Acta vet. scand. 1978, *19*, 497—505.

From the State Veterinary Serum Laboratory, Copenhagen V, Denmark.

THE P_{24}^{37} MODIFICATION OF THE INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS-SERUM NEUTRALIZATION TEST

By

V. Bitsch

BITSCH, V.: *The P_{24}^{37} modification of the infectious bovine rhinotracheitis virus-serum neutralization test.* Acta vet. scand. 1978, *19*, 497—505. — The applicability of a modified infectious bovine rhinotracheitis constant-virus/varying-serum neutralization test, with preincubation of virus-serum mixtures at 37°C for 24 hrs. (P_{24}^{37} test) as against 1 hr. in the conventional (P_{1}^{37}) test, was elucidated by examination of about 5000 bovine sera. The sensitivity of the test was studied mainly in parallel P_{24}^{37}/P_{1}^{37} testings of sera from animals in two infected herds and of sera taken from a bullock during one month following nasal infection. In agreement with conclusions in previous papers, the P_{1}^{37} test appeared to be inadequately sensitive, and the P_{24}^{37} test regularly gave titers that were about 4 in $\log_2$ higher than the P_{1}^{37} titers, which means that the P_{24}^{37} test is about 16 times as sensitive as the conventional test. From examinations of 4902 sera from four different groups of cattle it was concluded that with the technique described and with the use of undiluted serum, the specificity of the P_{24}^{37} test would be as good as 0.999. The observed high sensitivity and specificity of the P_{24}^{37} test give high diagnostic values of both negative and positive reactions.

infectious bovine rhinotracheitis; virus-serum neutralization; neutralization test.

The conventional test for demonstration of virus-neutralizing antibody (VNA) in serum to infectious bovine rhinotracheitis (IBR) virus, with 100 TCID50 of virus as test dose and preincubation of virus-serum mixtures at 37°C for 1 hr. (P_{1}^{37} test), has proved to be of inadequate sensitivity (cf. *Bitsch* 1973a). Higher sensitivity was obtained with the P_{1}^{37} test by using lower test doses of virus and larger amounts of serum (*Bitsch* 1970, *Huck & Woods* 1972). With serum doses of 0.5 ml tested against

approx. 10 TCID50, a virus-neutralizing effect could be demonstrated in a relatively high proportion of sera that were negative in a conventional test. Other observations strongly suggested that positive results obtained by this modified test were significant and that even further improvement of the sensitivity would be desirable (*Bitsch* 1973a). Subsequent studies showed that prolongation of the preincubation from 1 to 24 hrs. at 37°C would augment the sensitivity considerably (*Bitsch* 1973b). Quantitative studies of the neutralization test with IBR virus had previously been made by *Mohanty & Lillie* (1965) and *House & Baker* (1971), but the influence of the length of preincubation and of temperature had never before been elucidated.

In a study of the neutralization reaction in virus-serum neutralization tests with sera collected late in the course of immunization (*Bitsch* 1978a), a direct proportionality could be demonstrated between antibody titer and length of preincubation. A regular deviation, however, was observed when preincubation periods were relatively short. As a result of this, a prolongation of the preincubation at 37°C from 1 to 24 hrs. should raise VNA titers, not by a factor of 24, but only by a factor of about 16. A direct relationship was found between preincubation temperature and logarithmic values of VNA titer. The results indicated that a sensitivity as obtained by preincubation at 37°C for 3 hrs. or more could be achieved at 20 or 4°C only after a fourfold and 16-fold increase in the length of preincubation, respectively. In a neutralization test for general use with the relatively heat-resistant IBR virus, preincubation should therefore preferably not be made at temperatures much lower than 37°C.

The aim of the present paper was to give an account of the P^{37}_{24} virus-serum neutralization test with IBR virus, particularly in respect of sensitivity and specificity. As there would seem to be no reason to doubt the specificity of moderate or high P^{37}_{24} titers (≥ 16), attention will mainly be focussed on the specificity of lower reactions.

