General avian serology

Vol. 1. Results interpretation of serological tests

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General avian serology

Vol. 1.	Results interpretation of serological tests
Vol. 2.	Introduction to practical avian serology - from the basics to the interpretation of test results
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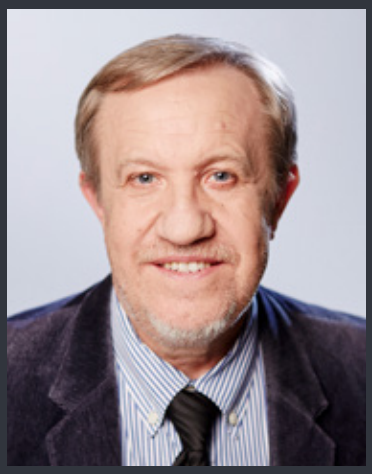


Contents

Serology – definition

Serology is dealing with the interactions between antigens and antibodies that occur *in vitro*. Serology includes different methods to demonstrate the presence of antigens and antibodies in serum or other biological material, thus, by identifying the pathogen or antibodies, it enables the diagnosis of infectious diseases (Gołąb et al., 2017).

Serological methods are commonly used in veterinary medicine, they are also one of the important tools for managing the health of poultry flocks (Butcher, 2018; Szeleszczuk, 1995).

Serological tests have been used in the diagnosis of infectious diseases of poultry for many decades. According to the review of the literature, tube hemagglutination, introduced nearly 110 years ago by Jones, was the first serological method used to diagnose *Salmonella Pullorum*-infected individuals, and at the same time the first serological test practically used in the diagnosis of poultry diseases (Jones, 1913). Over time, other serological tests were introduced to identify antibodies and antigens in serum, swabs or albumin (e.g. seroneutralization test, precipitation test, hemagglutination inhibition test). A major breakthrough in the use of serological methods in poultry practice took place in the second half of the 1980s, when the first commercial IDEXX ELISA tests appeared on the US market (7).

Serological tests

In avian pathology, serological tests are used to diagnose infections (serodiagnostics) and to assess post-vaccination response (serovaccinology). These tests are also very often used by poultry pathologists to determine the prevalence of diseases in a population (seroepidemiology).

As mentioned, several methods can be used to detect antibodies and antigens in avian serology (Fig. 1.), but in practice ELISA and the hemagglutination inhibition (HI) test are most often used, which allow not only to answer what antibodies are present in the serum, but also how many antibodies are contained in it (ELISA) or what is their hemagglutination activity. Currently, the plate agglutination test and the agar gel precipitation test (qualitative tests) are less frequently used, although they can still be very useful in some diseases. This applies in particular to the plate agglutination test used in the diagnosis of mycoplasmas or the agar gel plate test (AGPT) test used to diagnose Marek's disease (Szeleszczuk, 1995).

The scientific basis for the introduction of ELISA for the diagnosis of poultry diseases was developed by a team of researchers from the US University of Maryland (Snyder et al., 1983; Snyder et al., 1983; Snyder et al., 1984; Snyder et al., 1986).

The use of the ELISA test for serological diagnosis of poultry diseases enabled the emergence of a new strategy in veterinary care over large-scale poultry farming by introducing serological monitoring to the broad practice (Snyder et al. 1985; Szeleszczuk, 1997).

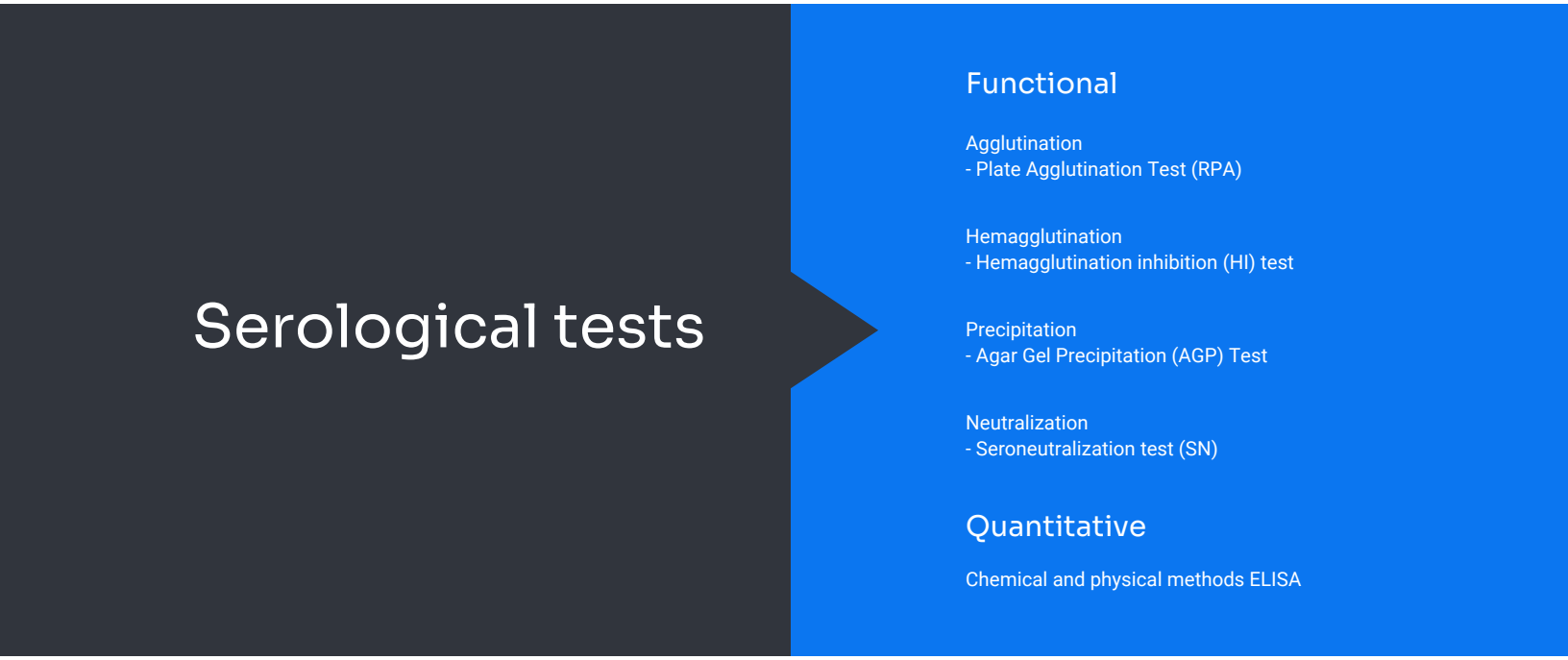


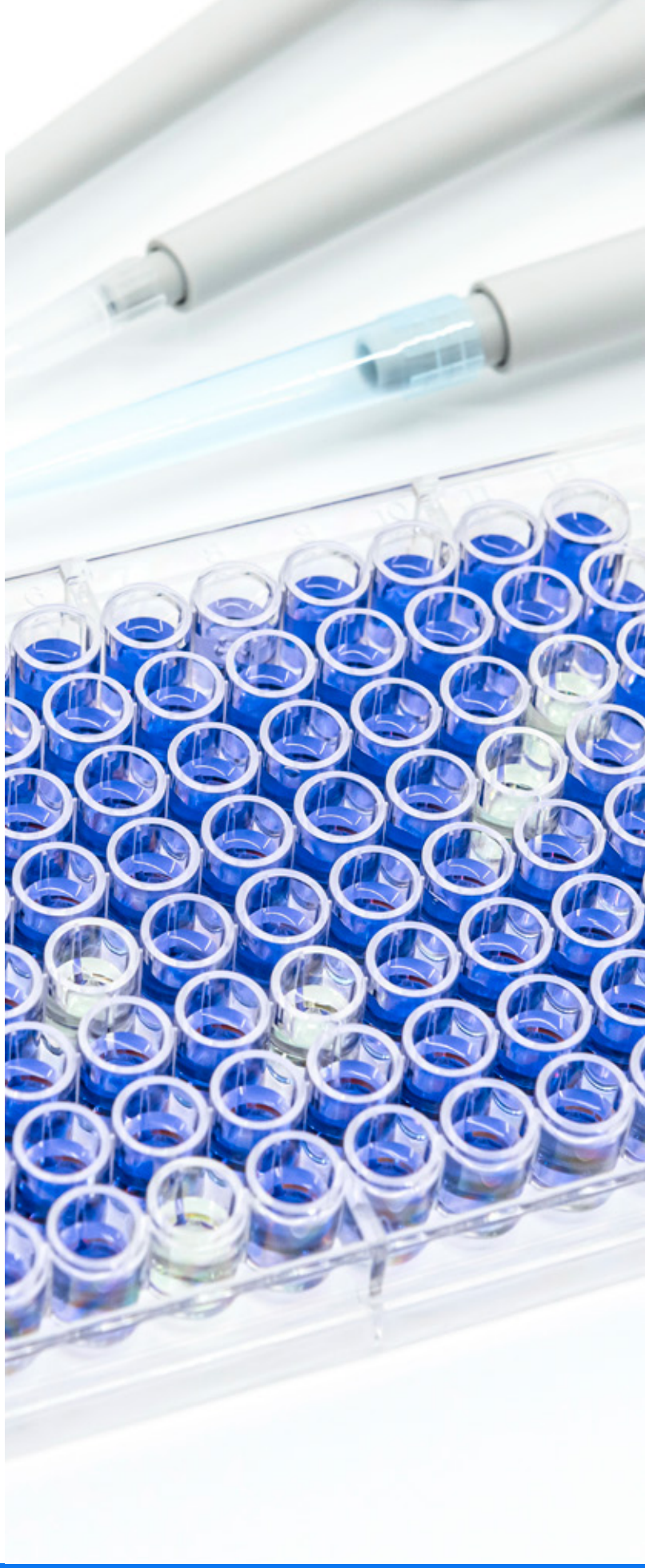
Fig. 1. Tests used in practical poultry serology

Immunoenzymatic test (ELISA)

In the serological diagnosis of poultry diseases, the enzyme immunoadsorption test is commonly used, most ofter referred as ELISA (Enzyme-Linked Immunosorbent Assay). The principle of the enzyme immunoassay was developed in 1971 independently by teams of scientists from the University of Stockholm (Engvall and Perlmann, 1971) and the research laboratory of the Dutch company NV Organon in Oss (van Weemen and Schuurs, 1971; Lequin, 2005). In a very short time, this test was applied to both scientific and practical research in veterinary medicine and food hygiene (Mallinson et al., 1988) and on viruses found in poultry like Newcastle disease virus, Gumboro disease virus, Reoviruses (Thayer et al., 1987). Currently, ELISA is a commonly used test in the diagnosis of many viral diseases in poultry and some bacterial infections (8). Since its introduction in 1995, this method has become a useful tool in the system of integrated veterinary care for intensive poultry production (Szeleszczuk, 1996).

ELISA tests are characterized by high sensitivity and repeatability of results. Therefore, they make it possible to detect even low concentrations of antibodies in the tested serum, undetectable by other serological tests. They are also characterized by a high degree of specificity, i.e. a low number of false positive results. In addition, it is a safe, quick and easy method that does not require expensive equipment; it is also technically simple, easy to automate and affordable.

