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Research Article

Characteristics of the Reactions in Tests for Antibodies to Viruses and Their Significance for Standard Assays and Adequate Routine Tests

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Submitted: 05 August 2023

Accepted: 04 November 2023

Published: 06 November 2023

ISSN: 2379-0636

Keywords

- Virus-Antibody Reactions
- Antibody Tests
- Test Sensitivity
- Standard Tests
- Virus Aggregation
- Virus Neutralization
- Complement-Dependent Virus Neutralization

Abstract

Interactions between antibodies and viruses are diverse and are used in different ways in tests for the demonstration of either virus or antibody. Three tests, each based on one of three different neutralization reactions by antibodies, and another two ELISA modifications of which one demonstrates a binding reaction and the other one a blocking reaction by antibodies, represent the five basic types of antibody tests.

These five tests are 1) the first-order virus neutralization test (f-ord-VNT), 2) the virus aggregation neutralization test (aggr-VNT), 3) the complement-enriched virus neutralization test (C-enr-VNT), 4) the conventional antibody ELISA (conv-ab-ELISA), and 5) the blocking antibody ELISA (bl-ab-ELISA).

Basic versions of these five tests are evaluated. The reaction in each test is described. The reacting antibodies in the highest concentration determining the antibody titer and the test sensitivity are defined as either neutralizing or non-neutralizing, and modifications with a sensitivity found optimal are evaluated for routine practical use and for the potential use as a reference or even gold standard assay (cf. Definitions).

In the f-ord-VNT with appropriately extended reaction, the reacting antibodies are exclusively neutralizing. The reaction is of first order, implying that the sensitivity is depending on the temperature and is proportional to the reaction time. A 37°C/24h configuration (reaction at 37°C for 24 hours), being approx. 16 times more sensitive than a 37°C/1h version, is judged to be the ideal reference and gold standard assay for measuring truly neutralizing antibodies to viruses.

In the aggr-VNT, the inactivation by aggregation is explosive and short-lasting. All antibodies to the various antigenic determinants react synergistically. The reaction is highly dependent on the antibody concentration and can readily be diluted away, but the sensitivity is low and not variable, and the test is of no significance for the demonstration of antibodies to viruses.

The reaction in the C-enr-VNT is explosive immediately after the addition of complement, but otherwise of first order with appropriately extended reaction times. The sensitivity of a 37°C/24h modification is equal to that of a conv-ab-ELISA with identical reaction conditions, but the test is more laborious and therefore not relevant for practical use.

The conv-ab-ELISA is a first-order assay, and the reacting antibodies in the highest concentration determining the test sensitivity are non-neutralizing. Aggregation of virions is impossible, and the sensitivity is directly proportional to the length of the reaction time. The 37°C/24h conv-ab-ELISA is an ideal reference and gold standard antibody assay for comparative measurements of the test sensitivity. Because of its high sensitivity, it is excellent for controlling questionable results by other tests, and it is well-suited for routine large-scale examinations.

The sensitivity of the bl-ab-ELISA is dependent on both the reaction temperature and time, but the reaction is not immediately of first order. An increase of the reaction time from 1 to 24 hours at 37°C will raise the sensitivity by approx. a factor of 4, but still the sensitivity is relatively high. The reactants can easily be varied in different ways to serve different objectives. In its basic configuration, it is the test best suited for large-scale antibody examinations in connection with the diagnosis and control of viral infections.

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 samples are examined. SARS-CoV-2 antibody tests are referred to in relevant sections.

Article Highlights

- The first analysis of the reactions in all five relevant tests for antibodies to viruses.
- Illustrates on basis of earlier results that the sensitivity of antibody tests generally is both variable and adjustable to very high levels following regular lines of reaction.
- Basic gold and reference standard antibody assays are presented in addition to recommended routine test modifications of relevant high sensitivity and specificity.

INTRODUCTION

Antibodies to important viral agents can be detected a very few days after infection. Demonstration of the presence of, or alternatively freedom from, antibodies may be relevant in the diagnosis of clinical disease but is of extreme importance in the control of viral infections. It is crucial that the antibody tests used for these purposes are reliable.

Very early findings regarding the neutralization of virus in virus-antibody mixtures were 1) that neutralization proceeded linearly with time when recorded semi-logarithmically (Andrews and Elford 1933) [1], 2) that the reaction rate was proportional to the antibody concentration (Burnet et al. 1937) [2], and 3) 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, from one assay to another, were elaborated merely on an empirical basis. It was commonly found that the reaction in a neutralization test at 4°C for one day, or at 37°C for 0.5 -1 hour, would give identical results and that the test sensitivity could not be further improved. The general concept was that virus-antibody interactions would lead to an equilibrium state.

The virus-antibody interactions in vitro have been analyzed comprehensively in four studies and analytical reviews by Bitsch (1978) [4], Bitsch and Eskildsen (1982) [5], Bitsch (2017) [6], and Bitsch (2022) [7]. Of greatest importance were 1) the finding that the test sensitivity was both variable and adjustable in accordance with regular lines of reaction and 2) the presentation of the antigen-antibody interaction formula for the reaction in first-order antibody assays.

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 a viral infection, IgG antibodies persist, so IgG antibodies in late post-infection samples will be the primary target for the antibody assays evaluated in the present analytical article.

In tests used for demonstration of antibodies to viruses, antibodies react with the antigen in three ways, which are neutralization reactions that can be considered to be natural, and in a further two ways which are only partly natural. In the following, the five antibody tests representing these fundamentally different reactions are evaluated in optimal modifications with respect to their functionality, i.e., their suitability for routine testing but also for their potential use as reference or even gold standard assays (see Definitions below).

