Active surveillance protocol for Antimicrobial Resistance in poultry population in Nepal, 2019
(Version-1)

Ministry of Agriculture and Livestock Development

Department of Livestock Services, Harihar Bhawan

May, 2019

Acronyms

AMR	Antimicrobial resistance
AMU	Antimicrobial use
AST	Antimicrobial susceptibility testing
BPW	Buffered Peptone Water
CBS	Central bureau of statistics
CLSI	Clinical and Laboratory Standards Institute
CVL	Central Veterinary Laboratory
E. coli	Escherichia coli
ESBL	Extended spectrum beta lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agricultural Organization
GLASS	Global Antimicrobial Resistance Surveillance System
GPS	Global Positioning System
QGIS	Quantum Geographic Information System
NAL	National Avian Disease Investigation Laboratory
OIE	World Organization for Animal Health
SPSS	Statistical Package for the Social Sciences
SAS	Statistical Analysis System
TWG- AH	Technical Working Group- Animal Health
WHO	World Health Organization

Contents

Introduction 6

Objectives 7

Sentinel sites for AMR surveillance in animal health sector 8

Target bacteria 8

Target antibiotics 9

Diagnostic testing 10

Surveillance plan 10

Target populations 10

Number of samples 11

Sampling locations and biological samples 17

Sampling logistics 19

Review sampling plan 20

Biosecurity practices when collecting samples 20

Sample transport 22

Culture, identification and AST 22

Confirmatory resistance testing (Central Veterinary Laboratory) 23

Isolate storage 23

Sample collection form 24

References 24

Annex 1. Kits for biosecurity practices during sampling 28

Annex 5: Laboratory testing 32

a. Sample preparation and enrichment 32

b. ESBL Positive Enterobacteriaceae 32

c. Escherichia coli 33

d. Salmonella 34

e. *Enterococcus* 35

f. Campylobacter 37

Annex 2: Storage of isolates 38

Short term storage 38

Long term storage 38

Recovery of isolates from long term storage 39

Annex 3. Equipment 39

Disclaimer: This protocol has been largely adopted from the document developed by a team of Joanna McKenzie, Roger Morris, Anne Midwinter and Sara Burgess of the School of Veterinary Medicine, Massey University, New Zealand in support from the Fleming Fund. This protocol has been tailor made for Nepal to conduct active surveillance in broilers and spent layer population in selected animal health surveillance sites in Kathmandu, Pokhara, Biratnagar and Chitwan with support from the Fleming Fund. The term AMR used in this document specifically refers to antibiotic resistance.

Introduction

The highest burden of the projected losses due to AMR will be on the developing countries including Nepal due to limited resources and capacities to tackle these problems. The real burden of AMR in Nepal is unknown, however there are evidences to suggest that the AMR problem is ever increasing. Limited laboratory-based surveillance data for humans available at National Public Health Laboratory (NPHL) and several independent research studies have shown increased resistance in bacteria such as *E. coli, Salmonella, Neisseria, Staphylococcus, Streptococcus, Shigella* and *Vibrio* against several antibiotics for example Amoxicillin, Amoxicillin-clavulanic acid, Cefixime, Ceftazidime and Cefotaxime, Ciprofloxacin, Nalidixic acid, Penicillin and Tetracycline (Basnyat et al., 2015; Chaudhary et al., 2016; Dhital et al., 2017; Joshi et al., 2018; Maharjan et al., 2017; Pokhrel et al., 2018; Shakya et al., 2016; Upreti et al., 2018; Zellweger et al., 2018).

In animal health side, the Central Veterinary Disease Investigation Laboratory (CVL) located at Kathmandu, five veterinary laboratories distributed across the country (Biratnagar, Janakpur, Pokhara, Surkhet, and Dhangadi), and National Avian Disease Investigation Laboratory (NAL), Chitwan perform bacterial culture, identification and antibiotic sensitivity testing on samples from poultry, milk, water and other samples brought to the laboratory for diagnostic purposes. These limited data and few other independent studies have suggested that common bacteria isolated from animals, such as *E. coli, Bacillus, Campylobacter, Salmonella, Pseudomonas, Staphylococcus* and *Streptococcus,* have developed resistance against commonly used antibiotics viz. Gentamicin, Amoxycillin, Enrofloxacin, Ceftriaxone, Amoxycillin and even to Colistin (CVL, 2017; Ghimire et al., 2014; Shrestha et al., 2010; Subedi et al., 2018), which is considered the last resort drug for multi-drug resistant gram negative bacilli such as *Acinetobacter baumannii* infections in humans (Paterson & Harris, 2016). However, there is no structured surveillance system in animal health sector to estimate the burden of AMR in livestock species in Nepal. The surveillance system needs to be strengthened to generate evidence which can guide the policy formulation to control the problem of AMR. A population-based surveillance system can give an estimate of AMR prevalence reflective of the target population. Therefore, it is necessary to develop a population-based laboratory surveillance system to estimate the AMR problem in animal health sector. The AMR surveillance data is necessary not only to safeguard the animal health but also to estimate the extent of zoonotic transmission of bacteria to humans.

Due to limited resources, it is not feasible to target all animal species and bacteria at once. The alternative is to strategically start the surveillance with one species and then gradually move to other species. In Nepal, among the livestock sector, poultry is the most commercialized sub-sector and has been rapidly growing in the last couple of decades. The total annual commercial chicken meat and egg production in Nepal is estimated to be 114,050 tons and 1.2 billion pieces respectively (CBS, 2014). Most of these commercial chicken products are consumed in major urban areas. The demand for chicken meat is ever increasing due to its relatively cheap price and easy accessibility. To meet these increasing demands, small to large poultry farms are being established all over the peri-urban areas of Nepal especially around Kathmandu valley, Chitwan, Pokhara, Biratnagar, Butwal and Dhangadi. Subsequently, antibiotics use is also increasing primarily for therapeutic purposes but occasionally as growth promoter and prevention purposes. Indiscriminate use of antibiotics in poultry industry is highly likely to contribute to AMR problem. Given these backgrounds, broiler and spent layer poultry population are proposed as the initial focus for AMR surveillance in livestock sector as consumption levels of poultry are generally highest or high compared with other meat sources and antibiotics are widely used in this sector, including some for which emergence of resistance in human is of concern.

This operating protocol has been prepared to conduct the active surveillance in poultry as the first step to strengthen AMR surveillance in animals. This document specifies structured active surveillance of specified poultry populations that helps to estimate AMR in poultry population that are as representative as possible of the target populations that are sampled.

Objectives

General Objective

Establish antimicrobial resistance surveillance in animal health sector primarily focusing on poultry to strengthen one health approach in AMR surveillance and response.

