CLINICAL SURVEILLANCE PLAN FOR ANTIMICROBIAL RESISTANCE IN MASTITIS CATTLE IN BHUTAN

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National Center for Animal Health, Department of Livestock
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GLOSSARY

1. Acronyms

AMR	Antimicrobial resistance
AmpC	AmpC beta-lactamases
AMU	Antimicrobial use
AST	Antimicrobial Susceptibility Test s
ATCC	American Type Culture Collection
BFDA	Bhutan Food & Drug Authority
CBP	Clinical Breakpoint
CIA	Critically Important Antimicrobial
CLSI	Clinical and Laboratory Standards Institute
DRA	Drug Regulatory Authority
ECOFF	Epidemiological Cut-off Values
EQAS	External Quality Assurance System
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
GLASS	Global AMR Surveillance System
G2C	Government to Citizen
H-MRSA	Hospital Associated Methicillin Resistant Staph Aureus
IALC	Integrated Agriculture and Livestock Census
L-MRSA	Livestock Associated Methicillin Resistant Staph Aureus.
MCC	Milk Collection Center
MPU	Milk Processing Unit
MIC	Minimum Inhibitory Concentration
MoH	Ministry of Health
MOU	Memorandum of Understanding
NCAH	National Centre for Animal Health
NDDC	National Dairy Development Center
NVRL	National Veterinary Reference Laboratory
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
PCU	Population Correction Unit
QC	Quality Control
RLDC	Regional Livestock Development Centre
RVH & EC	Regional Veterinary Hospital & Epidemiology Center
SOP	Standard operating procedures

WHO	World Health Organization

2. Definitions

- **Dzongkhag:** Districts
- **Gewog:** Sub-districts or blocks
- **Laboratory Information Management system (LIMS):** Laboratory Information Management System is the online database system designed to efficiently manage the information of all the veterinary laboratory activities in Bhutan
- **WHONET:** WHONET is free Windows-based database software developed for the management and analysis of microbiology laboratory data with a special focus on the analysis of antimicrobial susceptibility test results.

CHAPTER1: INTRODUCTION

In Bhutan, dairy farming is the predominant livestock production activity, supported by a favorable social environment and minimal religious stigma. However, most dairy farming operations remain at a subsistence level, with only a few commercial ventures. Such management practices often face challenges, particularly the prevalence of diseases.

Understanding the pathogen profile for mastitis is critical to effective management strategies. Identifying the specific pathogens responsible for clinical and subclinical mastitis at both the processing unit and farm levels provides valuable insights into disease dynamics. This knowledge enables the development of targeted treatment protocols, ensuring the appropriate and judicious use of antimicrobials to minimize the risk of resistance. Furthermore, a detailed pathogen profile can inform preventive measures by identifying likely reservoirs and transmission pathways of infection, allowing for the implementation of strategic interventions.

Bhutan's bovine milch population totals 66,309, producing 43,829 metric tons (MT) of milk annually, contributing 5.28% to the country's Gross Domestic Product (GDP) (1). The dairy sector is supported by 244 dairy farmer groups and 18 dairy farmer cooperatives (2)Milk Processing Units (MPUs) primarily process milk into cottage cheese and butter, although only a limited number of MPUs are equipped with milk pasteurization units.

Mastitis is a common issue encountered in Bhutan's dairy sector, but national-level prevalence data are not yet available. Studies conducted in specific dairy potential areas indicate a high prevalence of the disease. For instance, a study at the government breeding farm *(NJBC)* in Samtse reported a 78.6% prevalence of mastitis within the herd (3)*)*, while another study documented a 24% prevalence of sub-clinical mastitis in Eastern Bhutan *(Sharma et al., 1998)*. The high prevalence is attributed to poor milking management practices, which increase the risk of mastitis in dairy cattle (4)

Antimicrobial resistance (AMR) has become one of the important global public health challenges. In 2021, an estimated 4.71 million deaths were associated with bacterial AMR (5) and additionally, by 2050, 10 million lives a year and a cumulative 100 trillion USD of economic output are at risk due the rise of drug-resistant infections.

Antibiotics are used in animals to maintain animal health and welfare, as well as food security. However, much of their global use is not for treating sick animals but instead either to prevent infections or simply to promote growth. The quantity of antibiotics used in livestock is vast and often includes those medicines that are important for humans. Whilst the direct use of antimicrobial agents in human health is recognised as a major contributor to antimicrobial resistance in human pathogens, there are circumstances where antimicrobials used in both food-producing and companion animals are key contributing factors.

Raw milk is frequently implicated to contain bacteria that have antibiotic resistant genes to cephalosporin, cephamycin, fluoroquinolone, carbapenem, peptide antibiotics and tetracycline (6). The treatment of mastitis consists of systemic as well as intra-mammary use of antibiotics. The commonly used antibiotics for treatment of mastitis in Bhutan include drugs, as outlined in

the Standard Treatment Guideline, Veterinary Drug Formulary and the Essential Veterinary Drug List, 2023:

1. Intramammary treatments: Procaine penicillin, sulfamerazine, strepto-penicillin, cefoperazone, cloxacillin-ampicillin, and Cefuroxime.
2. Systemic treatments: Oxytetracycline (OTC), gentamicin, cephalexin, strepto-penicillin, and cloxacillin-ampicillin.

Among these, drugs such as Cefoperazone and Gentamicin are classified as critically important antimicrobials (CIAs) under the WHO categorization and TRAFFIC light signal listing. Their usage warrants cautious stewardship to mitigate the risk of antimicrobial resistance (AMR). This underscores the urgent need to conduct AMR surveillance in Bhutan to generate evidence-based insights and guide informed decisions for veterinary practitioners and farmers. Prioritizing the use of antimicrobial classes of lesser importance for human health while reserving critically important drugs for exceptional cases can help preserve their efficacy for future needs.

Unlike dairy practitioners in many parts of the world, dairy farmers in Bhutan commonly rear their cattle in bedded environments. Bedding materials such as pine leaves, oak leaves, straw, fern leaves, and sawdust are frequently used, primarily to produce manure for agricultural purposes, reflecting Bhutan's emphasis on integrated farming systems. However, these bedding practices may contribute to an increased risk of bovine mastitis by creating favorable conditions for harboring mastitis-causing pathogens. Consequently, such management practices have been associated with prevalence of mastitis cases, highlighting the need for improved hygiene and disease control measures, considering the usage of effective and appropriate antimicrobials.

Surveillance and reporting of antimicrobial resistance (AMR) and antibiotic usage have become global health priorities. Bhutan, building on the first phase of Fleming Fund support for active AMR surveillance in poultry, is initiating a clinical surveillance program for AMR in milk from cows with mastitis during this second phase.

This program will enable targeted antibiotic use by identifying mastitis-causing pathogens and their resistance patterns, ensuring effective treatment while reducing antimicrobial misuse. It will also support resistance monitoring, providing critical insights into AMR trends and emerging threats, thereby facilitating timely interventions.

Moreover, this surveillance initiative aims to strengthen government animal health services by enhancing their capacity to implement AMR surveillance in livestock. By fortifying both epidemiology and laboratory components, the program lays a foundation for conducting robust active and passive AMR surveillance across all livestock species.

1.1 Objectives

Bhutan has a total bovine population of 265,567, of which 66,309 are milch cattle (1), representing 29.78% of the total bovine population. Milk is a vital source of protein and nutrition for the Bhutanese population. However, while farmers regularly seek clinical treatment for cattle with mastitis, the collection of relevant samples through passive surveillance is rare, often leading to empirical antimicrobial therapy. Furthermore, farmers do not always observe the drug withdrawal period after treating mastitis, increasing the risk of antimicrobial-resistant (AMR) bacteria in milk. Nationally, there is no planned active surveillance for AMR in mastitis, resulting in a lack of baseline data on the AMR situation related to mastitis in the country.

The objective of the active surveillance program is to collect milk samples from cows with clinical or subclinical mastitis, as these cattle enter the human food chain and could contribute to AMR transmission through the consumption of unpasteurized milk. The data generated through this active surveillance will provide valuable insights into the major pathogens causing mastitis and their resistance patterns. This will form a foundation for future work related to AMR in dairy cattle. Additionally, given the minimal data available on AMR in mastitis pathogens from passive surveillance, this program will collect samples for culture, identification, and antimicrobial susceptibility testing (AST) to provide a more comprehensive understanding of the AMR trends in dairy animals.

