophoresis (CCIE). The numbers refer to log 2 values of the dilutions. The measured titer in CCIE was estimated to 8 (i.e. $1:2^8$ or 1:256). The titer in ICCIE was estimated to 10 (1:1024) and the titer in CCLAIE was estimated to 12 (1:4096).

Table 2. Log 2 values of mean titres and standard deviation of 10 positive sera titrated in two-fold dilutions in counter current immunoelectrophoresis (CCIE), indirect counter current immunoelectrophoresis (ICCIE) and counter current line absorption immunoelectrophoresis (CCLAIE). The titration data were recorded both on unstained and stained plates.

	Unstained		Stained	
	mean	(SD)	mean	(SD)
CCIE	8.1	(0.9)	9.2	(1.6)
ICCIE	9.1	(1.2)	11.1	(1.3)
CCLAIE	10.8	(1.5)	12.5	(1.7)

controls) up to a dilution of $1:10^7$. The choice of this RIA, however, was soon realized to be a mistake for the following reason: We first analysed the binding values of approximately 100 sera

from a farm with no positive animals as judged by CCIE testing. The average binding value + 3 standard deviations was defined as the limit between negative and positive sera (i.e. assuming a normal distribution of binding recordings less than 1 % of negative sera would be recorded as positive). The negative sera, however, were found to give very large deviations, which means that the marginal value was so high that we later experienced that sera recorded as weakly positive in the 3 agar tests were recorded as negative in the RIA test. We therefore decided not to proceed with the RIA studies.

DISCUSSION

The purpose of the study was to find a suitable alternative diagnostic method to CCIE for the ongoing AD mass screening program in Denmark. As discussed in the introduction, the CCIE is a fast, easy and quite sensitive method and therefore suitable for the mass screening program. But being a direct binding assay, falsely positive reactions are found (see the introduction and *Alexandersen et al.* 1985).

Generally, precipitation reactions in gels have the advantage over methods like RIA and ELISA that the precipitation reactions (patterns) can be seen directly. Trained personnel can often differentiate whether a precipitate is true or false. In RIA or ELISA, radioactive counts or colour intensities are recorded solely. False positive reactions (for example false immunoglobulin containing precipitates on the plastic surface) can give the same final radioactive counts as a real positive reaction. There are no visualization of these reactions. Only values are given. It is likely that this is what happened in the RIA in this study. The negative sera used in this assay were not all of the best quality. During the transport from the farm to the test laboratory the samples sometimes are exposed to rather extreme temperature shifts. In the winter time they are sometimes frozen (hemolysed) and in the summer time exposed to rather high temperatures. These conditions could cause some protein to denature and it is easier for denatured protein to precipitate on glass or plastic surfaces. Such denaturation might have happened with the sera from the A farm which gave so high standard deviation in the RIA.

The inhibition techniques such as CCLAIE and ICCIE have the advantage over the direct binding techniques (such as CCIE), that they offer a specificity control. In the two inhibition techniques used in this study, a precipitate is always expected to form during the electrophoresis, between the added antibody and the antigen. Only if antibody is present in the serum sample, the line is broken (in CCLAIE) or disappears (in ICCIE).

The CCLAIE has two advantages over ICCIE. It is easier to perform (no mixing of serum with antigen as in ICCIE) and it is more sensitive (see Table 2). As soon as this was realized we decided not to continue the ICCIE tests, as can be seen from Table 1 where only a few sera were tested in the ICCIE.

The 1 serum which was diagnosed as positive among the 272 sera from the negative farm (Table 1) deserves a comment. The farm was found negative for at least 3 years and if ADV had entered the farm, it is very likely that more animals would have been seropositive. The most likely explanation of this positive reaction is to to be found under category 2, 3 or 4 as mentioned in the introduction. This positive reaction illustrates that with the CCLAIE as with other serological techniques, false positive sera can be found, but with the 0.4 % frequency as found in the present material it sould be considered a minor problem, but certainly a problem which should be kept in mind when the CCLAIE results are interpreted.

The CCLAIE also offers an additional feature which the other precipitation techniques do not have in the experimental setup used in this study. The CCLAIE test can detect free viral antigen. In very few cases free viral antigen is found in sera from newly ADV infected mink. This can be seen in the CCLAIE test because a small plateau (or a rocket) is seen on the line precipitate between antigen and antibody, where normally small deflections are seen for negative sera and large deflections for positive sera.

The general conclusion of the study is that CCLAIE is the best alternative method to CCIE for a mass screening program. Not only it is the most sensitive of the electrophoretic methods used in this study, but it also offers a specificity control of a given precipitate. A developed precipitate should always fuse with the expected precipitate between the added antigen and the antibody, which under all circumstances should develop between the troughs. The serum containing wells will always cause a

slight inhibition (deflection) of the antigen-antibody precipitate. A trained technician will, however, soon be able to distinguish as true breakage of the line (caused by a positive serum) from the deflection caused by the serum containing well.

In addition to the positive features of the CCLAIE test mentioned above the higher sensitivity of this test compared to to the CCIE test meant that 9 % more sera were diagnosed as positive (Table 1). Because of this higher sensitivity it is likely to assume that farms with only few positive animals will become free of ADV infection faster than if the CCIE test is used alone.

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SAMMENDRAG

Modstrøms linie absorption immun-elektroforese er en alternativ diagnostisk screening-test til modstrøms-immun-elektroforese i mink-plasmacytose udryddelsesprogrammer.

Modstrømselektroforese er den diagnostiske metode, der i dag bruges i mink plasmacytose udryddelsesprogrammet for danske minkfarme. Det har vist sig, at der er et stigende behov for en alternativ diagnostisk metode specielt til at benytte ved evaluering af mulige falske positive reaktioner i modstrømselektroforese. Vi sammenlignede analyseresultater fra 3321 negative og positive mink sera i den indirekte modstrømselektroforese, modstrøms linie absorption immunelektroforese og radio immun assay med analyseresultater fra modstrømselektroforese og fandt, at modstrøms linie absorption immunelektroforese er det bedste alternativ til modstrømselektroforese i mink plasmacytose udryddelsesprogrammet. Modstrøms linie absorption immun-elektroforese viste sig ikke blot at være nyttig til evaluering af mulige mistænkelige positive reaktioner i modstrømselektroforese, men den viste sig også at have en større følsomhed end modstrømselektroforese.

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Reprints may be requested from: B. Aasted, the Department of Veterinary Virology and Immunology, Royal Veterinary and Agricultural University, 13 Bülowsvej, DK-1870 Frederiksberg C, Denmark.