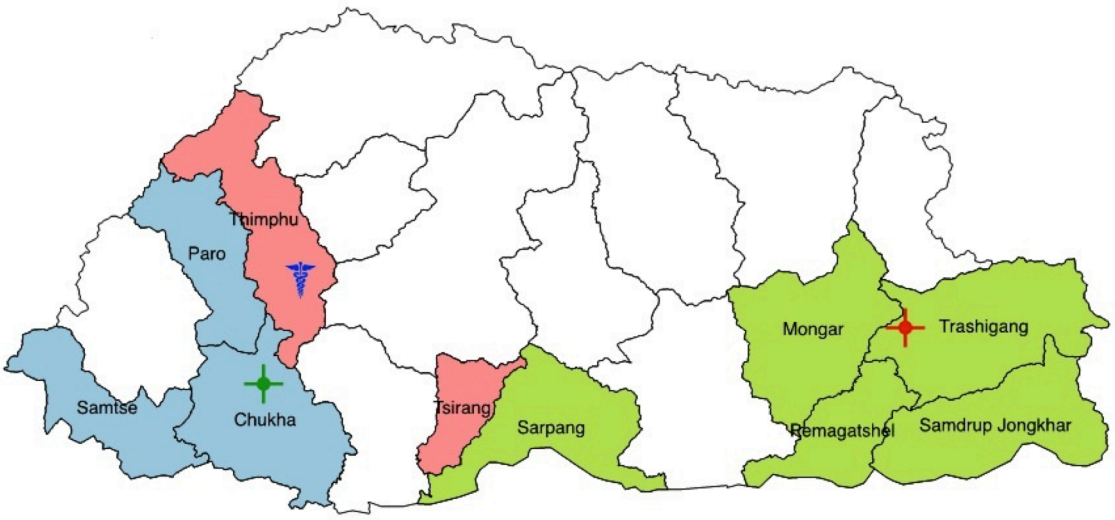
Surveillance Plan for AMR in Commercial Broiler and Layer Chicken Population in Bhutan



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GLOSSARY

1. Acronyms

AMR	Antimicrobial resistance
AGISAR	WHO Advisory Group on Integrated Surveillance of Antimicrobial resistance
AmpC	AmpC beta-lactamases
AMU	Antimicrobial use
API	Analytic Profile Index
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
BAFRA	Bhutan Agriculture and Food Regulatory Authority
CBP	Clinical Breakpoint
CLSI	Clinical and Laboratory Standards Institute
DRA	Drug Regulatory Authority
ECOFF	Epidemiological Cut-off Values
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
GPS	Global Positioning System
GLASS	Global AMR Surveillance System
G2C	Government to Citizen
IQAS	Internal Quality Assurance System
LIMS	Laboratory Information Management System database
MIC	Minimum Inhibitory Concentration
MoH	Ministry of Health
MOU	Memorandum of Understanding
NCAH	National Centre for Animal Health
NVL	National Veterinary Laboratory
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
QC	Quality Control
RLDC	Regional Livestock Development Centre
SOP	Standard operating procedures
WHO	World Health Organization

2. Definitions

Dzongkhag: District
Gewog: County

Laboratory Information Management system (LIMS): Laboratory Information Management System is the online database system designed to efficiently manage the information of all the veterinary laboratory activities in Bhutan

WHONET:WHONET is free Windows-based database software developed for the management and analysis of microbiology laboratory data with a special focus on the analysis of antimicrobial susceptibility test results.

CHAPTER 1: INTRODUCTION

Antimicrobial resistance (AMR) is a global health concern as new resistance mechanismsare emerging and resistant infections are spreadingglobally, threateningour ability to treat common infectious diseases, resultingin prolonged illness, disability, and death. It is increasing at an alarming rate and has become one of the important global public health challenges. Currently, 0.7 million people die of resistant infections every year(O'Neill, 2014). It is estimated that by 2050, drug-resistant infections could cause 10 million deaths per year costing up to 100 billion USD globally if we do not find a proactive solution now to slow down the rise of drug resistance(O'Neill, 2016).

Bhutan is equally affected by drug resistance emergence and its spread. Despite various policies, institutional mechanisms and implementation strategies put in place to rationalize the use of antibiotics, there are reports of antimicrobial resistance in human as well as in animal sectors. For instance, two ESBL (CTX-M-15) producing *E. coli* strains were isolated amongst 83 faecal samples from breeding pigs (Sharma et al., 2017). Isolates of *Salmonella* from imported chicken carcasses, showed resistance to nalidixic acid (96%) amoxicillin (12%), cephalexin (6%), ciprofloxacin (2%) and sulphamethoxazole-trimethoprim (2%)(Ellerbroek et al., 2010). Further, with the increasing demand for livestock and livestock products, the establishment of intensive livestock farming to meet the government's drive to food self-sufficiency, the use of antibiotics is increasing in Bhutan with at least 30% of the total annual medicines budget spent on procurement of antimicrobials.

Antimicrobials are used in animals to maintain animal health and welfare, as well as food security. However, much of their global use is not for treating sick animals but instead either to prevent infections or simply to promote growth. The quantity of antimicrobials used in livestock is enormous and often includes those medicines that are important for human health. While the direct use of antimicrobials in human health is recognised as a major contributor to antimicrobial resistance in human pathogens, there are circumstances where indiscriminate use of antimicrobials in both food-producing and companion animals are key contributing factors(Karen L Tang et al., 2017). Livestock farming is an integral part of livelihood in Bhutan. Among other species, chicken is extensively reared in Bhutan with a total population of 1.10 million constituting 69.45% of the total livestock population (Livestock statistic of Bhutan 2018). With the commercialization of the poultry industry, the use of antimicrobials in poultry have also increased like in other parts of the world.

Surveillance is one of the cornerstones of infectious disease management, however, this has been often ignored and remained under-resourced in the fight against AMR. Surveillance and reporting of antimicrobial resistance and antibiotic usage have become a global health priority. However, there is no formal AMR surveillance system in the livestock sector in the country. Fortunately, Bhutan received the UK government-based Fleming Fund grant which is focusing on building the foundation for AMR and AMU surveillance. Under the current grant, active surveillance of antimicrobial resistance in zoonotic bacteria of healthy broiler and layer chicken population will be carried out. The active surveillance programme is

designed to contribute to strengthening & enabling the 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 active and passive AMR surveillance in all livestock species. The implementation of the surveillance program and information generated thereof will contribute to a One Health approach to AMR surveillance in the country.

The core of an effective surveillance system is the integration of human, animal and agriculture programs within a One Health framework.This surveillance programme will generate 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 Working Group. Multi-sectoral review of the results will help to identify potential links between AMR in humans and animals which can be investigated in-depth through a multisectoral approach future surveillance and research. This document is prepared in line with the guideline provided in the Protocol for active AMR surveillance in commercial broiler and layer chicken populations for the Fleming Fund Country Grants Programme developed by Mott MacDonald(McKenzie et al., 2019).

1.1 Objectives of the poultry AMR surveillance in Bhutan

- To enhance AMR surveillance capacity and skills through strengthening the focal institutions for different components of AMR surveillance system
- To estimate the prevalence of resistance in *Escherichia coli, Salmonella, Campylobacter, Enterococci* from poultry layer and broiler against WHO, OIE and FAO priority antimicrobials.
- To generate baseline information and inform policy decisions on AMR mitigation in animals and humans.
- To strengthen the Institutional linkages & collaboration among One Health stakeholders using One Health approach on AMR.
- To disseminate the information generated from the AMR surveillance in further designing the subsequent phase of AMR surveillance in animals
- To obtain isolates for a national bacterial culture collection (biorepository) for future reference

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

2.1. Surveillance type

Active surveillance of antimicrobial resistance in zoonotic bacteria carried in the gut of healthy broiler and layer chicken population in semi-commercial and commercial poultry farms in three of the surveillance sites under National Veterinary laboratory (NVL) at National Centre for Animal Health (NCAH), Serbithang Regional Livestock Development Centres (RLDC) Tsimashamand Kanglung, Bhutan.

2.2. Surveillance area

The areas in which the samples are collected for surveillance are defined as the *surveillance areas*. The surveillance area is identified based on the location of surveillance site laboratories prioritising high chicken producing area. The National Veterinary Laboratory (NVL), Serbithang and RLDCs Tsimasham and Kanglung are identified as surveillance site laboratories. The microbiology capacity for AMR diagnostic of the surveillance site laboratories has been strengthened through Fleming Fund Country grant. The NVL also serves as National AMR reference laboratory for animal health.