As per the national categorization of the cattle farms in the country most cattle farmers are backyard, migratory and semi-commercial farming type, and so the study has taken into consideration the surveillance in both the categories of the cattle farms viz backyard, semi-commercial farms and government owned farms.
The following are the objectives of the mastitis AMR surveillance in Bhutan:

1. **Understanding Pathogen and Antimicrobial Susceptibility Profiles:** To identify the target pathogens responsible for mastitis and their antimicrobial susceptibility in dairy cattle in Bhutan.
2. **Guiding Treatment Regimens:** To develop evidence-based recommendations for effective treatment of mastitis and rational use of antibiotics for animal health workers and dairy practitioners.
3. **Guide management approaches to reduce mastitis:** To identify appropriate management practices that farmers can implement to reduce the incidence of mastitis and thus reduce the need for antimicrobial use.
4. **Supporting National Veterinary Frameworks:** To generate scientific evidence for reviewing a national veterinary drug formulary, standard treatment guidelines, and an essential veterinary drug list in Bhutan.

5. **Informing AMR Policies and Programs:** To provide data that informs national policies and programs to mitigate antimicrobial resistance (AMR) in both animal and human health sectors.
6. **Establishing a National Biorepository:** To collect and preserve bacterial isolates in a national culture collection for future research and investigation.

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

2.1 Target populations

Lactating dairy cattle producing milk and milk products for human consumption are proposed as the target populations for this round of AMR surveillance in dairy cattle. This is because consumption of milk and its product is high for dietary requirements and calories compared with other sources, thus, milk is an important source of foodborne infections globally; and antimicrobials are widely used in this sector, including some which are critical for human health. This protocol assumes that the potential risks for dairy cattle contributing to AMR in humans are high compared with other ruminant livestock species, for most countries.

The focus of surveillance is on both backyard and semi-commercial dairy sector of high producing regions in Bhutan. The backyard dairy sector is included in these rounds of surveillance, because the milk from the dairy sector is pooled at the milk cooperatives/collection sites. Samples will be collected from mastitis positive dairy cattle.

The target population for the surveillance is representative dairy cattle from dairy Dzongkhags. The initial screening of farm-level bulk milk samples for mastitis will be done at the Milk Collection Centers followed by tracing of individual dairy cattle with mastitis from the positive herds. All the milking cattle that are actively producing milk for human consumption and are positive to mastitis (clinical as well as sub-clinical) shall be sampled for surveillance. The surveillance aims to address public health and effective treatment concerns linked to antimicrobial use in dairy farming, especially for treatment of mastitis. In Bhutan, both intramammary infusion and parenteral forms of antibiotics are often prescribed without AST resorting to an empirical treatment regime. The AMR data from this initiative will inform policy makers and animal health practitioners to rationalize the intervention measures to ensure responsible antimicrobial use, safeguard human health, animal health, environmental health and sustain the dairy industry.

Moreover, when targeting the population for surveillance, several key factors were considered, particularly in the context of Bhutan's dairy farming practices. These factors include:

1. Proximity to Surveillance Laboratories: This is crucial, as the target sample is fresh milk, which is highly susceptible to contamination and is perishable.

2. Density of Dairy Population: Ten districts were selected for surveillance based on their high milk production and concentration of dairy cooperatives (MCC & MPU).

3. Geographical Location and Dairy Density: Areas with a high dairy population were prioritized based on dairy population in the surveillance sites. In addition, sites located along the border with India (Samtse, Chukha, Samdrup Jongkhar), to ensure comprehensive sampling.

4. Breed and Farming System: Representation of the major breeds of dairy cattle and the different farming systems in place were also considered in the selection of surveillance sites.

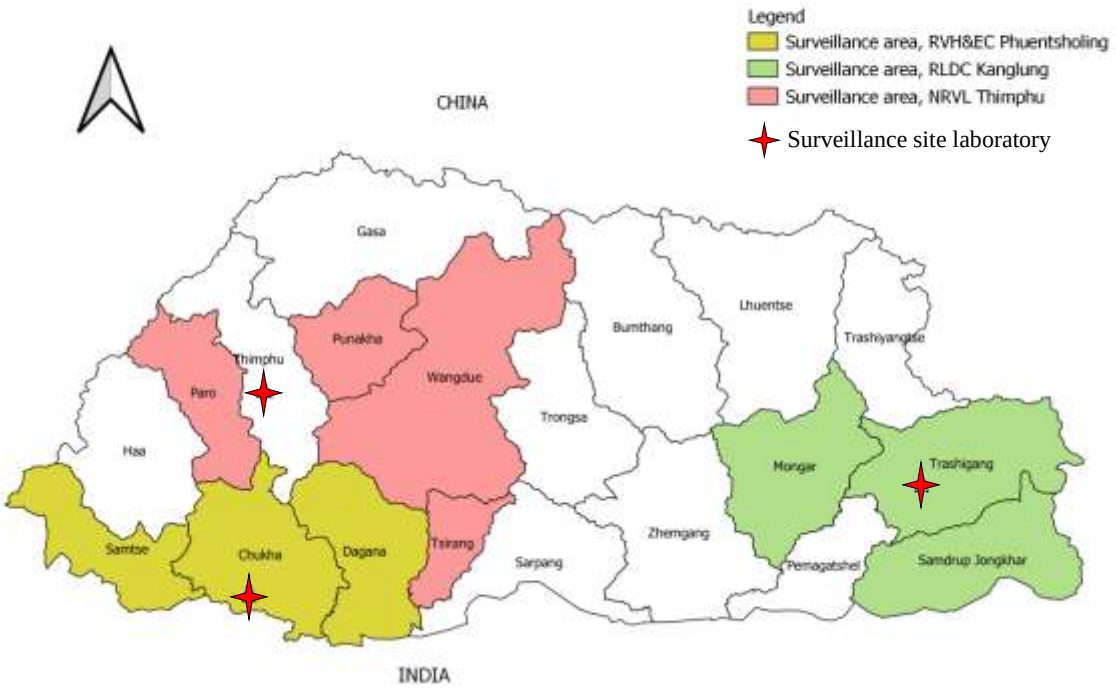


Figure 1 Map showing locations of the surveillance site laboratories and the surveillance areas

2.2 Laboratories and surveillance sites

There are currently three animal health laboratories that can support the scope and capacity for isolation, identification and AST in the country. This includes a National Referral Veterinary Laboratory (NRVL) at the National Center for Animal Health (NCAH), Thimphu, Regional

Livestock Development Center (RLDC), Kanglung, and Regional Veterinary Hospital and Epidemiology Center (RVH&EC), Phuntsholing.

The areas in which the samples are collected for surveillance are defined as the surveillance site and laboratories as the sentinel site for isolation, identification and AST. Each surveillance site is selected around a regional laboratory for convenience of transportation of samples and accuracy of testing. Sites include high milk-producing districts that can represent the dairy population across the country.

2.3 Target bacteria

The target bacteria for dairy surveillance are primarily pathogenic bacteria causing mastitis inflicting economic impact to the dairy farmers. There is also risk associated with mastitis (clinical and sub-clinical) in the transmission of pathogens to humans through consumption of milk, and indirectly through excretion from the animals and un-weaned calves which harbor resistant bacteria. *Escherichia coli* (mastitis caused due to environmental contamination and unhygienic milking practice) is a priority organism listed in the WHO Global AMR Surveillance System *(GLASS)(WHO, 2015).*

Escherichia coli is a leading cause of acute clinical mastitis in dairy cattle world-wide *(Shpigel et al., 2008; Blum et al., 2017; Geo et al., 2017).* S*taphylococcus spp*. causes chronic mastitis and is usually a subclinical infection. *Staphylococcus aureus* also causes gangrenous mastitis and udder impetigo. *Streptococcus spp (Streptococcus Agalactia)* is an important mastitis pathogen because of its highly contagious nature and its ability to degrade milk quality having high somatic cell counts and decrease milk production. The average duration of *streptococcal* mastitis is 12 days but can be prolonged for more than 300 days. *Streptococcus uberis* and *Streptococcus agalactia* are well known pathogens in inducing chronic mastitis *(PMC, Pub Med Central, National Library of Medicine).*

The six bacterial pathogens included in the first round of Mastitis AMR surveillance are *Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Staphylococcus aureus, coagulase negative Staphylococcus spp,* and *Escherichia coli.* These target pathogenic bacteria for the first round of AMR surveillance are present in milk and may be associated with transmitting antimicrobial resistant infections to humans through direct or indirect transmission of resistant bacteria or resistance genes.

