Active Surveillance Protocol for Antimicrobial Resistance in Poultry Population in Nepal

(Version 2.0)

Ministry of Agriculture and Livestock Development
Department of Livestock Services,

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Disclaimer

Text used within this protocol entitled "Active surveillance protocol for Antimicrobial Resistance in Poultry Population in Nepal, 2024" has been updated from the "Active surveillance protocol for Antimicrobial Resistance in Poultry Population in Nepal, 2019'"and other sources including but not limited to: National Action Plan on Antimicrobial Resistance, FAO, WHO and WOAH reference documents, Flemming initiative and experience gained from the past surveillance activities.

Development of the earlier version of the protocol was largely adapted 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 with support from the Fleming Fund. The protocol mostly focusses on conducting active surveillance in broilers, spent layer population and backyard poultry in selected sites in Koshi, Madesh, Bagmati, Gandaki, Lumbini, Karnali and Sudur Paschim provinces.

In the updated protocol, some AMR data and information are updated where applicable and sample size for surveillance has been revised based on the previous AMR surveillance results. The term AMR used in this document specifically refers to antibiotic resistance. This plan shall be reviewed as and when needed by the AMR-TWG and updated as decided by DLS.

'Active surveillance protocol for Antimicrobial Resistance Population in Poultry Population in Nepal, 2019' is hereby replaced. All the activities done, and the actions taken under this shall be claimed to have been done and taken under this.

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

Content

Introduction

The projected burden and losses due to Antimicrobial Resistance (AMR) is high in the developing countries due to limited resources and capacities to tackle these problems. Actual burden of AMR in Nepal is not estimated yet, however, evidence generated from past works in various sectors suggest that the problem of AMR is increasing and health care associated cost in public health and farm level infection control cost is growing high. Laboratory-based surveillance data 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 (NADIL), Chitwan perform bacterial culture, identification and antimicrobial susceptibility testing on samples from poultry, milk, water and other samples brought to the laboratory for diagnostic purposes. In addition, some laboratories in the province, district and local levels also perform bacterial culture and sensitivity testing. CVL, NADIL, Veterinary Laboratory Biratnagar, Veterinary Laboratory Pokhara and Veterinary Laboratory Surkhet have also been conducting active surveillance of AMR since 2021. From the animal sources, *E. coli, Klebsiella* spp., *Bacillus* spp., *Campylobacter* spp., *Salmonella* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Streptococcus* spp. are routinely identified from both active surveillance and clinical samples. These pathogens have developed resistance against commonly used antibiotics viz. Gentamicin, Amoxycillin, Enrofloxacin, Ceftriaxone, Amoxycillin (CVL AMR Newsletetr 2023, CVL, 2017; Ghimire et al., 2014; Shrestha et al., 2010; Subedi et al., 2018). Multi-drug resistance (MDR) in *E. coli* (57%), *Proteus* spp. (36%), *Pseudomonas* spp. (48%) and *Klebsiella* spp. (36%) were reported from CVL in the samples of poultry, cattle and buffalo

(CVL, 2023). Multi drug Resistance (MDR) *E. coli* isolates from poultry were found to be resistant to ciprofloxacin, tetracycline (97%), levofloxacin (95%), ampicillin (93%) and gentamicin (83%). The surveillance system in animal health is progressing and is generating evidence which is critical for guiding policy formulation to control the problem of development of AMR in animal health sector.

A population-based surveillance can give an estimate of AMR prevalence reflective of the target population. In Nepal, among the livestock sector, poultry is the most commercialized sub-sector and has been rapidly growing since last couple of decades. The total annual commercial chicken meat and egg production in Nepal is estimated to be 204,923 tons and 1.33 billion pieces respectively (MoALD, 2023). 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, Makwanpur, Bara, Pokhara, Biratnagar, Butwal, Dang and Dhangadi. Subsequently, use of antimicrobials is also increasing primarily for therapeutic purposes but sometimes also for preventive purposes. Indiscriminate use of antibiotics in poultry industry is very likely to contribute to AMR problem. Given these backgrounds, broiler and spent layer poultry population have been prioritized 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 is of concern in public health globally. For better estimates of resistance prevalence, the surveillance system developed has been extended to include wider geographical areas based on poultry value chain.

This operating protocol has been prepared to conduct the active surveillance in poultry as a building block for strengthening AMR surveillance in animals. This document specifies structured active surveillance of specified poultry populations that helps to estimate and monitor AMR in poultry that are as representative as possible of the target populations that are sampled.

Objectives

General Objective

Strengthen AMR surveillance in animal health sector primarily focusing on poultry to contribute for national AMR surveillance and response.

Specific Objectives

1. Conduct population-based active surveillance for antimicrobial resistance in poultry.
2. Isolate the target bacteria and determine their antimicrobial resistance status.
3. Evaluate the temporal and spatial distribution of the bacterial isolates and their antimicrobial resistance pattern in poultry population.
4. Maintain a national biorepository of bacterial isolates at CVL for further investigation.
5. Share information with one health stakeholder (in one health platform) for strengthening one health approach in AMR surveillance and response.
6. Identify possible risk factors that may influence AMR for further investigation.
7. Establish system for identification and analysis of AMR genes in target bacteria.