A total of 10 Dzongkhags are selected as a surveillance area. The Dzongkhags falling under the jurisdiction of each surveillance site laboratory are the surveillance area of the respective laboratory. Although Tsirang and Sarpang Dzongkhags do not fall under any surveillance sites laboratories, they are also included as surveillance areas given the high number of poultry farms in these two Dzongkhags. Tsirang Dzongkhag is included as one of the surveillance Dzongkhags of NVL while Sarpang Dzongkhag is allocated for RLDC Kanglung. Each surveillance site laboratory is the in-charge of a surveillance area allocated. The details of the surveillance area for each surveillance site laboratory are given in the table below;

Table 1. Showing the surveillance area (Dzongkhags) of each surveillance site laboratory

Sl.No.	Surveillance site Laboratory	Surveillance Area (Dzongkhags)	Incharge of the Surveillance Area
1.	NVL Serbithang	Thimphu, & Tsirang	NCAH Serbithang
2.	RLDC Tsimasham	Chhukha, Samtse & Paro	RLDC Tsimasham
3.	RLDC Kangling	Mongar, Trashigang, Pemagtashel,Samdrupjongkhar& Sarpang	RLDC Kangling

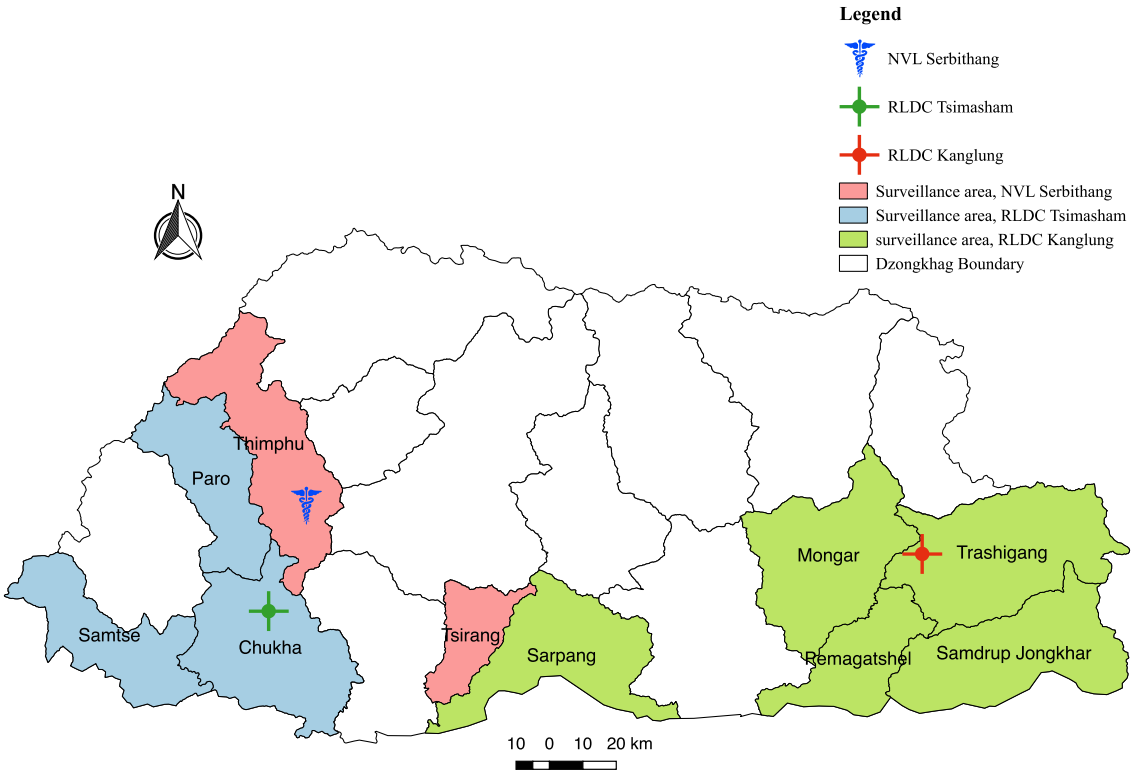

Figure 1. Map showing locations of the surveillance site laboratories and the surveillance areas

2.3 Target population

The target population is the entire set of units for which the surveillance data are to be used to make inferences. All the healthy broiler and layer chickens at the end of their production cycle intended for food consumption in the semi-commercial and commercial farms of the surveillance area are identified as the target population. Chicken is selected for the first phase of the Fleming fund AMR surveillance in animals since the consumption of chicken products is generally high compared with other protein sources and chickens are an important source of foodborne infection globally. Further, antimicrobials are widely used in this sector including some of which is a concern to human health (Van Boeckel Thomas et al., 2015). Therefore, it is assumed that the potential risks for contributing to AMR in humans are highest in chickens compared with other livestock species for the country as is the case for most countries.

As an important objective is to identify resistant bacteria and resistance elements that may spread from chickens to human via the food chain, samples will be collected from chickens sold for meat at the end of their production cycle as near as possible to the point at which they enter the food chain. Therefore, the broiler and layer birds will be sampled at the age of 45 to 50 days and 60 to 70 weeks respectively. Further, the sample will be only collected from healthy chickens. Sampling sick chickens will be avoided as these may not represent the status of resistance in bacteria carried by healthy chickens that enter the food chain.

The focus of this surveillance is on the semi-commercial and commercial broiler and layer production systems, prioritising the high chicken-producing areas in the country. The definition of semi-commercial and commercial of broiler and layer chicken farm is based on classification of Department of Livestock, Bhutan as shown below.

Table 2. Classification of poultry farm based on flock size

Type of species	Classification based on size of farm (No. of birds)		
	Backyard	Semi-Commercial	Commercial
Broiler	<500	500-5000	>5000
Layer	<100	100-1000	>1000

2.4. Target bacteria

The target bacteria for this 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.

Following are the four bacteria included in this surveillance

Table 3. Bacteria selected for the 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)(WHO, 2015). *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(OIE, 2018) and the AGISAR program(AGISAR, 2017).

2.5 Target antimicrobials

The aim of this AMR surveillance 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 below, has been selected from the critically and highly important antimicrobial classes for humans identified by WHO(WHO, 2018) and as recommended by FAO (OIE, 2019). 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 guidelines willbe used for testing and interpretation of resistance in humans.

Table 4. Antimircobial panel selected for the surveillance

Antimicrobial class	***E. coli***	***Salmonella* spp.**	***Campylobacter* spp.**	***Enterococcus* spp.**
Amioglycoside	Gentamicin	Gentamicin	Gentamicin Streptomycin	Gentamicin Streptomycin
Amphenicol	Choramphenicol	Choramphenicol		Chloramphenicol
Carbapenem	Meropenem and Ertapenem	Meropenem and Ertapenem		
3rd generation cephalosporin's	Ceftriaxone/ cefotaxime	Ceftriaxone/ cefotacime		
4th Generation cephalosporin's	Cefepime			
Quinolones	Ciprofloxacin Nalidixic acid	Ciprofloxacin Perfloxacin	Ciprofloxacin Nalidixic acid	
Macrolides			Erythromycin	Erythromycin
Glycopeptides				Vancomycin
Glycylcylines				Tigecycline
Oxazolidinones				Linezolid
Penicillins	Ampicillin	Ampicillin	Ampicillin	Ampicillin
Beta-lactam/beta-lactamase inhinitor combination	amoxicillin-clavulanic acid	amoxicillin-clavulanic acid	amoxicillin-clavulanic acid	amoxicillin-clavulanic acid
Polymixins	Colistin**	Colistin**		
Streptogramins				Quinupristin-dalfopristin*
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	Tetracycline
Sulpjonamides/Trimethropim	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.*

CHAPTER 3: SAMPLING PLAN

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

3.1 Sampling locations

A sampling at collection points in the chicken marketing chain such as abattoirs or live chicken markets is generally the cost-effective location to collect samples. However, in Bhutan where a high proportion of chickens are not sold through abattoirs or live chicken market, sampling directly from the farm is considered.

3.2. Sampling frame

The sampling frame is a list of sampling locations within the sureveillance areas from which samples will be collected. All the semi-commercial and commercial broiler and layer farms in the surveillance area are the sampling frame. The list of semi-commercial and commercial broiler and layer farmswere collected from the 10 districts in the surveillance areas and is shown below:

Table 5. Number of broiler and layer farms stratified by production level type in surveillance area

Districts	No. of existing Broiler farms		No. of existing Layer farms	
	Semi-commercial	Commercial	Semi-commercial	Commercial
chukha	13	1	20	5
Samtse	51	2	62	8
Paro	0	0	20	5
Thimphu	0	0	15	8
Tsirang	29	0	90	25
Sarpang	30	1	65	50
Pema Gatshel	0	0	17	4
T/Gang	0	0	7	3
S/Jongkhar	5	0	15	2
Mongar	0	0	14	6
Total	**128**	**4**	**325**	**116**

At the time of collecting the lists of broiler and layer farms from surveillance area, there are 441 layer farms (325 semi-commercial farms and 116 commercial farms) and 132 broiler farms (128 semi-commercial farms and 4 commercial farms) with the birds in stock. A required number of farms will be selected from the above farms as per the sampling plan.

