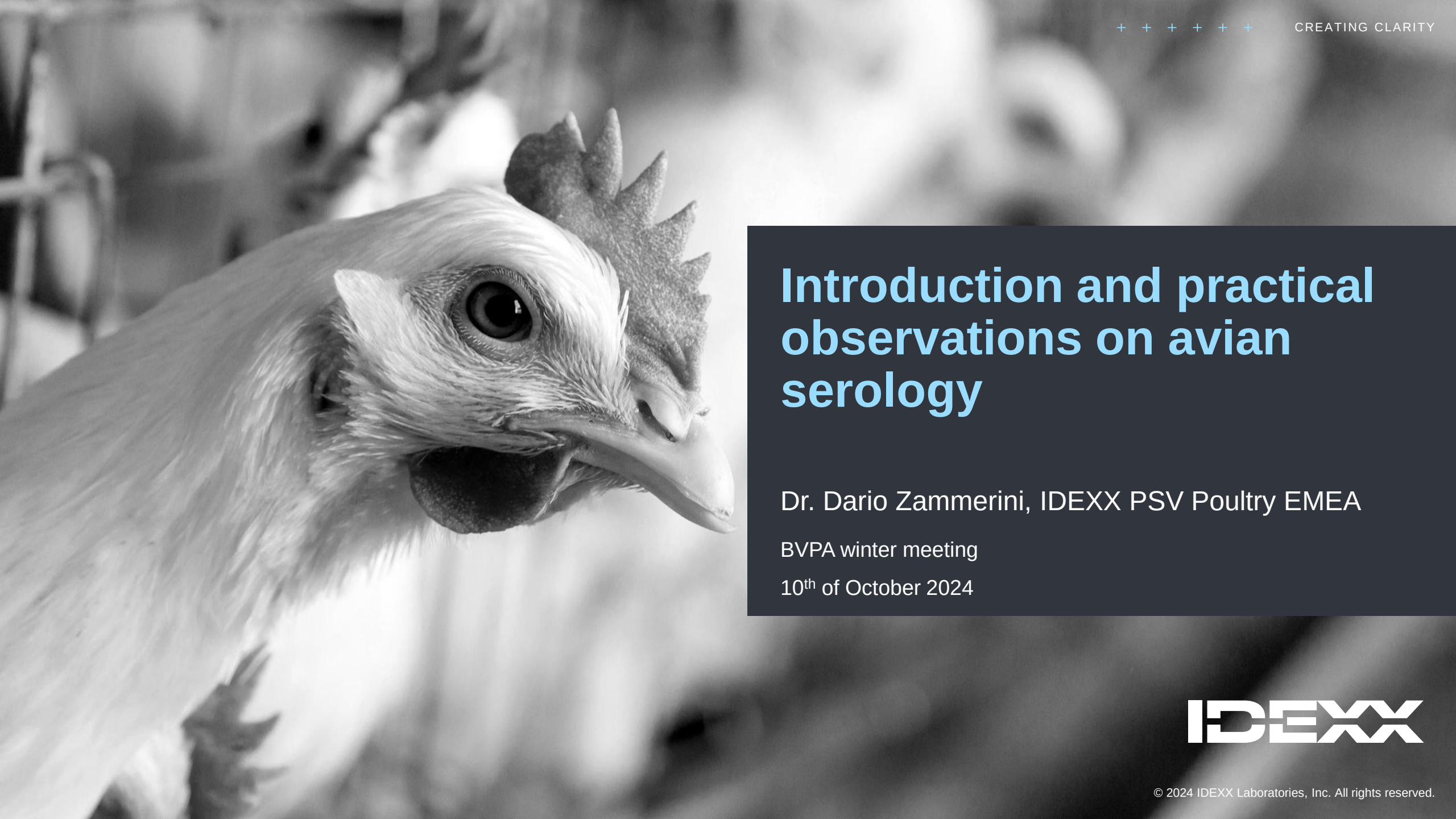


Introduction and practical observations on avian serology

Dr. Dario Zammerini, IDEXX PSV Poultry EMEA

BVPA winter meeting

10th of October 2024

Agenda

Review the importance of serology

1. Why do we test? Why serology in poultry?
2. Know good sampling
3. Understand ELISA results and the importance of baselines
4. Conclusions

Why do we test? Why serology in poultry?

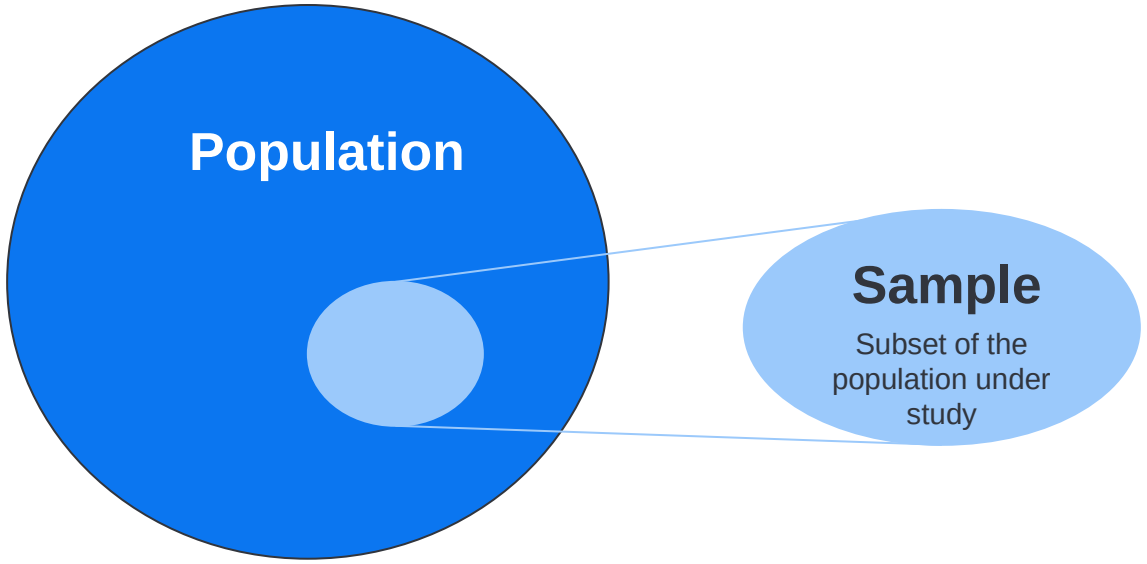
Diseases surveillance and prevention

- Surveillance involves continuous observation to determine the absence or presence of a disease in a flock.
- We can control a disease by active and passive surveillance methods.
- **Active surveillance:** performing routine and systematic **monitoring** to verify flock status.
- **Passive surveillance:** on-site flock evaluations, signs of disease identification, post-mortem examinations and samples for lab diagnosis → increased mortality, clinical signs and/or egg production drops.
- Laboratory testing is part of diseases surveillance and prevention.

