Acta vet. scand. 1976, *17*, 142—152.

From the State Veterinary Serum Laboratory, Copenhagen, and the State Veterinary Institute for Virus Research, Kalvehave Denmark.

A COMPARATIVE EXAMINATION OF SWINE SERA FOR ANTIBODY TO AUJESZKY VIRUS WITH THE CONVENTIONAL AND A MODIFIED VIRUS-SERUM NEUTRALIZATION TEST AND A MODIFIED DIRECT COMPLEMENT FIXATION TEST

By

V. Bitsch and *M. Eskildsen*

BITSCH, V. and M. ESKILDSEN: *A comparative examination of swine sera for antibody to Aujeszky virus with the conventional and a modified virus-serum neutralization test and a modified direct complement fixation test.* Acta vet. scand. 1976, *17*, 142—152. — Sera of pigs from élite breeding herds, of boars and sows collected at slaughter-houses, and of pigs from herds known to be infected, were examined for antibody to Aujeszky virus. The conventional and a modified virus-neutralizing antibody (VNA) test and a modified direct complement fixation (CF) test were employed. In simultaneous titrations of positive sera the modified VNA test gave titers approx. 4 $\log_2$ units above the titers obtained by the conventional test. The conventional VNA test was found insufficiently sensitive. Unspecific neutralization in the modified VNA test was infrequent in serum dilution 1/2 and rare in dilution 1/4. The CF tests on sera of slaughter sows and animals from known infected herds showed a remarkable consistency with the VNA tests. Inconsistent results were obtained with but few sera. Abt. 5 % of the sera could not be examined because of complement fixation with control antigen.

Aujeszky's disease; virus-serum neutralization; complement fixation.

In the conventional tissue culture test for demonstration of virus-neutralizing antibody (VNA), a certain quantity (100 TCID 50) of virus is mixed with varying amounts of serum and incubated for a period of 1 hr. at 37 C (a P_1 or P_1^{37} test) before inoculation onto tissue cultures. Even with undiluted serum, however, this test will not always allow demonstration of low, e.g. early, levels of Aujeszky VNA. Since logarithmic values of

the VNA titers and the corresponding incubation periods of virus-serum mixtures are practically linearly related within the range from 1 to 24 hrs. (*Bitsch*, to be published), the use of a prolonged virus-serum preincubation period (P), e.g. 24 hrs. (a P_{24} or P_{24}^{37} test) would be expected to result in improvement in sensitivity.

Demonstration of antibody in heat-inactivated swine serum by complement fixation (CF) is generally unsatisfactory unless the guinea-pig complement is supplemented with unheated normal serum (*Cowan* 1963, *Boulanger et al.* 1965, *Switzer* 1972) or with the porcine complement component C1q (*Eskildsen* 1975a). Furthermore, in lower dilutions most porcine immune sera exhibit a haemolytic prozone, which may mask a specific CF. The prozone effect can be eliminated by mercaptoethanol treatment of serum, and a direct complement fixation test, modified by addition of porcine C1q to the complement diluent and pretreatment of the test serum with mercaptoethanol, has proven useful in the demonstration of early serum antibody in pigs experimentally infected with Aujeszky virus (*Eskildsen* 1975b).

The present examination of swine sera for antibody to Aujeszky virus was undertaken in order to study the sensitivity and specificity of the conventional VNA-P_1 test, the VNA-P_{24} test, and the modified CF test.

MATERIAL

Several series of samples were collected for examination. Of the following five groups the two first were examined for VNA only, while the rest were examined for CF antibody as well.

I. Élite breeding herds. All adult pigs in 24 herds, i.e. 521 animals, from a certain area in Jutland were tested early in 1974. Since élite breeding herds had earlier been subjected to serological examination for Aujeszky's disease, and regulations aiming at preventing introduction of the infection been imposed upon the breeders, the sera were expected to be negative.

II. Slaughter boars. Blood samples from a total of 506 boars were collected early in 1975 at slaughter-houses on the islands Funen and Langeland, where Aujeszky's disease had occurred sporadically.

III. Slaughter sows. The samples, 335 in all, were collected on two different occasions in 1975 at a slaughter-house on Zealand, where Aujeszky's disease had occurred endemically for many years.

IV. Herd FH. The breeding stock, 32 sows and one boar, were blood-sampled in August 1975. Seven months earlier there had been deaths from the disease in the litters of four gilts. One of the gilts had been severely paralysed for more than one week, and the fattening pigs had shown loss of appetite for one or two days. The houses were low and overcrowded, and the airing was extremely bad.

