A Protocol for Active AMR Surveillance in Poultry

Towards a One Health AMR Surveillance System: protocol for active AMR surveillance in commercial broiler and layer chicken populations for the Fleming Fund Grants Programme.

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Acronyms

AGISAR	WHO Advisory Group on Integrated Surveillance of Antimicrobial resistance
AmpC	AmpC beta-lactamases
AMR	Antimicrobial resistance
AMU	Antimicrobial use
API	Analytic Profile Index
AST	Antimicrobial Susceptibility Test
ATCC	Amercian Type Culture Collection
CLSI	Clinical and Laboratiory Standards Institute
EQAS	External quality assurance system
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
FFCG	Fleming Fund Country Grants
GLASS	Global AMR Surveillance System
MM	Mott MacDonald
OIE	World Organisation for Animal Health
SOP	Standard operating procedures
WHO	World Health Organization

1. Introduction

This document has been prepared to assist with designing an active surveillance programme for antimicrobial resistance (AMR) in bacteria carried by healthy chickens that may contribute to AMR in humans. It is designed to strengthen a One Health approach to AMR surveillance.

The protocol is intended for use by technical specialists working in organizations involved with the Fleming Fund Grants programme; for example, government departments and institutions and country grantees implementing the programme as part of their country grant. It is designed to align with AMR surveillance guidelines prepared by the UN Food and Agriculture Organization (FAO) (currently in preparation), the World Organization for Animal Health (OIE)[1] and the World Health Organization's (WHO) GLASS,[2] AGISAR,[3] and Tricycle programmes (Mathieu et al., 2017).[4]

- Section 2 presents the objectives of this AMR surveillance programme supported by the Fleming Fund Country Grants programme.
- Section 3 presents pathway diagrams showing the preparations and planning that need to be undertaken for laboratories and for sample collection before beginning the AMR surveillance testing.
- Section 4 describes target chicken populations.
- Section 5 describes target laboratories, surveillance areas, bacteria and antimicrobials for testing, and the rationale for selection of these.
- Section 6 presents guiding principles for designing an AMR surveillance plan to meet the specified objectives.
- Section 7 provides a detailed guide to designing a sampling plan that will help ensure the results provide a robust estimate of prevalence of AMR in the target bacteria species from chicken populations within the surveillance areas.

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[1] OIE Terrestrial Animal Health Code (2018). Chapter 6.8 Harmonisation of national antimicrobial resistance surveillance and monitoring programmes (http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_antibio_harmonisation.htm)

[2] World Health Organization. Global Antimicrobial Resistance Surveillance System: manual for early implementation 2015. apps.who.int/iris/bitstream/10665/188783/1/9789241549400_eng.pdf

[3] Integrated surveillance of antimicrobial resistance in foodborne bacteria. WHO Advisory Group on Integrated AMR Surveillance (AGISAR). http://apps.who.int/iris/bitstream/handle/10665/255747/9789241512411-eng.pdf;jsessionid=24D7C1D9656F19FCD4CE8E0600C14126?sequence=1

[4] Note – the Tricycle protocol has been developed as a one health blue-print for examining levels of resistance to select drugs and bacterial pathogens and commensals in humans, chicken and the environment. It is due for release in late-2019.

- Section 8 provides advice on biosecurity measures to be taken when collecting samples.
- Section 9 presents guidance for preparation of standard operating procedures (SOPs), training and trialling sample collection before beginning surveillance.
- Section 10 provides guidance for reviewing the sampling plan after a few months.
- Section 11 outlines the responsibilities of the AMR reference laboratory and the regional/provincial surveillance laboratories with respect to diagnostic testing.
- Section 12 presents recommended methods for enriching samples, culturing and identifying the target bacteria to assist countries with defining reagents and consumables for the surveillance programme using either the Clinical and Laboratory Standards Institute (CLSI; https://clsi.org/) or European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) guidelines. It is important that either the CLSI or EUCAST guidelines are used in both human health and animal health laboratories within each country to ensure harmonisation of antimicrobial susceptibility testing (AST) in the target bacteria isolated from humans and chicken.
- Annexes 1 – 4 provide guidelines to support preparation of the sampling plan.
- Annex 5 gives an example of sample size calculations.
- Annex 6 gives guidance on how to prepare a country-specific plan for active AMR surveillance in commercial broiler and layer chickens based on the guidelines in this protocol.

The active surveillance for zoonotic AMR risks in chickens guided by this protocol is designed to contribute to strengthening government animal health services' ability to implement AMR surveillance in livestock. It will lay the foundations for future AMR surveillance by strengthening the epidemiology and laboratory components of the surveillance system. The capacity developed through this programme can be applied to conducting both active and passive AMR surveillance in all livestock species.

The implementation of this protocol and the AMR information generated will contribute to a One Health approach to AMR surveillance. Both the human health and animal health sectors can conduct many aspects of the programme collaboratively, including procurement of equipment, reagents, consumables and Amercian Type Culture Collection (ATCC) strains to ensure high quality products are purchased for both sectors; training in diagnostic testing methods for the bacteria that are common to AMR surveillance in humans and animals; and implementation of laboratory quality assurance schemes.

Importantly, this surveillance programme generates information on AMR patterns in chickens that can be considered together with AMR patterns in humans, providing a set of results for

review by a multi-sectoral One Health AMR surveillance technical team. Multi-sectoral review of the results will help to identify potential links between AMR in humans and animals which can be investigated in more depth through future surveillance and research. Future surveillance may involve more tightly integrated collection of samples in humans, animals and the environment such as supported by the Tricycle and AGISAR programmes (Mathieu et al., 2017; AGISAR, 2017), as well as progressively gathering baseline information on AMR across all farmed animal and aquatic species, food and the environment. This multi-sectoral approach will contribute to the design of evidence-based policies and programmes to mitigate AMR.

This protocol acknowledges that the development of integrated One Health surveillance of AMR is a long-term goal, and will require a step-wise approach. This protocol is therefore put forward as a potential starting point. Once the protocol is competently implemented, it is expected that additional species (both bacterial and livestock), additional approaches to surveillance and a greater degree of integration will be possible.

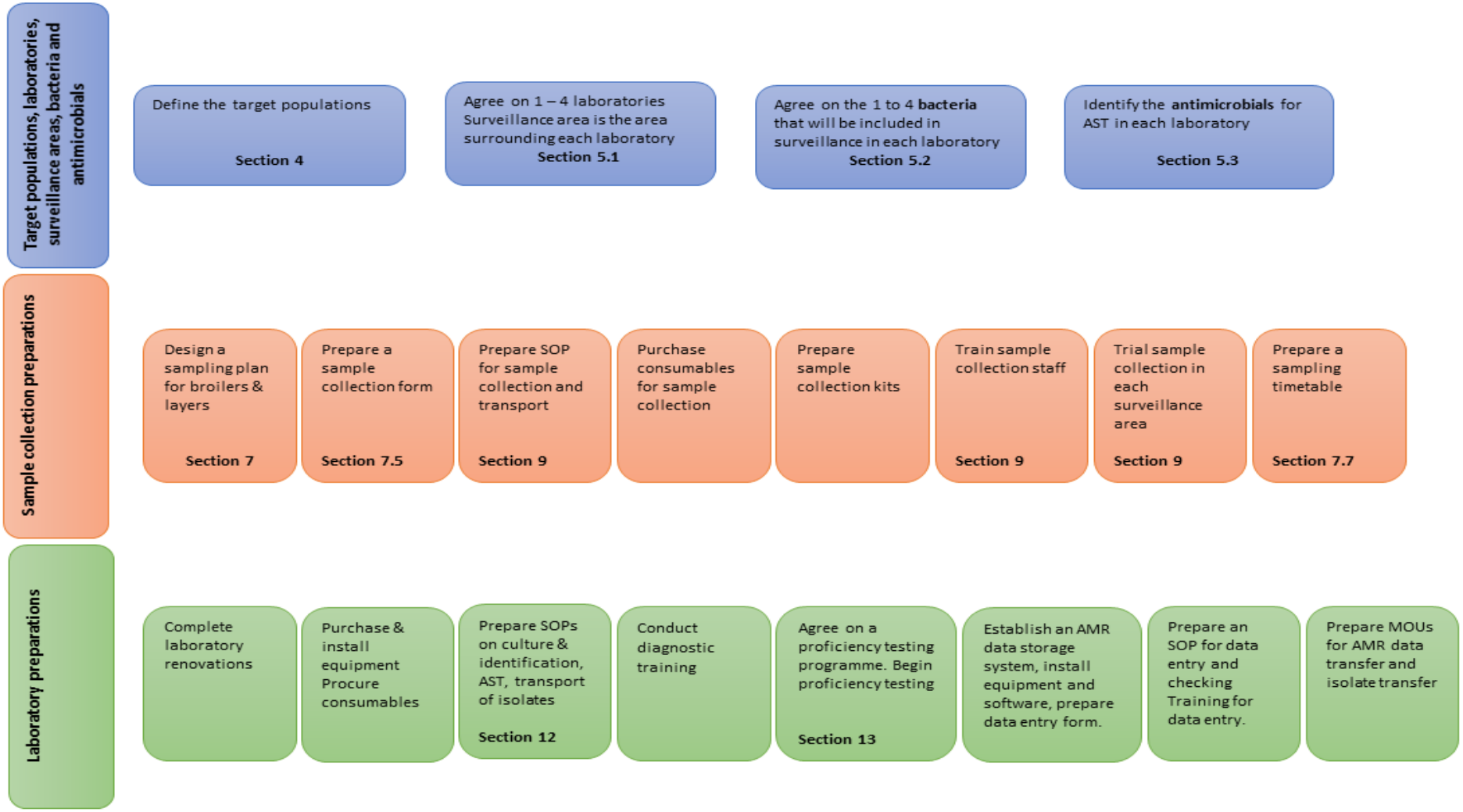
2. Objectives

The objectives of the protocol for AMR surveillance in chickens are to:

1. Strengthen all components of the AMR surveillance system, including epidemiological skills (sample design, data analysis and data reporting), sample collection and processing, laboratory diagnostic capability and data management.
2. Estimate prevalence of resistance in priority zoonotic bacteria to antimicrobials that have been specified by the WHO as critical for use in humans, in broilers and layers produced for human consumption in high chicken-producing areas of the country.
3. Generate baseline estimates of AMR prevalence in broiler and layer populations that are produced for human consumption against which results of subsequent rounds of surveillance can be compared to detect new AMR patterns and identify trends in prevalence of AMR.
4. Strengthen a One Health approach by producing AMR surveillance results from the animal health sector which can be shared and assessed alongside those from the human health sector.
5. Inform priorities and design of the next stage of AMR surveillance in animals and/or other sectors to improve understanding of risks for humans associated with AMR and antimicrobial use (AMU) in animals.
6. Inform policies and programmes for AMR mitigation in animals and humans.
7. Obtain isolates for a national bacterial culture collection (biorepository) for future investigation.

3. Planning and preparations before beginning AMR surveillance

Figure 1: Planning and preparation steps to prepare both the laboratories and the sample collection teams for the AMR surveillance. This diagram acts as a road map showing the relevant sections of the protocol to support each step.



4. Target populations for AMR surveillance

Broiler and layer chickens intended for food consumption are proposed as the target populations for this initial round of AMR surveillance in animals. This is because consumption of chicken products is generally high compared with other protein sources; chickens are an important source of foodborne infections globally; and antimicrobials are widely used in this sector, including some which are a concern to human health (Van Boeckel et al., 2015). This protocol assumes that the potential risks for contributing to AMR in humans are highest in chickens compared with other livestock species, for most countries.

Broilers and layers may have different AMR patterns given the differences in life cycle and management practices. Therefore a separate sampling plan needs to be designed for broilers and layers so that separate inferences can be made about the prevalence of AMR in the target bacteria in each population.

Samples will be collected from chickens sold for meat, including broilers and culled layer hens which have reached the end of their laying period and are sold for meat consumption. In the protocol we have used the term ‘spent hens’ to refer to layer hens sold for meat. We have used the term “broiler” to refer to chicken meat breeds throughout this document. However, other meat breeds such as sonalis, kuroilers and cockerels may be included in countries where these are produced in significant numbers.

The focus of the initial round of surveillance is on commercial and semi-commercial broiler and layer production systems, prioritising the high chicken-producing areas in each country. Backyard chickens may be included in future rounds of surveillance, but for the purposes of this protocol they are not considered a priority.

The FAO classification system[5] described in Table 1 provides a useful basis for categorising chicken production into sectors based on biosecurity practices and level of commercialisation. Pathogen prevalence, AMR prevalence, and antimicrobial use may vary according to these sectors, possibly resulting in sector-specific AMR patterns. It is therefore important to design a sampling plan that includes layers and broilers from all top 3 FAO sectors, when and where this is possible. In some countries it may not be possible to sample chickens from the highly commercial FAO sector 1 farms where access to these chickens is limited.

[5] http://www.fao.org/docs/eims/upload/224897/factsheet_productionsectors_en.pdf

Table 1: FAO classification system for chicken production sectors based on biosecurity and levels of commercialisation.

Sector	Description
Sector 1	Industrial integrated system with high biosecurity levels and chickens/products marketed commercially, e.g. farms that are part of an integrated broiler production enterprise with clearly defined and implemented standard operating procedures for biosecurity.
Sector 2	Commercial chicken production system with moderate to high biosecurity systems and chicken/products usually marketed commercially, e.g. farms with chickens kept indoors continuously, strictly preventing contact with other chickens or wildlife.
Sector 3	Commercial chicken production system with low to minimal biosecurity and chicken/products sold in live chicken markets e.g. a caged layer farm with chickens in open sheds, a farm with chickens spending time outside the shed, a farm producing both chickens and waterfowl.
Sector 4	Village or backyard production with minimal biosecurity and chicken/products consumed locally.

5. Target laboratories, surveillance areas, bacteria and antimicrobials

5.1. Laboratories and surveillance areas

The initial Fleming Fund Country Grant will support capacity building of 1 to 4 animal health laboratories in each country. This includes a national diagnostic laboratory, which is likely to become the national AMR reference laboratory for animal health, and up to three regional or provincial laboratories.

The areas in which the samples are collected for surveillance are defined as the *surveillance areas*. Each surveillance area should be based around a regional or provincial laboratory. Hence, selection of regional or provincial laboratories should consider the following criteria relating to the areas in which they are located:

- High chicken-producing regions.
- AMR surveillance is conducted in the human population.
- Represent geographic coverage of the country.

Selection of the laboratories that will receive support through the Fleming Fund Country Grant is likely to have taken place during the Fleming Fund positioning activities, in consultation with relevant government stakeholders.

5.2. Target bacteria

The target bacteria for the first round of AMR surveillance are zoonotic, pathogenic and commensal bacteria that are carried in the gastro-intestinal tract of healthy chickens and which may potentially be associated with transmitting antimicrobial resistant infections to humans through direct or indirect transmission of resistant bacteria or resistance elements such as plasmids (Table 2).

Table 2: Zoonotic pathogenic and commensal bacteria carried in the gastro-intestinal tract of healthy chickens to be considered for inclusion in the first round of AMR surveillance.

Bacteria
Escherichia coli
Salmonella spp.
Campylobacter spp.
Enterococcus spp. (E. faecium and E. faecalis)

E. coli and *Salmonella spp* are priority organisms listed in the WHO Global AMR Surveillance System (GLASS).[6] *Campylobacter* is an important zoonotic pathogen in humans and *Enterococcus spp* are commensal organisms that may act as an indicator for resistance patterns associated with Gram-positive organisms. This group of target bacteria is consistent with the focal bacteria recommended by OIE[7] and the AGISAR programme.[8]

5.2.1. Selection of bacteria and diagnostic tests for individual laboratories

All laboratories selected for the AMR surveillance programme may not have the capacity or capability to culture and conduct AST in all four bacterial species. Therefore, the surveillance activities need to be customised to the capacity and capability of individual laboratories. Within a country, different laboratories may test for a different number of target bacterial species. Some might only culture and conduct AST on *E. coli* or *E coli* and *Salmonella* spp, while others might culture, identify and conduct AST on all four bacterial species. In countries with very low laboratory capacity, some laboratories may just culture and identify a subset of the target bacterial species and send isolates to the national reference laboratory for AST.

For example, a national laboratory with good bacteriology capacity and capability may culture, identify and conduct AST for all four bacteria, while a regional laboratory that has had little bacteriology experience may focus initially on *E. coli* and then gradually increase the range of bacteria as appropriate for the capability and resourcing of the laboratory.

The recommended order of priority for building capacity to culture, identify and conduct AST in the target bacteria is:

E. coli → Salmonella spp → Enterococcus spp → Campylobacter spp

E. coli is the priority bacteria for strengthening diagnostic capability in animal health surveillance laboratories, followed by *Salmonella spp*. Laboratories that have the capability to reliably grow and identify *E. coli* and *Salmonella spp* may then be supported to include

[6] World Health Organization. Global Antimicrobial Resistance Surveillance System: manual for early implementation 2015. apps.who.int/iris/bitstream/10665/188783/1/9789241549400_eng.pdf

[7] OIE Terrestrial Animal Health Code (2018). Chapter 6.8 Harmonisation of national antimicrobial resistance surveillance and monitoring programmes (http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_antibio_harmonisation.htm)

[8] Integrated surveillance of antimicrobial resistance in foodborne bacteria. WHO Advisory Group on Integrated AMR Surveillance (AGISAR). http://apps.who.int/iris/bitstream/handle/10665/255747/9789241512411-eng.pdf;jsessionid=24D7C1D9656F19FCD4CE8E0600C14126?sequence=1

Enterococci and/or *Campylobacter spp* in their programme. *Campylobacter spp* are more challenging to culture hence it may only be feasible for more experienced laboratories to grow these. Future rounds of surveillance may include other bacterial species such as *Staphylococcus aureus* and *Klebsiella pneumoniae*.

5.3. Target antimicrobials

5.3.1. Critical antimicrobials for use in humans

The aim of the AMR surveillance programme presented in this document is to contribute to understanding the risks to human health that may be associated with the use of antimicrobials and AMR in chickens. To achieve this, the panel of antimicrobials for AST in each of the four bacteria, shown in Table 3, has been selected from the critically and highly important antimicrobial classes for humans identified by WHO.[9] Targeting resistance to antimicrobials that are critically important to humans contributes to the One Health AMR surveillance system, allowing comparison of AMR and antimicrobial usage (AMU) patterns in animals with those in humans, to identify potential links between AMR in the animal and human populations. Given the outcome of interest in this surveillance is resistance in the bacteria carried by healthy chickens that would occur if they were to infect humans, CLSI or EUCAST guidelines must be used for testing and interpretation of resistance in humans.

Table 3: Antimicrobials selected from WHO's critically and highly important antimicrobial classes for humans, for which resistance should be tested in the specified zoonotic pathogenic and commensal bacteria cultured from broilers and layers.

Antimicrobial Class/Antimicrobial	*E. coli*	*Salmonella spp.*	*Campylobacter spp.*	*Enterococcus spp.*
Aminoglycosides	Gentamicin		Gentamicin Streptomycin	
Amphenicol	Chloramphenicol	Chloramphenicol		
Carbapenem	Meropenem/Imipenem AND Ertapenem	Meropenem/ Imipenem AND Ertapenem		
3rd Generation Cephalosporins	Ceftriaxone	Ceftriaxone		
4th Generation Cephalosporins	Cefepime			
Quinolones	Ciprofloxacin Nalidixic acid	Ciprofloxacin Pefloxacin	Ciprofloxacin Nalidixic acid	
Macrolides			Erythromycin	
Glycopeptides				Vancomycin

[9] https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/

Antimicrobial Class/Antimicrobial	*E. coli*	*Salmonella spp.*	*Campylobacter spp.*	*Enterococcus spp.*
Glycylcyclines				Tigecycline
Oxazolidinones				Linezolid
Penicillins	Ampicillin	Ampicillin	Ampicillin	Ampicillin*
Polymixins	Colistin**	Colistin**		
Streptogramins				Quinupristin-dalfopristin*
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	
Sulphonamides/ Trimethoprim	Co-trimoxazole	Co-trimoxazole		

*Interpretation depends on species. **AST for colistin is to be conducted in the Animal Health AMR reference laboratory in countries where there is either capability to conduct minimum inhibitory concentration AST methods and/or equipment to conduct automated AST.

6. Guiding principles for AMR surveillance in chickens

Important factors that need to be considered when designing active AMR surveillance in healthy broilers and layers are described below.

6.1. Sustainable surveillance plan

To meet the objective of monitoring trends in AMR prevalence over time and detecting emergence of new resistance patterns, it is important to design a cost-effective sampling plan that may be more feasible for governments to sustain in the long-run. A repeatable population-based sampling plan will facilitate comparison of results over time. Sampling at collection points in the chicken marketing chain, such as abattoirs or live chicken markets, is generally the most cost-effective location to collect samples. However, in countries or poultry sectors within a country where a high proportion of chickens are not sold through abattoirs or live chicken markets, it may be necessary to collect samples directly from farms.

6.2. Sample chickens about to enter the food chain

Samples should be collected from broilers and layers at the end of their production cycle, as near as possible to the point at which they enter the food chain, as an important objective is to identify resistant bacteria and resistance elements that may spread from chickens to humans via the food chain.

6.3. Sample healthy chickens

Samples should only be collected from healthy chickens. Sampling sick chickens should be avoided as these may not represent the status of resistance in bacteria carried by healthy chickens that enter the food chain.

6.4. One sample per farm

The most precise estimates of AMR prevalence are obtained by maximising the number of farms of origin from which chickens are tested and testing a single isolate of each target bacteria per farm, when there is a fixed total number of samples (Yamamoto et al., 2014; Persoons et al., 2011; Regula et al., 2005). Therefore, when sampling chickens at abattoirs, slaughter points or markets the aim is to **sample a single chicken per farm during the surveillance period, as much as possible.** By sampling 1 chicken per farm it is more economically feasible to collect samples from a larger number of farms that are supplying chickens to the abattoir and/or market, achieving good coverage of the population.

Note, if sampling chickens on farms it may be feasible to collect a pooled sample by combining samples from multiple chickens into a single sample.

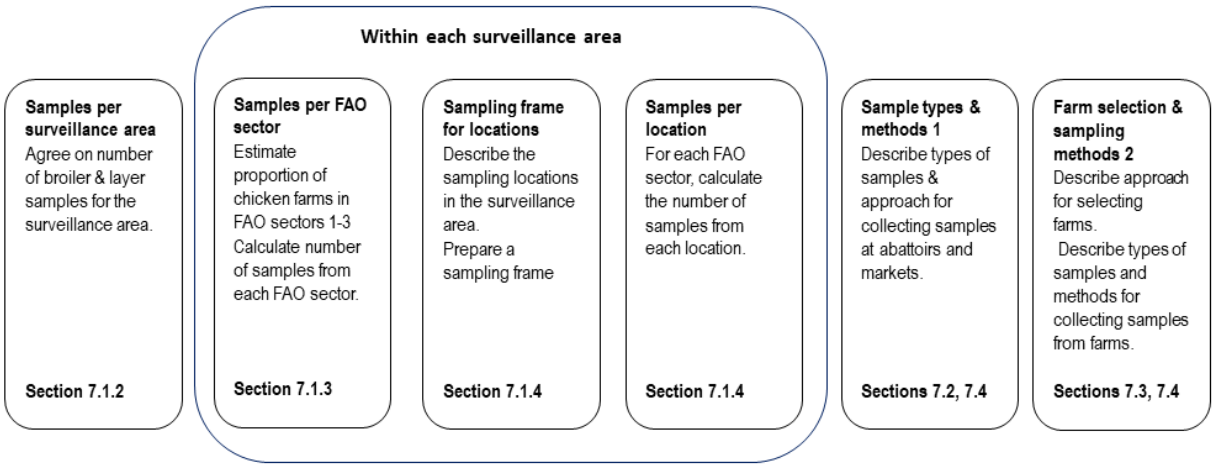
7. Designing a sampling plan

Having identified the laboratories, surveillance areas, target bacteria and antibiotics the next step is to design a sampling plan. A separate sampling plan should be prepared for broilers and for layers in each surveillance area.

The sampling plan should include a description of the following components:

1. The number of samples to be collected at the following four levels:
    a. National level.
    b. Surveillance area level.
    c. FAO sector level.
    d. Sampling location level.
2. Justification for the number of samples to be collected for each level.
3. The types of samples and methods for collecting samples for each type of sampling location.
4. A sampling timetable.

Figure 2. Steps for designing the sampling plan with a reference to the relevant section in the protocol that provides guidelines for each step.



7.1. Number of samples to be collected at each level

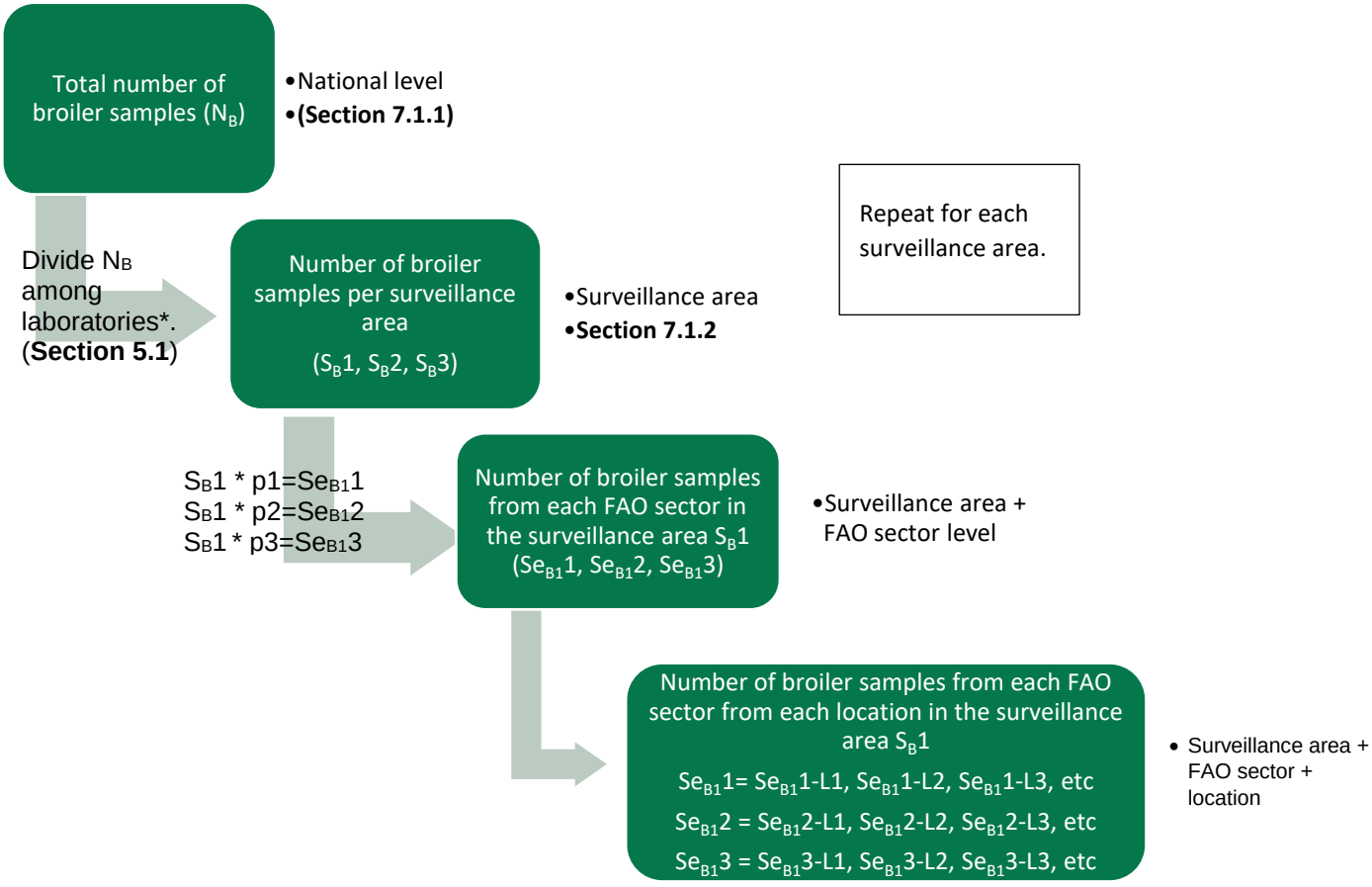
The number of broiler and layer samples needs to be calculated at the four levels described above, as illustrated in Figure 3:

- National level
- Surveillance area level
- FAO sector level
- Sampling location level

To avoid bias in the sample, a stratified sampling plan is recommended following the principle of **probability proportional to size**. Numbers should be stratified on the basis of both the FAO sector and the size of sampling locations as detailed in the sections below. The diagram shows an example for calculating the number of broiler samples in one surveillance area (S_B1). This needs to be repeated in all other surveillance areas for broilers and for layers.

Note that the calculations for number of samples to be collected at location level do not apply when samples are collected directly from farms (see Section 7.3).

Figure 3: Diagram showing the levels at which sample numbers need to be calculated, with stratification and sampling proportional to size for FAO sectors and locations within each surveillance area.



*One laboratory = one surveillance area

p1, p2, p3: estimated proportion of farms in FAO sectors 1, 2, 3 in surveillance area (S_B1).

Se1=FAO Sector 1, Se2=FAO sector 2, Se3=FAO sector 3.

L1, L2, L3 = locations 1, 2 and 3.

A working example of Figure 3 is provided in Annex 5.

7.1.1. Number of broiler and layer samples at the national level

The number of isolates required to estimate prevalence of resistance amongst the isolates for a fixed level of confidence varies with the expected prevalence and the desired level of precision, as shown in Table 4. Highest numbers of samples are required to estimate prevalence levels of 50% for a given precision; if a more precise estimate is required, the sample size increases.

Table 4: Number of isolates required to estimate prevalence of resistance to a specific antimicrobial in a particular bacterial species with a 95% confidence level, for two levels of precision (5% and 10%). (Extracted from OIE Terrestrial Animal Health Code).[10]

Expected AMR prevalence	Number of bacterial isolates needed	
	Desired precision	
	10%	5%
10%	**35**	**138**
20%	61	246
30%	81	323
40%	92	369
50%	**96**	**384**
60%	92	369
70%	81	323
80%	61	246
90%	35	138

Note that Table 4 indicates the number of bacterial isolates required for the estimation of prevalence of resistance in that bacterial genus or species, not the number of chickens to sample. The number of chickens that need to be sampled to produce the target number of bacterial isolates will depend on the prevalence of infection (i.e. prevalence of carriage) of each species in the population being sampled. For example, if the bacteria of interest is present on 50% of farms, then chickens from twice the number of farms need to be sampled to produce the number of isolates needed to achieve the prevalence estimates in Table 4, which can be extremely challenging to achieve in some countries. The prevalence of the different bacteria is likely to be highly variable, ranging from high prevalence levels for *E. coli* and lower prevalence levels for *Salmonella spp*. At the time of designing the sampling plan for this first round, it is unlikely that the prevalence of target bacteria will be known, a priori. Expected ranges of prevalence for the different bacteria were identified from a range of

[10] OIE Terrestrial Animal Health Code (2015). Chapter 6.7 Harmonisation of national antimicrobial resistance surveillance and monitoring programmes (http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_antibio_harmonisation.htm)

publications from studies conducted in Asia and/or Africa (Table 5). Published data was not available for the prevalence of commensal *E. coli* in these regions and data has been taken from a Canadian study (Lebert et al., 2017).

Table 5: Expected farm-level prevalence of the target bacterial infections in chickens.

Bacteria	Table Header	Table Header
E. coli	~ 100%	Lebert et al (2017)
Salmonella spp	7% – 65%	Tu et al (2015); Afema et al (2016); Kagambega et al (2019)
Campylobacter spp	50% – 90%	Bester et al. (2008; 2012) ; Carrique-Mas et al (2014); Kagambega et al (2019)
Enterococcus spp	50% - 65%	Ngbede et al (2017)

A national sample size of approximately 400 broilers and 400 layers is likely to yield approximately 400 isolates of *E. coli* and lesser numbers of the other bacteria, depending on the prevalence of bacteria in the broiler and layer populations. This should give precise estimates for all AMR prevalence values in *E. coli* and other bacteria present at a high prevalence. However, the lower number of isolates for the lower-prevalence bacteria such as *Salmonella spp* and *Campylobacter spp* may result in less precise AMR prevalence estimates, although the precision will depend on how close the estimated AMR prevalence is to 50% in those bacteria (Table 4).

In this first round of surveillance it is impractical to collect a sample size large enough to provide precise estimates of prevalence of resistant bacteria among bacteria present at a low prevalence in chickens, such as *Salmonella spp*. We have set a target sample size of 400 for broilers and 400 for layers based on the statistical reasons described above and on logistical reasons. For example, we estimate that in a country with 3 or 4 surveillance laboratories, it is achievable to collect and test 800 samples (400 broilers and 400 layers) within 12 months . The frequency of sampling and number of samples collected per sampling can be adjusted to the circumstances in each country. Some considerations for organising the timetable are provided in section 7.7.

National number of broiler samples $N_B = 400$

National number of layer samples $N_L = 400$

In countries in which there is only one or two animal health surveillance laboratories and a small number of chicken farms it may not be feasible to collect and/or test 800 samples from broilers and layers, in which case the number of samples should be adjusted according to the individual country situation. The results on prevalence of each bacterial species and of

antimicrobial resistance within each species that are generated from this round of surveillance will be useful to inform the design of future AMR surveillance in chickens.

7.1.2. Number of samples in each surveillance area

The number of samples from broilers and layers to be tested in each surveillance area can be based on the following considerations:

- Equally divided between all laboratories included in the AMR surveillance network and their respective surveillance areas, OR
- Varied according to the distribution of broilers and layers across the surveillance areas, OR
- Varied according to individual laboratory capacity.

Number of broiler samples per surveillance area (e.g. 3 surveillance areas)

$N_B = S_B1 + S_B2 + S_B3 = 400$

National number of layer samples per surveillance area (e.g. 3 surveillance areas)

$N_L = S_L1 + S_L2 + S_L3 = 400$

7.1.3. Number of samples in each FAO sector

The AMR prevalence may vary significantly between broiler and layer farms in the different FAO sectors. It is thus important to ensure that the estimate of AMR prevalence is not biased by over-representation of chickens from any one of the 3 FAO sectors in the sample. Therefore, when designing the sampling plan it is important to ensure that the proportion of chickens from each FAO sector in the sample for each surveillance area is roughly the same as the overall proportion of farms within each FAO sector in the surveillance area.

To achieve this, it is helpful to roughly calculate, within each surveillance area, the proportion of broiler farms within each FAO sector and the proportion of layer farms within each FAO sector. Table 6 shows an example of how to calculate the proportion of farms in each sector.

Table 6: An example showing how to calculate the proportion of chicken farms within each FAO sector.

FAO sectors	Number of farms in each sector	Proportion of farms in each sector
Sector 1	20	0.02^{p1}
Sector 2	180	0.18^{p2}
Sector 3	800	0.80^{p3}
Total	**1000**	**1.00**

p1 = 20/1000; p2 = 180/1000; p3 = 800/1000

Proportion of farms in each FAO sector

Note that the proportion of farms in each sector does not need to be exact. Countries may not have data showing the number of farms in each of the FAO sectors.
Thus, a **rough estimate** of the proportion of farms in each sector is adequate, based on local knowledge of the chicken production sector.

Number of broiler samples per FAO sector for surveillance area S_B1

$S_B1 = Se_B1 + Se_B2 + S3_B3$

(Se = FAO sector)

7.1.4. Number of samples per sampling location

To capture an adequate number of samples from chickens in all 3 FAO sectors it is likely that samples will need to be collected from a combination of sampling locations within each surveillance area, including: abattoirs, smaller slaughter points, and/or live chicken markets (preferably collect samples from a slaughter point in the market). The following factors should be considered when selecting the mix of locations for sampling:

Approximately 80% of the target broiler and layer populations from each of the FAO sectors 1 to 3 are slaughtered or sold through the sampling locations.

Locations should be reasonably accessible, allowing for collection of samples from multiple locations within one day.

Locations should represent geographic distribution within the surveillance area.

The first priority is to collect samples from abattoirs. However, additional samples may need to be collected from other locations to obtain the required number of samples in each of the FAO sectors.

In countries, or areas within countries, where the majority of broilers and/or ‘spent hens’ are not slaughtered at abattoirs or slaughter points and/or are not regularly sold through live chicken markets it may be necessary to collect samples directly from farms.

The decision tree in Annex 2 may help identify the mix of locations at which samples can be collected in each surveillance area to ensure an adequate number of samples is collected for all three FAO sectors for both broilers and layers.

Sampling frame

Prepare a sampling frame of the sampling locations in each surveillance area to help calculate the number of samples to be collected from each location.
A sampling frame lists each abattoir, slaughter point and live bird market within each surveillance area, together with details of: the total number of chickens slaughtered or sold through each and information on the catchment area of chickens sold at each location.
A template is provided in *Annex 1* to assist with preparing a sampling frame for each surveillance area.
If sampling directly from farms, a different sampling frame is needed as described in Section 7.3.

The number of samples for each FAO sector collected from each location should be roughly proportional to the number of chickens within the sector that are processed or sold through each location.

It is important to be mindful that some abattoirs may slaughter large numbers of chickens that originate from a small number of very large farms, which may be more likely to occur at abattoirs slaughtering chickens from FAO sector 1 farms. The number of samples from such abattoirs needs to be adjusted to avoid sampling chickens from the same farm multiple times.

The sampling frame is used to work out the number of samples to collect from each location, following the principle of sampling probability proportional to size.

Sampling probability proportional to size

The numbers of chickens sampled from each location do not have to be exactly proportional to the number of chickens or number of source farms slaughtered through each location.
The aim is to collect the largest number of samples from the abattoirs or markets with the largest number of source farms and fewer samples from those with fewer source farms.

The table in Annex 3 provides an example of a sampling plan showing the number of samples to be collected from chickens in each FAO sector at each location.

7.2. Sample collection from abattoirs and markets

It is important to design and implement the sampling plan so that, as much as possible, an individual farm is only sampled once (i.e. one chicken per farm) during the study period.

When collecting samples from an abattoir, information should be obtained from the abattoir regarding the farm or farms of origin of each group of chickens present at the time of sampling.

A single chicken should be sampled from a group of chickens which all come from the same farm, and details about the farm of origin recorded (see details in section 7.5).

A single chicken should be sampled from a group of chickens that originate from mixed farms, and details about the catchment area of the chickens recorded (see details in section 7.5).

This may be challenging in locations where traders/vendors sell groups of chickens sourced from multiple farms and it is difficult to identify the origin of individual chickens. In some cases, multiple vendors within a market may sell chickens from the same farm. Information on the farm of origin or the area of origin of sampled chickens should be collected and used to minimise the chance of sampling chickens from the same farms within the same or future visits to the location. If there is a high chance that multiple vendors in the same market are selling chickens from the same farm, then only a single broiler and a single layer should be sampled at the market during any one visit.

A further consideration when sampling chickens at live chicken markets is whether the vendor has treated the chickens with antibiotics. Vendors should be asked if they have treated the chickens with antibiotics in the past few days. Chickens that have been treated with antibiotics by the vendor should not be sampled.

7.3. Sample collection from farms

If sufficient numbers of samples cannot be collected from abattoirs, slaughter points or live bird markets for each FAO sector of broilers and layers then some samples may need to be collected directly from farms. However, caution should be taken not to sample from farms that sell to abattoirs or markets that are being sampled.

The selection method for farms needs to be considered within each FAO sector. If there are only one or two FAO sector 1 farms in the surveillance area, then both of these should be sampled. However, for the FAO sectors which have a high number of farms then the two-stage sampling process described below should be used to identify farms for sampling.

Simple random sampling of farms within a surveillance area is unlikely to yield a list of farms that is practical to work with as the selected farms may be widely distributed across the area. If that is so, **a two-stage sampling process** may be used, with the first stage being selection of a small number of administrative areas within the surveillance area and the second stage being selection of farms within the selected administrative areas, as described below.

Stage 1:

a. Prepare a sampling frame of all small administrative areas of interest (e.g. municipalities) within the surveillance area.

b. Randomly select a small number of administrative areas from the sampling frame and determine a number of farms to sample in each area. The number of administrative areas to select depends on the number of farms within administrative areas. For example, if there is a large number of farms in the administrative areas, then a smaller number of administrative areas needs to be randomly selected.

Stage 2:

a. Select a number of farms from each FAO sector within each selected administrative area.
The number of farms selected for sampling within each FAO sector, across selected administrative areas should add up to the total number of farms to be sampled in each FAO sector for broilers and layers within the surveillance area.

It may be possible to collect one pooled sample from multiple chickens when sampling directly from farms to increase the represenativesness of the sample. If an abattoir is present on the farm then whole caeca should be collected if possible. However, if this is not possible then samples should be collected from the shed that houses the oldest group of chickens. Samples should preferably be collected from chickens that are within 1 week of being slaughtered for human consumption (see details below). Boot swabs are a practical method for collecting a representative sample from the shed floor, where it is possible to walk around within the shed housing the birds to be sampled (see 7.4.4). Sampling faecal deposits is another option (see 7.4.3).

7.4. Biological sample types

7.4.1. Caecal samples

Collecting whole caeca is preferred as this will ensure the results will be more representative of on-farm antimicrobial use as there is less opportunity for environmental contamination of samples.

Collecting whole caeca is preferable to taking a swab of the caecal content as the higher volume of material in the whole caecum is likely to increase the chance of detecting the bacteria of interest if they are present (Funk et al., 2000). Caeca can be collected from chickens during the slaughter process at abattoirs and/or slaughter points.

Collection of whole caeca

The intact caecum plus contents should be collected by clipping at the ileal-caecal junction and at the caecal-colon junction and placing the entire caecum plus contents in a sterile whirl-pak or leakproof zipper bag.
The bag should be labelled with the sample identification number, location and date and placed in a cool box with ice packs for transport to the laboratory.
To avoid cross-contamination between chickens from different farms, the sampler should wear a new set of gloves and use a fresh scalpel blade for removing the caecum from each chicken.
Note: Samples should only be collected from healthy chickens and not from chickens showing signs of illness.

7.4.2. Cloacal swabs

If it is not practical to collect whole caeca, then cloacal swabs should be collected from live chickens. While the likelihood of growing bacteria present in the chicken may be slightly lower in cloacal swabs compared with caecal samples due to the smaller amount of faecal material collected (Funk et al., 2000), the chance of environmental contamination of samples is less compared to sampling faecal deposits from the floor of chicken cages.

When testing for *Campylobacter spp*, a cloacal swab should be collected from two birds. One swab should be stored in normal transport medium for testing *E. coli, Salmonella spp and Enterococcus spp* while the second swab should be stored in pre-packed transport medium specific for *Campylobacter spp* testing. If chickens are in a group of birds from mixed farms, it doesn't matter if the two sampled birds are not from the same farm.

Collection of cloacal swabs

If resources are available it is most convenient to use swabs that come within a tube of gel or liquid transport media.
Alternatively, sterile swabs can be used for cloacal sampling.
Wearing a pair of plastic gloves, gently insert the swab into the cloaca of the restrained chicken and rotate to collect as much faecal material as possible on the swab.
Place the swab and faecal material back into the tube containing the transport media or, if not using pre-prepared tubes, place the swab in a tube with 0.85% normal saline.
Stopper the tube, label the tube with the sample identifier, location and date and store in a rack in a cool box for transport to the laboratory.
If testing for *Campylobacter spp*, use a separate swab that has been pre-packed in a gel-based transport medium that contains charcoal, to collect a cloacal sample from a different chicken in the same group.
Ensure that a new set of gloves is worn for sampling each bird.

7.4.3. Faecal samples (live chicken markets or abattoirs)

If it is not feasible or publicly acceptable to collect caecal or cloacal samples, then one faecal deposit should be sampled per group of chickens. It is important that very fresh faecal deposits are sampled to reduce the loss of bacteria through desiccation and exposure to

oxygen (this is especially important if testing for *Campylobacter spp.*) and also to minimise the opportunity for environmental contamination of the samples. It is preferable to sample a deposit that is excreted while the sampler is present, and as much as possible try to extract the part of the sample that is not in contact with the floor and/or sides of the cage.

7.4.4. Boot swabs (on-farm sampling)

Boot swabbing is a useful method for collecting samples from broiler and layer farms, where it is possible to walk amongst the birds within a shed. On layer farms, samples should be collected from hens that are at the end of their laying period and about to be culled as 'spent hens'. On broiler farms, samples should be collected from birds that are close to the point of slaughter.

A new pair of boot swabs must be worn to collect faecal material from the floor of each shed that is sampled. See details in the box below for collecting samples using boot swabs.

Sampling using boot swabs

Boot swabs are boot covers made of absorptive material that are worn over the sampler's boots. They are designed to collect faecal material from the floor by walking around the shed that houses the chickens to be sampled.

Before use, the surface of the boot swabs should be moistened using sterile recovery diluent e.g. Maximum Recovery Diluent (MRD) (0.8% NaCl, 0.1% peptone in sterile deionised water). This can be applied by placing the boot swab in a plastic bag containing the recovery diluent or by pouring the recovery diluent into the boot swab before putting on or spraying onto the boot swab after it has been put on. Pre-moistened boot swabs are also available for purchase.

The sampler should avoid sampling in the doorway or entrance to the shed, hence the boot swabs should be put on once the sampler is inside the shed. It is extremely important that the sampler has clean boots and wears a new pair of plastic boot covers over the top of the boots and under the boot swab for each sampling event, to prevent contamination from the sampler's boots.

It is also important to ensure that the boot swabs do not come in contact with any disinfectant (boot dips) if the sampler's boots were disinfected prior to sampling, as this will kill any bacteria in the faecal material collected using the boot swab.

Wearing the boot swabs, the sampler should walk back and forth across the area of the shed where the birds are most dense, without covering floor area that has already been sampled. It is important to avoid sampling wet swampy areas in the sheds.

When sampling has been completed, and before walking back to the entrance of the shed, the sampler should carefully remove the boot swabs whilst wearing a pair of clean plastic gloves. The boot swabs should be turned inside out as they are removed to retain the faecal material inside. The swabs should then be placed in a sterile bag or jar.

Label the bag with the sample identification number, farm name and date then place in a cool box with ice packs, ensuring the sample does not directly touch the ice packs.

The gloves and plastic boot covers should be disposed after each sampling event.

It may be difficult to collect adequate samples by walking around the floor of the shed with boot swabs when layers are caged. In such cases, the boot swabs may be applied to the

sampler's hands and used as 'drag swabs' i.e. they are dragged across the areas where faecs has accumulated under the cages.

7.4.5. Faecal samples (on-farm sampling)

If testing for *Campylobacter spp,* in addition to the boot swabs we recommend collecting samples from 5 faecal deposits at different locations in the shed, using swabs pre-packed in charcoal-containing transport medium. Use a separate swab for each deposit and place each back in the swab's container. Swabs from an individual farm should be pooled at the laboratory.

7.5. Sample collection form

Complete a **sample collection form** for each sample to capture descriptive information that will help correctly interpret the AMR results. Forms should include the information shown below.

It is important to ensure that a **unique sample identification numbering system** is put in place between all surveillance laboratories so that every sample, regardless of its origin, has a unique sample ID. Ensure that the ID number written on the form matches the ID number on the sample tube.

Sample collection form*

Sample identification number
Sample collection date and time
Name of sampler
Location type (abattoir, slaughter point, live chicken market, farm, other)
Name of location (GPS coordinates if a GPS is available)
Name of city/town/village
District
Province/region
Species (chickens)
Production type (broiler, kuroiler, cockerel, layer)
Breed
Age of chicken in weeks (approximate, if known)
Name and location details of owner of the sampled chicken (if known)
Other farm details for the sampled chicken (if known) e.g. number of chickens on the farm, FAO sector (sectors 1 – 3)
If farm of origin is not known, collect information on the location of the catchment area for chickens in the group containing the sampled chicken
Type of sample (caecal, faecal, cloacal, boot swab, other)
Pooled sample – yes/no. If yes, how many individual samples are combined in the pool.

Additional information for abattoirs/slaughter points:

Average number of chickens slaughtered at the location per day
Average number of source farms supplying chickens to the location per day
Catchment area for the abattoir or slaughter point (rough description of the districts from which chickens have originated on the sampling day, if known)

Additional information for live chicken markets:

Average number of vendors in the market per day
Average total number of chickens sold at the market per day
Name of the vendor selling the sampled chicken
Average number of chickens sold per day at the vendor's stall
Catchment area for the vendor's chickens (rough description of the district from which vendor's chickens have originated on the sampling day, if known)

Additional information for farms:

Owner of the farm (Name, address, phone number if available)
FAO sector classification (sectors 1 – 3)
Number of chickens on the farm
Number of management units on the farm (refers to the number of different groups raised independently of each other e.g. layer houses or broiler sheds)
Number of chickens in the shed that was sampled
Date the chickens in this shed were most recently treated with antimicrobials, and name of the antimicrobial (if known)
Source of chicks (name of hatchery if known)

* AMU data is not included in this sample collection form as this protocol relates to AMR surveillance. However, if samples for AMR surveillance are collected on farms, AMU data may be collected simultaneously.

7.6. Sample transport

Store all samples in secure containers in a cool box with ice packs while all samples are being collected. Ensure that there is no direct contact between ice and the sample. Keep chilled (<10^{o}C), but not freezing, to prevent overgrowth of samples. Freezing of samples should be avoided as it may kill the bacteria or affect the carriage of plasmids.

Samples should ideally be transported to the laboratory on the day of collection. If not, they must be stored in a refrigerator at 4 - 8^{o}C and transported to the laboratory the next day.

7.7. Sampling timetable

A sampling timetable should be prepared for each surveillance area showing the number of samples to collect from each location by date. The timetable needs to ensure that the days of sample collection, the number of samples collected and the frequency of sampling match the laboratory's capacity to process samples.

7.7.1. Days of sampling

It is likely to be most practical to collect samples on the first two days of the working week so isolates can be grown and identified by the end of the week and laboratory staff will not be required to work weekends.

7.7.2. Number of samples

The number of samples collected on each sampling day needs to match the capacity of the laboratory to process samples collected for surveillance in addition to their routine workload. It will vary with the number of different bacteria that a laboratory is testing for the surveillance programme.

7.7.3. Frequency of sampling

A two-weekly cycle of sample collection may utilise laboratory capacity most effectively. In this way isolates can be cultured and identified and have AST performed in the first week, then additional testing/isolate storage can be performed in the second week.

Sampling should be distributed across the main climatic seasons of the year to capture any seasonal variability in chicken production systems and associated antimicrobial use.

7.7.4. Sampling locations

It may be most efficient to collect samples from a range of locations on an individual sampling day. For example, from multiple abattoirs, live chicken markets and/or farms.

8. Biosecurity practices when collecting samples

Sample collectors must apply good biosecurity practices[11] when collecting samples to avoid spreading disease from one location to another. This is extremely important when sampling from farms, both to ensure that pathogens are not spread between farms and to avoid farmers associating a disease outbreak that occurs by chance following sampling with the presence of the samplers on their farm.

The practices described below relate to sampling from farms. However, similar practices of cleaning and disinfection before and after sampling should also be followed when collecting samples from abattoirs, slaughter points and live bird markets.

When making an appointment to collect samples from a farm, check with the farmer that there is no evidence of infectious disease spreading between chicken on the farm. Do not collect samples from a farm where there are signs of illness affecting a group of chickens in one or more sheds on the farm. Check again with the farmer when arriving at the farm to ensure that no new disease problems have arisen. If the chickens have begun to show signs of illness between the time of making the appointment and arriving at the farm, do not enter the farm and arrange a time to return for sample collection when the chicken are healthy.

We strongly recommend using Virkon™ S as the disinfectant of choice. Some alternative disinfectants are rapidly inactivated by heat (such as normal temperatures in tropical locations) or by contact with organic matter, and therefore are not effective in killing the required range of organisms.

Virkon™ S disinfectant

Virkon™ S is a broad spectrum veterinary disinfectant suitable for organic farming that kills bacterial strains, the viruses likely to be present in a chicken shed, and other pathogens, including fungi. Resistance is not a problem with this disinfectant.
It is effective against chicken virus diseases, such as avian influenza, Newcastle disease and avian laryngotracheitis, and bacteria such as *E. coli*, *Salmonella spp*., *Staphylococcus spp* and against *Mycoplasma gallisepticum*.
Prepare a solution of 1:100 (10 grams of Virkon™ S to every 1 litre of water) and place in a sealed container which can be used as a boot dip at the farms. Replace solution once it has either become dirty or after a period of 4–5 days.
Source: http://virkon.com/fileadmin/user_upload/Virkon_S_Poultry_LXS_VIV_V2.pdf (page 10)

[11] OIE Terrestrial Animal Health Code. Chapter 6.5 Biosecurity procedures in chicken production. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_biosecu_poul_production.htm

The following biosecurity practices must be implemented:

1. The minimum number of people needed to undertake sampling should enter the farm. Ideally this is two people – one collecting the samples and a second recording the sample details.
2. The samplers' vehicle should be parked outside the farm gate and not driven onto the farm.
3. Clean rubber boots, overalls, hair nets and gloves must be worn by all samplers who are entering a farm. A separate set of clean overalls, hair nets and gloves should be worn for each farm.
4. The materials required for collecting faecal, caecal or boot swab samples should be placed into a plastic box with a lid which is dedicated for carrying sampling materials and samples for each farm. The necessary materials required for sampling chickens on an individual farm should be transferred to the box before entering the farm, the outside of the box washed with Virkon™ before and after entering the farm. After returning to the vehicle the samples should be transferred from the sample collection box to a cool box with ice packs for chilling during transport.
5. **Before entering the farm**, all samplers must undertake the following measures that demonstrate good biosecurity practices are being applied.
    a. Put on a new set of overalls and a new hair net for each farm.
    b. Scrub boots with soap and water.
    c. Brush boots carefully with Virkon™ solution or dip boots into the container of Virkon™ solution.
    d. Scrub the outside of the box containing the sampling materials using the Virkon™ solution.
    e. Wash hands using soap and water or rub hands with alcohol-based hand sanitiser.
    f. Apply a new set of gloves.
6. **Immediately after leaving the farm** and before entering your vehicle all samplers must undertake the following:
    a. Remove gloves and dispose in a rubbish bag.
    b. Scrub boots with soap and water to remove all manure, dust and dirt.
    c. Brush clean boots with Virkon™ solution or dip boots into the container of Virkon™ solution.
    d. Remove hair net and place in a rubbish bag.
    e. Remove overalls, place in a secure plastic bag and tie the bag for storage in the vehicle and disinfection/washing when back to base.

f. Scrub the outside of the box with soap and water if faecal material or dust are present.
g. Brush the outside of the box containing the samples using the Virkon™ solution.
h. Wash hands using soap and water or rub with alcohol-based hand sanitiser.

At the end of the sampling day, used overalls should be disinfected in Virkon™ S if significantly contaminated with faeces or other waste, then washed with standard laundry detergent before being used by samplers on subsequent farms.

Items to be carried in the vehicle for implementing biosecurity practices

The following items are to be carried in the vehicle to implement biosecurity practices when collecting samples from chicken farms, abattoirs and live chicken markets:

- Sufficient number of overalls, hair nets and gloves so that every sampler can wear a clean set for every location visited during a single sampling day.
- Premixed Virkon™ solution carried in a sealed container in which rubber boots can be dipped
- Bucket
- Soap
- Scrubbing brush
- Large container of water
- Disposable paper towels
- Rubbish bag for paper towels, used hair nets and gloves
- Plastic bag for used overalls

9. SOPs, training and trialling sample collection

Once the sampling plan has been agreed, SOPs need to be prepared with details for how to implement sampling at each type of location as follows:

- Biosecurity practices.
- Selection of chickens for sampling.
- Collection of samples from chickens.
- Labelling samples.
- Recording of sample details in sample collection form.
- Sample transport.

Once SOPs have been prepared sample collection staff should be trained in all aspects of sample collection as detailed in the SOP.

Following training, staff in each surveillance area should undertake supervised trial sample collections to ensure that they are competent in all aspects of the process.

10. Review sampling plan

The experience with sample collection and results of antimicrobial susceptibility testing should be reviewed after the first 2-3 months of sampling. If necessary adjust the sampling plan based on findings of the review.

Some examples of points to consider in the review are presented below.

1. Can the laboratory manage the number and frequency of samples being collected?
2. Is the quality of samples being received by the laboratories suitable for diagnostic testing?
3. Does the prevalence of each bacterial genus or species match what is expected?
4. If the prevalence is lower than expected, is this because of sample quality, quality of reagents used in the laboratory, and/or quality of sample processing, culture and/or identification methods in the laboratory?
5. If the prevalence is higher than expected, is this because of cross-contamination between samples at some point in the sample collection and laboratory testing process, or recording errors?

11. Responsibility of national AMR reference and regional/provincial surveillance laboratories

11.1. Regional/provincial surveillance laboratories

Culture, identification and AST should be conducted for each bacterial isolate against the panel of antimicrobials listed in Table 3, using disk diffusion or broth dilution methods (including automated). Disk diffusion is not a suitable method to test for resistance to colistin and AST for colistin should be conducted at the national AMR reference laboratory using minimum inhibitory concentration or automated analysis.[12]

Detailed instructions for culture and identification of the target bacteria are provided in Section 12.

Detailed instructions for AST are not provided in this document and should be obtained from the EUCAST or CLSI guidelines. The national AMR surveillance committee needs to decide which system will be used and ensure harmonisation across both human and animal diagnostic laboratories in the country. Laboratories should ensure that they are using the most up to date version of the guidelines. EUCAST may be preferred as a subscription is not required.

Given that the major objective of conducting AST in bacteria in this surveillance programme is to determine resistance of the bacteria if they are transmitted to humans, the guidelines for AST relating to humans need to be used.

In addition to testing resistance patterns in pure isolates of the targeted bacterial species/groups, each sample should be tested for the presence of Extended Spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* by plating directly onto selective media (see Section 12 for details).

11.2. AMR reference laboratory

For all isolates, manual (e.g. Analytic Profile Index (API) or other biochemical testing) and/or automated methods (e.g. mass spectrometry, Vitek II system, BD Phoenix system) for identification and susceptibility testing are acceptable.

[12] EUCAST, 2016. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group.

11.2.1. Colistin

AMR reference laboratories will conduct testing for resistance to colistin in *E. coli* and *Salmonella spp* using an automated analyser or minimum inhibitory concentration methods as disk diffusion or e-test methods are not reliable for colistin.[13]

11.2.2. ESBL and carbapenemase resistance

Detection of extended-spectrum β-lactamase (ESBL)-producing and carbapenem-resistant bacteria in animal populations is very important given the critical importance of third generation cephalosporins to human medicine and growing concern about carbapenem resistance.

The AMR reference laboratory should perform confirmatory phenotypic testing for ESBL production and carbapenem resistance on any *Salmonella* and *E. coli* isolate that show resistance to cefotaxime or ceftazidime in the initial testing conducted by surveillance laboratories.

Additional testing should be performed to differentiate the resistance pattern into one of the following four categories: (1) ESBL phenotype, (2) AmpC phenotype, (3) ESBL + AmpC phenotype, 4) carbapenem phenotype.[14]

AMR reference laboratories will receive training in these additional testing methods.

11.2.3. Salmonella serotyping

Where possible, *Salmonella* isolates from humans and animals should be serotyped as this is an important first step to identifying any association between resistance patterns in *Salmonella* in humans and animals. This may be conducted at both human and animal health reference laboratories if they have the capacity, or it may be conducted at a single national *Salmonella* reference laboratory.

[13] EUCAST, 2016. Recommendations for MIC determination of colistin (polymyxin E) As recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group

[14] EUCAST Guidelines for detection of resistance mechanisms and specific resistances of clinical and/or subclinical importance, 2013.
http://aurosan.de/images/mediathek/servicematerial/EUCAST_detection_of_resistance_mechanisms.pdf

12. Laboratory testing

Laboratories should develop standardised operating procedures (SOPs) for each aspect of sample processing, bacterial identification, AST, reporting, storage and transport. SOPs should take into account local capacity and should be developed with reference to national /international guidelines (for example, https://www.gov.uk/government/collections/standards-for-microbiology-investigations-smi#bacteriology). Laboratories should develop identification pathways for the relevant bacteria: the methods chosen will depend on availability of equipment/reagents, and staff familiarity with procedures. Some guidance is given below, but this should not replace the development of in-house SOPs.

12.1. Sample preparation and enrichment

For *E. coli, Salmonella spp* and *Enterococcus spp*:

Pulverise the caecum and caecal contents in each bag with a rubber mallet and mix well

Add 1 gram of a caecal or faecal sample to 9 ml sterile **Buffered Peptone Water (BPW)** in a 50 ml sterile tube with a lid.
Add a cloacal swab to 10 ml BPW in a 50 ml sterile tube with a lid.

Add the boot swabs from an individual farm to a bag containing 225ml BPW.

Mix well but do not shake, to avoid spillage.

For *Enterococcus spp*

Add 1 ml of BPW mixture to approximately 10 ml **Azide Dextrose Broth** (Pleydell et al., 2010)
Incubate aerobically at 35 °C for 18-24 hours

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For *E. coli, Salmonella spp*:

Incubate the 10ml BPW mixture (or the remaining 9 ml if sub-sampled for *Enterococcus*) aerobically at 34-38ºC for 16-20 hours

Continued next page

For C*ampylobacter* spp (ISO 10272-1:2017):[15]

Dip a sterile swab in the bag of well mixed caecal content
Or
Take the faecal swabs already collected in charcoal-containing transport media

Add the caecal or faecal swab to 10 ml of Bolton broth in a 50 ml sterile tube with a lid.

Faecal swabs collected from the same farm can be pooled into one tube with 10 ml Bolton broth

Incubate at 42°C for 48 hrs in a microaerobic atmosphere using one of the options below:

1. a microaerobic gas pack (such as CampyGen ™ or CampyPak ™) in an anaerobic jar
2. an anaerobic jar gassed with pre-mixed microaerobic (5% O_2, 10% CO_2, 85% N_2) gas mix (Hunt et al., 2001)
3. a variable atmosphere incubator if one is available in the laboratory.

Continued on page 48

[15] https://www.sis.se/api/document/preview/922034/

12.2. Isolation and identification

For *E. coli, Salmonella spp*:
Continued from incubation of BPW enrichment mixture

To screen for ESBL-producing Enterobacteriaceae:

Subculture

Inoculate the enriched BPW onto <u>one</u> of the three types of agar listed below using a sterile swab or 10 ul inoculum and streak for single colonies.

1. CHROMagar™ ESBL or Brilliance ™ ESBL agar
2. MacConkey agar mixed with 1 mg/L of ceftriaxone
3. MacConkey agar mixed with 1 mg/L ceftazidime PLUS 1 mg/L cefotaxime.

Bacterial growth on any of these plates is indicative of possible ESBL-mediated resistance.

We recommend using CHROMagar, but either of the alternative approaches is acceptable if you are already using these in your lab. Ensure you calculate consumables for the one option.

Incubate aerobically at 37 °C for 18-24 hours

Isolate purification

If there is growth on the selective plate, subculture one typical *E. coli* and one *Klebsiella pneumoniae* colony to non-selective media such as **Blood agar** or **Nutrient agar**.

Incubate at 35 °C for 18-24 hours, aerobically.

Continued next page

For *E. coli*:

Subculture

Inoculate the enriched BPW onto MacConkey agar using a sterile swab or 10 ul inoculum and streak for single colonies.

Incubate plates aerobically at 37°C for 18-24 hours.

Incubate at 37 °C for 18-24 hours, aerobically.

Isolate purification

Subculture one typical *E. coli* colony from the plate to non-selective media such as **Blood agar** or **Nutrient agar.**

Incubate at 35 °C for 18-24 hours, aerobically.

Continued next page

For *Salmonella spp (ISO 6579-1*:2017)

Enrichment in selective media

Either of the following two options:

1. Transfer 100 µl of enriched BPW to 10 ml of warmed Rappaport-Vassiliadis soy peptone.

   Incubate aerobically at 41.5 °C, preferably in a waterbath, for 21-27 hours.

   Or
2. Transfer 1 ml of enriched BPW to 10 ml of tetrathionate broth + iodine.

   Incubate aerobically at 35 °C for 20-24 hours.

Subculture

Subculture selective broths to XLD with novobiocin.

Incubate aerobically at 37 °C for 21-27 hours.

Isolate purification

Subculture one typical *Salmonella* colony from the plate to non-selective media such as **Blood agar** or **Nutrient agar.**

Incubate at 35 °C for 18-24 hours, aerobically.

Continued next page

For ESBL-producing Enterobacteriaceae:

Identification

E. coli can be identified by the following methods:

1. Initial growth/fermentation indicators (oxidase negative, lactose fermentation, able to grow on MacConkey agar), and then
2. Confirmation by further biochemical testing (e.g. indole positive, urease negative). Commercial analytical profile identification (API) systems or triple sugar-iron can also be used.
3. Alternatively, commercial systems (e.g. analytical profile identification (API) or triple sugar-iron testing) can be used.

K. pneumoniae can be identified by the following methods:

1. Initial growth/fermentation indicators (oxidase negative, lactose fermentation, mucoid colony morphology, able to grow on MacConkey agar), and then
2. Further differentiated by additional biochemical testing (e.g. indole negative, urease positive).
3. Alternatively, commercial systems (e.g. API)

For *E. coli:*

Identification

E. coli can be identified by the following methods:

1. Initial growth/fermentation indicators (oxidase negative, lactose fermentation, able to grow on MacConkey agar), and then
2. Confirmation by further biochemical testing (e.g. indole positive, urease negative).
3. Alternatively, commercial systems (e.g. analytical profile identification (API) or triple sugar-iron testing) can be used.

For *Salmonella spp:*

Identification

Salmonella spp can be identified by the following methods:

1. Biochemical testing (e.g. triple-sugar iron agar, API systems) followed by,
2. Serology (Salmonella poly-O and poly-H antisera agglutination)

For *Enterococcus spp*:
Continued from incubation of Azide dextrose broth enrichment mixture

Subculture

Subculture from Azide dextrose broth to Slanetz and Bartley selective agar.

Incubate at 35 °C for 18-24 hours, aerobically.

Isolate purification

Subculture one typical *Enterococcus* colony from the plate to **Blood agar*** or **Nutrient agar**.

Incubate at 35 °C for 18-24 hours, aerobically.

Identification

Biochemical testing: catalase negative**, PYR and bile aesculin positive.

There is no need to identify *Enterococci* to species level at this stage, although species can be inferred from susceptibility/non-susceptibility to amoxicillin and quinupristin/dalfopristin

* If using blood agar, note that Enterococcus can be α-, β- or non-haemolytic, and haemolysis pattern therefore cannot be used for identification.

** Some strains may be weakly catalase positive

For *Campylobacter spp:*
Continued from incubation of Bolton broth enrichment mixture

Isolate purification

Subculture one typical *Campylobacter* colony from the plate to **Blood agar**.

Incubate in microaerophilic conditions at 42 °C for 24-48 hours.

Identification

Campylobacter can be identified by characteristic colony morphology (oil-slick appearance) on charcoal-containing agar, oxidase positivity and Gram stain appearance (seagull morphology.

Further identification may not be required, however, *C. jejuni* can be distinguished from *C. coli* using a Hippurate test. *C jejuni* are usually hippuricase positive while *C. coli* are not.

12.3. Antimicrobial susceptibility testing

Test isolates for antimicrobial susceptibilities to the panel of antimicrobials listed in Table 3 using disk diffusion as per EUCAST or CLSI guidelines.

12.4. Isolate storage

Store ALL isolates regardless of AST results on an agar slant.

12.5. Isolate transport to reference laboratory

All isolates should be safely transported to the AMR reference laboratory once a month for additional testing and storage in a national biorepository.

13. Proficiency Testing and use of ATCC strains for Internal Quality Assurance

13.1. Proficiency testing

A system for proficiency testing should be in place in AMR reference and surveillance laboratories to ensure reliable diagnostic results are produced in laboratories after training in culture, identification and antimicrobial susceptibility testing has been completed, and once the laboratories have received their good quality reagents and consumables.

Suggested steps towards proficiency testing are described below.

1. The reference laboratory should first ensure they can produce repeatable AST results by testing each of the target bacteria against all the antibiotics listed in Table 3 for each bacteria in the panel. Each bacteria and antibiotic should be tested in triplicate.

   The laboratory should ensure it can produce repeatable results before developing the proficiency testing panel to send to the other laboratories.

2. Initially, the AMR reference laboratory should send a panel of known isolates of the target bacteria relevant to each surveillance laboratory, with their identity and resistance profile disclosed, and request the laboratory to test each against the full panel of antibiotics listed for each isolate. This will enable the laboratories to test that they can identify the bacteria and achieve the known AST results.

   A standard form should be sent with each panel of isolates for the regional laboratories to complete the AST results, to ensure standardisation in the reporting of results across all laboratories.

3. Subsequently an isolate with identity and resistance undisclosed should be sent by the AMR reference laboratory with the request to culture and identify the bacteria and test against the appropriate panel of antibiotics.
4. Laboratories should report the results to the reference laboratory. If the results are correct, sampling may proceed. If the results are incorrect, the reference laboratory should review and recommend corrective actions to be taken, before repeating the proficiency testing.
5. This process may be repeated quarterly in the first year to ensure the laboratories are performing reliably. Subsequently, the proficiency testing may be conducted at longer intervals.

Any issues should be addressed after each round of proficiency testing.

AMR reference laboratories should participate in an External Quality Assurance Scheme (EQAS) to ensure they are producing reliable results.

13.2. Use of ATCC strains in Internal Quality Assurance

ATCC strains should be tested once a week, and additionally for every new batch of media.

The zone diameter should be recorded for each ATCC strain each time it is tested. This information should be examined for consistency.

Any issues identified in the reliability of testing should be investigated and rectified before further testing is conducted for the AMR surveillance programme.

Caring for your ATCC strains

Note: a bacterial culture should never be sub-cultured more than 5 times.
It is important to make aliquots of the ATCC strains when they are first received, and to sub-culture each aliquot a maximum of 5 times then autoclave and discard it.

References

Afema J.A., Byarugaba D.K., Shah D.H., Atukwase E., Nambi M, Sischo W.M., 2016. Potential sources and transmission of Salmonella and antimicrobial resistance in Kampala, Uganda. PLOS ONE DOI:10.1371/journal.pone.0152130.

Bester, L.A., Essack, S.Y., 2008. Prevalence of antibiotic resistance in Campylobacter isolates from commercial chicken suppliers in KwaZulu-Natal, South Africa. Journal of Antimicrobial Chemotherapy 62, 1298-1300.

Bester, L.A., Essack, S.Y., 2012. Observational Study of the Prevalence and Antibiotic Resistance of Campylobacter spp. from Different Chicken Production Systems in KwaZulu-Natal, South Africa. Journal of Food Protection 75, 154-159.

Carrique-Mas J.J., Bryant J.E., Cuong N.V., Hoang N.V.M., Dung T.T.N., Duy D.T., Hoa N.T., Thompson C., Hien V.V., Phat V.V., Farrar J., Baker S., 2014. An epidemiological investigation of Campylobacter in pig and chicken farms in the Mekong delta of Vietnam. Epidemiology and Infection 142, 1425-1436.

Funk, Julie A., Davies, Peter R. and Nicholls, Monica A., 2000. The effect of fecal sample weight on detection of *Salmonella enterica* in swine feces. Journal of Veterinary Diagnostic Investigation 12, 412–418.

Hunt, J. M., Abeyta, C. & Tran, T. 2001. In Bacteriological Analytical Manual, Center for Food Safety and Applied Nutrition.

Kagambega, A., Thibodeau, A., Trinetta, V., Soro, D.K., Sama, F.N., Bako, E., Bouda, C.S., N'Diaye, A.W., Fravalo, P., Barro, N. 2019. Salmonella spp. and Campylobacter spp. in chicken feces and carcasses in Ouagadougou, Burkina Faso. Food Science and Nutrition 6, 1601–1606.

Lebert, L., Martz, S-L., Janecko, N., Deckert, A. E., Agunos, A., Reid, A., Rubin, J. E., Reid-Smith, R. J., McEwen, S. A. 2017. Prevalence and antimicrobial resistance among *Escherichia coli* and *Salmonella* in Ontario smallholder chicken flocks. Zoonoses Public Health 65, 134–141.

Marshall J., Heffernan H., Benschop J., Burgess S., 2018. Monitoring and surveillance for antimicrobial resistance in foods – planning phase. Report prepared for the Ministry of Primary Industries, New Zealand. January 2018. Pp 24.

Mathieu J., Aidara-Kane A., Andremont A., 2017. The ESBL Tricycle Antimicrobial Resistance Surveillance Project: A Simple One Health Approach to Global Surveillance. AMR Control 55 – 58. (http://resistancecontrol.info/2017/the-esbl-tricycle-amr-surveillance-project-a-simple-one-health-approach-to-global-surveillance/)

Ngbede, E.O., Raji, M.A., Kwanashie, C.N., Kwaga, J.K.P. 2016. Antimicrobial resistance and virulence profile of enterococci isolated from chicken and cattle sources in Nigeria. Tropical Animal Health Production 49(3), 451-458.

Persoons, D., Bollaerts, K., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Butaye, P., Catry, B., Haesebrouck, F., Dewulf, J. 2011. The importance of sample size in the determination of a flock-level antimicrobial resistance profile for Escherichia coli in broilers. Microbial Drug Resistance 17 (4), 513-519.

Pleydell, E., Rogers, L., Kwan, E. & French, N. 2010. Evidence for the Clustering of Antibacterial Resistance Phenotypes of Enterococci Within Integrated Chicken Companies. Microb. Ecol. 59, 678–688.

Regula, G., Lo Fo Wong, D.M.A., Ledergerber, U., Stephan, R., Danuser, J., Bissig-Choisat, B., and Stärk, K.D.C. 2005. Evaluation of an antimicrobial resistance monitoring program for campylobacter in chicken by simulation. Preventive Veterinary Medicine 70, 29–43.

Tu L.T.P., Hoang N.V.M., Cuong N.V., Campbell J., Bryant J.E., Hoa N.T., Kiet B.T., Thompson C., Duy D.T., Phat V.V., Hien V.B., Thwaites G., Baker S., Carrique-Mas J.J., 2015. High levels of contamination and antimicrobial resistant non-typhoidal Salmonella serovars on pig and chicken farms in the Mekong Delta of Vietnam. Epidemiology and Infection 143, 3074-3086.

Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences of the United States of America 112, 5649-5654.

Yamamoto, T., Hayama, Y., Hidano, A., Kobayashi, S., Muroga, N., Ishikawa, K., Ogura, A., Tsutsui, T., 2014. Sampling strategies in antimicrobial resistance monitoring: evaluating how precision and sensitivity vary with the number of animals sampled per farm. PLOS ONE 9 (1), | e87147.

Annex 1 Sampling frame for abattoirs, slaughter points and live bird markets

[Enter name of the surveillance area]

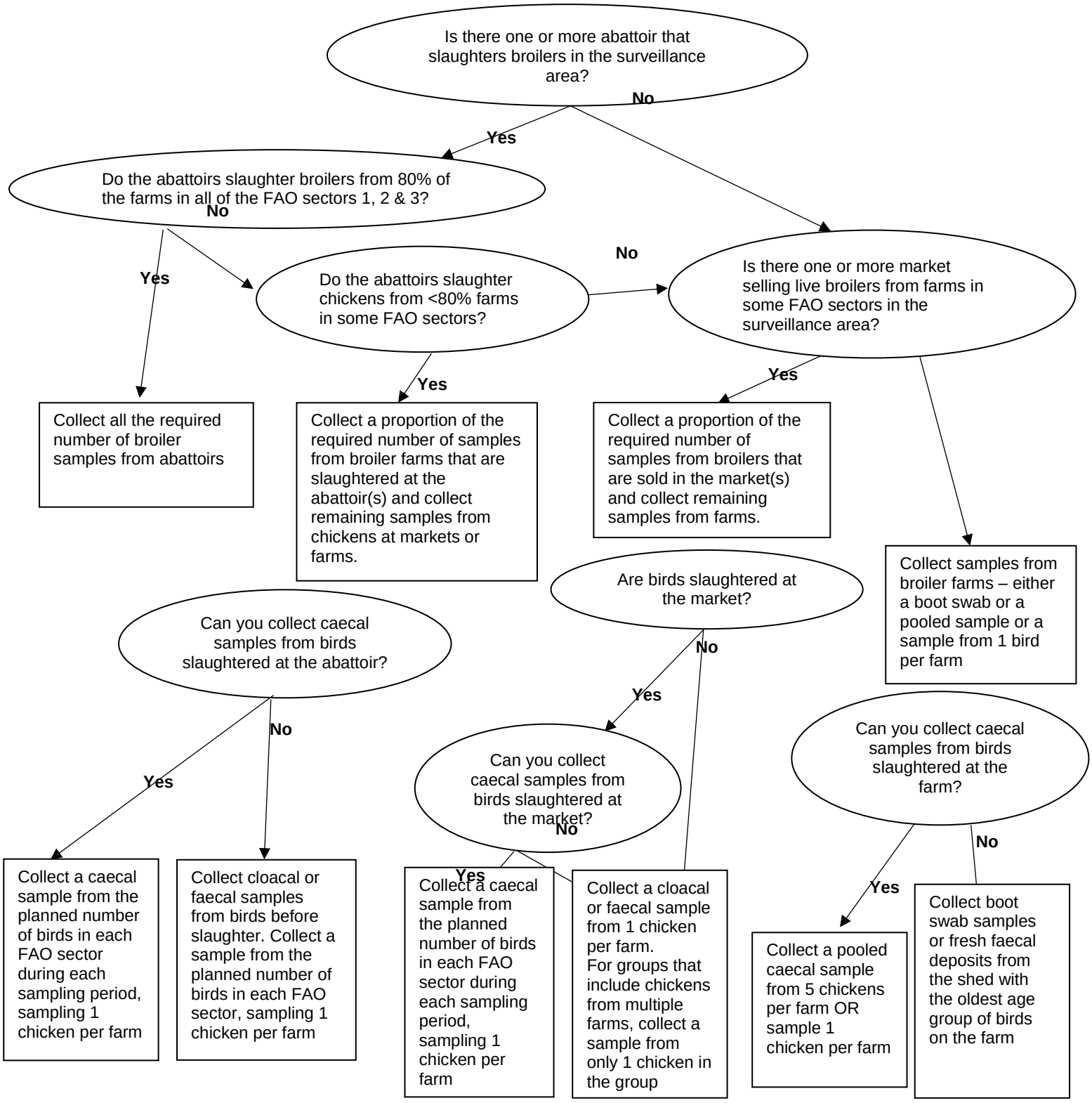
Name of abattoir, slaughter point or LBM	Classification • Abattoir (AB) • Slaughter point (SP) • Live bird market (LBM) • Other (describe)	Location (town name)	Numberof chickens slaughtered or sold per day (approx.)	Catchment area	Number of BROILER farms supplying the location (approx)	If information is available, add whether chickens are from sector 1, 2 or 3 broiler farms	Number of LAYER farms supplying the location (approx)	If information is available, add whether chickens are from sector 1, 2 or 3 layer farms

Definition of FAO sectors.

Sector	Description of FAO Sectors
Sector 1	Industrial integrated system with high biosecurity levels and chicken/products marketed commercially, e.g. farms that are part of an integrated broiler production enterprise with clearly defined and implemented standard operating procedures for biosecurity.
Sector 2	Commercial chicken production system with moderate to high biosecurity system and chicken/products usually marketed commercially, e.g. farms with chickens kept indoors continuously, strictly preventing contact with other chickens or wildlife.
Sector 3	Commercial chicken production system with low to minimal biosecurity and chicken/products sold in live chicken markets e.g. a caged layer farm with chicken in open sheds, a farm with chicken spending time outside the shed, a farm producing both chickens and waterfowl.
Sector 4	Village or backyard production with minimal biosecurity and chicken/products consumed locally.

Annex 2 Decision tree to guide selection of sampling locations

Decision tree to guide selection of sampling locations for AMR surveillance using broilers as an example. A similar process should be followed to identify sampling locations for layers.



Annex 3 Example sampling plan table

The table below provides an example of a sampling plan table that shows the number of samples to collect per location. Enter the number of samples to be collected from each location for each FAO sector, for broilers and layers. For farm locations, the number represents the number of farms to be sampled in each area.

Sampling plan for broilers and layers in [Enter name of the surveillance area]

Enter number of samples to be collected from each location in the cells below.

Location Identifier	**Locations by type**	FAO Sector 1 (broilers)	FAO Sector 2 (broilers)	FAO Sector 3 (broilers)	**Total broilers**	FAO Sector 1 (layers)	FAO Sector 2 (layers)	FAO Sector 3 (layers)	**Total layers**
L1	AB 1	# chickens	# chickens	# chickens		# chickens	# chickens	# chickens	
L2	AB 2	# chickens	# chickens	# chickens		# chickens	# chickens	# chickens	
L3	SP 1	# chickens	# chickens	# chickens		# chickens	# chickens	# chickens	
L4	LBM 1	# chickens	# chickens	# chickens		# chickens	# chickens	# chickens	
L5	LBM 2	# chickens	# chickens	# chickens		# chickens	# chickens	# chickens	
L6	LBM3	# chickens	# chickens	# chickens		# chickens	# chickens	# chickens	
L7	Farm area 1	# farms	# farms	# farms		# farms	# farms	# farms	
L8	Farm area 2	# farms	# farms	# farms		# farms	# farms	# farms	
L9	Farm area 3	# farms	# farms	# farms		# farms	# farms	# farms	
	TOTAL								

AB = abattoir; SP – slaughter premises; LBM = live bird market

Annex 4 Equipment

Essential equipment

1. Autoclave, with temperature recording device(s), capable of holding 121°C for 30 minutes within a waste load. Separate autoclaves should be used for media preparation and waste disposal.
2. Incubator, 35 °C, aerobic (capable of holding 35 +/- 2 °C)
3. Refrigerator or cold room capable of holding 2-8 °C
4. Freezer, -80 °C for reference laboratories and -20 °C for regional laboratories (not 'frost-free')
5. Calibrated thermometers or temperature recording devices (ones capable of recording maximum and minimum temperatures are preferable), one for each incubator/fridge/freezer
6. Water bath / stirrer capable of holding 42 °C +/- 0.2 °C
7. McFarland 0.5 and 2.0 standards
8. Analytic balance (0.01g)
9. Forceps or disk dispensers for applying antimicrobial disks (note that disk dispensers are brand-dependent)
10. Coplin jars or similar for ethanol sterilizing scissors/clippers/forceps
11. Metal scissors/clippers (for cutting swabs)
12. Bunsen burner(s) or Bacti Cinerator (or similar)
13. Callipers or ruler (for measuring zone sizes)
14. Loops, nichrome or disposable plastic
15. Calibrated pipette capable of measuring 100 µl
16. Oil-immersion light microscope
17. Oven or microwave oven for drying desiccant / plates
18. Conductivity meter for water QC
19. pH meter for water QC
20. Quality control organisms as specified by the relevant EUCAST or CLSI standards

21. Triple packaging specimen transport kits (UN 2814 compatible)

Additional recommended equipment

1. Campygen system and anaerobic jars for culture of *Campylobacter spp.*
2. Incubator, 42 °C, aerobic (capable of holding 42 +/- 2 °C) for culture of *Campylobacter spp.*
3. Stainless Steel Petri Dish Can
4. Carboy for dispensing distilled water

Annex 5 Working example of sample size calculations.

Example with four surveillance areas

Total number of broiler samples (400)

Assumed equal distribution of broiler samples at sites

Number of broiler samples per surveillance area (e.g 4 Sites)

(100+100+100+100) =400

- S_B1 = number of broiler samples for surveillance area 1.
- $Se_{B1}1$ = number of broiler samples for FAO Sector 1 in Surveillance area S_B1
- Example proportions in FAO sectors 1 – 3 are:

P1=0.2, P2=0.3, P3=0.5

Number of broiler samples from each FAO sector in the surveillance area S_B1

$S_B1 = Se_{B1}1 + Se_{B1}2 + Se_{B1}3$

$S_B1 = (S_B1*P1)+ (S_B1*P2)+(S_B1*P3)$

((100*0.2)+(100*0.3)+(100*0.5)) =100

- Example proportion of samples for each sector from each location are:
- $Se_{B1}1$ (Sector 1): L1=0.5, L2=0.3, L3=0.2.
- $Se_{B1}2$ (Sector 2): L1=0.2, L2=0.5, L3=0.3
- $Se_{B1}3$ (Sector 3):L1=0.1, L2=0.3,L3=0.6
- This example assumes only 3 locations but there could be many more or only 2. This will depend on each country

Number of broiler samples from each FAO sector from each location in the surveillance area S_B1

$Se_{B1}1= Se_{B1}1*L1+ Se_{B1}1*L2+ Se_{B1}1*L3$

$Se_{B1}1= ((20*0.5)+(20*0.3)+(20*0.2))=20$

$Se_{B1}2 = ((30*0.2)+(30*0.5)+(30*0.3))=30$

$Se_{B1}3 = ((50*0.1)+(50*0.3)+(50*0.6))=50$

Example with three surveillance areas

- National level
- **(Section 7.1.1)**

Total number of broiler samples (400)

- Surveillance area
- **Section 7.1.2**

Number of broiler samples per surveillance area (e.g 3 Sites)

(133+133+134) =400

- Surveillance area + FAO sector level

Number of broiler samples from each FAO sector in the surveillance area S_B1

$S_B1 = Se_{B1}1 + Se_{B1}2 + Se_{B1}3$

$S_B1 = (S_B1*P1)+ (S_B1*P2)+(S_B1*P3)$

((133*0.2)+(133*0.3)+(133*0.5)) =133

- Surveillance area FAO sector + location

Number of broiler samples from each FAO sector from each location in the surveillance area S_B1

$Se_{B1}1= Se_{B1}1*L1+ Se_{B1}1*L2+ Se_{B1}1*L3$

$Se_{B1}1= ((27*0.5)+(27*0.3)+(27*0.2))=27$

$Se_{B1}2 = ((40*0.2)+(40*0.5)+(40*0.3))=40$

$Se_{B1}3 = ((66*0.1)+(66*0.3)+(66*0.6))=66$

Annex 6 Guidelines to prepare a country-specific AMR surveillance plan to be supported by the Fleming Fund

Recommended chapter headings and content for a country-specific AMR surveillance plan in poultry to be funded by the Fleming Fund Country Grant are presented below.

CHAPTER 1: INTRODUCTION

- Background about AMR (situation in livestock; worldwide, [enter name of region] and in [enter country name]).
- Problem statement (Why is AMR an issue in[enter country name]).
- Justification (Why is it necessary to do this AMR surveillance).
- Scope (The scope will be zoonotic bacteria carried in the gut of healthy chickens).
- Aim of active AMR Surveillance in commercial broilers and layers in[enter country name].
- Objectives of the poultry AMR surveillance in [enter country name] e.g to estimate prevalence of resistance to specific antibiotics amongst the isolates of each bacteria/bacterial group etc.

(Refer to Section 2 of the AMR surveillance protocol for examples of objectives)

CHAPTER 2: TARGET POPULATIONS, LABORATORIES, SURVEILLANCE AREAS, BACTERIA AND ANTIMICROBIALS

- **Surveillance type:** Active surveillance.

(*See Introduction of the AMR surveillance protocol*)

- **Surveillance area**: i.e settings (Districts selected for surveillance).

Name the laboratories included in the Fleming Fund AMR surveillance network, that will conduct AST and define the geographic area that each of the laboratories will be in charge of surveying (each laboratory is in charge of a “surveillance area”).

(Refer to Section 5 of the AMR surveillance protocol)

- **Target population**.

Target population:

- which species and production types (broiler and/or layers) will be included.

- which FAO sectors will be included (FAO sectors 1 – 3).

(Refer to Section 4 of the AMR surveillance protocol)

CHAPTER 3: SAMPLING PLAN

A separate sampling plan should be prepared for broilers and layers for each surveillance area.

(*Refer to Sections 6 and 7 of the AMR surveillance protocol*)

- **Sampling locations** (sampling points for the target population eg. abattoirs, live bird markets, farms).

Indicate the type of location(s) from which samples will be collected in the surveillance areas: abattoirs? markets? farms? other? One type of location or several?

- **Sampling frame**: The sampling frame is a list of sampling locations within the surveillance areas from which samples will be collected (i.e. this can be a list of abattoirs, markets, farms or other sources mentioned for sampling).

(Refer to Annex 1 of the AMR surveillance protocol for a template to prepare a sampling frame).

In order to allow sampling proportional to size (see below) within the surveillance area, the sampling frame should provide details of geographic location and FAO sector (for abattoirs, markets and farms), size (for abattoirs and markets), and catchment area for abattoirs, slaughter points and markets.

If sampling on farms: The sampling frame for stage 1 (R*efer to Section 7.3 of AMR surveillance protocol*) should include a list of the administrative areas. For stage 2, if a list of farms is not available as a sampling frame for selecting farms within the chosen administrative areas, explain how farms will be selected. *(Annex 2 in the AMR surveillance protocol can guide the selection of sampling locations)*

- **Sample size** (**number of samples – section 7)**.

As the aim is to take one sample per farm, the total number of samples = number of farms to sample. Even if samples are taken at markets or abattoirs the aim is for each sampled chicken to have been raised in a different farm, and for each farm to be sampled once throughout the surveillance period.

Within each surveillance area, sampling is based on a stratified sampling proportional to size approach. The two strata which are sampled proportional to size are the FAO sector and the sampling location.

The following numbers need to be calculated (as illustrated in the figure below):

- Total number of samples per production type (i.e. for layers and broilers).

(*Refer to section 7.1.1 of the AMR surveillance protocol for details on the total sample size per production type)*

- Number of samples per production type and surveillance area (Number of samples that each laboratory will process): decide whether each laboratory will be responsible for equal numbers of samples or a number proportional to the proportion of chickens produced in the laboratories' surveillance area, or a number decided according to laboratory capability and capacity.

(*See Section 7.1.2 of the AMR surveillance protocol for guidelines).*

- Within each surveillance area, number of samples per production type and stratum (e.g. FAO sector).

(*See Section 7.1.3 of the AMR surveillance protocol for guidelines)*

- Within each FAO sector within each surveillance area, number of samples per sampling location, ensuring that for each FAO sector, sampling probability is roughly proportional to the number of chicken farms from each FAO sector that are supplying the location.

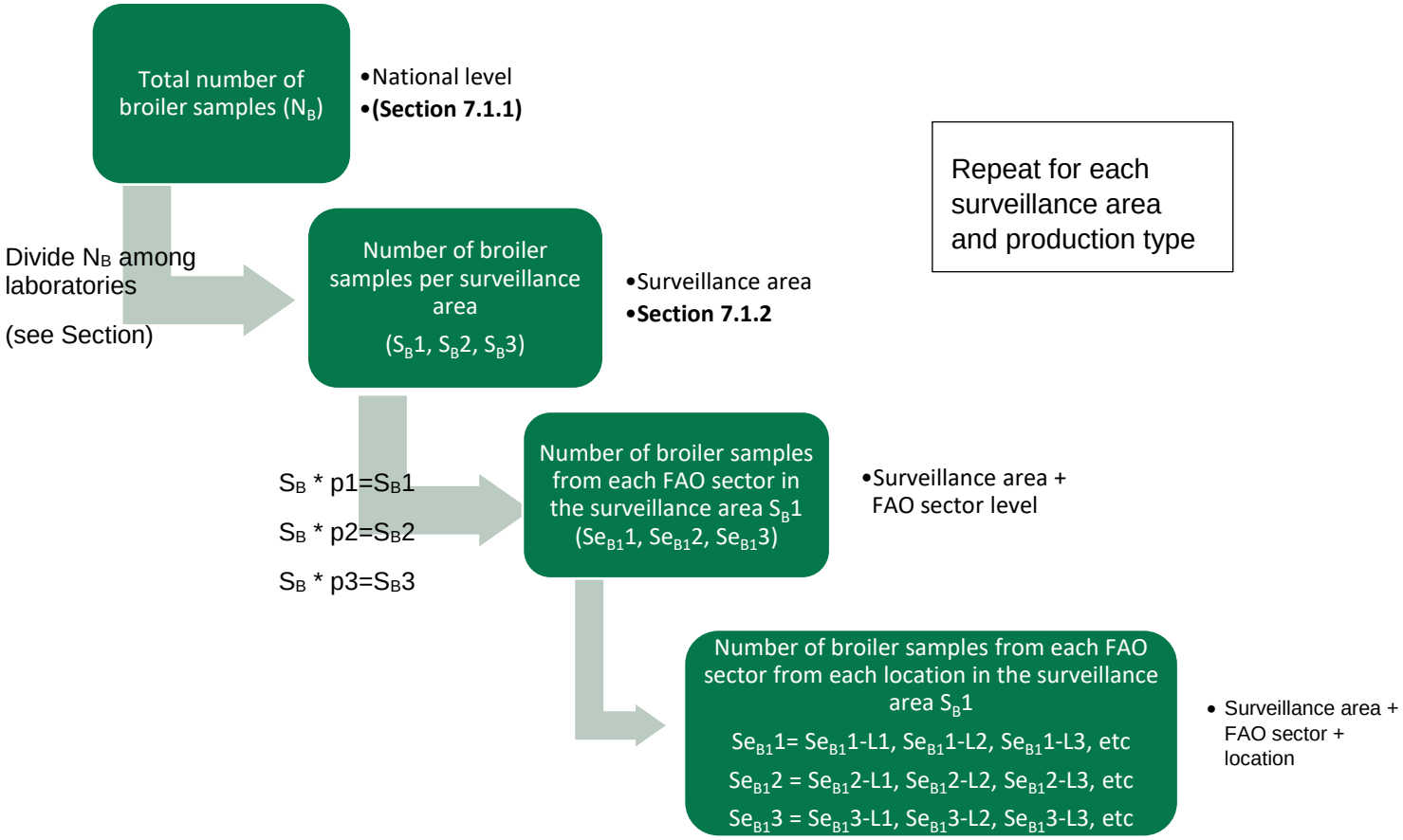
(See Section 7.1.4 of the AMR surveillance protocol for guidelines).

- As much as possible, only one sample should be taken per farm, whether sampling is done at abattoirs or markets. If chickens from multiple farms are grouped together, with no way of knowing their origins (e.g. at a live bird markets with poor traceability), one sample is taken from the group of chickens. As every country is different, it is not possible to provide standard advice on how to select chickens. Knowledge of the local chicken production and marketing system are absolutely key to appropriate selection of chickens.

  A single pooled sample may be collected from farms.

(Refer to Section 7.2 and 7.3 of the AMR surveillance protocol for details)

Example of sample size calculation at the four different levels: national, surveillance area, FAO sector and location:



p1, p2, p3: proportion of FAO sectors 1, 2, 3 farms in surveillance area considered. These proportions do not need to be exact. However, it is important to ensure that the proportion of samples from each FAO sector within the sample population is roughly equivalent to the proportion in the population – e.g. a few, many, very many.

- **Sampling location selection method**.

(See section 7.4 of the AMR surveillance protocol)

- **Prepare a sampling timetable**.

  Prepare a timetable for collecting samples, taking into consideration the following logistical factors.

  - Days of sample collection.
  - Number of samples per sampling day.
  - Frequency of sample collection.
  - Sampling locations.

(*Refer to section 7.7 of the AMR surveillance protocol for details*)

- **Sample collection, packaging, labelling, transportation, storage and disposal**.

At this stage the following should be detailed:

  - Sampling plans.

(*Refer to AMR surveillance protocol for details and examples on Abattoir/slaughterhouse/on farm/market sampling plans*)

  - The type of sample to be collected from each location: caecum or faecal or other; this may differ according to sampling location.

(*Refer to Section 7.4 of the AMR surveillance protocol for details and SOPs for collection*)

  - Biosecurity practices when collecting samples adapted to sampling locations and risks.

(R*efer to Section 8 of the AMR surveillance protocol for clarification*)

  - Sample labelling methods.
  - Sample packaging.
  - Sample transportation methods.
  - Sample collection form (develop questionnaire and attach in annex).

(*Refer to Section 7.5 AMR surveillance protocol for details and a draft that can be modified to country-specifics)*

  - Create a checklist (and attach in annex) of equipment and materials to take when collecting samples to minimise the chances of forgetting things.

CHAPTER 4: LABORATORY PROCEDURES (*refer to Sections 11, 12 and 13 of the AMR surveillance protocol for details)*

In this section the following need to be described and detailed:

- Selection of bacteria included in the plan.
- Sample processing (written SOPs): how the sample ID and details will be recorded, stored before identification, how and when the sample is to be stored or transported or discarded, etc.
- Laboratory technique for culture and identification of selected bacteria (SOP for *E. coli, Salmonella, Campylobacter, Enterococcus*). Example SOPs which can be adapted for local use can be found at https://www.gov.uk/government/collections/standards-for-microbiology-investigations-smi#bacteriology

- Panel of antimicrobials to be tested for selected bacteria (develop in table form).

(*Refer to Table 3 in the AMR surveillance protocol*)

- Methods for Antimicrobial Susceptibility Testing (AST) (SOP).
- Criteria for interpretation of AST (CLSI or EUCAST).
- Reporting format (quantitative - zone diameter (mm) and/or MIC).
- Quality Assurance (QA) and Quality control (QC) of AMR testing.
- Laboratory data management (LIMS, WHONET).
- Detail the EQAS and proficiency testing programme for all laboratories.

(*See Section 13 of the AMR surveillance protocol*)

CHAPTER 5: DATA MANAGEMENT AND QUALITY CONTROL FOR AMR FIELD AND LABORATORY DATA

In this chapter, the following procedures should be detailed and described:

- Data entry methods and responsibilities (Laboratory and field staff).
    - How will field data (demographic data) collected for each sample be stored (WHONET? Other software?) and who will enter the data?
    - How will AST results be stored in the laboratories and who will enter the results?
- WHONET or an alternative database system will have been established in each laboratory for storing the AST results and demographic data collected for each sample and staff trained in its use.
- SOP for AMR data transfer from regional laboratories to the AMR reference laboratory. Details: what data, how to extract from the regional laboratory database, how to send to the reference laboratory, how often, who is responsible. Are any transfer agreements/MOU needed?
- Details for data transfer between reference laboratory and the person/team in charge of data analysis. Are any transfer agreements/MOU needed?
- Data collation and validation: methods and responsibilities.
- Data management and analysis: methods and responsibilities.
- Interpretation: methods and responsibilities.
- Dissemination of data: methods and responsibilities. Who will be responsible for sharing, what information, with whom, how often, etc. Are any specific agreements/MOU needed?

CHAPTER 6: ROLES AND RESPONSIBILITIES OF THE ANIMAL HEALTH SERVICES

- Roles and Responsibilities of laboratories, field veterinary services and national veterinary services (Who does what?).
    - National Reference laboratory.

(*Refer to Section 11 of the AMR surveillance protocol*)

    - Surveillance sites (or « regional laboratories »).

(*Refer to Section 11 of the AMR surveillance protocol*)

    - Field veterinary services (e.g. sample collection).
    - Epidemiology unit (e.g. sampling plan design, data analysis and reporting).
- Ethics: ethics approval must be sought according to national guidelines, confidentiality of farmers' data should be ensured.
- Annexes (including schematic drawing of a sampling and testing strategy).