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Review Article

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Antigen-Antibody Interactions *in vitro*: I. The Characteristics of Reactions in Tests for Antibodies to Viruses and Their Significance for Standard Assays and Adequate Routine Tests

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Abstract

Interactions between antibodies and viruses are diverse and function in different ways in tests demonstrating virus or antibody. Three antibody tests based on three different neutralization reactions by antibodies and another two ELISA modifications, one demonstrating a binding reaction and the other a blocking reaction by antibodies, represent five different antigen-antibody interactions used for demonstrating antibodies to viruses.

These five tests are the *first-order virus neutralization test*, the *virus aggregation neutralization test*, the *complement-enriched virus neutralization test*, the *conventional antibody ELISA*, and the *blocking antibody ELISA*. Basic versions of these tests are evaluated. The reaction in each test is described. The reacting antibodies in the highest concentration measuring the antibody titer and the test sensitivity are, where relevant, defined as either neutralizing or non-neutralizing, and modifications adjusted to possess an appropriately high sensitivity are evaluated for routine use and for the potential application as a reference or even gold standard assay, cf. *Definitions*.

The reaction in a *first-order virus neutralization test* is slowly progressing and enduring with increasing reaction times and the reacting antibodies are exclusively neutralizing. The test sensitivity is temperature-dependent and directly proportional to the reaction time. The very sensitive 37°C/24h configuration (reaction at 37 °C for 24 hours) is concluded to be the ideal reference and gold standard assay measuring neutralizing antibodies to viruses.

In the *virus aggregation neutralization test*, the inactivation of the virus by aggregation is prompt and short-lasting. All antibodies to the various antigenic determinants on the virus, neutralizing or non-neutralizing, react synergistically. The reaction depends strongly on the antibody concentrations, i.e., largely on the number of antigenic determinants on the virion. The test sensitivity is low and practically not adjustable, because of which the test is unsuited for demonstrating antibodies to viruses. This test is almost similar to a conventional 37°C/1h neutralization test, which by many has been considered a gold standard assay for demonstrating neutralizing antibodies.

In the *complement-enriched neutralization test*, the reacting antibodies of the highest concentration are non-neutralizing The reaction is almost instantaneous immediately after the addition of complement due to a prompt reaction with antigen-antibody complexes formed, but otherwise of first order with extended increasing reaction times following the first-order binding of non-neutralizing antibodies to the virus. The sensitivity

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of a 37°C/24h modification is high and for IgG antibodies equal to that of a conventional antibody ELISA with identical reaction conditions. However, it is laborious, because of which the latter will be a better alternative.

The *conventional antibody ELISA* is basically a first-order assay, and the reacting antibodies of the highest concentration determining the antibody titer and test sensitivity are non-neutralizing. Aggregation of virions is impossible, and the sensitivity is directly proportional to the length of the reaction time. A 37°C/24h configuration will be the ideal reference and gold standard antibody assay for measuring titers of non-neutralizing antibodies in the highest concentration and test sensitivities.

The sensitivity of the *blocking antibody ELISA* depends on the reaction temperature and time, but the reaction is not of first order. Increasing the reaction time from 1 to 24 hours in a herpesvirus test raises the sensitivity by approx. a factor of 4. The reaction rate is decelerating with increasing reaction time but still the sensitivity is relatively high with extended reaction. With herpesviruses, the sensitivity of a 37°C/24h test is twice that of the 37°C/24h first-order neutralization test, although 4 times lower than that of the conventional antibody ELISA. In its basic configuration, it is the test best suited for large-scale antibody examinations in diagnosing and controlling viral infections. The reactants can be varied to serve different objectives.

The sensitivity and specificity of 37°C/24h modifications of the three antibody tests found of special value are regularly over 99 percent when undiluted serum/plasma samples are examined.

SARS-CoV-2 antibody testing is referred to in relevant sections.

Keywords: Virus-antibody reactions, antibody tests, test sensitivity, standard tests, virus aggregation, virus neutralization, complement-dependent virus neutralization.

Article Highlights

- An analysis of the reactions in five tests for antibodies to viruses based on five different antigen-antibody interactions.
- The sensitivity of antibody tests is documented to be usually variable and adjustable to very high levels following regular lines of reaction.
- Recommendable reference and gold standard antibody assay modifications are presented in addition to routine test variants of relevant high sensitivity and specificity.

1. Introduction

Antibodies to important viral agents can be detected a few days after infection. Demonstrating the presence of, or freedom from, antibodies is important in diagnosing clinical disease and controlling viral infections. Antibody tests used for these purposes must be reliable.

Early investigations into the virus-neutralizing effect of antibodies showed that neutralization in a mixture of virus and antibody under certain conditions proceeded linearly with time (*Andrews and Elford* 1933) [1], that the reaction rate was proportional to the antibody concentration (*Burnet et al.* 1937) [2], and that the reaction was temperature-dependent (*Dulbecco et al.* 1956) [3]. These findings, however, do not appear to have influenced the construction of virus-antibody assays for practical use, which were elaborated merely on an empirical basis. It was commonly found that the reaction in a neutralization test at 4 °C overnight, or at 37 °C for 1 hour, would give identical results and that the test sensitivity could not be further improved. Despite the early findings [1,2], it became generally acknowledged that virus-antibody interactions in antibody tests would lead to an equilibrium state, cf. *Sections 2.1 and 2.2*.

The virus-antibody interactions *in vitro* have been analyzed comprehensively in three studies and analytical reviews by *Bitsch* 1978 [4], *Bitsch and Eskildsen* 1982 [5], and *Bitsch* 2017 [6]. Of greatest importance were 1) that the test sensitivity generally was both variable and adjustable to high levels following regular lines of reaction, 2) the presentation of the formula for the regular antigen-antibody interactions *in vitro*, and 3) that the virus-inactivating aggregation by particularly non-neutralizing antibodies also followed certain recognizable lines of reaction.

Blood is the antibody medium most often examined in antibody investigations because of the highest levels of the IgM and IgG antibodies, but while IgM antibodies usually disappear shortly after the defeat of a viral infection, IgG antibodies persist.

In relevant tests used for demonstrating antibodies to viruses, antibodies react with the antigen in three neutralization tests in ways that can be considered natural, and in two ELISAs in partly natural ways. In the following, the five antibody tests representing these fundamentally different reactions are evaluated in basic modifications concerning their functionality, *i.e.*, their reaction characteristics, suitability for routine testing, and potential use as reference or even gold standard assays, cf. *Definitions* below. The characteristics of the antigen-antibody reactions in these tests are described in *Section 2,* relevant issues regarding the test sensitivity and specificity are presented in *Section 3*, and basic modifications of the five tests are further evaluated in *Section 4*.

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Background

In 1970, Denmark was the first country to require freedom from the BoHV-1 infection in artificial insemination bull centers. All centers had been cleared of the infection and studies of the virus-antibody reaction were undertaken to ensure the reliability of the control tests used. When preliminary investigations had demonstrated the huge advantage of a neutralization test with extended reactions at 37 °C, a test modification with reaction at 37 °C for 24 hours used on undiluted serum became the test for the control of animals to be admitted to the centers.

Over 2-3 decades after 1980, the three most costly, widespread respiratory *SuHV-1*, *BoHV-1*, and *bovine viral diarrhea* infections were eradicated in Denmark. Blocking antibody ELISA modifications with reaction at 37 °C for close to 24 hours were chosen for examination of undiluted serum/plasma or cow's milk because of sufficiently high sensitivity, simplicity, and applicability for automation. However, for the final stages of eradicating the retrovirus infection *enzootic bovine leukosis*, the conventional antibody ELISA with reaction at 37 °C for close to 24 hours was used, because its essentially higher sensitivity was needed for the bulk tank milk testing, cf. *Section 4.4*.