Sentinel sites for AMR surveillance in animal health sector

Laboratories functioning under the Department of Livestock Services (DLS) will function as surveillance sites for AMR surveillance. The Central Veterinary Laboratory (CVL) will also function as the referral and coordinating laboratory besides one of the surveillance sites for active surveillance. Laboratories beyond the DLS system can also be included as collaborators for the surveillance activities.

Based on the objective of the surveillance, context, target population, area of coverage, resource availability and timeframe, the Technical Working Group will decide the surveillance sites participating in the surveillance.

Target bacteria

The target bacteria for the AMR surveillance in poultry are zoonotic bacteria, animal pathogens and commensal gut bacteria and which may potentially be associated with transmitting AMR to animals and 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 is one of the common isolated organisms in the bacterial culture from different types of samples brought at the laboratories. *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. However, *Campylobacter* is a more challenging bacterium to culture, it may only be feasible for more experienced laboratories to grow this organism. Therefore, for the purpose of active surveillance CVL will be performing *Campylobacter* culture and susceptibility testing.

[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

Target antibiotics

The panel of antibiotics were selected for each four bacteria following WHO's critically and highly important antimicrobial classes for humans and also considering WOAH list of antimicrobial agents of veterinary importance as mentioned in Table 1, for which resistance shall be tested in the specified zoonotic, pathogenic and commensal bacteria cultured from broilers and layers.

TABLE 1 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			
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		
Macrolides				Erythromycin

Diagnostic testing

Detailed instructions for culture and identification of the target bacteria are provided in Annex 5. Antibiotic Sensitivity Testing (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 CVL. 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).

A proportion of all isolates (10%) from the participating laboratories will be tested at the CVL as part of Quality Assurance (QA) of isolation and identification procedures.

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 5 for details).

Surveillance scheme

The surveillance will be methodically designed on a periodic basis. The Technical Working Group (TWG) will determine the surveillance sites, target population, sample size and the sampling framework. These decisions will be guided by the context, timeframe, availability of resources and the overarching objectives of surveillance.

The detailed surveillance scheme is presented in Annex 8.

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 samples from the same place, seventy five percent of the samples will be collected from market. In addition to this, the slaughterhouses can provide information on the size and location of the source farm as well. The remaining twenty five percent of the samples will be collected directly from the farm.

1. Abattoir/slaughterhouse sampling

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

Pooled caecal samples

If individual consignments of birds at slaughterhouses could be identified and collector can guarantee that the birds within an individual consignment have all originated from the same farm, pooling caecal samples from 5 birds into one pooled sample is recommended. 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-pack. 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

If it is not possible to guarantee that the birds within an individual consignment originate from the same farm, then 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.

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 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.

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 minimizes 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.

***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.

Review of 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 design.

Biosecurity practices when collecting samples

Sample collectors must apply good biosecurity practices 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 donot 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 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 sanitizer. 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 (<10ºC), 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 4ºC and transported to the laboratory the next day.

Culture, identification and AST

Follow the process as mentioned in Annex 5 for culture and identification of the bacteria.

Confirmatory resistance testing (to be performed at CVL)

Colistin

AMR reference laboratories shall conduct testing for resistance to colistin in *E coli* and *Salmonella* using broth dilution, as disk diffusion or e-test methods are not reliable test for colistin[2].

ESBL and Carbapenemase resistance

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

The AMR reference laboratory 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[2].

Salmonella serotyping

CVL will serotype *Salmonella* isolates from animals, for testing the most common serotypes likely to be present in Nepal.

Isolate storage

Details for storage of isolates in regional and reference laboratories is mentioned in Annex 2.

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 as mentioned in Annexes 2,3 and 4.

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: The sample collection team should ensure that the ID number written on the form matches the ID number on the sample tube/pack.

[2]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

Data management and analysis

The data on sample (sample collections forms) will be collected in tablet/mobile using appropriate data collecting app and paper records of the same will be kept. The data on AST of the samples will be recorded in paper ledger and in electronic format in the LIMS system. The data will be analyzed using MS Excel or any appropriate statistical software at frequent intervals 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 in the LIMS.

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

1. **Sample collection**

   Date: Sample collection time:

2. **Sample code:**

3. **Name of respondent:** Contact No:

4. **Name of farm:**

5. **Address:**

   Province: District: Local level: Ward no.:

6. **GPS location:** Latitude/Longitude

7. **Type of poultry (Multiple choice):**

   Backyard chicken/Commercial broiler/Commercial layer/Broiler parent/Layer parent/Duck/ Turkey/Kalij/Kuroiler/Others

8. **Source of chicken (Hatchery):**

9. **Farm classification:** Small (less than 500)/Medium (500-1500)/Large (More than 1500)

10. **Age of poultry (in weeks):**
11. **Flock size:**
12. **Number of management units on the farm:**
13. **Species sampled (Only one):**

    Backyard chicken/Commercial broiler/Commercial layer/Broiler parent/Layer parent/Duck/ Turkey/Kalij/Kuroiler/Others
14. **Type of sample (Any one):** Caecal/Feces
15. **Pooled sample:** Yes/No