- Prevention programs should include:
  - Biosecurity.
  - Vaccination.
  - Evaluation of production results.
  - Regular flock examinations.
  - Examination of culled and dead birds.
  - Periodic serological flock profiling (monitoring program).

Monitoring program

- **Active surveillance → monitoring program:** collect data that describes the pathogen in a population.
- The monitoring program is designed to accumulate **statistically reliable prevalence data over time**, to indicate a change in the incidence of the pathogen!
- A monitoring program is also used to assess vaccines scheme compliance and to set a flock profile → **baselines.**
- In both cases, the **sampling methodology** and the **number of samples** must be representative of the population.



Why testing in poultry?

Notifiable diseases and eradication programs

- Notifiable diseases testing usually regulated by national control programs (NCP).
- OIE notifiable diseases:
  - AI: NCP regulates the testing, but also auto-control testing.
  - NDV: no NCPs. Birds usually vaccinated and tested for vaccination control and disease monitoring.
- Eradication programs:
  - MG and MS: usually, auto-control programs to obtain Mycoplasma-free parents' stock.
- Food Safety:
  - Salmonellosis: NCPs following EU/national legislation.
  - EU legislation includes 2 types of sampling: official controls and those by initiative of each food business operator.

Discretionary testing – vaccination and disease control

- Commonly monitoring programs for vaccination follow up and to check the health status of the flocks.
- The diseases monitored and the number of tests are related to:
  - Value of the birds.
  - Vaccination program.
  - Disease's prevalence and economic impact.
  - Cost.

Importance of discretionary testing

Objectives of a regular/good monitoring program

- **Reduce costs** by increasing the efficiency of feed utilization and decreasing the incidence of disease.
- Detection of subclinical problems.
- **Early detection** of disease outbreaks (increasing field challenge).
- Identification of new emerging problems.
- Detection of unnoticed production losses.
- **Serology tests are the most used in poultry**.
  - Determine **freedom from specific pathogens**.
  - **Monitor vaccine and vaccination process**.

Why serological monitoring of vaccinations?

- **Justify the investment** in the right vaccine.
- Vaccine failures often occur due to poor handling or application.
- **Ensure proper vaccine take** to maximize the vaccine benefits.
- **Monitor disease pressure** to adjust vaccinations.
- Allow for corrective action if take is not satisfactory.
- Avoid costly disease and suboptimal performance.
- Determine right vaccination date (IBD).
- Sometimes to check if another vaccination is necessary (e.g., CAV).

Know good sampling

Correct sampling for monitoring

- The key points to achieve a good monitoring are
  - Use of a **statistically valid sampling method** so that the samples are representative of the flock.
  - Collect, transport, handle and process the samples correctly so that they are of the needed uniform quality.
  - **Select the correct testing technique** so that the results are reliable, reproducible and repeatable.
- The statistical validity of the sampling method depends on two main conditions
  - Proper **samples size** to accurately represent the flock.
  - Selecting **random birds** for sampling.
- It is very important to **provide flock information to the laboratory**: age, current vaccination program and clinical history.

Correct sampling for monitoring – Sample size

- The **number of samples** to be collected **is as important as random sampling** in determining the true mean flock titer or freedom from a disease.
- Sample size is a function of the percentage positive (or negative) birds in a population and the required certainty/confidence level. Not a function of the test specs!
- At random sampling: $n = \{1 – (1 – \alpha)^{1/D}\}\{N – 0,5\ (D – 1)\}$
  - ✓ n = number of samples
  - ✓ α= required certainty of detection the infection
  - ✓ D = number of infected animals
  - ✓ N = herd size
- In practice the number of samples to collected from a flock is the number that is accepted as statistically correct.
- For this reason, 24 to 30 samples are ideal and will accurately reflect the infection rate with a 90-95% confidence level.
- If we are screening for a slow spreading disease, like mycoplasma, we may need to test more samples (≥ 60) because there will be fewer positives in the flock.

Statistically correct: 24-30 samples!

Correct sampling for monitoring

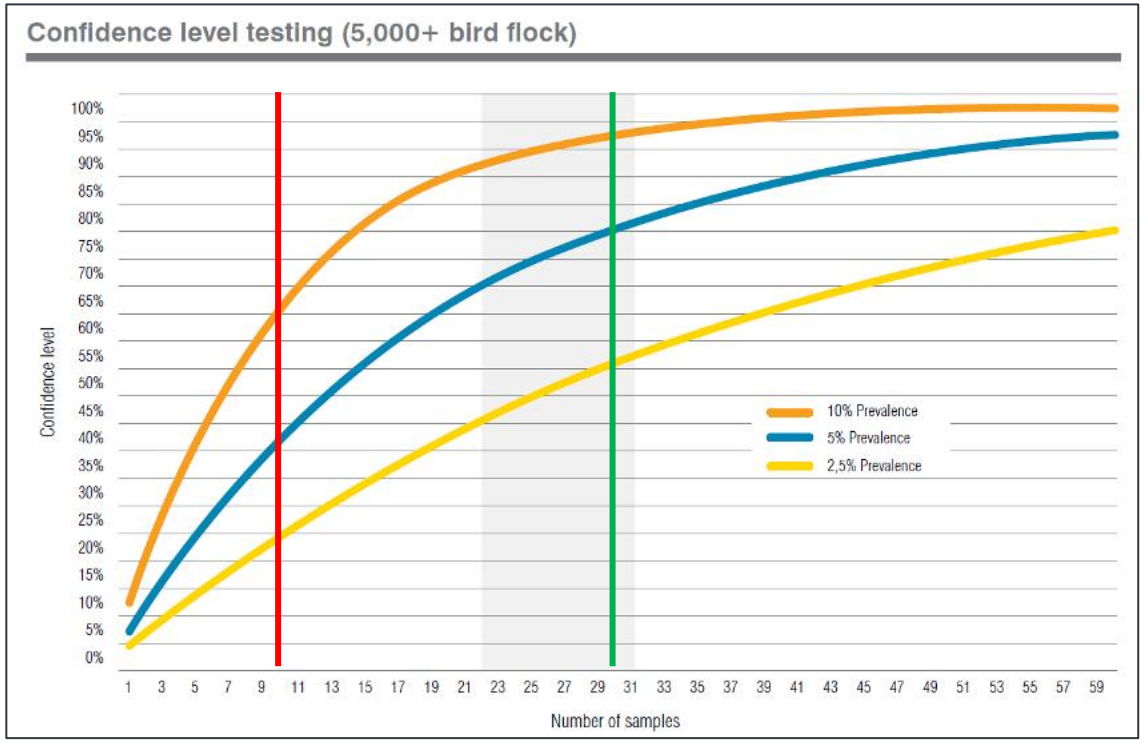
- Tables and graph show that more samples are needed if the disease is less prevalent, but flock size has little impact.
- Example:
  - ✓ if 10% of the birds in a house are positive for a disease then testing 30 random samples will give you an accurate estimate of the infection rate 95% of the time
  - ✓ if you test only 10 birds the confidence level drops to 65%, so that there is 1 in 3 chance of missing the disease.