Specific Objectives

1. Institute population-based active surveillance for antimicrobial resistance in poultry.

2. Isolate the target bacterial pathogens and determine their antimicrobial resistance status in poultry.
3. Evaluate the temporal and spatial distribution of the bacterial isolates and their antimicrobial resistance pattern in broiler and spent layers population.
4. Create a national biorepository of bacterial isolates from animal origin at the CVL for further investigation.
5. Share information with human health sector (NPHL) for strengthening one health approach in AMR surveillance and response.
6. Identify possible animal-associated risk factors that may influence AMR resistance for further investigation.

Sentinel sites for AMR surveillance in animal health sector

Four sites have been selected for AMR surveillance in animal health sector. These four sites were selected for surveillance as these sites have high human and poultry population density thereby increasing the chance of zoonotic transmission as well as offers major geographic coverage. The CVL will serve as the reference laboratory whereas NAL, Chitwan, and Veterinary laboratories in Biratnagar and Pokhara will serve as the sentinel sites for surveillance (Annex 4, Figure 1). The CVL will be responsible for leading active surveillance in Kathmandu valley (Annex 4, Figure 2) whereas NAL will lead surveillance in Chitwan (Annex 4, Figure 3). The Veterinary Laboratories at Pokhara and Biratnagar will lead surveillance in Kaski (Annex 4, Figure 4) and Morang/Sunsari (Annex 4, Figure 5) districts respectively.

Target bacteria

The target bacteria for the first round of AMR surveillance are zoonotic bacteria, including pathogens and commensal gut bacteria of poultry and which may potentially be associated with transmitting AMR to humans. Specifically, the target bacteria are:

1. Escherichia coli (E. coli)

2. Salmonella spp

3. *Enterococcus (E. faecium and E. faecalis)*

4. Campylobacter spp

E. coli and *Salmonella spp.* are priority organisms listed in the WHO's Global AMR Surveillance System (GLASS)[1]. *Campylobacter* is an important zoonotic pathogen of people and *Enterococcus* is a commensal organism that may act as an indicator for resistance patterns associated with gram positive organisms. *E. coli* is the initial priority for strengthening diagnostic capability in animal surveillance laboratories, followed by *Salmonella*. Laboratories that have the capability to reliably grow and identify *E. coli* and *Salmonella* may include *Campylobacter* in their programme. Since *Campylobacter* is a more challenging bacterium to culture, it may only be feasible for more experienced laboratories to grow this organism. Therefore, only CVL will be performing *Campylobacter* culture and susceptibility testing in the beginning.

Target antibiotics

The panel of antibiotics were selected for each four bacteria following WHO's critically and highly important antimicrobial classes for humans as mentioned in Fleming fund guideline (Table 1) , for which resistance should be tested in the specified zoonotic, pathogenic and commensal bacteria cultured from broilers and layers.

Table 1.The panel of target antibiotics for each of the four bacteria

Antibiotic Class/antibiotic	***E. coli***	***Salmonella spp.***	***Campylobacter spp.***	***Enterococcus spp.***
Aminoglycosides	Gentamicin /Amikacin		Gentamicin Streptomycin	Gentamicin
Amphenicol	Chloramphenicol	Chloramphenicol Florphenicol		
Carbapenem	Meropenem Ertapenem	Meropenem Ertapenem		
Cephalosporins III	Ceftriaxone	Ceftriaxone		
Cephalosporins IV	Cefepime			

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

Quinolones	Ciprofloxacin	Ciprofloxacin	Ciprofloxacin	Ciprofloxacin
Glycopeptides				Vancomycin
Glycylcyclines				Tigecycline
Oxazolidinones				Linezolid
Penicillins	Ampicillin	Ampicillin	Ampicillin	Ampicillin
Polymixins	Colistin	Colistin		
Synercids				Quinupristin-dalfopristin
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	Tetracycline
Sulphonamides /Trimethoprim	Co-trimoxazole	Co-trimoxazole		

Diagnostic testing

Detailed instructions for culture and identification of the target bacteria are provided in Annex 1. AST will be conducted for each bacterial isolate against the panel of antibiotics listed using modified Kirby Bauer's disk diffusion method, with an exception of colistin. Disk diffusion is not a suitable method to test for resistance to colistin and AST for colistin will be conducted using the broth dilution method at the Central Veterinary Laboratory. After the sensitivity tests, the diameter of the inhibition zone shall be measured, recorded and result will be interpreted as Sensitive (S), Intermediate (I) or Resistant (R) following CLSI guidelines (Veterinary Supplement Document VET01/VET08).

In addition to testing resistance patterns in pure isolates of the target bacterial species/groups, each sample will be tested for the presence of Extended Spectrum Beta-Lactamase (ESBL) positive Enterobacteriaceae by plating directly onto selective media (see Annex 1 for details).

Surveillance plan

Target populations

The target population for sampling for the first round of AMR surveillance in animals will be poultry used for meat purpose:

- Broilers
- Spent/ culled layer hens
- Backyard birds

The sampling shall be done to represent birds produced from small, medium, large farms and small subset of backyard birds(around 10% of the sample). For this surveillance, following farms sizes would be considered as small, medium, and large farms which was finalized via expert opinion in technical working group meeting based on poultry farming scenario of Nepal which corresponds with commercial broiler and layers farm size in Nepal.

Table 2. Classification of farm sizes for broilers and layers

Birds	**Farm sizes (number of birds)**		
	Small	**Medium**	**Large**
Broiler	<500	500- 2500	>2500
Layer	<1000	1000- 2500	>2500

Number of samples

The prevalence of resistance to the various antibiotics in the different bacteria/bacterial groups is unknown. The number of isolates required to estimate prevalence of resistance amongst the isolates vary with the expected prevalence and the level of precision required for the prevalence estimate, as shown in Table 3. Highest numbers of samples are required to estimate prevalence levels of 50% for a given precision and sample size increases with higher levels of precision for the estimate.

Table 3. Number of bacterial isolates required to estimate prevalence of resistance to a specific antibiotic in an organism for two levels of precision (5% and 10%) with a 95 % confidence level. (Extracted from OIE Terrestrial Animal Health Code)

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: Table 3 tells the number of bacterial isolates required for the estimation of prevalence of resistance in that bacterial genus or species, not the number of samples to be taken. The number of birds that need to be sampled to produce the target number of bacterial isolates will depend on the prevalence of each bacterial infection in the population being sampled. For example, if the expected prevalence of the organism of interest in the population is 50% of farms, then the number of samples which are needed for the prevalence estimates is double the number shown in the Table 3. The prevalence of the different bacteria is highly variable, ranging from high prevalence levels for *E coli* and lower prevalence levels for *Salmonella spp*. Expected ranges of prevalence for the different bacteria are shown in 4.