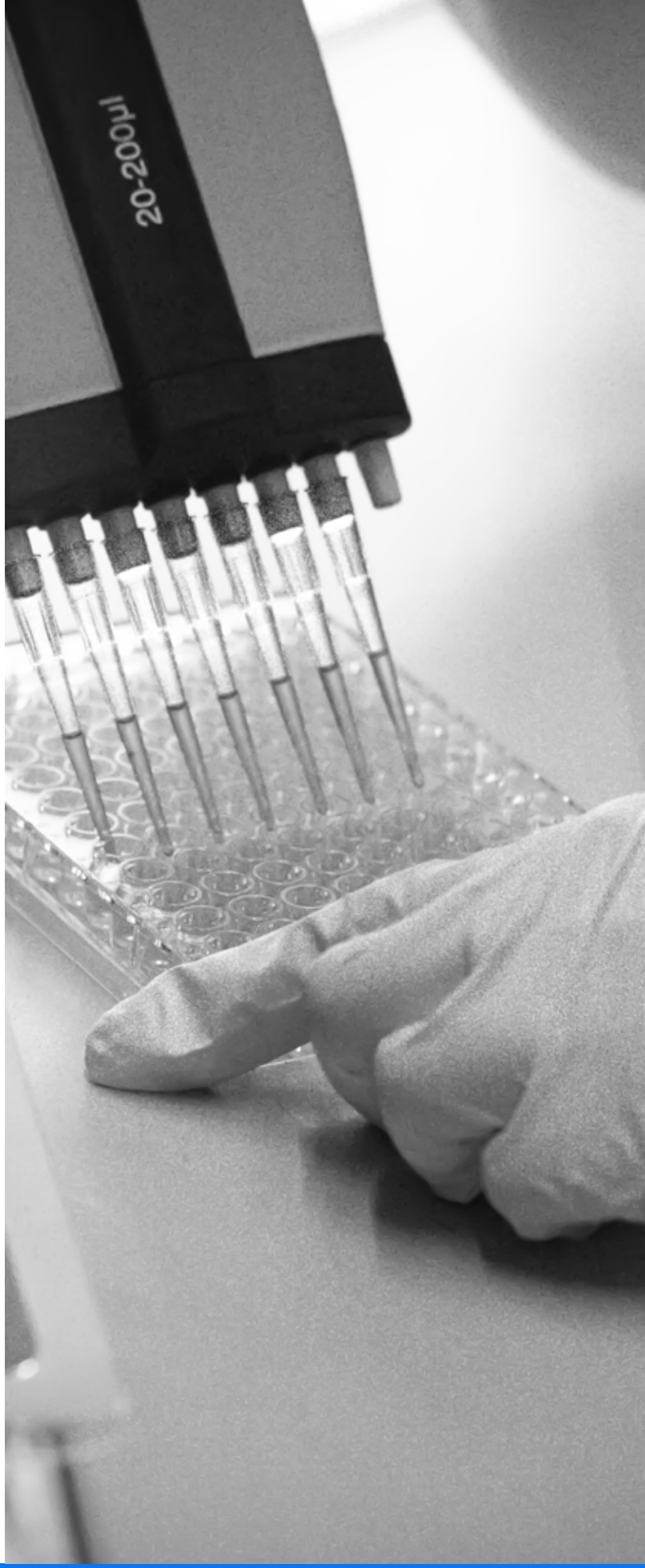
The wide application of this test in practice was mainly determined by the possibility of full automation of its conduct and the speed of performing determinations; it is possible to test 92 sera with different antigens in approx. 2 hours. The use of microtiter plates and com-



mercially available multi-channel pipettes, washers and spectrophotometers adapted to this format enable quick and precise testing even in small laboratories (Kępska et al., 2018). On the other hand, with the use of specialized laboratory robots in large diagnostic facilities, the tests can be fully automated. All these advantages mean that ELISA has an advantage over such classic techniques as the precipitation test or the neutralization test. Since the development of the enzyme immunoassay, many variants have been developed, which have been used both in clinical diagnostics and in basic research (14). In poultry serology, IDEXX kits use to detect antibodies indirect ELISA (Fig. 2) and blocking format test (Fig. 4), and for antigen detection use the sandwich test format.

Basic characteristics of the ELISA test (Gut-Winiarska et al., 2001):

a) antigen or antibody are bound in the solid phase by passive adsorption to the plastic well surface of the microtiter plate;
b) while one of the reagents is bound in the solid phase, further reagents are added and co-incubated, and their excess is removed by washing;
c) one of the reagents (serum against chicken antibodies - indirect ELISA or specific antigen serum-blocking and indirect sandwich format) used in the test is covalently bound to the enzyme. The addition of a substrate suitable for the enzyme results in a colored, soluble product that can be quantified by optical density (OD) measurement. In the indirect test, antibody concentration (titer) increases with increasing OD value (Fig. 3.), while in the direct competition test - blocking ELISA, the relationship is inverse, the concentration of antibodies increases with the decrease in the value of optical density (Fig. 5.).



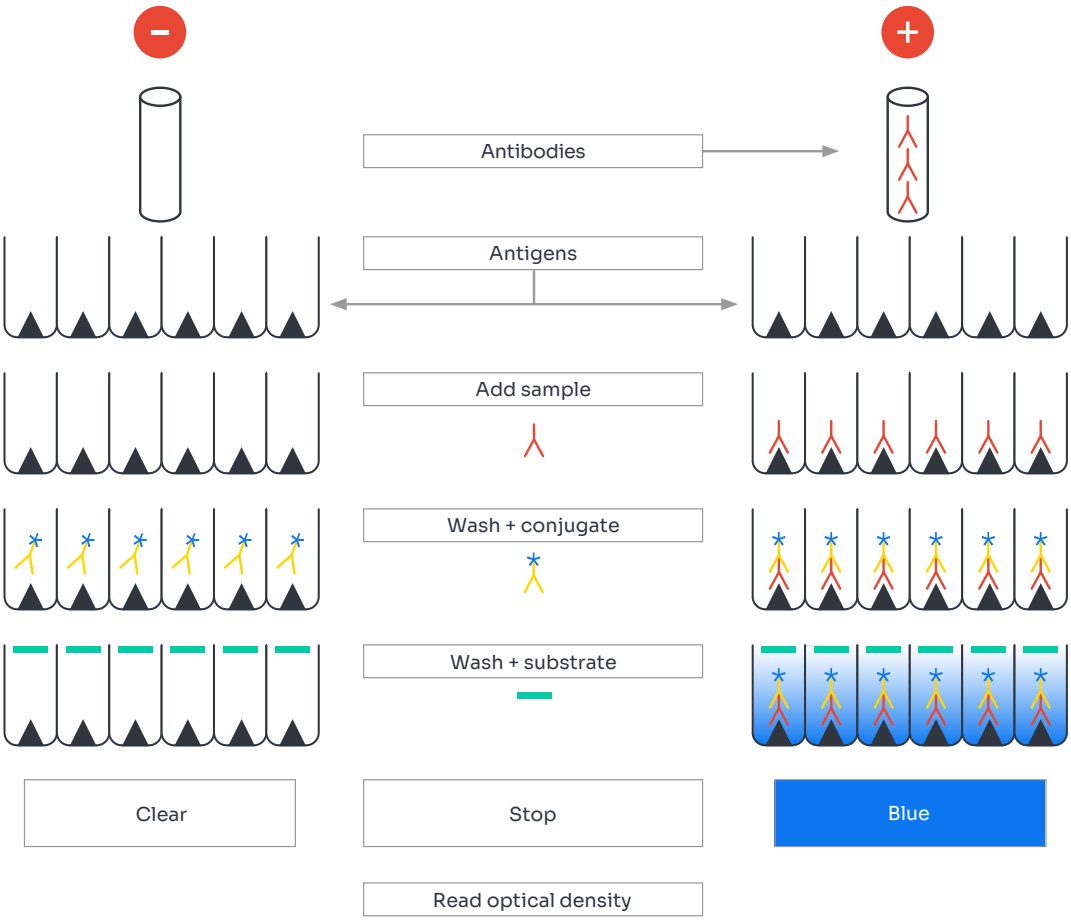


Fig. 2. Scheme of a non-competitive indirect ELISA*

*The conjugate is enzyme-labeled anti-chicken antibodies

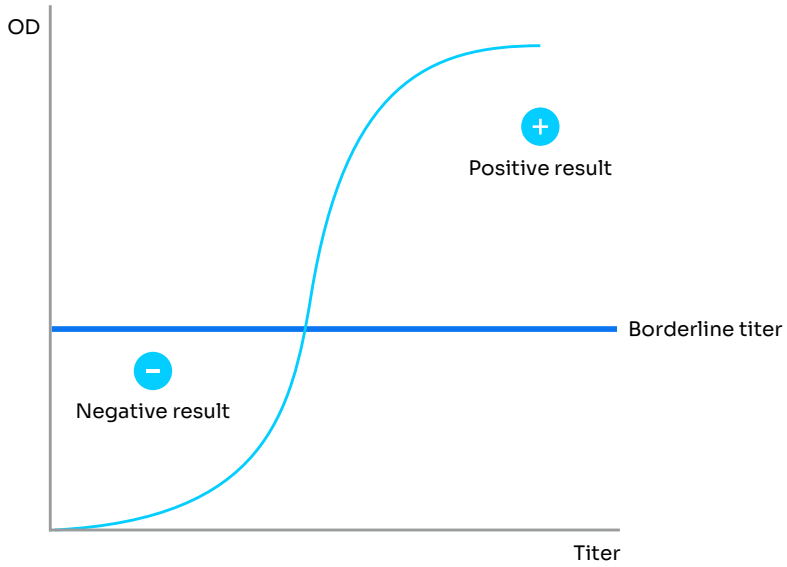


Fig. 3. Relationship between antibody concentration (titer) and optical density (OD) in an indirect test