Disease prevalence(%); at a 90% confidence level

Sample size based on birds/house	20%	15%	10%	5%	2%	1%	0.5%	0.1%
100	10	14	20	37	69	90	100	101
5,000	11	15	22	45	113	224	439	1,845
50,000	11	15	22	45	114	229	458	2,250

Disease prevalence(%); at a 95% confidence level

Sample size based on birds/house	20%	15%	10%	5%	2%	1%	0.5%	0.1%
100	13	17	25	45	78	95	100	101
5,000	14	19	29	59	147	290	564	2,253
50,000	14	19	29	59	149	298	596	2,950



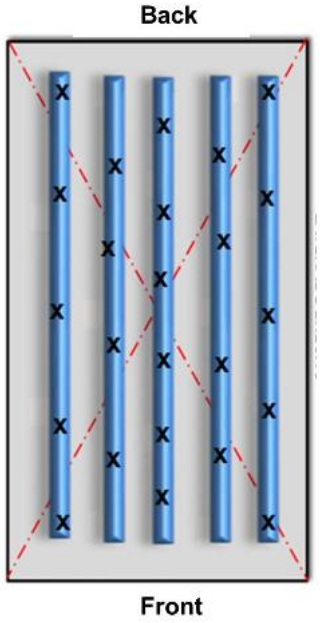
Sample size for vaccination control

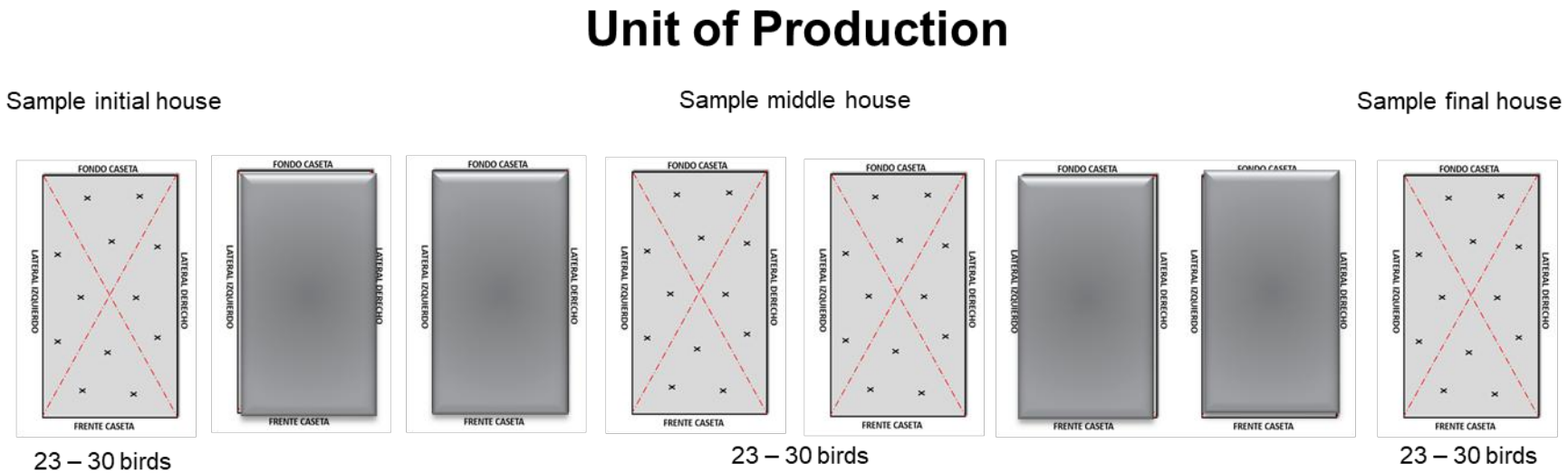
- 24-30 sample range is a statistical calculation that factors in disease prevalence and desired confidence regardless of flock size.
- With fewer than 24 samples there is a greater chance of missing the disease or misinterpreting vaccine status.
- Accuracy is very low with just a few samples but improves rapidly reaching 95% at around 30 samples.