V. Herd GC. Thirty sows, i.e. abt. two fifths of the breeding animals, were blood-sampled in August 1975. The disease had been diagnosed five months before in a litter of a gilt. The animals were housed in several buildings equipped with ventilation appliances.

METHODS

VNA. Primary or secondary pig kidney cells, deriving from SPF piglets, were cultured in plastic tubes. Maintenance medium was Earle solution with 2 % bovine serum, 1.8 ml in each tube. The virus used was a Danish laboratory strain: DaS67.

The conventional VNA-P_1 test with virus-serum preincubation at 37 C for 1 hr. and a VNA-P_{24} test with preincubation for 24 hrs. were used.

The serum samples were screened by the VNA-P_{24} test. Serum undiluted and in some series also in dilution 1/2, was mixed with an equal amount of virus suspension with 100 TCID 50 per 0.1 ml. After incubation at 37 C for 24 hrs. two tissue culture tubes were inoculated each with 0.2 ml of the virus-serum mixture.

Sera showing complete virus neutralization on screening were titrated by a twofold serum dilution procedure with inoculation of two tubes from each dilution. Hanks balanced salt solution with 2 % bovine serum was used both for the serum dilutions and for the virus suspension. VNA-P_1 and VNA-P_{24} titer recordings were based on simultaneous titrations. Final readings were taken after five days. Fifty % endpoints were calculated after Kärber.

CF. Direct complement fixation was conducted with microtiter equipment using four 100 % haemolytic units of complement. A preparation of porcine C1q was added to the complement diluent, and the serum was pretreated with mercaptoethanol at 56 C for 20 min. (cf. *Eskildsen* 1975b, *Eskildsen & Overby* 1976). Medium from primary swine kidney cell cultures infected with the Danish Korsør-B/62 strain of Aujeszky virus served as anti-

gen, while medium from non-inoculated cell cultures was used as control. The sera were screened in dilution 1/2 with 2 units of antigen. Sera giving partial or complete complement fixation were retested in dilutions 1/2 to 1/128. Titers were recorded as reciprocals of the highest dilutions giving 0 to 50 % haemolysis.

RESULTS

I. Élite breeding herds. Of 521 sera, 24 (4.6 %) showed virus neutralization in both tubes when screened in a VNA-P_{24} test with undiluted serum. In the subsequent titration, 13 sera gave P_{24} titers ≤ 1, while two, five, two, one, and one (11 sera, 2.1 %) had titers of, respectively, 1.4, 2, 2.8, 4, and 16. Thus only two sera showed P_{24} titers ≥ 4. All were negative by the VNA-P_1 test.

II. Slaughter boars. Of 506 sera, 425 had VNA-P_{24} titers < 1, 16 had titers of 1—1.4 and 65 titers ≥ 2. Of these 65 sera 63 were titrated. The $\log_2$ mean P_{24} titer was 6.67. The P_1 and P_{24} titer distributions are shown graphically in Fig. 1. Only four sera had P_{24} titers in the range from 2 to 11, while seven had titers of 16—22. Of these seven sera, five had P_1 titers of 1—1.4, while two had titers < 1.

III. Slaughter sows. Of 335 sera, 285 showed no virus neutralization in the VNA-P_{24} test with undiluted serum, while 11 neutralized in one of the tubes and 39 in both tubes. On titration, five of the 39 sera gave P_{24} titers < 2, two gave a titer of 4, and

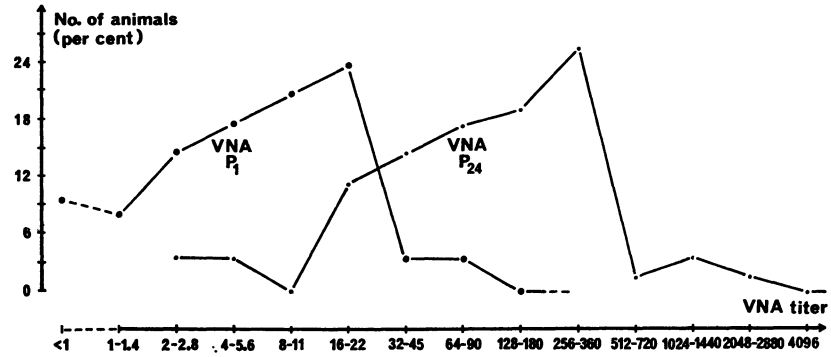

Figure 1. Antibody to Aujeszky virus in slaughter boars. The distribution of virus-neutralizing antibody (VNA) titers of 63 sera as obtained by the conventional test with virus-serum preincubation for 1 hr. (P_1) and by a test with preincubation for 24 hrs. (P_{24}). Sera with P_{24} titers < 2 omitted.