3.3 Total sample size and allocation of number of samples

The number of broiler and layer samples are calculated at two levels stratified by the production capacity i.e. semi-commercial or commercial;

- National level
- Surveillance area level

Although it is important to consider FAO sectors while designing a sampling plan as the AMR prevalence may vary significantly between broiler and layer farms in the different

FAO sectors, however such classification of farms is not practised in Bhutan. Further, it will be inconvenient to classify existing farms into different FAO sectors. Hence, FAO sector is not taken into account while designing this sampling plan.As an alternative, sampling of the layer and broiler populations will be stratified on flock size as follows: semi-commercial (>500 – 5000 birds) and commercial (>5000 birds).

3.3.1 Number of broiler and layer samples at national level

The number of isolates required to estimate the prevalence of resistance amongst the isolates for a fixed level of confidence varies with the expected prevalence and the desired level of precision. The sample size was determined based on the assumption of 50% prevalence to get the maximum sample size as the prevalence of AMR of isolates is not known a priori; and 95% confidence level and 0.05 absolute precision. Based on the above, a total of 400 samples each for broiler and layer chickens need to be sampled. 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 will give precise estimates for all AMR prevalence values in *E. coli* and other bacteria present at high prevalence. However, the lower number of isolates for the lower-prevalence bacteria such as *Salmonella* and *Campylobacter spp* may result in less precise AMR prevalence estimates.

In this first round of surveillance, it is impractical to collect a sample size large enough to provide precise estimates of the 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 including logistical reasons.

3.3.2.Number of samples in each surveillance area

The AMR prevalence may vary between different production systems based on the flock size. While designing the sampling plan, it is important to ensure that the proportion of chickens from each production system in the sample for each surveillance area is roughly the same as the overall proportion of farms within each production system in the surveillance area. Therefore, the national level sample size of 400 broilers and 400 layers will be divided among three surveillance site laboratories following the principle of probability proportional to the number of farms in the respective surveillance sites and stratified by production system (semi-commercial and commercial type). The total number of samples allocated to each surveillance sitelaboratory will be further divided among the Dzongkhags under the respective surveillance site laboratory following the same principle as mentioned above.The total numbers of samples need to be collected for broiler and layer at each level is shown in Table 6.

3.3.3.Number of samples per sampling location

Since we will be collecting samples on-farm, sampling locations are the semi-commercial and commercial broiler and layer farms in the surveillance area.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 (Persoons et al., 2011; Yamamoto et al., 2014). Therefore, as far as possible, only one sample will be collected from each farm.

Layer samples

Out of 441 layer farms in the surveillance area, 400 layer farms will be selected for the surveillance. Since the number of commercial layer farm is very less compared to semi-commercial farms, all the 116 commercial farms will be included. The remaining 284 farms required will be selected from 325 semi-commercial farms using a random sampling method.

Broiler samples

There are only 132 broiler farms (128 semi-commercial farms and 4 commercial farms) in the surveillance area. Therefore, all the farms will be included in the surveillance. In order to get the required samples of 400 for the entire surveillance, 132 broiler farms will be sampled upto 3 times over the period of one year.Sincethe production cycle of broiler is short i.e., 45 days on an average, broiler farmers stock their farm atleast 3 times a year. Therefore, from each farm, samples can be collected three times from three different batches at the end of each production cycle. However, if there are more number of farms meeting the inclusion criteria at the time of sample collection, these farms will also be included in the sampling frame and samples will be collected as per the sampling design.

Table 6. Showing the total number of samples needs to be collected by surveillance site laboratories

Surveillance site laboratories	**District**	**No. of broiler samples***		**No. of layer samples****	
		Semi-commercial	**commercial**	**Semi-commercial**	**commercial**
NVL, Serbithang	Thimphu	0	0	13	8
	Tsirang	88	0	79	25
	Sub-total	**88**	**0**	**92**	**33**
		88		**125**	
		213			
RLDC, Tsimasham	Chhukha	39	3	17	5
	Samtse	155	6	54	8
	Paro	0	0	18	5
	Sub-total	**194**	**9**	**89**	**18**
		203		**107**	
		310			
RLDC, Kanglung	Pema Gatshel	0	0	15	4
	T/Gang	0	0	6	3
	S/Jongkhar	15	0	13	2
	Mongar	0	0	12	6
	Sarpang	91	3	57	50
	Sub- total	**106**	**3**	**103**	**65**
		109		**168**	
		277			
Total		**388**	**12**	**284**	**116**

Total	**400**	**400**

* *for broiler samples, 3 samples from each farm sampled over the period of one year. One sample per farm per visit. Preferably 3 samples should be collected from 3 different batches and from different sheds.*

***for layer sample, one sample per farm*

3.4 Sampling timetable

A sampling timetable is prepared for each surveillance area showing the numbers of samples to be collected from the farms by month. This timetable is prepared 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.

Table 7. Sampling timetable

Surveillance site laboratories	**No of samples**												
	2020							**2021**					**Total**
	Jun	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	
NVL, Serbithang	18	18	18	18	18	18	18	18	18	18	17	16	**213**
RLDC Tsimasham	26	26	26	26	26	26	26	26	26	26	25	25	**310**
RLDC Kanglung	24	23	23	23	23	23	23	23	23	23	23	23	**277**

3.4.1 Days of sampling

Samples will be collected at any time of the week aiming to reach the samples to the lab during the first/second day of the working week. However, it should be planned properly so that the samples are not stored for more than 72 hours before processing.

3.4.2 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.

3.4.3. 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.

3.5. Sampling of bird on farm

A bird will be randomly selected from the farm for the sample collection.*Please refer checklist of equipment and materials to take when collecting samples (annexure 1)*

3.6. Biological sample

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. Further, 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, Davies, & Nichols, 2000). Caeca

will be collected through the purposive culling process where the chickens are purchased during the survey.*Please refer SOP for a culling of birds (annexure 2)*

3.6.1. Sample collection from 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. This bag should be placed inside a second ziplock for transport to the laboratoy to ensure there is no leakage and cross-contaminationof samples in the cool box.The bag should be labelled with the sample identification number, location and date and placed in a cool box with ice packs for transportation to the laboratory. To avoid cross-contamination between the samples 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.

The samples from the caeca for Campylobacter isolation will be further submitted to National Veterinary Laboratory from all the regional surveillance sites using the Charcoal containing transport medium mantained at a temperature at 4 ^{o}C (should not be frozen). These samples need to be transported within 72 hours (2 to 3 days) from the surveillance sites to the national laboratories.*Please refer SOP for handling of sample for Camolyobacter isolation (Annexure 3)*

3.7 Information collection during sampling

During the sample collection, a sampler should also collect descriptive information that will help correctly interpret the AMR results. The sample collection form has been developed and should be used to collect the information. *Please refer sample collection form (Annexure 4)*.Since the samples for AMR surveillance will be collected on farms, AMU data will be also collected. However, AMU data is not included in this sample collection form as separate AMU surveillance will be also carried as one of the country grant activities. This AMU surveillance will also includes the farms selected for AMR surveillance.

A unique sample identification numbering system has been developed so that every sample has a unique sample ID. Ensure that the ID number written on the form matches the ID number on the sample package.

3.9 Sampling labelling methods

3.9.1. Samples from the Field

The samples will be clearly labelled using a permanent marking pen. If possible, labels will be prepared prior to the sampling. The information will be placed in a plastic envelope on the outside of the shipping container and will always accompany the samples to the laboratory. The microbiology unit will record the data of the sampling. The sample ID number will contain first three letters of Dzongkhag, Gewog, species, year and followed by number, example: Thi/Mew/Chi-LA/2020/01 for layer and Thi/Mew/Chi-BR/2020/001 for Broiler. The labelling will be done following the standard code of Dzongkhags and Geogs.

Please refer standard code of Dzongkhags and Geogs (annexure 5). Any unlabeled samples received will be rejected.

3.9.2. Isolates from Laboratory

The isolates once identified by the microbiology unit will be labelled with the same sample ID number receivedfrom the field mentioning the organism and/ use a barcode to store the samples.

3. 10 Sample packing

Samples must be packed in a primary and secondary container/leak proof zipper bag so that the samples do not pose any threat to persons or animals during shipment. Double layer of protection is required to ensure that the biological contents in the container do not contaminate other samples or environment in case of leakage or spillage.

The recommended procedure for packing caeca is as follows:

- Both the caeca are put in a primary container (zip-lock plastic bag) and placed in a well-labelled seconadry container (zip-lock plastic bag). The secondary packaging is placedin an outer container, this will be cool boxes (foam boxes) / UN boxes with ice gel packs. Ensure that there is no direct contact between the 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

- Sample collection form and a list of samples or materials will be enclosed in an envelope, enclosed in a plastic bag and placed between the secondary packaging and outer box

3.11 Sample transportation

The samples should be sent safely to the laboratory as soonas possible by the fastestavailable means.The samples shouldideally be transported immediately or within 24 hours under refrigerated condition. If not, they must be stored in a refrigerator at 4-8^{o}Cand transported to the laboratory the next day. The laboratory analysis should begin immediately after the sample reaches the laboratory. All the samples sent to the laboratory should strictly comply with packaging instruction mentioned above.