Definitions

- A reference standard antibody test is a modification recommended by authorities, not seldom for practical use but especially for the indication of 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 the indication of the lowest recommendable level of test sensitivity.
- A gold standard antibody test is a certain test modification with a specified viral antibody activity and an optimal high sensitivity, which is generally acknowledged, irrespective of the type of virus, to be the test for comparison, when other tests are evaluated with regard to their sensitivity.
- The reacting antibodies of the highest concentration are of special significance. They are the antibodies to an antigenic determinant measured by the test in question and found to be of the highest concentration. These antibodies determine the titer of an antibody sample, which also is an indicator of the test sensitivity. Unfortunately, very little appears to be known about the relative concentrations in blood of antibodies to the various antigenic determinants. Antibodies to different antigenic determinants on the virus may have the same level of concentration.

Points of Notice

- Contrarily to what seems to be widely recognized, the attachment of antibody molecules to their antigenic determinants will under physiological conditions result in firm and irreversible bindings. Antigen-antibody bindings do not lead to an equilibrium state. The sensitivity of antibody assays may consequently be both variable and adjustable [4,6].
- Antibodies can be divided into two groups, i.e., neutralizing antibodies that are capable of inactivating viruses 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 [4,6].
- The diversity of virus-antibody reactions enables the configuration of complex virus and antibody tests being both rapid and sensitive [6,7]. Such modifications, however, cannot be readily performed at most laboratories and are not suited for practical use.

THE CHARACTERISTICS OF THE REACTIONS IN FIVE DIFFERENT TYPES OF ANTIBODY ASSAYS

The tests representing five different antigen-antibody reactions are

- The first-order virus neutralization test (f-ord-VNT), based simply on inactivation of virus by the binding of neutralizing antibodies to their antigenic neutralization determinant 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 one reactant block for the binding of a third detecting reactant, usually a specific antibody conjugate.

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, i.e., caused by 1) the binding 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 explosive and short-lasting and is described below [4,6].

The neutralization caused by the binding of neutralizing antibodies to their antigenic neutralization determinant is monovalent and slowly progressing, following the lines of the formula of antigen-antibody interactions in first-order antibody assays,

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

where k_{st} is the standard reaction rate, Ab and Ag are antibody and antigen titers, T is the reaction time, and q is a co-determiner of the reaction rate, a particular log-log antibody/antigen ratio, varying with the reaction temperature. In the 1978 study, q was found to be 0.15 at 37°C but 0.24 at 4°C [4,6]. The formula shows directly that an antibody/time relationship is of first order, while an antigen/time relationship is exponential, depending on the value of the factor q (Figure 1).

From this formula will be seen that the antibody titer (Ab) is directly proportional to the period of reaction (T). In other words, the sensitivity of a first-order assay 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°C. In the following, a test with a reaction at, for example, 37°C for 24 hours will be designated a 37°C/24h modification.

The widely different characteristics of the aggregation and the first-order neutralization reaction, one being explosive and short-lasting and the other one being enduring and slowly progressing, implies that one and the same test can be used to measure the titer of both the aggregating antibodies and the neutralizing antibodies: 1) with a very short reaction time, only the aggregation titer will be recorded, while 2) with appropriately extended reaction periods, no aggregation reaction but only the monovalent neutralization by the neutralizing antibodies will be measured.

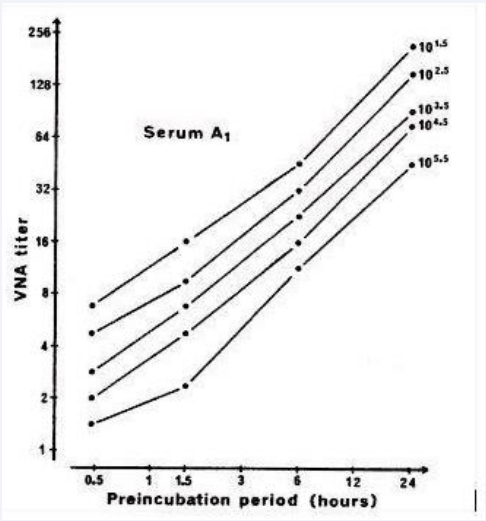

Figure 1 Kinetics of virus neutralization at 37°C in a dilution series of a serum sample (IgG) with short and extended reaction periods and varying virus concentrations. From [4].
Virus: BoHV-1. VNA: virus-neutralizing antibody. Preincubation period: reaction time. The neutralization curves are identical, 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 reaction. Other investigations in the 1978 study with extended reaction 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.

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 non-neutralizing and the neutralizing antibodies. For herpes viruses, exclusively the first-order reaction by the neutralizing antibodies (IgG) is shown with reaction at 37°C for more than 2-3 hours. The initial neutralization caused by aggregation explains, 1) why the increase of the reaction period at 37°C from 1 to 24 hours in a herpesvirus neutralization test does not raise antibody titers, or the test sensitivity, by a factor of 24 as indicated by the antigen-antibody interaction formula above, but only by a factor of 16-18, and 2) why only with reaction times exceeding 2-3 hours at 37°C, the neutralization observed proceeds as a first-order reaction [4-7]. A titer measured in an f-ord-VNT is determined by the reacting antibodies of the highest concentration, which are truly neutralizing antibodies, cf. Definitions.

Reaction characteristics summarized

- The neutralization in a conventional neutralization test is bi-factorial, caused both by 1) the simple virus aggregation being explosive and short-lasting and 2) the first-order reaction by neutralizing antibodies being bound monovalently to their antigenic neutralization determinant.

- Due to the relatively slow progression of the first-order reaction, the neutralization caused specifically by the binding of neutralizing antibodies to their antigenic neutralization determinant will not be observed with very short reaction periods.
- With appropriately extended reaction periods, however, only neutralization by the neutralizing antibodies will be seen. A titer recorded, and correspondingly also the test sensitivity, will be directly proportional to the length of the reaction period in accordance with the antigen-antibody interaction formula.
- The reacting antibodies of the highest concentration with extended reaction are neutralizing antibodies.
- The factor q in the antigen-antibody interaction formula depends on the temperature to such a degree that optimal sensitivity will be achieved at a reaction temperature not below 37°C.

The Virus Aggregation Neutralization Test

Simple virus aggregation is defined as aggregation by antibodies without involvement of complement. As explained above, this reaction predominates in conventional neutralization tests with relatively short reaction periods. The aggregation reaction is explosive and short-lasting, because the antibodies to the various antigenic determinants, predominantly the non-neutralizing ones, react immediately and synergistically. The reaction observed thereafter will be the neutralization by the first-order binding of neutralizing antibodies to their antigenic neutralization determinant [4,6].

In the 1978 study [4], the very early and regular over-neutralization phenomenon was not immediately explainable, but after the report by Brioen et al. (1983) [8], it could be concluded that this reaction was in fact neutralization by virus aggregation [6].

In a dilution series of a polyclonal antibody sample examined in a neutralization test, a virus aggregation reaction will have been diluted away long before the first-order neutralization reaction by neutralizing antibodies, so aggregation is highly dependent on a sufficient antibody concentration [4,6].

The explosive virus-aggregating reaction can be considered almost independent of the reaction time and temperature, because of which the sensitivity of an aggr-VNT will be low and practically non-adjustable. Measurement of the titer of virus-aggregating antibodies should be performed with a very short reaction period to eliminate the influence from the slowly progressing first-order neutralization by the neutralizing antibodies.

Reaction characteristics summarized

- The simple in vitro aggregation of viruses by di- and polyvalent antibodies is explosive and short-lasting.
- All antibodies to various antigenic determinants react synergistically.
- Simple aggregation is the predominant reaction in neutralization tests with short reaction periods, but will not be observed in tests with appropriately extended reaction. Simple aggregation titers shall be measured in neutralization tests with a very short reaction period.
- In virus-antibody mixtures, the aggregation reaction is highly dependent on the antibody concentration and can readily be diluted away.
- The reaction is dependent on the total concentration of antibodies and their valency but is not adjustable by changing the reaction conditions. The test sensitivity will accordingly be rather low.

The Complement-Enriched Neutralization Test

The complement component C1q is hexavalent, so its aggregation potency is extraordinary. It will attach to the Fc region of antibodies, but only to 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 immediately, if being present in adequate quantities, attach to these virus-antibody complexes and neutralize them by including them into aggregates [5,6].

Samples to be examined in neutralization tests are regularly heated at 56°C for 30 min. in order to inactivate complement, so this complement-dependent reaction, functioning regularly in vivo, will not be seen in a conventional neutralization test, unless complement is added deliberately.

In a C-enr-VNT, the supplementary neutralization by aggregation will proceed in two steps. Immediately after the addition of complement, the reaction will be explosive, because complement will almost instantly bind to preformed virus-antibody complexes, while the aggregation reaction thereafter will be of first order, following the first-order binding of the non-neutralizing antibodies to their antigenic determinants, cf. Figure 2. The log-log coefficient slope 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).

The binding reaction by antibodies, neutralizing as well as non-neutralizing, will be of first order with extended reaction periods [4-6]. For herpes viruses, 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 linked to the reaction with non-neutralizing antibodies. With extended reaction conditions, the reaction for both neutralizing and non-neutralizing antibodies is monovalent and of first order. For the non-neutralizing antibodies, one antibody molecule bound to a virion will inactivate the virion by means of complement, and for neutralizing antibodies it is generally accepted that one molecule attached to the antigenic neutralization determinant will have the same effect. It will therefore be logical to conclude that not only the titer but also

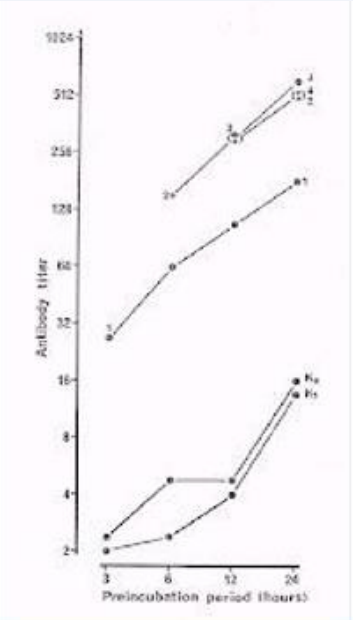

Figure 2 The effect of complement on the progression of virus neutralization in dilution series of an early convalescent-phase serum sample. From [5].
Virus: SuHV-1. Preincubation period: reaction time. Serum from an experimental pig was taken 13 days after nasal infection. Virus-serum mixtures were incubated at 37°C, and titers were recorded by inoculation of cultures after reaction for 3,6,12, and 24 hours. K0 and K1: no complement (K0) or heat-inactivated complement (K1) was added at the start of virus-serum incubation. For the reactions 1-1, 2-2, 3-3, and 4, complement was added at the start of incubation, and after 5, 11, and 23 hours, respectively. The log-log coefficient slope 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).