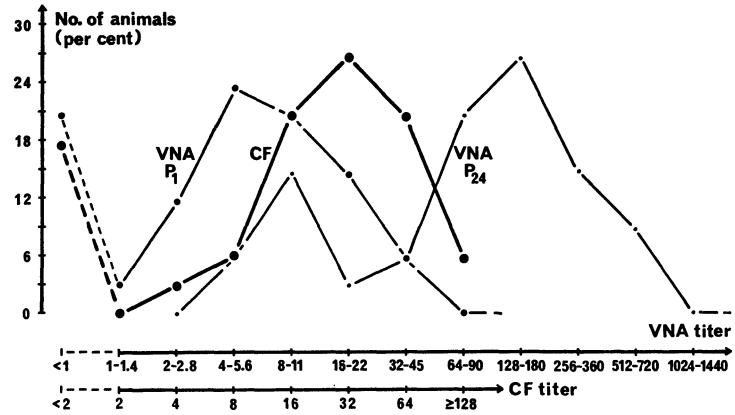

Figure 2. Antibody to Aujeszky virus in slaughter sows. The distribution of virus-neutralizing antibody (VNA) titers of 34 sera as obtained by the conventional test with virus-serum preincubation for 1 hr. (P_1) and by a test with preincubation for 24 hrs. (P_{24}), and of complement fixation (CF) titers of the same sera as obtained by a modified direct test. Sera with P_{24} titers < 2 omitted.

five gave titers of 8—11. Twelve of the sera were negative in the P_1 test, while the rest, 27 sera, were positive. The $\log_2$ mean P_{24} titer of 34 sera with P_{24} titers ≥ 2 was 6.25.

In the CF test 286 sera were negative. Twenty-seven sera gave titers ≥ 8, three gave a titer of 4, and one a titer of 2. The VNA-P_1, VNA-P_{24}, and CF titer distributions are shown graphically in Fig. 2. Sera giving inconsistent or low titers by the VNA and CF tests are recorded in Table 1 together with similar sera from Herds FH and GC. Eighteen sera (5.4 %) were found unsuitable for CF test, giving CF with control antigen in serum dilution 1/4 to 1/32. One of these sera was VNA-positive.

IV. Herd FH. The whole breeding stock had VNA-P_{24} titers ≥ 11 and except for three animals, CF titers ≥ 8. Two of these three animals had a CF titer < 2 and the third a titer of 4 (Table 1). The titer distributions for all 33 sera are shown in Fig. 3. The $\log_2$ mean P_{24} titer was 7.88.

V. Herd GC. In the VNA-P_{24} screening with serum undiluted and in dilution 1/2, 11 sera showed titers < 1, while five had titers of 1—1.4. Titer distributions of the remaining 14 sera with P_{24} titers ≥ 2 are shown in Fig. 4. The $\log_2$ mean P_{24} titer of these sera was 5.29. The lower titer level was reflected also in the CF titers. Ten sera had CF titers ≥ 8, two a titer of 4, and one a titer of 2.

Table 1. Titers of 26 sera originating from slaughter sows (S), Herd FH, and Herd GC, and giving inconsistent or inconclusive results by the virus-neutralizing antibody (VNA) tests and/or the complement fixation (CF) test.

	Sample	Serum titer			Assessment*
		VNA-P_{24}	VNA-P_1	CF	
Positive in both VNA tests, negative in the CF test	S 1-004	64	2	<16**	positive
	S 2-031	64***	4	<2	positive
	S 2-096	128***	4	<2	positive
Negative in the VNA-P_{24} test, positive in the CF test	GC 8	<1	<1	4	inconclusive
	S 1-055	<1	<1	2	negative
	S 2-001	<1	<1	4	inconclusive
	S 2-072	<1***	<1	16	inconclusive
	S 2-106	<1***	<1	4	inconclusive
Positive in the VNA-P_{24} test (low titers), positive in the CF test (low titers)	FH 11	11	1	4	positive
	GC 6	22	1	4	positive
	GC 7	5.6	<1	2	inconclusive
	GC 12	45	1.4	4	positive
	GC 25	16	1	8	positive
	GC 27	22	<1	4	positive
	S 1-091	4	<1	4	inconclusive
	S 1-114	8	<1	16	positive
	S 1-127	11	<1	8	positive
	S 2-067	16	1.4	8	positive
Positive in the VNA-P_{24} test (low titers), negative in the CF test	FH 7	22	1.4	<2	positive
	FH 15	11	<1	<2	positive
	GC 15	8	<1	<2	positive
	GC 29	11	<1	<2	positive
	S 1-014	11	<1	<2	inconclusive
	S 1-109	4	<1	<2	inconclusive
	S 1-158	8	<1	<2	inconclusive
	S 2-092	8	<1	<2	inconclusive