    If yes, how many individual samples are combined in the pool:
16. **Number of chickens in the sampled unit:**
17. **Other species on the farm (Multiple choice):** Cattle/Buffalo/Sheet/Goat/Pig/Fish/Others
18. **Name of sampler:**

Annex 3. Sample collection form for live bird market

1. **Sample collection**

   Date: Sample collection time:
2. **Sample code:**
3. **Name of respondent:** Contact No:
4. **Name of live market:**
5. **Address:**

   Province: District: Local level: Ward no.:
6. **GPS location:** Latitude/Longitude
7. **Type of poultry (Multiple choice):**

Backyard chicken/Commercial broiler/Commercial layer/Broiler parent/Layer parent/Duck/ Turkey/Kalij/Kuroiler/Others

8. **Age of poultry (in weeks):**
9. **Species sampled (Only one):**

   Backyard chicken/Commercial broiler/Commercial layer/Broiler parent/Layer parent/Duck/ Turkey/Kalij/Kuroiler/Others
10. **Type of sample (Any one):** Caecal/Feces
11. **Pooled sample:** Yes/No

    If yes, how many individual samples are combined in the pool:
12. **Average number of chickens sold per day:**
13. **Is slaughtering done at the market?** Yes/No
14. **Catchment area for the vendor's poultry:**
15. **Other species on the farm (Multiple choice):**

Cattle/Buffalo/Sheet/Goat/Pig/Fish/Others 16.

Name of sampler:

Annex 4. Sample collection form for slaughterhouse/ slaughter place

1. **Sample collection**

   Date: Sample collection time:
2. **Sample code:**
3. **Name of respondent:** Contact No:
4. **Name of slaughterhouse/slaughter place:**
5. **Name of market:**
6. **Address:**

   Province: District: Local level: Ward no.:
7. **GPS location:** Latitude/Longitude

8. **Type of poultry (Multiple choice):**

   Backyard chicken/Commercial broiler/Commercial layer/Broiler parent/Layer parent/Duck/ Turkey/Kalij/Kuroiler/Others

9. **Soure of the poultry:**

   Province: District: Local level:

10. **Age of poultry (in weeks):**

11. **Species sampled (Only one):**

    Backyard chicken/Commercial broiler/Commercial layer/Broiler parent/Layer parent/Duck/ Turkey/Kalij/Kuroiler/Others

12. **Type of sample (Any one):** Caecal/Feces

13. **Pooled sample:** Yes/No

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

14. **Average number of chickens slaughtered per day:**

15. **Catchment area of slaughterhouse/place for poultry supply:**

16. **Other species on the farm (Multiple choice):**

    Cattle/Buffalo/Sheet/Goat/Pig/Fish/Others

17. **Name of sampler:**

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 ±1ºC 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 RappaportVassiliadis 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 6. 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 byproducts 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 35º – 37ºC.

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 22º – 25ºC 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 35º – 37ºC.

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 -80ºC. Long term storage of isolates at -20ºC 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 35-37ºC. Perform at least one sub-culture before using the isolate to inoculate a test.

Annex 7. 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. Coplin jars or similar for ethanol sterilizing scissors/clippers/forceps
11. Scissors/clippers, metal (for cutting swabs)
12. Bunsen burner(s) or Bacti-Cinerator (or similar) if gas is not available.
13. Callipers or ruler (for measuring zone sizes)
14. Loops, nichrome or plastic disposables
15. Micropipette capable of measuring 100 µl
16. Microscope light, with oil-immersion objective
17. Quality control organisms as specified by the relevant CLSI standards Other equipment
    1. Oven or microwave oven for drying (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 8. Surveillance scheme 2024/25

Target Population:

The target population for sampling for the AMR surveillance will be poultry used for meat purposes. This will consist of three categories:

- Broilers

- Spent/Culled layer hens
- Backyard and other commercial birds reared for meat (e.g., Turkey, Kalij, Quail).

The sampling shall be done to represent all categories mentioned in the target population; a larger proportion of the samples will be from broilers and spent layers and a small proportion will be from backyard and other commercial birds.

Number of samples:

The number of isolates required to estimate/(monitor) prevalence of resistance vary with the expected prevalence and the level of precision required for the prevalence estimate as shown in table 2. Also, the prevalence of resistance varies between isolates collected in active surveillance (the target population being apparently healthy birds intended for human consumption) and in passive surveillance (samples from sick birds submitted to the laboratories). The number of samples is decided based on prevalence records of previous surveillance.

TABLE 2. 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

For the estimation of sample size for the active surveillance in 2024/25, the findings from previous active surveillance (2022/23) have been used. The number of isolates for the designated prevalence of AMR (result of active surveillance 2022/23) is determined using table 2. The total number of samples is then calculated based on the desired number of isolates and prevalence of an indicator bacteria (result of active surveillance 2022/23). From the active AMR surveillance conducted during 2022/23, the prevalence of three bacteria is presented in table below:

TABLE 3. PREVALENCE OF TARGET BACTERIA (ACTIVE AMR SURVEILLANCE 2022/23)

Bacteria	**Prevalence**
E. coli	70.2%
Salmonella spp.	11.9%
Enterococcus spp	55.3%

The highest prevalence of resistance was seen in *E. coli* isolates against ciprofloxacin. 63.4% of the isolates showed resistance against Ciprofloxacin. Considering the prevalence of AMR as 60%, the desired number of isolates for surveillance with 5% precision would be 369 (from table 2).