Table 4. Expected prevalence of different bacterial infections in poultry

Bacteria	**Expected prevalence in poultry population**
E. coli	80 - 100%
Salmonella	5 – 20%
Campylobacter	80%
Enterococcus	50%

A national sample size of approximately 400 for broilers and 400 for layers is likely to yield 380-400 isolates of *E coli* and lesser numbers of the other bacteria. This will give precise estimates for all AMR prevalence values in *E coli*. However, the lower number of isolates for the lower-prevalence bacteria such as *Salmonella* may result in less precise AMR prevalence estimates, although the precision will depend on how close the estimated prevalence is to 50%.

Number of samples per surveillance site

Since all the four identified surveillance sites are major poultry producing area, samples will be collected from these four sites as mentioned in Table 5. The target population for the first round of AMR surveillance in animals will be poultry used for meat purpose i.e. broilers, layers, and small number of backyard birds. However, the exact number of samples to be collected from the farm is dependent upon the scenario of the farms and capacity of the laboratory with total sample size of 400 broilers, 400 layers and 80 backyard poultry.
Based on the availability, equal number of broilers and layers samples will be collected from the sites.

Table 5. Number of samples to be taken by each laboratory

Laboratory	**Broiler**	**Layer**	**Backyard**	**Total**
CVL	125	125	20	270
NAL	125	150	20	295
Veterinary Laboratory, Biratnagar	50	50	20	120
Veterinary Laboratory, Pokhara	100	75	20	195
Total	400	400	80	880

Number of samples per sub-sector within each surveillance area

The samples will be collected from slaughterhouses/wet bird market and farms. Sampling at collection points in the chicken marketing chain, such as slaughterhouse or wet bird market is

generally the most cost-effective location to collect samples. So, in total, 70% of the samples will be collected from the market (live bird market, slaughterhouses/places/slabs).There is also the trend to sell chicken directly from the farm so remaining 30% of the samples will be collected directly from the farms. It will also help to collect epidemiological information of the area. In both types, the birds ready for selling or slaughtering will be chosen. An individual farm/production unit shall only be sampled once during the study period, regardless of whether samples are collected from the farm, the slaughterhouse or live bird markets. Besides broilers and layers, 20 poultry from backyard poultry rearing system will also be sampled from each surveillance sites during sampling. The detail sampling plan has been shown in Table 6.

Table 6. A sampling plan (number of samples to be collected from different farms)

Laboratories	Type of birds	Small farm		Medium farm		Large farm		Total
		Slaughterhouse /live bird market	Farm	Slaughterhouse /live bird market	Farm	Slaughterhouse /live bird market	Farm	
Central Veterinary Laboratory (CVL)	**Broilers**	30	12	30	12	29	12	125
	Layers	30	12	30	12	29	12	125
	Backyard	20						20
	Total							**270**
Regional Veterinary Laboratory (RVL), Pokhara	**Broilers**	24	10	23	10	23	10	100
	Layers	17	8	17	8	17	8	75
	Backyard	20						20
	Total							**195**
Regional Veterinary Laboratory (RVL), Biratnagar	**Broilers**	12	5	12	5	11	5	50
	Layers	12	5	12	5	11	5	50
	Backyard	20						20
	Total							120
	Broilers	30	12	30	12	29	12	125

National Avian Laboratory (NAL) Chitwan	**Layers**	35	15	35	15	35	15	150
	Backyard	20						20
	Total							**295**
	GRAND TOTAL							880

Note: A broiler and a layer may be sampled from the same vendor in a live bird market if they are held in different cages and the faecal sample can be linked directly to the birds in a cage.

Sampling locations and biological samples

The primary locations of sampling shall be market (slaughterhouses/ slaughter places/ slaughter slabs/ live poultry market), poultry farms and small subset of backyard poultry. Slaughterhouses receive poultry from different types of poultry farms, therefore making it easier to collect sample from the same place,70 percent of the samples will be collected form market. In addition to this, the slaughterhouses can provide information on the size and location of the source farm as well. Remaining 30 percent of the samples will be collected directly from the farm. 20 birds will be sampled from backyard poultry rearing system from each surveillance sites during sampling.

1. Abattoir/slaughterhouse sampling

Caecal samples shall be collected from birds during the slaughter process at slaughterhouses and/or slaughter points.

Pooled caecal samples

Where you can identify individual consignments of birds at slaughterhouses and guarantee that the birds within an individual consignment have all originated from the same farm, we recommend pooling caecal samples from 5 birds into one pooled sample. This will increase the likelihood of identifying the presence of organisms that may be in the flock at lower levels, such as *Salmonella* (Cowling et al., 1999; Christensen and Gardner, 2000; Skov et al., 1999).

Collection of caecal samples

The intact caecum plus content should be collected by clipping at the ileal-caecal junction and at the caecal-colon junction and placing the entire caecum plus content in a whirl-pak. Caeca should be collected from 5 birds at different points within the group and pooled into one bag, i.e. samples should not be collected from 5 birds in a row in the slaughter system.

Note: Samples should be collected from healthy birds and not from birds showing signs of illness.

Non-pooled caecal samples

However, if it is not possible to guarantee that the birds within an individual consignment originate from the same farm, then a caecal sample should be collected from only one bird. Pooled samples must not be collected in such circumstances.

Pooled faecal samples

If it is not practical to collect caecal samples from birds during slaughter, then faecal samples should be collected from birds before slaughtering/killing. Within a consignment of birds from the same farm, collect 10 fresh faecal deposits and place these together in one whirl-pack. If there are multiple cages of birds from the same farm, distribute faecal deposit collection across the different cages to ensure that faeces come from different birds.

Non-pooled faecal samples

If you cannot guarantee that a consignment of birds originates from the same farm, i.e. the consignment is made up of birds collected from different farms, collect only one faecal deposit from the group – do not collect a pooled sample from multiple birds.

2. On-farm sampling

If sampling is conducted on a farm, faecal material from fresh **faecal deposits** should be collected following guidelines below[2].

Shed selection: Samples should be collected from the shed that houses the oldest birds i.e. those that are closest to being sold for consumption. If there are multiple sheds with birds of the same age, then randomly select one shed for sampling.

Pooled samples: Each sample should contain faeces from 10 fresh faecal deposits pooled into the one sample. The fresh faeces should be sampled from different points in the shed also ensuring that areas where birds congregate are sampled. The aim of collecting from different points in the shed is to ensure that the faecal samples come from different birds.

[2]Government of Canada. Chapter 5 Design and Methods. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2015 Annual Report. Public Health Agency of Canada, Guelph, Ontario. 2017.

Collection of faecal samples

A sterile disposable spoon or a sterile tongue depressor should be used to scoop up as much faecal material as possible from a fresh faecal dropping, without contacting the portion of the deposit that is in contact with the shed floor. This minimises contamination of the sample with bacteria in the shed environment.

Fresh faecal deposits should be collected from 10 different points in the shed and pooled into one container.