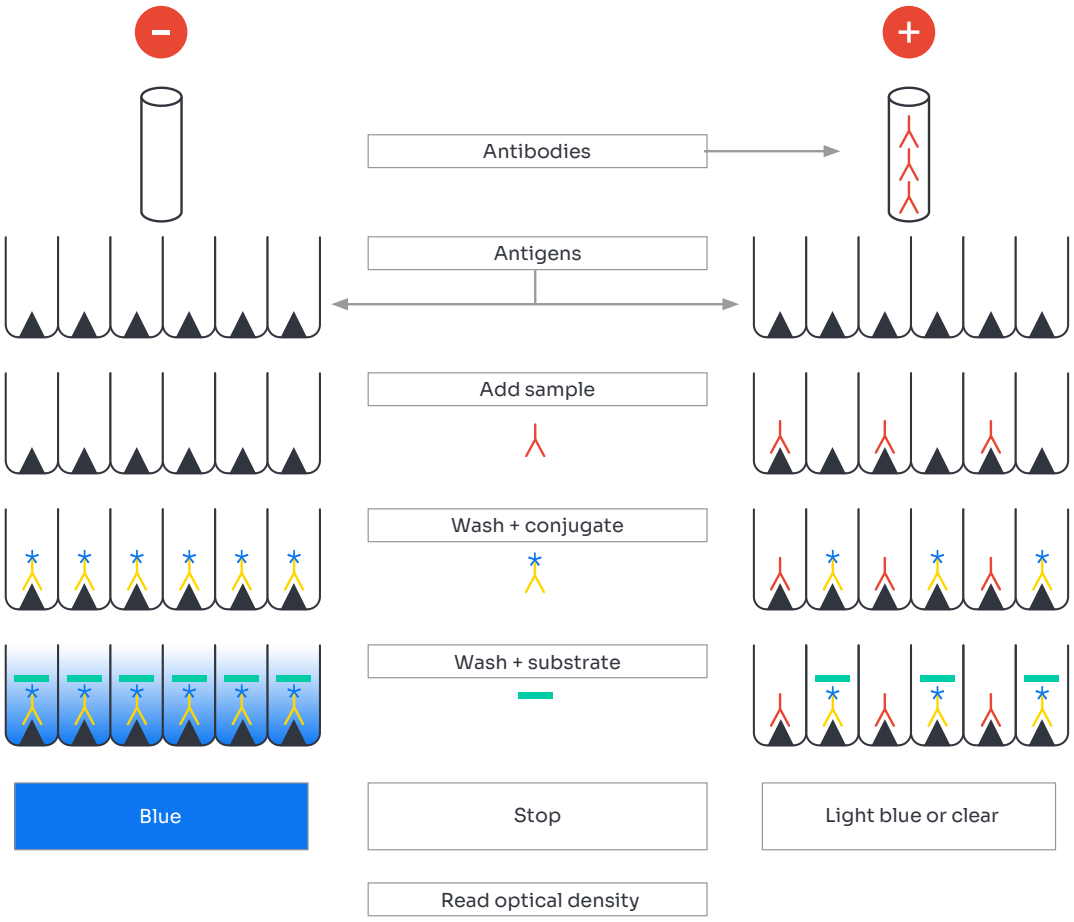


Fig. 4. Scheme of a direct competition test - blocking ELISA

** The conjugate is enzyme-labeled anti-platelet antigen antibodies

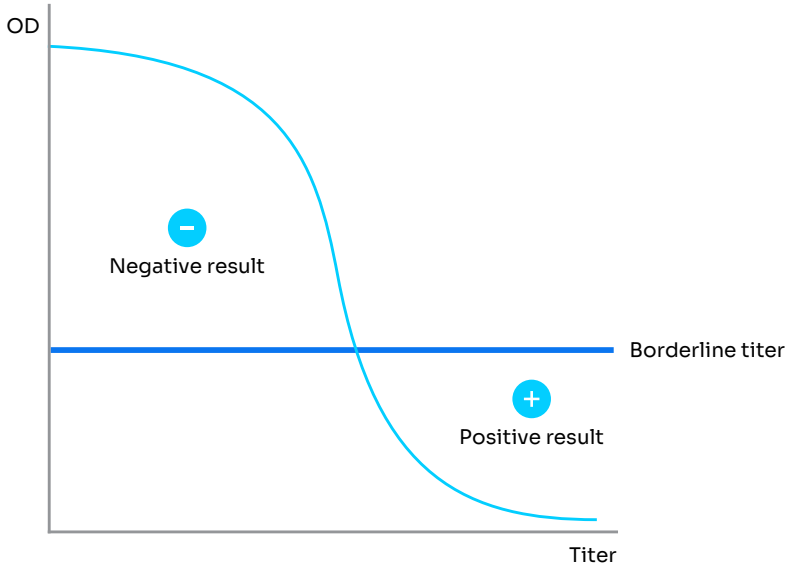


Fig. 5. Relationship between antibody concentration (titer) and optical density (OD) in the blocking ELISA

xChekPlus* test acronym	Pathogen	Name of the test	Test description	Validated species	S/P or S/N ratio cut-off
AE*	Avian encepha-lomyelitis virus	IDEXX AE Ab Test	ELISA test for the detection of antibody to avian encephalomyelitis virus (AE) in chicken serum.	Chicken	S/P > 0,2
AI*	Avian influenza virus	IDEXX AI Ab Test	ELISA test for the detection of antibody to Avian Influenza Virus (AI) in chicken serum.	Chicken	S/P > 0,5
Aims*	Avian influenza virus	IDEXX AI MultiS-Screen Ab Test	ELISA test for the detection of antibody to avian influenza virus (AI) in chicken, turkey, duck, ostrich, and goose serum	Chicken, turkey, duck, ostrich, and goose	S/P > 0,5
ALV-J*	Avian leukosis virus subgroup J	IDEXX ALV-J Ab Test	ELISA test for the detection of antibody to avian leukosis virus, subgroup J in chicken serum.	Chicken	S/P > 0,6
APV*	Avian pneumovirus	IDEXX APV Ab Test	ELISA test for the detection of antibody to avian pneumovirus (APV) in chicken and turkey serum.	Chicken and turkey	S/P > 0,2
CAV**	Chicken anemia virus	IDEXX CAV Ab Test	ELISA test for the detection of antibody to Chicken Anemia Virus (CAV) in chicken serum	Chicken	S/N < 0,6
IBV*	Infectious bronchitis virus	IDEXX IBV Ab Test	ELISA test for the detection of antibody to infectious bronchitis virus (IBV) in chicken serum.	Chicken	S/P > 0,2
IBD*	Infectious bursal disease virus	IDEXX IBD Ab Test	ELISA test for the detection of antibody to infectious bursal disease virus (IBD) in chicken serum.	Chicken	S/P > 0,2
IBD-XR*	Infectious bursal disease virus	IDEXX IBD-XR Ab Test	ELISA test with enhanced dynamic range for the detection of antibody to infectious bursal disease (IBD) in chicken serum	Chicken	S/P > 0,2

Tab. 1. IDEXX ELISA tests for serodiagnosis of poultry diseases (8)
*Indirect (no-competitive) ELISA
**Blocking (competitive) ELISA
***Antigen Capture ELISA (Indirect Sandwich ELISA)

xChekPlus* test acronym	Pathogen	Name of the test	Test description	Validated species	S/P or S/N ratio cut-off
INFAvian*	Avian influenza virus	IDEXX Influenza A Ab Test	ELISA test for the detection of antibody to influenza A virus in animal serum	Chicken, turkey, falcon, flamingo, quail, Japanese quail, laughing gull, house finch, starling, and herring gull	S/P > 0,5
LLAB*	Avian leukosis virus subgroups A and B	IDEXX ALV Ab Test	ELISA test for the detection of antibody to avian leukosis virus (ALV-subgroups A and B) in chicken serum	Chicken	S/P > 0,4
LLAG***	Avian leukosis virus all subgroups	IDEXX ALV Ag Test	ELISA test for the detection of avian leukosis virus Antigen p27 in chicken serum, cloacal swabs, or albumin samples	Chicken	S/P > 0,2
MG*	*Mycoplasma gallisepticum*	IDEXX MG Ab Test	ELISA test for the detection of antibody to *Mycoplasma gallisepticum* (Mg) in chicken and turkey serum.	Chicken and turkey	S/P > 0,5
MMt*	*Mycoplasma meleagridis*	IDEXX MM Ab Test	ELISA test for the detection of antibodies to *Mycoplasma meleagridis* (Mm) in turkey serum.	Turkey	S/P > 0,5
MS*	*Mycoplasma synoviae*	IDEXX MS Ab Test	ELISA test for detecting of antibodies to *Mycoplasma synoviae* in chicken and turkey serum.	Chicken and turkey	S/P > 0,5
MS/MG*	*Mycoplasma synoviae/ Mycoplasma gallisepticum*	IDEXX MG/MS Ab Test	ELISA for for the detection of antibody to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* (Mg/Ms) in chicken and turkey serum	Chicken and turkey	S/P > 0,5

Tab. 1. IDEXX ELISA tests for serodiagnosis of poultry diseases (8)
*Indirect (no-competitive) ELISA
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xChekPlus* test acronym	Pathogen	Name of the test	Test description	Validated species	S/P or S/N ratio cut-off
NDV*	Newcastle disease virus	IDEXX NDV Ab Test	ELISA test for the detection of antibody to Newcastle disease virus (NDV) in chicken serum.	Chicken	S/P > 0,2
NDVt*	Newcastle disease virus	IDEXX NDV-T Ab Test	ELISA test for the detection of antibody to Newcastle disease virus (NDV) in turkey serum.	Turkey	S/N < 0,2
ORT*	*Ornitho-bacterium rhinotracheale*	IDEXX ORT Ab Test	ELISA test for the detection of antibody to *Ornithobacterium rhinotracheale* (ORT) in chicken and turkey serum	Chicken and turkey	S/P > 0,4
PM*	*Pasteurella multocida*	IDEXX PM Ab Test	ELISA test for the detection of antibody to *Pasteurella multocida* (Pm) in chicken serum	Chicken	S/P > 0,2
PMt*	*Pasteurella multocida*	IDEXX PM-T Ab Test	ELISA test for the detection of antibody to *Pasteurella multocida* (Pm) in turkey serum	Turkey	S/P > 0,2
REO*	Avian reovirus	IDEXX REO Ab Test	ELISA test or the detection of antibody to avian reovirus (REO) in chicken serum.	Chicken	S/P > 0,2
REV*	*Reticuloendo-theliosis virus*	IDEXX REV Ab Test	ELISA test for the detection of antibody to *Reticuloendotheliosis virus* (REV) in chicken serum.	Chicken	S/P > 0,2
SE Ab X2*	*Salmonella enteritidis*	IDEXX SE Ab X2 Test	ELISA test for the detection of antibody to *Salmonella Enteritidis* (SE) in chicken serum.	Chicken	S/P > 0.2

Tab. 1. IDEXX ELISA tests for serodiagnosis of poultry diseases (8)
*Indirect (no-competitive) ELISA
**Blocking (competitive) ELISA
***Antigen Capture ELISA (Indirect Sandwich ELISA)

In poultry serology, due to the large number of birds in a flock, up to tens of thousands, it is necessary to use the methods used in statistics to determine the number of samples to be tested.

Statistic deals with the principles and methods of generalizing the results obtained from a random sample to the entire population (that is, the population from which the samples were taken). This procedure is called statistical inference. Proper random selection of appropriate quality samples is important (Moczko et al., 1998). The first element facilitating the analysis of the obtained results is the division into titer groups, statistically called a distribution series; it is the basic statistical way of presenting the distribution of test results. In poultry serology using ELISA, it is created by dividing (assigning) the obtained results into (up to) 18 categories (groups of titers) and specifying the number of sera belonging to each of these categories. Imaging the results in the form of a histogram allows for a clear presentation and enables quick orientation in the variability of titers, which facilitates the evaluation of the obtained result.

For practical purposes in poultry serology the statistics parameters are most often used. These inform us about the average tirer value (AMT, GMT), i.e. the average number of antibodies in a specific volume of serum, and they measure the variability/dispersion of antibodies level in the population under study (SD, CV%).

Box 1. Statistics

Selected statistical parameters used in serological tests graphs

In addition to the central tendency described by the mean titer (AMT, GMT), scatter is a fundamental sample characteristic in serology testing in poultry. A poultry flock consists of many individuals whose reaction to contact with the antigen leads to different levels of serological response. This diversity is a natural phenomenon resulting from the genetic structure of a given individual or the current efficiency of its immune system. Therefore, the obtained, different results of serological tests must be processed using indicators describing the degree of their differentiation. In the case of elaborating the results of serological tests from a given population (flock), it is assumed that they have the form of the so-called normal distribution, otherwise known as the Gaussian distribution, illustrated by the Gaussian curve, which distribution is the most important theoretical probability distribution in statistics (Moczko et al., 1998). The normal distribution is also the most intuitive statistical distribution. In short, it describes a situation in which most cases are close to the average result, and the more a given result deviates from the average, the less numerous it is. The simplest way to say is that in populations with a normal distribution, most serum titers are close to the mean value, and the further we move away from the mean result, the fewer sera there are (Fig. 6).

The most commonly used measures of dispersion in medical research are: variance, standard deviation and coefficient of variation (Andrasiak et al., 2018). Dispersion (scattering, scattering, dispersion) is the variation in the observed values of a variable; it is the greater, the more these values deviate from the central tendency, i.e., the mean titer. The desired probability function of the random variable has the shape of a normal curve (Gaussian Function). Gaussian curve (Fig. 6.) describes a normal distribution in which the curve is symmetrical about the axis determined by the mean value and reaches its maximum here (high and low titer values occur less frequently, the more they differ from the sample mean).