3.12 Biosecurity practices when collecting samples adapted to sampling locations and risk

Strict biosecurity measures should be followed throughout the sample collection, packaging andshipping process to avoid spreading of disease from one location to another. This is extremely important to ensure that pathogens do not spread between farms and also to avoid farmers associating a disease outbreak with the sampling event if any were to occur by chance after sampling teams visit to the farm. 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.

Disinfection:

Virkon™ S is a recommended 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 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.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.

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. All samplers who are entering a farm must wear clean rubber boots, overalls/lab coat, hairnets and gloves. A separate set of clean overalls; hairnets and gloves should be worn for each farm.
4. The materials required for collecting the 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 packed as described above for transportation.
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 hairnet 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 sanitizer.

f. Put on a new set of latex gloves.
g. Put on second pair of gloves before handing the animal for sampling.

After collecting sample and before leaving the farm dispose off the outer gloves.

6. Immediately after leaving the farm and before entering your vehicle all samplers must undertake the following:
    a. Scrub boots with soap and water to remove all manure, dust and dirt.
    b. Brush clean boots with Virkon™ solution or dip boots into the container of Virkon™ solution.
    c. Remove hair net and place in a rubbish bag.
    d. Remove overalls, place in a secure plastic bag and tie the bag for storage in the vehicle and disinfection/washing when back to base.
    e. Scrub the outside of the box with soap and water if faecal material or dusts are present.
    f. Brush the outside of the box containing the samples using the Virkon™ solution.
    g. Remove gloves and put in disposable waste bag. -
    h. Wash hands using soap and water or rub with alcohol-based hand sanitizer.
    i. 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.

Please refer the items list to be carried in the vehicle to implement biosecurity practices when collecting samples (annexure 6)

CHAPTER 4: LABORATORY PROCEDURES

4.1. Sample processing

1. Verification of quality;
    - Once a sample enters the laboratory, the samples will be verified for proper labeling, adequate in quantity, in good condition and appropriate for test requested. The test request must be complete and include all necessary information.
    - Record sample information into a register or spreadsheet

2. Recording of sample details

    The laboratory will keep a register (log) of all incoming samples to record all the information required like any other samples coming to laboratory. A master register will be maintained at the reception and a separate register at microbiology unit.

    Assign the sample a laboratory identification number/ Registration number; write the number on the sample and the requisition form. A excel spreadsheet will be used to record all the epidemiological information collected in sample collection form for this AMR surveillance as well as AMR result.

    The register will capture following information:
    - Sample ID
    - Date and time of collection
    - Location of sample collection
    - Date and time the sample was received in the laboratory
    - Sample type
    - Owner name and demographic information as required
    - Laboratory assigned identification
    - Tests to be performed.

The spreadsheet will capture all the information recorded in sample collection form which cannot be recorded in register, LIMS or WHONET. This information will be used in analysis of AMR results.

3. Rejection of samples

    The laboratory will establish rejection criteria and follow them closely. The reasons for the rejection will be recorded in the logbook. It is sometimes difficult to reject a sample, but a poor sample will not allow for accurate results.

    - The following are rejection criteria for samples: Broken or leaking tube/container
    - Unlabeled sample
    - Insufficient sample information/details
    - Mismatch in sample and sample information form
    - Sample collected in wrong tubes/container (e.g. using the wrong preservatives or a non-sterile container)

- Insufficient sample quantity for the test requested
- Prolonged transport time or other poor handling during transport

4. Storage before identification
   All the samples received will be refrigerated at 2-4 ^{o}C until it is processed for testing. Once the tests are completed and bacteria are identified, stored samples can be discarded.

5. Processing caecal contents
   Pulverise the caeca and content with a rubber mallet and mix well while in the Ziplock bag. Collect a swab from the well-mixed caecal content and add to a tube of charcoal-containing transport media then refrigerating before sending to NVL for Campylobacter culture.
   Add 1gm of caecal content to 9ml BPW, homogenize well. This initial suspension will be used for culturing of E. coli, Salmonella and Enterococci. For Enterococci, transfer 1ml of the initial suspension to Enterococci enrichment medium/ azide dextrose broth. Incubate both the initial suspension and azide dextrose broth at 37°C for 18- 28hours as per the SOP.

Table 8. Distribution of initial suspension

Volume	Utilization	Related SOPs
10ml(~ 1g)/Swab	For the detection of *Campylobacter spp*	Refer SOP
10ml(~ 1g)	For the detection of *E. coli, ESBL*	Refer SOP
	For the detection of *Salmonella spp*	Refer SOP
	For the detection of *Enterococcus spp*	Refer SOP

Samples received via surveillance laboratories for the culture of *Campylobacter spp* shall be processed immediately as per the SOP for isolation and identification of Campylobacter spp.

4.2 Bacterial Isolation

Once the samples are received by the surveillance laboratory, the sample will be processed by the microbiology unit as mentioned under processing. The samples will be then processed for isolation and identification of the target bacteria (*E. coli, Salmonella spp, Enterococcus spp and Campylobacter spp*) as per the standard operating procedure for isolation and identification of *E. coli, Salmonella spp, Enterococcus spp* and *Campylobacter spp*developed by National Veterinary Laboratory (NVL).

4.3 Antimicrobial Susceptibility Testing (AST)

There are various methods for *in vitro* AST (disk diffusion, e-test, agar dilution, and broth macro-dilution). The main methods used are the disk diffusion, dilution susceptibility testing methods and molecular methods. At present, the surveillance laboratories use the disk diffusion method only.

Disk diffusion method is technically simpler to perform, less expensive and useful for guiding treatment in a clinical setting, this procedure results in an inhibitory zone diameter (mm) which is used for classifying isolates into categories relating to clinical sensitivity of

the antimicrobials i.e. susceptible, intermediate, or resistant. While the inhibitory zone diameter data can be used for surveillance and monitoring, the results are less precise, especially for measuring low levels of resistance, compared with minimum inhibitory concentrations generated from broth and/or agar dilution methods. *Refer SOP for Disk diffusion method developed by NVL.*

The regional laboratories will conduct AST using disk diffusion, and the NVL will conduct AST (including MIC) via Viket machine for all the isolates from all surveillance laboratories to provide AMR surveillance data.

4.3.1. Interpretation of tests results

Two different types of interpretive criteria are available; clinical breakpoints (CBPs) and epidemiological cut-off values (ECOFFs). Depending on the type of the method used for AST, the interpretation criteria will be applied as per the CLSI guidelines.
To compare the AST results for the human and animal isolates, the data should be interpreted with CBPs. The priority is assigned to CLSI CBPs. For antimicrobials where CLSI CBPs do not exist, EUCAST CBPs should be used.

4.3.2. Extended spectrum Beta lactamase (ESBL) producing Salmonella spp and E. coli

Detection of ESBL producing *Salmonella spp* and *E. coli* are considered very important. Ceftriaxone or cefotaxime is included in the harmonized panel for routine monitoring. *Salmonella spp.* or *E. coli* isolates that are resistant to ceftriaxone should be further confirmed for ESBL-production. The phenotypic confirmatory test requires the use of ceftazidime and cefotaxime alone, and in combination with clavulanate. *Refer the SOP for detection of ESBL producing Enterobacteriaceae.*

4.4. Isolate storage

Regional laboratories should store all the isolates regardless of AST results on an agar slant before transporting to the NVL. *Refer the SOP for isolate storage and transport.*

4.5 Isolate transport to reference laboratory

All isolates should be safely transported to the AMR reference laboratory (NVL) once a month for additional testing and storage in a national biorepository. *Refer the SOP for isolate storage and transport.*

4.6 Quality Control (QC) in antimicrobial susceptibility testing

The QC for AST aims to ensure that the only variable in the test is the microorganism's properties determining its reaction to an antimicrobial drug. However, AST is vulnerable to other factors that may influence the results such as the quality of media and reagents, the viability of microorganisms being tested, and the person performing the test. Hence, the goals of a QC programme for AST are to monitor and ensure consistency of;

- The precision/repeatability and accuracy of the susceptibility test procedure
- The performance of reagents and the viability of microorganisms used in the test and
- The performance of the persons who carry out the tests and interpret the results.

Reference strains example *Escherichia coli* ATCC 25922,*Campylobacter jejuni ATCC® 33560,* and *E. faecalis ATCC® 29212* will be used for quality control. The disk diffusion ranges for QC strains and the frequency of QC testing are described in the SOP for kirby-bauer disk diffusion susceptibility test developed by NVL.*Refer SOP for Disk diffusion method developed by NVL.*

4.7 Laboratory data management (Spread sheet)

The storage of raw (primary, non-interpreted) data is essential to allow the evaluation in response to various kinds of questions including those arising in the future. Results will be maintained in excel spread sheet (along with the epidemiological data) as well as in WHONET (only AMR data) by the surveillance laboratories and recorded quantitatively:
a) asinhibition zone diameters in millimeters, and b) as MICs in micrograms per milliliter for those isolates that are tested with both methods.

4.8 EQAS and proficiency testing programme for all laboratories

For quality and uniformity of microbiological procedures, all surveillance laboratories will use the same international guideline such as the CLSI. Laboratories will follow the same procedures in testing and reporting, with a harmonized SOP in all the procedures.

4.8.1. 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.

Steps towards proficiency testing are described below;

1. The reference laboratory should first ensure that they can produce repeatable AST results by testing each of the target bacteria against all the antibiotics listed in *Table.4* 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 surveillance laboratories to test that they are able to identify the bacteria and achieve the known AST results. A standard form should be sent with each panel of isolates for the surveillance 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. Proficiency testing may be conducted atleast once a year. Any issues should be addressed after each round of proficiency testing. AMR reference laboratory should participate in an External Quality Assurance Scheme (EQAS) to ensure that they are producing reliable results.

4.8.2. 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.

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

5.1 Data entry and analysis

The detail information of the sample is collected using sample collection form in hard copies during the sample collection and entered in LIMS and WHONET. However, LIMS cannot capture all the demographic data collected. LIMS is currently used online database system designed to efficiently manage the information of all the veterinary laboratory activities in the country. It is only used to generate laboratory results in a standard format for dissemination like any other laboratory test for this program and record keeping. Even the WHONET cannot capture all the demographic data as we could not set fields for which information is collected. Therefore, a spreadsheet in excel will be also maintained to record all the demographic data and results along with WHONET database. *Refer reporting format (Annexure 15).*

The Surveillance data will be in the electronic format/spreadsheet and stored in WHONET for ease of sharing and further data analysis. The data will be collected and analyzed at regional and national level.

The field data for any samples for AMR surveillance will be collected using the sample collection form and accordingly updated in the spreadsheet by the respective surveillance site laboratories, which are the two regional laboratories (RLDC Tsimasham and RLDC Kanglung) and the National Veterinary Laboratory (NCAH Serbithang). The samples will be subjected to different tests such as identification, isolation and AST in the respective laboratories and the results obtained are updated in spreadsheet.

Simultaneously the details of the samples with results are entered in WHONET offline database which will generate the AST findings with concrete analysis, which can be further, updated using the analytical tools available in the database. Subsequently, the surveillance laboratories will update the AST findings from WHONET on excel spreadsheet.

The final analysis of the AMR surveillance from all three surveillance laboratories will be done first at the animal health level by NCAH. Further, the National AMR Program for the country will compare the results from Animal Health and Human Health at the National level.

WHONET and LIMS will be established in each laboratory for storing the AST results of each sample and staffs will be trained in its use. The AMR findings will be interpreted at three levels comprising of susceptible, intermediate and resistance based on the WHONET analysis done automatically by the database software.

The data from WHONET can be exported in csv format for more complete analysis of AMR data. The laboratories should save the data from WHONET in csv file and sent it to AMR reference laboratories through email, where the overall merging of the data from different levels is possible through the system. Since the WHONET is not set up to capture all the demographic data collected, the excel spreadsheet should be also sent to AMR reference laboratory (NVL) along with WHONET csv file for compilation into a national data set. The

transfer of data and compilation will follow a regular procedure without any requirement for agreements and MOU within the government settings.

5.2 Data collation, validation and dissemination

The collation and validation of the AST data will be done at respective surveillance laboratories and national AMR reference laboratory. The final data analysis and reporting for the animal AMR surveillance will be done by national AMR reference laboratory at NCAH Serbithang based on the excel spreadsheet and WHONET database shared/viewed through the database. The data should be regularly cross-checked between excel spreadsheet and WHONET database at the surveillance laboratory level as well as at the AMR reference laboratory after receiving the data from the surveillance laboratories. It is extremely important that the sample ID is recorded in all the data sets as this is the key ID for linking laboratory results and demographic data.

AMR reference laboratory, NVL Serbithang will do the overall data interpretation at the animal setting while it will be done by the National AMR Program with the support of AMR technical working group for the country after including both the information from human and animal health. The analysed information will be disseminated to different stakeholders like poultry farmers, policymakers, department of Livestock, BAFRA, MOH, pharmaceutical suppliers/companies, veterinarians, Para veterinarians, livestock and poultry consumers, media and other relevant stakeholders. The raw data including the analysed report will also be submitted to National AMR Program for the final generation of the country report after the inclusion of human AMR surveillance report.

The detail data management till the dissemination of information is summerised in Table 9.

Table 9. Methods and responsibilities of AMR data management at diffeent levels

Data type	Data entry	Data collation and validation	Data management	Analysis and Interpretation	Information desimination
Details of sample: 1. Sample ID 2. Sample type 3. Sample source 4. Bird type 5. Age of birds 6. Location	Sample collection team shall compile demographics of sample in sample collection form	Information collected in sample shall be collated into sample submission form for submission to laboratories of surveillance sites	Sample demographics will be entered into MS Excel spreadsheet and stored as soft copy. A printed copy shall be maintained for the same.	Basic analysis on sample collection progress shall be performed by team leader	The sample collection team shall forward the details of samples to surveillance laboratory
Details of organism isolation and identification: 1. Colony character 2. Basic test 3. Biochemical test	Laboraory technicians at surveillance laboratory shall enter all the details of laboratory results for each isolate in in WHONET and	All the details of isolation and identification process shall be entered into WHONET and spreadsheetat respective surveillance	Isolation and identification records shall be maintained at surveillance laboratory	Basic analysis on the consistencies of isolate and reference organism. This activity shall be performed by respective	Surveillance laboratory shall forward these information to national reference laboratory along with isolate. Details of

4. Isolate naming	spreadsheet. Recovered isolate shall be archived with standard identification process	laboratory. Results of isolate shall be compared with that of reference organism		surveillance laboratory	isolation and identification shall be sent in both electronic as well as paper copy
Details of AST from DD 1. Panel of antibiotics 2. Zone diameter of isolate 3. Zone diameter of reference organism	Surveillance laboratory and National Veterinary Labshall enter isolate details for each sample (including samples from which no isolates were retrieved) in WHONET and spreadsheet: - Laboratory ID - Sample ID - Selected panel of antibiotics - Zone diameter of both isolate and reference organism	Surveillance laboratory shall collate cummulative data on AST from all the isolates	Surveillance laboratory shall be the custodian of AST data at their level	Surveillance laboratory shall use WHONET to analyse AST data and draw some preliminary interpretation	Surveillance laboratory shall pass AST data to national reference laboratory in both electronic (WHONET csv file and excel spreadsheet) as well as paper copy
Details of AST from MIC 1. Panel of antibiotics 2. MIC of isolate 3. MIC of reference organism	National reference laboratory shall update the records for each sample and isolatein WHONETand spreadsheet with: - Selected panel of antibiotics - MIC of both isolate and reference organism	National reference laboratory shall collate cummulative data on MIC data from all the isolates	National reference laboratory shall be the custodian of DD and MIC AST data at national level	National reference laboratory shall use WHONET to analyse AST (both DD and MIC) data and interpretate AMR ststus at national level	National reference laboratory shall pass DD and MIC AST data to AH TWG for epidemiological analysis. These result should then go to the NCC for comparision with HH results

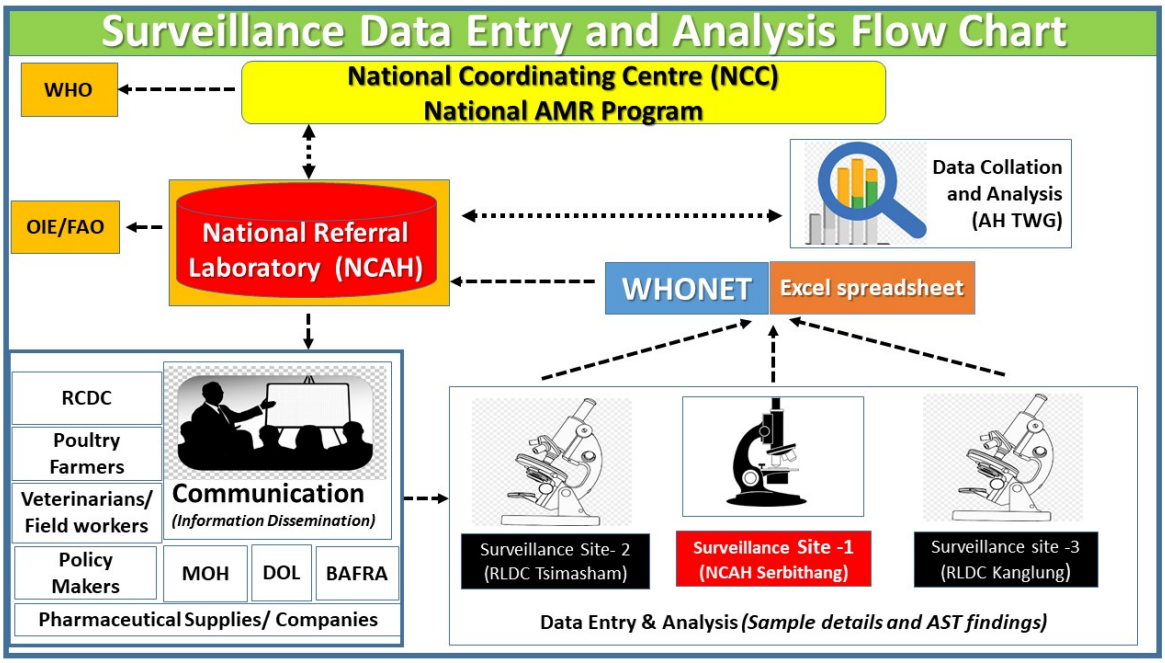

Figure 2. Flow chart showing collection, entry and analysis including the information dissemination

CHAPTER 6: ROLES AND RESPONSIBILITIES OF THE ANIMAL HEALTH SERVICES

6.1 Roles of AMR reference laboratory

1. The National Center for Animal Health at Serbithang is identified as the AMR National reference laboratory.
2. Provide leadership and technical support for the laboratories in the two Regional Livestock Development Centres that are participating in the surveillance program and the NFTL.
3. Develop and upgrade SOPs for microbiology and AST.
4. Train and mentor microbiology technicians in RLDCs and NFTL in culture, identification and AST methods.
5. Support the other labs in implementing good quality systems and will run an EQAS involving the surveillance labs.
6. Maintain an inventoried national biorepository of isolates produced by all labs in the surveillance network.
7. Maintain an ATCC strain collection.
8. Collate & verify AMR surveillance diagnostic data from the contributing laboratories
9. Participate in an international EQAS
10. Develop the capability to undertake the following more advanced diagnostic methods:
    a. ESBL- acquired AmpC (pAmpC) and/or carbapenemase-producing organism confirmation
    b. *Salmonella* spp serotyping
    c. Minimum Inhibitory Concentration (MIC) tests on a subset of isolates showing resistance on disk diffusion tests to identify epidemiology cut-off values (ECOFF) which ensures the comparability of data over time at the country level and also facilitates the comparison of resistance patterns between countries.
11. Implementation of biosafety and biosecurity measures
12. Coordinate and implement the safe transport of samples and isolates between the laboratories.
13. Maintain a national database of verified AMR results and associated demographic data in WHONET.
14. Maintain and share quarterly and annual reports of AMR and AMU surveillance results with the MOAF AMR and AMU Surveillance TWG, the NATC and the RLDCs.
15. Extend G2C database for electronically recording prescription and antibiotic use data in Dzongkhag Veterinary Hospitals.

6.2 Roles of regional surveillance laboratory

1. Coordinate and carryout AMR surveillance in liaising with surveillance sites within their region.
2. Produce reliable quality bacterial culture, identification and Antibiotic Susceptibility Test (AST) results for *E. coli*, *Salmonella* and *Enterococci*.
3. Collect good quality samples from healthy layer hens and broilers are regularly for culture and AST, according to the agreed schedule.
4. Collect good quality samples from healthy layers and broiler chicken and send enough quantity to NVL for culture and AST for *Campylobacter spp* according to the agreed schedule.
5. Collect appropriate samples for AMR testing that are labelled appropriately, transported in a safe manner, arrive at the laboratory in good condition for diagnostic testing, and are accompanied by appropriate demographic information that is labelled to match the samples.
6. Implementation of biosafety and biosecurity measures
7. Safe transport of samples and isolates between the laboratories.
8. Maintain accurate data on sample demographics and laboratory results in the LIMS, WHONET and an excel register, and send this to NVL monthly.

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ANNEXURE

Annexure1. Checklist of Equipment and Materials to take when collecting Samples

- Sample collection form
- Leak proof zip-lock plastic bags
- Scissors
- Scalpel blade
- Sterile swabs
- Sterile sample tube with charcoal containing transport medium (for Campylobacter)
- Sterile rubber mallet
- Cool box/UN box
- Labeling stickers
- Permanent marker pen
- Ice packs
- Biohazard plastic bags

Annexure 2. SOP for Slaughter and disposal of poultry after sample collection

This SOP covers the guidelines and steps for humane culling and safe disposal of carcass including other materials after proper sample collection. The team comprises of laboratory technician with one-hired labour. Each culling member must be provided with adequate protective measures, which includes a facemask, Nitrile gloves.

Materials required

The slaughter group should carry personal protective equipment, heavy-duty trash bags, small plastic bags, roll of paper towels, water-proof notebook and pen.

Culling Procedures

The bird in the farm premises will be taken to the isolated locations identified by the culler after purchasing the poultry from the owner. The team will identify the site for culling and disposal of carcass. To minimize the handling and reduce stress on the poultry, they should be preferably culled on the farm. The welfare aspect during the culling must be ensured. The method chosen for slaughter of poultry must be safe and humane. Neck dislocation is considered a humane method of poultry euthanasia and is the most common method for killing birds. The neck dislocation can effectively carry out using hands or with burdizzos, forceps, or pliers.

Following steps will be followed for a neck dislocation:

- Place the bird breast-down on a flat surface (or hold the bird against your hip).
- Use one hand to hold both wings behind the bird's back.

- Using your other hand to hold the head between your middle and ring fingers, with the middle finger on the back of the chicken's head.
- Sharply turn the head 90 degrees while at the same time pulling it firmly and quickly away from the body (in a motion like stretching the neck). You will feel the vertebra separate.
- Hold the bird in this position until flapping stops.

After collecting the samples, the carcass should be properly packed for disposal. Ideally, the carcass should be disposed of on-site by burial. While burying, make sure that the wild animals or dogs cannot access the carcass. If the team could not find a proper place for burial, the carcass can be packed in bio-hazard plastic bag and taken to the laboratory for disposal into biological pits. All th contaminated materials and surroundings should be decontaminated using available disinfectants.

Annexure 3. SOP on handling samples for Camplyobacter isolation

Introduction

This SOP targets the thermo tolerant *Campylobacter* species relevant to human health. The most frequently encountered and relevant to human health species are *C.jejuni* and *C.coli.* This document covers the sample collection, storage and transportation of caecal content from regional laboratories to national veterinary laboratory for Campyloabcetr isolation.

Objective

To obtain quality and standardized samples from all the regional laboratories for Campylobacter isolation

Procedure

At the Regional Laboratories a swab of the caecal content for campylobacter isolation should be taken on the same day the sample is collected.

1. Pulverize the caeca and content with a rubber mallet and mix well while in the ziplock bag.
2. Collect a swab from the well-mixed caecal content and add to a tube of charcoal-containing transport media. To be done first before the sample is processed for other organism.
3. Label the swab as per the standard format
4. Refrigerate before sending to NVL for Campylobacter culture (do not freeze)

Sample Transport

The refrigerated swabs for Campylobacter culture should be sent to NVL weekly, ensuring that samples are stored in the transport medium for no longer than one week (if possible, within 72 hours after collection) before being processed at NVL.

The Regional Laboratories should target to send samples on such days (Eg. Monday) that the samples reach the NVL during the start of the weeks. The samples that reach on Thrusday/Friday/Saturday and Sunday will not be entertained.

Annexure 4. Sample Collection Form

Sl.No	Details	Information
A: Details of the sample collector		
1	Name of sampler and designation:	
2	Contact details:	
***B:** Details of the farm*		
1	Name of the Owner:	
2	Farm type (backyard/semi-commercial/commercial):	
3	Farm location (GPS coordinates):	
4	Farm size (number of birds):	
5	Production type (broiler/layer):	
6	Name of town/village:	
7	Name of the sub-District (Geowg):	
8	District:	
9	Contact no:	
C: Details of the sample		
1	Sample ID number (Dzongkhag/Gewog/Year/Species/No) *(E.g.; Thi/Mew/2020/Chi-LA or BR/01)*	
2	Collected from broiler or layer	
3	Sample collection date and time:	
4	Breed:	
5	Age of chicken in weeks (approximate):	
6	Type of sample (Whole Caeca, faecal, cloacal, boot swab, others):	
7	Date when the chickens in this shed were most recently treated with antimicrobials, and name of the antimicrobial:indication	
8	Number of management units on the farms (sheds)	
9	Number of chickens in the shed that was sampled:	
10	Source of chicks (name of hatchery if known):	
11	Source of feed fed to chicken (Name of Commercial feed and address including contact no.)	
D: Additional information for broiler		
1	**Chicken reared and slaughtered annually**	
1.1	No. of broilerreared annually	
1.2	Estimate of Chicken produced annually (in MT)	

Sl.No	Details	Information
2	**Destination (place) of processed Chicken**	
2.1	Destination and Quantity (MT)	
2.2	Destination and Quantity (MT)	
2.3	Destination and Quantity (MT)	
2.4	Destination and Quantity (MT)	
E	*Additional Information for layers*	
1	No layers reared annually	
2	Total egg produced annually (in million)	
3	Destination and Quantity of eggs (in millions)	
3.1	Destination and Quantity of eggs (in millions)	
3.2	Destination and Quantity of eggs (in millions)	
4	Annul/Two yearly total spent hens disposed (nos)	
5	Destination and quantity of spent hens (MT)	
5.1	Destination and quantity of spent hens (MT)	
5.2	Destination and quantity of spent hens (MT)	
5.3	Destination and quantity of spent hens (MT)	

Annexure 5. Standard Code of Dzongkhags and Gewogs

Dzongkhag	Dzongkhag code	Gewog	Geog code
Thimphu	Thi	Mewang	Mew
		Kawang	Kaw
		Genekha	Gen
		Dagala	Dag
		Soe	Soe
		Naro	Nar
		Lingzhi	Lin
		Chang	Cha
Paro	Par	Hungrel	Hun
		Naja	Naj
		Tsento	Tse
		Dogar	Dog
		Dopshari	Dop
		Doteng	Dot
		Lamgong	Lam
		Lungnyi	Lun
		Shaba	Sha
		Wangchang	Wan
Chhukha	Chh	Chapcha	Cha
		Samphelling	Sam
		Phuentsholing	Phu

		Lokchina	Lok
		Metakha	Met
		Dungna	Dun
		Darla	Dar
		Getana	Get
		Bongo	Bon
		Gelling	Gel
		Bjabcho	Bja
Samtse	Sam	Phuntshopelri	Phu
		Dophuchen	Dop
		Dumtoed	Dum
		Tashichhoeling	Tas
		Tendrul	Ten
		Pemaling	Pem
		Norgaygang	Nor
		Sangngagchoeling	San
		Tading	Tad
		Norbugang	Nor
		Yoeseltse	Yoe
		Namgyacholing	Nam
		Ugyentse	Ugy
		Denchukha	Den
		Samtse	Sam
Tsirang	Tsi	Serigithang	Ser
		Tsholingkhar	Tsh
		Tsirang Toe	Tsi
		Semjong	Sem
		Rangthaling	Ran
		Phuentenchu	Phu
		Patshaling	Pat
		Mendrelgang	Men
		Kilkhorthang	Kil
		Gosarling	Gos
		Doongalagang	Doo
		Barshong	Bar
Sarpang	Sar	Senggye	Sen
		Gakidling	Gak
		Shompangkha	Sho
		Dekidling	Dek
		Samtenling	Sam
		Gelephu	Gel
		Chhudzom	Chh
		Jigmecholing	Jig
		Sershong	Ser
		chuzanggang	Chu
		Tareythang	Tar

		Umling	Uml
Mongar	Mon	Tshakaling	Tsh
		Tsamang	Tsa
		Thangrong	Tha
		Silambi	Sil
		Shermuhung	She
		Saling	Sal
		Ngatshang	Nga
		Narang	Nar
		Mongar	Mon
		Khengkhar	Khe
		Jurmey	Jur
		Gongdue	Gon
		Drepong	Dre
		Dremitse	Drm
		Chaskhar	Cha
		Chali	Chl
		Balam	Bal
Tashigang	Tas	Khaling	Kha
		Kangpara	Kan
		Kanglung	Kal
		Bidung	Bid
		Bartsham	Bar
		Yangneer	Yan
		Udzorong	Udz
		Thrimshing	Thr
		Shongphu	Sho
		Samkhar	Sam
		Sakteng	Sak
		Radhi	Rad
		Phongmey	Pho
		Merak	Mer
		Lumang	Lum
Pema Gatsgel	Pem	Dungmin	Dun
		Decheeling	Dec
		Choekhorling	Che
		Norbugang	Nor
		Khar	Kha
		Chongshing	Cho
		Yurung	Yur
		Shumar	Shu
		Chhimung	Chh
		Zobel	Zob
		Nanong	Nan
Samdrup Jongkhar	Saj	Wangphu	Wan
		Orong	Oro

	Gomdar	Gom
	Samrang	Sam
	Martsalla	Mar
	Pemthang	Pem
	Phuntshothang	Phu
	Lauri	Lau
	Serthi	Ser
	Langchenphu	Lan
	Dewathang	Dew

Annexure 6. Items list to be carried in the Vehicle to implement Biosecurity Practices when collecting Samples

- ✓ *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 hairnets and gloves*
- ✓ *Plastic bag for used overalls*
- ✓ *First aid box*

Annexure 7. Laboratory submission form

Name of the Laboratory

Sender Ref No: Sender: RLDC Ref. No:
Date sent: Date received:

DETAILS OF OWNER
Owner:
Village: Geog: Dzongkhag:
Contact No. Date collected

DETAILS OF ANIMALS
Sample IDNo: Species: Breed: Age: Sex:

SPECIMENS
Caecal content: Caecal content swab for Campylobacter:

Purpose: For AMR surveillance in layer and broiler chicken

CASE HISTORY*
Nos. affected: Nos. dead: Nos. at risk (household/farm level):
Length of illness:
Symptoms:
Summary of PM findings (if performed):

Treatment:
Vaccination:
Disease suspected:
Examination requested (choose from below):

LABORATORY SECTION REFERRED

☐ Parasitology ☐ Clinical Pathology (Bio-chemistry/Haematology)
☐ Toxicology ☐ Bacteriology ☐ Serology/Virology/Molecular
☐ Anatomical Pathology (Postmortem/Histopathology)

Laboratory findings of the laboratory

Laboratory test requested

Submitted by (name): Signature: Designation:

**No need to fill case history part since the samples are collected from healthy chicken for AMR surveillance purpose*

Annexure 14. SOP for Bacterial Isolate Transportation

1. Introduction

Maintaining the stock culture by sub-culturing on the medium appropriate for growth is very essential for further confirmatory tests and stock. Sub-culturing should be done four times a year to maintain viability.

Slant cultures are preferred to broth (i.e. liquid medium) cultures because the first sign of contamination is much more readily noticed on an agar surface. Slanting the surface of the agar gives the bacteria a greater surface area for the growth in a test tube. Furthermore, slants are created in test tubes that can be capped, which minimizes water loss since the media contains high moisture.

The slants are useful in maintaining and transport of pure cultures. These slants can be transported at ambient temperature to the national referral labs for further confirmatory tests. However, the guidelines recommended should be followed for packaging and transportation of the isolates from regional to national referral laboratories and UN guidelines for transporting to international laboratories.

2. Purpose:

The purpose of this SOP is to describe the procedure for maintaining pure culture and transportation of bacterial isolates from the Surveillance Laboratories to the National Referral Laboratory (NRL) and further to International Laboratories.

3. Equipment/materials:

- Autoclave
- Pipettes
- Petri plates
- Test Tubes
- Erlenmeyer flask
- Cryovials

4. Chemicals/reagents

4.1 Nutrient agar

4.2 Mueller–Hinton (MH) agar with 2% horse blood

5. Procedure:

5.1 Transport of isolate from Surveillance Laboratories to the National Referral Laboratories

5.1.1 Inoculation of slant

- Inoculate the slant by transferring pure isolates (Refer preparation of slants in annex) with an inoculating loop from a single-colony microorganism on a plate to the slant's surface.
- Move the loop across the surface of the slant and recap the tubes.
- Incubate the slant until there is evidence of growth, then put the tube in a refrigerator.

For growing strict aerobes, it may be necessary to slightly loosen the cap for incubation (but close securely before storage) if there is insufficient air in the headspace.
For easy handling, plastic cryovials of 2ml size could be used filling with about 1ml semi solid nutrient agar and inoculating with culture and after about 24hrs of incubation, transport to national referral lab with proper labelling and packing as described below.

The transport of isolates of *Campylobacter* a true microaerophilic bacterium under aerobic conditions is often unsuccessful. MH agar with 2% horse blood, suitable transport vials, and an optimum temperature of 2 ± 2°C provided survival of three *Campylobacter* type strains for at least one month under atmospheric conditions.

5.1.2 Packaging of samples

Samples must be packed in a primary and secondary container so that the samples arrive in good condition and do not present any hazard to persons or animals during shipment. It is essential that the contents of containers, which break or leak during transit do not contaminate the outside layer of the package.

The recommended procedure for packing samples is as follows:

- Samples must be put a primary container (glass or plastic tubes or bottles) with screw caps and wrapped with paraffin film or adhesive tape individually in order to prevent leakage of fluid. The wrapping of bottles or primary containers should be carried out in clean surroundings.

- The primary container must be packed in water tight secondary packaging, which should be a strong crushproof and leak-proof metal container. The container should contain absorbent cotton wool sufficient to absorb the entire contents of the primary container.

- The secondary packaging must be placed in an outer container. This should be a polystyrene foam box covered with a hard box or other appropriate container.

- Sufficient information and a list of samples or materials should be enclosed in an envelope, enclosed in a plastic bag and placed between the secondary packaging and outer box.

- It is recommended that a freezer box is put outside the secondary packaging to ensure that all materials are kept cool during shipment. These packs should be pre-frozen at -20 degrees centigrade before packaging.