Definitions

- *A reference standard antibody test* is a test modification, often recommended for practical use but mainly for indicating a recommended or required level of sensitivity.
- *A reference standard antibody test sample* contains antibodies to a specified virus and is acknowledged by authorities for indicating the lowest acceptable level of test sensitivity.
- A g*old standard antibody test* is a certain test modification with a specified antibody activity and an optimal high sensitivity, which is acknowledged, irrespective of the type of virus, to be the test for comparison when other tests are evaluated concerning their test characteristics.
- Tests are principally performed as *screenings* or as *titrations*, where a dilution series of the antibody medium is tested to determine a given end-point of reaction. Tests referred to in the following are usually titrations.
- *The reacting antibodies of the highest concentration* in an antibody medium are of special significance. These antibodies determine the titer of an antibody sample, which also is a measure of the *test sensitivity*.
- Antibodies can be divided into two groups, *i.e.*, *neutralizing antibodies* that are capable of inactivating viruses *in vitro* by being bound to their antigenic neutralization determinant on the virion and *non-neutralizing antibodies* which are unable to neutralize the virus simply by being attached to their antigenic determinant.

Points of notice

- Contrarily to what appears to be widely acknowledged, the attachment of antibody molecules to their antigenic determinants will under physiological conditions result in firm and irreversible bindings. Antigen-antibody interactions do not lead to an equilibrium state. The sensitivity of antibody assays may consequently be both variable and adjustable to high levels [4,6].
- Very little appears to be known about the relative concentrations in blood of antibodies to the various antigenic determinants on virions.
- The diversity of virus-antibody reactions enables the configuration of ELISAs demonstrating virus or antibody, which by the incorporation of appropriate aggregation reactions will be both rapid and very sensitive [6]. Such modifications, however, cannot be readily performed at most laboratories and are presently not suited for practical use.

2. The characteristics of the reactions in five antibody assays representing five different virus-antibody reactions

The antibody tests representing five different antigen-antibody interactions relevant for reaction analyses are:

- *The first-order virus neutralization* test (f-ord-VNT), based on inactivation of virus by the binding of neutralizing antibodies to their antigenic neutralization determinants on the virion.
- *The virus aggregation neutralization test* (aggr-VNT)*,* based on simple aggregation of virions made possible because of the di- or polyvalency of antibodies.
- *The complement-enriched virus neutralization test* (C-enr-VNT), based on supplementary neutralization of virions coupled with non-neutralizing antibodies through the aggregation of such virus-antibody complexes by the complement component C1q.
- *The conventional antibody ELISA* (conv-ab-ELISA), based on the identification of antibodies bound to immobilized antigenic determinants.
- *The blocking antibody ELISA* (bl-ab-ELISA), based on the demonstration of antibodies, which by reaction with their antigenic determinants block for the binding of a detecting reactant, usually a specific antibody-enzyme conjugate.

2.1. The first-order neutralization test

The neutralization by antibodies in a conventional neutralization test, where complement in test samples has been inactivated, is bi-factorial, caused by 1) the binding

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of neutralizing antibodies to their antigenic determinant on the virus and 2) simple aggregation of virions by the di- or polyvalent antibodies. This second reaction is rapid and short-lasting and is described in *Section 2.2* [4,6].

The regular neutralization caused by the binding of neutralizing antibodies to their antigenic neutralization determinant is monovalent and slowly progressing but enduring in conventional neutralization tests with increasing reaction times, following the lines of the formula of the regular antigen-antibody interactions *in vitro* not comprising aggregation [4],

$$k_{st} = \frac{[Ab][Ag]^q}{T}$$

where k_{st} is the standard reaction rate factor, Ab and Ag are antibody and antigen titers, T is the reaction time, and q is a co-determiner of the reaction rate factor, a particular log-log antibody/antigen binding ratio that is independent of the reacting antigen and antibody concentrations but varying with the reaction temperature. In the 1978 herpesvirus study, q was found to be approx. 0.15 at 37 °C but 0.24 at 4 °C [4,6]. Three of the four independent variables determining the standard reaction rate factor, the antigen and antibody titers and the reaction time and temperature, are shown directly in the formula, while the temperature is shown indirectly by the factor q being temperature-dependent. This factor q might be considered a genuine antibody-antigen affinity/avidity factor.

The formula illustrates that the reaction in an antibody test not including aggregation will be of first order, implying that an antibody titer and the test sensitivity will be directly proportional to the length of the reaction time, whereas in an antigen test the titer/reaction-time relationship will be exponential, depending on the value of the factor q. With q values essentially below 1 as found in the 1978 herpesvirus study, the sensitivity of an antigen ELISA will be considerably increased with extended reaction periods.

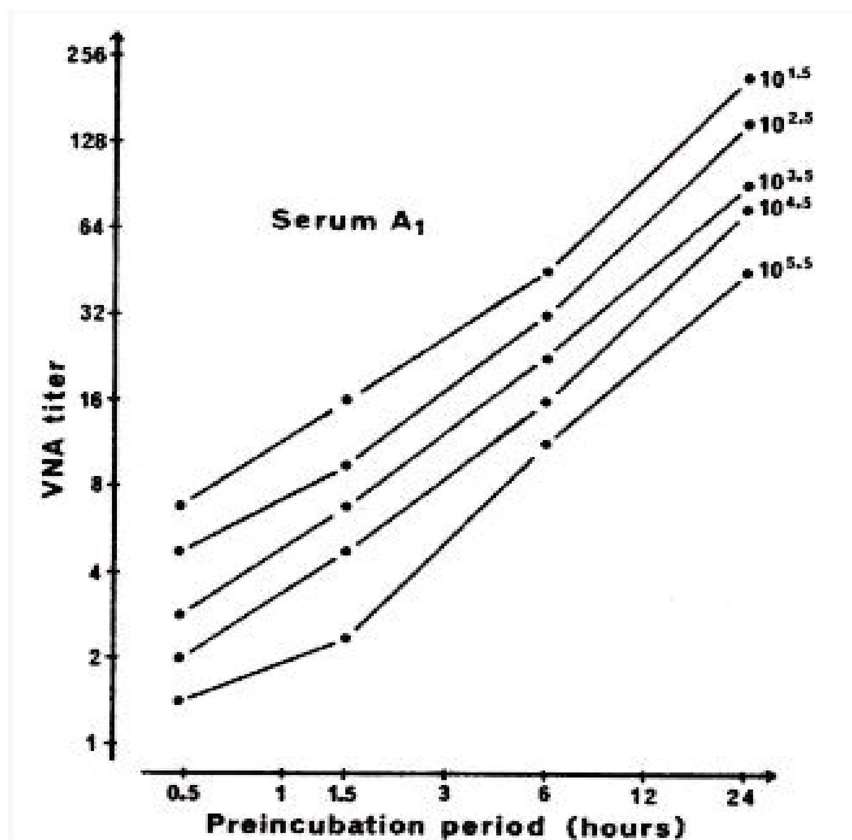

Figure 1: Kinetics of virus neutralization at 37 °C for a late-infection (IgG) serum sample in the dilution series of neutralization tests with extended reaction periods and varying virus concentrations. From *Bitsch* 1978 [4].

Virus: BoHV-1. VNA: virus-neutralizing antibody. Preincubation period: reaction time.
The neutralization lines are identical, varying only with the virus concentration. From 2 to 3 hours onwards, the log-log lines are linear with a slope coefficient of 1, which is characteristic of a first-order antigen-antibody binding reaction. Other measurements in this study documented a strictly log-log linear relationship with a slope coefficient of 1 for reactions up to at least 48 hours at 37 °C, 4 days at 26 °C, and 8 days at 15 and 4 °C.