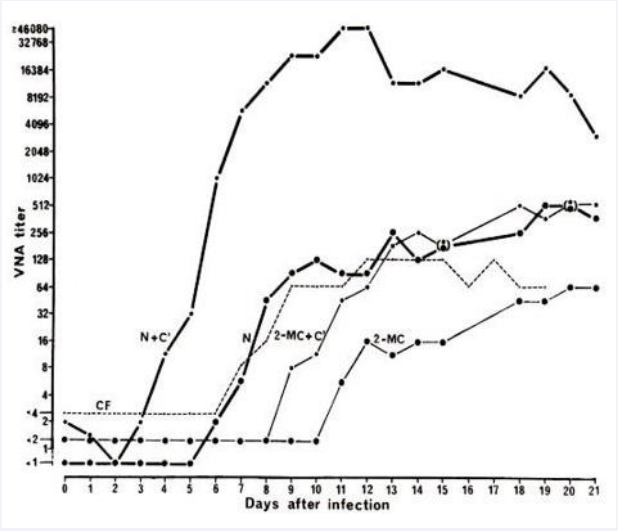

Figure 3 The appearance of non-neutralizing and neutralizing IgM and IgG antibodies in blood during the first 21 days after experimental nasal infection. From [5].
Virus: SuHV-1. VNA: virus-neutralizing antibody titer. Tests: the first-order neutralization test and the complement-enriched neutralization test. The porcine serum samples were heated at 56°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°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).
N: Titers of neutralizing IgM antibodies
N+C': Titers of non-neutralizing IgM antibodies
2MC: Titers of neutralizing IgG antibodies
2MC+C': Titers of non-neutralizing IgG antibodies

the concentration of reacting non-neutralizing herpesvirus antibodies of the highest concentration is eight times higher than that of the neutralizing antibodies.

For IgG antibodies, an optimal herpesvirus C-enr-VNT will show a sensitivity 8 times higher than that of an f-ord-VNT with identical reaction conditions, 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. For early convalescent-phase samples (IgM), the titers were extremely high, being over 10.000, 9-11 days after infection for the animal tested. The overall effect of the complement component C1q is that all non-neutralizing antibodies are effectively converted into neutralizing ones [5-7].

Reaction characteristics summarized

- The hexavalent complement component C1q will immediately, if present in adequate concentrations, bind to preformed antigen-antibody complexes and neutralize them by aggregation.
- In a C-enr-VNT with extended reaction, the binding between non-neutralizing antibodies and their antigenic determinants proceeds as a reaction of first order.
- Two different reaction rates are seen in a C-enr-VNT. After addition, complement will react explosively with preformed antibody-virus complexes, but thereafter the reaction will 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 in a C-enr-VNT, determining the test sensitivity, are non-neutralizing. In samples with predominantly IgG, they are the same non-neutralizing antibodies determining the titer in a conv-ab-ELISA, because of which these tests share the same high sensitivity.
- The higher sensitivity of the C-enr-VNT as compared to the f-ord-VNT indicates that the concentration of the reacting non-neutralizing antibodies of the highest concentration is higher than that of the neutralizing antibodies.
- The reaction temperature in a C-enr-VNT should not be below 37°C.
- For early convalescent-phase serum (IgM), the aggregation effect of C1q is huge.
- The overall effect of complement is that all non-neutralizing antibodies are effectively converted into neutralizing ones.

The Conventional Antibody ELISA

After viral infection, IgM antibodies appear a few days later and will usually be non-demonstrable after some weeks. IgG antibodies, however, will appear some days after the IgM antibodies and will regularly persist for life, cf. Figure 3. A conv-ab-ELISA can be modified in different ways, but for the sake of simplicity, only a basic configuration will be considered, where a whole antigen preparation is coated directly onto the ELISA plates and the detecting reagent will identify specific antibodies of any immunoglobulin isotype.

In a conv-ab-ELISA, virus aggregation by antibodies is impossible because the antigens have been immobilized. The individual reactions between the various antibodies and their antigenic determinants, and also the combined effect of these reactions, will therefore be of first order from the start to the end of the reaction period. 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 herpes viruses, 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, when 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 [5,7,9,10]. The unique sensitivity of a conv-ab-ELISA as compared to a highly sensitive f-ord-VNT can therefore be simply explained by the fact that non-neutralizing antibodies are the reacting antibodies found in the highest concentration. The reacting antibodies of the highest concentration in 37°C/24h modifications of the C-enr-VNT and the conv-ab-ELISA are identical, because of which these two tests share the same high sensitivity,

As mentioned earlier, the factor q in the first-order reaction formula is temperature-dependent to such a degree that the optimal reaction temperature for first-order antigen-antibody tests should regularly not be below 37°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 a f-ord-VNT, appears to be due to the fact that also non-neutralizing antibodies bound to the antigen are recorded, 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 they share the same extremely high sensitivity.
- The reaction temperature of a conv-ab-ELISA should not be below 37°C.

The Blocking Antibody ELISA

In the simplest form of the bl-ab-ELISA, a whole antigen preparation is coated directly to the wells of the microtiter plates. The reactant in the detecting reagent, the 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 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 for the detecting enzyme conjugate. The blocking reaction is rather complex [6,7].

With whole antigen coated to the plates and a selected high test sensitivity, antibody-positive samples with moderate to high antibody concentrations will all give a close to 100 percent blocking reaction, and only over a limited antibody concentration range of 5-6 logarithmic base-2 units, varying reactions are seen. Over this interval from a negative to a moderately positive reaction, the log-log reaction rate is linear, however with a slope coefficient different from 1 (Figure 4). The majority of positive samples will always give a maximal blocking reaction in screening examinations with a test of acceptable sensitivity (Figure 5).

In examinations of 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 [12], demonstrated a sensitivity dependency on both reaction time and temperature, but the reaction did

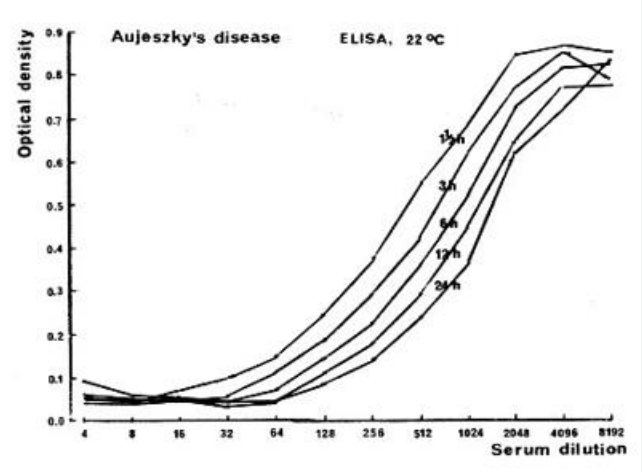

Figure 4 The kinetics of the reaction in a blocking antibody ELISA. From [6]. Virus: SuHV-1. The plates were coated with a whole antigen preparation and a polyclonal antibody was used for the detecting enzyme conjugate. The highest optical density corresponds to no blocking effect by specific antibodies, while reduced optical density shows a blocking effect by antibodies. 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. Over a limited range, from a negative to a moderately antibody-positive reaction, a linear relationship is seen between logarithmic values of antibody titer and reaction period. The log-log titer-time ratio is lower than 1 (decelerating rate). Increase of the reaction time from 1.5 to 24 hours raised titers approximately by a factor of 4.

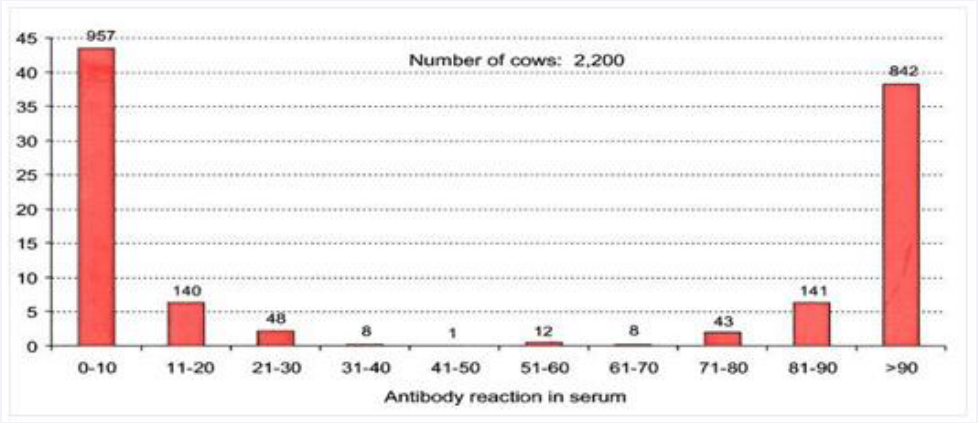

Figure 5 Distribution of blocking antibody ELISA reactions from screening examinations of serum from all 2200 cows in 36 dairy and 77 non-dairy herds on the island of Samsø performed 1992. From [11].
Virus: Bovine viral diarrhea virus, a pestivirus. All samples were tested undiluted and with reaction for close to 24 hours (overnight) at 37°C. The abscissa shows the percentage of blocking of the enzyme reaction, and the ordinate shows the percentage of samples.

not follow strictly the lines of a first-order test. Kramps et al. [13], 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 is depending on both the reaction time and temperature, although to different degrees than in a first-order test. The test sensitivity is variable and adjustable.
- 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 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.

SENSITIVITY AND SPECIFICITY OF ANTIBODY ASSAYS

After the early studies of antigen-antibody interactions [1-3], it became generally accepted that virus-antibody interactions would lead to an equilibrium state, but such a condition would imply that the sensitivity of antigen-antibody assays would neither be variable nor adjustable.

The 1978 study [4] was the first investigation documenting that the reaction in a neutralization test was bi-factorial. The early and fast reaction was termed the over-neutralization reaction and the enduring, slowly progressing reaction was identified as the first-order antibody neutralization reaction. The complete formula for the antigen-antibody interaction in this first-order antibody test was presented, and the test sensitivity was shown to be both variable and adjustable. Accordingly, tests of earlier unknown high sensitivity could be elaborated. The antigen-antibody interaction formula is shown above.

The test sensitivity will naturally be decreased by a factor of 10 if a test sample is examined in the dilution of 1:10 instead of undiluted. It was a personal experience that test samples always should be examined in the highest possible concentration, usually undiluted. But most importantly, regarding specificity, it was found that even a highly increased sensitivity by changed reaction conditions never in any way tended to give unspecific reactions. Figure 6 illustrates, how a high sensitivity of the f-ord-VNT, increased by a factor of 16, 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 for all practical diagnostic or control purposes be sufficiently high to enable a reliable differentiation between truly antibody-positive and antibody-negative individuals. The sensitivity and specificity should therefore be practically 100 percent. Terms like diagnostic or predictive values of test sensitivity and 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 simply unacceptable and should be replaced or modified in order to fulfil 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 6. It is generally accepted that antibody responses will be higher after severe clinical

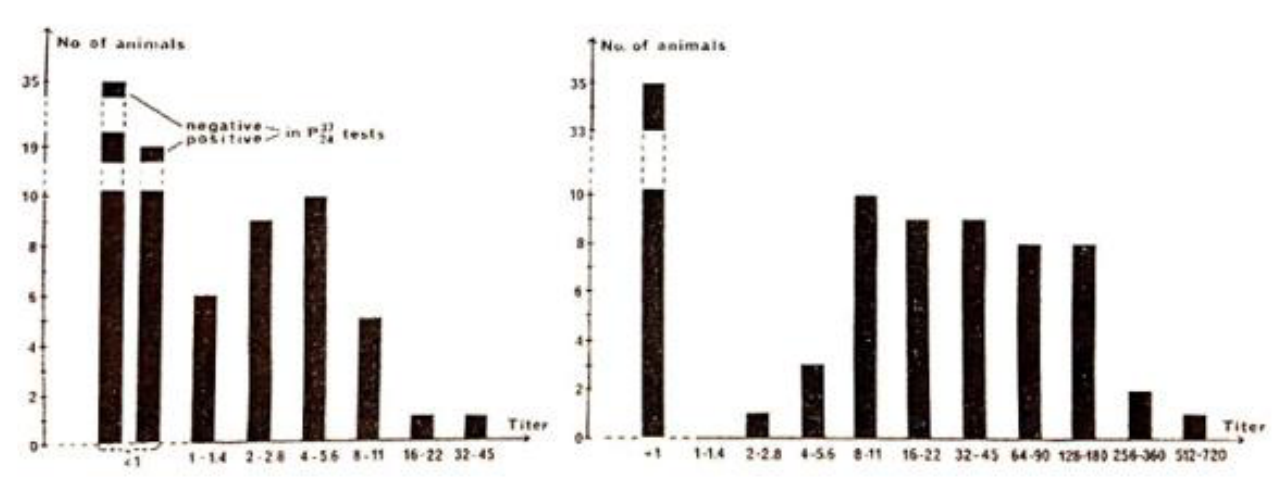

Figure 6 Virus-neutralizing antibody titers in serum of 86 animals from 2 cattle herds. From [9].
Virus: BoHV-1. Ordinate: number of samples. Abscissa: antibody titers. The sera were tested undiluted and in dilutions to determine the antibody titers. Two different test modifications were used. Left: 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 reactions.

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 full variation of antibody levels in a population represented. If, for example, the samples are selected only among individuals having shown clinical disease, a calculated test sensitivity will most likely be misleadingly high. The sensitivity of a new test modification can also be evaluated in comparative examinations with another test with a well-characterized reaction and a documented high sensitivity, a reference or gold standard antibody test, or simply by examination of a reference standard antibody test sample.

High test sensitivity will regularly not give any reduction in specificity, so standard antibody assays should be performed with the optimal highest sensitivity, although still under conditions allowing easy and practical performance. In other words, the sensitivity of a reference or gold standard antibody assay should ideally be higher than that required for a test for routine use. A 37°C/1h conv-ab-ELISA might be found sufficiently sensitive in many circumstances, but the particular condition that the sensitivity of, for example, a 37°C/24h modification is 24 times higher, implying that antibody levels 24 times lower can be detected, indicates that extended reaction should be chosen for a gold standard assay. High reactions in screenings are regularly not questioned. Varying high enzyme reactions are not strictly proportional to the antibody concentration, and only titrations can show the relative levels of the reacting antibodies of the highest concentration.

An important detail related to the results shown in Figure 5 is that the animals with the lowest antibody-positive blocking reactions from 40 to 70 percent 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 also been observed for other viral infections and underlines that whenever the identification of any earlier infected animal is crucial, e.g., in control of herpesvirus infections, the application of a highly sensitive antibody test for control examinations is essential.

Sensitivity and specificity of antibody assays summarized

- Tests for diagnostic and control purposes should be safe. The sensitivity and specificity of antibody assays 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.
- An optimal, highly sensitive modification of a test for the demonstration of antibodies to one virus will usually - appropriately adapted - be optimal also for other viruses.
- An even highly increased sensitivity by changed reaction conditions will not reduce the test specificity.

EVALUATION OF OPTIMAL MODIFICATIONS OF FIVE DIFFERENT ANTIBODY TESTS FOR PRACTICAL USE

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 6, 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 very close to 100 %. 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, indicating that no comprehensive spreading of the infection had occurred in these two herds for a couple of years [9].

In 2008, the World Organization for Animal Health (WOAH) acknowledged the 37°C/24h f-ord-VNT [9] 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 seen from 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). In 1976, however, Bitsch and Eskildsen [10], 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 beyond 99 percent in line with the BoHV-1 assay.

The reacting antibodies of the highest concentration in a f-ord-VNT with extended reaction time will be neutralizing antibodies. As mentioned above, it does not seem to be documented at present, if only one such determinant may exist on a virion. Monoclonal antibodies to various antigenic determinants may have been incorrectly identified as neutralizing by authors not paying due attention to the general ability of antibodies to neutralize by aggregation. The 37°C/24h f-ord-VNT appears to be extremely well-suited for a gold standard assay demonstrating specifically neutralizing antibodies.

Considerations regarding 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, has often in literature been considered the golden standard neutralization assay and is also by many believed to reflect exclusively the neutralization reaction caused by the binding of neutralizing antibodies to their antigenic neutralization determinant. This test modification has also been used as a reference standard antibody test in SARS-CoV-2 investigations [14,15]. But as judged from the regular lines of antigen-antibody interactions documented in the studies with herpesviruses, a major part of this short-term neutralization reaction may be due to the aggregation by non-neutralizing antibodies. It might be topical for this reference neutralization assay to be revised to achieve consistency with the first-order reaction lines. A 37°C/24h f-ord-VNT will guarantee no interference from non-neutralizing antibodies and a considerable improvement of the test sensitivity.

Overall conclusions regarding the f-ord-VNT

- The reacting antibodies of the highest concentration in an f-ord-VNT with extended reaction are neutralizing antibodies.
- The reaction is enduring, following the lines of the formula for the regular antigen-antibody interaction, implying that the sensitivity is highly dependent on the reaction temperature and directly proportional to the reaction time.
- The 37°C/24h f-ord-VNT is highly sensitive and appears ideal for a gold standard antibody assay for the demonstration of especially neutralizing antibodies to a broad variety of viruses.

The Aggregation Neutralization Test

The special aggr-VNT is actually a traditional neutralization test but shall be performed with a very short reaction period that will prevent interference from the first-order neutralization. The test sensitivity is not adjustable and will be relatively low. The herpesvirus studies [4,6] illustrated that a 37°C/1h neutralization test will show the combined effect of two completely different neutralization reactions. The reaction seen was mainly neutralization by aggregation caused predominantly by non-neutralizing antibodies. A 37°C/1h neutralization test is widely accepted as a reference standard antibody test, apparently also by the WHO and the WOAH, 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. The aggr-VNT has a low sensitivity and 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.

Overall conclusions regarding the aggr-VNT

- The simple aggregation of viruses by antibodies is explosive and short-lasting.
- The reaction can be measured in a neutralization test with a very short reaction time, which will not allow the first-order neutralization by the attachment of neutralizing antibodies to their antigenic neutralization determinant to be seen.
- The sensitivity is relatively low, especially because the test sensitivity is not immediately adjustable.
- The simple aggr-VNT is not relevant for use in the diagnosis and control of viral infections.

The Complement-Enriched Neutralization Test

In a C-enr-VNT, the polyvalent 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 preformed virus-antibody

complexes will be explosive but thereafter, it will continue as a first-order reaction [5,6]. High sensitivity 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.

Complement will react with both IgM and IgG antibodies, and a titer is determined by the IgM or IgG non-neutralizing antibodies of the highest concentration, but the responding isotype will not be immediately identified. If found appropriate, a positive serum/plasma sample can be examined with and without inactivation of IgM antibodies possibly being present. If then identical titers are recorded, the reaction can be considered to be due to IgG antibodies, while a higher titer for the non-treated sample will be due to non-neutralizing IgM antibodies, cf. Figure 3. With late post-infection IgG serum/plasma samples, the test sensitivity is identical to that of the conv-ab-ELISA and is determined by the same non-neutralizing antibodies of the highest concentration. One minor complication connected with the use of complement is that it may cause a very low degree of unspecific neutralization 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., after more than one hour but not later than 15 minutes before the end of the reaction time [5].

The characteristics of the reaction in the C-enr-VNT are well-known and described in detail above. The neutralization with extended reaction is of first order. The test sensitivity is adjustable and can be very high, i.e., as judged from the herpesvirus studies, regularly 8 times higher than that of the f-ord-VNT. For early convalescent-phase samples, the sensitivity may be extremely high. Because of its high sensitivity, 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 conv-ab-ELISA identifies the same non-neutralizing antibodies as the reacting antibodies in the highest concentration and is equally sensitive.

Overall conclusions regarding the C-enr-VNT

- The reaction in a C-enr-VNT will be explosive immediately after the addition of complement. Thereafter, neutralization with extended reaction will proceed as a first-order reaction with a sensitivity being proportional to the length of the reaction time.
- For convalescent-phase serum/plasma, relative titers of IgM and IgG antibodies can be determined by testing with and without inactivation of the IgM antibodies.
- The reacting antibodies of the highest concentration, determining the test sensitivity, are non-neutralizing.
- For optimal sensitivity, complement should be added not earlier than after one hour of reaction and not later than 15 minutes before the end of the reaction time.
- The 37°C/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.

The Conventional Antibody ELISA

The reaction in the conv-ab-ELISA follows the formula for the antigen-antibody interaction, and the sensitivity will be directly proportional to the reaction time. The dependence on temperature is so significant that sensitive tests always should be performed at a temperature not below 37°C. In the f-ord-VNT, only neutralizing antibodies are active at extended reaction but in the conv-ab-ELISA, the detecting reagent will bind also to non-neutralizing antibodies. The antibodies of the highest concentration in late post-infection samples, which determine the titer of a sample and also the test sensitivity, will regularly be non-neutralizing and be identical to the reacting antibodies of highest concentration in a C-enr-VNT. The 37°C/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 the conv-ab-ELISA are described above. The reaction is of first order, the sensitivity is adjustable, the 37°C/24h modification is extremely sensitive, it is simple, and it is applicable for automation. So, the 37°C/24h conv-ab-ELISA will be extremely well-suited for a gold standard antibody assay for demonstrating levels of non-neutralizing antibodies 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. It was anticipated that the prevalence would be very low. Because of the persistent-viremia character of this infection, antibody levels in blood and milk are relatively high. A reference standard test sample was used for indication of the required test sensitivity. Using an antibody ELISA, dairy herds could be tested twice, with a specified time interval, on milk in pools of samples from maximally 20 cows. But by using reaction at 37°C for close to 24 hours, the sensitivity was raised to a level allowing test of milk samples in pools representing more than 400 cows [6]. The Danish dairy herds could consequently be controlled primarily on bulk tank milk samples.

It is worthy of note that a first-order antibody test performed with reaction at 37°C but with a reaction time deviating a very few hours from 24 hours will have a sensitivity deviating only slightly from that of a 37°C/24h test. Routine examinations in control of infections in the veterinary field have therefore usually, for practical reasons, been performed, not with strictly 24 hours of reaction, but with reaction at 37°C overnight, implying a reaction time of more than 18 hours. Bulls at artificial insemination bull centres 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 involving reaction at 37°C for close to 24 hours.

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 not be below 37°C.
- The reacting antibodies of highest concentration in late post-infection serum/plasma samples, determining the antibody titer and the test sensitivity, are regularly non-neutralizing. The test sensitivity is identical to that of the C-enr-VNT with the same reaction conditions.
- Modifications with extended reaction at 37°C are extremely sensitive and specific, and are well-suited for automation. Screenings with a conv-ab-ELISA should usually be performed on undiluted test samples.
- The 37°C/24h modification of the conv-ab-ELISA will be ideal for a universal gold standard antibody assay for comparative measurements of test sensitivity.

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 37°C/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 [12], BoHV-1 [6], and bovine viral diarrhea virus [11] infections because of 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 required higher sensitivity. The test sensitivity and specificity are extremely 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. 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 regarding SARS-CoV-2

In 2020, Tan et al. [14] reported the elaboration of a bl-ab-ELISA for the demonstration of SARS-CoV-2 antibodies. It was named a surrogate neutralization test. The agent 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. [15] published a simpler version allowing reaction in the ELISA plates, where the coating agent was the RBD viral protein while ACE2 was the reactant in the enzyme conjugate. A third version may appear in the future, where the reactant in the detecting enzyme conjugate is replaced with a polyclonal, or alternatively a monoclonal, specific viral antibody.

Other bl-ab-ELISA variants have shown a high specificity and can be performed with a very 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. 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. It appears likely that a test modification with an appropriately high sensitivity will be found relevant 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. But 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, it is extremely well-suited for large-scale routine examinations and for a reference standard antibody test.
- Screenings should preferably be performed on undiluted samples.
- The bl-ab-ELISA is very simple and can easily be modified to serve special objectives.

CONCLUDING CONSIDERATIONS

Background

In 1970, Denmark was the first country to introduce freedom from the BoHV-1 infection in artificial insemination bull centers. All centers had been cleared of the infection. Studies of the virus-antibody reaction were undertaken to ensure that control tests were reliable, and when preliminary investigations had demonstrated the huge advantage of a neutralization test with extended reactions, the 37°C/24h test used on undiluted serum became the standard antibody test for control of animals to be admitted to the centers. Over a couple of decades after 1980, the three 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 routine examination of serum/plasma or cow's milk because of sufficiently high sensitivity, simplicity, and applicability for automation. For the final stages of the eradication of enzootic bovine leukosis, however, the conv-ab-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.

Evaluation of Tests

Regarding the relative sensitivity of 37°C/24h herpesvirus antibody tests used on late post-infection serum/plasma,

- The f-ord-VNT was found 16 times more sensitive than a 37°C/1h VNT, i.e., not fully 24 times more sensitive because of a remaining aggregation reaction recorded after 1 hour of reaction,
- The bl-ab-ELISA was 2 times more sensitive than the f-ord-VNT, but 4 times less sensitive than the conv-ab-ELISA, while
- The conv-ab-ELISA and the C-enr-VNT were found equally sensitive. The IgG titers by the C-enr-VNT shown in Figure 3 were 8 times higher than those of the f-ord-VNT, while for early convalescent-phase serum, extremely high titers for non-neutralizing IgM antibodies were recorded: blood samples collected 11 and 12 days after nasal infection were significantly positive in dilution 1:10.000.

In the present analysis, one of the five types of antibody tests evaluated, the aggr-VNT was found irrelevant for use in context with diagnosis and control of viral infections, mainly because the test sensitivity was low and not variable. The remaining four types of antibody tests with adjustable sensitivity all have important advantages, but only in modifications with high sensitivity.

The 37°C/24h f-ord-VNT will be ideal for a gold standard assay. Complement in test samples is routinely inactivated, and no neutralization by aggregation whatsoever will be shown. It measures titers of the reacting antibodies in the highest concentration, which will be truly neutralizing. The test sensitivity is approx. 16 times higher than that of the 37°C/1h neutralization test, as illustrated in investigations with herpes viruses.

The 37°C/1h neutralization test is widely accepted as a gold standard neutralization test. The herpesvirus studies, however, documented that the neutralization reaction at 37°C for only1 hour was bi-factorial including also inactivation by aggregation caused by mainly non-neutralizing antibodies. The two main problems associated with the 37°C/1h neutralization test are 1) that the sensitivity is low and 2) that two completely different reactions are regularly involved. Consequently, it does not fulfill requirements to neither a reference nor a gold standard antibody assay.

The 37°C/24h C-enr-VNT demonstrates exactly the same non-neutralizing antibodies in the highest concentration in late post-infection serum/plasma as the conv-ab-ELISA, but the examination is a more laborious and tedious process, so, despite its extremely high sensitivity, this test does not seem to be relevant for a reference or gold standard antibody test.

The 37°C/24h bl-ab-ELISA is the simplest antibody test for routine use. Its sensitivity is high, although not quite at the level of that for the conv-ab-ELISA. Its applicability for routine use is unique. Accordingly, the 37°C/24h version will - appropriately adapted to the type of virus - be an adequate reference standard antibody test for a great variety of viruses.

Short Summary

- The 37°C/24h f-ord-VNT is ideal for use as the gold standard test for demonstration of truly neutralizing antibodies. It is also well-suited for use as a reference standard antibody test for the indication of a level of sensitivity recommended.
- The aggr-VNT has a fixed low sensitivity. It is not suited for practical purposes related to the diagnosis and control of viral infections.
- The 37°C /24h C-enr-VNT has a very high sensitivity equal to that of the conv-ab-ELISA. The reacting antibodies in the highest concentration are non-neutralizing. It is laborious and tedious and therefore not relevant for a standard antibody test or for routine use.
- The 37°C /24h conv-ab-ELISA is extremely sensitive. The reacting antibodies of the highest concentration are non-neutralizing. It is well-suited for both a gold and a reference standard assay and a modification with reaction at 37°C for close to 24 hours is further well-suited for routine testing.
- The 37°C/24h bl-ab-ELISA is in its basic configuration not a gold standard assay, but it is sensitive and well-suited for a reference standard antibody test. A modification with reaction at 37°C for close to 24 hours is particularly well-suited for large-scale routine testing.

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