2.4 Target Antimicrobials

The aim of this AMR surveillance is to understand the antibiotic profile of the pathogens causing mastitis in cattle, guide the formulation of effective treatment regimes with antibiotics that are not critically important for human health, guide improved management of mastitis to reduce the need for antimicrobial treatments and promote rational use of antibiotics in the field. To achieve this, the panel of antibiotics are selected based on the recommended antibiotics for mastitis

treatment that are available in Bhutan and those for which there is available interpretive criteria for disk diffusion and treatment options for mastitis. The target antibiotics for specific bacteria are identified in Table 1

Table 1: Antibiotic panel selected for surveillance of each of the mastitis pathogens

Antimicrobial class	E. coli	*Staphylococcus aureus*	Coagulase negative *Staphylococus* spp.	Streptococcus spp.
Penicillins	Ampicillin	Penicillin G	Penicillin G	Penicillin G
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	Tetracycline
Sulfonamides/Trimethoprim	Co-trimoxazole	Co-trimoxazole	Co-trimoxazole	Co-trimoxazole
2nd generation cephalosporins		Cefoxitin	Oxacillin	
3rd generation cephalosporin	Cefotaxime			
Beta-lactam/beta-lactamase inhibitor combination	Amoxicillin-clavulanic acid	Amoxicillin-clavulanic acid	Amoxicillin-clavulanic acid	Amoxicillin-clavulanic acid
Quinolones	*Ciprofloxacin*	Norfloxacin	Norfloxacin	Norfloxacin

CHAPTER 3: SAMPLING PLAN

3.1. Sampling plan

The areas in which the samples are collected for surveillance are defined as surveillance sites. The surveillance site is identified based on the location of surveillance site laboratories, prioritizing districts with high dairy cattle density with the possibility of mastitis cases.

The National Reference Veterinary Laboratory (NRVL), Serbithang and Regional Veterinary Hospital & Epidemiology Center, (RVH&EC), Phuntsholing, and Regional Livestock Development Center, (RLDC), Kanglung are identified as surveillance site laboratories. The NRVL also serves as the National AMR reference laboratory for animal health.

A total of 10 Districts are selected as surveillance sites. The district falling under the jurisdiction of each surveillance site laboratory is the surveillance site of the respective laboratories. Although Tsirang and Dagana do not fall under any surveillance site laboratories, they are also included as surveillance areas given the high number of cattle in the Dzongkhags and proximity to surveillance site laboratories. Each surveillance site laboratory is the in-charge of an allocated surveillance site. Based on the capacities of each surveillance site laboratories within the allocated timeframe, a total of individual milk samples positive to CMT is targeted as per the table 2.

Table 2. The surveillance sites and sample size for sample collection.

Sl.No	Surveillance Sites (Laboratory)	Surveillance Sites (District)	Surveillance Sites Lead	Sample Size (n=420)
1	National Referral Veterinary Laboratory (West, West Central)	Paro, Punakha, Wangdue phodrong, Tsirang	NRVL, NCAH	180
2	Regional Veterinary Hospital & Epidemiology Center, Phuntsholing (South West region)	Chukkha, Samtse, Dagana	RVH&EC, Phuntsholing	120
3	Regional Livestock Development Center, Kanglung (East, South East)	Trashigang, Monggar, Samdrup jongkhar	RLDC, Kanglung	120

The target site for the screening of milk samples to identify herds containing cows with clinical or subclinical mastitis is milk collection points and/or milk collection centers. This is done to reduce the logistic cost and also to capture the maximum animals with mastitis under different production systems. This is further substantiated with the fact that the members of the MCP/MCC are from different production systems such as backyard, semi-commercial and commercial in nature. Such an approach also reduces the time taken for sampling and garners efficiency in the execution of the plan.

3.2 Sample collection process

The milk sampling is conducted at two levels. The first level of sample collection is from the individual farm bulk milk samples from different Milk Collection Centers/Points, in which the pooled milk from one owner with more than one cow is subjected for the California Mastitis Test CMT test. If the bulk milk sample is positive to CMT with CMT score of 2 or 3 then the herd is visited and individual animals from that herd are subjected to CMT test. Additionally, all the farmers will be asked if his/her cows are showing signs of mastitis. Herds in which the bulk milk sample is negative to the CMT but the farmer reports the cows showing signs of mastitis will aslo be visited and individual cows tested with CMT.

At individual cow level any milk sample positive to CMT (T/1/2/3) is subjected to laboratory test for bacterial isolation and identification as per the SOP on mastitis pathogen detection and AST. The milk sample collection process is described in the flowchart in Figure 2

For example, for a regional laboratory, visiting approximately five herds (at the individual level) per day, with at least three cows testing positive for CMT, would yield 15 samples. In this scenario, it would require eight visits to different collection points among the allocated sampling sites. Similarly, for the national laboratory, 12 visits would be required to meet the target of 180 samples. However, if the required number of isolates (e.g., 100 bacterial species of *Staphylococcus aureus*, *Streptococcus* species, and/or *E. coli*) is not achieved, further sampling would be necessary.

Figure 2. Pictorial flow chart for sample collection

3.3 Data Collection

The information on both bulk and individual milk samples will be collected using the respective submission sheets during the sample collection process. Additionally, demographic information, including details of the animals, farming system, management practices, and treatment history of the affected cows will be recorded for each individual farm using the EPICOLLECT 5 app, as provided in the annexure. This data will be further utilized to develop and plan practitioner engagement programs.

Sample size

We estimate a sample size of approximately 100 isolates of each of the 3 main bacterial species to obtain an estimate of resistance to the different antimicrobials of interest, based on a design prevalence of 50% and a precision of 10%. In reality we expect the prevalence of resistance to be 20 – 30% thus 100 isolates will give us a more precise result than 10%. It will also allow for a slightly inflated sample size to allow for within-herd clustering of pathogens causing mastitis and their AMR patterns.

During a pilot study involving samples from 25 cows from 10 herds, S. aureus was grown in 44%(11/25), Streptococcus species in 32% (8/25), non-coagualse staphylococcus species in 1.2 % (3/25) and mixed infection with 1.6 % (4/25). Based on these results we have taken a conservative estimate that 420 samples will need to be tested to produce approximately 100 isolates of each of the major bacterial species. .

3.4 Sampling timetable

The milk sample collection from different surveillance sites as per the projected sample size is scheduled from January to June 2025. The sampling timetable for nine districts under three surveillance laboratory sites are mentioned in Table 3.

Table 3. The sampling timetable for the surveillance laboratories

Sl.no	Sites	Activities Sampling	Jan 2025	Feb 2025	March 2025	April 2025	May 2025	June 2025
1	NCAH, west	Paro						
		Punakha, Wandgue						
		Tsirang						
2	RVH&EC,	Chhukha						

	central & south	**Samtse**						
		Dagana						
3	**RLDC, Kanglung**	**Tashigang**						
		Mongar						
		Samdrup jongkhar						

3.5 Sample labeling

The samples will be clearly labeled using a permanent marking pen. The information will be placed in a plastic envelope on the outside of the shipping container and will always accompany the samples to the laboratory. The microbiology unit will record the data of the sampling. The sample ID number will contain first three letters of Dzongkhag, continuous number followed by clinical status of the animal example: Par-01-S or Par-02-C , S- subclinical, positive CMT but no obvious signs or symptoms, C – clinical, obvious clinical signs of mastitis, e.g. clots, discolouration of milk, systemic illness of cow). Any unlabeled samples, spilled/leaking samples received will be rejected by the laboratory.

3.6 Sample packing

Milk samples must be first collected in a sterile primary container such as vials which are leak proof. The primary container will be placed in a secondary container to avoid direct contact with the ice pack and placed in a cool box/thermocool box. A double layer of protection is required to ensure that the biological contents in the container do not contaminate other samples or the environment in case of leakage or spillage.

3.7 Sample transportation

The samples should be sent safely to the laboratory as soon as possible by the fastest available means. The samples should ideally be transported immediately or within 24 hours under refrigerated condition. If not, they must be frozen at -20 deg C and stored in a frozen state till it

reaches the laboratory for culture. Samples must not be frozen, thawed and re-frozen. The laboratory analysis should begin immediately after the sample reaches the laboratory. All the samples sent to the laboratory should strictly comply with packaging instructions mentioned above.

3.8 Biosecurity practices when collecting samples

Strict biosecurity measures should be followed throughout the sample collection, packaging and shipping process while in the individual dairy farms. The main objectives in observing biosecurity practices during milk sample collections is to prevent the spread of disease from farm to farm and also prevent the contamination of environmental areas. The visitor will wear single use disposable gloves and shoe covers during sample collection in the farm and dispose once the task is completed.

