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POULTRY DIAGNOSTIC AND ANALYSIS LABORATORY

SEROLOGICAL SAMPLING MANUAL

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HOW SEROLOGIC MONITORING CORRELATES TO GOOD SAMPLING

In serological monitoring processes, a valid result can only be obtained through correct sampling. To put it more precisely, a reliable and more accurate estimate is only possible through proper sampling of the subject flock. The key to achieving this is:

1) Using a statistically valid sampling method so that the sample can become representative;

2) Collecting, transporting, handling and processing the samples correctly so that they are of the desired uniform quality; and

3) Selecting the correct testing technique so that the results are reliable, reproducible and repeatable.

SAMPLING METHODS

Using statistically valid sampling methods is vital in terms of the reproducibility and reliability of the serological analysis results and/or the true mean flock titre. The statistical validity of a sampling method is dependent on two main conditions:

1) Selecting random animals for sampling,

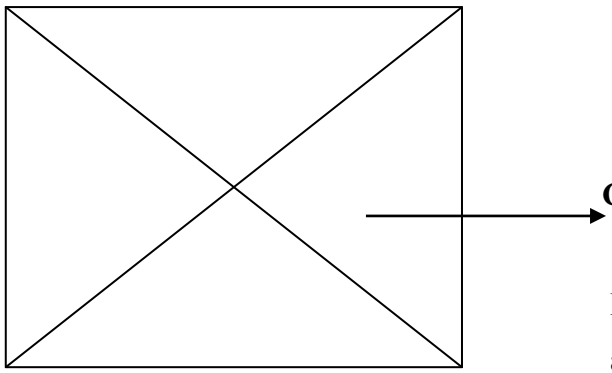
2) Taking enough number of samples to accurately represent the flock.

Random selection of animals for sampling means each animal in the flock has equal chances of being selected for sampling. Of course, this is easier said than done; however, in practice, there are various ways to follow these requirements as closely as possible in a poultry house (see Figure 1 below).

Random sampling is extremely important as it yields a mean of the effects known as "Uncontrollable Variables" in statistics (examples to uncontrollable variables include factors such as non-equal volume of vaccine intakes via drinking water, varying ratios and quantities of feed consumed by each bird, and disparities in the temperature and/or humidity distribution inside the house). Here is another example to paint a clearer picture: Let us assume that we have a poultry house with a malfunctioning drinking system. The drinking system is out of order in the front section, which is the first half of the house, but works perfectly fine in the back section, which is the second half of the house. Mass vaccination via drinking water is performed using this drinking system. If we collect samples only from the first half of the house, we may arrive at the conclusion that our flock has not received any vaccination. Similarly, if we collect samples only from the second half, we might conclude that our entire flock has been properly vaccinated.

If we collect samples via random sampling across the entire house, we will see a bimodal distribution of vaccination graphic in which a part of the flock is vaccinated and another part is free of any vaccination, which would represent the actual situation in the house.

Figure 1. Random sampling in a house where birds range free and fed outdoors.



For correct sampling of 24 birds, 12 birds should be sampled along each corner line; for example, if corner lines are 60 m long, sampling should be done by selecting 1 bird at every 5 meters throughout the line.

If collecting samples from cages or nests, these cages or nests should first be given numbers; then, numbers should be randomly selected and sample should be taken from 1 bird from each cage or nest corresponding to each selected number.

COLLECTING, TRANSPORTING AND PROCESSING SERUMS

Another important aspect for ensuring uniformity during and after sampling is the proper collection, transportation and processing of samples. Following the suggestions below will help in ensuring correct collection, transportation and processing of samples:

- For Elisa tests, minimum 2-3 ml of blood should be collected in a disposable plastic tube suitable for serum collection. 2-3 ml of blood can yield 0.5 - 0.75 ml of serum.
- The serum amount indicated above will not be sufficient for a Haemagglutination Inhibition Test (HI). For HI tests, at least 10 ml of blood should be collected in larger plastic blood collection tubes. If this is not possible, blood should be collected in a few Eppendorf tubes from the same bird to ensure minimum 5 ml of overall serum per bird. Some tests may require more serum than others. Some types of tests can be performed without any problems if the serum amount required for an HI test is taken as a basis.

- Disposable (single-use) syringes should be used for each sample.
- Blood samples that have foam on the surface should not be sent to the laboratory as they will not yield any serum.
- If possible, serum should be separated from whole blood via centrifuging; otherwise, the sample tubes should be left in slanted position to clot naturally (by leaving at room temperature for 1-2 hours).
- If the farm where samples are collected is located at a distance that allows the sample to reach the laboratory within 24 hours after collection, samples can be sent as whole blood, without separating the serum; however, this will not guarantee a serum yield from the sample.
- SAMPLES MUST BE IDENTIFIED AND LABELLED CORRECTLY (Company Name, Farm Name, Flock Code, Flock Age, Poultry Type etc.)
- Before dispatching, samples can be stored in short-term containers (for < 24 hours) at 5± 3°C in refrigerators.
- -22± 3°C freezers can be used for long term storage using airtight, screw top type micro-centrifuge tubes (Eppendorf 1.5 ml) or micro-plates coated with airtight film, both of which should be properly labelled.
- Samples that have been frozen and thawed multiple times may not be suitable for testing.
- Haemolysed or cloudy sera are not suitable for some tests. In such cases, new samples should be collected!
- Samples that change colour or develop an odour a short while after collection (which can be seen especially in blood samples collected from processing plants) should not be sent to the laboratory. The proteolytic enzymes in such samples tend to cause changes in Elisa results.

NUMBER OF SAMPLES/SAMPLE SIZE

The number of samples to be collected is as important as random sampling in determining the true mean flock titre that will represent a flock. Number of samples is also of great importance in ensuring the uniformity of the values obtained from a flock. Typically, the more variability a flock has in titre values (non-uniformity), the more number of samples will be needed to yield the correct mean value. Hence, the most correct way to check for the optimum sample number is by checking the mean values in a flock's history.

In practice, the number of samples to be collected from a flock is the number that is accepted as statistically correct, for example a mixture of the cost of the analysis to the company. However, reducing sample numbers to save money when creating this mixture may result in serious negative impacts on the reliability and accuracy of results.

Table 1 quite nicely explains how mean titre values are likely to be affected if smaller groups are created through random sampling from a single group containing 100 samples collected from a day old broiler flock. These 100 serum samples were put through an IBD-Gumboro test using the Elisa kit of a commercial company. The mean titre value found to be 4057 with a CV (coefficient of variation) % of 48. Vaccination Day Prediction (VDP) was done using the Log_2 and Deventer Log_2 methods, which found days 19 and 21. Then, samples were divided into various groups using random selection, and mean titre values and CV% have been calculated for each group, and then their VDP was done using Log_2 and Deventer Log_2 methods. This procedure was done for a total of 16 times.

Table 1. Role of the Number of Samples in Vaccination Day Prediction (VDP) and Determination of Mean Flock Titre Using the Log2 Method

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Group Sample numbers	Mean Titre		CV%		VDP	
	Lowest	Highest	Lowest	Highest	Lowest	Highest
46	3712	4449	42	58	20	22
30	3333	4779	37	57	20	22
23	3388	4753	40	64	20	22
15	3390	4973	33	62	19	22
10	2374	5260	25	71	17	23
5	2221	5371	23	71	16	23

As clearly seen in the Table, once the sample number decreases, an increase occurs in the deviation in mean titre, in CV% and in VDP. If we consider this for 23 samples, the mean titre value will be between 3388 and 4753, CV will be between 40 and 64%, and VDP will be between days 20 and 22. If we consider 10 samples, the estimated mean titre will fluctuate between 2374 and 5260, and the CV will be between 25% and 71%! Similarly, as can be seen from the data given here, the deviation in mean titre is not that big between 23 samples and 46 samples. However, when the number of samples drops under 23, deviations in estimated mean titre increase dramatically. The increase in variability as the sample number decreases also results in a loss of confidence with regard to vaccination day predictions and mean titre calculations.

Table 2 shows what percentage of the mean titre estimated according to the size of each sample group falls within the true mean titre range of 20%, which is valid for 100 samples. Moreover, it shows which of the VDP done according to the size of each sample group match the actual mean vaccination day with a variation of maximum 1 day.

Table 2. Correlation between sample size and % reliable predictions of the true population mean

Group Sample numbers	Mean Titre	VDP corresponding % with mean variation of +/- 1	
	4057 with +/- 20% variation, as %	LOG_2	Deventer LOG_2
46	100%	100%	100%
30	100%	100%	100%
23	100%	88%	100%
15	88%	88%	94%
10	50%	43%	57%

- True Population Mean Titre (TPMT) was 4057, within which estimated mean titres were identified with +/- 20% variations for acceptable estimates.
- True mean VDP was found as day 19 with the Log_2 method and day 21 with the Deventer Log_2 method; estimated mean VDP was defined with a variation of 1 day for acceptable estimates.

CONCLUSION FOR SAMPLE SIZE

The number of samples that should be collected is estimated around 23 based on the criteria of reliability and reproducibility. When tests were done with less than 23 samples, considerable drops were observed in the reliability and reproducibility of VDP and mean titre values.

In the light of the above data, 23 samples are considered sufficient to draw a profile that can serve as a baseline for a flock or for VDP, in view of both ease of application and statistical accuracy. If no previous study has been done to serve as a baseline for the flock, sample sizes smaller than this will not work in drawing a general flock profile, and may lead to wrong conclusions for the existing flocks.

Compromising from sample size for economic gain will cause inaccuracies in the calculation of mean titre and negatively affect their reliability, possibly leading to wrong decisions that may prove irreversible.

When monitoring for diseases, sample size should be determined according to percentage of infection and sampling frequency. If titres are to be identified at low (< 2-5%) infection rate (for example Salmonella monitoring), the number of samples may have to be way over 23. Table 3 gives more detailed information on this.

Table 3. Monitoring Programme: Correlation between sample size, flock size and infection rate

NUMBER OF SAMPLES REQUIRED TO IDENTIFY WITH 95% RELIABILITY ANY CONTAMINATION OR PREVALANCE OF DISEASE AT VARIOUS SEVERITY LEVELS

Flock Size (N)	CONTAMINATION RATE (n= number of samples)						
	20% n	10% n	5% n	2% n	1% n	0.5% n	0.1% n
20	10	15	19	48	20	20	20
50	12	22	34	77	50	50	50
100	13	25	44	94	96	100	100
200	13	26	45	105	158	190	200
500	14	28	55	128	225	349	500
1000	14	28	56	138	258	450	950
5000	14	28	58	146	290	564	2253
10000	14	28	58	147	294	581	2588
100000	14	28	58	148	299	596	2995

In order to capture an infection rate of 10% in a flock of 5000-10000 birds, 28 random samples should be collected. In order to capture an infection rate of 2% in a flock of 5000-10000 birds, 147 random samples should be collected.

Revision 3. Revision status moved to the back page.
Revision 2. Table 2 and 3 were updated.