* See Discussion.

** Could not be examined with higher serum concentrations because of complement fixation with control antigen.

*** Retesting of remaining serum from the CF examination gave negative results (≤ 2.8) for samples S 2-072 and S 2-106, and positive results (> 64) for samples S 2-031 and S 2-096.

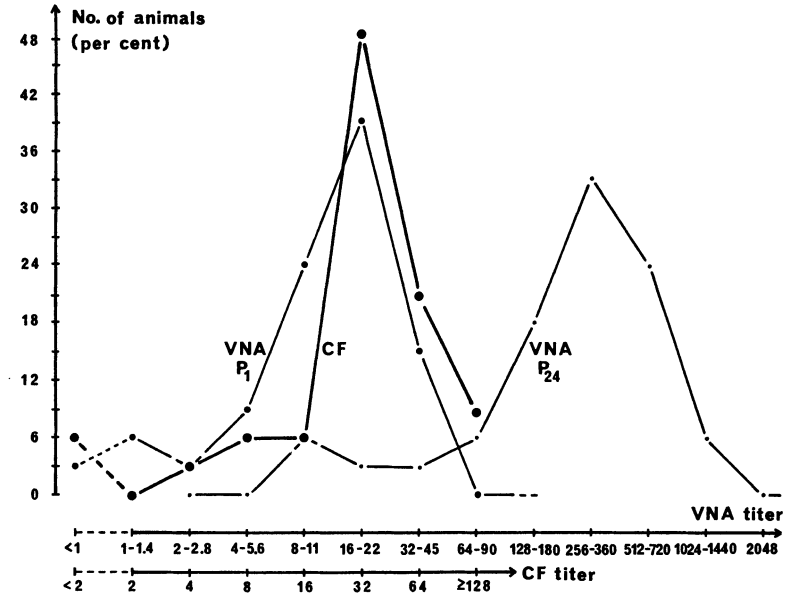

Figure 3. Antibody to Aujeszky virus in Herd FH. The distribution of virus-neutralizing antibody (VNA) titers of 33 sera as obtained by the conventional test with virus-serum preincubation for 1 hr. (P_1) and by a test with preincubation for 24 hrs. (P_{24}), and of complement fixation (CF) titers of the same sera as obtained by a modified direct test.

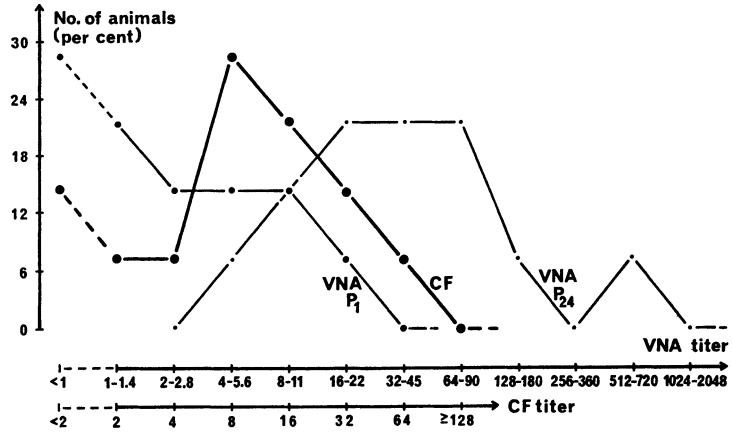

Figure 4. Antibody to Aujeszky virus in Herd GC. The distribution of virus-neutralizing antibody (VNA) titers of 14 sera as obtained by the conventional test with virus-serum preincubation for 1 hr. (P_1) and by a test with preincubation for 24 hrs. (P_{24}), and of complement fixation (CF) titers of the same sera as obtained by a modified direct test. Sera with P_{24} titers < 2 omitted.