Number of samples	Real percentage of 'well vaccinated' birds (95% certainty interval)						
	Positive test result (positive or on a good level)						
	All pos.	-1	-2	-3	-4	-5	- 6
30	100 88-100	97 83-100	93 78-99	90 73-98	87 69-96	83 65-94	80 61-92
24	100 86-100	96 79-100	92 73-99	88 68-97	83 63-95	79 58-93	75 53-90
18	100 81-100	94 73-100	89 63-99	83 59-96	78 52-94	72 47-90	67 41-87
10	100 69-100	90 55-100	80 44-97	70 35-93	60 26-88	50 19-81	40 12-74
6	100 54-100	83 36-99	67 22-96	50 12-88	33 4-78	17 1-64	0 0-46
5	100 48-100	80 28-99	60 15-95	40 5-85	20 1-72	0 0-52	

Correct sampling for monitoring - random selection

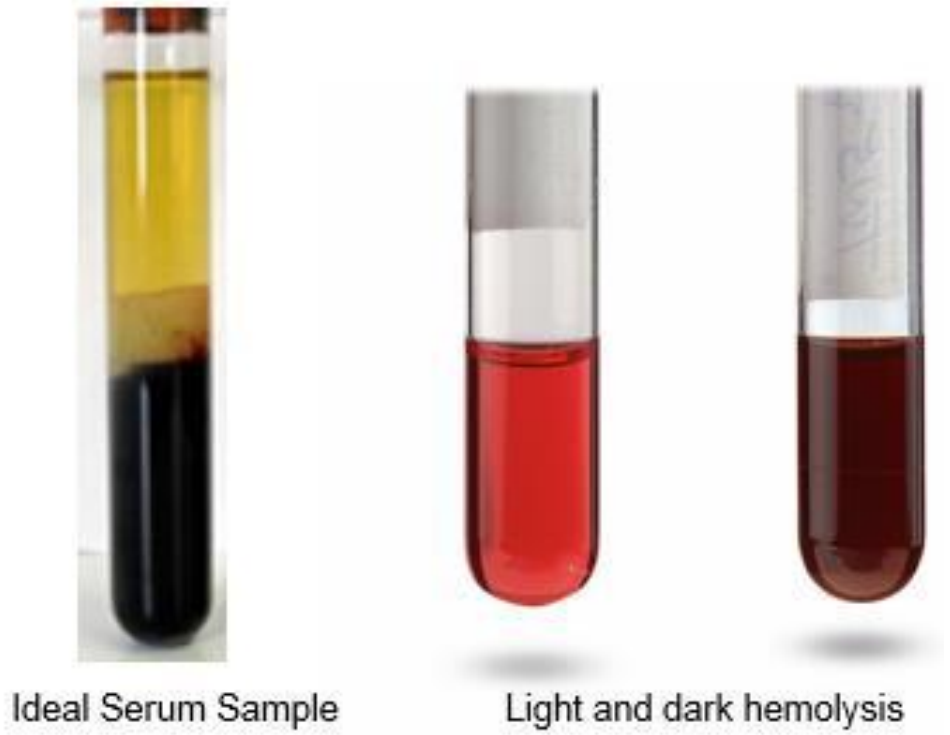
- The sampling needs to be representative of the flock!
- Random selection inside the house is the best way to obtain it.
- Collecting samples from multiple locations within the poultry house, averages out the effects of variation within the flock.
- **Sampling one house does not represent the entire farm!**





Basic sampling – good blood samples

- It is important to keep birds as calm as possible during bird collection and blood sampling.
- No more than 1% of the body weight of the bird should be collected in blood at one time.
- The tube should not have anticoagulant in it for serology.
- Wait for a clot to form at room temperature before refrigerating.
- Goal: one single large clot in the tube, which can easily be separated from the serum.
- Good storing before sending and transportation are critical!!!

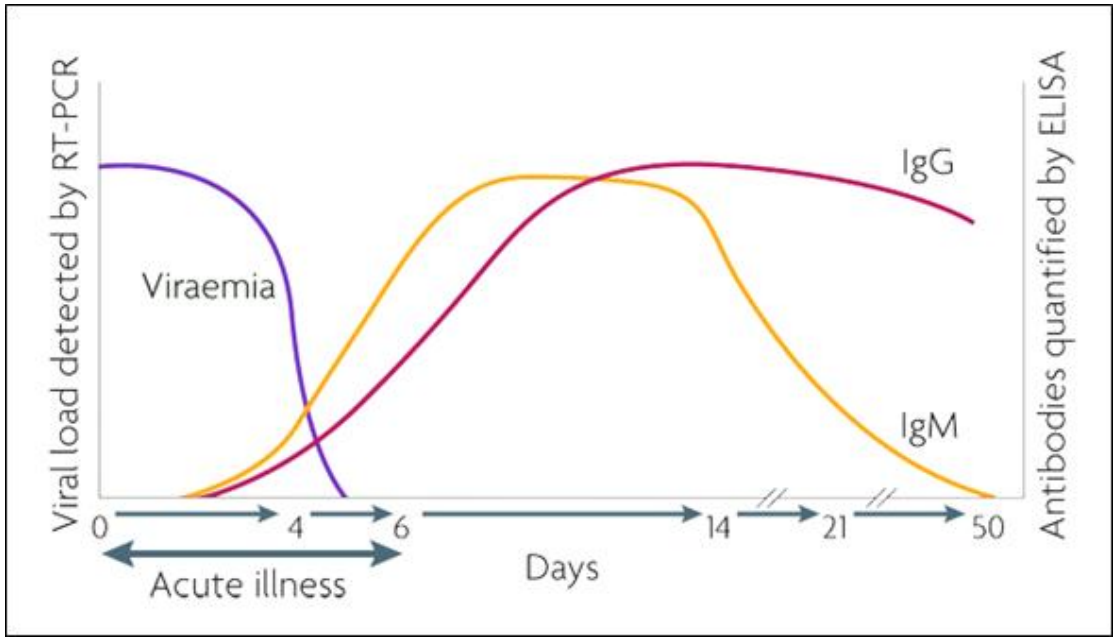


Ideal Serum Sample

Light and dark hemolysis

Select the correct test - right timing is crucial!

- PCR usually positive from 48-72h after infection.
- ELISA only after 14-21 days. For some virus/bacteria seroconversion may happen after 8wks.



Time	PCR results	ELISA results	Interpretation
Day 1	+	-	Clinically sick birds.
Week 1	+	-	Acute infection. Not detectable IgG.
Week 2	+	+	Ongoing infection. Decrease in viral load.
Week 5	-	+	End acute phase (chronic).