The total number of samples to yield the desired number of isolates depends on the prevalence of the bacteria and would be calculated as:

$$Number\ of\ samples\ (N) = \{(number\ of\ desired\ isolates) \times \frac{1}{(prevalence\ of\ \ bacteria)}\}$$

To obtain 369 isolates of *E. coli* whose prevalence is 70%, 527 samples would be needed. A sampling plan for 565 samples would thus give precise estimates for AMR prevalence values in *E coli*. However, with these number of isolates the estimates of AMR prevalence in lower-prevalence bacteria such as *Salmonella* may be less precise.

Surveillance sites and catchment areas:

For the active AMR surveillance 2024/25, seven laboratories have been identified as surveillance sites which would collect samples from their respective catchment areas.

TABLE 4. SURVEILLANCE SITES AND CATCHMENT AREAS

Surveillance site (Laboratory)	**Catchment Area (Districts)**
CVL	Kathmandu Valley, Kavrepalanchok, Dhading, Nuwakot
NAL	Chitwan, Makwanpur, Nawalparasi
VL Biratnagar	Morang, Sunsari, Jhapa
VL Janakpur	Dhanusha, Siraha, Bara
VL Pokhara	Kaski, Tanahu, Lamjung
VL Surkhet	Surkhet, Dang, Banke
VL Dhangadhi	Kailali, Kanchanpur

Number of samples per surveillance site:

The total number of samples per surveillance sites have been decided on the basis of the poultry population in the catchment area and resources in the laboratory and is presented in table below.

TABLE 5. NUMBER OF SAMPLES TO BE COLLECTED AT SURVEILLANCE SITES

Surveillance site	Broiler	Layer	Backyard/others	Total
CVL	55	55	10	120
NAL	45	45	10	100
VL, Pokhara	40	40	10	90
VL, Biratnagar	25	25	5	55
VL, Janakpur	25	25	5	55
VL, Surkhet	40	30	10	80
VL, Dhangadhi	30	30	5	65
Total	**260**	**250**	**55**	**565**

Number of samples per surveillance site per sub sector:

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, 75% 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 25% 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. The detail sampling plan has been shown in the table 6.

(Note: Farms included in AMU survey, if present within the area and timeframe will be prioritized for sampling for AMR surveillance. Farm size would also be considered while sampling, to represent different farm size categories as per the AMU survey protocol.)

TABLE 6. SURVEILLANCE SAMPLING PLAN (NUMBER OF SAMPLES FROM DIFFERENT SOURCES)

Lab	Type of birds	Farms	Slaughter Place	Live Market	Total	Total for site
CVL	Broiler	15	20	20	55	120
	Layer	15	20	20	55	
	Backyard and others	10			10	
VL, Biratnagar	Broiler	5	10	10	25	55
	Layer	5	10	10	25	
	Backyard and others	5			5	
VL, Janakpur	Broiler	5	10	10	25	55
	Layer	5	10	10	25	
	Backyard and others	5			5	
NADIL	Broiler	10	20	15	45	100
	Layer	10	20	15	45	
	Backyard and others	10			10	
VL, Pokhara	Broiler	10	15	15	40	90
	Layer	10	15	15	40	
	Backyard and others	10			10	
VL, Surkhet	Broiler	10	15	15	40	80
	Layer	6	12	12	30	
	Backyard and others	10				
VL, Dhangadhi	Broiler	7	13	10	30	65
	Layer	7	13	10	30	
	Backyard and others	5				