The 126 sera with VNA-P_1 titers ≥ 1 showed a mean titer increase of 4.15 $\log_2$ units in the P_{24} test. An improvement of 3 $\log_2$ units was obtained for eight sera, of 3.5 for 21, of 4 for 44, of 4.5 for 33, of 5 for 19, and 5.5 for one serum.

In all, 398 sera were subjected to comparative CF — VNA examination. Of these sera, 312 were negative in both tests (CF and VNA-P_{24} titers ≤ 2), and divergent results were recorded for just eight sera (Table 1). Five of these eight sera, having CF titers ≥ 2, gave a VNA-P_{24} titer < 1, and of the three other sera, showing VNA-P_1 titers of 2 or 4, two gave a CF titer < 2, while one was unfit for CF test because of complement fixation with control antigen.

As will have appeared from the preceding, results of the CF and VNA tests were consistent in the majority of cases. Assuming equivalence of a CF titer of 2 with a VNA-P_1 titer of 1, which are the sensitivity limits of the two tests, the CF titers were generally from 0 to 1 $\log_2$ higher than VNA-P_1 titers. Calculated on the basis of 60 sera with CF titers ≥ 8 and VNA-P_1 titers ≥ 2, the mean titer difference was 0.6 $\log_2$ unit. Fifty-five % of the 60 sera showed CF titers of 0.5 or 1 $\log_2$ above the VNA-P_1 titers, and for 82 % of the sera the CF — VNA-P_1 difference deviated less than 1 $\log_2$ from the mean difference.

DISCUSSION AND CONCLUSIONS

Incubation of virus-serum mixtures at 37 C for 24 hrs. instead of 1 hr. resulted in a titer increase for positive sera of approx. 4 $\log_2$ units. The variation noted — 93 % of the sera showed an increase deviating by less than 1 $\log_2$ from the mean increase of 4.15 — hardly exceeds what could be expected to arise from titration errors. This would seem to suggest that the titer increase obtained by incubating the virus-serum mixture at 37 C for 24 hrs. instead of 1 hr. is the same for all positive sera. This is consistent with the notion that, expectedly, the titer increase should be 4.59 $\log_2$ units ($\log_2 \frac{24}{1} = 4.59$) and a somewhat lesser actual increase be ascribable to presence in the P_1^{37} test of virus-antibody complex which will dissociate on dilution (*Bitsch*, to be published).

The possibility of unspecific neutralization in the VNA tests is illucidated by the examination of 24 élite breeding herds. All

sera were negative in the conventional P_1 test. In the P_{24} test 95 % showed no neutralization with undiluted serum, and just two samples neutralized to titers $\geq$ 4. This is in accordance with results obtained by examination of several thousands of Danish SPF swine sera and hundreds of sera of boars in artificial insemination centres. In VNA-P_{24}^{37} tests with infectious bovine rhinotracheitis virus, unspecific neutralization is extremely rare even with undiluted serum (*Bitsch* 1975).

The virus neutralization rate for swine sera with unspecific reaction has been found equivalent to that of immune serum (*Bitsch*, unpublished). Since P_{24}^{37} titers of 16—22 may be unspecific, the same may be true of P_1^{37} titers of 1—1.4. On the other hand, from the results recorded in Herds FH and GC, and to some extent also from the results of examination of slaughter sows and boars, it is obvious that such titers will regularly be indicative of previous infection. Even P_{24} titers of 8—11 must often be regarded specific (Table 1). The eight sera from Herds FH and GC with P_{24} titers from 8 to 22 must be interpreted as definitely positive, simply because unspecific neutralization in serum dilution 1/8 is rare, cf. the present examination of élite herds and the examination of SPF pigs referred to above. It should be noted also that the specificity of the low P_{24} titers in some of the sera from slaughter sows and infected herds is substantiated by CF titers, and that as far as boar sera are concerned the titer distribution strongly suggests that even the seven reactions with P_{24} titers of 16—22 are specific (Fig. 1).

The concordance between CF and VNA titers in the comparative titrations is striking and might suggest that the same antibody is involved in both tests. The divergent results in Table 1, however, might indicate that in a few cases either CF or VNA titers have been influenced by some other factor, possibly related to low concentrations of early or late antibody. With further improvement of the CF test such inconsistencies may perhaps be eliminated.

The main conclusion as regards the examination for VNA is that the conventional P_1^{37} test is not sensitive enough at low antibody levels. In such cases a P_{24}^{37} test will be advantageous. However, the P_{24}^{37} test may give unspecific neutralization with undiluted serum and serum dilution 1/2, but very seldom with dilutions 1/4 and 1/8. Still, the P_{24}^{37} test with undiluted serum

or serum diluted 1/2 is a valuable tool in cases when it is of crucial importance to ascertain freedom from infection, be it in whole herds or in individual animals such as pigs in quarantine stations and boars before admission to artificial insemination centres (*Bitsch* 1974).

Results obtained by examination with a modified direct CF test have shown a remarkable accordance with results obtained by the VNA test. In previous experiments, referred to in the preceding, the CF test has been found useful for demonstration of early antibody in experimentally infected pigs. In the present work late antibody has been demonstrated at different levels in serum from naturally infected pigs. Still, a further refinement of the test would seem desirable, especially with a view to eliminating fixation of complement with control antigen.

REFERENCES

Bitsch, V.: La maladie d'Aujeszky au Danemark. (Aujeszky's disease in Denmark). Cah. Méd. vét. 1974, *43*, 211—219.

Bitsch, V.: The infectious bovine rhinotracheitis virus infection in cattle in Denmark, with regard to distribution, epidemiologi, importance, diagnosis, and control. Bull. Off. int. Epiz. 1975, XLIII[e] Sess. gén., Rapp. no. 105.

Boulanger, P., M. Appel, G. L. Bannister, G. M. Ruckerbauer, K. Mori & D. P. Gray: Hog cholera. III. Investigation of the complement-fixation test for the detection of the virus in swine tissue. Canad. J. comp. Med. 1965, *29*, 201—208.

Cowan, K. M.: Immunologic studies on African swine fever virus. II. Enhancing effect of normal bovine serum on the complement-fixation reaction. Amer. J. vet. Res. 1963, *24*, 756—761.

Eskildsen, M.: Complement fixation with porcine serum antibodies. Enhancing effect of porcine C1q on complement fixation with IgG prepared from porcine pseudorabies immune serum. Acta path. microbiol. scand., Sect. C 1975 a, *83*, 315—324.

Eskildsen, M.: Demonstration of antibodies against pseudorabies virus in swine serum by a modified direct complement fixation test. Bull. Off. int. Epiz. 1975 b, XLIII[e] Sess. gén., Rapp. no. 213.

Eskildsen, M. & E. Overby: Serological diagnosis of classical swine fever. A comparison of a modified direct complement fixation test with an immunofluorescence plaque neutralization test in the diagnosis of experimental subclinical infection. Acta vet. scand. 1976, *17*, 131—141.

Switzer, W. P.: Mycoplasmal pneumonia of swine. J. Amer. vet. med. Ass. 1972, *160*, 651—653.

SAMMENDRAG

En sammenlignende undersøgelse af svinesera for antistoffer mod Aujeszky-virus med den konventionelle og en modificeret virus-serum-neutralisationstest og en modificeret direkte komplementbindingstest.

Til undersøgelse for Aujeszky-antistoffer indsamledes blodprøver af svin i eliteavlsbesætninger, af søer og orner på slagtehuse og af svin i besætninger, hvor Aujeszky's sygdom havde været diagnosticeret. Den konventionelle og en modificeret virus-serum-neutralisationstest og en modificeret direkte komplementbindingstest blev anvendt. Ved simultane titreringer gav den modificerede neutralisationstest titre, som var fire $\log_2$-enheder højere end titrene opnået med den konventionelle test. Den konventionelle test fandtes at være utilstrækkeligt følsom. Uspecifik neutralisation med den modificerede neutralisationstest forekom ikke ofte med serumfortyndingen 1/2 og var sjælden med fortyndingen 1/4. Resultaterne fra komplementbindingstesten, som blev anvendt ved serumprøverne af slagte-søer og -orner og af svin i inficerede besætninger, viste en bemærkelsesværdig overensstemmelse med resultaterne fra undersøgelsen for virusneutraliserende antistoffer. Uoverensstemmelse sås kun ved meget få sera. Omkring fem % af prøverne kunne ikke undersøges ved komplementbindingstesten, idet de viste komplementbinding med kontrolantigenet.

(Received February 20, 1976).

Reprints may be requested from: V. Bitsch, the State Veterinary Serum Laboratory, Bülowsvej 27, DK-1870 Copenhagen V, Denmark.