Box 2. Statistics

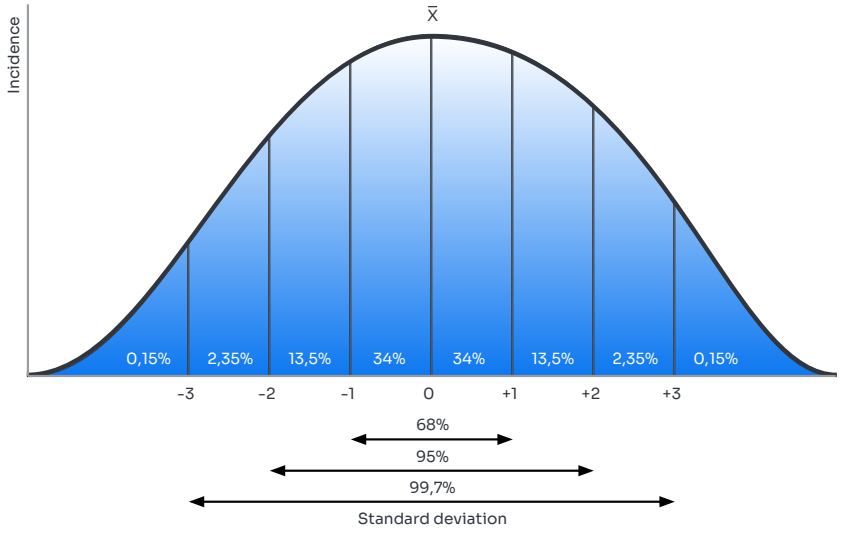


Fig. 6. Theoretical Gaussian curve describing the normal distribution

As you can see, about 68% of the observations are close to the mean, within one standard deviation of the mean (a measure of distance in the language of statistics). As you move away from the mean, the Gaussian curve descends. As much as 95% of observations are in the range of -2 to +2 standard deviations from the mean value. Extreme values (at the ends of the Gaussian curve) are represented by a negligible percentage of observations (Fig. 8.). This means that when the distribution of antibody levels is close to the normal distribution, we can say that there are no anomalies in the tested sample, the obtained data are normal, there are few extreme observations, and a significant part of the observations is concentrated around the mean. The occurrence of such anomalous distributions means that the properties of the normal distribution cannot be applied to them, and thus many statistical tests cannot be used, because their results may be distorted by the occurrence of a non-standard distribution of results (Fig. 9.). In practice, the normal distribution of results is most often disturbed by combining chicks from different parent flocks with different immunological status in the hatchery. This practice, sometimes referred to as chicks mixing, makes it very difficult to determine the proper timing of vaccinations, e.g., against Gumboro disease (Fig. 10.).

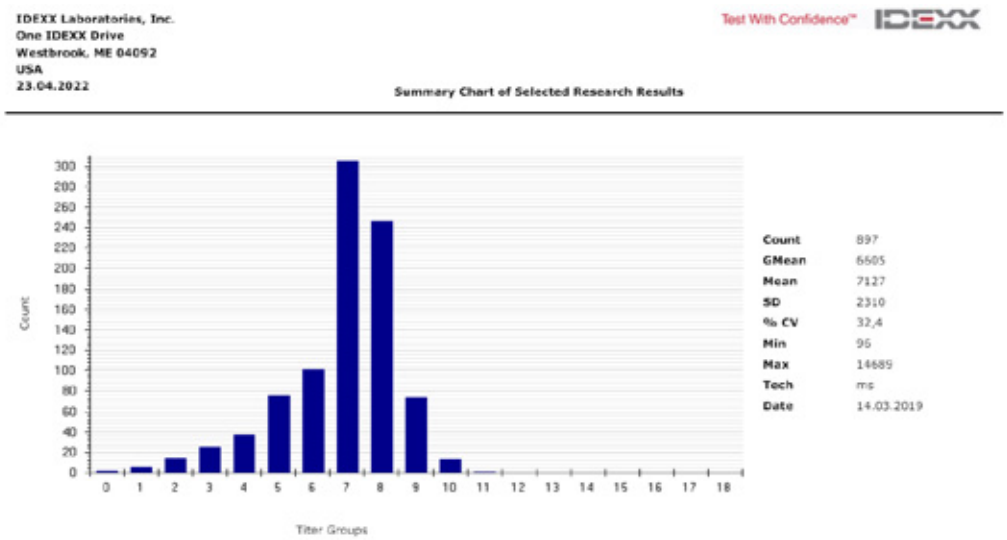


Fig. 7. Distribution of IBD virus antibody titers in 39 flocks of day-old broiler chicks

As can be seen from the data in Fig. 7., of the 897 sera tested from day-old broiler chicks, only two sera belonged to group zero and only one to group 11. More than 35% of the sera were in the 7th titer group (the range of titers in this group is 6,000 - 7,999) and the arithmetic mean titer was 7,127. In the range of 2,507 - 11,747 (about twice the SD value), there were approx. 95% of the tested sera.

In the presented example, it can be observed that the more measurements we make, the more the resulting curve will resemble a symmetrical bell. If (which is of course impossible) we analyzed a group with an infinite number of cases, we would get a normal distribution. In practice, the sample we study always has a limited number of elements, but it is worth remembering that the more elements we include in the study, the better the actual distribution of the examined variable in the analyzed population will be reproduced. At this point, it should be strongly emphasized that too few samples tested may cause the results of the serological test to be unreliable and misleading. In practice, it is recommended to collect 23-30 blood samples, regardless of herd size.

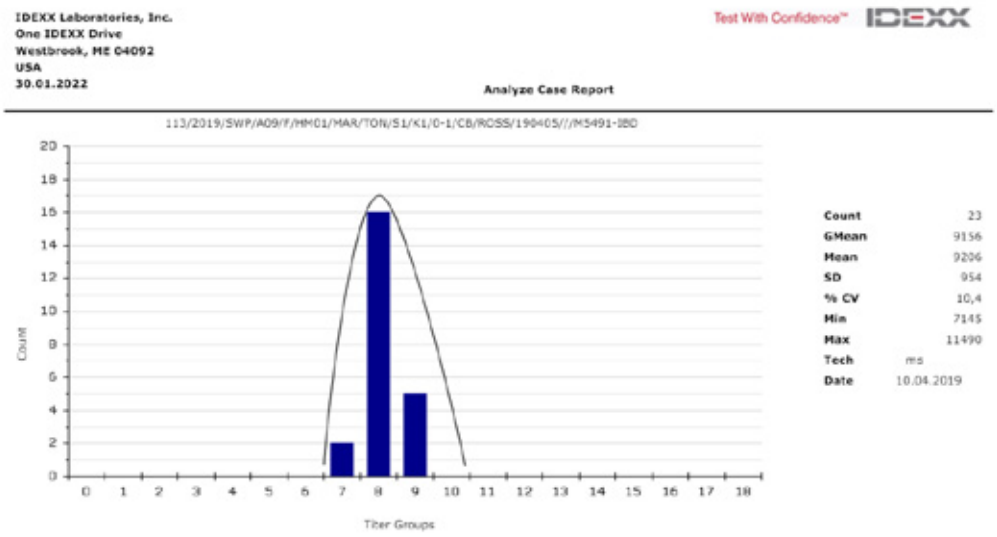


Fig. 8. Example of distribution of ELISA results in a flock of day-old broiler chicks from a very well IBD vaccinated parent flock

In Fig. 8. the effect of examining a flock of one-day-old broiler chicks coming from a very well-protected parent flock was illustrated. Noteworthy is the very low value of the coefficient of variation (CV - 10.4%) and the difference between GMT and AMT of only 50 and the very low value of the standard deviation (954). In such a herd, scheduling vaccination against Gumboro disease will not be a problem.

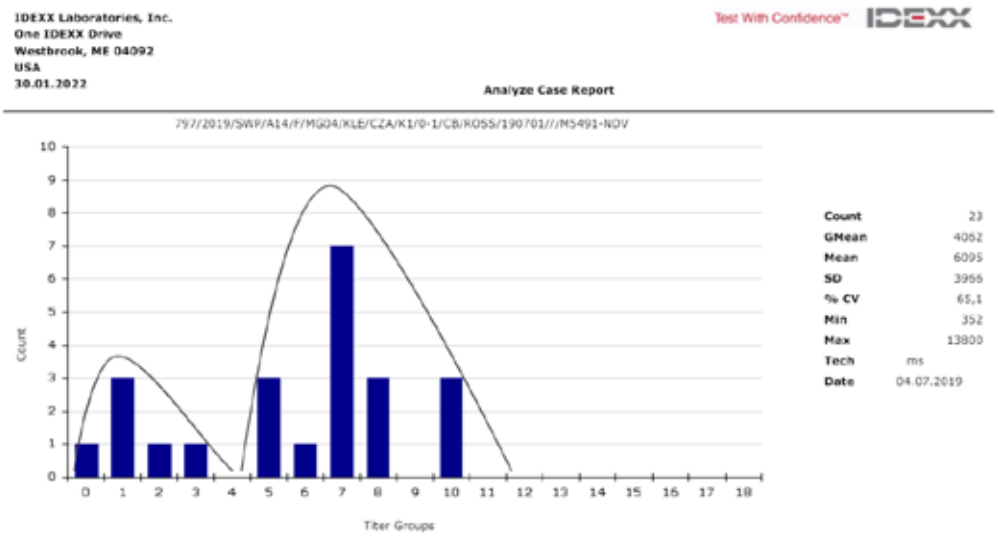


Fig. 9. An example of the distribution of results obtained in the ELISA test in a flock of day-old broiler chicks from a very poorly NDV vaccinated parent flock.

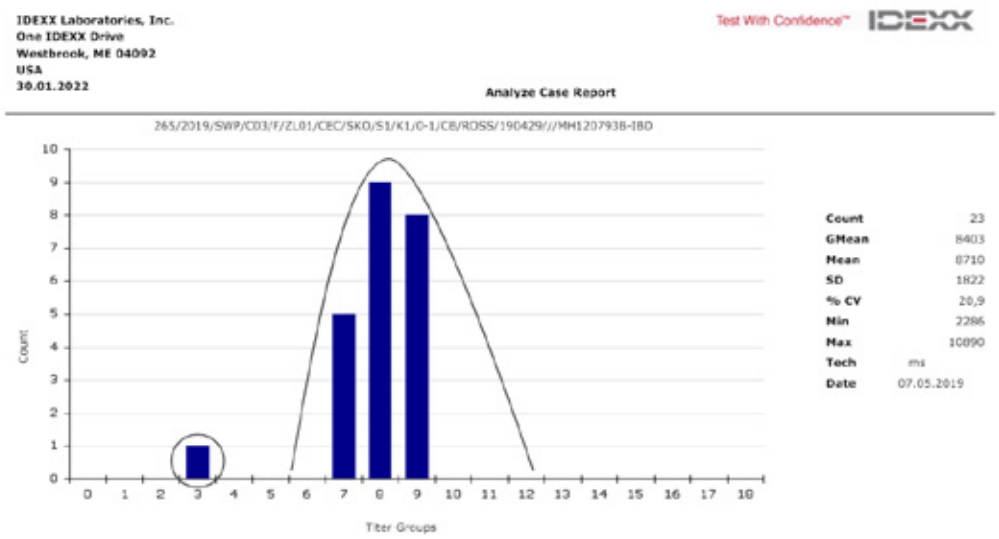


Fig. 10. Example of the variability of serological test results in the case of chicks from different parents' flocks.

To describe the data obtained in quantitative serological tests, it is necessary to use descriptive statistics to better understand the level of specific antibodies in the population, and its distribution. The values obtained because of the use of descriptive statistics are also the basis for further, more advanced methods of statistical analysis, such as testing differences between groups or examining relationships between selected indicators (e.g., the level of specific antibodies against a given pathogen and the performance of the flock).