5.1.3 Transport of isolates

- The specimens should be forwarded to the laboratory by the fastest method available.
- If they can reach the laboratory within 48 hours, samples should be sent refrigerated.
- Infectious substances, which can include diagnostic specimens, are not permitted to be shipped as checked luggage or as carry on luggage and must be shipped as cargo.

5.2 Transport of isolate from National Referral laboratories to the International Laboratories

5.2.1 Freeze Drying (Lyophilisation)

In this method, the culture is rapidly frozen at a very low temperature (around -70°C) and then dehydrated by vacuum. Lyophilized or freeze-dried pure cultures are then sealed and stored in the dark at 4°C in refrigerators.

Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years. Freeze-drying method is the most frequently used technique by culture collection centres.

5.2.2 Lyophilisation Process

- In this process the microbial suspension is placed in small vials.
- A thin film is frozen over the inside surface of the vial by rotating it in mixture of dry ice (solid carbon dioxide) and alcohol, or acetone at a temperature of -78^{o}C.
- The vials are immediately connected to a high vacuum line. This dries the organism while still frozen.
- Finally, the ampules are sealed off in a vacuum with small flame.
- To revive microbial cultures, it is merely necessary to break open the vial aseptically, add a suitable stale medium, and after incubation make further transfers.
- The process permits the maintenance of longer number of cultures without variation in characteristics of the culture and greatly reduces the danger of contamination.

5.2.3 Packaging & Transport of culture isolates

Prior import authorization should be sought from the country of the laboratories to be referred. For referral, the culture isolate is consigned as diagnostic specimen (UN3373). UN 3373, covers 'Diagnostic Specimens or Clinical Specimens or Biological Substances Category B'. This category has a lower risk and packages containing these specimens should be labelled as 'Diagnostic Specimens or Clinical Specimens or Biological Substances Category B'; a Declaration of Dangerous Goods is not needed.

i. Infectious substances assigned to UN 3373 'Diagnostic Specimens' must be packed in good quality packaging, which must be strong enough to withstand the shocks and loadings normally encountered during transport. Packaging must be constructed and closed so as to prevent any loss of contents, which might be caused under normal conditions of transport.

ii. The packaging must consist of three components:
   - a primary receptacle;
   - a secondary packaging; and
   - a rigid outer packaging.

iii. For liquid substances:
   - The primary receptacle(s) must be leak-proof and must not contain more than 1 litre; the secondary packaging must also be leak-proof;
   - Adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the primary receptacle(s);
   - If multiple primary receptacles are used, they should be individually wrapped or separated so as to prevent contact;
   - The primary receptacle or the secondary packaging must be capable of withstanding without leakage an internal pressure of 95 kPa in the range of –40°C to 55°C (–40°F to 130°F);
   - The outer packaging must not contain more than 4 litres. This quantity excludes ice, dry ice, or liquid nitrogen when used to keep specimens cold.

iv. For solid substances:
   - the primary receptacle(s) must be sift-proof and must not exceed the outer packaging weight limit; the secondary packaging must be sift-proof;
   - adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the primary receptacle(s);
   - except for packages containing body parts, organs or whole bodies, the outer packaging must not contain more than 4 kg. This quantity excludes ice, dry ice or liquid nitrogen when used to keep specimens cold;
   - if there is any doubt as to whether or not residual liquid may be present in the primary receptacle during transport then packaging suitable for liquids, including absorbent materials, must be used.

v. An itemised list of contents must be enclosed between the secondary packaging and the outer packaging.

vi. If shipped at ambient temperatures or higher, the primary receptacle must have a positive means of ensuring that it is leak proof, such as a leak proof seal, heat seal or skirted stopper. If screw caps are used, they should be sealed with parafilm or tape.

vii. Prefrozen packs or dry ice can be packed around the secondary receptacle. If dry ice is used, there must be an internal support to secure the secondary receptacle in the original position after the dry ice has been dissipated. The outer packaging must permit the release of carbon dioxide.

viii. Packages containing diagnostic or clinical specimens are not required to have the net quantity marked on the outside of the package. However, where dry ice is used as a refrigerant, the net quantity of dry ice must be shown.

ix. The primary and secondary receptacles must be put into a shipping container with adequate cushioning material.

x. The packaging must be able to withstand a 1.2 metre drop test. (There are additional strength requirements for packaging used for UN 2900 and UN 2814 specimens.)

xi. At least one surface of the outer packaging must have a minimum dimension of 100 mm × 100 mm.

xii. For transport, the label 3373 must be displayed on the external surface of the outer packaging on a background of a contrasting colour and must be clearly visible and legible. The mark must be in the form of a square set an angle of 45° (diamond-shaped) with each side having a length of at least 50 mm, the width of the line must be at least 2 mm, and the letters and numbers must be at least 6 mm high. The proper shipping name “Diagnostic specimen”, “Clinical specimen” or “Biological substance category B” in letters at least 6 mm high must be marked on the outer package adjacent to the diamond-shaped mark.

6. Safety

The samples should be considered as Infectious since the bacterial pathogens could be zoonotic in nature.

7. References

- Basic Practical Microbiology, A Manual, Society for Microbiology 2006, ISBN 0 95368 383 4
- https://microbeonline.com accessed on 2020
- OIE terrestrial manual 2008, Chapter 1.1.1 collection and shipment of diagnostic samples.
- OIE terrestrial manual 2008, Chapter 1.1.3 Transport of Biological Materials.
- IremOmurtag, Fuat Aydin, Peter Paulsen, Friederike Hilbert & Frans J.M. Smulders(2011).Simple media and conditions for inter-laboratory transport of

*Campylobacter jejuni*isolates,Veterinary Quarterly,31:2,73-75,DOI: 10.1080/01652176.2011.585797.

Annexure: Composition

8.1 Nutrient agar:

Beef extract	3g
Peptone	5g
Agar	9-18g
Distilled water	1000ml

Preparation of Nutrient agar slant

Nutrient agar and broth are available commercially in powdered (free-flowing, homogeneous) form.

1. Dissolve the dehydrated medium in the appropriate volume of distilled water i.e., 23 gm dehydrated nutrient agar (*see the manufacturer instruction*) in 1000 ml distilled water.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder
3. Sterilized the medium by autoclaving (121°C for 15 min)
4. Dispense the medium into tubes *(i.e. 3 ml to make nutrient agar slopes)*. Leave the agar medium to solidify. Making sure the medium inside the tubes is at a slanted position relative to the test tubes.
5. Date the medium and give it a batch number.
6. Store in a cool dark place.

pH of medium: The pH of Nutrient Agar should be within the range of pH 7.2-7.6 at room temperature.

8.2 Mueller Hinton agar

Acid Hydrolysate of Casein	17.5gm
Beef Extract	17.5gm
Starch	1.5gm
Lysed Horse Blood	20.0ml
Agar	17.0gm

Annexure 15. Result Submission Excel Spreadsheet Form

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
1		Details of sample collector		Details of the Farm									Details of the Sample						Shed details		Source details		Antimicrobials used			Additional information for broiler						Additional information for layers			
2	Sl.No	Name	Contact No	Owner name	Farm type	Farm location (GPS)	Farm size	**Broiler/Layer**	Village	Geog	District	Contact details	Sample ID	collected from broiler or layer	collection date & time	Breed	Age(weeks)	Type of sample	No. of chickens in the shed (Sampled)	No of sheds in the farm	Chicks	Feed	Date	Antimicrobial name	Indication	No of broiler reared annually	MT of chicken produced annually	Destination and Quanity (MT)	Destination and Quanity (MT)	Destination and Quanity (MT)	Destination and Quanity (MT)	Total layerreared annually	Total egg produced annually (in million)	Destination and Quantity of eggs (in millions)	Annul/Two yearly total spent hens disposed (nos)
3																																			
4																																			
5																																			
6																																			
7																																			
8																																			
9																																			
10																																			
11																																			

	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF	BG	BH	BI	BJ	BK	BL	BM	BN	BO	BP	BQ	BR	BS	BT	BU
1	Laboratory Information				Antimicrobial discs (Disk Diffusion)																										ESBL Antimicrobial discs						Sample refferal	Remarks
2	Sample Registration/submission No.	Culture Media	Biochemical tests	Organism Isolated	**Gentamicin**	**Sensitive, Intermediate, Resistant (SIR)**	**Chloramphenicol**	**SIR**	**Meropenem**	**SIR**	**Ertapenem**	**SIR**	**Ceftriaxone**	**SIR**	**Cefotaxime**	**SIR**	**Cefepime**	**SIR**	**Ciprofloxacin**	**SIR**	**Nalidixic acid**	**SIR**	**Pefloxacin**	**SIR**	**Streptomycin**	**SIR**	**Vancomycin**	**SIR**	**Tigecycline**	**SIR**	**ceftazidime**	**ceftazidime/Caluvanate**	**SIR**	**cefotaxime**	**cefotaxime/clavulanate**	**SIR**	Yes, When	
3																																						
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