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The sensitivity of an f-ord-VNT is both variable and adjustable. Another important relationship is that the factor q is temperature-dependent to such an extent that sensitive first-order antibody tests always should be performed with a reaction temperature not below 37 ºC. The sensitivity of a test with reaction at 37 ºC for 24 hours was not achieved in tests at 22 or 4 ºC until after 4 and 16 days of reaction, respectively [4]. In the following, a test with a reaction at, for example, 37 ºC for 24 hours will be designated a 37ºC/24h modification.

The widely different characteristics of the simple virus aggregation reaction without interference by complement and of the first-order neutralization reaction, the first being rapid and short-lasting and the other enduring and slowly progressing, means that one and the same test can be used to measure the titer of both the aggregating antibodies and the neutralizing antibodies. With a very short reaction time, only the aggregation titer will be seen, while with appropriately extended reaction periods only the neutralization by the neutralizing antibodies will be recorded.

The reaction time required for a neutralization test to show exclusively the first-order reaction by neutralizing antibodies depends on the relative titers of the reacting non-neutralizing and neutralizing antibodies. For herpesviruses, exclusively the first-order reaction by the neutralizing antibodies (IgG) is shown with reaction at 37 ºC for more than 2-3 hours (*Figure 1*). A remaining aggregation reaction measured after 1 hour of reaction explains why an increase of the reaction from 1 to 24 hours does not raise antibody titers, or the test sensitivity, by a factor of 24 as indicated by the antigen-antibody interaction formula above, but by a factor of 16-18 [4,6,9].

A titer measured in an f-ord-VNT is determined by the reacting antibodies in the highest concentration, which are neutralizing, cf. *Definitions*.

Reaction characteristics summarized

- The neutralization in a conventional neutralization test is bi-factorial, caused by 1) the simple virus aggregation without interference from complement being practically instantaneous and short-lasting and 2) the first-order reaction by neutralizing antibodies being bound monovalently to their antigenic neutralization determinant, which is enduring and slowly progressing with increasing reaction times.
- Due to the slow progression of the first-order reaction, the neutralization caused by the binding of neutralizing antibodies to their antigenic neutralization determinant will not be observed with short reaction periods.
- With appropriately extended reaction times, only neutralization by the neutralizing antibodies will be seen. A titer recorded and the test sensitivity will be directly proportional to the length of the reaction period in accordance with the regular antigen-antibody interaction formula.
- In a herpesvirus neutralization test, the first-order reaction became evident in a neutralization test with reaction times exceeding 2-3 hours at 37 ºC, irrespective of the antibody levels in the samples tested.
- The reacting antibodies of the highest concentration with extended reaction are neutralizing.
- The factor q in the antigen-antibody interaction formula depends on the temperature to such a degree that an optimal sensitivity will not be achieved under practical conditions at a reaction temperature below 37 ºC.

2.2. The virus aggregation neutralization test

In the 1978 study [4], the very early and regular "over-neutralization" phenomenon was not immediately understood, but after the report by *Brioen et al.* 1983 [7], it could be concluded that this reaction was neutralization caused by simple virus aggregation by the di- and polyvalent antibodies without interaction from complement [6].

As explained in *Section 2.1*, simple aggregation without involvement of complement predominates in conventional neutralization tests with short reaction periods. The aggregation reaction is almost instantaneous and short-lasting, because the antibodies to the various antigenic determinants, predominantly the non-neutralizing ones, react *promptly* and *synergistically*. The reaction observed with appropriately extended reaction times will be the neutralization by the first-order binding of neutralizing antibodies to their antigenic neutralization determinant, cf. *Section 2.1* [4,6].

The rapid virus-aggregating reaction can be considered to be almost independent of the reaction time, because of which the sensitivity of an aggr-VNT will be low and practically non-adjustable. A measurement of the titer of virus-aggregating antibodies should be performed at 37 ºC with a very short reaction period to eliminate the influence from the slowly progressing first-order neutralization by the neutralizing antibodies, cf. *Section 2.1*. The reactants should be heated to 37 ºC before being mixed.

Reaction characteristics summarized

- The simple in vitro aggregation of viruses in a neutralization test by di- and polyvalent antibodies is rapid and short-lasting.
- All antibodies to various antigenic determinants aggregate viruses synergistically.
- The simple aggregation is the predominant reaction in neutralization tests with short reaction periods but will not

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be observed in tests with appropriately extended reaction.

- Simple aggregation titers shall be measured in neutralization tests with a very short reaction period and the reactants should be heated to 37 °C before being mixed.
- The reaction is dependent on the total concentration of antibodies, which largely depends on the number antigenic determinants. The test sensitivity cannot be adjusted to acceptable levels.

2.3. The complement-enriched neutralization test

The complement component C1q is hexavalent, so its aggregating potency is quite extraordinary. It will attach to the Fc region of antibodies that have been sensitized by being coupled with their antigenic determinant. If virions have been bound to a non-neutralizing antibody molecule, the polyvalent component C1q will promptly, if being present in adequate quantities, attach to these virus-antibody complexes and neutralize them by including them in aggregates [5,6].

Samples to be examined in neutralization tests are regularly heated at 56 °C for 30 min. to inactivate complement, so the complement-dependent neutralization functioning *in vivo,* will not be seen in a conventional neutralization test.

In a C-enr-VNT, the supplementary neutralization by aggregation will proceed in two steps. Immediately after the addition of complement to the virus-antibody mixtures, the reaction will be prompt, because complement will practically instantly bind to the virus-antibody complexes formed immediately, whereas the continuing aggregation reaction with extended reaction times will be of first order, following the subsequent first-order binding of the non-neutralizing antibodies to their antigenic determinants, cf. *Figure 2*.

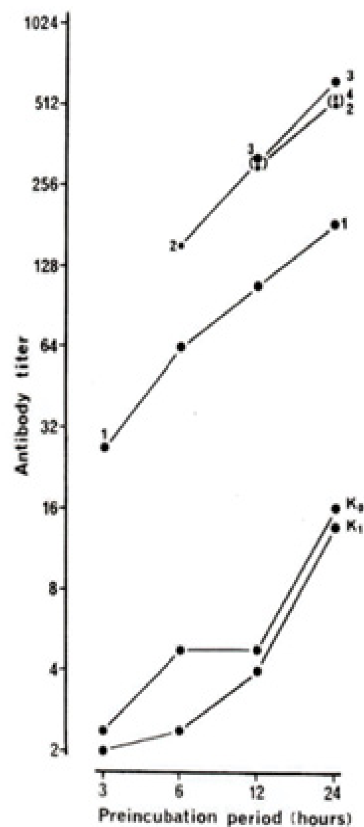

Figure 2: The effect of complement on the progression of neutralization in dilution series of an early convalescent-phase serum sample tested in virus neutralization tests. From *Bitsch and Eskildsen* 1982 [5].

Virus: SuHV-1. Preincubation period: reaction time.
Serum was collected 13 days after nasal infection. Virus-serum mixtures were incubated at 37 °C, and titers were recorded by inoculation of cultures after reaction for 3, 6, 12, or 24 hours. K_0 and K_1: no complement (K_0) or heat-inactivated complement (K_1) was added at the start of virus-serum incubation. For the reactions 1-1, complement was added at the start of incubation and for reactions 2-2, 3-3, and 4, complement was added one hour before inoculation of cultures, i.e. after 5, 11, or 23 hours of reaction, respectively.
The log-log slope coefficient of all complement neutralization lines is 1, documenting a first-order, monovalent reaction. An optimal effect is not obtained if complement is added at the start of virus-serum incubation (neutralization line 1-1).