For Campylobacter, a swab moistened in transport medium should be used to collect faecal material from the same 10 faecal deposits and placed directly into charcoal-containing Amies or charcoal-containing other-transport-media (like a Copan 114C).

3. Live bird market sampling

When sampling birds in live bird markets, fresh **faecal deposits** should be sampled as described above.

Single samples: collect one fresh faecal sample from one cage of broilers or one cage of layers sold by each vendor in the market. Note: faecal deposits should only be sampled from cages that contain only broilers or only layers and not from cages that contain birds of mixed types. Do not pool samples from multiple birds per vendor as the birds are likely to have originated from different farms.

If a vendor is selling both layers and broilers, it is possible to collect a sample from both the broilers and the layers sold by the same vendor if the two groups of birds are held in separate cages and the faecal sample collected is associated with the relevant cage of birds. In this case, the faecal sample from the broiler and the layer cage must be stored separately and the relevant information collected for each sample (see below).

Sampling logistics

Days of sampling: The samples will be collected 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 during the weekend.

***Frequency of sampling*:** Sampling shall be done on a three-week cycle. This way sampling will be distributed across the climatic seasons of the year and capture any seasonal variability in poultry production systems and associated antibiotic use.

Number of samples: On each sampling period, 5-10 samples will be collected.

Sampling locations: Samples will be collected from a range of locations on an individual sampling day. For example, from multiple slaughterhouses, live bird markets and/or farms. Sample one or more farm consignments/live markets per sampling day.

Figures 1 show the sampling plan (number of samples to be collected from different sectors).

Review sampling plan

The experience with sample collection and results of antibiotic sensitivity testing will be reviewed after the first 2-3 months of sampling to identify if any changes need to be made to the sampling plan.

Biosecurity practices when collecting samples

Sample collectors must apply good biosecurity practices[3] when collecting samples from farms to avoid spreading disease from one farm to another or the handlers. This is extremely important to both ensure that disease isn't spread between farms and to avoid farmers associating a disease outbreak that occurs following sampling with the presence of the samplers on their farm.

When making an appointment to collect samples from a farm, check with the farmer /owner of the poultry farm that there is no evidence of infectious disease spreading between birds on the farm. Do not collect samples from a farm where there are signs of illness affecting a group of birds in one or more sheds on the farm. Check again with the farmer when arriving at the farm

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

to ensure that no new disease problems have arisen. If the birds 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 birds are healthy.

It is recommended to use DLS approved virucidal disinfectants (hereafter "virucidal").
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 gumboots, 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 or caecal 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 birds on an individual farm should be transferred to the box before entering the farm, the outside of the box washed with disinfectant before and after entering the farm. After returning to the vehicle, samples should be transferred from the sample collection box to a polystyrene box 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 set of clean overalls that have first been disinfected in virucidal. If contaminated with faeces or other waste, then wash with standard laundry detergents after visiting a farm. A new hair net should also be worn.
    b. Scrub gumboots with soap and water if they are not clean.
    c. Brush gumboots carefully with virucidal solution or dip boots into the container of virucidal solution.
    d. Scrub the outside of the box containing the sampling materials using the virucidal solution.
    e. Wash hands using soap and water or rub hands with hand sanitizer.
    f. Apply gloves.

6. **Immediately after leaving the farm** and before entering your vehicle all samplers must undertake the following:
    a. Remove gloves and dispose of in rubbish bag.
    b. Scrub gumboots with soap and water to remove all manure, dust and dirt.
    c. Brush clean boots with virucidal solution or dip boots into the container of virucidal solution.
    d. Remove hair net and place in a rubbish bag.
    e. Remove overalls and place in a large plastic bag for storage in the vehicle and 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 virucidal 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 virucidal. If significantly contaminated with faeces or other waste, then washed with standard laundry detergent before being used by samplers on subsequent farms.

Sample transport

Store all samples in secure containers in polystyrene boxes with ice pads while all samples are being collected. Keep chilled (<10^{o}C), but not freezing, to prevent overgrowth of samples.
Note: never freeze samples as this may kill the bacteria.
Samples should ideally be transported to the laboratory on the same day of collection. If they cannot be transported on the same day, they must be stored in a refrigerator at no more than 4^{o}C and transported to the laboratory the next day.

Culture, identification and AST

Annex 1 provides instructions for culture and identification of the bacteria.

Confirmatory resistance testing (Central Veterinary Laboratory)

Colistin

AMR reference laboratories will conduct testing for resistance to colistin in *E coli* and *Salmonella* using broth dilution, as disk diffusion or e-test methods are not reliable for colistin[4].

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 will perform confirmatory phenotypic testing for ESBL production and carbapenem resistance on any *Salmonella* and *E. coli* isolate that shows resistance to cefotaxime or ceftazidime in the initial testing conducted by surveillance laboratories.

Confirmatory testing should differentiate the resistance pattern into one of the following four categories: (1) ESBL phenotype, (2) AmpC phenotype, (3) ESBL + AmpC phenotype, 4) carbapenem phenotype[5].

Salmonella serotyping

Reference laboratories will serotype *Salmonella* isolates, testing for the most common serotypes likely to be in the country.

Isolate storage

Annex 2 provides details for storage of isolates in regional and reference laboratories, following guidelines recommended in the WHO Tricycle project.

[4]EUCAST, 2016. Recommendations for MIC determination of colistin (polymyxin E) As recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf

[5]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

Sample collection form

Sample collection form for each sample will be completed to capture descriptive information that will help correctly interpret the AMR results. Forms will 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 the area from which it comes, has a unique sample ID.

Note: Ensure that the ID number written on the form matches the ID number on the sample tube.

Data management and analysis Plan

The data recorded entered in the paper-based ledger shall be entered ideally in the WHONET. Until, laboratory staffs are trained to use the WHONET, the data will be stored in the Microsoft Excel sheet. The data will be analysed at frequent interval to present the trends during the bi-monthly meeting of the TWG-AH and other higher-level AMR committees. A dashboard shall be used for quick data visualization. For longer term data analysis using epidemiological information collected during sampling, appropriate statistical software such as Epi Info, SPSS, SAS or R shall be used.

Data dissemination / reporting plan.

It would help to make a flow diagram.

References

1. Adeyi, Olusoji O.; Baris, Enis; Jonas, Olga B.; Irwin, Alec; Berthe, Franck Cesar Jean; Le Gall, Francois G.; Marquez, Patricio V.; Nikolic, Irina Aleksandra; Plante, Caroline Aurelie; Schneidman, Miriam; Shriber, Donald Edward; Thiebaud, Alessia. 2017. Drug-resistant infections: a threat to our economic future (Vol. 2) : final report (English). Washington, D.C.: World Bank Group. Available at: http://documents.worldbank.org/curated/en/323311493396993758/final-report (accessed November 25, 2018).