MATERIAL AND METHODS

Serological tests. The conventional P^{37}_{1} and the P^{37}_{24} constant-virus/varying-serum neutralization test, with preincubation of virus-serum mixtures at 37°C for 1 and 24 hrs., respectively, were employed. A suspension containing 100 TCID50/0.1 ml of

the Danish DaB69 strain of IBR virus was mixed with an equal amount of test serum. Undiluted serum was used in screening tests, twofold dilutions in titrations. Of each virus-serum mixture, 0.2 ml was inoculated into each of two tubes with low passage calf kidney cell culture in maintenance medium. This medium was as described elsewhere (*Bitsch* 1978a), and Hanks balanced salt solution with 2 % IBR-negative bovine serum and 50 µg neomycin per ml was used for virus suspensions and serum dilutions. In comparative titrations, the virus suspension and the serum dilutions were preheated to 37°C. All sera were inactivated at 56°C for 30—60 min. and stored below —20°C. VNA titers were recorded as the serum dilution factors corresponding to the 50 % endpoints of neutralization (after Spearman-Kärber).

Test material. Blood samples collected from 86 animals in Herds CMa and JB in January 1974, and from a 3-year-old bullock during one month following experimental nasal infection, were selected for a comparative examination by the two tests. The number and age of animals tested in the two herds appear from Table 1.

Table 1. Distribution of 86 animals in Herds CMa and JB in January 1974 with respect to age and serological reaction (P_{24}^{37} test).

Herd	Number of samples	Animals positive	Age distribution (years)							
			≥ 7	6	5	4	3½	3	2½	≤2*
CMa	40	24	4/0**	3/0	4/0	4/0	0/0	6/0	2/0	1***/16
JB	46	27	9/0	4/0	6/0	5/1	3/1	0/6	0/4	0/7

* Age group ≤ 2 years only incompletely represented.
** Animals positive/animals negative.
*** IBR-positive bull calf, 1 year of age, with titer of 128.

The following four groups of animals were examined by the P_{24}^{37} test only:

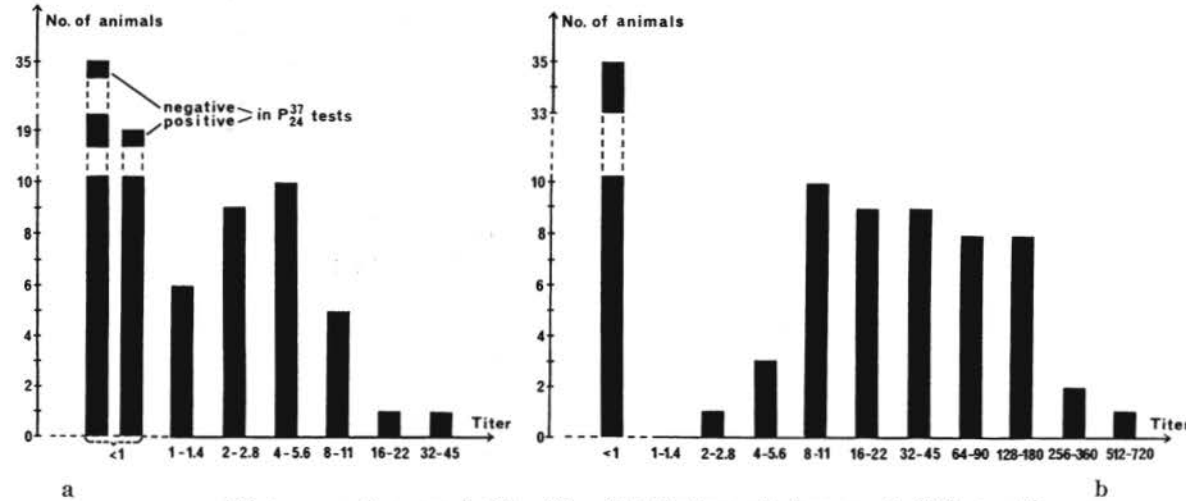
1. *Bulls and bullocks intended for introduction into AI centres.* In the period from January 1974 through June 1977, 1731 samples were examined. The majority of animals were under 1 year of age.
2. *Bulls and bullocks at AI centres.* In the autumn of 1976 all animals (1037) at 28 centres were tested (cf. *Autrup & Bitsch* 1978).

3. *Pregnant heifers,* generally 2 to 2½ years of age. In the spring of 1977, 1589 animals from 776 herds in Jutland were tested.
4. *Herds with a history of IBR virus infection.* In the spring of 1977, 546 animals from 12 herds were examined. Each of these herds had a history of infection, which is described elsewhere (*Bitsch* 1978b).