The following are some of the minimum biosecurity measures observed in the farm while doing sample collection:

1. The minimum number of people should enter the farm, ideally two people with one person for collecting the samples and the other person for recording and assisting the first person during the sample collection.
2. The vehicle should be parked outside the farm gate.
3. The sample collection team shall wear clean rubber boots, overalls/lab coat, and gloves with biohard bags and minimum sampling items into the farm.
4. Within the farm, the same gloves can be used after washing and sterilizing using alcohol.
5. After the completion of sample collection, the used gloves, shoe cover and other accessories shall be removed and burnt or packed in biohazard bags for disposal at the surveillance site.
6. Thoroughly scrub the boots with soap and water using antiseptics such as Glutaraldehyde.
7. Before exiting the farm properly wash hands using soap and water or rub with alcohol-based hand sanitizer.
8. The reusable overalls should be disinfected in disinfectant and then then washed with standard laundry detergent before being used by samplers in subsequent farms.

CHAPTER 4: LABORATORY PROCEDURES

4.1 Processing of Milk Samples

In refrigerated samples, bacteria will be concentrated in the fat layer. Hence, such samples should be warmed to 25°C at room temperature or incubator for about half an hour and should be gently inverted before plating.

4.2 Bacterial Isolation

The detailed instructions on culture identification for mastitis pathogens are provided in annexure on SOP for the Detection of Mastitis Path**ogens.**

4.3 Antimicrobial Susceptibility Testing (AST)

The surveillance laboratories will conduct AST using a disk diffusion method for the mastitis pathogens as per the *SOP for Disk diffusion method.*

4.4 Interpretation of tests results

Two different types of interpretive criteria will be used: clinical breakpoints (CBPs) and epidemiological cut-off values (ECOFFs) as per the standard guidelines (CLSI/EUCAST). The data should be interpreted with CBPs. The priority is assigned to CLSI CBPs. For antimicrobials where CLSI CBPs do not exist, EUCAST/ECOFFS will be used as per the SOP 5: CLSI Disk Diffusion for mastitis pathogens.

4.5 Isolate storage and transportation

Surveillance laboratories should preserve the isolates after identification on 20% glycerol solution at -80oC or ultra low freezer. Surveillance laboratories should culture all the isolates regardless of AST results on a non-selective agar (Sheep blood agar for *Streptococcus spp.*) and transport to the NRVL. The isolates should be transported to the NRVL on a monthly basis by the surveillance laboratory for national biorepository. The detailed instructions on Isolate transportation are provided in annexure as SOP for the Isolate transportation.

The NRVL should revive the culture upon arrival and preserve the isolates through lyophilization or 20% glycerol solution at -80degC.

4.6 Quality Control (QC) in antimicrobial susceptibility testing

ATCC strains should be tested once a week, and additionally for every new batch of media. The zone diameter should be recorded for each ATCC strain each time it is tested. This information should be examined for consistency. Any issues identified in the reliability of testing should be investigated and rectified before further testing is conducted for the AMR surveillance programme.

This ensures the performance of reagents and the viability of microorganisms used in the test. Reference strains used are *Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Streptococcus pneumoniae ATCC 49619*

CHAPTER 5: DATA MANAGEMENT

The recording of raw (primary, non-interpreted) data is essential for future references. Results will be maintained in excel spread sheets in respective surveillance sites including the inhibition zone diameters in millimeters, and in MICs in micrograms per milliliter for those isolates that are tested with both methods. The surveillance laboratories will send the raw data to the NRVL on a monthly basis.

5.1 Data entry and analysis

Data Entry and Analysis

All sample-related information, whether collected via EpiCollect 5 or recorded on hard copy spreadsheets, is updated into an electronic Excel spreadsheet for further data analysis at the regional and national levels. Field data for samples collected as part of AMR surveillance are recorded using the sample collection forms and subsequently updated in the spreadsheets by the respective surveillance laboratories. These laboratories include the two regional facilities (RLDC Kanglung and RVH&EC Phuentsholing) and the National Referral Veterinary Laboratory (NRVL) at NCAH Serbithang. The final analysis of AMR surveillance data from all three laboratories is conducted at the NRVL.

AMR findings are interpreted at three levels—susceptible, intermediate, and resistant—based on CLSI and EUCAST guidelines. Data transfer and compilation follow established procedures within government settings, without the need for additional agreements or MOUs.

5.2 Data collation, validation and dissemination of AST data

The collation and validation of the AST data will be done at respective surveillance laboratories and national AMR reference laboratory. The final data analysis and reporting for the animal AMR surveillance will be done by the national AMR reference laboratory at NCAH Serbithang based on the excel spreadsheet. The data should be regularly cross-checked in excel spreadsheets at the surveillance laboratory level as well as at the AMR reference laboratory after receiving the data from the surveillance laboratories.

The analysed information will be disseminated from NCAH to different stakeholders such as dairy farmers, policymakers, department of Livestock, BFDA, MOH, pharmaceutical suppliers/companies, veterinarians, Para veterinarians, livestock and dairy consumers, media and other relevant stakeholders. The flow of information from the sample collection and testing till the dissemination of the information to the practitioner engagement is shown in Fig…

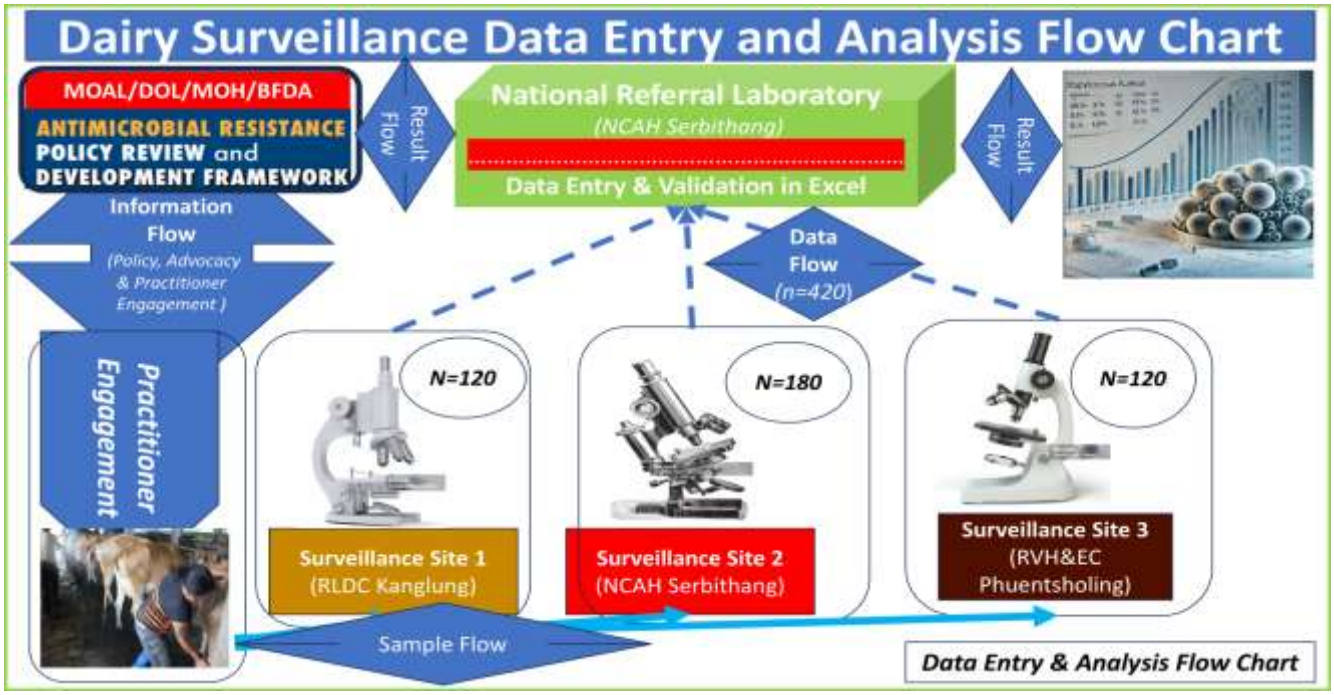


Figure 2. Flow chart showing collection, entry and analysis including the information dissemination

The detailed data management till the dissemination of information is summarized in Table 9.