Annex 9. Maps of surveillance sites and catchment area

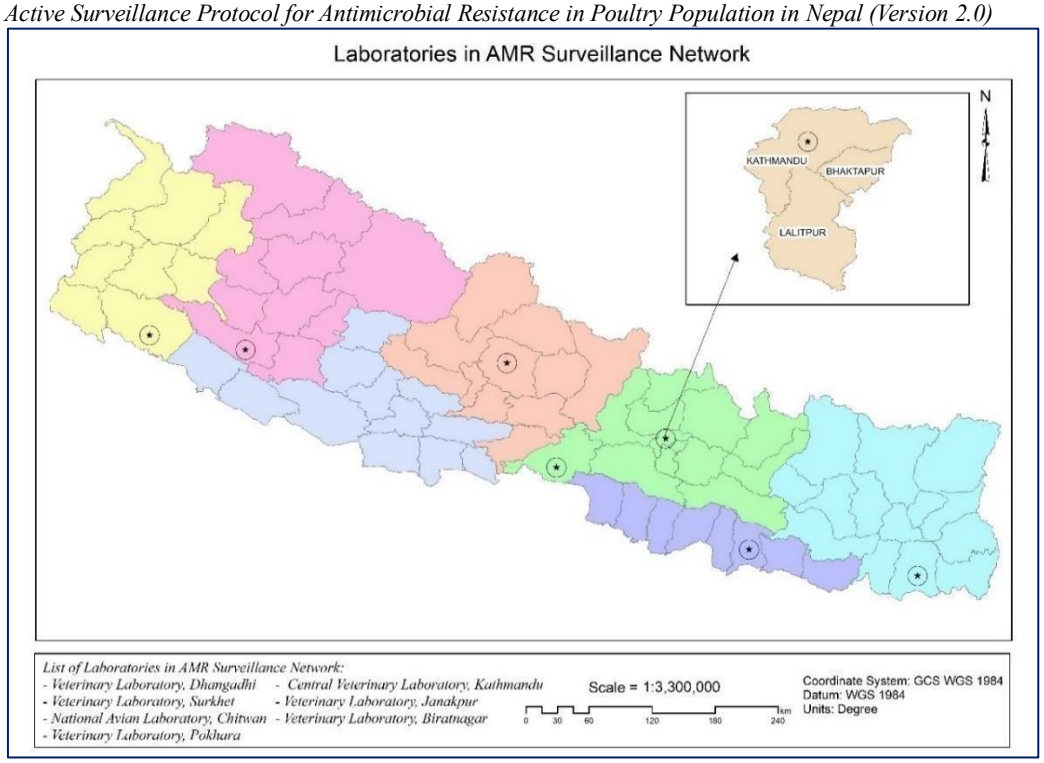

FIGURE 1. LABORATORIES IN THE AMR SURVEILLANCE NETWORK

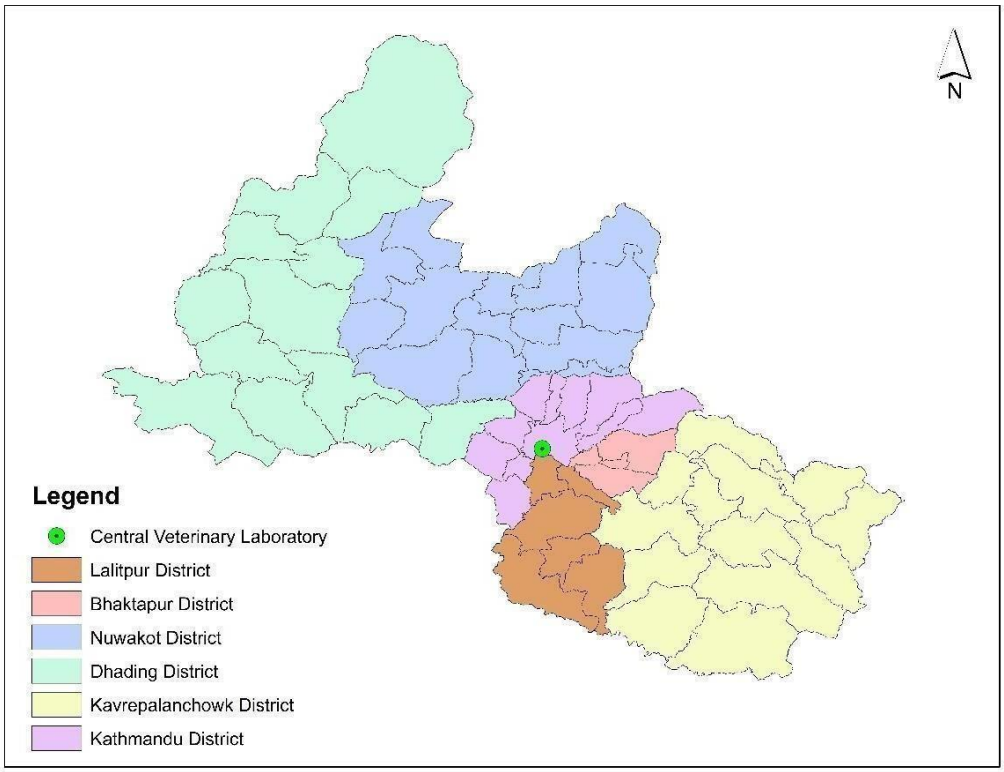