2. Basnyat, B., Pokharel, P., Dixit, S., & Giri, S. (2015). Antibiotic Use, Its Resistance in Nepal and Recommendations for Action: A Situation Analysis. Journal of Nepal Health Research Council, 13(30), 102–111.
3. Cowling, D. W., Gardner, I. A., & Johnson, W. O. (1999). Comparison of methods for estimation of individual-level prevalence based on pooled samples. Preventive Veterinary Medicine, 39(3), 211-225.
4. CBS (2014). Summary Report & Major Findings: Nepal Commercial Poultry Survey 2071/72, Thapathali, Kathmandu.
5. Chaudhary, P., Bhandari, D., Thapa, K., Thapa, P., Shrestha, D., Chaudhary, H. K., … Gupta, B. P. (2016). Prevalence of Extended Spectrum Beta-Lactamase Producing Klebsiella Pneumoniae Isolated From Urinary Tract Infected Patients. Journal of Nepal Health Research Council, 14(33), 111–115.
6. Christensen, Jette, and Ian A. Gardner. "Herd-level interpretation of test results for epidemiologic studies of animal diseases." Preventive veterinary medicine 45, no. 1-2 (2000): 83-106.
7. Dhital, S., Sherchand, J. B., Pokharel, B. M., Parajuli, K., Mishra, S. K., Sharma, S., … Rijal, B. (2017). Antimicrobial susceptibility pattern of Shigella spp. isolated from children under 5 years of age attending tertiary care hospitals, Nepal along with first finding of ESBL-production. BMC Research Notes, 10(1), 192. https://doi.org/10.1186/s13104-017-2512-1
8. FAO, 2015. The FAO Action Plan on Antimicrobial Resistance 2016-2020. Available at: http://www.fao.org/policy-support/resources/resources-details/en/c/459933/ (accessed November 20, 2018).
9. Ghimire, L., Singh, D. K., Basnet, H. B., Bhattarai, R. K., Dhakal, S., & Sharma, B. (2014). Prevalence, antibiogram and risk factors of thermophilic Campylobacter spp. in dressed porcine carcass of Chitwan, Nepal. BMC Microbiology, 14, 85. https://doi.org/10.1186/1471-2180-14-85
10. Hunt, J. M., Abeyta, C., & Tran, T. (2001). Laboratory Methods - BAM: Campylobacter. In Bacteriological Analytical Manual (8th ed.). Center for Food Safety and Applied Nutrition.
11. Joshi, R. D., Khadka, S., Joshi, D. M., Shrestha, B., Dangal, G., Acharya, K. P., … Dongol, Y. (2018). Antimicrobial Sensitivity Trend in Blood Culture Positive Enteric Fever. Journal of Nepal Health Research Council, 16(2), 228–232.
12. Maharjan, S., Rayamajhee, B., Shreshtha, A., & Acharya, J. (2017). Serotyping and

antibiotic susceptibility patterns of Vibrio and Shigella isolates from diarrheal patients visiting a Tropical and Infectious Diseases Hospital in central Nepal. BMC Research Notes, 10(1), 626. https://doi.org/10.1186/s13104-017-2967-0

13. Paterson, D. L., & Harris, P. N. A. (2016). Colistin resistance: a major breach in our last line of defence. The Lancet. Infectious Diseases, 16(2), 132–133. https://doi.org/10.1016/S1473-3099(15)00463-6
14. Pokhrel, B., Koirala, T., Shah, G., Joshi, S., & Baral, P. (2018). Bacteriological profile and antibiotic susceptibility of neonatal sepsis in neonatal intensive care unit of a tertiary hospital in Nepal. BMC Pediatrics, 18(1), 208. https://doi.org/10.1186/s12887-018-1176-x
15. O'Neill, 2014. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. Available at: https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf (accesses November 20, 2018).
16. Shakya, G., Acharya, J., Adhikari, S., & Rijal, N. (2016). Shigellosis in Nepal: 13 years review of nationwide surveillance. Journal of Health, Population, and Nutrition, 35(1), 36. https://doi.org/10.1186/s41043-016-0073-x
17. Shrestha, A., Regmi, P., Dutta, R. K., Khanal, D. R., Aryal, S. R., Thakur, R. P., … Singh, U. M. (2010, August). First report of antimicrobial resistance of Salmonella isolated from poultry in Nepal. Veterinary Microbiology. Netherlands. https://doi.org/10.1016/j.vetmic.2010.04.015
18. Skov, Marianne N., B. Carstensen, N. Tornøe, and Mogens Madsen. "Evaluation of sampling methods for the detection of Salmonella in broiler flocks." Journal of Applied Microbiology86, no. 4 (1999): 695-700.
19. Subedi, M., Luitel, H., Devkota, B., Bhattarai, R. K., Phuyal, S., Panthi, P., … Chaudhary, D. K. (2018). Antibiotic resistance pattern and virulence genes content in avian pathogenic Escherichia coli (APEC) from broiler chickens in Chitwan, Nepal. BMC Veterinary Research, 14(1), 113. https://doi.org/10.1186/s12917-018-1442-z
20. Upreti, N., Rayamajhee, B., Sherchan, S. P., Choudhari, M. K., & Banjara, M. R. (2018). Prevalence of methicillin resistant Staphylococcus aureus, multidrug resistant and extended spectrum beta-lactamase producing gram negative bacilli causing wound infections at a tertiary care hospital of Nepal. Antimicrobial Resistance and Infection Control, 7, 121. https://doi.org/10.1186/s13756-018-0408-z

21. Zellweger, R. M., Basnyat, B., Shrestha, P., Prajapati, K. G., Dongol, S., Sharma, P. K., … Karkey, A. (2018). Changing Antimicrobial Resistance Trends in Kathmandu, Nepal: A 23-Year Retrospective Analysis of Bacteraemia. Frontiers in Medicine, 5, 262. https://doi.org/10.3389/fmed.2018.00262
22. WHO, 2015. Global Action Plan. Available at: https://www.who.int/antimicrobial-resistance/global-action-plan/en/ (accessed November 20, 2018).

Annex 1. Kits for biosecurity practices during sampling

Items to be carried in the vehicle for implementing biosecurity practices

The following items are to be carried in the vehicle and used during sample collection. Sufficient number of overalls gloves and mask so that every sampler can wear a clean set for every farm visited during a single sampling day.

- Premixed virucidal solution carried in a sealed container in which gumboots can be dipped
- First aid box
- Bucket
- Soap
- Scrubbing brush
- Large container of water
- Disposable paper towels
- Rubbish bag for paper towels,
- Plastic bag for used overalls

Annex 2. Sample collection form for poultry farm

Sample collection form for poultry farm

1. Sample collection: Date Time:
2. Sample identification number: (Any format?)
3. Address of farm
   District:
   Province:
4. Species: Chicken

a. Production type (Circle One): Broiler Spent (or culled) layer Backyard Other

b. Breed:

5. Age of bird in weeks (approximate, if known):
6. Type of sample (Circle One): Caecal Faeces
7. Pooled sample (Circle One): Yes No

If yes, how many individual samples are combined in the pool:

8. Farm classification (Circle One): Small Medium Large
9. Flock size:
10. 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):
11. Number of chickens in the shed that was sampled:
12. Source of chickens (name of hatchery if known):
13. Other species on the same farm (Circle): Ducks Geese Guinea fowl Goats Sheep Cattle Buffalo Pigs Fish

Annex 3. Sample collection form for live bird market

Sample collection form for live bird market

1. Sample collection: Date Time:
2. Sample identification number:
3. Name of sampler:
4. Address of live market
a. District:
b. Province:
5. Species: Chicken
a. Bird type (Circle One): Broiler Spent (or culled) layer Backyard Other
b. Breed:
6. Age of bird in weeks (approximate, if known):
7. Type of sample (Circle One): Caecal Faeces
8. Pooled sample (Circle One): Yes No
If yes, how many individual samples are combined in the pool:
9. Average number of chickens sold per day:
10. Is slaughtering done at the market (Select One)? Yes No
11. Catchment area for the vendor's poultry:
12. Other species sold by the vendor (Circle): Ducks Geese Guinea fowl
Goats Sheep Cattle Buffalo Pigs Fish

Annex 4. Sample collection form for slaughterhouse/ slaughter place

Sample collection form for slaughterhouse/ slaughter place

1. Sample collection: Date Time:
2. Sample identification number:
3. Name of sampler:
4. Address of slaughterhouse
c. District:
d. Province:
5. Species: Chicken\
Address of the farm:
. District:
Province:

a. Bird type (Circle One): Broiler Spent (or culled) layer Backyard Other
b. Breed:
6. Age of bird in weeks (approximate, if known):
7. Type of sample (Circle One): Caecal Faeces
8. Pooled sample (Circle One): Yes No
If yes, how many individual samples are combined in the pool:
9. Average number of chickens slaughtered per day:
10. Catchment area of slaughterhouse/place for poultry supply:
11. Other species slaughtered in same premises (Circle): Ducks Geese Guinea fowl Goats Sheep Cattle Buffalo Pigs Fish

Annex 5: Laboratory testing

a. Sample preparation and enrichment

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

The pooled caeca from one bag are pulverised with a rubber mallet and mixed well.

Or

The pooled faecal deposits in one bag are mixed well.

1 gram of caecal or faecal sample is added to 9 ml Buffered Peptone Water (BPW) in a sterile screw capped tube (50ml tubes recommended).

Samples should be gently mixed but not be shaken to avoid spillage.

- **For *E coli and Salmonella*:** Incubate for 16-24 hours at 36 ±1^{o}C aerobically.
- **For *Enterococcus*:** a sub-sample of the BPW must be mixed with Azide Dextrose Broth or Brain Heart Infusion BEFORE the BPW mixture is incubated (see details below under e. Enterococcus).

For Campylobacter: The swab used to collect faecal material placed in charcoal-containing transport medium (or a swab mixed through the pooled caecal sample) is added to 9 ml of an enrichment broth (e.g. Bolton broth) in a sterile screw capped tube (50ml tubes recommended).

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
   or
2. an anaerobic jar gassed with pre-mixed microaerobic (5% O_2, 10% CO_2, 85% N_2) gas mix[8]
   or
3. a variable atmosphere incubator if one is available in the laboratory.

b. ESBL Positive Enterobacteriaceae

Subculture

Following incubation, inoculate the enriched Buffered Peptone Water onto one 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

OR

2. MacConkey agar mixed with 1 mg/L of cefotaxime

OR

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 resistance.

Isolate purification

Subculture at least **four** typical *Enterobacteriaceae* colonies from the plate to non-selective media such as Blood agar or Nutrient agar.

Incubate at 36 ±1 °C for 18-24 hours, aerobically.

Tentative Identification

Enterobacteriaceae can be identified by the following options:

1. Roughly identified by bacteriology (oxidase, lactose fermentation, able to grow on MacConkey agar), and then

2. Further differentiated by further biochemical testing (such as triple sugar-iron agar, lysine decarboxylase, urease, Indole, SIM, citrate utilisation, motility).

All purified isolates should be stored and sent to the AMR reference laboratory for confirmatory testing of the specific type of resistance.

c. Escherichia coli

Subculture

Following incubation, inoculate the enriched Buffered Peptone Water onto MacConkey agar using a sterile swab or 10 ul inoculum and streak for single colonies. Incubate plates aerobically at 36 ±1 °C for 18-24 hours.

Isolate purification

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

Incubate at 36 ±1 °C for 18-24 hours, aerobically.

Identification

E. coli can be identified by the following options:

1. Roughly identified by bacteriology (oxidase, lactose fermentation, able to grow on MacConkey agar), and then
2. Further differentiated by further biochemical testing (such as triple sugar-iron agar, lysine decarboxylase, urease, motility).

Antimicrobial susceptibility testing

Test the three isolates for antimicrobial susceptibilities by disk diffusion using the panel of antibiotics listed in **Error! Reference source not found.1** as prescribed by CLSI.

All purified isolates, regardless of AST results, should be stored and sent to the AMR reference laboratory for confirmatory testing of the specific type of resistance.

---

d. Salmonella

Subculture 1

Either of the following two options:

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

   Incubate aerobically at 42 °C, preferably in a water bath for 20-24 hours.

OR

2. Transfer 1 ml of enriched Buffered Peptone Water to 10 ml of tetrathionate broth + iodine.

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

Subculture 2

Subculture selective broths to XLD (with or without novobiocin).

Incubate aerobically 20-24 hours at 35 °C.

Isolate purification

Subculture **three** typical colonies from the plates to non-selective media such as Blood agar or Nutrient agar and incubate at 35 °C for 18-24 hours, aerobically.

Identification

Salmonella can be identified by the following options:

1. Bacteriology (triple-sugar iron agar, urea, lysine decarboxylase; add others from E coli)
2. Serology (Salmonella OH antisera-based agglutination)

Antimicrobial susceptibility testing

Test isolates for antimicrobial susceptibilities by disk diffusion against the panel of antibiotics listed in **Error! Reference source not found.1** as prescribed by CLSI.

All purified isolates, regardless of AST results, should be stored and sent to the AMR reference laboratory for confirmatory testing of the specific type of resistance.

e. ***Enterococcus***

Before BPW is incubated, add 1 ml of Buffered Peptone Water mixture to approximately 10 ml Azide Dextrose Broth[1] (OR Brain Heart Broth + 3 mg/L vancomycin)[2]

Incubate broth for 18-24 hours at 35°C.

Subculture

1. Subculture broth to selective agar such as Slanetz and Bartley (Pleydell, 2010). Incubate 18-24 hours at 42°C aerobically.

AND

2. Subculture broth to CHROMagar™ VRE (Peltroche-Llacsahuanga, et al., 2009) OR Brilliance™ VRE (Gouliouris et al. 2016) and incubate 18-24 hours at 37°C aerobically.

Isolate purification

Subculture **3-4** colonies (if present, choose colonies that appear different) from each plate (if present) to Blood agar or Nutrient agar.

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

Identification

Enterococci will be identified by catalase test with PYRase.

There is no need to identify Enterococci to species level at this stage.

Antimicrobial susceptibility testing

Test isolates for antimicrobial susceptibilities by disk diffusion against the panel of antibiotics listed in **Error! Reference source not found.** as prescribed by CLSI.

All purified isolates, regardless of AST results, should be stored and sent to the AMR reference laboratory for confirmatory testing of the specific type of resistance.

f. Campylobacter

Subculture

Subculture the incubated enrichment broth onto charcoal-containing agar such as mCCDA.

Incubate plates at 42°C for 40-48 hours in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2), using either of the following options:

4. a microaerobic gas pack (such as CampyGen ™ or CampyPak ™) in an anaerobic jar

   OR

5. an anaerobic jar gassed with pre-mixed microaerobic (5% O_2, 10% CO_2, 85% N_2) gas mix (Hunt et al., 2001).

   OR

6. a variable atmosphere incubator if one is available in the laboratory.

Isolate purification

Subculture one typical colony from the plates to non-selective media such as Blood agar. Incubate at 42 °C for 24-48 hours, microaerobically.

Identification

Presumptive *Campylobacter* can be identified by characteristic colony morphology on charcoal-containing agar, oxidase, catalase and cell shape/motility (preferably by dark-field microscopy).

C. jejuni can be distinguished from *C. coli* using a Hippurate test. *C jejuni* are usually hippuricase positive while *C. coli* are not.

Antimicrobial susceptibility testing

Test isolates for antimicrobial susceptibilities to the panel of antibiotics listed in Table 1**using disk diffusion as prescribed by CLSI.**

All purified isolates, regardless of AST results, should be stored and sent to the AMR reference laboratory for confirmatory testing of the specific type of resistance.

Annex 2: Storage of isolates

Short term storage

All pure isolates other than *Campylobacter* should be inoculated onto the surface of a nutrient agar for short term storage. Blood agar, tryptone soy agar (TSA) and heart infusion agar (HIA) are examples of good storage media for enteric organisms. Carbohydrate-containing media (e.g. Kligler iron agar (KIA) or triple sugar iron agar (TSI) should not be used because acidic by-products of metabolism quickly reduce viability of the organisms.

Place tubes of medium that are still hot after autoclaving in a slanted position to provide a short slant and deep butt (2 – 3 cm). To inoculate, stab the inoculating needle to the butt of the medium once or twice, and then streak the slant. Incubate the culture overnight at 35^{o} – 37^{o}C.

Seal the tube with cork stoppers that have been soaked in hot paraffin or treated in some other way to provide a tight seal. Store cultures at 22^{o} – 25^{o}C in the dark.

Campylobacter isolates should be stored on a heavily inoculated swab in charcoal-containing transport medium in a cool location and shipped to the reference laboratory preferably within a week.

Long term storage

Isolates should be regularly sent to the AMR reference laboratory to be stored in a national biorepository maintained within a -80 freezer. Isolates should be stored in glycerol broth as follows:

I. Inoculate a TSA or HIA slant (or other non-inhibitory, salt-containing growth medium) and incubate at 35^{o} – 37^{o}C.
II. Harvest cells from the slant and make a suspension in the freezing medium.
III. Freezing medium is prepared with Tryptic Soy Broth (TSB) with glycerol in a proportion of 2:1. The medium is dispensed in cryogenic screw-capped vials (cryovials) and autoclaved.

IV. A thick growth of bacteria is picked with a sterile loop from a non-selective medium and emulsified in the freezing medium. The cryovials can then be snap-frozen in liquid nitrogen or stored at -80^{o}C. Long term storage of isolates at -20^{o}C is not recommended as some organisms will lose viability at this temperature.

Note: glass ampoules should never be used for freezing in liquid nitrogen because they can explode upon removal from the freezer.

Recovery of isolates from long term storage

- Remove the frozen cultures from the freezer and transfer to a bio- safety cabinet or a clean area if a cabinet is not available.
- Using 1 ul sterile disposable plastic loop, scrape the top-most portion of the culture and transfer to a growth medium, being careful not to contaminate the top or inside of the vial.
- Re-close the vial before the contents thaw and return to the freezer. With careful technique, transfers can be successfully made from the same vial several times.
- Incubate 18-24 hours at 35-37^{o}C. Perform at least one sub-culture before using the isolate to inoculate a test.

Annex 3. Equipment

Essential equipment

1. Autoclave, with temperature recording device(s), capable of holding 121 °C for 30 minutes within a waste load.
2. Incubator, 35 °C, aerobic (capable of holding 35 +/- 1 °C)
3. Incubator, 36 +/- 1°C, aerobic (capable of holding 37 +/- 1 °C)
4. Incubator, 42 °C, aerobic (capable of holding 42 +/- 1 °C), tall enough to fit anaerobic jars (if used)
5. Refrigerator or cold room capable of holding 2-8 °C
6. Freezer, -80 °C for reference laboratories and -20 °C for regional laboratories (not 'frost-free')
7. Calibrated (or capable of being calibrated) thermometers or temperature recording devices (ones capable of recording maximum and minimum temperatures are preferable), one for each incubator/fridge/freezer

8. McFarland 0.5 and 2.0 standards
9. Disk dispensers with desiccant (NB they are disk-brand dependent) or desiccator for storing opened antimicrobial disk cartridges.
10. Forceps (metal) if disk dispensers are not used.
11. Coplin jars or similar for ethanol sterilizing scissors/clippers/forceps
12. Scissors/clippers, metal (for cutting swabs)
13. Bunsen burner(s) or Bacti-Cinerator (or similar) if gas is not available.
14. Callipers or ruler (for measuring zone sizes)
15. Loops, nichrome or plastic disposables
16. Micropipette capable of measuring 100 µl
17. Microscope light, with oil-immersion objective
18. Quality control organisms as specified by the relevant CLSI standards

Other Equipment

1. Oven or microwave oven for drying desiccant (alternatively the 42 °C incubator can be used)
2. Stirred water bath capable of holding 42 °C +/- 0.2 °C
3. Dark-field microscope
4. Anaerobic jars/boxes (essential if doing *Campylobacter)*

Annex 4. Maps of surveillance sites

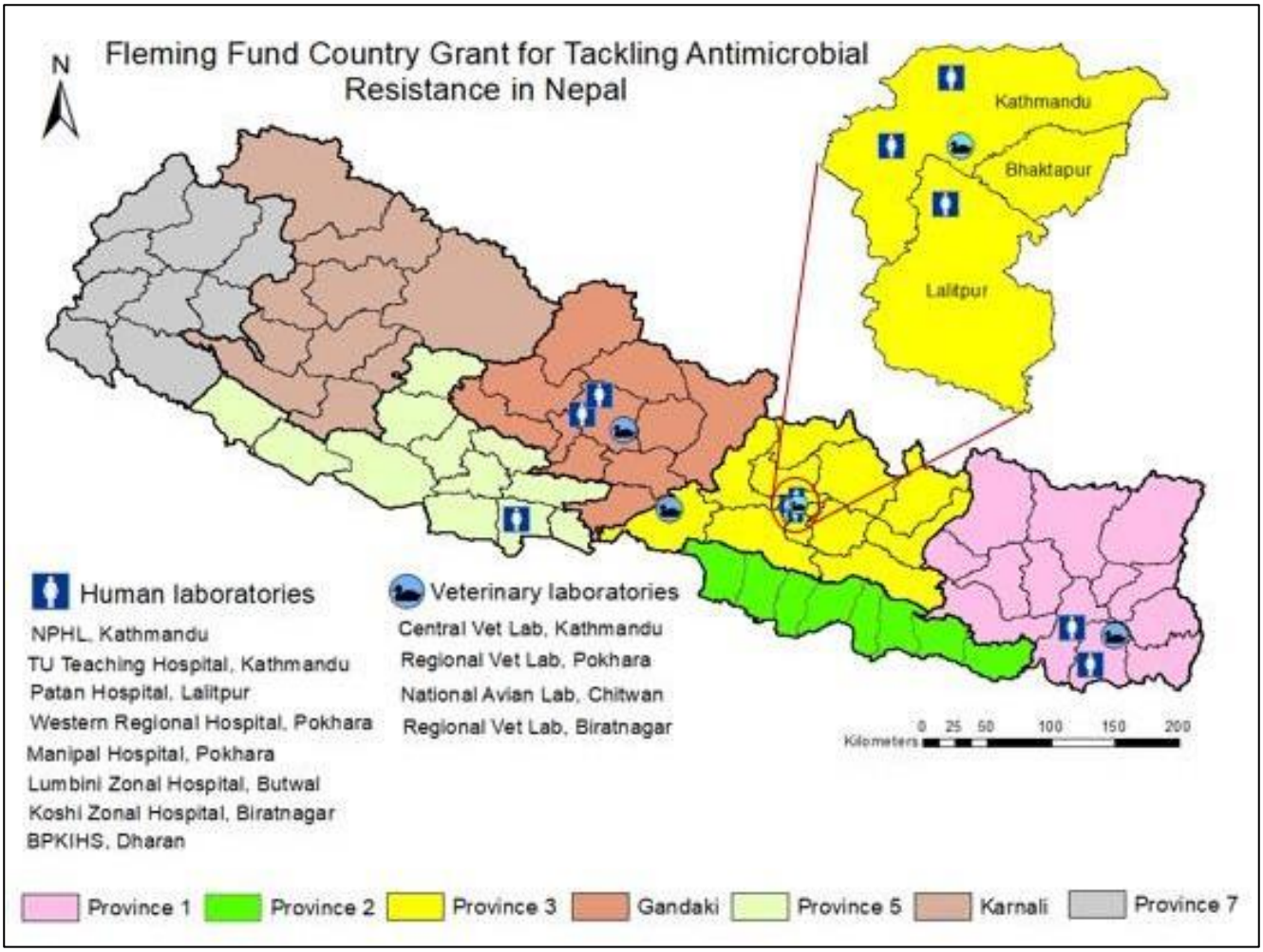

Figure 1: Distribution of human and animal surveillance sites supported by Fleming Fund. The original locations have been moved slightly to avoid overlapping of laboratories in the map.

Figure 2: Map of Kathmandu valley with location of Central Veterinary Laboratory.

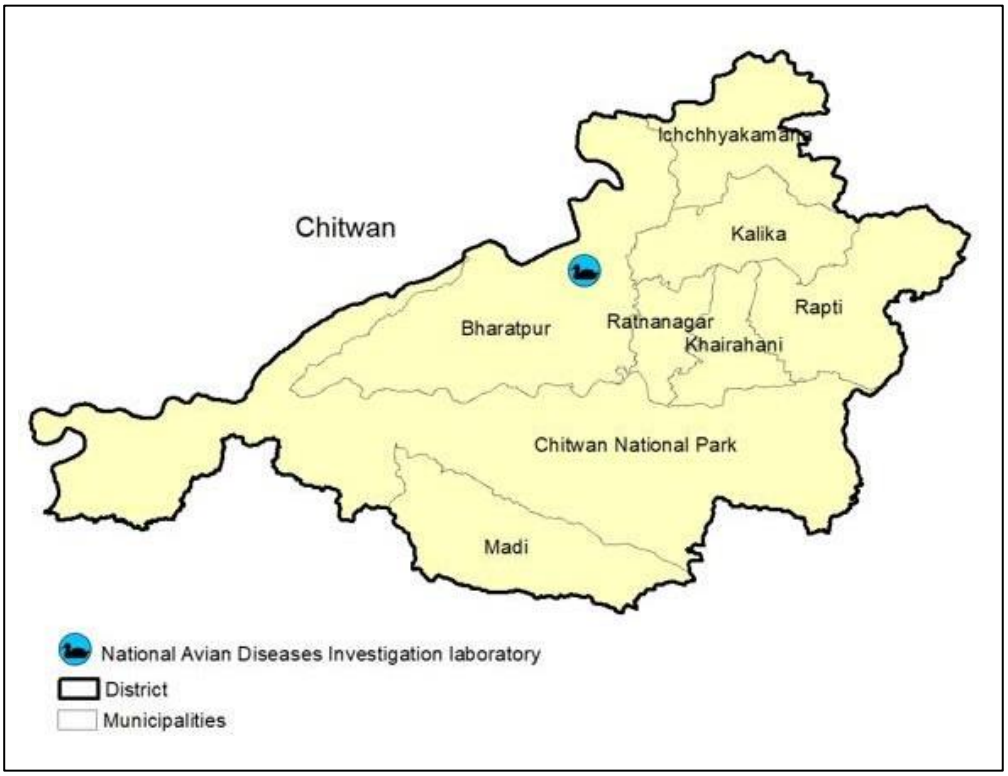

Figure 3: Map of Chitwan district with location of National Avian Laboratory.

Figure 4: Map of Kaski district with location of Veterinary Laboratory, Pokhara.

Figure 5: Map of Morang district with location of Veterinary Laboratory, Biratnagar.