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The binding reaction by antibodies, neutralizing as well as non-neutralizing, will be of first order with extended reaction periods [4,5,6]. For herpesviruses, complement will raise the titer of IgG antibodies in serum by a factor of approx. 8, cf. *Figure 3*. Titers are determined by the reacting antibodies of the highest concentration, and the supplementary neutralization by complement is due to the reaction with non-neutralizing antibodies. The binding reaction for both neutralizing and non-neutralizing antibodies with extended reaction periods is monovalent and of first order. For non-neutralizing antibodies, one antibody molecule bound to a virion will inactivate this virion by aggregation in cooperation with the complement component C1q, and for neutralizing antibodies it must be assumed that one molecule attached to an antigenic determinant on the virion will neutralize it. It will therefore be logical to conclude that the concentration of the reacting non-neutralizing herpesvirus IgG antibodies of the highest concentration is approx. eight times higher than that of the neutralizing antibodies. Furthermore, the total concentration of aggregating non-neutralizing IgG antibodies will be many times higher, largely proportional to the number of related antigenic determinants.

For herpesvirus IgG antibodies, an optimal C-enr-VNT shows a sensitivity 8 times higher than that of an f-ord-VNT, cf. *Figure 3*. In this context, it is noteworthy that also the first-order herpesvirus conv-ab-ELISA is approx. 8 times more sensitive than a f-ord-VNT, see later in *Section 2.4*.

For acute infection-phase samples with predominating IgM non-neutralizing antibodies, the titers in a 37°C/24h C-enr-VNT were extremely high, 10.000 or higher from 8 to 15 days after infection, cf. *Figure 3*, and a significant IgM neutralizing titer was recorded as early as 4 days after nasal infection.

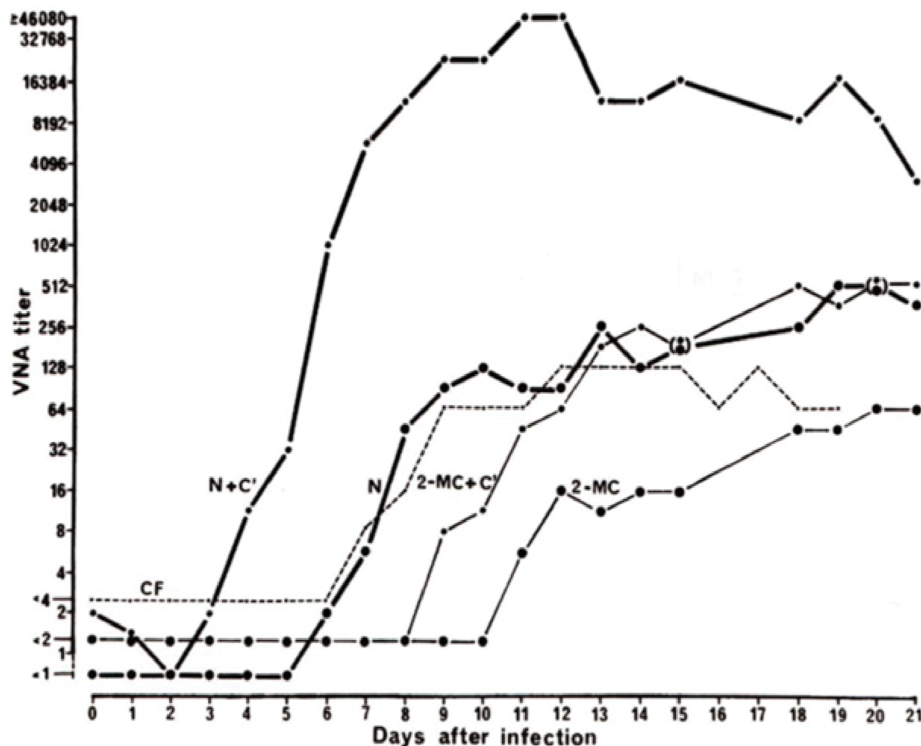

Figure 3: The appearance of non-neutralizing and neutralizing IgM and IgG antibodies in blood during the first 21 days after experimental nasal infection. From *Bitsch and Eskildsen* (1982) [5].

Virus: SuHV1. VNA: virus-neutralizing antibody titer. Tests: the first-order neutralization test and the complement-enriched neutralization test. The porcine serum samples were heated at 56 ^{o}C for 30 min. and tested, either untreated (N) or treated with 2-mercaptoethanol (2-MC), which will inactivate the IgM antibodies, but leave IgG antibodies unchanged. In both cases, the sera were tested with and without the addition of complement (C´). The virus-serum mixtures were incubated at 37 ^{o}C for 24 hours and, where used, complement was added after 23 hours of reaction. Results from a complement fixation test are also shown (CF). As should be expected, the CF titer line follows the one for the titers of the reacting non-neutralizing IgM antibodies in the highest concentration, although at a lower level of sensitivity.

Symbols: N+C': titers of non-*neutralizing* IgM antibodies

N: titers of *neutralizing* IgM antibodies

2MC+C': titers of non-*neutralizing* IgG antibodies

2MC: titers of *neutralizing* IgG antibodies

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One particular effect of the complement component C1q in addition to the prompt aggregation is that all non-neutralizing antibodies are effectively converted into neutralizing ones, also inactivating viruses in neutralization tests with extended reaction times with the same rate as neutralizing antibodies [5,6].

Reaction characteristics summarized

- The hexavalent complement component C1q will in adequate concentrations bind promptly to antigen-antibody complexes and neutralize them by aggregation.
- Thereafter, the reaction will with increasing extended reaction times be of first order, following the continuing first-order binding of non-neutralizing antibodies to their antigenic determinants .
- The reacting antibodies of the highest concentration determining the antibody titer and the test sensitivity, are non-neutralizing. In samples with predominantly IgG antibodies, they are the same non-neutralizing antibodies determining the titer measured in a conv-ab-ELISA, because of which these tests share the same high sensitivity with identical reaction conditions.
- The sensitivity 8 times higher when measuring IgG antibodies of the C-enr-VNT as compared to the f-ord-VNT indicates a concentration of the reacting non-neutralizing IgG antibodies of the highest concentration, which is 8 times higher than that of the neutralizing antibodies.
- The reaction temperature in a C-enr-VNT should not be below 37 ^{o}C.
- For acute infection-phase serum with IgM antibodies, the neutralizing aggregation effect by C1q is huge.
- One special effect of complement is that all non-neutralizing antibodies are converted into neutralizing ones, also inactivating the virus in neutralization tests with extended reaction times with the same rate as the neutralizing ones.

2.4 The conventional antibody ELISA

After viral infection, IgM antibodies appear after a few days and will usually be non-demonstrable after some weeks, whereas IgG antibodies will appear some days after the IgM antibodies, cf. *Figure 3*, and will regularly persist for life. An antibody ELISA can be modified in different ways, but for the sake of simplicity, only a basic configuration will be considered here, where a whole antigen preparation is coated directly onto the ELISA plates and the detecting reagent will show specific IgG antibodies.

In a conv-ab-ELISA, virus aggregation by antibodies is impossible because the antigens have been immobilized. The reactions between the antibodies and their antigenic determinants will therefore be of first order. In other words, if the reaction time is increased from for example 1 to 24 hours, the test sensitivity, or a measured antibody titer, will be raised by a factor of 24, cf. the antigen-antibody interaction formula above [4,6].

For herpesviruses, the titer of non-neutralizing IgG antibodies was found to be 8 times higher than that of the neutralizing ones, cf. *Figure 3*. Consistently, the sensitivity of the conv-ab-ELISA used on late post-infection samples was found to be generally 8 times higher than that of the f-ord-VNT in comparative examinations with identical reaction conditions [5,8]. The unique sensitivity of a conv-ab-ELISA as compared to a highly sensitive f-ord-VNT can therefore be simply explained by the circumstance that the reacting antibodies in the highest concentration are non-neutralizing. These reacting antibodies will be those of the highest concentration also in the C-enr-VNT, implying that 37°C/24h modifications of these two tests can be considered to share the same high sensitivity, cf. *Section 2.3*.