Two groups of parameters play a key role in descriptive statistics: central tendency parameters and dispersion parameters. All central tendency parameters estimate the most typical value of the parameter characterizing the studied group, but each of them focuses on a different aspect of its distribution. In serology the most used parameters of central tendency are the arithmetic mean and the geometric mean. The central tendency parameters determine a certain value that is most representative for the tested sample, but it is obvious that characterizing the titers of a poultry flock only with this parameter is definitely insufficient (Andrasiak et al., 2018). Therefore, the concept of dispersion parameters was introduced, which characterize the degree of dispersion of results around the central tendency measure. The larger the value of the dispersion parameter, the more the results are scattered around the value of central tendency. In general, this also means that the value of central tendency is less representative of such results because they vary widely. Small values of the dispersion parameter allow, in turn, to consider the central tendency value as a good representative of the entire sample. In the case of serological assays, dispersion parameters provide information on how diverse the test population is in terms of antibody titers. The primary parameter of dispersion used in serology is variance. This parameter expresses the degree of dispersion of the random variable (antibody titer) around the mean value. The greater the variance, the greater the scatter of the variable (Moczko et al., 1998).

The variance of the denominators $x_1, x_2, ..., x_n$ is the arithmetic mean of the squared deviations from their arithmetic mean:

$$\sigma_2 = (x_1 - \overline{X}) 2 + (x_2 - \overline{X}) 2 + ... + (x_n - \overline{X}) 2n$$

The standard deviation is the square root of the variance. The standard deviation determines approximately how much all statistical units of a given population differ on average from the arithmetic mean of the variable under study. It is assumed that the value of the standard deviation for a normally distributed population should not be greater than 50% of the mean value. The standard deviation provides us with the necessary knowledge about whether the results in a particular group of results are like each other - whether the group of titers is similar or different, and if so, to what extent (Moczko et al., 1998).

How to read ELISA test results obtained with xChekPlus* software

Laboratories performing tests using IDEXX ELISA kits provide the results in the form of a template printout, consisting of a typical histogram (graph view with the given statistics and legend - description below the graph) and a data table (statistical data, controls, internal controls, verification wells, comments, lot number and test expiration date) [Fig. 11]. This form of presenting test results enables easy analysis of the obtained results of the serological test. In addition, a statistical summary may also be included. In the upper left corner of the test result, there is the data of the laboratory performing the test and the date of printing the report (this is not the date of the test).

We start the analysis of the test result by reading the description of the tested material. It is located above the histogram and can have a very different form, but it always contains the research direction in the form of an acronym (Tab. 1.). A description identifying the flock is printed here. This is extremely important information for the correct interpretation of the result. Although the recording solutions used by specific laboratories differ in form, they should allow full identification of the ordering party and the tested material.

The system of describing/marking samples in serological monitoring tests of poultry flocks in the country is not unified, which is often an obstacle to a full analysis of the serological test result. Very often, in practice, the correct interpretation of the serological monitoring result is hindered by the lack of additional information relevant for this assessment, such as the age of the birds or the vaccination program.
The recommended structure of material and stock description is given in Tab. 2. and the description scheme most often used in practice is contained in Tab. 3.

1.	2.	3.	4.	5.	6.
Laboratory (usually with logo and address)	Test number according to laboratory procedures	Submitter	Sampling date	Referring veterinarian	Owner - identification data

7.	8.	9.	10.	11.	12.	13.
Farm - location data	Age of birds on sampling date	Production type	Breed	Hatching Date	Purpose of the test	Vaccination program

Tab. 2. Recommended sample coding system for serological testing

Order number	Owner	Sampling date	Species/ Breed	Principal	Material	Material description	Date of submission	Object

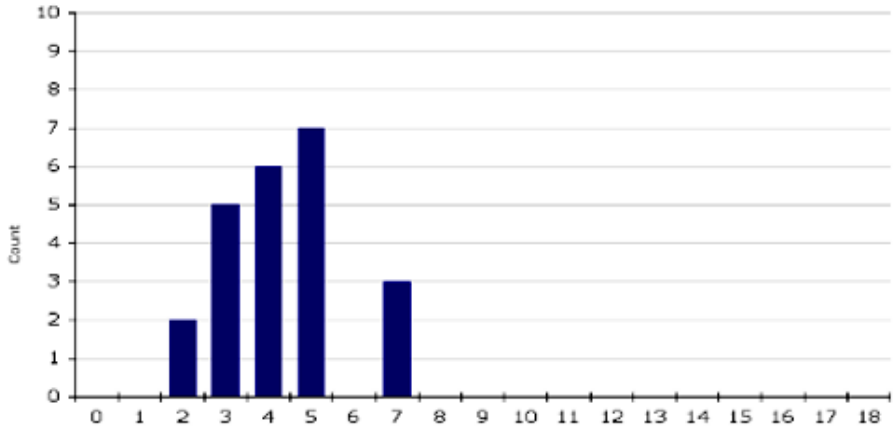
Tab. 3. The most used description in practice of serologically tested samples taken from a poultry flock.

IDEXX Laboratories, Inc.
One IDEXX Drive
Westbrook, ME 04092
USA
23.04.2022

Test With Confidence™ IDEXX

Analyze Case Report

591/2019/SWP/B07/T/HM01/MAR/TON/S1/K1/6-4/CB/ROSS/190405///M5491QX-IBD



Count	23
GMean	3543
Mean	3803
SD	1428
% CV	37,5
Min	1633
Max	7151
Tech	ms
Date	06.06.2019

Assay	Date	Count	AMean	% CV	Age	Case ID	Comment
IBD	06.06.2019	23	3803	37,5	6-4	591/2019/SWP/B07/T/HM01/MAR/TON/S1/K1	

Case number	591/2019/SWP/B07/T/HM01/MAR/TON/S1/K1/6-4/CB/ROSS/190405/// M5491QX - 06.06.2019-001 IBD	Kit Lot	Unknown	Expiration	20.12.2011

IBD - 06.06.2019 - ms

Comments

	Well	O.D.	S/P	Titer	Group	Results
Neg	A1	0,044				
Neg	A2	0,042				
Pos	A3	0,260				
Pos	A4	0,287				
1	E3	0,601	2,421	6005	7	Pos
2	E4	0,289	1,067	2459	3	Pos
3	E5	0,669	2,716	6807	7	Pos
4	E6	0,450	1,766	4257	5	Pos
5	E7	0,345	1,310	3075	4	Pos
6	E8	0,401	1,553	3702	4	Pos
7	E9	0,345	1,310	3075	4	Pos
8	E10	0,698	2,842	7151	7	Pos
9	E11	0,372	1,427	3376	4	Pos
10	E12	0,481	1,900	4612	5	Pos
11	F1	0,404	1,566	3736	4	Pos
12	F2	0,226	0,794	1781	2	Pos
13	F3	0,322	1,210	2821	3	Pos
14	F4	0,399	1,544	3679	4	Pos
15	F5	0,297	1,102	2547	3	Pos
16	F6	0,472	1,861	4509	5	Pos
17	F7	0,431	1,683	4041	5	Pos
18	F8	0,471	1,857	4497	5	Pos
19	F9	0,292	1,080	2492	3	Pos
20	F10	0,212	0,733	1633	2	Pos
21	F11	0,476	1,879	4555	5	Pos
22	F12	0,261	0,946	2156	3	Pos
23	G1	0,472	1,861	4509	5	Pos

	S/P	Titer	Log2
AMean	1,584	3803	12
GMean	1,492	3543	12
SD	0,546	1428	1
% CV	34,4	37,5	4,7
Min	0,733	1633	11
Max	2,842	7151	13

Histogram

Samples data

Statistics

Fig. 11. Report of IBD test results performed using xChekPlus* software

In order to facilitate the tests coding, a proper recording system was proposed, the use of which enables the full use of the xChekPlus* software to generate reports that are extremely useful in managing the flock health status (Szeleszczuk, 2012). If you are working with the xChekPlus* software, use the “Comments” record to encode information about your vaccination program. The rules for coding the vaccination program are given in Tab. 4. An example of a printout of the result, considering the full coding system of information about the herd and the vaccination program, is given in Fig. 12.

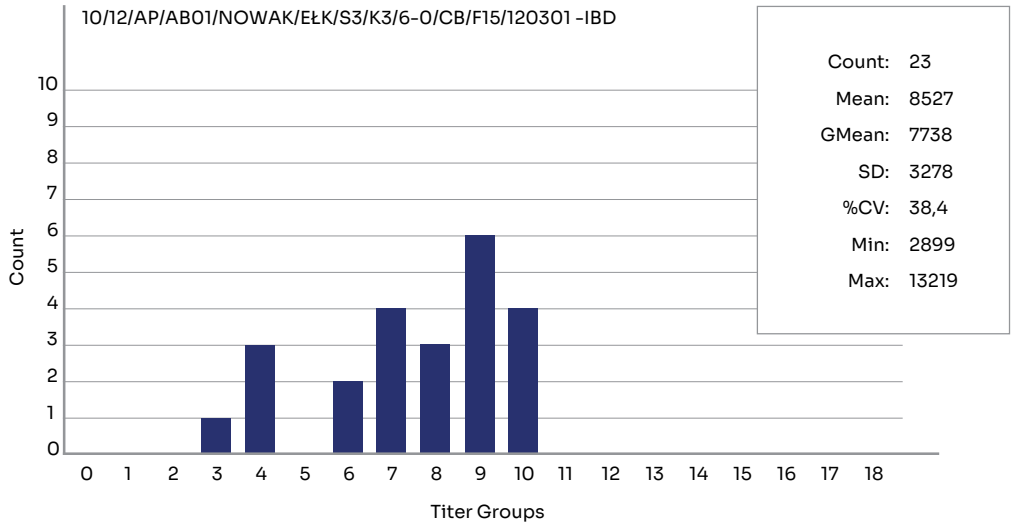


Fig. 12. A printout of the result of the test for antibodies to Gumboro disease virus

Code	Assay	Date	Count	GMean	CV	Age
AND	IBD	27	23	7738	38,4	6–0

Case Comment

10/12/22/AP/AB01/Jan Nowak/Elk/S3/K3/6-0/CB/F15/120301/P/ ///M1HI//N1AS//I1MS//I10VW//G18HW/G24HW

1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18.

Description of code elements:

1.	10/12/22/	Laboratory serial number
2.	AP/	Submitter code
3.	AB01/	Veterinarian code
4.	Jan Nowak/	Name and surname of flock owner - we do not code!
5.	Elk/	Farm location
6.	S3/ K3/	Sector House
7.	6–0/	Age of birds on sampling day (weeks-days)
8.	CB/	Type of birds (commercial broiler)
9.	F15/	Production line
10.	120301/	Date of chicks hatching YY/MM/DD, e.g., March 1, 2012
11.	P/	Purpose of the study - P - problem in the herd
12.	///	Three slashes separate flock data from the vaccination program
13.	M1HI//	Vaccination against MD at day 1 with injectable HVT vaccine
14.	N1AS//	Vaccination against ND at day 1 with asymptomatic vaccine by spray
15.	I1MS//	Vaccination against IB at day 1 with Mass vaccine by spray
16.	I10VW//	Vaccination against IB at day 10 with a variant vaccine by drinking water
17.	G18HW/	Gumboro disease vaccination at day 18 with an intermediate plus vaccine by drinking water
18.	G24HW	Vaccination against Gumboro disease at day 24 with an intermediate plus vaccine by drinking water

Code	Disease	Acronym	Type of vaccine	Vaccination technique
N	Marek's disease	MD	H = HVT R = Rispens	I = injection
N	Newcastle disease	ND	A = asymptomatic L = lentogenic	S = spray W = in drinking water
I	Infectious bronchitis	IB	M = Mass V = variant	S = spray W = in drinking water
G	Gumboro disease	IBD	P = intermediate H = intermediate plus	W = in drinking water
A	Metapneumovirus infections	APV		S = spray W = in drinking water
R	Reoviral infections	REO		I = injection
C	Coccidiosis	Coccidiosis		S = spray W = in drinking water

Tab. 4. Vaccination codes for broiler flocks

The most important advantage of the proposed sample coding system is the ability to quickly search for results of interest, stored in the databases of programs used to generate test results. With proper coding, it is possible to prepare various reports, e.g., collecting the results of blood tests from the age of 6 weeks in one chart., taken by a specific submitter, vaccinated against Gumboro disease.

In statistics, 2D histograms are a graphical representation of the frequency distribution of a selected variable(s), in which the columns (bars) are plotted above the group ranges (denominator groups 0-18), and the height of the columns is proportional to the number of sera in each titer group. All sera with titers below the cut-off titer (negative) are in group 0.
In the given example, there are 23 tested sera in this group.

Quantity (on the Y axis)

The number of sera with a titer belonging to a given titer group.

Group of titers (on the X axis)

Titer group number. To facilitate the recording and analysis of serological monitoring data, the obtained titer was grouped into titer groups according to the rule given in Table 6.

	Range of changes in the group			Result/antibody concentration
Titers group	All tests except MS, MG, REV, LLAB	Ms/Mg, Mg	REV, LLAB	
0	0-396	0-1076	0-844	Negative/none
Cut off titer				
1	397-999	1097-1499	845-999	
2	1000-1999	1500–1999	1500–1999	Positive/Low
3	2000-2999	2000–2999	2000–2999	
4	3000-3999	3000–3999	3000–3999	
5	4000-4999	4000–4999	4000–4999	
6	5000-5999	5000–5999	5000–5999	Positive/Average
7	6000-7999	6000–7999	6000–7999	
8	8000-9999	8000–9999	8000–9999	
9	10000-11999	10000–11999	10000–11999	
10	12000-13999	12000–13999	12000–13999	
11	14000-15999	14000–15999	14000–15999	Positive/High
12	16000–17999	16000–17999	16000–17999	
13	18000–19999	18000–19999	18000–19999	
14	20000–21999	20000–21999	20000–21999	
15	22000–23999	22000–23999	22000–23999	
16	24000–27999	24000–27999	24000–27999	Positive/Very high
17	28000–31999	28000–31999	28000–31999	
18	32000	32000	32000	

Tab. 6. Titer groups in the IDEXX indirect ELISA.

Next to the histogram, there are basic statistical data, most often used when analyzing the test result.

Quantity

The number of sera tested in the performed test.
The recommended number of sera is 23 for the serological diagnosis of viral diseases, and 60 for the diagnosis of bacterial infections. Testing less than 18 sera per flock does not give reliable information about the flock.

Geometric mean titer – GMT

The geometric mean of the titers is the nth root of their product. The geometric mean in statistics is most often used to calculate the average severity of changes. In serology, the geometric mean of titers is most often used, as it better reflects the distribution of titers in the studied population of birds.
The geometric mean of the denominator x_1, x_2, x_3, ..., x_n is expressed by the formula:

$$GMT = \sqrt[n]{x_1 \times x_2 \times x_3 \times x_n}$$

x_1 – serum titer 1
x_2 – serum titer 2
x_3 – serum titer 3
x_n – serum titer n
n – quantity of tested sera

Arithmetic mean

Arithmetic mean titer (AMT), sometimes referred to as mean titer, by default - mean titer.
The arithmetic mean of the titers is the sum of these titers divided by their number. The arithmetic mean belongs to the classic average measures and expresses the average level of the observed feature.
The arithmetic mean of x_1 , x_2 , x_3 , ..., x_n is given by:

$$AMT = \frac{x_1 + x_2 + x_3 + x_n}{n}$$

x_1 – serum titer 1
x_2 – serum titer 2
x_3 – serum titer 3
x_n – serum titer n

SD

Standard deviation – standard deviation is a classic measure of variability, next to the arithmetic mean, the most frequently used concept used in the language of statistics. Standard deviation (SD), defined as the square root of the quotient of the sum of squares of deviations of the variable (X) from the arithmetic mean of the set ($\bar{x}$). Intuitively, the standard deviation tells you how widely the values of a quantity are spread around its mean, the greater the standard deviation, the greater the variance in the studied population. It is a measure of the distance of individual results from the average. The further a given result is from the mean in units of standard deviation, the more unusual it is, i.e., it is inconsistent with the normal distribution.

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(x - x)^2}{N - 1}}$$

% CV

Coefficient of variation - is a classic and extremely useful measure of titer distribution variability in the analysis of serological test results. The coefficient of variation is a relative measure, i.e., it depends on the size of the arithmetic mean, as it expresses the variability of a feature in relation to the value of this mean. The coefficient of variation is usually expressed as a percentage.

The coefficient of variation is defined by the formula:

$$CV = \frac{SD \times 100}{x}$$

The coefficient of variation characterizes the variability of the result. If the CV is less than 40%, it is assumed that the dispersion of the results, i.e., the differentiation of changes between individual individuals, is satisfactory, which may mean that all birds in the flock reacted similarly to the vaccine administration. A high coefficient of variation means that the variation of titers between individuals in the flock is too large, which is not a favorable phenomenon. The valuation of the coefficient of variation value is particularly important when determining the timing of vaccination against Gumboro disease. Reliable determination of the optimal date of vaccination is possible only when the coefficient reaches the value 40%. In samples taken from birds vaccinated with live vaccines, the value of this ratio should not exceed 60%.

Min

It is short for “minimal” - antibody titer. The assessment of the minimum titer value is important, for example, when evaluating the results of day-old chicks, as it allows to assess whether all tested individuals were sufficiently protected against early infection.

Max

Itis short for “maximum” antibody titer. The assessment of the maximum titer value is important, for example, when evaluating the results of tests in vaccinated birds suspected of infection with field virus. Very high maximal titers indicate that such a high serological response cannot be expected from the applied vaccination program, therefore they may confirm clinical symptoms characteristic of a given disease.

Operator

Details of the person performing the test.

Data

The date the test was performed is shown here.

07.03.2022

Analyze Case Report

Case number	1144/2019/SWP/A14/T/MG04/KLE/CZA/K1/5-5/CB/R			Kit Lot	Unknown
IBV - 21.08.2019 - ms				Comments	
	Well	**O.D.**	**S/P**	**Titer**	**Group**	**Results**
Neg	A1	0,042				
Neg	A2	0,040				
Pos	A3	0,296				
Pos	A4	0,280				
1	A5	0,226	0,749	1672	2	Pos
2	A6	0,145	0,421	892	1	Pos
3	A7	0,177	0,551	1195	2	Pos
4	A8	0,438	1,607	3843	4	Pos
5	A9	0,649	2,462	6115	7	Pos
6	A10	0,054	0,053	93	0	Neg
7	A11	0,049	0,032	54	0	Neg
8	A12	0,076	0,142	272	0	Neg
9	B1	0,087	0,186	367	0	Neg
10	B2	0,095	0,219	437	1	Pos
11	B3	0,244	0,822	1850	2	Pos
12	B4	0,087	0,186	367	0	Neg
13	B5	0,368	1,324	3110	4	Pos
14	B6	0,085	0,178	349	0	Neg
15	B7	0,099	0,235	472	1	Pos
16	B8	0,060	0,077	140	0	Neg
17	B9	0,060	0,077	140	0	Neg
18	B10	0,174	0,538	1167	2	Pos
19	B11	0,150	0,441	939	1	Pos
20	B12	0,111	0,283	580	1	Pos
21	C1	0,083	0,170	332	0	Neg
22	C2	0,239	0,802	1800	2	Pos
23	C3	0,132	0,368	771	1	Pos

	S/P	**Titer**	**Log2**
AMean	0,518	1172	9
GMean	0,303	622	9
SD	0,573	1416	2
% CV	110,5	120,8	18,3
Min	0,032	54	6
Max	2,462	6115	13

Tab. 7. IBV result data table of 23 sera collected from a flock of 40-day-old broilers.

Next to the histogram, the result of the ELISA test is presented as a table data (Tab. 7). The TEST MATERIAL field contains information about the test, which enables its identification, the reason for testing and the code of the technician performing the test.

The first column contains data describing the results of testing control sera - positive and negative in duplicate, followed by the numbers of the tested sera, and in the second column their location in a specific well on the microtiter plate.

Optical density

It is worth emphasizing that the only variable determined empirically in the ELISA test is the optical density value in the third column of the data table. Optical density (OD), sometimes called extinction or absorbance, although not quite correctly, is a physical quantity described by the tenth logarithm of the quotient of the intensity of a monochromatic beam entering the absorbing medium and the intensity of the beam passing through this medium (according to the Lambert-Beer law). In simpler terms, optical density for transparent materials is measured by the ratio of the intensity of light that has passed through a particular layer of a substance to the light incident on it. Theoretically, it is assumed that the

concentration of a substance is one of the most important factors affecting the amount of light absorbed. The second is the length of the path that light travels through the sample, which is conditioned by the width of the measuring cuvette, and the last one is the extinction coefficient of this substance. According to the Lambert--Beer law, the intensity of the light transmitted (transmittance) through the test solution decreases exponentially if each of the three factors is increased. It is therefore assumed that the level of color intensity, depending on the concentration of the enzyme reaction product, is proportional to the concentration of antibodies in the sample. As mentioned earlier, during the next stage of the ELISA test, after adding the appropriate substrate, the enzyme contained in the conjugate catalyzes the reaction, the product of which can be quantified by the spectrophotometric method that measures the color intensity. Parallel to the tested samples, analogous reaction steps are performed for calibration sera with known concentration of the tested antibody (positive and negative controls), thanks to which it is possible to determine the concentration of antibodies in the tested samples.

S/P ratio

Since the rate of the enzyme reaction is dependent on the conditions under which the assay is performed, especially on the temperature in the laboratory, the relative amounts of antibodies in the test samples are calculated by reference to the positive control, corrected for the OD values of the negative control sera. In a laboratory where the test is performed at a higher temperature, the OD values are higher than those found in a laboratory performing the test at a lower temperature. A comparison of OD values may therefore be unreliable. Regardless of the test conditions, the result expressed as the value of the S/P ratio in indirect tests is comparable, because the proportions are maintained. For this purpose, the OD value of the test serum, i.e., the sample taken for testing, is divided (S, sample) by the OD value of the positive control serum of the kit (P, positive). The value of the coefficient is calculated from the formula:

$$S/P = \frac{\text{OD Tested Serum - OD Mean value Negative Controls}}{\text{OD Mean value Positive Controls - OD Mean value Negative Controls}}$$

S/N ratio

In the blocking ELISA format, the S/N ratio is calculated. Regardless of the test conditions, the result expressed as the value of the S/N ratio in the blocking format tests is comparable, because the proportions are maintained. The value of the S/N ratio is calculated from the formula:

$$S/N = \frac{\text{OD Tested Serum - OD Mean value Positive Controls}}{\text{OD Mean value Negative Controls - OD Mean value Positive Controls}}$$

Cut-off titer

The so-called the cut-off titer is the value of the titer of S/P or S/N ratio at which the result is considered positive. This value is set by the kit manufacturer. It is usually determined empirically by testing a large number of positive and negative samples. Values are selected that maximize the sensitivity and specificity of the test. Depending on the direction of the examination and the type of test, it ranges from 0.2 to 0.6. For example, samples with an S/P greater than 0.2 (titer greater than 396) when tested with the IDEXX IBD Ab assay are considered positive.

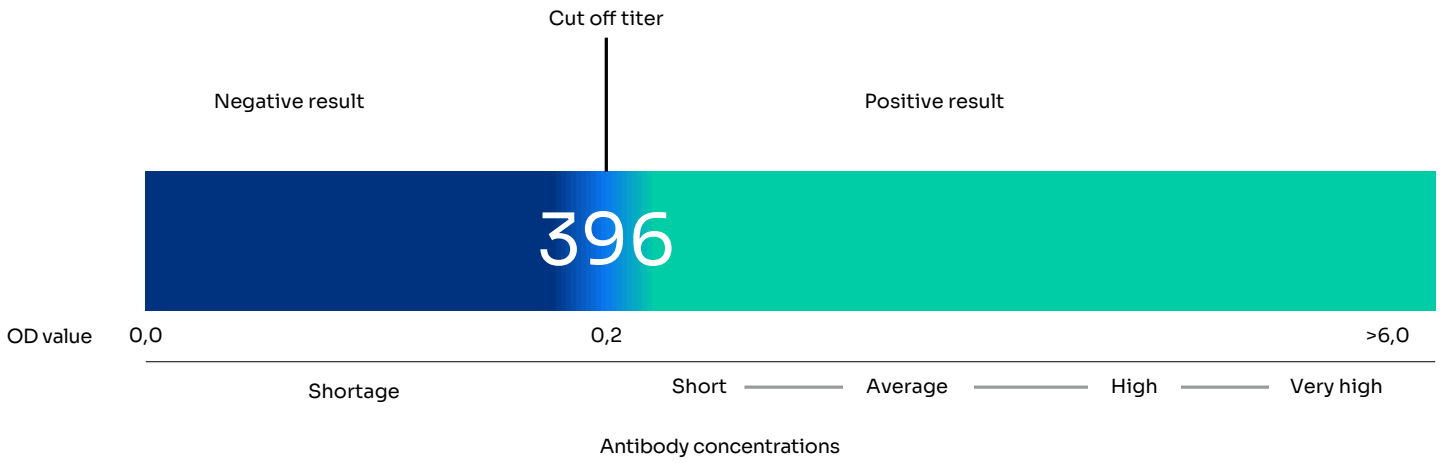


Fig. 13. Principle of borderline determination in indirect ELISA

Titer

The titer is one of the basic parameters assessed during the interpretation of the serological result. The titer is calculated according to the formula provided by the test manufacturer, and it varies depending on the formula used by the test manufacturer to calculate it. All formulas are based on the OD value and the S/P ratio calculated on this basis.
The sample algorithm has the following formula:

$$\text{Titer } (\log_{10}) = 1{,}09\ (\log_{10} S/P) + 3{,}36^{*}$$

**Refers to the S/P ratio at a 1:500 dilution to the end point, defined as the reciprocal of the highest dilution that gives a reading above the cut off value.*

In indirect tests by IDEXX, the cutoff titer is usually 396 (dilution - 1: 500; S/P > 0.2) [Tab. 6.].

As shown in Figure 14, depending on the ELISA manufacturer's titration formula, the results of the same sera from different manufacturers may vary significantly, which should be considered when interpreting test results. If comparisons are necessary, use S/P (or S/N) values that are independent of the titer calculation formula.

The next column in the table of results contains information to which group of titers the test result of a given serum belongs.

The last column gives the interpretation of the result.

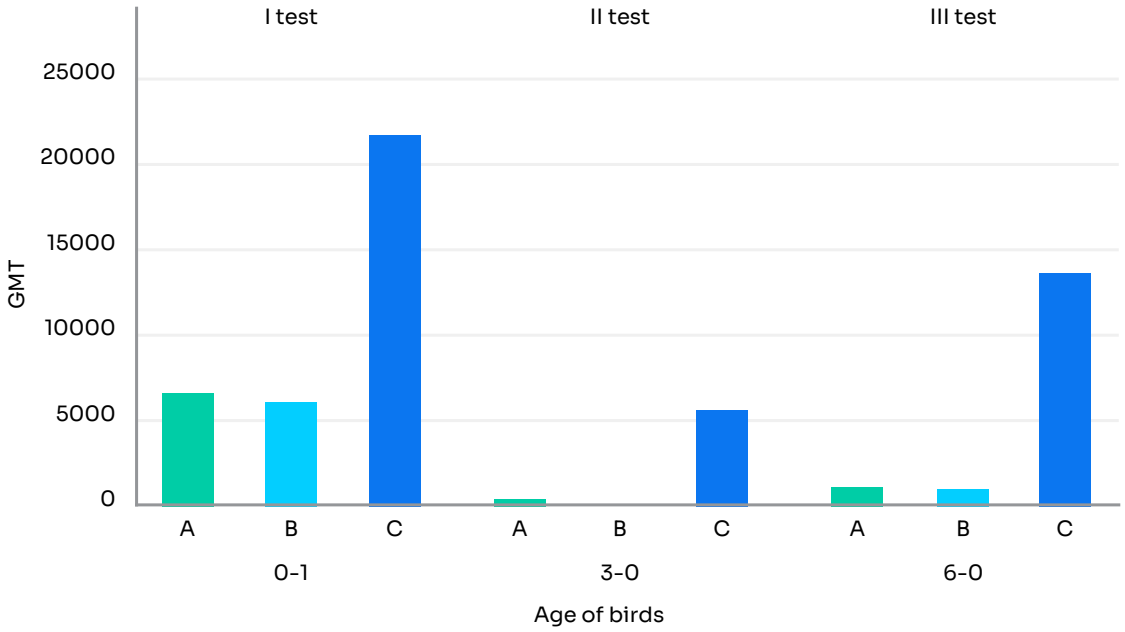


Fig. 14. The level of antibodies against IBDV in serological monitoring performed using tests from 3 manufacturers (A, B and C) in 1. day on 3. and 6. week in a herd vaccinated against Gumboro disease (Szeleszczuk et al., 2014)

Hemagglutination inhibition test (hemagglutination inhibition assay - HI test)

In poultry practice, the hemagglutination inhibition test is also widely used for the serodiagnosis of infectious diseases caused by hemagglutinating pathogens. It is a serological test used to detect antibodies formed after spontaneous infection, as well as post-vaccination antibodies. It uses the fact that hemagglutinin of surface viral receptors has a clumping effect on red blood cells (Hirst phenomenon). Regarding poultry diseases, this feature has e.g., Infectious Bronchitis virus, Newcastle disease, Egg Drop Syndrome 1976, and Avian Influenza virus. The HI test is also used in the serodiagnosis of mycoplasma infections. Specific antibodies, which are a humoral immune response to infection or vaccination, can inhibit agglutination by binding to hemagglutinins of pathogens, i.e., they have the ability to inhibit pathogen-induced erythrocyte agglutination. If the test serum contains specific antibodies against haemagglutinin, the addition of the test pathogen causes an antigen/antibody reaction. This is visible after the addition of erythrocytes. They are no longer agglutinated as the reaction is inhibited (Fig. 15.).

The technique of performing the HI test consists in introducing the test serum into the wells of the microplate and making its successive dilutions, and then adding the pathogen of a certain concentration (e.g., 4 hemagglutinating units) and a suspension of red blood cells (Fig. 16.). If the tested serum contains specific antibodies directed against this pathogen, its hemagglutinating properties are blocked, and as a consequence the blood cells settle in a compact group at the bottom of the well, forming a nub. If there are no antibodies specific for a specific subtype in the tested serum, then the pathogen, due to its ability to agglutinate blood cells, causes them to settle at the bottom of the well in the form of a characteristic mesh. The result is read visually. The antibody titer is the reciprocal of the last serum dilution that completely inhibits hemagglutination. The strengths of the test are: the possibility of selecting a specific antigen for testing depending on the changing epizootic situation, the possibility of simultaneous detection of antibodies to different subtypes of

pathogens and the low price of the tests. The weaknesses of the test are: subjectivity of the result due to visual reading, the likelihood of cross-reactions and the possibility of false-negative results, and the lack of established standards for evaluating results for specific disease entities.

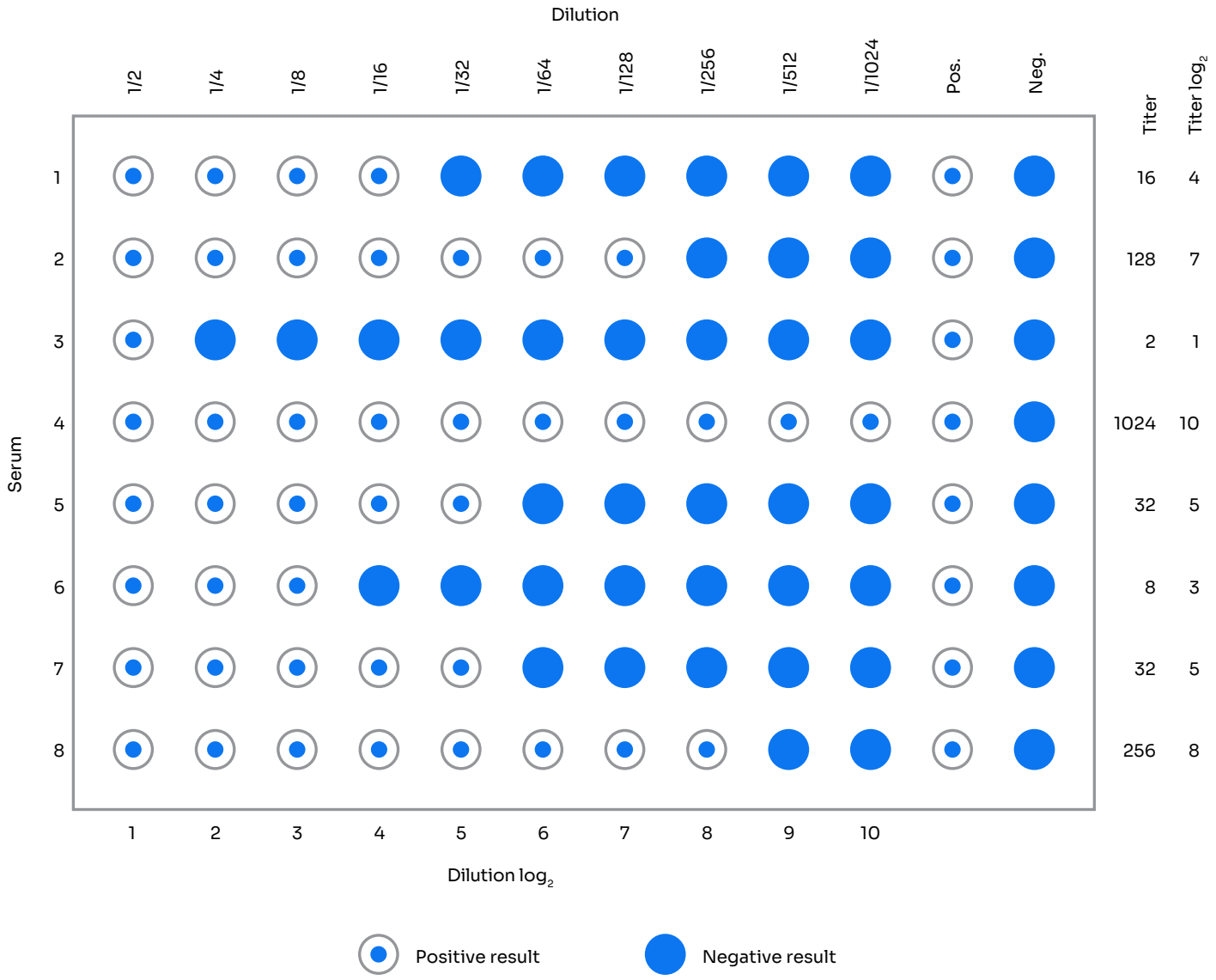
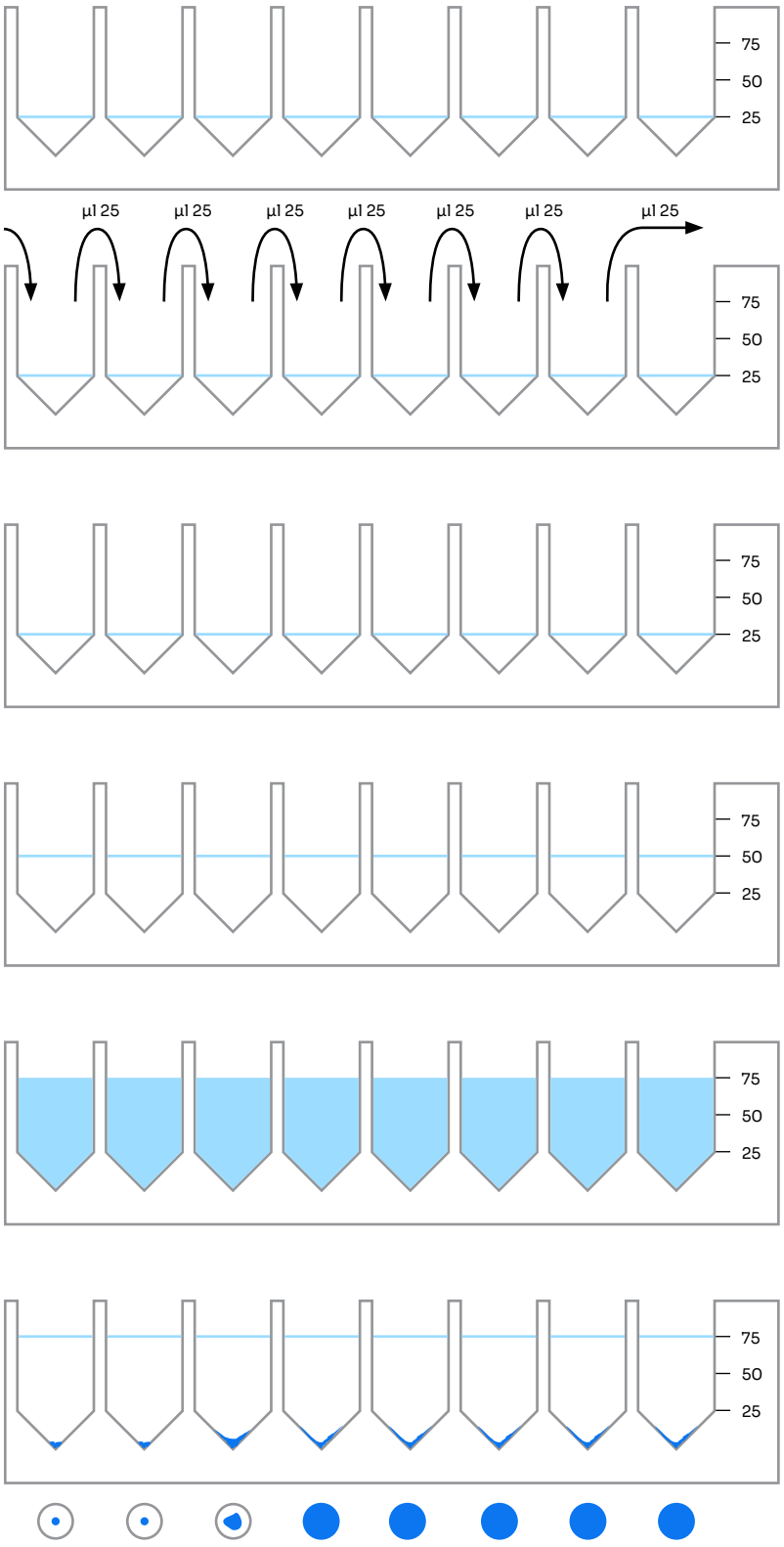


Fig. 15. Rules for reading the result of the hemagglutination inhibition test



1. Add 0.025 ml of PBS to all wells of each row; add 0.025 ml of test serum (or standard) to the first well

2. Make two-fold dilutions of the serum

3. To doubling dilutions of serum (0.025 ml/well) add 4 U. HA virus antigen in a volume of 0.025 ml

4. Mix and incubate for exactly 20 minutes at room temperature

5. Add 0.025 ml of 1% blood cell solution to all wells

6. Mix, leave until the blood cells sink to the bottom of the well for approx. 40 minutes at temp. room (approx. 20°C) or at temp. 4°C (when the ambient temperature is too high) and read the result 1:4 titre 4/2 $\log_2$

Fig. 16. Hemagglutination inhibition test (HI test)

How to read hemagglutination inhibition test results

Laboratories performing the HI test usually present the test result in a tabular form, attaching a graphical presentation of the results in the form of graphs (Fig. 18.). Detailed analysis of the result depends on the disease entity and type of production as well as the age of the birds. Guidance that may be useful in analyzing hemagglutination inhibition test results is provided in Table 8.

Serum number	HI titer	Log_2 Titers	Result
1	8	3	Neg.
2	8	3	Neg.
3	16	4	Pos.
4	8	3	Neg.
5	16	4	Pos.
6	16	4	Pos.
7	8	3	Neg.
8	16	4	Pos.
9	8	3	Neg.
10	8	3	Neg.
11	8	3	Neg.
12	8	3	Neg.
13	32	5	Pos.
14	8	3	Neg.
15	8	3	Neg.
16	8	3	Neg.
17	8	3	Neg.
18	8	3	Neg.
19	8	3	Neg.
20	8	3	Neg.
21	8	3	Neg.
22	8	3	Neg.
23	8	3	Neg.

Statistical summary:

Mean: $log_2 3.3$

Pos/Neg: 5/18

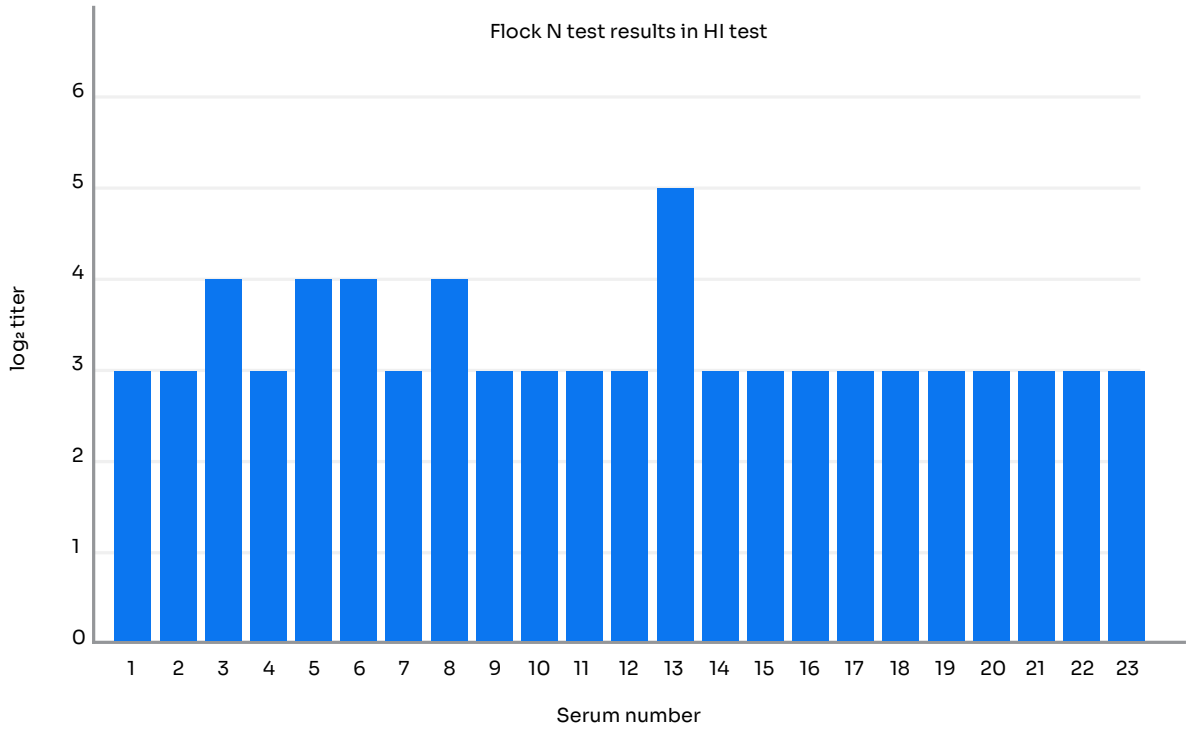
Max.: $log_2 5$

Min.: $log_2 3$

Interpretation of the test result:

Positive sample: $log_2 > 3$

Fig. 17. An example of HI test results.



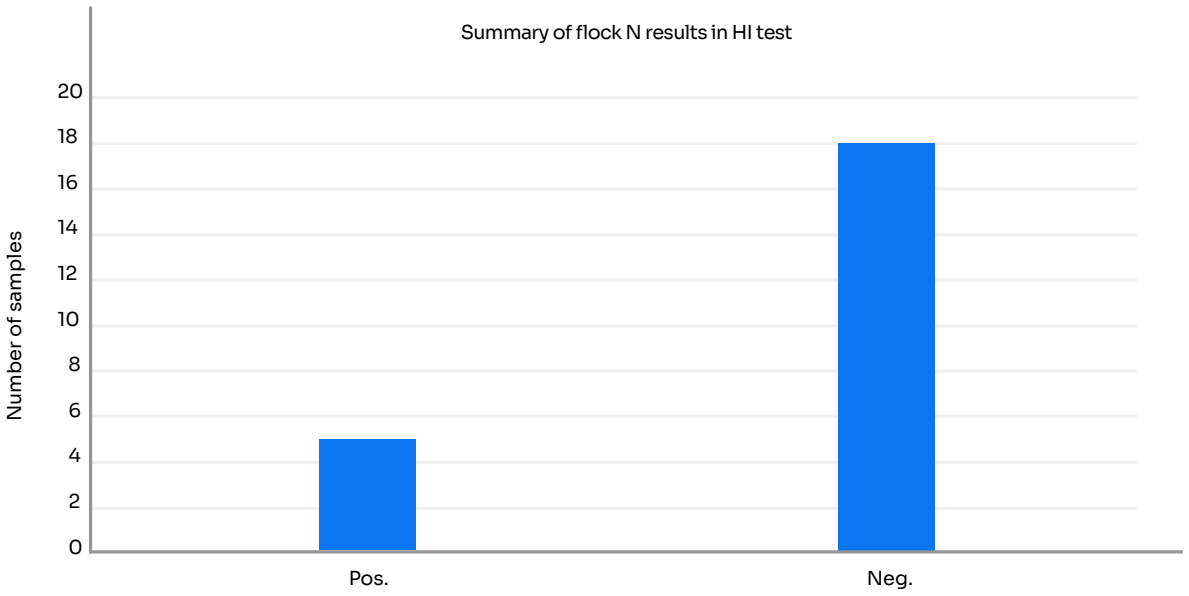


Fig. 18. An example of HI test results.

Titer	Log_2 titers	Interpretation	
2	1	No immunity. Lack of protection - in case of infection possible clinical signs	Negative test result
4	2		
8	3	Poor immunity. Partial protection - mild symptoms	Fisrt vaccination with live vaccines
16	4		
32	5		
64	6	Protection against mortality. No clinical symptoms	Revaccination with live vaccines
128	7		
256	8		
512	9		
1024	10	Field virus infection - disease	Live and inactivated vaccines – intensive program
2048	11		
4096	12		
8192	13		

*Depending on the pathogen, titers of 4 ($log_2 2$) to 8 ($log_2 3$) are considered negative.

** Titers >1024 usually confirm contact with the field germ, although it is more reliable to test pairs of sera and determine the seroconversion rate (WS >10).

Tab. 8. General indications for interpreting the results obtained in the HI test.

Literature

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