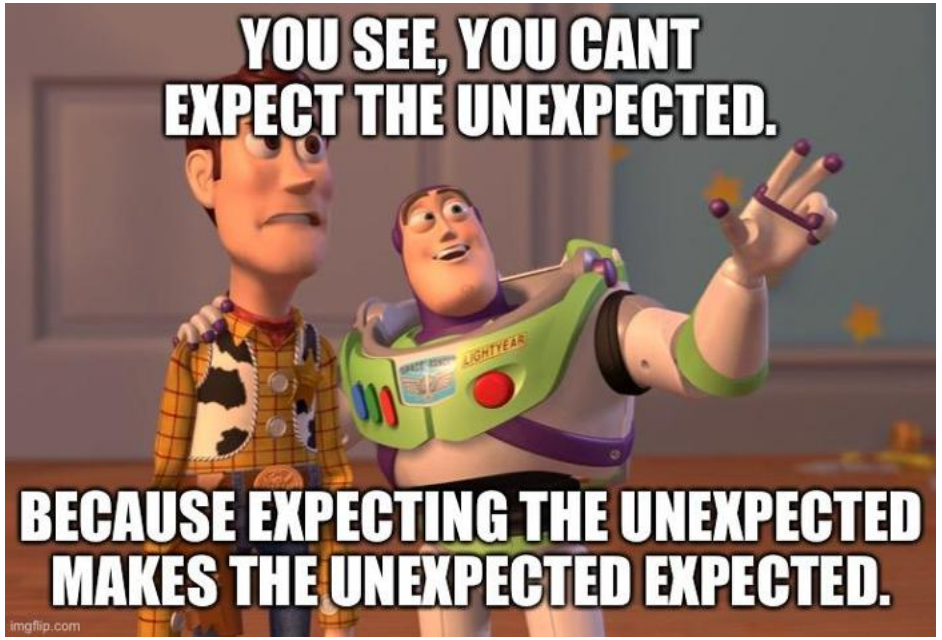
Understand ELISA results and the importance of baselines

Understanding ELISA results

- When comparing results keep in mind that **different ELISA will have different cut off, sensitivity and specificity**.
- Higher titers with ELISA does not always means higher protection (e.g., IgA not included).
- Live respiratory vaccines (IBV, ND) may give also a local immunity response, that can not be measured in ELISA.
- Furthermore, for many bacteria, immunity is primarily cell-mediated and it is not measured by ELISA.
- ELISA are not always strain specific: high titers may not guarantee protection against a different strain.
- Leave always 3-4 weeks between same live vaccination (particularly if same strain) otherwise we may waste vaccine and over stimulating the birds, and the titers may also be lower!
- **Baseline are very important** for your ELISA interpretation!
- Regional Specific Baseline: the same titer can mean protection for one farm but not enough to protect another farm.

Why there is a variation in my ELISA titers?

- Caused by normal biological variation
  - Contact with field pathogen (not always results in infection).
  - Changes in vaccination (type, time, administration, etc.).
  - Immunosuppression.
- Caused by human or instrument errors
  - Improper sample collection, storage or handling.
  - Improper ELISA technique.
  - Improper (malfunction) instruments.
- Assay validity - check positive and negative controls
  - Duplicate controls.
  - Stick to insert information.



ELISA results calculation and titers

Indirect ELISA: ratio S/P (Sample to Positive)

$$S/P = \frac{\text{Sample Mean} - NC\bar{x}}{PC\bar{x} - NC\bar{x}}$$

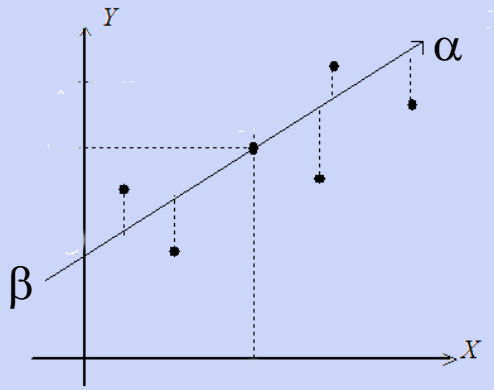
Titers

Y= a (X) + b

a = gradient

b = intercept

$$\text{Log}_{10}\,\text{Titer} = 1.09\,(\log_{10} S/P) + 3.36^{*}$$



Blocking ELISA: ratio S/N (Sample to Negative)

$$S/N = \frac{\text{Sample A(650)}}{NC\bar{x}}$$

+ What is an ELISA titer?

The result of a mathematical formula which translates a relative color intensity (S/P) of a single dilution of the serum into a number = **the titer**

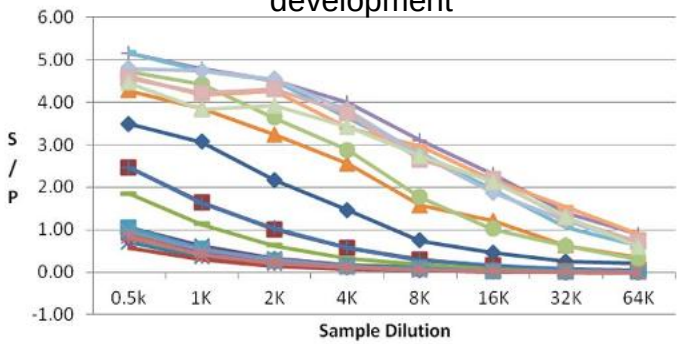
Indirect ELISA result transformation

Sample Optical Density → Compare to Positive Control → **S/P Ratio** → Use equation of the line to generate titer value → **Titer** → In xChekPlus* assign titers to groups → **Titer Groups**

$$S/P = \frac{\text{Sample Mean} - NC\bar{x}}{PC\bar{x} - NC\bar{x}}$$

$$\text{Log}_{10}\,\text{Titer} = 1.09\,(\log_{10} S/P) + 3.36^{*}$$

Equation derived early in kit development



Group 0	396
Group 1	397
Group 2	1000
Group 3	2000
Group 4	3000
Group 5	4000
Group 6	5000
Group 7	6000
Group 8	8000
Group 9	10000
Group 10	12000
Group 11	14000
Group 12	16000
Group 13	18000
Group 14	20000
Group 15	22000
Group 16	24000
Group 17	28000
Group 18	32000

Why different ELISA titers?

