1. Guidelines for on-farm sampling for targeted surveillance to certify disease freedom, diagnosis in case of mortalities and for analysis of mortalities caused by unknown aetiology

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1. Guidelines for on-farm sampling for targeted surveillance to certify disease freedom, diagnosis in case of mortalities and for analysis of mortalities caused by unknown aetiology

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1.1. Introduction

Sampling on the farm is an important first step for assessing the disease situation within a group of animals, a farm, a region or a country. Although it might be possible to sample and analyse all animals in question for a small population like a group of broodstock of some hundred fish, this is not achievable or cost-effective for populations with tens of thousands of individuals. A sampling strategy designed according to the purpose of any particular investigation is therefore fundamental for gathering the information we are aiming for, and to ensure that the conclusion about the population drawn from the samples is statistically valid. Depending on the purpose, sampling can be performed randomly (probability sampling) in order to obtain samples that give representative information about a population or a subpopulation, or sampling can be non-random or purposive (non-probability sampling) in order to increase the probability of finding the condition of interest such as the detection of disease.

A thorough description of sampling principles and sampling applications is given in the survey toolbox for aquatic animal diseases by Cameron (2002).

1.2. Sampling elements

Sampling requires a good knowledge of the population to be sampled, either on a national scale for nation-wide surveillance programmes or at farm level when the on-farm disease/infection

status is of interest. Therefore, in order to perform proper sampling, the following principles must be taken into consideration:

- Characteristics (of the population of interest) that influence the disease status e.g. reared species, number of fish, cages/tanks, stocking density, disease history, the disease/infection status, farm location, epizootiology data of the region
- Units to be sampled
- Disease characteristics
- Test characteristics (sensitivity (Se) and specificity (Sp))

Sampling procedures also relate to the particular target fluid and/or organ(s) to be sampled from the chosen individuals and on how these biopsies should be handled and the tissues transported. These are discussed in the relevant sections dealing with the particular diseases/pathogens.

1.3. Sampling for diagnostic confirmation and disease detection purposes

When a disease outbreak is under investigation, purposive sampling of target fish, which are most likely to test positive for the infectious pathogen, or disease under investigation, are sampled. Such target fish can be freshly dead fish, moribund fish, or fish that exhibit disease symptoms, or simply behave differently than the rest of the group. The probability of detection depends on the number of samples collected, the prevalence of the pathogen in the population, and the diagnostic test sensitivity and specificity. A larger number of samples is usually required when moribund fish, or fish with overt symptoms are rare and the prevalence of the pathogen is expected to be low, especially during the early stage of an infection. Such cases require diagnostic tests with high sensitivity.

1.4. Sampling to certify the disease-free status

When the aim is to demonstrate freedom from a specific pathogen, it is important to sample the fish that are most likely to carry the pathogen. Such fish may be freshly dead or moribund or showing signs of disease that may be connected to the pathogen in question. In addition, it is also important to identify the most susceptible age group and perform sampling when environmental conditions promote infection by the particular pathogen (e.g. water temperature).

1.5. Sample size calculation and examples

A larger sample size reduces sampling error and increases the likelihood that the sample accurately reflects the population of interest. The minimum required number of samples that need to be collected for analyses depends on a number of factors and the final sample number is often a decision based on a balance of:

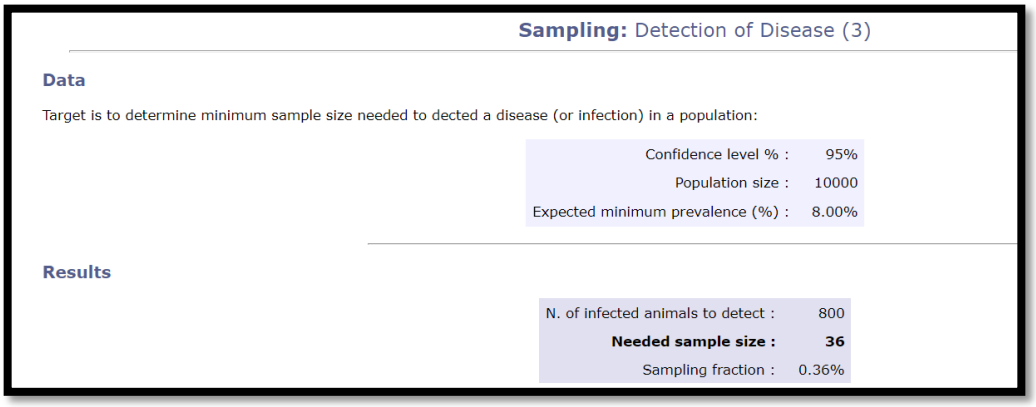
- the required degree of confidence of the results (i.e. consequences of missed cases or false positive cases),
- the sensitivity and specificity of the diagnostic test,
- available resources (economic, personnel, laboratory capacity).

Online tools (i.e. WinEpi, FreeCalc - EpiTools) are available to perform sample size calculation for different sampling purposes. Examples using the WinEpi tool (http://winepi.net/winepi2) are shown below (de Blas and Muniesa 2010). These examples have been modified in accordance with the course on Application of Epidemiology in Aquatic Animal Health in Zaragoza (Spain),

25th February – 1st March 2019, as part of MedAID project (http://www.medaid-h2020.eu/index.php/event/advanced-couse-on-application-of-epidemiology-in-aquatic-animal-health/).

Case 1. Sample size calculation for disease detection

In a sea cage with 10,000 seabass, the veterinary services want to check if the nervous necrosis virus (NNV) is present in the population. If present, the assumption is that the prevalence would be at least 8%. The aim is also to be 95% certain that the sampling would give a correct answer (confidence level of 95%). Using the WinEpi tool, the minimum number of fish required for detecting NNV in this situation is 36, given that the diagnostic test is 100% sensitive and specific and the fish are randomly sampled. Thirty-six fish are 0.36% of the total population (sampling fraction). It must be kept in mind that most tests are not perfect (Sp=Se=100%), so 36 is the lowest number that should be sampled.



Sampling: Detection of Disease (3)

Data

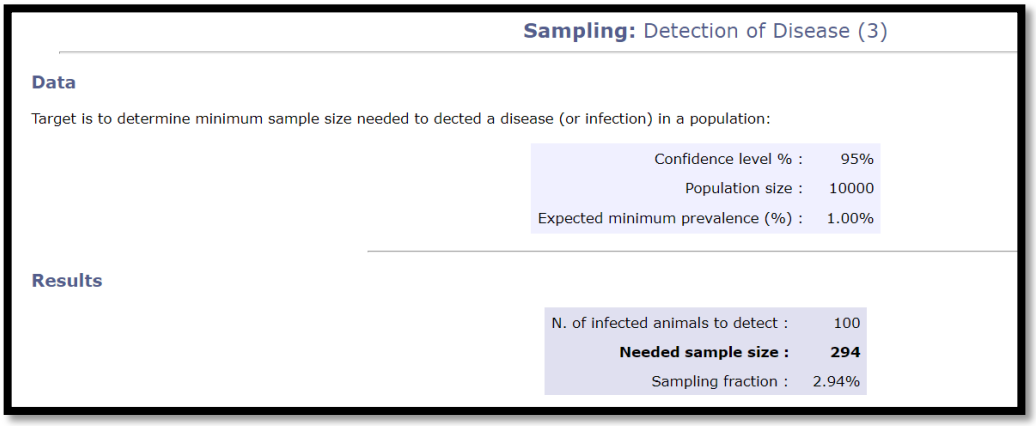
Target is to determine minimum sample size needed to dected a disease (or infection) in a population:

Confidence level % :	95%
Population size :	10000
Expected minimum prevalence (%) :	8.00%

Results

N. of infected animals to detect :	800
Needed sample size :	**36**
Sampling fraction :	0.36%

Then, for the same size population they want to initiate an early detection programme to detect the infection at an early stage, at an expected prevalence of 1% and with 95% confidence level. Using the WinEpi tool, the minimum number of fish required to sample is 294 under the same assumptions as above. A larger sample size is needed to detect a possible infection at a lower prevalence.



Sampling: Detection of Disease (3)

Data

Target is to determine minimum sample size needed to dected a disease (or infection) in a population:

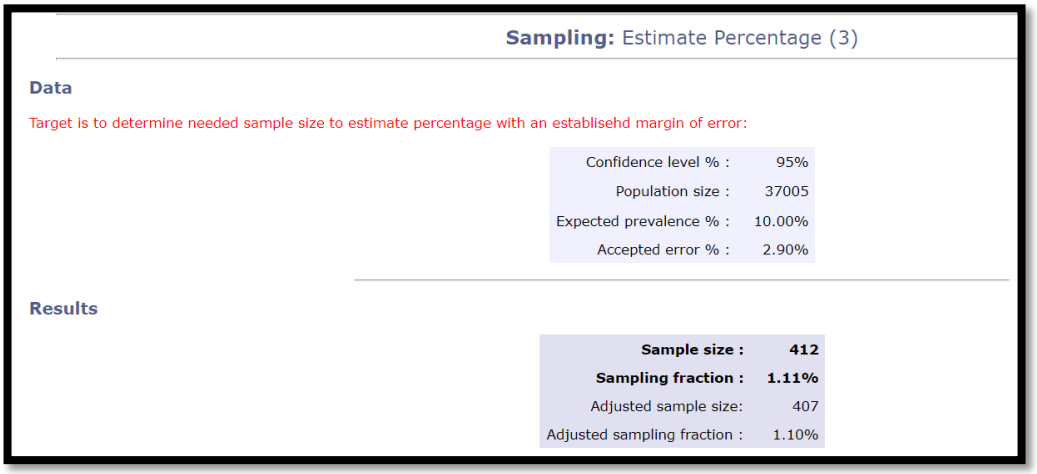
Confidence level % :	95%
Population size :	10000
Expected minimum prevalence (%) :	1.00%

Results

N. of infected animals to detect :	100
Needed sample size :	**294**
Sampling fraction :	2.94%

Case 2. Sample size calculation for prevalence estimation

The veterinary services want to estimate the prevalence of vibriosis in a cage with 37,000 seabream, assuming an expected prevalence of 10% with a precision of 2.9% (10% +/-2.9%), and a confidence level of 95%. Using WinEpi to calculate the sample size for estimation of prevalence, the sample size required is 412.



Sampling: Estimate Percentage (3)

Data

Target is to determine needed sample size to estimate percentage with an establisehd margin of error:

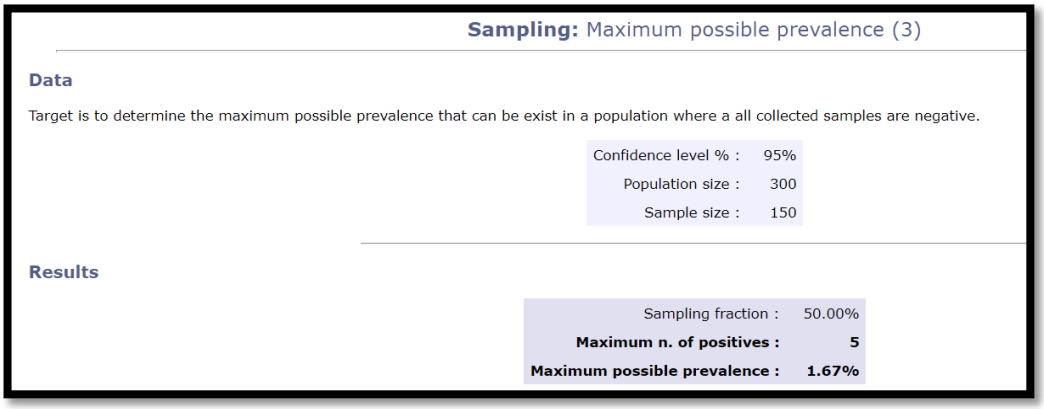
Confidence level % :	95%
Population size :	37005
Expected prevalence % :	10.00%
Accepted error % :	2.90%

Results

Sample size :	**412**
Sampling fraction :	**1.11%**
Adjusted sample size:	407
Adjusted sampling fraction :	1.10%

Case 3. Sample size to calculate the maximum possible prevalence

A fish health veterinarian suspects that a hatchery of 300 gilthead seabream might have been infected with *Vibrio alginolyticus*. Half of the population is inspected, and all are found to be negative. Based on information from half the population, we want to know what the maximum possible prevalence is if the population is still infected with 95% level of confidence. Using WinEpi, and the 150 negative samples, the maximum possible prevalence would be 1.7 % (5 positive fish).



Sampling: Maximum possible prevalence (3)

Data

Target is to determine the maximum possible prevalence that can be exist in a population where a all collected samples are negative.

Confidence level % :	95%
Population size :	300
Sample size :	150

Results

Sampling fraction :	50.00%
Maximum n. of positives :	**5**
Maximum possible prevalence :	**1.67%**

1.6. Sampling examples given by OIE and EU

OIE (2018) has provided a table on how to interpret the test results from sampled fish given specific test criteria (Table1). In the example of testing 330 fish using a design prevalence of 5% (Table 1, in bold), we can expect as many as 23 fish to test false positive when the Sp=95%. This means that there is a 95% confidence that the prevalence in the population is 5% or less given that all 23 are confirmed negative.

In many cases we do not know the Se and/or Sp. For demonstrating freedom (or a maximum prevalence), all positives should therefore be confirmed true or false positives.

Table 1.1. Examples of how to interpret test results at a given design prevalence of 5% (OIE, 2018)

Design prevalence (%)	Sensitivity (%)	Specificity (%)	Sample size (no. of fish)	Maximum number of expected false positives
2	100	100	149	0
2	100	95	1671	98
2	95	100	157	0
2	95	95	1854	108
5	100	100	59	0
5	100	95	330	23
5	95	100	62	0
5	95	95	351	24
10	100	100	29	0
10	100	95	105	9
10	95	100	30	0
10	95	95	109	9

EU (2015) has laid down rules for sampling by Member States in connection with the disease status of the Member States, or zones or compartments thereof for the non-exotic aquatic animal diseases (Table 2). These rules also define sampling procedures for surveillance over time, which is not a part of this manual.

Table 1.2. Screening for confirming disease status according to EU legislation

Design prevalence	Number of fish	Frequency	Confidence Interval
2%	150	Once a year	95%
5%	75	Once a year for two years	95%
10%	30	Once a year for four years	95%

1.7. Some reflections on sampling and sampling size

A tailored sampling strategy is an important criterion to achieve a reliable conclusion about the disease status in a population. The sampling procedure applied should therefore always accompany the result report. By focusing on the subpopulation of fish at risk of having the infection, one can increase the prevalence in the sampled population and increase the probability of finding positive fish.

One sampling is, however, just a snapshot at the time of sampling. To maintain knowledge of the disease status it is important to have proper information about biosecurity and disease history (risk of disease introduction), and have frequent samplings as shown in the examples in Table 2.

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