Table 4.. Methods and responsibilities of AMR data management at different levels

Data type	Data entry	Data collation and validation	Data management	Analysis and Interpretation	Information dissemination
Details of sample: 1. Sample ID 2. Sample type 3. Sample source 4. Animal Type. 5. Age of animal 6. Parity 7. Stage of	Sample collection team shall complete details of sample in sample collection form	Information collected in sample shall be collated into sample submission form for submission to laboratories of	Sample demographics will be entered into MS Excel spreadsheet and stored as soft copy. A printed copy shall be maintained	Basic analysis on sample collection progress shall be performed by team leader	The sample collection team shall forward the details of samples to surveillance laboratory

Data type	Data entry	Data collation and validation	Data management	Analysis and Interpretation	Information dissemination
Lactation		surveillance sites	for the same as a back up		
Details of organism isolation and identification: 1. Colony character 2. Basic test 3. Biochemical test 4. Isolate naming	Laboratory technicians at surveillance laboratories shall enter all the details of laboratory results for each isolate in the Excel spreadsheet. Recovered isolate shall be archived with standard identification process.	All the details of the isolation and identification process shall be entered into a spreadsheet at the respective surveillance laboratory. Results of isolate shall be compared with that of reference organism.	Isolation and identification records shall be maintained at surveillance laboratory	Basic analysis on the consistencies of isolate and reference organisms will be done in each of the surveillance laboratories. This activity shall be performed by respective surveillance laboratory	The surveillance laboratory shall forward this information to the national reference laboratory along with isolate. Details of isolation and identification shall be sent in both electronic as well as paper copy

Data type	Data entry	Data collation and validation	Data management	Analysis and Interpretation	Information dissemination
Details of AST from DD 1. Panel of antibiotics 2. Zone diameter of isolate 3. Zone diameter of reference organism	Surveillance laboratory and National Veterinary Lab shall enter isolate details for each sample (including samples from which no isolates were retrieved) in spreadsheet: - Laboratory ID - Sample ID - Selected panel of antibiotics - Zone diameter of both isolate and reference organism	Surveillance laboratory shall collate cumulative data on AST from all the isolates	Surveillance laboratory shall be the custodian of AST data at their level	Surveillance laboratory shall use Excel spread sheet to analyze AST data and draw some preliminary interpretation	Surveillance laboratory shall pass AST data to national reference laboratory in excel spreadsheet as well as paper copy
Details of AST from MIC 1. Panel of antibiotics 2. MIC of isolate 3. MIC of reference organism	National reference laboratory shall update the records for each sample and isolate in the spreadsheet with: - Selected panel of antibiotics - MIC of both isolate and reference organism	National reference laboratory shall collate cumulative data on MIC data from all the isolates	National reference laboratory shall be the custodian of DD and MIC AST data at national level	National reference laboratory shall use Excel Sheet to analyse AST (both DD and MIC) data and interpret AMR status at national level	National reference laboratory shall pass DD and MIC AST data to AH TWG for epidemiological analysis. These result should then be available for sharing with relevant

Data type	Data entry	Data collation and validation	Data management	Analysis and Interpretation	Information dissemination
					stakeholders.

CHAPTER 6: ROLES AND RESPONSIBILITIES OF SURVEILLANCE LABORATORIES

6.1 Roles of AMR reference laboratory

1. The Laboratory Services Unit at National Center for Animal Health at Serbithang is identified as the National reference laboratory for AMR.
2. Provide leadership and technical support for the laboratories in the Regional Livestock Development Centre and Regional Veterinary Hospital, Epidemiology Center & National Food Testing Laboratory that are participating in the surveillance program.
3. Develop/ upgrade SOPs for microbiology including AST.
4. Train and mentor microbiology technicians in Regional Livestock Development Centre and Regional Veterinary Hospital & Epidemiology Center & National Food Testing Laboratory in culture, identification and AST methods.
5. Support the other labs in implementing good laboratory quality systems and will run an EQAS involving the surveillance labs.
6. Maintain inventories of national biorepository of the isolates produced by all labs in the surveillance network.
7. Collection and maintaining ATCC strains.
8. Collate, compile & verify AMR surveillance data submitted by surveillance laboratories.
9. Participate in international EQASIA
10. Develop the capacity to undertake the following advanced diagnostic methods:

    a. ESBL- acquired AmpC (pAmpC) and/or carbapenemase-producing organism confirmation.

    b. Minimum Inhibitory Concentration (MIC) tests on a subset of isolates showing resistance on disk diffusion tests to identify epidemiology cut-off values

(ECOFF) which ensures the comparability of data over time at the country level and also facilitates the comparison of resistance patterns between countries.

11. Implementation of biosafety and biosecurity measures.

12. Coordinate and implement the safe transport of samples and isolates between the laboratories.

13. Maintain a national database of verified AMR results and associated demographic data in WHONET.

14. Maintain and share quarterly and annual reports of AMR and AMU surveillance results with the MOAL; AMR and AMU Surveillance with TWG, NATC and the Regional Laboratories.

6.2 Roles of regional surveillance laboratory

1. Coordinate and carry out AMR surveillance in liaising with surveillance sites within their region and the assigned areas.
2. Produce reliable quality bacterial culture, identification and Antibiotic Susceptibility Test (AST) results for *E. coli, Staphylococcus and Streptococcus.*
3. Collect good quality milk samples from dairy cows for culture and AST and submit to the national referral laboratory, as per the schedule.
4. Collect appropriate samples for AMR testing that are labelled appropriately, transported in a safe manner, arrive at the laboratory in good condition for diagnostic testing, and are accompanied by appropriate demographic information that is labelled to match the samples.
5. Implementation of biosafety and biosecurity measures.
6. Safe transport of samples and isolates between the laboratories.
7. Maintain accurate data on sample demographics and laboratory results in the LIMS, WHONET and an excel register, and send this to NVL monthly.

REFERENCES

1. National Statistics Bureau (NSB) RGoB. <IALC-2023-Report.pdf>. 2024.
2. Report on the nationwide monitoring and evaluation of farmers' groups and cooperatives in bhutan. Thimphu; 2023.
3. Tshering D, Gyem K. Prevalence of Clinical and Sub-clinical Mastitis in Lactating Dairy Cows at the National Jersey Breeding Centre, Samtse, Bhutan. Bhutan Journal of Natural Resources and Development. 2023;2(1):33-9.
4. Dendup T, Dorji J. Milking Management Practices by Small Scale Dairy Farmers of Trashiyangtse District in Bhutan. 2020;4:54-9.

5. Naghavi M, Vollset SE, Ikuta KS, Swetschinski LR, Gray AP, Wool EE, et al. Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. The Lancet. 2024;404(10459):1199-226.
6. Tóth AG, Csabai I, Krikó E, Tőzsér D, Maróti G, Patai Á V, et al. Antimicrobial resistance genes in raw milk for human consumption. Sci Rep. 2020;10(1):7464.

ANNEXURES

BULK SAMPLE RESULT SUBMISSION FORM-A

Details of Sample collector				**Name:**			**Contact Details:**			
Sl.No.	**Milk Collection Point (MCP)details**						**Details of Sample**			**Remarks**
	Name MCP	**Owner name**	**Village**	**Geog**	**District**	**No of milch animals**	**Tank No.**	**Collection date & time**	**CMT test Score**	**Individual animals selected for Screening *(Yes/No)***
1										

INDIVIDUAL ANIMAL SAMPLE RESULT SUBMISSION FORM- B

Details of Sample collector: Name….....Contact Details:…..... Date .....

Sl No	Owner Name	No of milch cows	Farm Type (B/SC/C)	Breed	Age (years)	No of lactation	Stage of lactation (Month of calving)	Clinical observation (Clinical or Subclinical or Normal)	Antibiotic course in recent time (Yes/ No)	CMT Score					Sample ID	Remarks
										RF	RH	LF	LH	Pool (culture)		

SOP 1:CALIFORNIA MILK TEST FOR BULK AND INDIVIDUAL COW SAMPLES

1. PURPOSE

The California Milk Test (CMT) is a qualitative measurement of the Somatic Cell Count (SCC) of milk. It is a diagnostic tool to aid detection of clinical and subclinical mastitis in dairy cattle. Mastitis (infection of the mammary tissue) causes the body to send white blood cells to the udder. These somatic cells are what the CMT measures. A good somatic cell count for bulk milk is below 200,000 cells/ml- a level to which mastitis in the herd is adequately controlled.

2. MATERIALS REQUIRED

The following minimum materials are required for the conduction of the tests:

- Recording sheet – attached as an appendix to this document
- CMT paddle
- Clean 5ml syringes
- CMT reagent
- Gloves

3. Procedure for Bulk milk sampling and testing

3.1 Wear gloves

3.2 Mark each well of the paddle with the farmers name for identification purposes

3.3 Collect approximately 3mls of milk from the farmers bulk milk- the ideal amount of milk is what remains in the paddle when it is tilted to nearly vertical, or to the line indicated in the CMT paddle if present

3.4 Add an equal amount of CMT reagent to each well in the paddle, or to the line indicated on the paddle.

3.5 Rotate the paddle in a circular motion to thoroughly mix the contents for no more than 10 seconds. The reaction will start to disintegrate after 20 seconds, and so a quick reading of results is required.