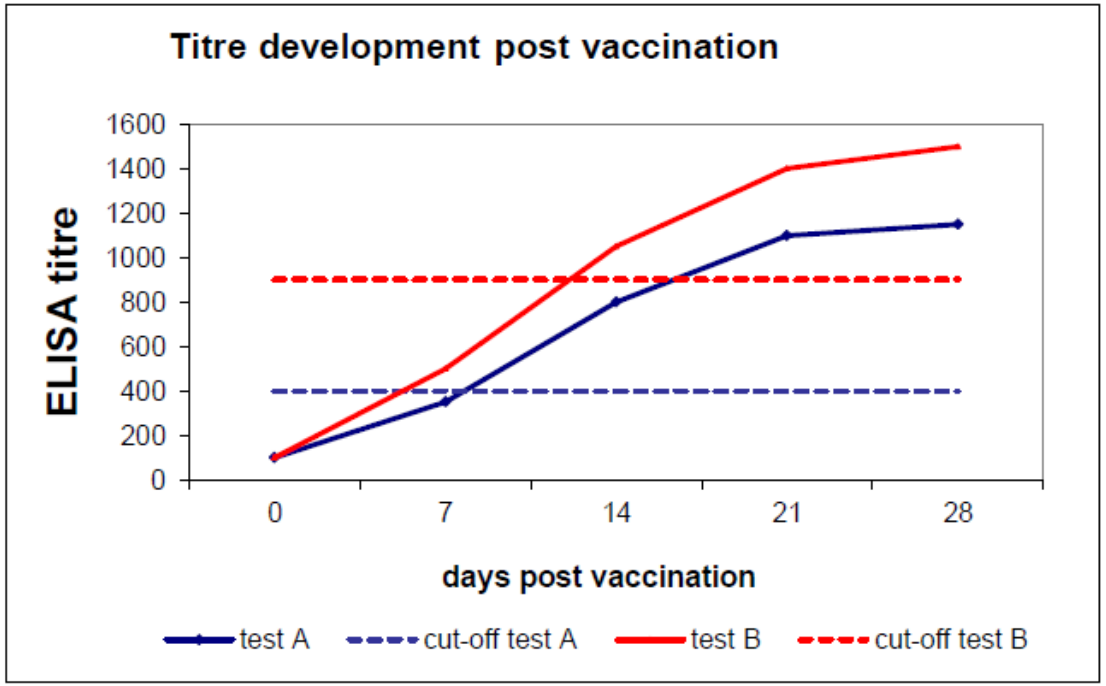
An ELISA kit with higher titers means a more sensitive test?

+ **Higher titers** sounds good: suggests many antibodies, high quality vaccination…it **only depends on the kit and the formula**!

+ Direct titer comparison is not possible!

Example

+ **Test A**: S/P > 0.2 positive sample.
+ Formula: $\text{Log}_{10}\text{Titer} = 1.09\ (\log_{10}\text{S/P}) + 3.36^{*}$
+ Positive titer > 396
+ **Test B:** S/P > 0.2 positive sample.
+ Formula: $\text{Log10 Titre} = 1.0 * (\text{log10 S/P}) + 3.62$
+ Positive titer > 834



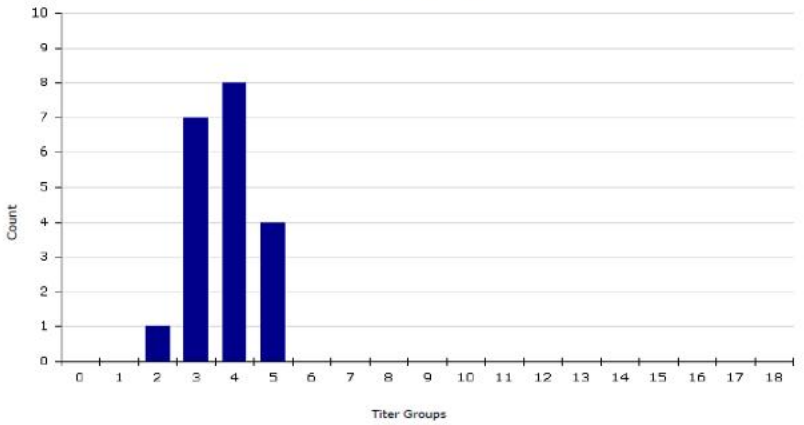
Understanding ELISA results and report

IDEXX Laboratories, Inc.
One IDEXX Drive
Westbrook, ME 04092
USA
18/06/2020

Test With Confidence™ IDEXX

Analyze Case Report

BA1021111001MM-IBD



Graph

Count	20
GMean	2998
Mean	3107
SD	793
% CV	25,5
Min	1539
Max	4448
Tech	JM
Date	22/08/2003

Statistics

Case BA1021111001MM - 8/22/03-003

IBD - 22/08/2003 - JM

	Well	O.D.	S/P	Titer	Group	Result
Neg	A1	0,042				
Neg	A2	0,040				
Pos	A3	0,358				
Pos	A4	0,329				
1	A5	0,562	1,722	4143	5	Pos
2	A6	0,330	0,955	2180	3	Pos
3	A7	0,417	1,243	2904	3	Pos
4	A8	0,313	0,899	2040	3	Pos
5	A9	0,377	1,111	2569	3	Pos
6	A10	0,251	0,694	1539	2	Pos
7	A11	0,454	1,365	3217	4	Pos
8	A12	0,597	1,838	4448	5	Pos
9	B1	0,435	1,302	3056	4	Pos
10	B2	0,349	1,018	2336	3	Pos
11	B3	0,548	1,676	4022	5	Pos
12	B4	0,554	1,696	4074	5	Pos
13	B5	0,446	1,339	3149	4	Pos
14	B6	0,467	1,408	3327	4	Pos
15	B7	0,539	1,646	3944	4	Pos
16	B8	0,537	1,640	3927	4	Pos
17	B9	0,430	1,286	3013	4	Pos
18	B10	0,321	0,926	2106	3	Pos
19	B11	0,464	1,398	3302	4	Pos
20	B12	0,411	1,223	2853	3	Pos

Controls

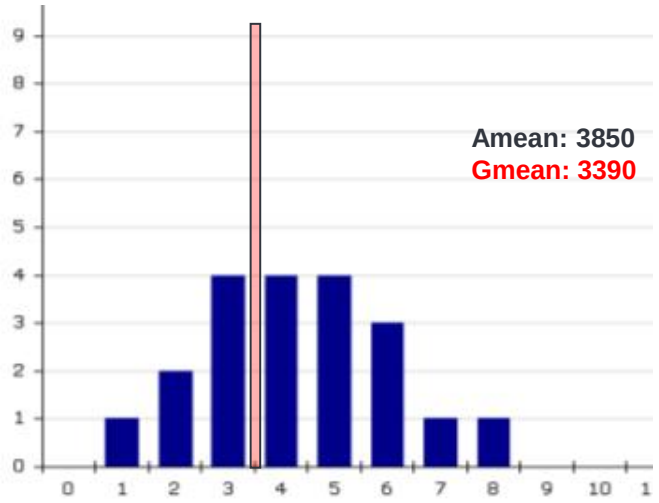
	S/P	Titer	Log2
AMean	1,319	3107	12
GMean	1,280	2998	12
SD	0,311	793	0
% CV	23,6	25,5	3,5
Min	0,694	1539	11
Max	1,838	4448	12

IDEXX

Understanding ELISA results and report

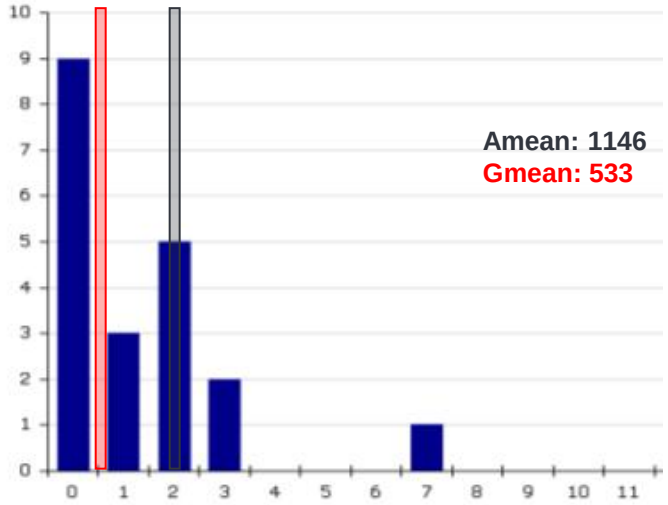
Arithmetic mean

- y = n1 + n2 + n3 + … nx / x
- It is very sensitive to extreme values.
- Mean titer of tested birds = approximate titer of average bird within the flock.
- The larger the sample size, the more reliable the mean.



Geometric mean

- the n^{th} root of the product of n numbers: $\bar{x} = \sqrt[n]{x_1 * x_2 .. x_n}$
- It considers all the values of the distribution.
- It is less sensitive than extreme arithmetic mean.



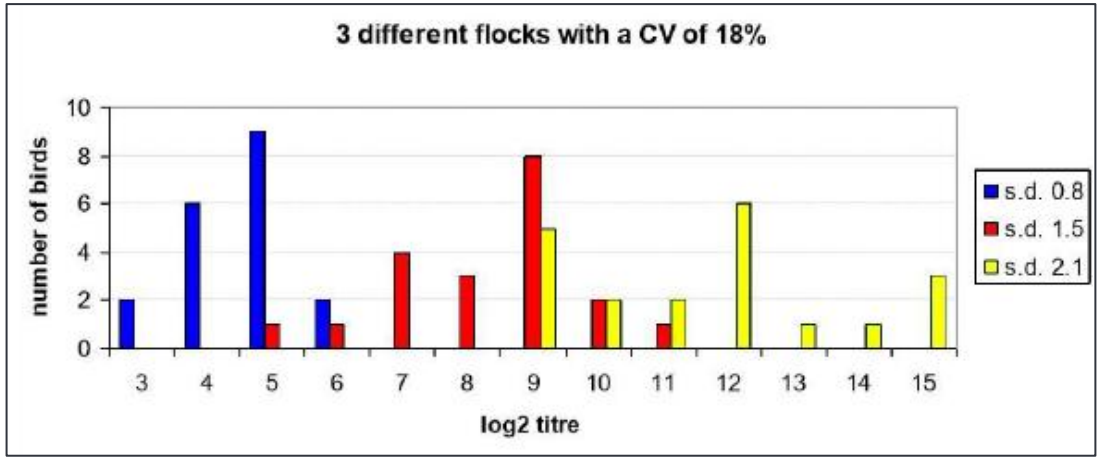
Understanding ELISA results and report

Standard deviation (SD)

- SD = $[\ \Sigma\ (x - y)^2]^{½}$
- A statistical measurement of the amount of variation around the mean titer value.
- A large SD indicates a large variation around the mean.
- The larger the sample size, the smaller the SD.

Coefficient of Variation (CV)

- CV = (SD/Mean titer) x 100
- Calculates the variation of the average individual, expressed in % deviation of the mean titer values.
- A flock with a **low CV (<40%)** has birds that show similar, uniform responses.
- A flock with a **high CV (>40%)** indicates more variability than normal.

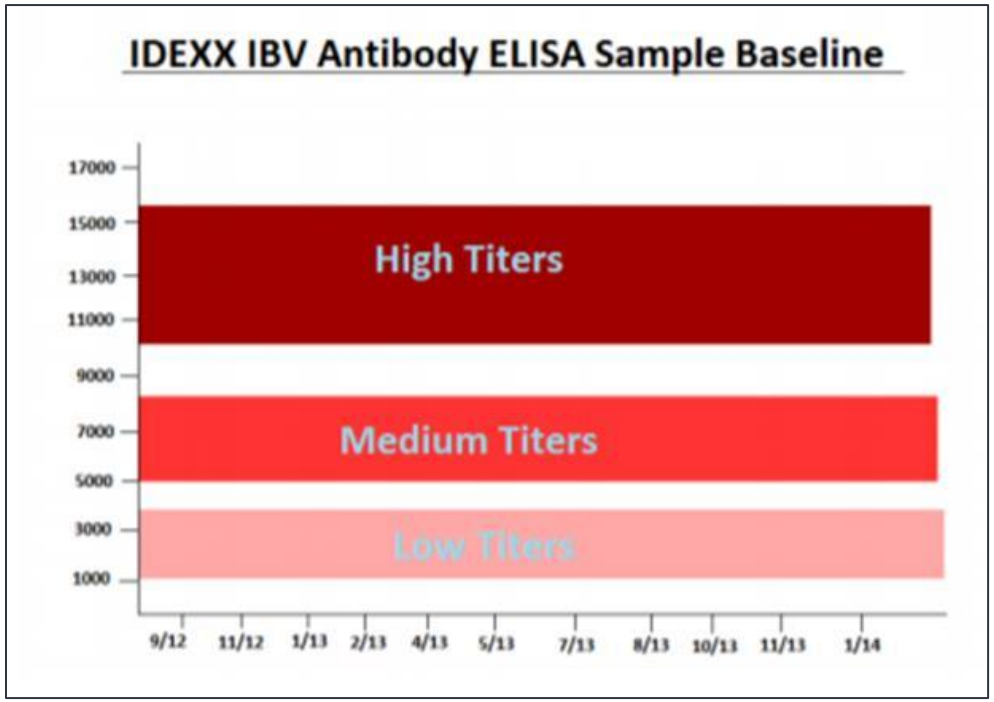


Guidelines for results interpretation are a starting point

Standard IDEXX Baseline for IBV for Broilers					
	Vaccine	Slaughter Age		Titers suspicious of field challenge **	CV %
		Titers at 30-35 days*	Titers at 35-42 days*		
IBV - Infectious Bronchitis	MDA (Maternal Antibodies)	1000-6500 (0 to 4 days old)			
	1x Mass or Dutch strain at hatchery	500-1200	600-1800	>3500	60%
	1x IB Primer	500-2100	500-2200	>4000	65%
	1x Dutch strain at the hatchery and 4/91 at 2 wks	700-3000	800-3500	>5500	55%
	1x Mass strain at hatchery and 4/91 at 2 weeks old	800-3000	800-3600	>5500	60%
	2x Dutch strain	700-2000	900-2500	>4500	60%
	2x Mass strain	700-2800	900-3550	>5500	60%
	2x H120 or Ma5+ 4/91	1000-4000	1200-4500	>7000	60%
	2x H120 or Ma5 + CR88	1000-4000	1200-4500	>7000	60%
	3x Live vaccines	900-3500	900-4000	>6500	55%
	No vaccination, no infection	<396		>396	
* Average min and max Titer. Field data collected from over 400 flocks					
Titers below the min Titer showed in the table may suggest not correct vaccination/review vaccine application					
Titers above the max Titer showed in the table may suggest presence of field challenge					
** Correctly vaccinated birds may show higher titer in presence of field challenge without showing clinical signs. It happens because the vaccine worked well and it is protecting the birds from clinical signs					

For a better results interpretation you need baselines

- A **baseline is a range of titers** which are set based on previous experiences to define an average of expected titers in flocks with known production parameters.
- They typically include low, medium, and high values.
- Up or down shifts are more noticeable with the use of a baseline.
- IDEXX color coded graph: yellow below the expected titers (in relation to the average baseline value), in green are as expected and red above.
- **It is important** that the baseline is specific to:
  - Type of birds
  - Age.
  - Vaccination program, schedule and type.
  - Geography
  - Temporality (seasons).

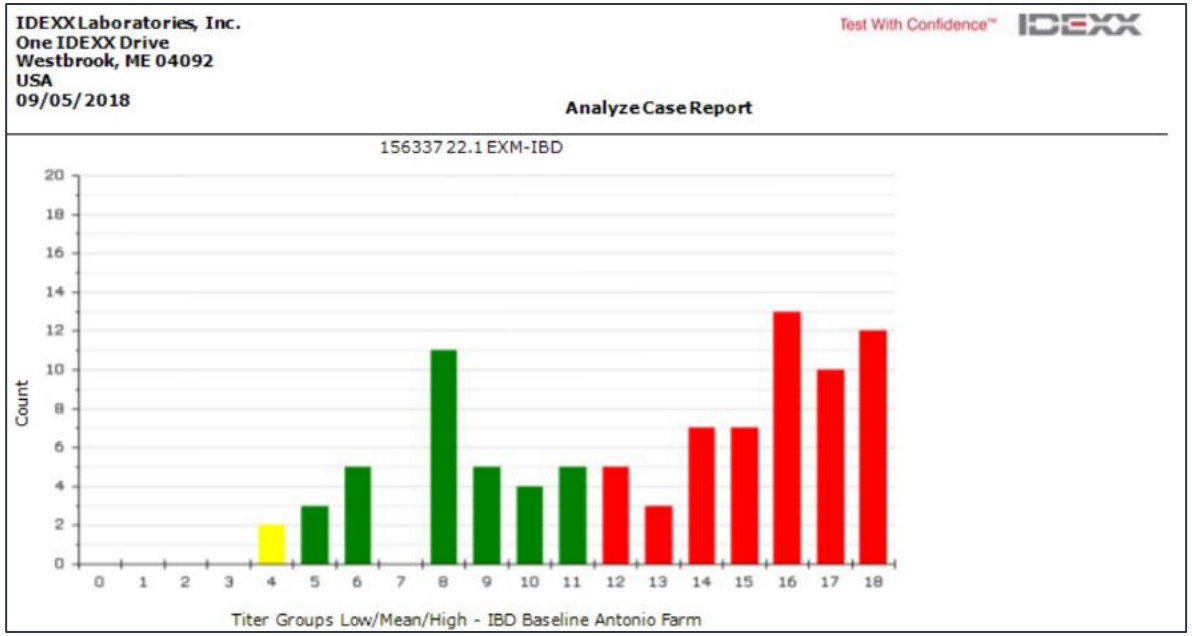


Build your baselines

- Keep in mind the sample size and sampling time.
- Confirm that is tested a statistically significant number of samples from each lot.
- When greater variability in titers is expected, it is advisable to increase the number of samples.
- Reducing the sample size can cause unreliable or erroneous results.

What do you need to create reliable baselines?

- A min of 24 samples/test and min 6 cycles with the same vaccination program for broilers and min 3 for breeders.
- Age of the birds and **age and status of the breeders.**
- Compare ELISA results to slaughter or eggs **production performances to correlate with the baseline.**



IDEXX

Conclusions

- Laboratory testing is crucial in diseases prevention and surveillance.
- The sampling methodology and the number of samples must be representative of the population!
- Sampling one house does not represent the all-entire farm!
- Taking 24 to 30 samples are ideal and will accurately reflect the infection rate with a 90-95% confidence level.
- Compromising the sample size for an economic gain may cause unreliable flock titers and misleading results.
- Use proper sample handling and proper ELISA techniques.
- Establish baselines.
- Good cooperation between veterinarians and laboratory will yield in added value to the poultry producer.

Check IDEXX books!

Thanks!!!

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