FIGURE 2. CENTRAL VETERINARY LABORATORY AND CATCHMENT AREA

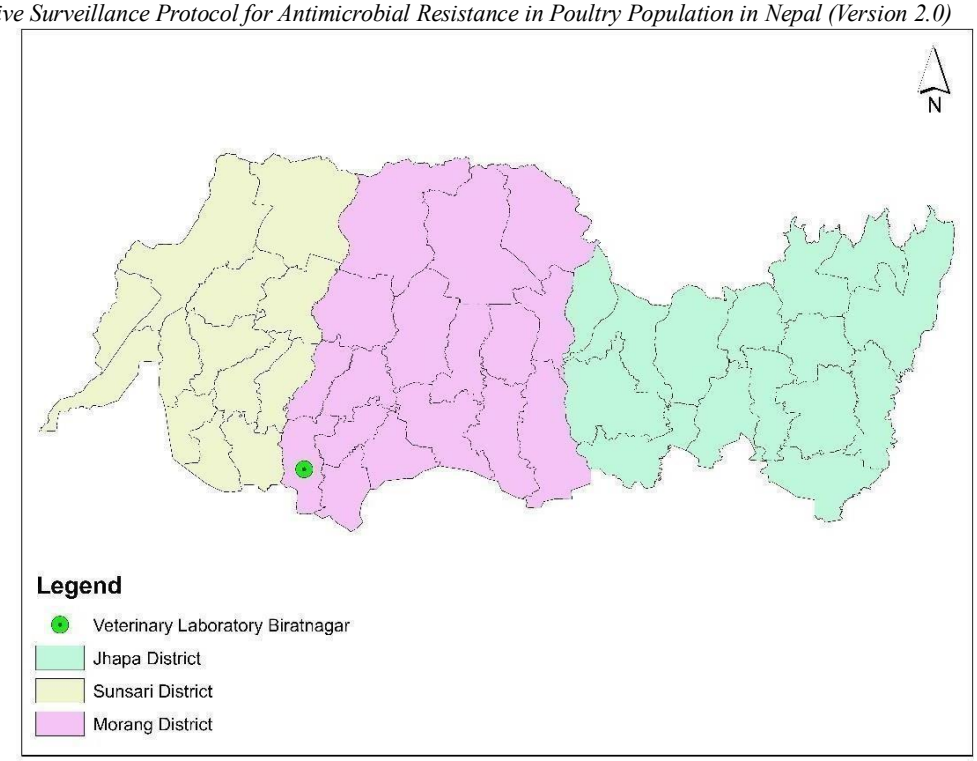

FIGURE 3. VETERINARY LABORATORY BIRATNAGAR AND CATCHMENT AREA

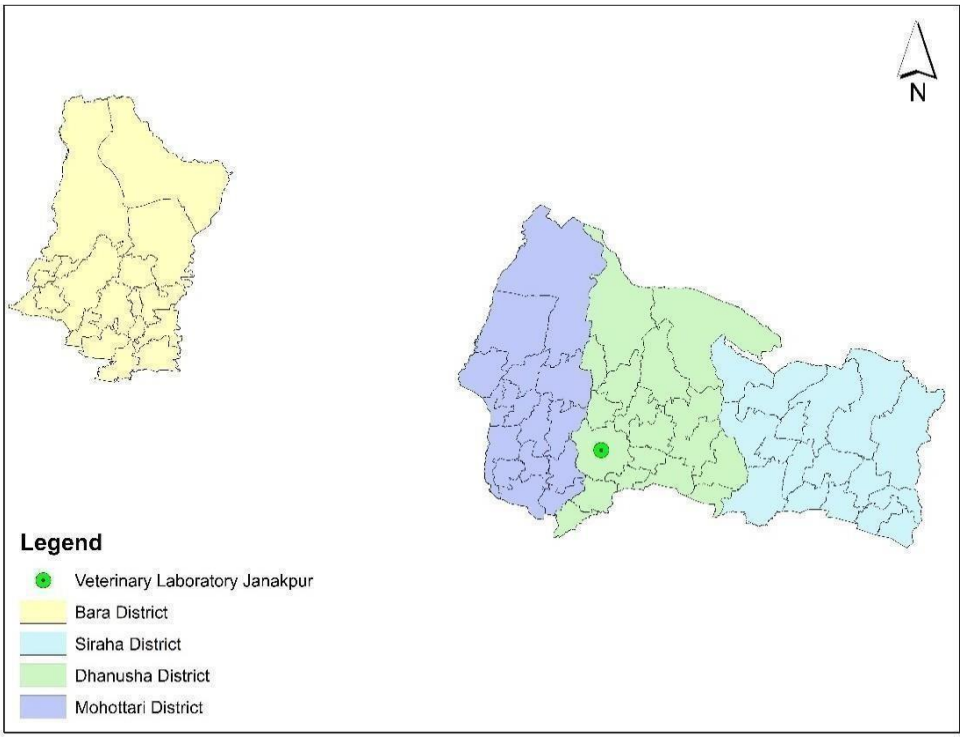

FIGURE 3. VETERINARY LABORATORY JANAKPUR AND CATCHMENT AREA

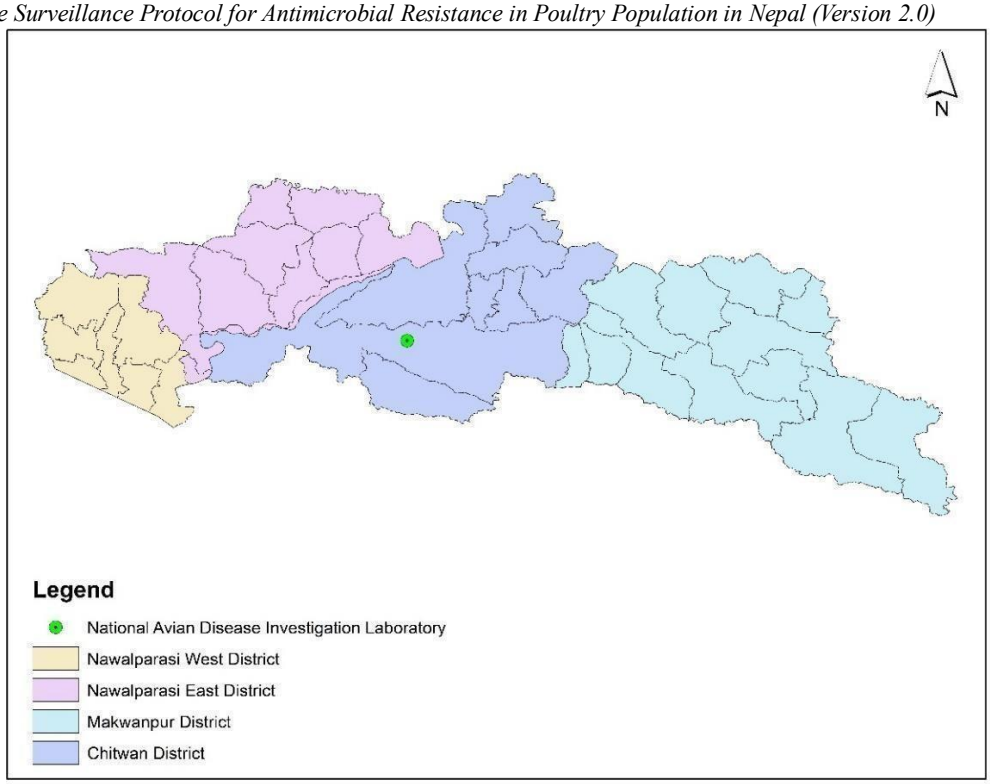

FIGURE 4. NADIL AND CATCHMENT AREA

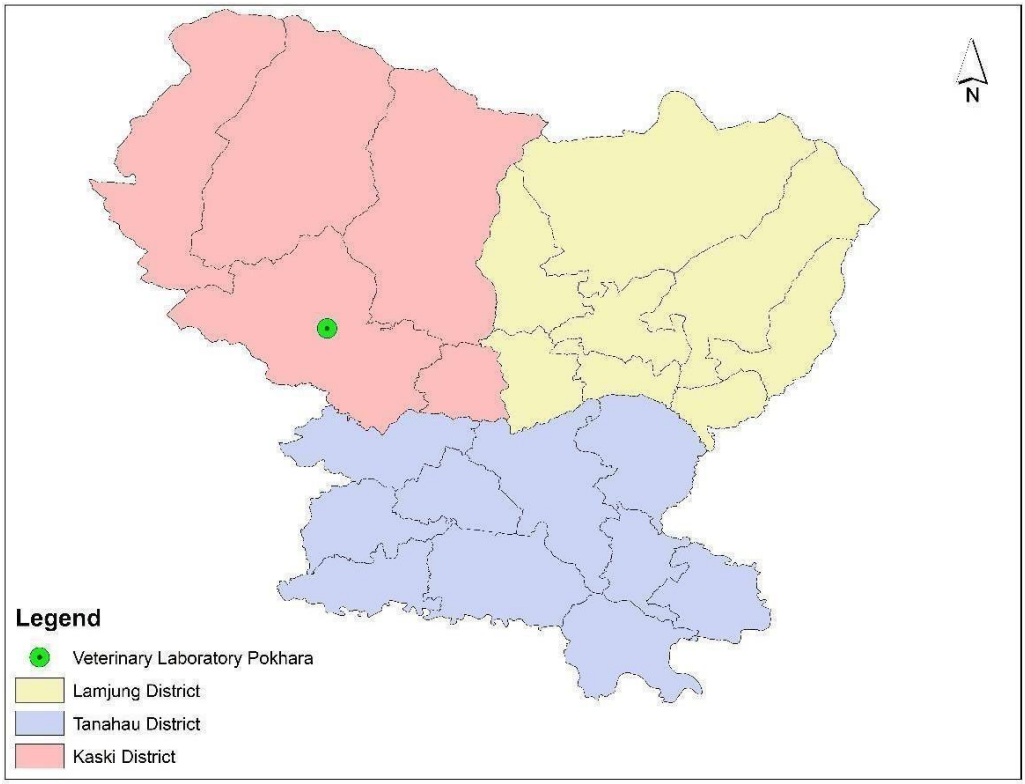

FIGURE 5. VETERINARY LABORATORY POKHARA AND CATCHMENT AREA

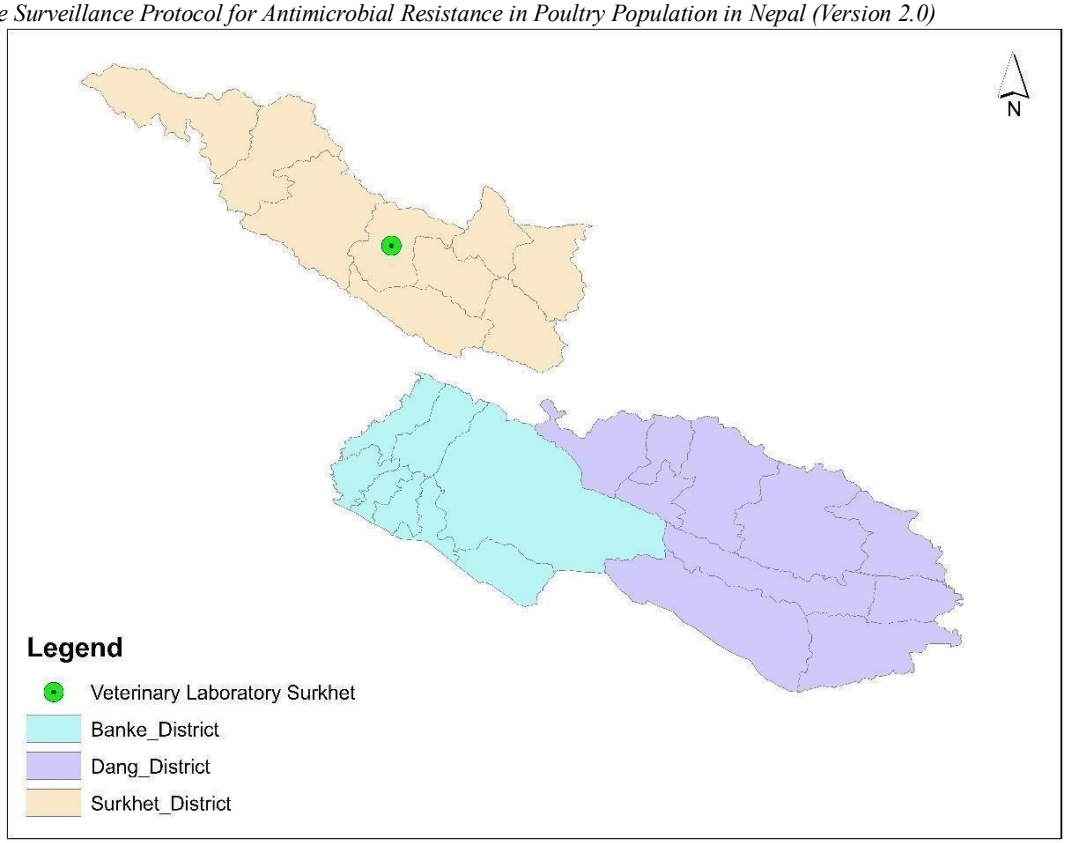

FIGURE 7. VETERINARY LABORATORY SURKHET AND CATCHMENT AREA

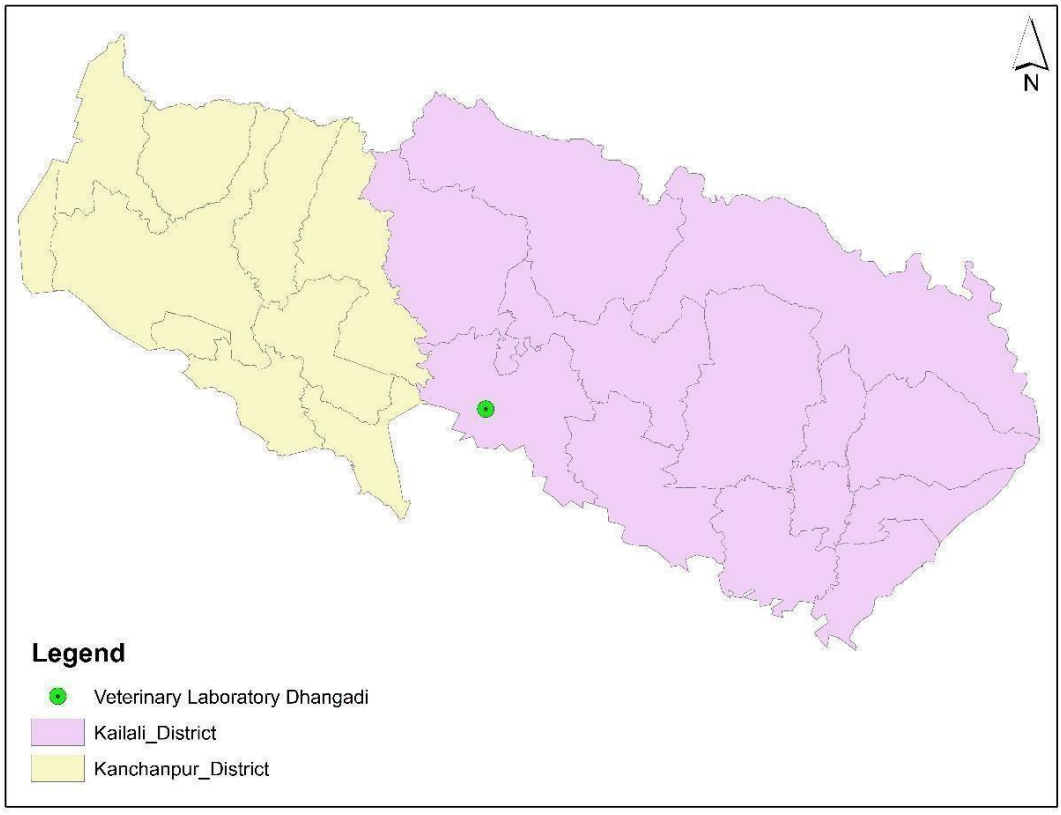

FIGURE 6. VETERINARY LABORATORY DHANGADHI AND CATCHMENT AREA

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