3.6 The reaction is visually scored depending on the amount of gel that forms.

3.7 Rinse paddle before next use.

3.8 The details of the sample collection, testing and recording of the results is recorded in Bulk Milk Sample Submission Form-A.

PROCEDURE FOR INDIVIDUAL COW SAMPLING

1.1 Wear gloves

1.2 Hold the paddle in the same way each time to identify which wells correspond to each quarter

1.3 Foremilk each quarter and discard the first few streams of milk

1.4 Draw 2-3 squirts of milk from each quarter into the corresponding well – to the line on the CMT paddle or what remains in the paddle when it is tilted almost vertically

1.5 Add an equal amount of CMT reagent or until the line indicated on the CMT paddle

1.6 Rotate the paddle in a circular motion and read the results of the test quickly- within 10-20 seconds.

1.7 The reaction is visually scored depending on the amount of gel that forms.

1.8 Record the result on the recording form- attached as an appendix to this document.

1.9 Quarters that test positive to the CMT should have a sterile sample taken for culture. See SOP for Sterile Collection Procedure for Milk Culture

1.10 Rinse paddle before next use.

4. Interpretation of the results

Any reaction of milk indicates subclinical or clinical mastitis.

CMT SCORE	**RESULT**	**CMT**
0	Negative	Milk remains liquid
Trace	Trace	Very slight thickening to solution
1	Weak Positive	Milk slightly mucoid
2	Positive	Mixture mucoid but can tip out a little liquid
3	Strong Positive	Mucoid mixture formed a gel.

6.

SOP 2: STERILE COLLECTION OF MILK SAMPLE FOR CULTURE

1. PURPOSE

Culture and antimicrobial susceptibility tests must be performed on milk samples collected from cows showing clinical or subclinical signs of mastitis. This helps to identify causative bacteria causing the mastitis and also identify susceptible antimicrobials which could be used for treatment.

2. MATERIALS REQUIRED

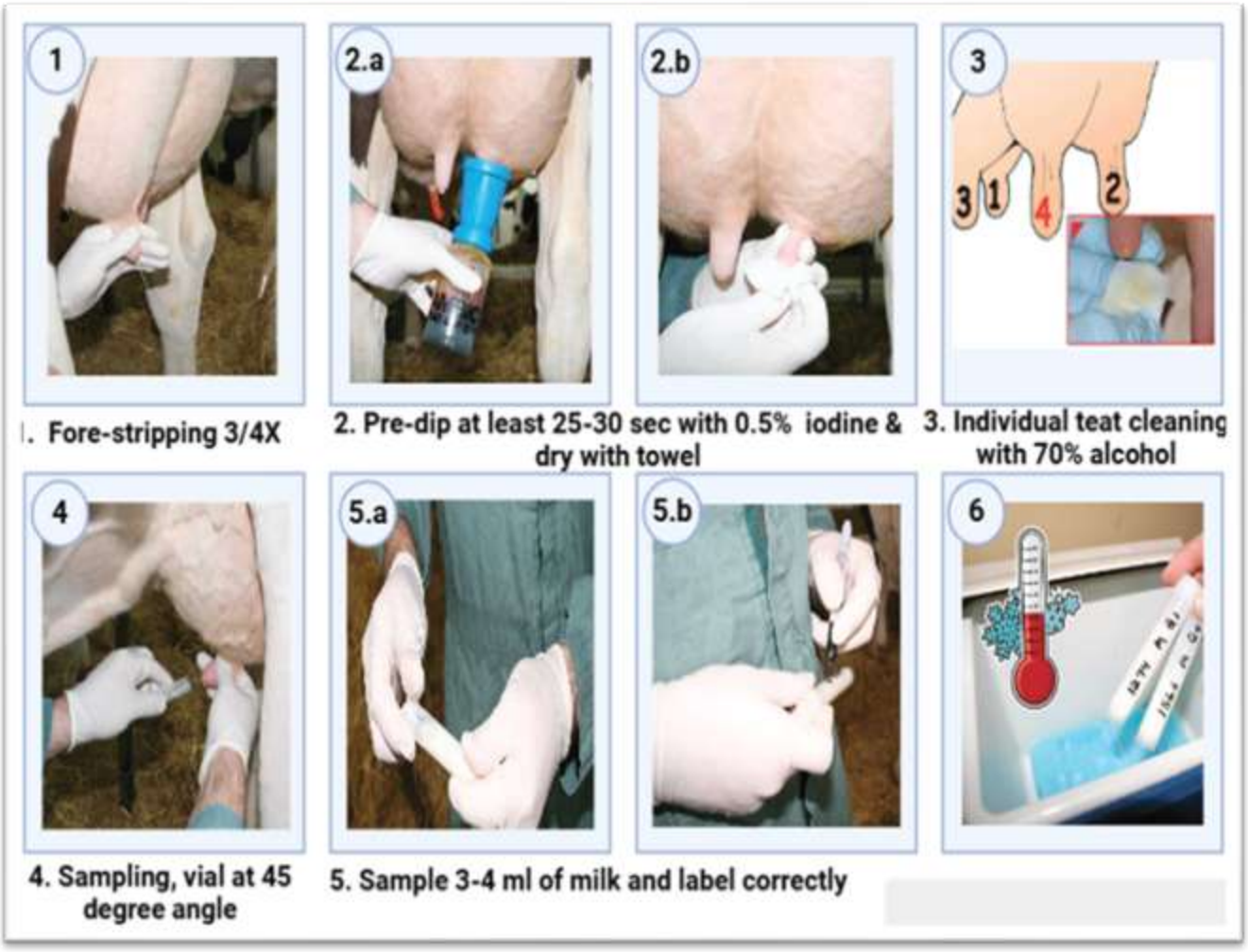
- Gloves
- Surgical spirit/ 70-80% isopropyl alcohol
- Cotton Wool/Gauze
- Paper towel
- Sterile Sample Pot
- Marker Pen
- Recording sheet
- Teat dip/Iodine
- Cool box (Ice packs)

Milk Sample Collection

Milk samples must be collected before milking or at least 1hr after milking. When individual quarters show clinical signs of mastitis, it should be recorded.

PROCEDURE

1. Wear gloves
2. Wash and or scrub the udder and teats to make free of debris
3. Predip teat with iodine and leave for 30 seconds
4. Dry each teat thoroughly with paper towel
5. Wipe teats with cotton wool soaked in isopropyl alcohol/surgical spirit
6. Strip the teat 5 times, discard milk and wipe again with surgical spirit
7. Strip the teat 4 times into the sterile sample vial and collect about 3-5ml of milk sample.
8. Put lid on straight away and label using code and date
9. If multiple quarters are affected, then strip each quarter into the same sample vial.
10. Record the details of samples collected into the spreadsheet
11. Place samples into the cooler on ice packs and transport to the laboratory within 24 hours or to the refrigerator as per the storage specifications.



13. CONSIDERATIONS

The samples should be labeled as follows:

- **DISTRICT – SAMPLE NUMBER – S/C** where in S means subclinical and positive CMT but no obvious signs or symptoms and C means clinical with obvious clinical signs of mastitis such as clots, discolouration of milk, systemic illness of cow. For example, milk collected from a cow from Paro can be recorded as PARO – 1 – S.

IMPORTANT CONSIDERATIONS

- Test scores will vary between operators, care should be taken to standardise the reading of the results.
- Cows that have recently calved (<10 days postpartum) may have a false positive reaction.

- Cows with acute clinical mastitis may have a false negative result due to destruction of somatic cells. Any cow with obvious clinical symptoms of mastitis should have a sample collected for culture.

Special Consideration:

1. Handle sample tubes properly to ensure sterility at all times to avoid contamination. Make sure only the milk samples come into contact with the inside of the tubes.
2. Samples taken from cows on antibiotic therapy should be recorded with the name of antibiotics in the form.
3. Sample tubes should be no more than ½ full and lids should be completely closed to avoid leakage or bursting upon freezing (milk expands when frozen).
4. Collect samples directly from teats as the bucket or other sources of samples carry over bacteria from previous cows.
5. The best time for sample is at milking time before the cow is milked. If not, it should be taken at least 1 hour after the last milking.
6. Label the sample tube with a permanent marker *before sample collection* as milk fat will cause the ink to smear.
7. For composite milk samples, try to collect the same volume of milk from each quarter.
8. Collect samples in a clean area as much as possible to avoid contamination. Avoid areas with massive air movement where bedding and dust can cause major contamination problems.
9. Make sure samples are cold or frozen until they are delivered to the lab to avoid excessive growth of bacteria, which can lead to misleading results.