The factor q in the antigen-antibody interaction formula is temperature-dependent to such a degree that the reaction temperature for a first-order antigen-antibody test should not be below 37 ^{o}C.

Reaction characteristics summarized

- The reaction in a conv-ab-ELISA is of first order.
- The test sensitivity is proportional to the length of the period of reaction.
- The relatively high sensitivity of the conv-ab-ELISA as compared to an f-ord-VNT found in investigations with herpesviruses is explained by the fact that also non-neutralizing antibodies bound to the antigen are measured, that the concentration of non-neutralizing antibodies is higher than that of the neutralizing, and that the reacting antibodies of the highest concentration are non-neutralizing.
- The reacting antibodies of the highest concentration are the same as for the C-enr-VNT, because of which these tests with identical reaction conditions share the same high sensitivity.
- The reaction temperature of a conv-ab-ELISA should not be below 37 ^{o}C.

2.5. The blocking antibody ELISA

In the basic and simple form of the bl-ab-ELISA, a whole antigen preparation is coated directly to the wells of the microtiter plates. The specific reactant in the detecting enzyme conjugate, will typically be identical to the antibody to be detected, so positive test samples are demonstrated by their ability to block for the binding of the detecting reagent to

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the antigen. Several variations are possible, for example, with a selected fraction of the virus used as the coated antigen or a monoclonal specific antibody used in the detecting enzyme conjugate. The reaction is somewhat complex [6].

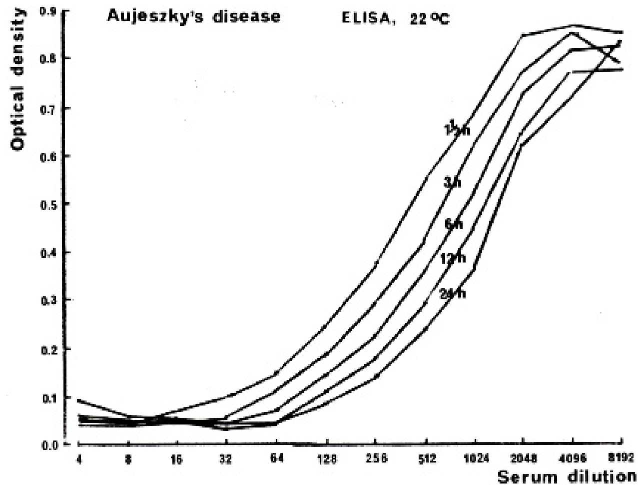

Figure 4: The kinetics of the reaction in a blocking antibody ELISA. From *Bitsch* 2017 [6].

Virus: SuHV-1.
The plates were coated with a whole antigen preparation and a polyclonal antibody was used for the detecting enzyme conjugate. The highest optical density corresponds to no blocking effect by specific antibodies, while reduced optical density shows a blocking effect by antibodies in the test sample. A twofold dilution series of a natural late post-infection antibody-positive serum sample was allowed to react for 1½, 3, 6, 12, and 24 hours. Reading titers by e.g. a 50% blocking reaction, the log-log titer-time ratio is linear with a slope coefficient lower than 1 (decelerating rate).

With whole antigen coated to the plates and an optimal test sensitivity, an antibody-positive sample with a moderate to high antibody concentration will give a close to 100 percent blocking reaction, and only over a limited antibody concentration range of 5-6 log base-2 units, varying reactions are seen. If titers are read by a 50% blocking endpoint, the reaction rate is linear, but with a log-log slope coefficient different from 1 (*Figure 4*). Increase of the reaction time from 1.5 to 24 hours raised titers approximately by a factor of 4 (decelerating rate).

In screening examinations of undiluted serum, the majority of positive samples will give a maximal blocking reaction with a test of acceptable sensitivity, cf. *Figure 6* [10].

With late post-infection serum samples, the sensitivity of the herpesvirus 37°C/24h blocking ELISA was found to be high, *i.e.*, twice that of the 37°C/24h f-ord-VNT, although 4 times lower than that of the 37°C/24h conv-ab-ELISA [6]. *Sørensen and Lei* 1986 [11] demonstrated for the bl-ab-ELISA a sensitivity dependency on both reaction time and temperature, but the reaction was not of a first-order. *Kramps et al.* (1994) [12] introduced a variant with a monoclonal antibody used for the detecting reagent and demonstrated high sensitivity. For all variants, test samples were tested undiluted in screenings. The highest sensitivity is obtained with extended reactions at a temperature not below 37 °C.

Reaction characteristics summarized

- The reaction in a basic variant of the bl-ab-ELISA with whole antigen and a polyclonal specific antibody for the detecting enzyme reagent is not of first order.
- The test sensitivity depends on both the reaction time and temperature. An increase in the reaction time at 37 °C from 1 to 24 hours will raise the sensitivity by a factor of approx. 4.
- The reaction rate is decelerating with increasing reaction times.
- The reaction temperature of a bl-ab-ELISA should be 37 °C, and routine screenings should be performed with a reaction time of close to 24 hours on undiluted samples.

3. Sensitivity and specificity of antibody assays

Early studies of antigen-antibody interactions demonstrated regular lines of reactions [1,2,3] but, nevertheless, it became generally acknowledged that virus-antibody interactions would lead to an equilibrium state. However, such a condition would imply that the sensitivity of antigen-antibody assays would neither be variable nor adjustable.

The 1978 study [4] documented that the reaction in a neutralization test was bi-factorial. An early fast reaction was termed *the "over-neutralization"* and an enduring, slowly progressing reaction was identified as a *first-order neutralization reaction*. The sensitivity of an f-ord-VNT test with extended reaction was shown to be both variable and adjustable, the formula for the regular antigen-antibody interaction was presented, and a hypothetical so-called *percentage law* [1], according to which the rate of neutralization would be independent of the virus concentration, was found invalid. On basis of the relationships demonstrated, tests of earlier unknown high sensitivity could be elaborated. The regular antigen-antibody interaction formula is presented above in *Section 2.1*.

The test sensitivity will naturally be decreased by a factor of 10 if a test sample is examined in the dilution 1:10 instead of undiluted. It was a personal experience that test samples always should be examined in the highest possible concentration, usually undiluted. Furthermore, regarding specificity, it was found that even a highly increased sensitivity by changed reaction conditions never in any way

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tended to give unspecific reactions. The results in *Figure 5* illustrate, how a high sensitivity of the f-ord-VNT, increased by a factor of 16-18, allowed a reliable differentiation between antibody-positive and antibody-negative individuals without any negative influence on the test specificity. The sensitivity of a reference standard antibody assay shall be sufficiently high to enable a reliable differentiation between truly antibody-positive and antibody-negative individuals. The sensitivity and specificity of tests for routine use shall therefore be practically100 percent.

Terms like *diagnostic* or *predictive* values of the test sensitivity or the test specificity are often used in evaluations of tests of limited sensitivity and specificity. But tests for diagnosis of viral infections with insufficient sensitivity and specificity are not acceptable and should be replaced or modified in order to fulfill the ideal requirements.

The sensitivity of a test is usually calculated after the examination of a sufficient number of antibody-positive samples. But antibody levels vary, cf. *Figure 5*. It is generally accepted that antibody responses are higher after severe clinical disease than after transient or even asymptomatic infections, so in order to achieve a true sensitivity estimation for a test, the samples should be selected with the aim to have the whole variation of antibody levels in a population represented. If, for example, test samples are selected only among individuals having shown clinical disease, a calculated test sensitivity is likely to be misleadingly high. The sensitivity of a new test modification can also be evaluated in comparison with another test with a known well-characterized antibody reaction and a documented sufficiently high sensitivity, a reference or gold standard antibody test, or simply by examining reference standard test samples.

Increased test sensitivity by changed reaction conditions will regularly not give any reduction in specificity, so standard antibody assays should be performed with the optimal highest sensitivity, although still allowing easy and practical performance. The sensitivity of a reference or gold standard antibody assay should be higher than that required for a test for routine use, and the reacting antibodies in the highest concentration should be specified, as least as neutralizing or non-neutralizing. A 37°C/1h conv-ab-ELISA might be found sufficiently sensitive for several purposes, but the particular circumstance that the sensitivity of for example a 37°C/24h modification is 24 times higher, indicates that extended reaction should be chosen for a gold standard assay.

High reactions in screenings are regularly not questioned, but it should be realized that varying screening reactions obtained in for example antibody ELISAs are regularly only over a short interval proportional to the antibody titer or concentration. Only titrations can show the relative levels of the reacting antibodies of the highest concentration determining the test sensitivity.

An important detail related to the results shown in *Figure 6* is that the animals with the lowest antibody-positive blocking reactions, from 40 to 70 percent of blocking, generally were the youngest antibody-positive animals in herds with no recent active infection. This indicates that these animals became infected during the latest spreading while having effective levels of maternal antibodies reducing the stimulus to antibody production. This particular condition has been observed also for other viral infections and underlines that whenever the identification of any earlier infected individual is essential, *e.g.,* in controlling herpesvirus infections in the veterinary field, the application of a highly sensitive antibody test for control examinations is crucial.

Sensitivity and specificity of antibody assays summarized

- The sensitivity of antibody assays for practical use is generally both variable and adjustable.
- Tests for diagnosing and controlling infectious diseases must be safe. The sensitivity and specificity of antibody assays for practical use should be optimal, practically 100 percent.
- The sensitivity of a test is indicated by the titer obtained for the reacting antibodies to antigenic determinants of the highest concentration.
- A gold or reference standard antibody test should have a high sensitivity exceeding that required for a safe routine test and the reacting antibodies of highest concentration should be characterized, at least as neutralizing or non-neutralizing.
- A highly increased sensitivity of an antibody assay by changed reaction conditions will regularly not reduce the test specificity.

4. Optimal modifications of antibody assays for practical use

4.1. The first-order neutralization test

It is of the utmost importance that the sensitivity of the f-ord-VNT is adjustable, being dependent on the reaction temperature and directly proportional to the reaction time. As illustrated in *Figure 5*, the increase in reaction time from 1 to 24 hours at 37 °C facilitated a safe differentiation between BoHV-1 antibody-negative and -positive individuals, indicating a sensitivity and specificity close to 100 %.

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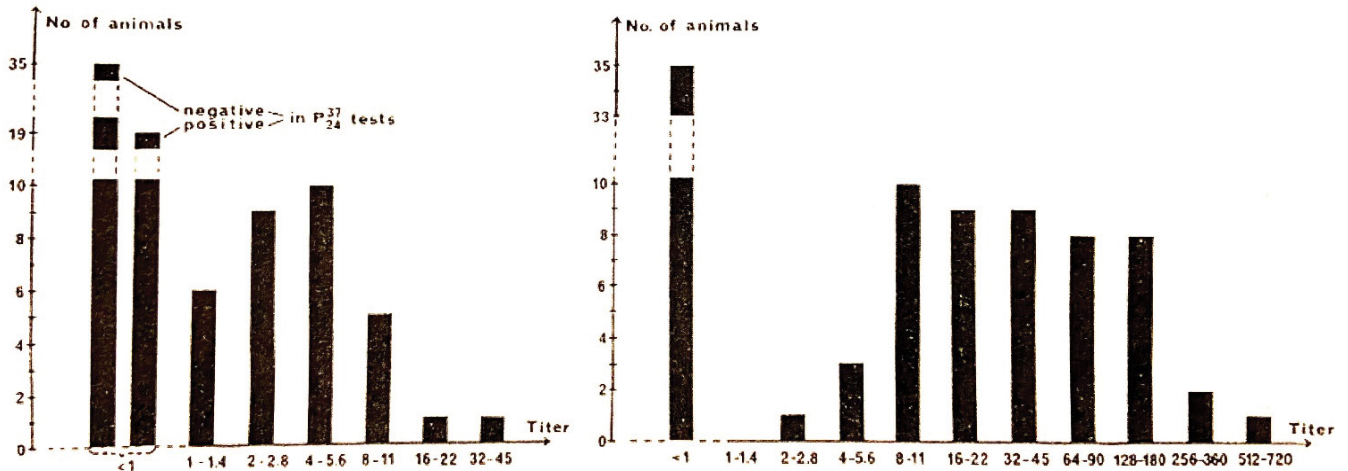

Figure 5: Virus-neutralizing antibody titers in serum of 86 animals from two cattle herds. From *Bitsch* 1978 [8].

Virus: BoHV-1. Ordinate: number of samples. Abscissa: antibody titer.
The sera were tested undiluted and in dilutions to determine the antibody titers. Two different test modifications were used.
Left: Titers obtained in a neutralization test after reaction at 37°C for 1 hour.
Right: Titers obtained in a neutralization test after reaction at 37°C for 24 hours.
The 37°C/24h test differentiated clearly between antibody-positive and antibody-negative individuals with even a "safety distance" between the negative and the lowest positive reaction. The average increase in sensitivity was by a factor of 16.

The average titer increase by raising the reaction time from 1 to 24 hours was by a factor of slightly more than 16 and not fully 24, because a remaining aggregation reaction was still recorded after 1 hour of reaction. The antibody-negative animals were all below 2-3 years old, and the only young antibody-positive animal (young calves under 6 months of age with possibly maternal antibodies were not tested) was a one-year-old bull calf with an unquestionable high antibody titer. A comprehensive spreading of the infection in these two herds could therefore be concluded not to have occurred in these two herds for a couple of years (*Bitsch* 1978) [8].

In 2008, the World Organization for Animal Health (WOAH) acknowledged the 37°C/24h f-ord-VNT [8] as the reference standard test for control of the BoHV-1 infection (*WOAH Terrestrial Manual 2021, Ch. 3.4.11*). But the general confusion concerning regular antigen-antibody interactions in tests is illustrated by the fact that the WOAH at the same time accepted a 37°C/0.5h SuHV-1 neutralization test as a reference standard antibody test (*WOAH Terrestrial Manual 2021, Ch. 3.1.2*). Earlier, in1976, *Bitsch and Eskildsen* [9] had documented that a 37°C/1h SuHV-1 neutralization test was insufficiently sensitive, whereas a 37°C/24h test would be adequate with sensitivity and specificity values over 99 percent in line with the BoHV-1 assay.

The reacting antibodies of the highest concentration in an f-ord-VNT with an appropriately extended reaction time are *neutralizing* antibodies. As mentioned above, it does not seem to have been documented at present, if only one or very few different neutralization determinants exist on a virion. Monoclonal antibodies to various antigenic determinants may have been incorrectly identified as neutralizing by authors not paying attention to the general ability of non-neutralizing antibodies to neutralize by aggregation.

A 37°C/24h f-ord-VNT will be the ideal gold standard assay demonstrating specifically neutralizing antibodies.

Considerations related to SARS-CoV-2

A 37°C/1h neutralization test, where neutralization after inoculation of tissue cultures can be recorded in slightly but insignificantly different ways, is often in literature considered the gold standard neutralization assay and is also by many believed to reflect exclusively neutralization caused by the binding of neutralizing antibodies to their antigenic determinant. This test modification has also been used as a reference standard antibody test in SARS-CoV-2 investigations [13,14]. But judged from the regular lines of antigen-antibody interactions documented in the studies with herpesviruses [4,6], a considerable part of this short-term virus neutralization reaction is due to virus aggregation by non-neutralizing antibodies.

It is topical to accept a reference or gold standard neutralization assay consistent with first-order reaction lines. A 37°C/24h f-ord-VNT will guarantee no interference from non-neutralizing antibodies and a sufficiently high test sensitivity for comparative examinations.

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Overall conclusions regarding the f-ord-VNT

- The reacting antibodies of the highest concentration in an f-ord-VNT with appropriately extended reaction are neutralizing antibodies.
- The reaction is enduring with increasing reaction times, following the lines of the formula for the regular antigen-antibody interactions without aggregation, implying that the sensitivity is dependent on the reaction temperature and directly proportional to the reaction time.
- The 37°C/24h f-ord-VNT is highly sensitive and will be ideal for a gold standard antibody assay for demonstrating neutralizing antibodies to a broad variety of viruses.

4.2 The aggregation neutralization test

The herpesvirus study [4,6] illustrated that a 37°C/1h neutralization test will show the combined effect of two completely different neutralization reactions (*Section 2.1*). The early reaction seen was mainly neutralization by aggregation caused predominantly by non-neutralizing antibodies.

The special aggr-VNT is actually a traditional neutralization test but shall be performed with a very short reaction period, 2-5 minutes,whicht will show the prompt aggregation reaction and prevent interference from the slow first-order neutralization. The virus and antibody preparations should be heated to 37 °C before being mixed.

The test sensitivity is not adjustable to acceptable levels and the test does not have advantages over other antibody assays. Not fulfilling the basic requirement for sensitivity, it is not suited for practical use in the diagnosis and control of viral infections.

A 37°C/1h neutralization test is widely accepted as a reference standard antibody test, apparently also by authorities, most probably because it is commonly believed that the reaction exhibited is by neutralizing antibodies and that the sensitivity of a neutralization test is not variable and adjustable.

Overall conclusions regarding the aggr-VNT

- The simple aggregation of viruses by antibodies is prompt and short-lasting.
- The neutralizing capacity of non-neutralizing antibodies by aggregation can be measured in a neutralization test with a very short reaction time, which will not allow the slowly progressing first-order neutralization by the attachment of neutralizing antibodies to their antigenic neutralization determinant to be seen.
- Reactants should be heated to 37 °C and an acceptable reaction time would be 2-5 minutes.
- The sensitivity is relatively low, especially because the test sensitivity is not immediately adjustable.
- The aggr-VNT is not relevant for use in diagnosing and controlling viral infections.

4.3. The complement-enriched neutralization test

In a C-enr-VNT, the hexavalent complement component C1q will practically instantly aggregate and inactivate virions that have been coupled with a non-neutralizing antibody. The reaction after the addition of complement with readily formed virus-antibody complexes will be practically instantaneous but, thereafter, it will continue as a first-order reaction with increasing reaction times following the first-order binding of non-neutralizing antibodies to antigenic determinants [5,6]. High sensitivity and a specified reaction with non-neutralizing antibodies can therefore be achieved by extended reaction at 37 °C, and the obvious choice of reaction time for a standard assay would be 24 hours, cf. *Figure 3.*

With late post-infection IgG serum samples, the test sensitivity is identical to that of the conv-ab-ELISA and determined by the same non-neutralizing antibodies of the highest concentration.

One minor complication associated with the use of complement is that it may cause a very low degree of unspecific virus inactivation with serum/plasma undiluted or in very low dilutions. But by exploiting a very high test sensitivity this will not be a problem. To optimize the test sensitivity, it is recommendable that complement is added late, *i.e.*, under practical conditions after more than one hour and not later than 15 minutes before the end of the reaction time [5].

The characteristics of the reaction in the C-enr-VNT are described in detail in *Section 2.3.* The neutralization with extended reaction is of first order. The test sensitivity is adjustable and will for a 37°/C/24h version be high, *i.e.*, as concluded from herpesvirus studies, for IgG antibodies regularly 8 times higher than the sensitivity of the f-ord-VNT. With acute-phase and early convalescent-phase samples with IgM antibodies, the sensitivity is extremely high.

A 37°/C/24h C-enr-VNT might be excellent for a reference or gold standard antibody assay measuring non-neutralizing IgG antibodies, but the test performance is demanding. The 37°/C/24h conv-ab-ELISA identifies the same non-neutralizing IgG antibodies of the highest concentration and is equally sensitive, cf. *Sections 2.3* and *4.4.*

Overall conclusions regarding the C-enr-VNT

- The reaction in a C-enr-VNT will be almost explosive immediately after the addition of complement to the virus-antibody mixtures. Thereafter, neutralization with extended reaction will proceed as a first-order reaction with increasing reaction times following the first-order binding of non-neutralizing antibodies to their antigenic determinants.

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- The reacting antibodies of the highest concentration, determining the test sensitivity, are non-neutralizing.
- For optimal sensitivity, complement should preferably be added not earlier than after one hour of reaction and not later than 15 minutes before the end of the reaction period.
- The 37ºC/24h C-enr-VNT would be suited for a standard antibody assay for the demonstration of especially IgG non-neutralizing antibodies, but the test performance is complicated and demanding, because of which the equally sensitive 37ºC/24h conv-ab-ELISA, is a better alternative.
- A 37ºC/24h C-enr-VNT will measure also IgM antibodies and its sensitivity is extremely high.

4.4. The conventional antibody ELISA

The reaction in the conv-ab-ELISA follows the formula for regular antigen-antibody interactions (*Section 2.1*), and the sensitivity will be directly proportional to the reaction time. Due to the temperature dependence, sensitive tests should be performed at a temperature not below 37 ºC.

In the conv-ab-ELISA, the detecting reagent will bind to all IgG antibodies. The antibodies of the highest concentration in late post-infection samples, determining the titer of a sample and also the test sensitivity, will be non-neutralizing and identical to the reacting antibodies of highest concentration in a C-enr-VNT, cf. *Sections 2.3* and *2.4*. The 37ºC/24h modifications of these two tests will be equally sensitive. Complement raised IgG antibody titers in a C-enr-VNT by approx. a factor of 8 (*Figure 3*), which is in agreement with the finding that the sensitivity of a herpesvirus conv-ab-ELISA is approx. 8 times higher than that of an f-ord-VNT with identical reaction conditions [6].

The characteristics of the reaction in a conv-ab-ELISA are described in *Section 2.4*. The reaction is of first order, the sensitivity is adjustable, the 37ºC/24h modification is extremely sensitive, it is simple, and it is applicable for automation. So, the 37ºC/24h conv-ab-ELISA will be extremely well-suited for a gold standard antibody assay for demonstrating levels of reacting non-neutralizing antibodies in the highest concentration and for comparative measurements of test sensitivity.

The advantage of a conv-ab-ELISA with adjustable sensitivity can be demonstrated by the following. In 1988, the EU Commission issued a directive (88/406) committing member countries to test their cattle herds for the presence of *enzootic bovine leukosis*, a retrovirus infection. A reference standard test sample was available for indication of the required test sensitivity. According to the directive, dairy herds could be tested using a conv-ab-ELISA on milk in pools of samples from maximally 20 cows. But by using reaction at 37 ºC for close to 24 hours, the sensitivity was raised to a level allowing test of milk samples in pools representing more than 400 cows. The sensitivity could be further improved by use of a longer reaction period if found relevant, e.g., 48 hours allowing testing pools of milk from 800 cows [6]. Danish dairy herds could consequently be controlled on bulk tank milk samples.

It is worthy of note that a first-order antibody test performed with reaction at 37 ºC, but with reaction for 18-20 hours instead of 24 hours, will have a sensitivity only slightly lower than that of a 37ºC/24h test. Routine examinations in control of infections in the veterinary field have therefore usually, for practical reasons, been performed, not with 24 hours of reaction, but with reaction at 37 ºC overnight.

Artificial insemination bull centers in EU countries must be free from the BoHV-1 infection, cf. the EU commission delegated regulation 2020/686. The reference standard antibody test for control is the conv-ab-ELISA or alternatively a bl-ab-ELISA, both in highly sensitive versions.

Overall conclusions regarding the conv-ab-ELISA

- The reaction in a conv-ab-ELISA is of first order. The test sensitivity is therefore adjustable and proportional to the reaction time. The reaction temperature should be 37 ºC.
- The reacting antibodies of highest concentration in late post-infection serum/plasma samples, determining the antibody titer and the test sensitivity, are non-neutralizing. The test sensitivity is identical to that of the first-order C-enr-VNT with the same reaction conditions.
- Modifications with extended reaction at 37ºC are extremely sensitive and specific, and are well-suited for automation. Test samples should be tested undiluted in screenings.
- The 37ºC/24h modification of the conv-ab-ELISA is ideal for a gold standard antibody assay for comparative measurements of test sensitivity.

4.5. The blocking antibody ELISA

The reaction in the bl-ab-ELISA is somewhat complicated and not of first order. Nevertheless, the sensitivity is adjustable and is improved by extended reaction. For 37ºC/24h herpesvirus tests, the sensitivity of the bl-ab-ELISA was twice that of the f-ord-VNT, although approx. 4 times lower than that of the conv-ab-ELISA and the C-enr-VNT.

In Denmark, a bl-ab-ELISA was selected for all routine antibody examinations in connection with the eradication of the *SuHV-1* [11], *BoHV-1* [6], and *bovine viral diarrhea virus* [10] infections because of a sufficiently high sensitivity and specificity and easy applicability for large-scale examinations, while for the final stages of the eradication of enzootic bovine *leukosis*, a conv-ab-ELISA was selected, because of a

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required higher sensitivity, cf. *Section 4.4*. The test sensitivity and specificity are high, practically 100 percent. All samples, serum/plasma or milk, were tested undiluted in screenings. A conv-ab-ELISA was used occasionally for follow-up examinations, or when a higher sensitivity was considered desirable.

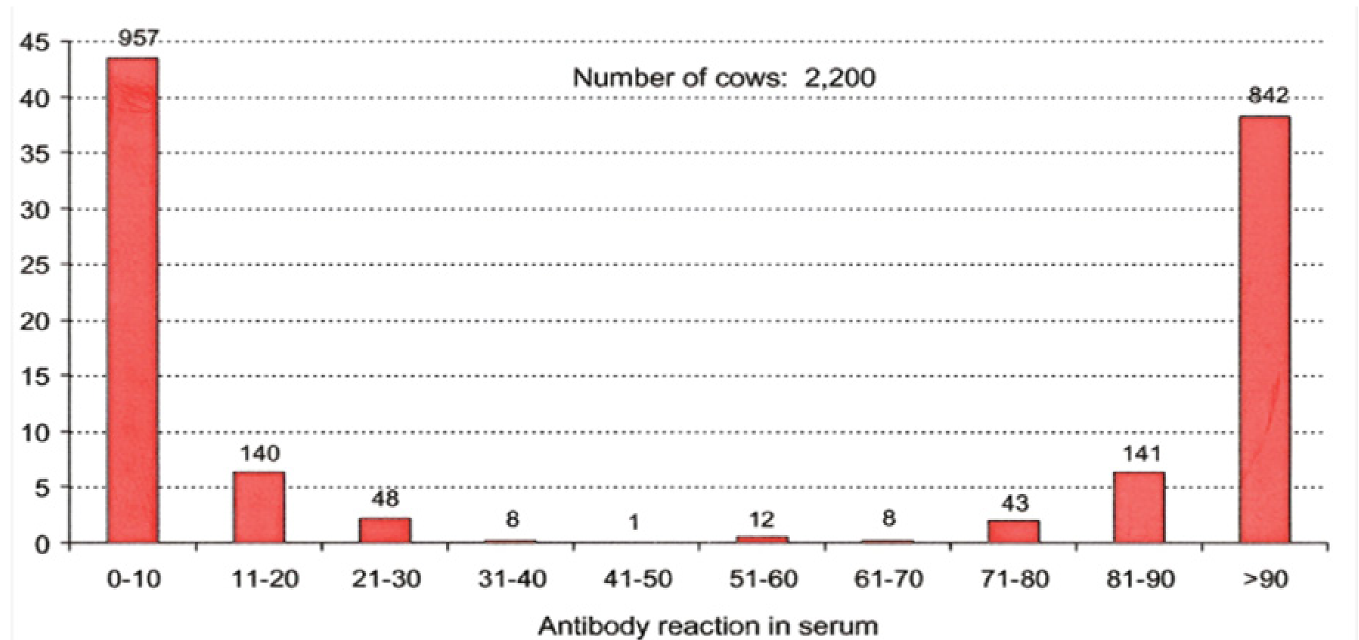

Figure 6: Distribution of blocking antibody ELISA reactions from screening examinations of serum from all 2200 cows in 36 dairy and 77 non-dairy herds on the island of Samsø performed 1992. From *Bitsch* 2020 [10].

Virus: Bovine viral diarrhea virus, a pestivirus. All samples were tested undiluted and with reaction for close to 24 hours at 37°C. The abscissa shows the percentage of blocking of the enzyme reaction, and the ordinate shows the percentage of samples.

The bl-ab-ELISA is in its basic form simple, very sensitive, specific, and applicable for automation. It is well-suited for routine, large-scale examinations. It can easily be modified to meet different objectives and has in one modification functioned as a reference standard antibody test (EU Commission delegated regulation 2020/686).

Considerations related to SARS-CoV-2

In 2020, *Tan et al.* [13] reported the elaboration of a bl-ab-ELISA demonstrating SARS-CoV-2 antibodies. It was named a surrogate neutralization test. The reactant coated to the plates was a human ACE2 receptor preparation, while the reactant in the detecting enzyme conjugate was the viral RBD protein.

In 2022, *Kolesov et al.* [14] published a simpler version allowing reaction in the ELISA plates, where the coated agent was the RBD viral protein while ACE2 was the reactant in the enzyme conjugate. A third simpler version may be presented in the future, where the ACE2 in the detecting enzyme conjugate is replaced with a specific antibody preparation.

Other bl-ab-ELISA variants have shown a high specificity and can be performed with a high sensitivity. The antibody-positive samples used for evaluation of the test sensitivity in these two bl-ab-ELISA versions did not seem to include post-infection samples from individuals with transient or asymptomatic infections, cf. *Section 3.* The samples were examined with a short reaction time and in dilutions 1:10 and 1:20, so the sensitivity of these tests may be considerably improved. A test modification with an appropriately high sensitivity is likely to be found well suited for a reference standard antibody test.

Overall conclusions regarding the blocking antibody ELISA

- The sensitivity of a bl-ab-ELISA is dependent on both the temperature and duration of the reaction. The reaction is not of first order.
- With reaction at 37 °C, an increase of the reaction time from 1 to 24 hours raised herpesvirus antibody titers by a factor of approx. 4. For 37°C/24h versions, the sensitivity of the bl-ab-ELISA was 2 times higher than that of the f-ord-VNT but 4 times lower than that of the conv-ab-ELISA.
- In a version with reaction at 37 °C for close to 24 hours, a bl-ab-ELISA is extremely well-suited for large-scale routine examinations and for a reference standard antibody test.

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- Screenings can be performed on undiluted samples.
- A bl-ab-ELISA is simple and can easily be modified to serve special objectives.

Author's Declaration

Publication involves no conflicts of interest related to persons, institutions, or corporations.

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