RESULTS

The comparative P^{37}_{1}/P^{37}_{24} testing

The titer distribution for the 86 sera from Herds CMa and JB, which were examined simultaneously by the two tests, are shown in Figs. 1a and 1b.



a b

Figures 1a and 1b. The distribution of virus-neutralizing antibody titers of 86 sera collected in 1974 from Herds CMa and JB and examined simultaneously by P^{37}_{1} (1a) and P^{37}_{24} tests (1b).

Nineteen sera which were negative in P^{37}_{1} tests showed P^{37}_{24} titers from 2.8 to 22. Thirty-two sera with P^{37}_{1} titers ≥ 1 showed titer rises as follows on P^{37}_{24} testing: 3 in $\log_2$ in two sera, 3.5 in two sera, 4 in 13 sera, 4.5 in 11 sera, and 5 in four sera. The mean titer improvement being 4.2 in $\log_2$, 94 % of the sera showed improvements that deviated less than 1 in $\log_2$ from the mean. In a subsequent testing the two sera that had shown a titer rise of 3 in $\log_2$ showed rises of 3.5 and 4, while the four sera with titer rises of 5 showed rises of 4 (2) and 4.5 (2).

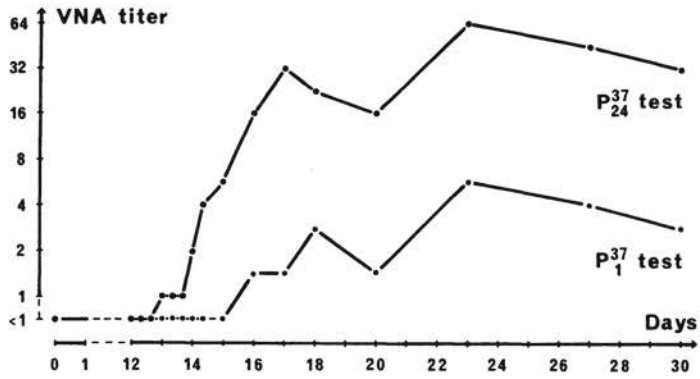


Figure 2. Virus-neutralizing antibody titers in sera drawn from a bullock during the first month following experimental nasal infection, as obtained simultaneously by P^{37}_{1} and P^{37}_{24} tests.

Also in the sera from the primary immunization phase of the bullock (Fig. 2) the P^{37}_{24} titers were about 4 in $\log_2$ higher than the P^{37}_{1} titers, and the fact that the first six P^{37}_{24}-positive sera were negative in the P^{37}_{1} test demonstrated the essentially higher sensitivity of the P^{37}_{24} test.

The P^{37}_{24} testing of selected groups of animals

Animals intended for introduction into AI centres. All the 1731 samples but 10 showed a titer of < 1. Of the positive animals three with titers of 32, 128, and 360, respectively, were adults, while the rest were calves from 1 to 4 months of age with titers of 4 (1), 5.6 (2), 8 (3), and 64 (1). The reaction in these calves was considered to be of colostral origin. Four of their mothers were tested and found positive. A fifth calf was from a known IBR-infected herd, and the remaining two, with titers of 8 and 64, were from an area where the infection was known to be prevalent.

Animals at AI centres. Two animals out of 1037 gave positive reactions: a bull (titer 180) from preputial washings of which IBR virus had been isolated in 1969, and a bullock at a different centre with a titer of 2 (cf. *Autrup & Bitsch* 1978). A subsequent serological examination of the herd of origin of the latter animal turned out negative. It was concluded that it was most probably uninfected. Ten months later the bullock gave a titer of < 1, but a P^{37}_{48} titer of 1.

Pregnant heifers. All animals (1589) but 18 showed titers of < 1. The 18 reactors were from 13 different herds located in areas served by previously infected centres (*Autrup & Bitsch*). The titer distribution is shown in Table 2. The animal with a titer of 4 was from the same herd as a heifer with a titer of 180. The reaction of 1.4 in one animal must remain inconclusive. The two animals with titers of 1 were retested after one month and in one of them not the slightest neutralizing effect could be observed, not even in a $P\frac{37}{48}$ test, while the other, which came from the same herd as one of the heifers with a titer of 32, gave a titer of 1.4.

Table 2. Distribution of $P\frac{37}{24}$ virus-neutralizing antibody titers in 18 IBR-positive sera found among 1589 sera from the group of pregnant heifers.

VNA titer	1	1.4	4	16	22	32	64	>64
Number of samples	2	1	1	2	3	2	2	5

Herds with a history of IBR virus infection. Of the total of 546 samples, 52 from seven different herds were seropositive. The titer distribution is shown in Table 3.

Table 3. Distribution of $P\frac{37}{24}$ virus-neutralizing antibody titers in 52 IBR-positive samples found among 546 samples in the 1977 examination of 12 herds with a history of previous IBR virus infection.

Herd	Number of samples	Titer distribution									
		1-1.4	2-2.8	4-5.6	8-11	16-22	32-45	64-90	128-180	256-360	512-720
JH	1	1									
JJu	3						1			1	1
KN	9					1	1	4	2	1	
CMø	12					7	1	1		2	1
CMa	6						1	2	1	1	1
JB	15		1	2	4	3	2	2		1	
CH	6				1		3			2	
	52	1	1	2	5	11	9	9	3	8	3

The animal in Herd JH with a titer of 1.4 was born in November 1967 and was the only animal left of those which had been exposed to infection during an extensive spreading in December

1968—January 1969. In November 1977 the animal was retested and titers of 2 and 2.8 were recorded at two successive examinations. Of the reactors in Herd JB the one with a titer of 2.8 was retested four months later the same result. In 1974 it had shown a titer of 4. An animal with a titer of 5.6 showed a titer of 2.8 one month later, while one with a titer of 4 showed a titer of 16 four months later. Three of the four animals with titers of 8 or 11 had shown titers of 8 to 22 in 1974; the fourth, a 3-year-old cow, had a titer of 32 on retesting one month after the first sampling. The animal in Herd CH with a titer of 11 was recorded positive in 1971 in a modified $P^{3}_{1}{}^{7}$ test at a titer corresponding to a $P^{3}_{24}{}^{7}$ titer of about 8.

DISCUSSION AND CONCLUSIONS

The results of the comparative $P^{3}_{1}{}^{7}/P^{3}_{24}{}^{7}$ testing are consistent with the principles of virus neutralization elucidated in a previous paper (*Bitsch* 1978a). In a similar examination of 126 porcine sera with antibody to Aujeszky virus, the titer rises recorded in $P^{3}_{24}{}^{7}$ tests deviated, in 93 % of the sera, less than 1 in $\log_2$ from the mean rise of 4.1 in $\log_2$ (*Bitsch & Eskildsen* 1976). The fact that extreme titer rises were not reproducible further substantiates the notion that with practically all sera the $P^{3}_{24}{}^{7}$ test will give titers about 4 in $\log_2$ over $P^{3}_{1}{}^{7}$ titers, with the possible exception of sera collected very early during primary immunization; but even with such sera the superiority of the $P^{3}_{24}{}^{7}$ test is obvious. In conclusion, the sensitivity of the $P^{3}_{24}{}^{7}$ test will generally be 4 in $\log_2$ over that of the conventional $P^{3}_{1}{}^{7}$ test.

High sensitivity may tend to give false positive reactions, but in the examinations of the animals entering AI centres and of the infected herds the findings strongly supported the notion — not least in the light of the generally low prevalence rate of the infection in Danish cattle (cf. *Bitsch* 1973a, 1975) — that even all low reactions were specific. In the 1974 examination of Herds CMa and JB, the titer distribution (Figs. 1a and 1b) and the distribution of infected and uninfected animals with regard to age (Table 1) strongly supported the same conclusion. Only one animal from an AI centre and two from the group of pregnant heifers gave reactions, of 1 to 2, which were assessed as inconclusive or falsely positive. As almost 5000 animals were examined and only a small proportion gave positive results, the probability

of a false positive reaction must be very low, presumably less than 0.001, and the specificity accordingly high, i. e. better than 0.999. For a sensitive serological test this appears to be an extremely high value.

A few very low reactions, with $P_{2}^{3}{}_{4}^{7}$ titers from 1 to 2.8, were assessed as undoubtedly positive, which implies that even a still higher sensitivity might be advantageous. And with the observed high specificity of the $P_{2}^{3}{}_{4}^{7}$ test an additional augmentation of the sensitivity would be safe in most cases. Yet, as there were no further indications of false negative reactions, cf. Fig. 1b and Table 1, it seems justified to consider the sensitivity of the $P_{2}^{3}{}_{4}^{7}$ test to be generally adequate.

In conclusion, the $P_{2}^{3}{}_{4}^{7}$ test with undiluted serum, being about 16 times as sensitive as the conventional $P^{3}{}_{1}^{7}$ test and showing a specificity of about 0.999 with the present technique, must be said to be a highly reliable serological tool, with high diagnostic values of both negative and positive reactions. The following key to interpretation of test results has been presented earlier (*Bitsch* 1975): titers < 1, negative; ≥ 1 but < 4, inconclusive; ≥ 4, positive. The results obtained in the present study have substantiated the validity of this key, but have also pointed out that in infected herds even low reactions should be regarded as specific.

REFERENCES

Autrup, E. H. & V. Bitsch: The occurrence, control, and eradication of the infectious bovine rhinotracheitis virus infection at artificial insemination centres in Denmark. Nord. Vet.-Med. 1978, *30,* 169—177.

Bitsch, V.: Improved sensitivity of the IPV/IBR virus-serum neutralization test. Acta vet. scand. 1970, *11,* 606—608.

Bitsch, V.: The IBR-IPV virus-serum neutralization test. Sensitivity and significance of the tissue culture tube test. Acta vet. scand. 1973a, *14,* 683—690.

Bitsch, V.: The IBR-IPV virus-serum neutralization test. Studies on the influence of the virus-serum incubation prior to inoculation. Acta vet. scand. 1973b, *14,* 767—769.

Bitsch, V.: The infectious bovine rhinotracheitis virus infection in cattle in Denmark with regard to distribution, epidemiology, importance, diagnosis and control. Bull. Off. int. Epiz. 1975, *84,* 95—105.

Bitsch, V.: An investigation into the basic virus-antibody neutralization reaction, with special regard to the reaction in the constant-virus/varying-serum neutralization test. Acta vet. scand. 1978a, *19*, 110—128.

Bitsch, V.: Persistence of infection with infectious bovine rhinotracheitis virus in Danish cattle herds. Nord. Vet.-Med. 1978b, *30*, 178—185.

Bitsch, V. & 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.

House, J. A. & J. A. Baker: Bovine herpesvirus IBR-IPV. The antibody virus neutralization reaction. Cornell Vet. 1971, *61*, 320—335.

Huck, R. A. & D. G. Woods: Serum neutralization tests with infectious bovine rhinotracheitis/infectious pustular vulvovaginitis IBR/IPV virus. Brit. vet. J. 1972, *128*, LXII—LXIII.

Mohanty, S. B. & M. G. Lillie: A quantitative study of the infectious bovine rhinotracheitis neutralization test. Amer. J. vet. Res. 1965, *26*, 892—896.

SAMMENDRAG

$P^{3\,7}_{2\,4}$-modifikationen af neutralisationstesten med infektiøst bovint rhinotracheitisvirus.

Anvendeligheden af en konstant-virus/varierende-serum-neutralisationstest modificeret ved præinkubering af virus-serum-blandingerne ved 37°C i 24 timer ($P^{3\,7}_{2\,4}$-test) i stedet for 1 time som ved den konventionelle ($P^{3,7}_{1}$) test er belyst ved undersøgelse af ca. 5000 kvægsera. $P^{3,7}_{1}$-testen viste sig i overensstemmelse med tidligere publicerede resultater at være utilstrækkelig følsom, og $P^{3\,7}_{2\,4}$-testen gav titre som var 4 $\log_2$-enheder højere end $P^{3,7}_{1}$-testens titre. På grundlag af undersøgelser af 4902 sera fra 4 forskellige grupper af kvæg konkluderedes det, at specificiteten af $P^{3\,7}_{2\,4}$-testen ved anvendelse af ufortyndet serum var omkring 0,999. De observerede høje værdier for sensitiviteten og specificiteten af $P^{3\,7}_{2\,4}$-testen giver særdeles høje diagnostiske værdier for både negative og positive testresultater.

(Received April 10, 1978).

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