References:

- Protocol for collection of bovine milk samples: OPTIBOV SOP version 20-11-2019

SOP 3: DETECTION OF MASTITIS PATHOGEN

1. Purpose

- To isolate and identify bacterial pathogens from milk samples causing mastitis.

2. Materials Required

- Inoculation loops (10 µL)
- Sheep Blood Agar

- Mannitol Salt Agar (MSA)
- MacConkey Agar
- Marker pen
- Sterile workspace
- Incubator
- Candle
- Anaerobic jar
- 3% hydrogen peroxide
- Esculin Agar
- 6.5% Sodium Chloride solution
- ATCC *Staphylococcus aureus* (control strain)
- Rabbit plasma

3. Procedure

i. Sample Preparation

- Warm the refrigerated milk samples to 25°C for about half an hour to dissolve the fat layer.
- Gently invert/shake the vials several times to ensure uniform mixing/ dispersing bacteria from milk fat.

ii. Inoculation of Plates

- Using a 10 µL inoculation loop, plate 20 µL (two loops) of the milk sample on the following media:
    - Sheep Blood Agar (SBA)
    - Mannitol Salt Agar (MSA)
    - MacConkey Agar
- Spread the inoculum evenly over half of each plate using separate loops to ensure well-isolated colonies.

iii. Incubation of Cultures

- **Sheep Blood Agar:** Incubate in increased CO_2 conditions by placing plates in an anaerobic jar with a lit tealight candle. Seal the jar with an airtight lid.
- **MSA and MacConkey Agar:** Incubate aerobically.
- Incubate all plates at 37°C for 18-24 hours, then examine for colonies of *Streptococci*, *Staphylococci*, or other organisms.
- Re-incubate plates for an additional 24 hours if required.

Examine for growth, morphologic features such as colony size, shape, color and hemolytic characteristics.

iv. Sub-culturing

- Isolate pure subcultures of morphologically distinct colonies on Sheep Blood Agar before proceeding to bacterial identification.

4. Bacterial Identification

Differentiate the bacteria based on the gram's reaction (Gram-positive or Gram-negative), cellular morphology and arrangements of the bacteria. Additional catalase test (Hydrogen Peroxide 3%) to be done for Gram positive cocci, followed by coagulase test for catalase positive.

- Follow the flowchart in Figure 1 for biochemical differentiation of *Staphylococcus* and *Streptococcus* species.
- For pink colonies on MacConkey Agar, conduct presumptive and confirmatory tests for *E. coli*.

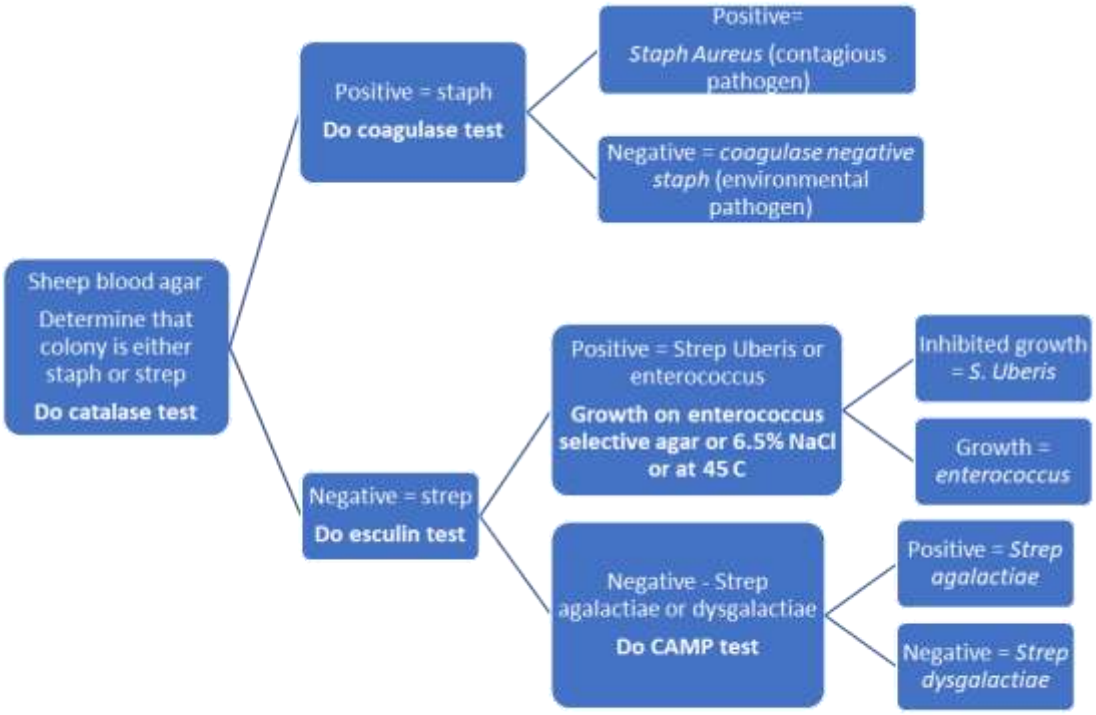


Figure 1 Biochemical tests for differentiation of Staphylococcus species and Streptococcus species from milk

Considerations

- Use *Staphylococcus aureus* ATCC 25923 as a control strain for all tests.
- Follow strict biosafety and biosecurity protocols, as milk pathogens can pose health risks to humans.
- Ensure accurate documentation of all sample details and test results in the laboratory records.

References:

Claxton, P. D., & Ryan, D. (1980). *Bovine Mastitis: Bacteriology*. Australian Standard Diagnostic Techniques for Animal Diseases. Arthur Webster Pty Ltd and NSW Agriculture.

SOP 4: ISOLATE TRANSPORTATION

1. Purpose

To obtain pure culture and transportation of bacterial isolates from the Surveillance Laboratories to the National Veterinary Referral Laboratory (NRL).

2. Material required

- Autoclave
- Pipettes
- Test Tubes
- Erlenmeyer flask
- Cryovials/10 ml tubes

3. Culture media and reagents

- Nutrient agar
- Sheep Blood agar

4. Procedure:

5.1 Transport of isolate from surveillance laboratories to the National Veterinary Referral Laboratories

5.1.1 Inoculation of slant

- Inoculate the nutrient agar slant/ blood agar slant with pure isolates using inoculating loop (nutrient agar slant for *Staphylococcus* spp and blood agar slant for *Streptococcus* spp). Streak the surface of the slant and recap the tubes.
- Incubate the slant until there is evidence of growth, then put the tube in a refrigerator.
- For growing strict aerobes, it may be necessary to slightly loosen the cap for incubation (but close securely before storage) if there is insufficient air in the headspace.
- For easy handling, plastic cryovials of 2ml size could be used filling with about 1ml semi solid nutrient agar and inoculating with culture and after about 24hrs of incubation, transport to national veterinary referral lab with proper labelling and packing as described below.

5.1.2 Packaging of isolates

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

The recommended procedure for packing samples is as follows:

- Samples must be put in a primary container (glass or plastic tubes or bottles) with screw caps and wrapped with paraffin film or adhesive tape individually in order to prevent leakage. The wrapping of bottles or primary containers should be carried out in clean surroundings.
- The primary container must be packed in water tight secondary packaging, which should be a strong crushproof and leak-proof metal container. The container should contain absorbent cotton wool sufficient to absorb the entire contents of the primary container.
- The secondary packaging must be placed in an outer container. This should be a polystyrene foam box covered with a hard box or other appropriate container.
- Sufficient information and a list of samples or materials should be enclosed in an envelope, enclosed in a plastic bag and placed between the secondary packaging and outer box.
- It is recommended that a freezer box is put outside the secondary packaging to ensure that all materials are kept cool during shipment. These packs should be pre-frozen at -20 degrees centigrade before packaging.

5.1.3 Transport of isolates

- The specimens should be forwarded to the laboratory by the fastest method available.
- If they can reach the laboratory within 48 hours, samples should be sent refrigerated.

5. Safety

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

6. References

- Basic Practical Microbiology, A Manual, Society for Microbiology 2006, ISBN 0 95368 383 4
- https://microbeonline.com accessed on 2020
- OIE terrestrial manual 2008, Chapter 1.1.1 collection and shipment of diagnostic samples.
- OIE terrestrial manual 2008, Chapter 1.1.3 Transport of Biological Materials.
- SOP 7, Isolate transportation, 2020 National Center for Animal Health, Department of Livestock, Thimphu

SOP 5: CLSI DISK DIFFUSION FOR MASTITIS PATHOGENS

1. Purpose

To describe the procedure for performing antibiotic susceptibility test for Staphylococcus aureus, other *Staphylococcus* species and *Streptococcus* species using CLSI disk diffusion method.

2. Material required

4.1 Antibiotic disc dispenser
4.2 Ruler/Zone scale/vernier caliper to measure inhibition zones
4.3 Vortex mixer
4.4 34˚ to 35˚ C ambient air incubator
4.5 Densitometer/Turbidity meter for McFarland's turbidity determination.
4.6 Sterile petri plates
4.7 Sterile cotton swab
4.8 Antimicrobial discs
4.9 Muller Hinton agar
4.10 Sheep blood

4.11 pH meter
4.12 Antibiotics discs
4.13 Normal Saline

3. Procedure

6.1 Preparation of Inoculum

a. Using sterile loop or swab, transfer four or five isolated colonies of similar colony morphology grown overnight from sheep blood agar to 5ml sterile normal saline aliquot.

b. Vortex for 15-20 seconds to mix well and adjust the turbidity to 0.5 McFarland turbidity standards, which is equal to $1.5x10^8$ CFU/ml.

Note- For *Streptococcus* species prepare using colonies from an overnight (18- to 20-hour) sheep blood agar plate incubated in 5% CO_2.

6.2 Inoculating Plates

6.2.1 Bring agar plates (MHA and MHA with 5% sheep blood) to room temperature before use, but avoid prolonged exposure to elevated temperatures. Agar plates should be freshly prepared with approximately 20ml of media in each plate.

6.2.2 Within 15 min of adjusting the inoculum, dip a sterile cotton swab into the inoculum and rotate it against the wall of the tube above the liquid to remove excess inoculum.

6.2.3 Swab entire surface of agar plate three times, rotating plate approximately 60 degrees between streaking to ensure even distribution. Avoid hitting sides of petri plate and creating aerosols.

6.2.4 Allow inoculated plate to stand for at least 3 minutes but no longer than 15 minutes before applying disks.

6.3 Application of Antibiotic disks to agar plate

6.3.1 Bring the antibiotic disk from refrigerator and allow it to equilibrate to room temperature. Do not use disks beyond expiry date

6.3.2 Using a disk dispenser apply appropriate antibiotics in the selected MHA/MHA with 5% sheep blood. Adjust the disk dispenser to appropriate height to avoid over/under pressing the disk.

6.3.3 Place no more than 5 disks on a 90-mm plate.

6.3.4 Do not relocate a disk once it has made contact with agar surface, because antibiotic diffusion begins instantly.

6.4 Incubation

6.4.1 Incubate plates within 15 minutes of disk application.

6.4.2 Invert plates and stack them no more than 5 high.

6.4.3 Incubate for 16 to 18 hours at 35±2˚C in an ambient air incubator for ***Staphylococcus*** **species** and at 35±2˚C with 5% CO_2 for 20–24 hours for ***Streptococcus*** **species.**

6.5 Reporting Results

6.6.1 Reference Range:

Using the available breakpoints from CLSI and ECOFF the following table were developed for interpretation.

Staphylococcus aureus

AB	**Disk content**	**Clinical breakpoint**				**ECOFF WT**
		Origin	**R**	**I**	**S**	
Penicillin	10	CLSI HUM/VET	28		29	
Ampicillin	10	CLSI VET	28		29	N/A
Oxacillin	1	CLSI VET	10	11-12	13	N/A
Cefoxitin	30	CLSI HUM	21		22	>22
Amoxicillin/Clav	20/10	CLSI VET	19		20	
Tetracycline	30	CLSI VET	14	15-18	19	>22
Erythromycin	15	CLSI VET	13	14-22	23	>21
Marbofloxacin	5	CLSI VET	14	15-19	20	N/A
Trim/Sulfa	1.25/23.75	CLSI VET	10	11-15	16	>17

*Use penicillin plus cefoxitin sensitivity to show sensitivity for cloxacillin, cephalosporins and amoxicillin-clavlate

Streptococcus spp

AB	**Disk content**	**Clinical breakpoint**				**ECOFF**
		Origin	**R**	**I**	**S**	**WT**
Penicillin	10	CLSI HUM			>24	
Ampicillin	10	CLSI VET	18	19-26	26	n/a
Oxacillin	1	-	-	-	-	
Cefoxitin	30	-	-	-	-	
Amoxicillin/Clav	20/10	CLSI VET	13	14-17	18	n/a
Tetracycline	30	CLSI VET	18	19-22	23	n/a
Erythromycin	15	CLSI VET	15	16-20	21	n/a
Marbofloxacin	5	CLSI VET	14	15-19	20	n/a
Trim/Sulfa	1.25/23.75	CLSI VET	15	16-18	19	n/a

*Penicillin sensitivity = cephalosporins, amoxicillin, ampicillin, amoxyclav

E.coli

AB	**Disk content**	**Clinical breakpoint**				**ECOFF**
		Origin	**R**	**I**	**S**	**WT**
Penicillin	10					
Ampicillin	10	CLSI VET	13	14-16	17	>14
Oxacillin	1					
Cefoxitin	30					

Amoxicillin/Clav	20/10	CLSI VET	13	14-17	18	>19
Tetracycline	30	CLSI VET	14	15-18	19	
Erythromycin	15					
Marbofloxacin	5	CLSI VET	14	15-19	20	
Trim/Sulfa	1.25/23.75	CLSI VET	10	11-15	16	>16

*Ampicillin predicts amoxycillin susceptibility

6.6.2 Reporting Format:

6.6.2.1 Report results as susceptible, intermediate, or resistant along with zone diameter according to laboratory practice and format.

6.6.2.2 Report any multidrug resistant (MDR) isolate to National Referral veterinary reference laboratory (NRVL) for further confirmation. (MDR- if the isolate are resistant to two or more than two antibiotics)

6.6.2.3 Perform ESBL testing if the isolates are resistant to 3rd generation cephalosporin (Cefotaxime) as per SOP for ESBL detection

4. Quality Control

7.1 QC Strains

Run reference strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus ATCC 25923* at all times before running the tests.

DEMOGRAPHIC AND AMU QUESTIONNAIRE FOR EPICOLLECT

Date of Collection: _____

Farm Information

- *Farm Name/ ID:* _____

- *Owner Name: _____*
- *Contact Number: _____*
- *Farm Address: _____*
- *Farm Size (Number of Lactating Animals): _____*

Type of farm- *Backyard farm*

Semi-coomercial

Commercial

Breed of Cattle

a. 01-Jersey Pure
b. 02-Jersey Cross
c. 03-Brown Swiss Pure
d. 04-Brown Swiss Cross
e. 05- 06-Mithun Pure
f. 07-Jatsha-Jatsham
g. 08-Yanku-Yankum
h. 09-Doeb-Doebum
i. 10-Doethra-Doethram
j. 11-Nublang-Thrabum
k. 12-Jaba
l. 13-Buffalo
m. 14-Yak 15-Zo/Zom (Goleng)
n. 15. Holestine Cross

Management Type:

☐ Free grazing ☐ Stall-fed ☐ Mixed

What type of cattle shed do you have?

improved (proper housing with concrete feeding trough and floor)
temporary shed (just roof with open sides.
traditional (rammed mud and timber)

Animal Details

- *Animal ID/Tag: _____*
- *Parity (Lactation Number): _____*
- *Stage of Lactation: _____*
- *Previous Mastitis Episodes (Yes/No): _____*

Treatment History

- *Have any of those antibiotics used in last 12 months for mastitis? (Yes/No): _____*

Date antibiotics was last used- calendar

Antibiotic treatment record form

Intramammary	**Please tick where applicable**	**Oral**	**Please tick where applicable**	**Parental**	**Please tick where applicable**
Pendistin SH/Vetclox/ streptopenicillin (Green tube)		Amoxicillin Trihydrate (2 white boli/strip)		Ampixillin Cloxacilline (Powder form in the vial need to diluted)	
		Cephalexin (2 white boli/strip)		Oxytetracycline LA (oily yellowish fluid)	
Cefoperazone (White tube)		Sulfadiazine and trimethoprim (2 white boli/strip)		Gentamicin (transparent fluid)	
				Benzathine Penicillin (white thick consistency after dilution)	

Treatment Duration

- *Outcome Prescribed By (Vet/Owner)*
- *Was the antimicrobial withdrawal period followed for milk? (Yes/No):*

1. *Clinical information CMT positive*
2. *CMT negative*

Sample ID: