Antimicrobial Resistance Surveillance Plan in Local Chicken Meat in Bhutan

Bhutan Agriculture & Food Regulatory Authority
BAFRA
Ministry of Agriculture and Forests
Royal Government of Bhutan

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Published by the Bhutan Agriculture and Food Regulatory Authority (BAFRA)

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Funding Support:

UKaid from the British people
The Fleming Fund

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INTRODUCTION

The discovery of antimicrobials, availability and use of antibiotics have benefited immensely in the area's public health, animal health, food safety. However, these immeasurable benefits are under enormous threat from the emerging and increasing antimicrobial resistance (AMR) as a result from the overuse and misuse of antimicrobial agents. Due to these, there is an urgent global call to prevent, control and manage AMR.

AMR is a global problem and Bhutan is equally affected by its emergence and spread. The limited studies conducted reveals resistance against commonly used antibiotics (nalidixic acid, ciprofloxacin, penicillin, and tetracycline) as high as 70% in humans. Similarly, in livestock sector, a resistance for nalidixic acid (96%), amoxicillin (12%), cephalexin (6%), ciprofloxacin (2%) and sulphamethoxazole-trimethoprim (2%) were detected in chicken carcass

In the current setting, only routine isolation and antibiotic susceptibility testing of clinical samples were conducted in human and animal sector to generate clinical reports to the respective clinicians. Rarely, some research related to AMR are carried out in both the sectors. Through the Fleming Fund Country Grant support, the surveillance of AMR in human, animal and healthy food animals are taken up as the priority to understand the burden of AMR for the prioritized bacteria in the country.

This surveillance plan covers the information on AMR surveillance for the chicken the retail meat shop from samples collection from the field to result interpretation at the lab including management of data. This will provide an unbiased estimate of the national prevalence of AMR at the retail for different bacterial/antimicrobial combinations. Retail surveillance provides a measure of human exposure to antimicrobial-resistant bacteria via the preparation and/or consumption of undercooked meat. Retail food represents a logical sampling point for surveillance of antimicrobial resistance because it is the endpoint of food animal production and consumption chain. The information obtained from this type of surveillance will be important for understanding the epidemiology of AMR in the food chain and for monitoring the impact of antimicrobial usage in animals and guide evidence-based actions to address AMR. Furthermore, the data can be used for risk analyses for both human and animal populations for the evaluation of interventions and has the potential to transform policies and practices.

ROLES AND RESPONSIBILITIES OF THE NATIONAL FOOD TESTING LABORATORY

1. Coordinate and carryout AMR surveillance in food in the surveillance sites.
2. Produce reliable quality bacterial culture, identification and Antibiotic Susceptibility Test (AST) results for E. coli, Salmonella, Enterococci and Campylobacter
3. Collect good quality and appropriate samples from retail meat shops and regularly carry out culture, identification and AST, according to the agreed schedule.
4. Implementation of biosafety and biosecurity measures for quality assurance
5. Maintain a national database of verified AMR results from food and associated demographic data in WHONET.
6. Maintain and share quarterly and annual reports of the results of AMR surveillance in food with the MOAF TWG the NATC and the National AMR Coordination Center
7. Safe transport of samples and isolates from NFTL to the reference laboratory for confirmatory testing and storage in the national biorepository.

Objectives

- To estimate the prevalence of Escherichia coli, Salmonella, Campylobacter, Enterococci resistance in local chicken meat sold in Thimphu against WHO, OIE and FAO priority antimicrobials in the country.
- Provide data that allow accurate comparisons with data from other sectors (livestock and human health) and other regional countries that use similar surveillance systems.
- To generate baseline information of AMR in local chicken meat
- Inform policy and programs on AMR management in food animals and humans.
- Inform decisions on designing subsequent phases of AMR surveillance in chicken meat and other livestock products.

TARGET POPULATIONS, LABORATORIES, SURVEILLANCE AREAS, BACTERIA AND ANTIMICROBIALS

Surveillance type

Active surveillance of antimicrobial resistance in Escherichia coli, Enterococcus, Salmonella, and Campylobacter in retail local chicken meat in Thimphu will be carried out by the National Food Testing Laboratory (NFTL).

Surveillance sites

The areas in which the samples are collected for surveillance are defined as the surveillance sites. For this first round of AMR surveillance in local chicken meat, Thimphu is selected as the surveillance site.

Most of the local chicken meat sold in Thimphu is sourced from Phuentsholing, Tsirang, and Sarpang districts. There is only one chicken slaughter plant, in Sampheling, which is supplied by one group of broiler chicken farmers in Sampheling geog. Farms outside this group slaughter chickens on their own farms. A few middlemen collect the chicken meat from the farms where they are processed and distributed to the meat shops.

There are a few large meat shops in Thimphu which operate as retail shops as well as supplying meat to other smaller meat shops. The major local chicken available in meat shops is supplied by Sampheling processing plant. The largest meat shop, Druk, obtains meat from approximately 10 different source farms. Most of the other larger retail meat shops obtain meat from multiple farms. The imported chicken meat in the meat shops can be easily distinguishable from local chicken meat as it is frozen state and labelled.

From the logistic perspective, the time taken for the collection and transport of the samples from Thimphu to the laboratory will be much easier considering the location of the NFTL in Yusipang, Thimphu.

The NFTL is the national reference lab for food testing in the country. The NFTL will conduct collection of the samples, culture, identification and antibiotic sensitivity testing (AST) for the AMR surveillance in chicken meat. The AST results and the isolates will be sent to the National Veterinary Laboratory for confirmatory and advanced testing, genotyping and biorepository.

The microbiology capacity for AMR diagnostic of the NFTL laboratory has been strengthened through Fleming Fund Country grant.

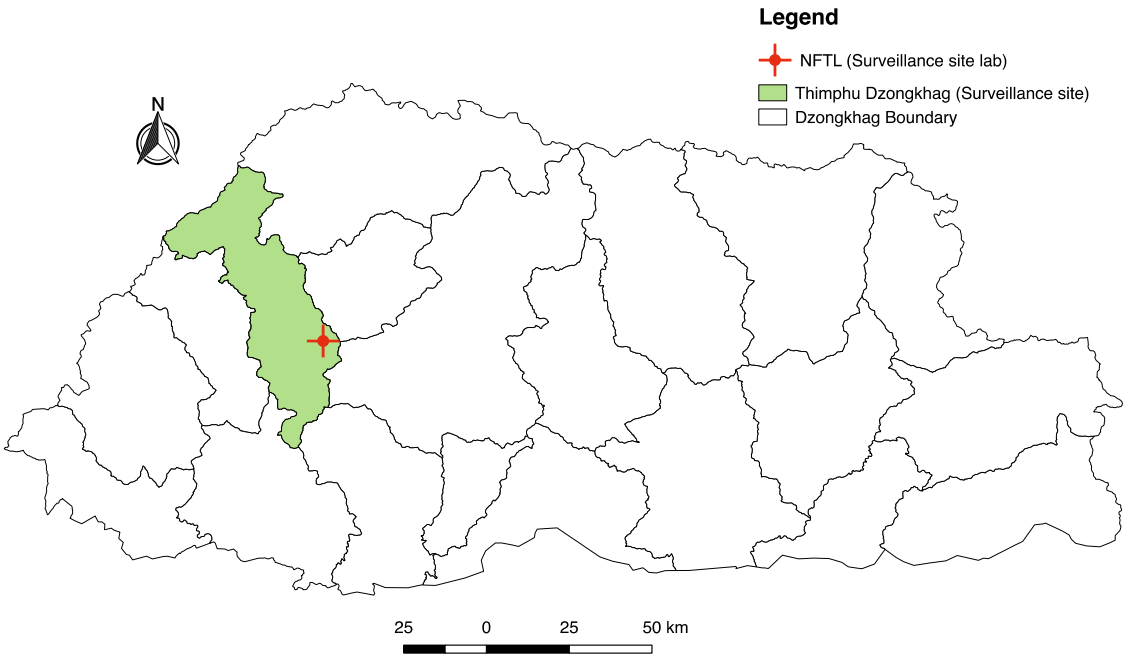

Figure 1. Map showing locations of the AMRsurveillance site & Laboratory

Target population

The target population is the entire set of units for which the surveillance data are to be used to make inferences. The target population for this first round of AMR surveillance in local chicken meat will be the local chicken meat sold in Thimphu. Chicken is selected for the first phase of the Fleming fund AMR surveillance as it is the important source of foodborne infection globally. In addition, the consumption of chicken products is generally high compared with other protein sources available in the country. Further, antimicrobials are widely used in this sector including some of which is a concern to human health (Van Boeckel Thomas et al., 2015). Therefore, it is assumed that the potential risks for contributing to AMR in humans are highest in chickens compared with other livestock species for the country as is the case for most countries.

Source population

The source population is the population from which the study subjects are drawn. Conceptually, all units in the source population should be listed and have a non-zero probability of being included in the study. The source population for AMR surveillance in local chicken meat are retail meat shops in Thimphu. Sampling Plan

The sampling plan should ensure that the sample is representative of the population of interest and meets the objective of the surveillance. An appropriate sampling strategy needs to be developed prior to collection of samples specifying the number of units to be sampled. The sampling plan will include the sampling methods and sample size including the method of sample collection. When developing the sampling plan for AMR surveillance in local chicken meat in Thimphu, different steps of the food chain, including source, processing, and retailing, are considered.

Generally, the sample size should be large enough to determine the prevalence or trends in existing and emerging antimicrobial resistance phenotypes and should be representative of the units of interest. The sample size takes into consideration the expected prevalence of the bacteria in the sample type, the expected prevalence of the resistance phenotype and the desired level of precision and confidence. Ideally, the sample size calculation should be based on independent samples. However, if there is any clustering at the establishment or retailing, the sample size should be adjusted accordingly. The number of isolates required to estimate the prevalence of resistance amongst the isolates for a fixed level of confidence varies with the expected prevalence and the desired level of precision. Highest numbers of samples are required to estimate prevalence levels of 50% for a given precision; if a more precise estimate is required, the sample size increases.

Table 1:Number of isolates required to estimate the prevalence of resistance to a specific antimicrobial in a bacterial species with a 95% confidence level, for two levels of precision (5% and 10%). (Extracted from OIE Terrestrial Animal Health Code10).

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

*Note that Table indicates the number of bacterial isolates required for the estimation of prevalence of resistance in that bacterial genus or species, not the number of animals/products sampled.

Sampling frame

The sampling frame is defined as the list of all the sampling units in the source population and it should contain the information about the source population that

enables you to draw a sample. The sampling frame for the AMR surveillance in local chicken meat is the registry of retail meat shops in Thimphu which sell local chicken meat, maintained with BAFRA, Thimphu office (Annexure 01).

As of 10th August 2020, there are 63 retail meat shops selling local chicken in Thimphu. 10 out of 63 retail shops are not currently selling meat. Therefore, a total of 53 retail meat shops will be included in the sampling frame. A required number of meat samples will be drawn from the above list as per the sampling strategy.

Sample size

A total of 300 meat samples will be tested in this first round of surveillance. The number was set at 300 local chicken meat samples to match the capacity of the National Food Testing Laboratory to process surveillance samples for 10 months. At the same time, according to the guide of the OIE Terrestrial Animal Health Code10, the 300-sample size will produce sufficient isolates to give a reasonable level of precision (within 10%) for the prevalence of AST in the more commonly isolated bacteria (Table 3). The number of isolates for lower-prevalence bacteria such as Salmonella and Enterococcus is unlikely to result in precise estimates of antimicrobial resistance. However, it is not feasible for the laboratory to test sufficient samples to isolate large numbers of Salmonella and Enterococcus in this first stage. This will be an exploratory stage that will strengthen the capability of the NFTL and at the same time produce some useful results to compare with the AMR results from chickens on farms (objective 3). It will also provide a source of isolates for genomic sequencing to identify the relationship between resistance in isolates found on meat and those in on-farm chickens and humans.

Table 2. Expected prevalence of target bacteria in local chicken meat and the expected number of isolates of each bacterial species from 300 meat samples(li et al 019, khan et al 2018, tyson et al, 2018)

Bacteria	**Predicted prevalence of bacteria on meat samples**	**Expected number of isolates from 300 meat samples**
E coli	0.40	120
Salmonella spp.	0.12	36
Enterococcus spp.	0.20	60
Campylobacter spp.	0.50	150

Number of samples per stratum

Since the surveillance site for the AMR surveillance in local chicken meat is in Thimphu, all the 300 samples will be collected from Thimphu. Going through the sampling frame, the retails meat shops vary in the volume of meat they sell per month

and the source of the meat. All retail meat shops in Thimphu source local chicken meat from Sarpang, Samtse, Chukha and Tsirang, therefore, the meat source will not introduce sampling bias. However, the volume of the local chicken meat handled by the retail meat shop varies considerably between the retail meat shops with the estimated mean of 552.4 KGs (SD ±1035.3 KGs) per month (Max – 6171, Min – 50 Kgs). Therefore, the sampling method used will be stratified sampling proportional to volume. The retails meat shops will be stratified based on the volume of local chicken meat sold per month and are classified into the following strata:

- Stratum 1: 50 – 499 KG/Month
- Stratum 2: 500 – 999 KG/Month
- Stratum 3: 1000+KG/Month

Based on the stratification of the retail meat shops, the number of samples to be collected from each stratum is given below:

Table 3. Samples collection distribution from each stratum

Stratum	**Average volume of local chicken sold/month**	**No of Retail meat shops**	**Total samples**	**Samples/Retail meat shop**
I	50-499 KGs	36	108	3 samples/meat shop/4months
II	500-999 KGs	22	132	1 sample/2 months
III	1000+ KGs	6	60	1 samples/month
	Total	64	300	

Note: The sampling frame is as of August 2020. However, during the time of sample collection and testing which is spread over 10 months from March 2021, there will be addition or subtraction of the list of retail meat shop which sells local chicken meat in the sampling frame. If one of the selected shops does not sell meat at the time of sample collection, then the sample will be adjusted from another shop with the same sample size stratum. Further, if there is an addition of shops to the sampling frame, the samples will be collected based on the strata.

Number of samples per retail shop in different strata

Since we will be collecting samples from the retail meat shop, the sampling location is the retail meat shop. The most precise estimates of AMR prevalence are obtained by maximizing the number origin from which chickens are tested and testing a single isolate of each target bacteria per origin when there is a fixed total number of samples (Persoons et al., 2011; Yamamoto et al., 2014). Therefore, we will include all the

registered retail meat shop in Thimphu. In the stratum I (50-499 KGs/ Month), there are 36 retail meat shops and we will be collected 3 samples per retail shop spread over 4 months period. Only one sample from each retail shop will be collected at one go. From the retail shop under stratum II (500-999 KGs/Month), at total of 132 samples from each retail shop (one samples per month for 10 months) will be collected. Similarly, a total of 60 samples from each retail shop (one samples per month from each shop) will be collected under the stratum III (1000+ KGs/month)

Sampling timetable

NFTL has the capacity to process 30 meat samples every month, in addition to its normal diagnostic testing load. A sampling timetable is prepared for the National Food Testing Laboratory showing the numbers of samples to be collected from retail meat shop by month. This timetable is prepared to ensure that the days of sample collection, the number of samples collected and the frequency of sampling match the laboratory's capacity to process samples in addition to the routine work.

The NFTL will collect 15samples every2 weeks from the surveillance sites based on the sampling distribution for each stratum. In this way, isolates can be cultured and identified and have AST performed in the first week, then additional testing/isolate storage can be performed in the second week. However, sample collection should be planned properly so that the samples are not stored for more than 72 hours before processing.

Table 4. Sample collection timetable

Stratum	No of samples										
	2020			2021							Total
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	
I (41 shops)	11	11	11	11	11	11	11	11	11	11	110
II (5 shops)	5	5	5	5	5	5	5	5	5	5	50
III (7l shops)	14	14	14	14	14	14	14	14	14	14	140
Total	30	30	30	30	30	30	30	30	30	30	300

Sampling of meat

A whole carcass of the chicken with skin will be randomly selected from the retail meat shop for the sample collection.

Inclusion Criteria – The fresh local chicken carcass from the retail meat shops in the sampling frame will only be collected.
Exclusion Criteria - The chicken carcass which are in frozen state (local & imported) and smoked (local) and imported are excluded from sampling from the surveillance sites.
Note: Ideally, the sample collection should be carried out early morning to get the fresh samples. Please refer checklist of equipment and materials to take when collecting samples (annexure 3)

Type of samples:

There are several options for the selection of the chicken meat samples. A whole carcass with skin will give the most representative information on resistant bacteria that are carried on retail chicken meat to which consumers are exposed. Chicken breast, leg and wings (with skin), are convenient and easy to handle but may not be as representative as the whole carcass. Similarly, sampling skin from around the neck area will capture bacteria on the carcass during the slaughter process but may not represent the bacteria over the whole carcass i.e. including bacteria from food handlers and the transport plus retail environment.

For the purpose of AMR surveillance in local chicken meat, a whole chicken carcass with skin is selected as an ideal sample type considering that the sample gives the most representative information on the resistant bacteria that are present in the chicken meat. The whole carcass sample will also yield more bacteria compared to other sample which is consistent with the objective of the AMR surveillance.

Sample collection from retail meat shop

After wearing the gloves, the whole chicken carcass with intact skin should be collected and placing the entire carcass in a sterile leak proof zip lock bag. This bag should be placed inside a second Zip lock for transport to the laboratory to ensure there is no leakage and cross-contamination of samples in the cool box. To avoid cross-contamination between the samples from different shops, the sampler should wear a new set of gloves and use a fresh sampling bag for collecting the whole chicken carcass.

Information collection during sampling

During the sample collection, a sampler should also collect descriptive information that will help correctly interpret the AMR results, using the prepared sample collection form. Please refer sample collection form (annexure 3).

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

Sample labelling methods

Samples from the Field

The samples will be clearly labelled using a permanent marker pen. If possible, label will be prepared prior to the sampling. The sample information will be placed on a plastic envelope on the sample packaging and transported in cool box. The microbiology unit will record the date of the sampling. The sample ID number will contain Strata No/Name of Meat shop - code)/sample Number. For example the sample id No. I/01/01 means I – strata number I, 01 – Meat shop number in strata I, 01 – sample number). Any unlabeled samples received will be rejected.

The sample labelling, packaging and transportation will follow as per the SOP (annexure No

Target bacteria

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

Following are the four bacteria included in this surveillance

Table 5. Bacteria selected for the surveillance

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

E. coli and Salmonella spp are priority organisms listed in the WHO Global AMR Surveillance System (GLASS)(WHO, 2015). Campylobacter is an important zoonotic pathogen in humans and *Enterococcus spp* are commensal organisms that may act as an indicator for resistance patterns associated with Gram-positive organisms. This group of target bacteria is consistent with the focal bacteria recommended by OIE(OIE, 2018)and the AGISAR program(AGISAR, 2017).

The same organisms are selected for the surveillance of AMR in layers and broilers semi and commercials in the country for comparison of the findings. Further, a standardized protocol for identification, culture and isolation and AST will be followed by the NFTL and animal health surveillance labs for comparability of the results.

Target antimicrobials

The aim of this AMR surveillance is to generate AMR information in understanding the risks to human health that may be associated with the use of antimicrobials and AMR in chickens. To achieve this, the panel of antimicrobials for AST in each of the four bacteria, shown in Table below, has been selected from the critically and highly important antimicrobial classes for humans identified by WHO(WHO, 2018) and as recommended by FAO(OIE, 2019) Targeting resistance to antimicrobials that are critically important to humans contributes to the One Health AMR surveillance system, allowing comparison of AMR and antimicrobial usage (AMU) patterns in animals with those in humans, to identify potential links between AMR in the animal and human populations. Given the outcome of interest in this surveillance is resistance in the bacteria carried by healthy chickens that would occur if they were to infect humans, CLSI guidelines will be used for testing and interpretation of resistance in humans.

Table 6. Antimircobial panel selected for the surveillance

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

Streptogramins				Quinupristin-dalfopristin*
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	Tetracycline
Sulpjonamides/Trimethropim	Co-trimoxazole	Co-trimoxazole		

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

Isolates from Laboratory

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

LABORATORY PROCEDURES

Sample receiption

- Verification of quality;
- Once a sample enters the laboratory, the samples will be verified for proper labeling, quality of the sample, adequate in quantity, and appropriateness for test requested. The test request must be complete and include all necessary information.
- Record sample information into a register or spreadsheet (annexure 12)
- Recording of sample details

  The laboratory will keep a register (spreadsheet) of all incoming samples to record all the information in the laboratory. A master register will be maintained at the reception and a separate register at microbiology unit.

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

  The register will capture following information:
  - Name of the sample collector
  - Name and address of the meat shop
  - Location of the meat shop (GPS)
  - Sample ID
  - Date and time of collection
  - Date and time the sample was received in the laboratory
  - Sample type (Broiler/layer)
  - Laboratory assigned identification number
  - Sample storage details
  - Tests to be performed

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

- Rejection of samples
  The laboratory will establish rejection criteria and follow them closely. The reasons for the rejection will be recorded in the logbook.

The following are rejection criteria for samples:

- Broken or leaking bag/container (that could cause cross-contamination)
- Unlabeled sample
- Insufficient sample information/details
- Mismatch in sample and sample information form
- Evidence of environmental contamination during transport of samples
- Insufficient sample quantity for the test requested
- Prolonged transport time, sample spoilage or other poor handling during transport

- Storage before identification
  All the samples received will be refrigerated at 2-4°C until it is processed for testing. Once the tests are completed and bacteria are identified, stored samples can be discarded.
- Processing chicken carcass:

Table 7. Distribution of initial suspension

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

Samples received via surveillance laboratories for the culture of *Campylobacter spp* shall be processed immediately as per the SOP for isolation and identification of *Campylobacter spp* (annexure 5)

Bacterial Isolation

The samples will be then processed for isolation and identification of the target bacteria (*E. coli, Salmonella spp, Enterococcus spp and Campylobacter spp*) as per the standard operating procedure for isolation and identification of *E. coli, Salmonella*

*spp, Enterococcus spp and Campylobacter spp*developed by National Veterinary Laboratory (NVL) (annexure 4, 5, 6, 7 & 8)

Antimicrobial Susceptibility Testing (AST)

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

Disk diffusion method is technically simpler to perform, less expensive and useful for guiding treatment in a clinical setting, this procedure results in an inhibitory zone diameter (mm) which is used for classifying isolates into categories relating to clinical sensitivity of the antimicrobials i.e. susceptible, intermediate, or resistant.While the inhibitory zone diameter data can be used for surveillance and monitoring, the results are less precise, especiallyfor measuring low levels of resistance, compared with minimum inhibitory concentrations generated from broth and/or agar dilution methods. Refer SOP for Disk diffusion method (annexure 9)

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

Interpretation of tests results

Two different types of interpretive criteria are available; human CLSI guidelines.

To compare the AST results for the human and animal isolates, the data should be interpreted with CBPs. The priority is assigned to CLSI CBPs. For antimicrobials where CLSI CBPs do not exist, EUCAST CBPs should be used.

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

Detection of ESBL producing Salmonella spp and *E. coli* are considered very important. Ceftriaxone or cefotaxime is included in the harmonized panel for routine monitoring. Salmonella spp.or E. coli isolates that are resistant to ceftriaxone should be further confirmed for ESBL-production. The phenotypic confirmatory test requires the use of ceftazidime and cefotaxime alone, and in combination with clavulanate. Refer the SOP for detection of ESBL producing Enterobacteriaceae (annexure 10)

Isolate storage

NFTL should store all the isolates regardless of AST results on an agar slant before transporting to the NVL. Refer the SOP for isolate storage and transport (annexure 11)

Isolate transport to reference laboratory

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

for isolate storage and transport (annexure 11)

Quality Control (QC) in antimicrobial susceptibility testing

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

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

Reference strains example Escherichia coli ATCC 25922, Campylobacter jejuni ATCC® 33560, and E. faecalis ATCC® 29212 will be used for quality control. The disk diffusion ranges for QC strains and the frequency of QC testing are described in the SOP for kirby-bauer disk diffusion susceptibility test developed by NVL.Refer SOP for Disk diffusion method developed by NVL (annexure 10)

Laboratory data management (Spread sheet)

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

EQAS and proficiency testing programme for all laboratories

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

Proficiency testing

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

Steps towards proficiency testing are described below;

- The reference laboratory should first ensure that they can produce repeatable AST results by testing each of the target bacteria against all the antibiotics listed in Table.4 for each bacteria in the panel. Each bacteria and antibiotic should be tested in triplicate. The laboratory should ensure it can produce repeatable results before developing the proficiency testing panel to send to the other laboratories.
- Initially, the AMR reference laboratory should send a panel of known isolates of the target bacteria relevant to each surveillance laboratory, with their identity and resistance profile disclosed, and request the laboratory to test each against the full panel of antibiotics listed for each isolate. This will enable the surveillance laboratories to test that they are able to identify the bacteria and achieve the known AST results. A standard form should be sent with each panel of isolates for the surveillance laboratories to complete the AST results to ensure standardization in the reporting of results across all laboratories.
- Subsequently, an isolate with identity and resistance undisclosed should be sent by the AMR reference laboratory with the request to culture and identify the bacteria and test against the appropriate panel of antibiotics.
- Laboratories should report the results to the reference laboratory. If the results are correct, sampling may proceed. If the results are incorrect, the reference laboratory should review and recommend corrective actions to be taken, before repeating the proficiency testing.
- Proficiency testing may be conducted at least once a year. Any issues should be addressed after each round of proficiency testing. AMR reference laboratory should participate in an External Quality Assurance Scheme (EQAS) to ensure that they are producing reliable results.

Use of ATCC strains in Internal Quality Assurance

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

DATA MANAGEMENT AND QUALITY CONTROL FOR AMR FIELD AND LABORATORY DATA

Data entry and analysis

The detail information of the sample is collected using sample collection form in hard copy during the sample collection and entered in LIMS and WHONET. However, LIMS cannot capture all the demographic data collected. LIMS is an online database system designed to efficiently manage the information of all laboratory activities carried out by BAFRA in the country. Even the WHONET cannot capture all the demographic data as there were limited fields set for which information is collected.

Therefore, an excel spreadsheet will be maintained to record all the demographic data and results along with WHONET database. Refer reporting format (Annexure).

The field data of samples for AMR surveillance will be collected using the sample collection questionnaire and accordingly updated in the spreadsheet. The samples will be subjected to different tests such as identification, isolation and AST and the results obtained are updated in spreadsheet.

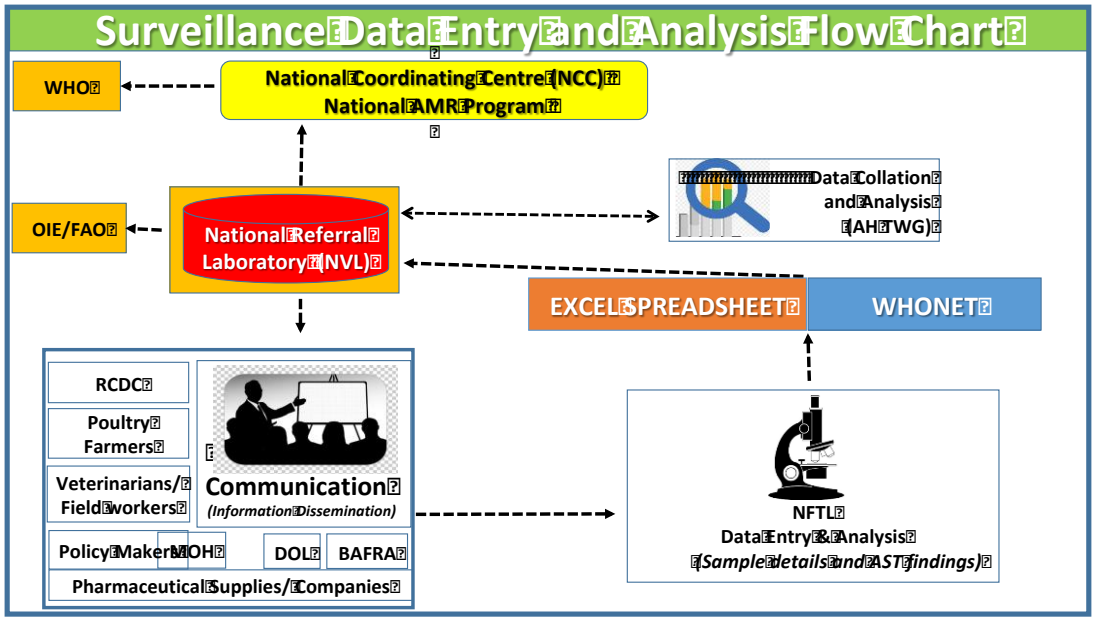

Figure 2. Flow chart showing collection, entry and analysis including the information dissemination

Simultaneously the details of the samples with results are entered in WHONET offline database which will generate the AST findings with concrete analysis, which can be further, updated using the analytical tools available in the database. Subsequently, the AST findings from WHONET will be updated on excel spreadsheet. Information in all 3 databases will be linked by a common sample ID entered into each database.

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

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

The data from WHONET can be exported in csv format for more complete analysis of AMR data. Since the WHONET is not set up to capture all the demographic data collected, the excel spreadsheet should be also sent to AMR reference laboratory

(NVL) along with WHONET csv file for compilation and analysis. The transfer of data and compilation will follow a regular procedure without any requirement for agreements and MOU within the government settings.

Data collation, validation and dissemination

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

AMR reference laboratory, NVL Serbithang will do the overall data interpretation at the animal setting while it will be done by the National AMR Program with the support of AMR technical working group for the country after including both the information from human and animal health. The analyzed information will be disseminated to different stakeholders like poultry farmers, policymakers, department of Livestock, MOH, pharmaceutical suppliers/companies, veterinarians, Para veterinarians, livestock and poultry consumers, media and other relevant stakeholders. The raw data including the analyzed report will also be submitted to National AMR Program for the final generation of the country report after the inclusion of human AMR surveillance report. The detail data management till the dissemination of information is summarized in Table 8.

Table 8. Methods and responsibilities of AMR data management at different levels

Data type	**Data entry**	**Data collation and validation**	**Data management**	**Analysis and Interpretation**	**Information desimination**
Details of sample: 1. Sample ID 2. Sample type 3. Sample source 4. Bird type	Sample collection team shall compile demographics of sample in sample collection form	Information collected in sample collection form shall be collated into sample submission form for submission to NFTL	Sample demographics shall be entered into MS Excel spreadsheet and stored as soft copy. A hard copy shall be printed and compiled as hard copy	Basic analysis on sample collection progress shall be performed by team leader	The sample collection team shall forward the details of samples to NFTL
Details of organism isolation and identification: 1. Colony character 2. Basic test 3. Biochemical test Isolate naming	Laboraory techncians at NFTL shall enter all the details of laboratory results for each isolate in WHONET and spreadsheet. Recovered isolate shall be archived with standard identification process	All the details of isolation and identification process shall be entered into WHONET and spreadsheet at NFTL. Results of isolate shall be compared with that of reference organism	Isolation and identification records shall be maintained at NFTL	Basic analysis on the consistencies of isolate and reference organism. This activity shall be performed at NFTL	NFTL shall forward these information to 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 desimination
Details of AST from DD 1. Panel of antibiotics 2. Zone diameter of isolate 3. Zone diameter of reference organism Name of standard used (CLSI, EUCAST)	NFTL shall enter isolate details for each sample (including samples from which no isolates were retrieved) in WHONET and spreadsheet: - Laboratory ID - Sample ID - Selected panel of antibiotics Zone diameter of both isolate and reference organism	NFTL shall collate cummulative data on AST from all the isolates	NFTL shall be the custodian of AST data at their level	NFTL shall use WHONET to analyse AST data and draw some preliminary interpretation	NFTL shall pass AST data to national reference laboratory in both electronic (WHONET csv file and excel spreadsheet) as well as paper copy
Details of AST from MIC 1. Panel of antibiotics 2. MIC of isolate 3. MIC of reference organism Name of standard used (CLSI. EUCAST)	National reference laboratory shall update the records for each sample and isolate in WHONET and spreadsheet with: - Selected panel of antibiotics MIC of both isolate and reference organism.	National reference laboratory shall collate cummulative data on MIC data from all the isolates	National reference laboratory shall be the custodian of DD and MIC AST data at national level	National reference laboratory shall use WHONET to analyse AST (both DD and MIC) data and interpretate AMR ststus at national level	National reference laboratory shall pass DD and MIC AST data to AH TWG for epidemiological analysis. These result should then go to the NCC for comparision with HH results

GLOSSARY

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

Definitions

Dzongkhag: District
Gewog: County

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

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

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ANNEXURE

Annexure 01: Sampling Frame

Sl No.	Name of Meat Shops	Location	Contact no.	Average volume of Local chicken (kg)/month	Meat collection areas
1	Gurung MS	Babesa	17428757	0	
2	MD MS	Changbangdu	77309321	0	
3	Phendeyling MS	Hongkong Market	17494679	0	
4	Three Brother MS	Hongkong Market	17705634	0	
5	Subba MS	Hongkong Market	17688096	0	
6	Dagana MS	Hongkong Market	77209607	0	
7	Lhams M/s	Hongkong Market	17459602	0	
8	Hema M/s	Hongkong Market	17562424	0	
9	TP MS	Sabji Bazar	17660307	0	Samtse, Gomtu, Pasakha
10	Samjana M/s	Sabji Bazar	17902517	0	Samtse
11	MS MS	Olakha	17800538	80	Samtse
12	Maya MS	Olakha	17885946	70	Pasakha
13	Olakha MS	Olakha	17456565	60	Pasakha
14	Roy MS	Olakha	17693635	50	Gomtu
15	Sagar M/s	Sabji Bazar	77373629	50	Tsirang, Samtse
16	Yk M/s	Babesa	17247559	150	Tsirang, Sarpang
17	RB MS	Changzamtok	17883398	150	Tsitrang, Gomtu,
18	Dipa MS	Olakha	17889911	150	Pasakha
19	Ghalley MS	Changzamtok	17663142	140	Tsirang, Pasakha

20	Mountain M/s	Lanjophaka	17608120	140	Tsirang,Pasakha
21	Tek Maya MS	Motithang	17660239	120	Tsirang, Samtse
22	Kancha MS	Olakha	17608120	100	Samtse
23	Sibsoo MS	Pamtsho	17602023	100	Samtse
24	Motithang MS	Motithang	17988686	240	Sarpang
25	Druk Star m/s	Dechencholing	17660239	220	Tsirang, Samste
26	Kersung M/s	Babesa	77389075	200	Pasakha
27	Babesa MS	Babesa	17758070	200	Tsirang, Wangdue,Samtse
28	Dimali MS	Babesa	17350636	200	Tsirang, Samtse
29	4 Star MS	Changidaphu	17712027	200	Gomtu
30	DB MS	Changzamtog	17698693	200	Tsirang Gomtu, Pasakha
31	Tendu MS	Changzamtok	17758176	200	Wangdue, Gomtu
32	OM MS	Hongkong Market	17608120	200	Tsirang, Samtse
33	BM MS	Olakha	77345968	200	Pasakha
34	Rai MS	Olakha	17648575	200	Pasakha
35	SM MS	Olakha	17617630	200	Pasakha
36	IB MS	Taba	17658339	200	Samtse
37	Shingden MS	Babesa	17927816	280	Samtse, Mendayala
38	XXX MX	Sabji Bazar	17666850	275	Tsirang, Sarpang, Gomtu
39	Himalayan MS	Sabji Bazar	17640205	260	Tsirang, Samtse,
40	Daga MS	Babesa	17356151	250	Tsirang Gomtu
41	KRT MS	Babesa	17916886	250	Pasakha
42	Ugyen M/s	Changzamtog	17978180	250	Samtse
43	Abi Maya MS	Kabesa	17976940	250	Gomtu
44	Chang MS	Olakha	17350636	250	Gomtu
45	JN M/s	Sabji Bazar	17615698	250	Tsirang, Chukha
46	CT MS	Hongkong Market	77601990	450	Thimphu

47	Makcha MS	Sabji Bazar	17258700	405	Tsirang, Gomtu, Sarpang, Pasakha
48	GM MS	Khasarapchu	17675641	400	Samtse
49	Tsirang MS	Pamtsho	17675467	400	Sarpang
50	Jimba MS	Chang Debsi	17779325	300	Tsirang, Samtse/ Pasakh
51	AM MS	Sabjee Bazar	17892899	300	Tsirang,Gomtu
52	Dragon MS	Sabji Bazar	17334201	640	Tsirang, Chukha
53	PR MS	Changjiji	17574787	500	Tsirang Gomtu/Pasakha
54	Dagana MS	Pamtsho	17593856	500	Pasakha
55	SNR MS	Sabji Bazar	17812170	920	Tsirang, Dagana, Gomtu
56	Wangyel MS	Sabji Bazar	17666640	824	Tsirang, Gomtu
57	Kaka MS	Sabji Bazar	17445595	6171	Tsirang, Wangdue, Sarpang
58	Druk MS	Hongkong Market	17666661	4509	Samtse
59	B.K MS	Sabji Bazar	17330923	1557	Tsirang, Chukha
60	DK MS	Sabji Bazar	17615698	1380	Tsirang, Dagana, , Pasakha
61	Karmaling MS	Sabji Bazar	17712027	1184	Samtse, Gomtu
62	Good Meals MS	Sabji Bazar	17280253	1119	Sarpang/Pasakha
63	BLDC	Chubachu		1381	

Annexure 02: Sample Information Collection Form

Sl.No	Details	Information
A: Details of the sample collector		
1	Name of sampler and designation:	
2	Contact no:	
B: Details of meat shop		
1	Name of Owner:	
2	Name of Meat Shop	
4	Location (GPS coordinates):	X - Y -
6	Location Name:	
9	Contact No:	
10	Shop Stratification No.	
C: Details of the sample		
1	Sample ID number (Strata No/Name of Meat shop(code)/date of sample collection/sample Number)	*(E.g.; I//A01/20092020/01)*
2	Point of sample collection	☐ Display Table ☐ Fridge ☐ Meat Van
3	Date and time of sample collection	
4	Date and time of slaughter	
5	Slaughter type	☐ Meat Processing Plan ☐ At farm premise
5	Carcass transport medium	☐ Freezer Van ☐ Puff box ☐ Thermo cool box ☐ Others specify

Sl.No	Details	Information
	Carcass transported with (meat other than chicken/vegetables)	☐ Other meat ☐ Vegetable
7	Type of carcass:	☐ Broiler ☐ Spent Layer
	Farm Category	☐ Commercial Farm ☐ Semi - Commercial Farm
8	Source of chicken (name of farmers group or individual farmer supplying chicken)	Farm owner: …….. Village: ……. Geog: ..... District: .....

Only for laboratory use below this line

……

…….……

NFTL/FF/REC/7.4/01

Review of Request

Registration No.: ……. **Received Date:** ……..							
Sl #	**Sample ID**	**Sample conditions upon receive (Yes✓), (No = X)**					**FTL Code**
		Fresh	Temperature maintained (2 to 8^{o}C)	Leakage	Sufficiently labeled	Sufficient sample quantity	

Received and reviewed by
(Name):_____

Signature: _____
Date:_____

Annexure 03: SOP for sample collection

Scope

This SOP describes the method for sampling of fresh local chicken samples from the retail meat shops for laboratory analysis. It includes following:

- Type of samples to be collected
- Sample information to be collected
- Labelling of the samples
- Sample packing and transportation to the laboratory

Objective

The objective is to detail the procedure for collecting local chicken carcass samples from the retail meat shop for laboratory analysis.

Principles

Sampling of fresh local chicken collection, packing and transportation to laboratory through aseptically approach to avoid contamination. The samples collected are sufficiently labeled and transported to laboratory for analysis fulfilling appropriated transportation requirements.

Sampling of meat from the retail meat shops

A whole carcass of the chicken with skin will be randomly selected from the retail meat shop for the sample collection.

- **Inclusion Criteria** – The fresh local chicken carcass from the retail meat shops in the sampling frame will only be collected.
- **Exclusion Criteria** - The chicken carcass which are in frozen state (local & imported) and smoked (local) and imported are excluded from sampling from the surveillance sites.

Note: Ideally, the sample collection should be carried out early morning to get the fresh samples.

Type of samples

There are several options for the selection of the chicken meat samples. A whole carcass

with skin will give the most representative information on resistant bacteria that are carried on retail chicken meat to which consumers are exposed. Chicken breast, leg and wings (with skin), are convenient and easy to handle but may not be as representative as the whole carcass. Similarly, sampling skin from around the neck area will capture bacteria on the carcass during the slaughter process but may not represent the bacteria over the whole carcass i.e. including bacteria from food handlers and the transport plus retail environment.

For the purpose of AMR surveillance in local chicken meat, a whole chicken carcass with skin is selected as an ideal sample type considering that the sample gives the most representative information on the resistant bacteria that are present in the chicken meat. The whole carcass sample will also yield more bacteria compared to other sample which is consistent with the objective of the AMR surveillance.

Sample collection from retail meat shop

a) Materials/items required:
   - Face mask
   - Sterile gloves
   - Sterile ziplock plastic bags
   - Cool box with ice packs
   - Marker pen
   - Stickers for labelling
   - Sample collection form

b) After wearing sterile gloves, the whole chicken carcass with intact skin should be collected and placing the entire carcass in a sterile leak-proof zip lock bag.

c) This bag should be placed inside a second Zip lock for transport to the laboratory to ensure there is no leakage and cross-contamination of samples in the cool box.

d) To avoid cross-contamination between the samples from different shops, the sampler should wear a new set of gloves and use a fresh sampling zip lock bag for collecting the new chicken carcass.

Information collection and Sample labeling

a) During the sample collection, a sampler should also collect descriptive information that will help correctly interpret the AMR results, using the prepared sample collection form. Please refer sample collection form.

b) The informations collection should include following details:

- Name of the sample collector
- Name and address of the meat shop
- Location of Meat shop (GPS)
- Date of slaughter
- Date of sample collection
- Date of sample submission to laboratory
- Sample type (Broiler/layer)
- Source of sample
- Sample storage details
- Test request

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

d) The sample ID number will contain Name of the Meat shop, date of collection followed by number, example: ST No.MS/27072020/01 (Where, ST No – Strata number, MS – name of the meat shop, 27072020 – Date/Month/Year, 01 – Sample Number).

e) The samples will be clearly labeled using a permanent marker pen. If possible, labels will be prepared prior to the sampling and sticked on the sample plastic bag.

f) The sample information will be placed on a plastic envelope on the sample packaging and transported in cool box. The microbiology unit will record the data of the sampling.

g) Any unlabeled samples received will be rejected.

Sample packing

a) Samples must be packed in a primary and secondary container/leak proof zipper bag so that the samples do not pose any threat to persons or animals during shipment.

b) The secondary packaging is then placed cool boxes (foam boxes) / UN boxes with ice packs to keep the samples chilled (<10oC), but not freezing, to prevent

overgrowth of samples. Freezing of samples should be avoided as it may kill the target organisms.

c) Sample collection form will be enclosed in an envelope, enclosed in a plastic bag and placed between the secondary packaging and outer box.

Sample transportation

a) The samples should be transported 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.

b) If not, they must be stored in a refrigerator at 4-8oC and transported to the laboratory very next day.

c) All the samples sent to the laboratory should strictly comply with packaging instruction mentioned above.

Sample processing of Chicken carcass

a) All the samples received in the microbiology unit should be stored at refrigerated condition (at 2-4^{o}C) until it is processed for analysis/testing.

b) For the analysis of received chicken carcass, 400ml of BPW is added to the carcass within the bag in which it was transported.

c) The bag is tightly sealed and the BPW mixed around so that it covers all surfaces of the carcass.

d) 1ml of BPW enrichment material is added to 9ml Boltons broth and incubated in microaerophilic conditions for Campylobacter (refer SOP for identification of Campylobacter).

e) 1 ml of BPW enrichment material is added to 9ml of Azide dextrose broth and incubated at 37°C for 18- 28hours (refer SOP for identification of Enterococcus).

f) 10 ml of BPW enrichment material into sterile container and incubated at 37 °C ± 1 °C for 18 h ± 2 h for identification of E.coli, Salmonella and ESBL Enteriobactericeae (refer SOP for E.coli, Salmonella)

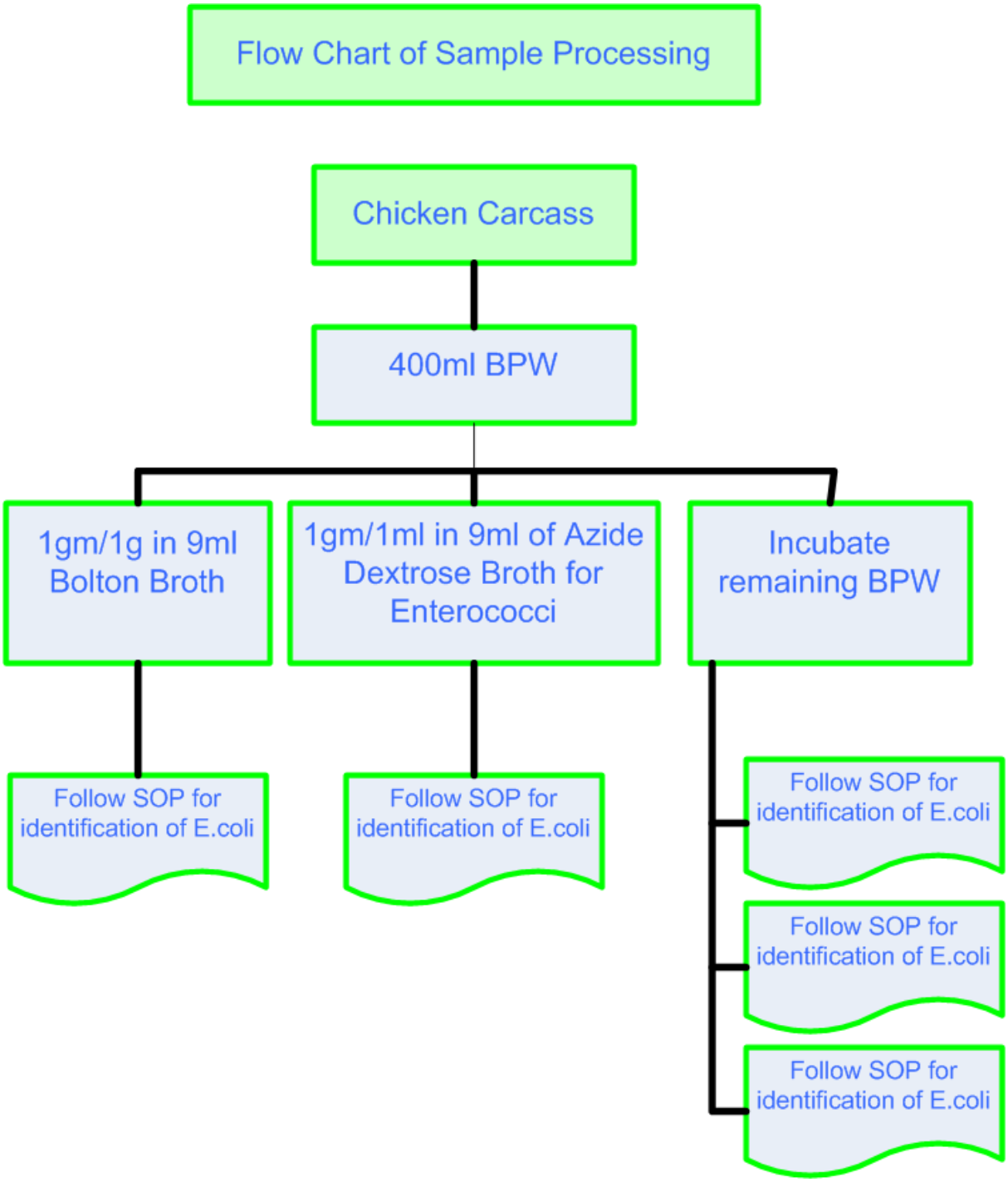


Figure: Flow chart of Chicken carcass Sample Processing

Annexure 04: SOP on handling samples for Camplyobacter isolation

Introduction

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

Objective

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

Procedure

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

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

Sample Transport

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

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

Annexure 05: SOP for Isolation and Identification of *Campylobacter spp.*

WARNING **--**In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Campylobacter* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

Disclaimer

This SOP is an adoption with national modifications and has been reproduced from ISO-, 10272-1:2017, Microbiology of food chain –Horizontal method for the detection and enumeration of Campylobacter spp.- Part 1: Detection method.

1. Introduction

Campylobacter jejuni is a major cause of foodborne illness causing human acute bacterial enteritis worldwide. Campylobacter spp. has long been associated with the cause of veterinary diseases, such as diarrhoea in cattle, and septic abortions in cattle and sheep. Their association with human blood cultures in the late 1950's was rare and hence Campylobacter spp. was deemed to be an opportunistic human pathogen. It is only in the last 30 years that these organisms have been recognized as a major cause of human illness. *Campylobacter jejuni* is now recognised as one of the main causes of bacterial foodborne disease in many developed countries with Campylobacter coli less frequently implicated. Foods of animal origin, in particular poultry, have been identified as significant sources of this enteropathogen as a result of infection and contamination at the pre-harvest and harvest levels. The handling and consumption of poultry meat has been previously linked to human illness (John, E. Moore, 2005)

This SOP targets the thermo tolerant Campylobacter species relevant to human health. The most frequently encountered and relevant to human health species are *C.jejuni*and *C.coli.* This document covers the isolation and identification of the Campylobacter species as per the international standard ISO 10272-1.

2. Scope

This document specifies a horizontal method for the detection of Campylobacter spp by enrichment and direct plating. It is application to

- Samples from primary production stage such as animal feces, dust and swabs.
- Products intended for human consumption
- Products intended for animal feeding
- Environmental samples in the area of food and feed production, handling

With this horizontal method most of *Campylobacter spp*. are to be detected. It also includes procedures for detection of *C.jejuni* and *C.coli.*

3. Objective

To identify the *Campylobacter spp* from laboratory samples by morphological and biochemical test..

4. Principle

Depending on the type of sample and the purpose of the tests, three different detection procedures can be used.

A. Detection procedure A: detection of *Campylobacter* by enrichment, in samples with low numbers of *Campylobacters* and low level of background micro flora and/ or with Stressed *Campylobacters;*
B. Detection procedure B; detection of *Campylobacter* by enrichment, in samples with low numbers of *Campylobacters* and high level of background micro flora;
C. Detection procedure C; detection of *Campylobacter* by direct plating, in samples with high numbers of *Campylobacters*.

The detection of *Campylobacter* requires two or three successive steps depending on the sample type.

4.1. Enrichment in selective liquid medium; the test portion is added to the liquid enrichment medium (Bolton broth and Preston broth).

4.2. Isolation on selective solid medium; from the enrichment medium two selective solid media are inoculated (mCCDA and Preston agar/Karmali agar)

4.3. Confirmation by biochemical test; suspect colonies are examined for morphology, motility and biochemical tests.

5. Equipment and Consumables

a. Incubators; temperature ranges 33°C - 37°C (35±2°C),
b. Incubators; temperature ranges 41°C -44°C,
c. Incubators; temperature ranges 24°C - 26°C (25±1°C)
d. Water bath
e. Sterile loops (10µl and 1 µl)
f. Microscope
g. Anaerobic jar; 2.5 L with rack
h. Micro aerobic gas pack; 0_2 content 5%±2%, co_2 content 10%±3%, and optional hydrogen ≤10% with balance nitrogen
i. Timer; countdown time 10min, 30min, 60min
j. Sterile petri dishes; 90mm
k. Media bottles: Capacity (250ml, 500ml, 1000ml)
l. Measuring cylinder: Capacity (5ml, 10ml, 100ml, 500ml, 1000ml)
m. Refrigerators; temperature ranges 3°C ± 2°C, 5°C ± 3°C
n. Sterileforceps
o. Bunsenburner
p. Spatula
q. Autoclave
r. Glass slides
s. Biosafety cabinet type II
t. Personal protective equipment

6. Culture media and Reagents

a. Bolton broth
b. Preston broth
c. Modified charcoal cefoperazone deoxycholate agar (mCCD agar)
d. Columbia Blood Agar
e. Grams stain kit
f. Catalase test reagents
g. Oxidase test reagents
h. Ninhydrin reagent
i. Hippurate tubes
j. Hippurate media

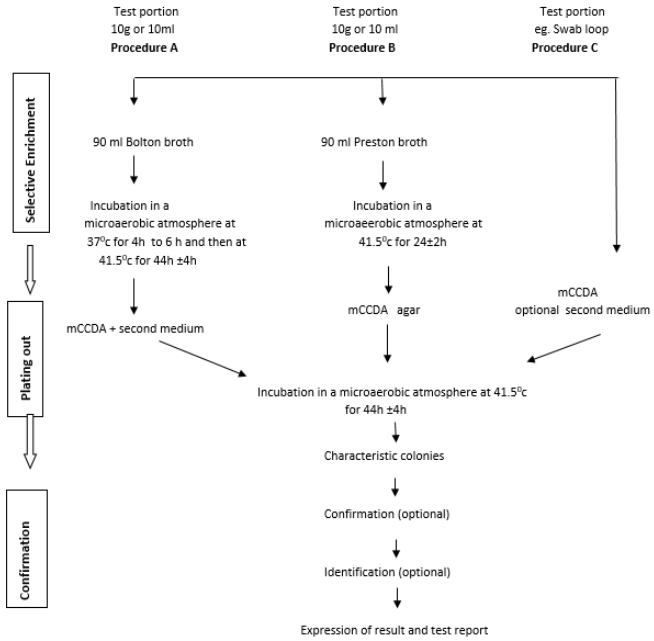
k. Indoxyl Acetate Strips

7. Sampling

Sampling is not part of the method specified in this document. Prepare the test sample in accordance with the sampling protocol dealing with the product concerned.

Note: *The samples for Campylobacter detection are mainly the caecum from animal, skin and meat from food item. Since the Campylobacter is very sensitive to freezing but survives best at low temperatures, samples to be tested should not be frozen but stored at* 3^0C *and subjected to analysis as rapidly as possible. Also take care to prevent the samples from drying.*

8. Procedure



8.1 Enrichment

Depending on the type of sample and purpose of the tests, one or more of three different detection procedures are used:

Detection procedure A:

Combine a quantity of 1ml of the non-selective enrichmemt medium (BPW) with 9ml of the selective enrichment medium (Bolton broth), so to obtain 1 in 10 dilution and homogenize.

Incubate in micro-aerobic atmosphere at 37°C for 4 h to 6 h, then at 41.5°C for 44 h $\pm$ 4 h

Note: This method is applied to samples with low numbers of *campylobacters* and low level of background micro flora and /or with stressed campylobacter, e.g. cooked or frozen products.

Detection procedure B:
Combine a quantity of 10g or 10ml of the sample with 90ml of the enrichment medium Preston broth, so to obtain 1 in 10 dilution and homogenize.

Incubate in micro-aerobic atmosphere at 41.5^{o}C for 24h ± 4 h

Note: This method is applied to samples with low numbers of *campylobacters* and high level of background micro flora, e.g. raw meats (including poultry) or raw milk.

Detection procedure C
For caecal or fecal samples, use a loop or sterile swab to bring some of the well mixed sample material onto the first half of a mCCDA plate. Use another loop to streak out on the second half on the plate.

Incubate the plate in micro-aerobic atmosphere at 41.5^{o}C for 44h ± 4 h

Note: *This method is applied to samples with high numbers of campylobacters, e.g. faeces, poultry caecal contents or raw poultry meat.*

If little information is available concerning the best method for particular type of sample to be tested, then use detection procedure C, in parallel with detection procedure A or B.

8.2 Plating out and Identification

i. Detection procedure A:
- Using culture obtained in the enrichment media, inoculate in mCCDA agar using sterile loop (10ul)
- Incubate the plate in micro aerobic atmosphere at 41.5^{o}C for 44h ± 4 h

ii. Detection procedure B:
- Using culture obtained in the enrichment media, inoculate in mCCDA agar using sterile loop (10ul) Incubate the plate in micro aerobic atmosphere at 41.5oC for 44h ±4 h

iii. Detection procedure A, B and C:
- After 44h±4 h of incubation, examine the plates for typical and /or suspect colonies of *Campylobacter*.
- Typical colonies are greyish on mCCD agar, often with a metallic sheen, and are flat and moist, with a tendency to spread.

8.3 Confirmation of Campylobacter
- As *Campylobacter* rapidly loses culture ability in air, follow the procedures described below without delay. For clear distinction between positive and negative confirmation reactions, it is important to use control strains.

8.3.1 Selection of colonies for confirmation

Select at least four typical or suspect *Campylobacter* colony from mCCDA and subculture onto non-selective blood agar (Columbia blood agar) in order to allow the development of well isolated colonies.

Incubate the plates in micro aerobic atmosphere at 41.5oC for 24 h to 48 h.

The suspect colony could be previewed for characteristic morphology and motility before streaking on blood agar.

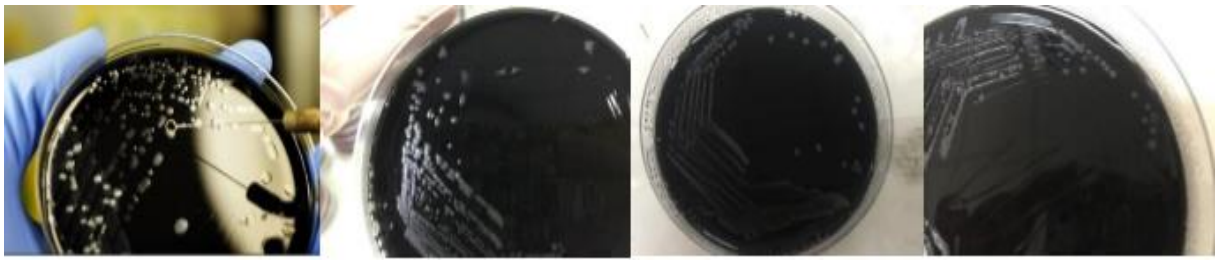


Figure 1: mCCDA plate showing typical colonies for *Campylobacter*

8.3.2 Confirmation tests

8.3.2.1 Gram staining;

- Examined the freshly grown colony from blood agar plate and perform gram staining.
- *Campylobacter spp* appears gram negative, spiral or rod or curved shaped bacilli.

8.3.2.2 Oxidase test;

- Using a sterile wooden stick pick well-isolated colony from blood agar plate and streak it on the moistened oxidase disc.
- Observe the colour change within 10 seconds.
- Campylobacter gives violet/deep blue colour within 10 seconds.

8.3.2.3 Study of aerobic growth at 25oC

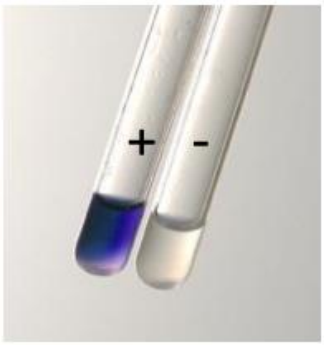
- Using pure colonies from blood agar, culture onto non-selective blood agar plate (Columbia blood agar) and incubate at 25oC for 44h + 4 h at aerobic condition.
- Observe for absence of growth
- No growth for Campylobacter

8.3.2.4 Catalase activity

- Using a sterile wooden stick or loop place a well isolated colony onto a clean glass
- Slide. Add a drop of catalase reagent (3% hydrogen peroxide).
- Observe for bubble formation.
- Note: Do not pick agar while performing the test. This gives false positive result.
- Immediate appearance of bubbles shows positive reaction for Campylobacter spp.

8.3.2.5 Hippurate hydrolysis test

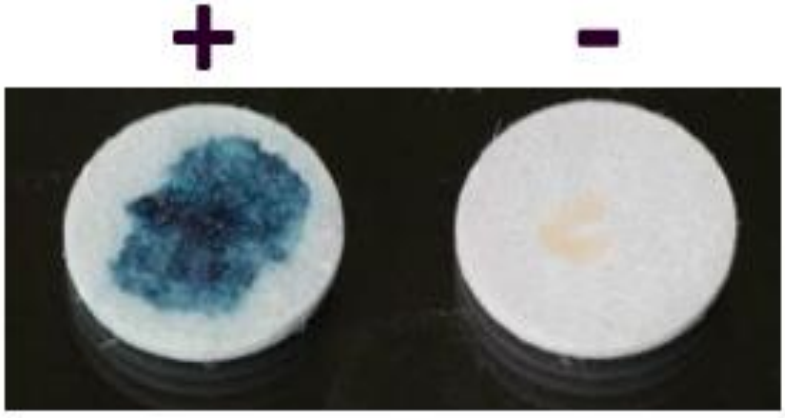
- Make up hippurate solution and ninhydrin reagent .
- Inoculate a loopful of culture into tube containing 0.4 ml of sodium hippurate solution (heavy inoculum).
- Incubate for 4 h at 37 C in a water bath(aerobic)
- Gently run about 0.2 ml of ninhydrin reagent down the inside wall of the tube so it forms a layer on the top (do not mix)
- Reincubate for 10-15 min at 37C
- Observe for colour change
  - A dark purple colour indicates positive reaction for Campylobacter spp.
  - No colour change or pale violet colour indicates a negative reaction



Note: The hippurate solution in water can be made and dispensed in advance if it can be stored frozen. Otherwise, it only lasts about a week in the fridge. The ninhydrin reagent can last up to six months at 'room temperature' in a brown (or tin-foil wrapped) bottle.

8.3.2.6 Indoxyl acetate hydrolysis test

- Place a loopful of colony on an indoxyl acetate disc and add a drop of sterile distilled water on the disc.
- Observe for colour change



- A dark blue colour change within 5-10 minutes indicates positive reaction for Campylobacter.
- No colour change indicates a negative reaction.

Table : Interpretation of primary and biochemical tests

Characteristic	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*

Morphology	Small curved bacilli	Small curved bacilli	Small curved bacilli	Small curved bacilli
Motility	Corkscrew motility	Corkscrew motility	Corkscrew motility	Corkscrew motility
Aerobic growth at 25ºC	-	-	-	-
Oxidase activity	+	+	+	+
Catalase activity	+	+	+	- Or weak
Hippurate hydrolysis	+ [a]	-	-	-
Indoxyl acetate hydrolysis	+	+	-	+
Key: + positive; - negative; [a] Some hippurate negative C.jejuni strains have been reported.				

9. Isolate Storage:

Inoculate and incubate the *Campylobacter* isolates in 5ml Luria bertani broth . Prepare 20% glycerol stock (as per ncah sop bacto39) and store at -80 ^{o}C for future references

10. Waste Disposal

As per the SOP for laboratory waste management

11. References

1. ISO 10272-1:2017(E) International Standard; Microbiology of the food chain-Horizontal method for detection and enumeration of *Campylobacter spp*.
2. Hands-on Training "Standardized and Harmonized Surveillance Methods for Antimicrobial Resistance in Food Animals in South Asia"- Isolation of Target bacteria for AMR surveillance, Chulalongkorn University.
3. https://doi.org/10.1051/vetres:2005012

Annexure Composition and Preparation of Culture Media and Reagents

1. Bolton broth

1.1 Composition	
Enzymatic digestion of animal tissues	10.5 g
Lactalbumin hydrolysate	5.0 g
Yeast extract	5.0 g
Sodium chloride (CAS NO.7647-14-5)	5.0 g

Sodium pyruvate (CAS NO. 113-24-6)	0.5 g
Sodium metabisulfite (CAS NO.7681-54-7)	0.5g
Sodium carbonate anhydrous (CAS NO. 497-19-8)	0.6g
α-ketoglutaric acid, monopotassium salt (CAS NO. 58485-42-0	1.0 g
Haemin(dissolved in 0.1% sodium hydroxide	0.01 g
D/H_2O	945

1.2 Antibiotic solution for Bolton broth

Composition	
Cefoperazone sodium salt	0.2 g
Vancomysin hydrochloride	0.2 g
Trimethoprim lactate salt (CAS NO.23256-42-0)	0.2g
Amphotericin B (CAS NO. 1397-89-3)	0.1g
Dissolve components in 5 ml of 50/50 ethanol/distilled water.	

1.3 Preparation complete Bolton broth

- Dissolve basic component or dehydrated complete basic medium in 945 ml of D/ water
- Autoclave the medium at 121^{o}C for 15 mins
- Cool to 50°C. Aseptically add 50 ml Lysed Horse Blood and 5 ml of antibiotic solution
- Mix well and distribute into sterile screw top containers
- Store at 7^{o}C for one week

Preston broth

Basic medium (composition)

Composition	
Enzymatic digestion of animal tissues	10.0 g
peptone	10.0g
Sodium chloride (CAS NO.7647-14-5)	5.0 g
D/H_2O	945ml

Antibiotic solution for Preston broth

Composition	
Polymixin B sulfate (CAS NO. 1405-20-5)	5000 IU
Rifampicin (CAS NO. 13292-46-1)	0.01g
Trimethoprim lactate salt (CAS NO.23256-42-0)	0.01g
Amphotericin B (CAS NO. 1397-89-3)	0.01g
Ethanol 95%	5ml

Preparation

- Dissolve the component in 5 ml of 95% ethanol

Preparation of complete Preston broth

- Dissolve basic component or dehydrated complete basic medium in 945 ml of D/ water
- Autoclave the medium at 121^{o}C for 15 mins
- Cool to 50°C. Aseptically add 50 ml Lysed Horse Blood and 5 ml of antibiotic solution
- Mix well and distribute into sterile screw top containers
- Store at 7^{o}C for one week

Sodium hippurate solution Composition

Sodium hippurate hydrate 10g

Phosphate Buffer Saline for Sodium hippurate

Composition	
Sodium chloride	8.5 g
Disodium hydrogen phosphate dehydrate (Na_2HPO_4 .$2H_2O$)	8.98g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	2.71g
Water to the final volume for	1000ml

Preparation

- Dissolve the sodium hippurate in PBS solution
- Sterilize by filtration
- Aseptically dispense 0.4 ml in tube
- Store at -20^{o}C

Ninhydrin solution Compositions

Composition	
Ninhydrin (CAS NO. 845-47-2)	1.75g
Acetone (CAS NO. 67-64-1)	25 ml
2-Butanol (CAS NO. 78-92-2)	25 ml

Preparation

- Dissolve ninhydrin in acetone/ 2- Butanol
- Store the solution dark at 5^{o}C for one week.

Indoxyl acetate discComposition

Composition	
Indoxyl acetate (CAS NO.608-08-2)	0.1g

Acetone	(CAS NO.67-64-1)	1 ml

Preparation

- Dissolve the indoxyl acetate in acetone
- Dispense 25µl to blank paper disc for diameter 0.6cm and 50 µl to blank paper disk for diameter 1.2 cm
- Let it dry at room temperature and store at 5^{o}C in dark container or tube having silica gel.

Annexure 06: SOP for Isolation and Identification of Enterococcus spp.

WARNING— In order to safeguard the health of laboratory personnel, it is essential that tests for Enterococci species are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that precaution is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice.

The selective media described in this standard operating procedure contains sodium azide. As this substance is highly toxic and mutagenic, precautions shall be taken to avoid contact with it, especially by the inhalation of fine dust during the preparation of commercially available dehydrated complete media. Azide-containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide (HN3) may be produced. Solutions containing azide can also form explosive compounds when in contact with metal pipework, for example from sinks. Azides can be decomposed safely by the addition of an excess of a saturated nitrite solution.

1. Introduction

Enterococci are commensal bacteria in the intestines of all animal and humans. They are commonly isolated from animal intestinal contents, faeces and relevant to human health. Commensal bacteria are exposed to antibiotics taken via feed and /or water, and could thus serve as reservoirs for transferable resistance determinants that may be transferred to other commensal and pathogenic bacteria in the animal or human gut.
Enterococcus faecium and *Enterococcus faecalis*represents commensal population of gram-positive bacteria.

2. Scope

This document specifies a horizontal method for detection of *Enterococci* spp. It is application to the following;

- Samples from the primary production stage such as animal faeces, ceacal content and swabs.
- Products intended for human consumption

With this horizontal method, most of *Enterococcus faecium* and *Enterococcus faecalis* are intended to be detected.

3. Objective

To isolate and identify *Enterococci* from laboratory samples by morphological and biochemical tests.

4. Principles

The detection of *Enterococci*requires three successive stages

4.1 Enrichment liquid medium

Brain hearth Infusion broth with 6.5% NaCl or Azide dextrose broth is used as enrichment medium to increase the number of desired organisms to detectable levels. The medium at ambient temperature is inoculated with the test portion, then incubated at the required temperature.

4.2 Plating out and identification

A loopful of the enriched cultures obtained in 4.1 are plated onto the selective solid medium Slanetz-Bartley agar. Slanetz-Bartley agar is selective medium which inhibits the growth of gram-negative organism due to the presence of sodium azide. Organism capable of reducing triphenyltetrezolium chloride (TTC) will produce red/pink colonies.

4.3 Confirmation

Colonies of presumptive Enterococci are sub-cultured and their identity is confirmed by appropriate biochemical test;

5. Equipment and consumables

a. Incubator: capable of maintaining 36°C ± 2°C
b. Incubator: capable of maintaining 44°C ± 0.5°C
c. Autoclave: capable of maintaining at 121°C ± 3°C
d. Hot plate or water bath: maintained at 100°C
e. Sterile loops (10ul and 1ul)
f. Microscope
g. Sterile petri dishes
h. Refrigerators
i. Sterileforceps
j. Bunsenburner
k. Spatula
l. Autoclave
m. Glass slides
n. Biosafety cabinet type II
o. Personal protective equipment

6. Culture media and reagents

Composition of culture media and reagents, their preparations are described in Annex B

a. Brain hearth infusion (BHI) broth
b. 6.5 % Normal Saline

c. Slanetz-Bartley agar
d. Sheep Blood Agar (SBA)
e. Grams stain kit
f. Catalase test reagents
g. Oxidase test reagents
h. Bile aesculin azide agar
i. Pyrrolidonyl Aminopeptidase (PYR) broth
j. PYR reagent

7. Sampling

Sampling is not part of the method specified in this document. Prepare the test sample in accordance with the sampling protocol dealing with the product concerned

8. Procedure

8.1.Enrichment method

- Combine a quantity of 1g/1ml (sample) in 10ml of enrichment medium, Brain heart infusion supplemented with 6.5% NaCl or azide dextrose broth and homogenize.
- Note: a sample from the BPW enrichment should be added to brain heart infusion or the azide dextrose broth **before** the BPW is incubated.
- Incubate at 37°C for 18- 28 h

8.2.Plating out and identification

- Using culture obtained in the enrichment media, inoculate onto Slanetz-Bartley agar using sterile 10 µl loop
- Incubate at 37°C for caecal sample for 18- 24 h aerobically
- Incubate at 42°C for food samples for 18- 24 h aerobically

Typical colonies for *Enterococci* on Slanetz-Bartley agar are

- Deep red colonies with golden reflection in certain angles of the light are Enterococcus faecalis.
- Pink/white colonies with a less or deeper red center are *Enterococcus faecium*
- Completely pink colonies are *Enterococcushirae*and *Enterococcusdurans.*

Figure 1: *Enterococcus faecalis* on Slanet-Bartley

8.3.Isolate purification

Select typical colonies from Slanetz-Barley agar and sub culture onto sheep blood agar for isolating pure colonies. Incubate at 37oC for 18- 24 h aerobically.

Observation of haemolytic pattern:

- *Enterococcus faecalis* shows gamma haemolysis (no haemolysis)
- *Enterococcusfaecium* shows alpha haemolysis(partial/greenish haemolysis).

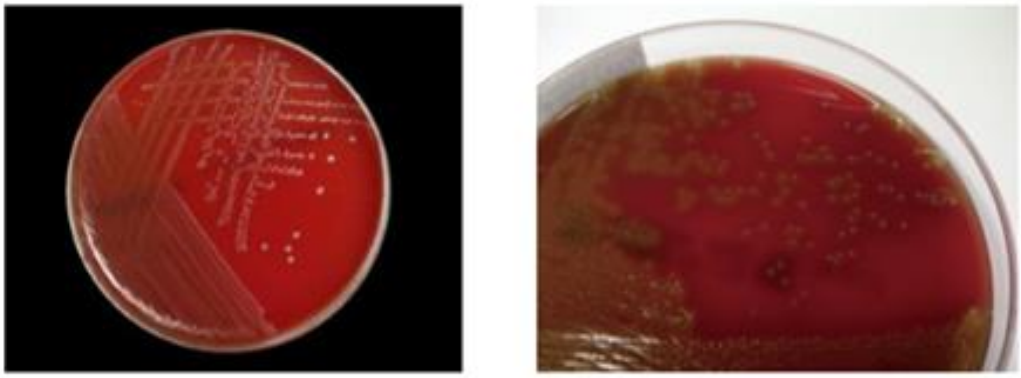


Figure 2: *Enterococcus faecalis* on blood agar showing no haemolysis, *Enterococcus faecium* showing alpha haemolysis.

8.4.Confirmation

8.4.1 Gram staining

- Examined the freshly grown colony from blood agar plate and perform gram staining.
- *Enterococci* cultures appears as gram positive cocci.

8.4.2 Oxidase test

- Using a sterile wooden stick pick well isolated colony from blood agar plate and streak it on the moistened oxidase disc.
- Observe the colour change within 10 seconds.
- Violet / blue colour indicates a positive reaction
- *Enterococci* cultures gives negative results with no colour change

8.4.3 Catalase activity

- Using a sterile wooden stick or loop place a well isolated colony onto a clean glass slide.
- Add a drop of catalase reagent (3% hydrogen peroxide) and observe for bubble formation
- *Enterococci* cultures does not produce bubbles giving negative results.

Note: Do not pick agar while performing the test. Gives false positive result.

8.4.4 PYR broth test

- Inoculate PYR broth with 3 to 5 colonies from 18-24 h pure cultures.
- Incubate the tube aerobically at 35°C to 37°C for 4 hours.
- Add 2-3 drops of PYR reagent and observe for colour change within 1-2 minutes.
- *Enterococci* culture produces red/pink colour indicating positive result.
- No colour change or blue colour indicates negative result.

8.4.5 Bile esculin test

- Inoculate/streak the surface of bile esculin medium with one to two colonies. Incubate the tube at 35°C to 37°C for 24 hours.
- Observe the colour change
- *Enterococci* produce black colour medium

Table: Interpretation of primary and biochemical tests

Characteristic	***Enterococcus faecalis***	***Enterococcus faecium***
Slanetz-Barley	Deep red colonies with golden reflection	Pink/white colonies
Blood agar	Gamma / no haemolysis	Alpha haemolysis
Morphology	Gram positive cocci	Gram positive cocci
Oxidase activity	-	-
Catalase activity	-	-
PYR broth test	Red colour	Red colour
Bile esculin test	Black medium	Black medium
Key: + positive; - negative;		

9. Isolate Storage

At reference laboratories (NVL), prepare 20% glycerol stock and store at -80°C for future references. At regional laboratories, prepare NA slant and transfer the isolates to NVL as per SOP for isolate storage and transportation.

10. Waste disposal

As per the SOP for laboratory waste management

11. References

1. ISO 7899-2: 2000 International Standard; Water quality detection and enumeration of intestinal *Enterococci*
2. Domig K. J et.al 2003, methods used for the isolation, enumeration, characterization and identification of *Enterococci* spp. Media for isolation and enumeration. International Journal of Food Microbiology 88 (2003) 147-164
3. Hands-on Training “Standardized and Harmonized surveillance methods for antimicrobial resistance in food animals in South Asia” Isolation of target bacteria for AMR surveillance, Chulalongkorn University

Composition and preparation of culture media and reagents

1. Slanetz-Bartley medium

Basal medium

Tryptone	20.0g
Yeast extract	5.0g
Glucose	2.0g
Dipotassium hydrogen phosphate	4.0g
Sodium azide	0.4g
Agar	8g-18g
D/H2O	1000 ml

2. TTC solution

2,3,5-Triphenyl-tetrazolium chloride	1g
D/H2O	100ml

Preparation of TTC solution

- Dissolve the indicator in water by stirring
- Sterilize by filtration using pore size 0.2πm
- Store the solution away from light
- Note: discard if pink tinge develops

Preparation of Slanetz-Bartleymedium (complete medium)

- Procedure
- Dissolve the ingredients (basal medium) in 1000ml of boiling water
- Once the dissolution is complete, heat for an additional 5 mins.
- Cool to 50°C - 60°C
- Note: do not autoclave the media
- Aseptically add 10 ml of TTC solution into the basal medium
- Adjust the PH at 7.2± 0.1 at 25°C
- Pour 20 ml of medium at 90mm sterile petri dish and allow to cool
- Store the media at dark for upto two weeks at 5°C ±3°C.

3. Bile Aesculin agar /slant

Composition

Peptone	14.0g

Bile salt	15.0g
Ferric chloride	0.5g
Aesculin	1.0g
Agar	14.0g

Preparation: As per the manufacturer instruction

4. Blood agar

COLUMBIA BLOOD AGAR BASE

Composition

Special peptone	23.0g
Starch	1.0g
Sodium chloride	5.0g
Agar	10.0g

Preparation: As per the manufacturer instruction

5. Brain Heart Infusion(BHI) with 6.5% NACL

Composition

Casein peptone	14.5 g
Meet peptone	7.0g
Sodium chloride	65.0g
Brain heart infusion	6.0g
Disodium phosphate	2.5g
Dextrose	2.0g

Preparation: As per manufacturer instruction

Annexure 07: SOP for Isolation and Identification of Escherichia coli

WARNING--In order to safeguard the health of laboratory personnel, it is essential that the whole of this method be carried out only by skilled personnel using good laboratory practices and preferably working in a containment facility. Care must be taken in the disposal of all infectious materials.

1. Introduction

The typical *Escherichia coli* is Gram-negative rod, motile, fermenting lactose with the production of gas and usually produces smooth, non-mucoid colonies on solid media.

Escherichia coli is one of the predominant facultative anaerobes in the human and animal gastrointestinal tracts. However, there are several pathogenic Escherichia coli strains that cause disease and variety of infections in both humans and animals through consumption of contaminated foods sources, water, etc.

2. Scope

This document specifies a horizontal method for the detection of *Escherichia coli* by enrichment and direct plating. It is application to

- Samples from primary production stage such as animal feces, dust and swabs.
- Products intended for human consumption
- Products intended for animal feeding
- Environmental samples in the area of food and feed production, handling

With this horizontal method most of *Escherichia coli* are to be detected.

3. Objective

To isolate and identify *Escherichia coli* from laboratory samples by morphological and biochemical tests.

4. Principles

Depending on the type of sample two different detection procedures can be used.

A. Detection procedure A: detection of *Escherichia coli* by direct plating in selective agar mediums, in samples with high numbers of *Escherichia coli*, (e.g., faeces and poultry caecal contents)

B. Detection procedure B: detection of *Escherichia coli* by enrichment in selective liquid medium for the resuscitation of the injured or sub-lethally damaged *Escherichia coli*.

The detection of *Escherichia coli* requires two or three successive steps depending on the sample type.

4.1. Enrichment in liquid medium; the test portion is added to the liquid enrichment medium (Buffered peptone water).

4.2. Plating out and Identification; from the enrichment medium two selective solid media are inoculated (MacConkey and Eosin Methylene Blue agar)

4.3. Confirmation by biochemical test; suspect colonies are examined for morphology, motility and biochemical tests.

5. Equipment and Consumables

a. Incubator (37±2°C)
b. Refrigerator
c. Bunsen burner
d. Autoclave
e. Microwave oven
f. Weighing Balance
g. Personal protective equipment.
h. Bio-safety cabinet type II
i. Conical flask/Media preparation bottle cap (250ml,500ml &1000ml)
j. Measuring Cylinder (50ml,100ml,500ml &1000ml)
k. Autoclave indicator strip
l. Petri dish (90mm size)
m. Test tube with cap (15ml)
n. Pipette (100-1000µl)
o. Graduated sterile pipette (10ml) or Automated pipette (1-10ml)
p. Rubber bulb /Pipette gun

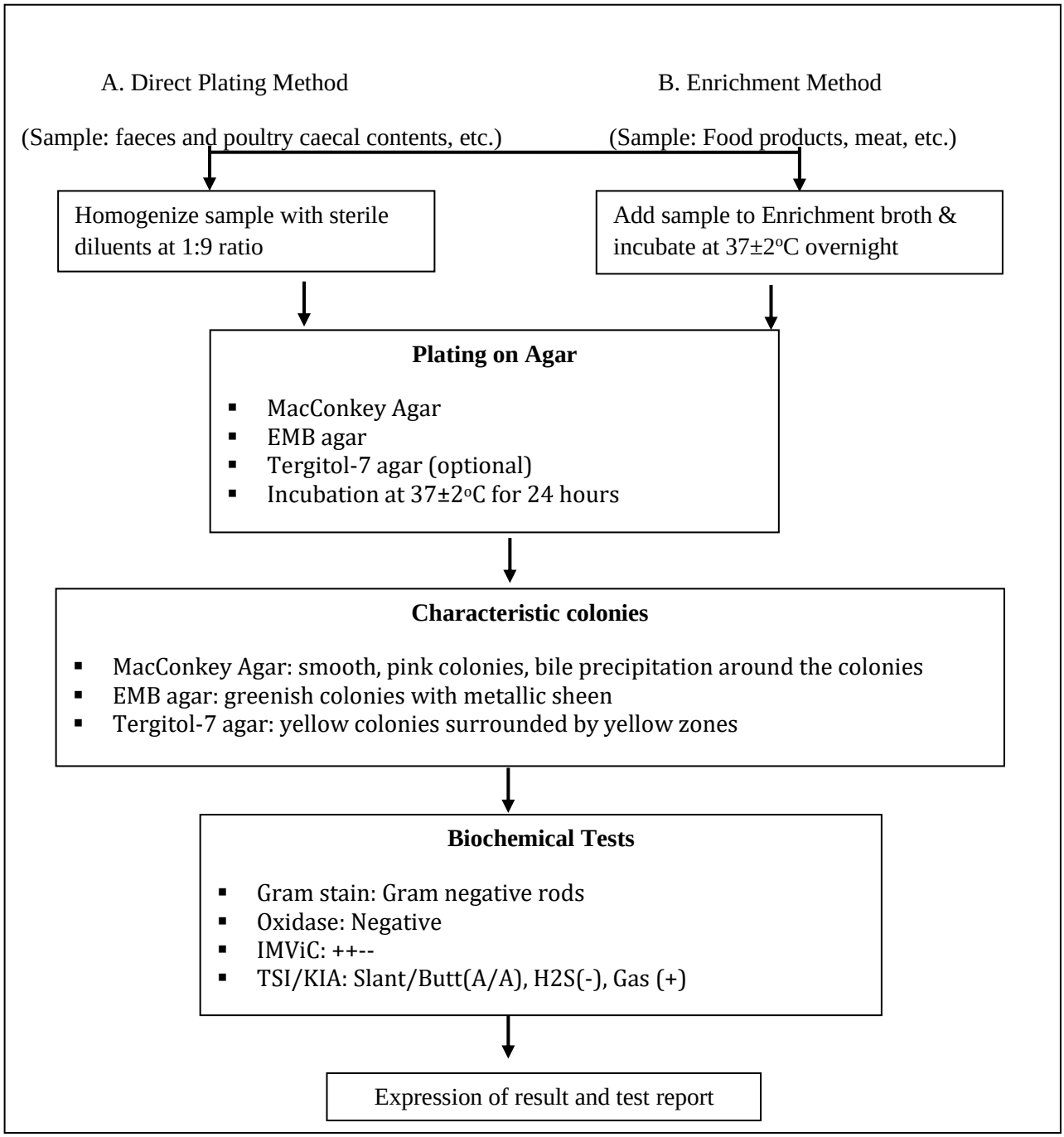
6. Culture media and Reagents

a. Buffered Peptone water
b. MacConkey agar
c. Eosin Methylene Blue Lactose (EMB) agar
d. Tergitol-7 agar
e. Nutrient agar
f. Triple Sugar Iron (TSI) agar or Kligler Iron Agar (KIA)
g. Tryptone broth
h. Methyl Red and Voges-Proskauer (MR-VP) broth
i. Simmon's citrate agar
j. Kovacs reagents
k. Methyl red indicator
l. Barritt's reagent A & B
m. Oxidase reagent or disc
n. Gram stain kits

7. Sampling

Sampling is not part of the method specified in this document. Prepare the test sample in accordance with the sampling protocol dealing with the product concerned.

8. Procedure



8.1 Sample preparation and Enrichment

- Caecum and caecal contents: Pulverize the caecum or caecal contents in each bag containing sterile buffered peptone water (BPW) at 1:9 ratio and mix well, avoid spillage.
- Add cloacal swab to 10ml of BPW in a sterile tube with a lid.
- For whole chicken carcass sample, take 10ml from te 400ml in the chicken bag and incubate overnight
- Boot swabs: add 225ml sterile BPW and mix well, avoid spillage.
- Foods of animal origin: Homogenize the sample with sterile BPW at 1:9 ratio, avoid spillage.

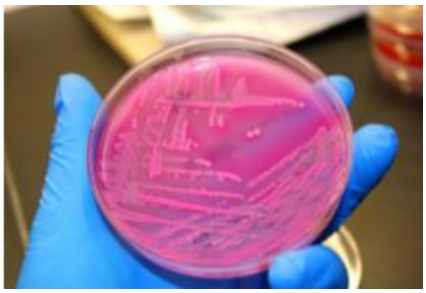
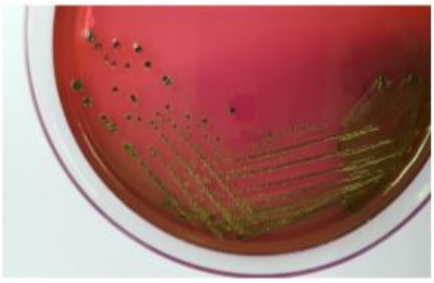
- Incubate the samples at 37±2^{o}C overnight.

8.2 Plating out and Identification

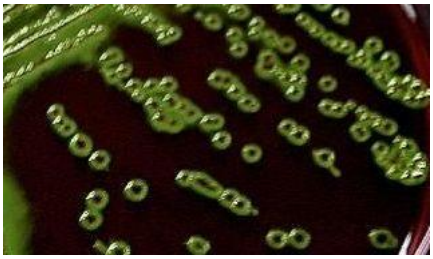
- After incubation, streak out a loopful on to each of the solid media MacConkey agar, EMB agar, and if available on Tergitol-7 agar.
- Incubate the plates at 37±2 ^{o}C overnight.
- Pick out and mark as many suspected colonies from the solid media as possible, but not less than 5, to investigate.

The suspected colonies are;

- MacConkey agar: smooth, pink colonies, bright pink halo due to lactose fermentation and bile precipitation around the colonies.

- EMB agar: greenish colonies with metallic sheen and dark purple center.



- Tergitol-7 agar: yellow colonies surrounded by yellow zones

8.3 Isolate purification

Streak the suspected colonies on nutrient agar plates and incubate at 37 ^{o}C for 24±2hours for the biochemical identification tests.

8.4 Confirmation Tests

8.4.1 Gram's Stain

- On a clean grease-free slide, prepare very light and thin smear covering small area.
- Fix the smear by passing to and fro over a flame and cool the slide.
- Cover the smear with the Gram's Crystal violet stain for 1 minute.
- Pour off the stain and wash with water and then cover with Gram's Iodine for 1 minute.
- Wash off the stain with Gram's Decolorizer until the dye ceases to stream out, wash in running water and apply Gram's Safranin for 1 minute.
- Wash with water and dry for examination under oil emersion.
- Escherichia coli are observed as Gram-negative rods.

8.4.2 Oxidase Test

- Use a loop to aseptically transfer a large mass of pure culture of bacteria to the disc or add 2-3 drops of the oxidase reagent on the organism and observe the reaction.
- A positive reaction is indicated by colour change to violet or purple within 10 seconds.
- A negative reaction is indicated by no coloration or light pink.
- Escherichia coli give negative oxidase reaction.

8.4.3 TSI or KIA agar

- Inoculate TSI or KIA medium by stabbing the strain into the butt and streaking the slop. Incubate at 37±2 oC for 24±2hours.
- Interpret the changes in the medium as follows:
  a) Butt:
     - Yellow: glucose positive (glucose fermentation);
     - Red or unchanged: glucose negative (no fermentation of glucose);
     - Black: formation of hydrogen sulphide;
     - Bubbles or cracks: gas formation from glucose;
  b) Slant surface:
     - Yellow: lactose and/or sucrose positive (lactose and/ sucrose fermentation);
     - Red or unchanged: lactose and sucrose negative (no fermentation of lactose or sucrose).
     - *Most Escherichia coli gives lactose fermenting reactions Acidic/Acidic (Slant/Butt) with gas (bubbles or cracks) formation and no formation of H_2S (blackening).*

8.4.4 Indole

- Inoculate a tube containing 5ml of the Tryptone/Tryptophan medium with the suspected colony. Incubate at 37oC for 24±2hours.
- After incubation, add 1 ml of the Kovacs reagents.
- The formation of a red ring (surface layer) indicates a positive reaction.
- A yellow-brown ring (surface layer) indicates a negative reaction.
- Escherichia coli give positive reaction.

8.4.5 Methyl Red Voges-Proskauer (MR-VP) Test

- Inoculate the medium and incubate at 37oC for 24±2hours.
- Divide the incubated medium in two tubes.

  a) **MR Test:** To one portion add 2 drops of methyl red solution.
     - A positive reaction is indicated by red colour.
     - Negative reaction is indicated by yellow colour.
     - Escherichia coli give positive reaction.

b) **VP Test:**

- To the second portion add 0.6 ml or 5-6 drops of Barritt's reagent A.
- Shake and add 0.2ml or 2 drops of Barritt's reagent B.
- Shake and slop the tube and observe for up to 30 minutes for the reaction.

- A positive reaction is indicated by pink colour.
- Negative reaction is indicated by yellow colour.
- *Escherichia coli give negative reaction.*

8.4.6 Citrate Test

- Inoculate the medium slant using a straight wire and incubate at 37±2oC for up to 24 hours for the growth of the organism.
- A positive reaction is indicated by colour change of the medium from green (neutral) to blue (alkaline) due to the citrate utilization by the organism.
- Negative reaction is indicated by no colour change in the medium.
- Escherichia coli give negative reaction

Table: Biochemical Test

Tests	TSI/KIA			Oxidase	Indole	MR	VP	Citrate
	Slant/Butt	Gas	H_2S					
E. coli	A/A	+	-	-	+	+	-	-

9. Isolate Storage

At reference laboratories (NVL), prepare 20% glycerol stock and store at -80°C for future references. At regional laboratories, prepare NA slant and transfer the isolates to NVL as per SOP for isolate storage and transportation.

10. Waste Disposal

As per the SOP for laboratory waste management

11. References

1. Indian Standard Methods for Detection of Bacteria Responsible for Food Poisoning; Part-I: Isolation, Identification and Enumeration of Escherichia coli (First Revision) – IS: 5887 (Part I) -1976
2. Protocol for active AMR surveillance in commercial broiler and layer chicken populations for the Flaming Fund Country Grants Programme. Version 1.1. Towards a One Health AMR Surveillance System. The Fleming Fund. September 2019.
3. Standard Operating Procedure for Detection and Identification of Escherichia coli in Fecal Specimen; Version: 1.00, Enteric and Invasive Disease Laboratory, RCDC
4. Hands-on Training "Standardized and Harmonized Surveillance Methods for Antimicrobial Resistance in Food Animals in South Asia"- Isolation of Target bacteria for AMR surveillance- Chulalongkorn University.

Media and Reagent Composition and Preparation

1. **Buffered peptone water**

Peptone[a]	10g
Sodium chloride	5.0g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)[b]	9.0g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5g

[a]For example, enzymatic digest of casein

[b] If disodium hydrogen phosphate with a different water content is used, amend the mass of the ingredient accordingly. For example, in case of anhydrous disodium hydrogen phosphate, use 3.57g

Preparation: As per the manufacturer's instruction

2. **MacConkey Agar**

Peptone	17.0g
Proteose peptone	3.0g
Lactose	10.0g
Bile salts	1.5g
Sodium chloride	5.0g
Neutral red	0.03g
Crystal violet	0.001g
Agar	13.5g

Preparation: As per the manufacturer's instruction

3. **EMB agar**

Peptic digest of animal tissue (Peptone)	10.0g
Dipotassium phosphate	2.0g
Lactose	5.0g
Sucrose	5.0g
Eosin-Y	0.4g
Methylene blue	0.065g
Agar	13.5g

Preparation: As per the manufacturer's instruction

4. **Tergitol-7 agar**

Preparation: As per the manufacturer's instruction

5. **Nutrient agar**

Meat extract	3.0g
Peptone	5.0g
Agar	9g to 18g[a]

[a] Depending on the gel strength of the agar

Preparation: As per the manufacturer's instruction

6. **TSI agar**

Beef/meat extract	3.0g
Peptone	20.0g
Yeast extract	3.0g
Lactose	10.0g
Sucrose	10.0g

Glucose	1.0g
Iron(III) citrate	0.3g
Sodium thiosulfate	0.3g
Phenol red	0.024g
Agar	12.0g

Preparation: As per the manufacturer's instruction

7. Tryptone/Tryptophan medium

Tryptone	10.0g
Sodium chloride	5.0g
DL-Tryptophan	1.0g

Preparation: As per manufacturer's instruction

8. Methyl Red and Voges-Proskauer (MR-VP) broth

Buffered peptone	7.0g
Dextrose	5.0g
Dipotassium phosphate	5.0g

Preparation: As per the manufacturer's instruction

9. Simmon's citrate agar

Ammonium dihydrogen phosphate	1.0g
Dipotassium hydrogen phosphate	1.0g
Sodium chloride	5.0g
Sodium citrate	2.0g
Magnesium sulfate	0.2g
Bromothymol blue	0.08g
Agar	13-15g

Preparation: As per the manufacturer's instruction

10. **Kovacs reagents (indole reagent**)

4-Dimethylaminobenzaldehyde	5.g
2-Methylbutan-1-ol or pentan-1-ol	75.0ml
Hydrochloric acid (p_{20} 1.18g/ml to 1.19g/ml)	25.0ml

Preparation:

- Dissolve the 4-Dimethylaminobenzaldehyde in the alcohol, by warming if necessary in a water bath maintained between 44-47^{o}C.
- Cool to room temperature and add the hydrochloric acid.
- Protect from light in a brown glass bottle and store at 3 ^{o}C±2^{o}C.
- The reagent shall be light yellow to light brown and free of precipitate.

11. Methyl red indicator

Methyl red	0.2g
Ethyl alcohol	60.0ml
Distilled water	40.0ml

12. Barritt's reagent A & B

Barritt's reagent A

a-naptholin	6.0g
95% ethyl alcohol	100ml

Barritt's reagent B

Potassium hydroxide	16.0g

Water	100ml

13. Oxidase reagent

N,N-Dimethyl p-phenylenediaminehydrochloride	0.15g
Distilled water	10.0ml

14. Gram stain kits

1. Gram's Crystal violet

Crystal violet	2.0g
Ethyl alcohol 95%	20.0ml

2. Gram's Iodine

Iodine	1.0ml
Potassium iodine	2.0ml
Distilled water	300.0ml

3. Safranin (0.5%w/v)

Safranin O	0.5g
Ethyl alcohol 95%	100ml

4. Gram's Decolourizer

Ethyl alcohol 95%	50.0ml
Acetone	50.0ml

Annexure 08: SOP for Isolation and Identification of Salmonella spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella* species, especially *Salmonella* Typhi and *Salmonella* Paratyphi, are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that precaution is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice.

Disclaimer

This SOP is an adoption with national modifications and has been reproduced from ISO 6579-1:2017, Microbiology of food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp.

1. Introduction

Salmonella has been recognized as an important zoonotic pathogen of economic significance in animals and humans. Salmonellosis is common and food animals; food of animal origin is often implicated as source of *Salmonella* as the leading causes of bacterial foodborne illness. Despite the conserved genetic background, molecular analysis has indicated successful evolution of the *Salmonella* genome in response to the environment, particularly to the selective pressure from antimicrobial agents. It is, therefore, important to monitor the prevalence and trends of antimicrobial resistance in foodborne bacterial strains in the food animals and food of animal origin that can eventually pass to the consumers along the farm-to-fork chain resulting in significant public health impact.

2. Scope

This document specifies a horizontal method for the detection of *Salmonella* spp. It is applicable to the following:

- Products intended for human consumption and the feeding of animals;
- Environmental samples in the area of food production and food handling;
- Samples from the primary production stage such as animal faeces, dust and swabs

With this horizontal method, most of the *Salmonella* serovars are intended to be detected. It also includes procedures for detection of *Salmonella* Typhi and *Salmonella* Paratyphi.

3. Objective

To isolate and identify *Salmonella* from laboratory samples using a combination of biochemical and serological tests

4. Principle

4.1. General

The detection of *Salmonella* requires four successive stages as specified in Annex A.

4.2. Pre-enrichment in non-selective liquid medium

Buffered peptone water at ambient temperature is inoculated with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h. For large quantities (e.g. 1 litre or more), it is recommended to pre-warm the BPW to 37 °C ± 1 °C before inoculation with the test

portion. Pre-enrichment is used to permit the detection of low numbers of Salmonella or injured Salmonella.

4.3. Enrichment in selective liquid media

Rappaport-Vassiliadis medium with soya (RVS broth) or Modified Semi-solid Rappaport-Vassiliadis (MRSV) agar and Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn broth) are inoculated with the culture obtained in 4.2

The RVS broth or the MSRV agar is incubated at 41.5 °C ± 1 °C in a waterbath for 24 h and the MKTTn broth at 37 °C ± 1 °C for 24 h.

Note: MSRV agar is intended for the detection of motile *Salmonella* and is not appropriate for the detection of non-motile *Salmonella* strains.

4.4. Plating out and identification

From the cultures obtained in 4.3, two selective solid media are inoculated:

— Xylose lysine deoxycholate agar (XLD agar);
— Brilliant Green Agar as the second selective media

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h. BGA agar is incubated according to the manufacturer's instructions.

Note: *Second plating-out medium could be used are - Bismuth Sulfite Agar (BSA), Deoxycholate Citrate agar (DCA), Xylose Lysine Tergitol 4 Agar (XLT_4), etc*

4.5. Confirmation

Colonies of presumptive *Salmonella* are sub-cultured and their identity is confirmed by means of appropriate biochemical and serological tests.

5. Equipment and consumables

a. Oven
b. Autoclave
c. Drying cabinet - capable of operating between 25°C and 50°C
d. Incubator - capable of operating in the range of 37°C and at 41.5°C ±1°C
e. Water bath - capable of operating at 37°C ±1°C; 41.5°C ±1°C and 47°C to 50°C
f. Refrigerator
g. Freezer – (-20°C ±5°C)
h. Sterile loops
i. pH-meter
j. Sterile tubes, bottles or flasks
k. Sterile graduated pipettes or automatic pipettes - 25ml, 10ml, 1ml and 0.1ml
l. Sterile petri dishes - 90mm and 140mm

Note: *It is recommended to use water bath containing an antibacterial agent because of the low infective dose of Salmonella*

6. Culture media, reagents and antisera

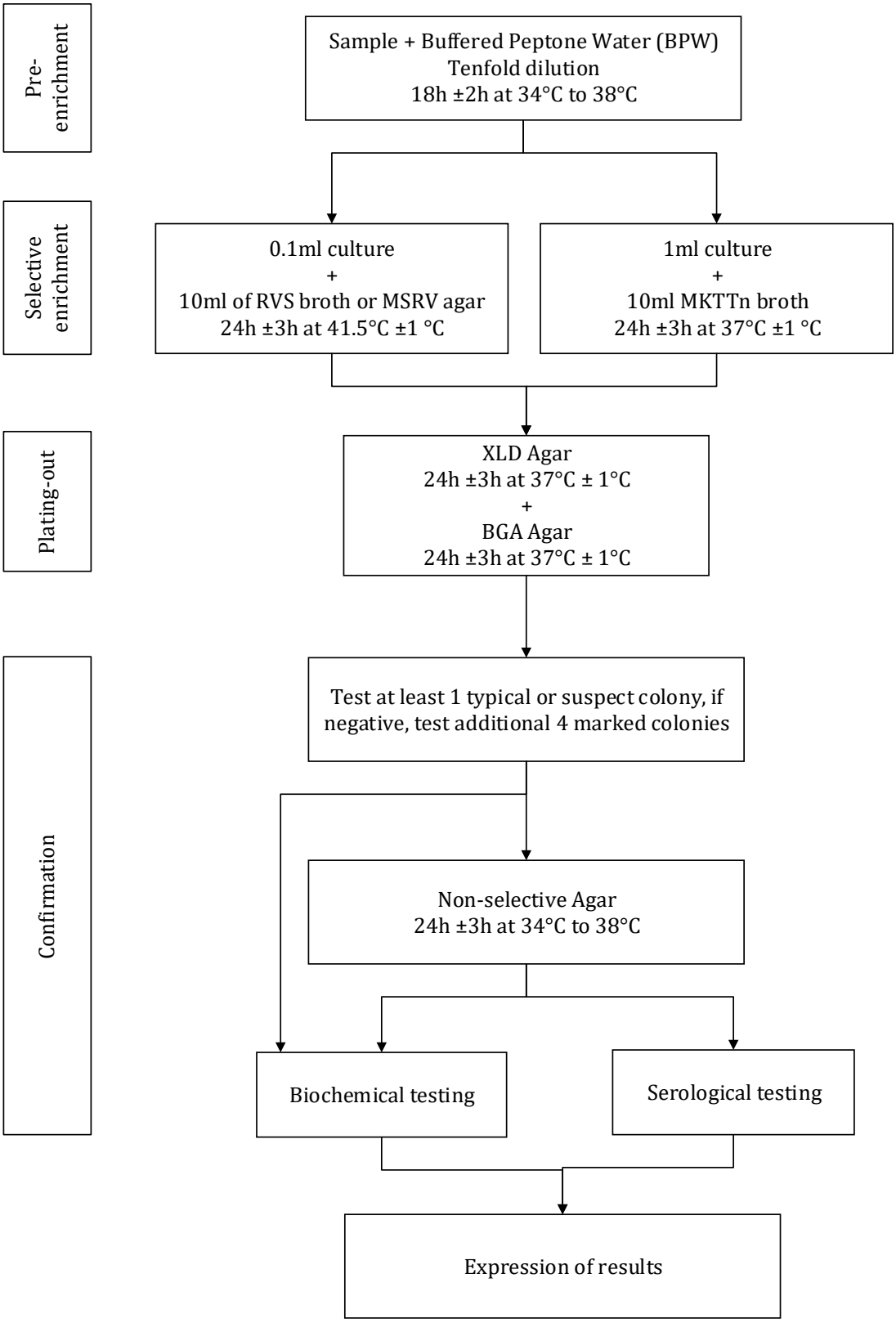
Composition of culture media and reagents, their preparation and quality assurance are described in Annex B

7. Sampling and preparation of test sample

Sampling is not part of the method specified in this document. Prepare the test sample in accordance with the sampling protocol dealing with the product concerned.

8. Procedure

Diagram of the *Salmonella* detection procedure



8.1. Test portion and initial suspension

For preparation of the initial suspension, use pre-enrichment medium specified in B.2 (buffered peptone water). Pre-warm the BPW to room temperature before use. Test portion

is added to a quantity of BPW to yield a tenfold dilution. For example, 25g test portion is mixed with 225ml of BPW. For large quantities (e.g. 1 l or more), it is recommended to pre-warm the BPW to 34°C to 38°C (37°C) before mixing it with the test portion

A. Boot socks

- Pooled boot socks are processed as a single sample.
- Add 225ml of BPW and mix the sample gently.
- Incubate the sample bag at 34°C to 38 °C (37°C) for 18h ±2h

B. Caecal samples

- Solid caecal contents/whole caeca – weigh appropriate amount and mix in BPW to maintain sample to BPW ratio of 1:9
- Caecal swabs/cloacal swabs - Mix individual caecal swab in 9ml of BPW
- Pooled caecal contents - 25g of pooled caecal content is mixed with 225 ml of BPW (pooled samples should not exceed 10 caecal samples)
- The sample is incubated at 34°C to 38 °C (37°C) for 18h ±2h

C. Broiler carcass (Neck skin/breast skin)

- 25g of carcass is mixed with 225ml of BPW, mix the sample gently
- The sample is incubated at 34°C to 38 °C (37°C) for 18h ±2h

D. Faecal samples

- Solid feces – weigh appropriate amount and mix in BPW to maintain sample to BPW ratio of 1:9
- Pooled fecal material - 25g of pooled fecal material is mixed with 225 ml of BPW
- The sample is incubated at 34°C to 38 °C (37°C) for 18h ±2h

E. Food, animal feed and environmental samples

- 25 gm of sample in 225ml of BPW
- The sample is incubated at 34°C to 38 °C (37°C) for 18h ±2h

Note: *This document is validated for test portions of 25g. A smaller test portions may be used without the need for validation provided that the ratio of sample to BPW is maintained at tenfold. Do not pool samples weighing more than 25g test portion*

8.2. Non-selective pre-enrichment

Incubate the initial suspension (8.1) between 37°C for 18h ±2h. It is permissible to store the pre-enrichment sample after incubation at 5°C for a maximum of 72h.

8.3. Selective enrichment

8.3.1. General

Allow the selective enrichment media, RVS broth or MSRV agar and MKTTn broth to equilibrate at room temperature if they were stored at a lower temperature. Minimize the particulate material from pre-enrichment into the selective enrichment media.

Note: *MSRV agar is intended for the detection of motile Salmonella strains and is not appropriate for the detection of non-motile Salmonella strains.*

8.3.2. Procedures for food, animal feed and environmental samples

Transfer 0.1ml of the culture obtained in 8.2 to a tube containing 10ml of the RVS broth or to the surface of MSRV agar plate. Inoculate the MSRV agar with one to three equally spaced spots on the surface of the medium

- Transfer 1ml of the culture obtained in 8.2 to a tube containing 10ml of MKTTn broth
- Incubate the inoculated RVS broth at 41.5 °C for 24h ±3h
- Incubate the inoculated MSRV agar plates at 41.5 °C for 24h ±3h. Do not invert the plates
- Incubate the inoculated MKTTn broth at 37°C for 24h ±3h
- Suspect MSRV plates will show a grey-white, turbid zone extending out from the inoculated drop.

8.4. Plating out

8.4.1. General

From the selective enrichment cultures (8.3), inoculate two selective isolation agar media. The first isolation medium is Xylose Lysine Deoxycholate Agar (XLD). The second isolation medium is Brilliant Green Agar (BGA).

Allow the XLD agar plates and BGA plates to equilibrate at room temperature if they were stored at lower temperature. If necessary, dry the surface plates before use.

8.4.2. Procedures for food, animal feed and environmental samples

From the culture obtained in the RVS broth (8.3.2), inoculate by means of a 10µl loop the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the BGA.

From positive growth obtained on the MSRV agar (8.3.2), determine the furthest point of opaque growth from the inoculation points and dip a 1µl loop just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV agar are extracted. Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the BGA agar plate.

From the cultures obtained in the MKTTn broth (8.3.2), inoculate by means of 10µl loop the surface of an XLD plate so that well-isolated colonies are obtained. Proceed in the same way with BGA plates.

- Incubate the XLD plates inverted at 37°C for 24h ±3h
- Incubate BGA plates at 37°C ±1°C for 24h ±3h

Typical colonies of *Salmonella* on XLD agar have a black centre and lightly transparent zone of reddish colour due to the colour change on the indicator. *Salmonella* in BGA plates appear pink-white colonies on red colour media

Note: To obtain well-isolated colonies, large size petri dishes with plating-out media (140mm) or two normal size plates (90mm) can be used. *Salmonella* H2S-negative variants growth on XLD agar are pink with dark pink centre. Lactose positive *Salmonella* grown on XLD agar are yellow with or without blackening.

8.5. Confirmation

8.5.1. General

The combination of biochemical and serological test result indicates whether an isolate belongs to the genus *Salmonella*. For serotyping of *Salmonella* strains, full serotyping is needed. For biochemical confirmation, alternative commercial formulations are available. These commercial formulations are allowed provided that the performance for biochemical confirmation of *Salmonella* is verified before use. Verify the biochemical reactions of the media of each biochemical test with well-characterized positive and negative control strains.

Note: *The recognition of colonies of Salmonella is, to a large extent, a matter of experience and their appearance can vary somewhat, not only from serovar to serovar, but also from batch to batch of the selective medium used.*

8.5.2. Selection of colonies for confirmation

Mark suspect colonies on each plate (8.4), select at least one typical or suspect colony for subculture and confirmation. If this is negative, select up to four more suspect colonies ensuring that these colonies are sub-cultured from different selective enrichment/isolation medium combination showing suspect growth.

Streak the selected colonies onto the surface of a pre-dried non-selective agar medium in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 37°C for 24h ±3h

Alternatively, if well-isolated colonies are available on the selective plating media (8.4), the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.

Note: Use pure cultures for biochemical and serological confirmation.

8.5.3. Biochemical testing

8.5.3.1. General

Inoculate the biochemical confirmation media with each of the cultures obtained from the colonies selected in 8.4 or 8.5.2. For confirmation of *Salmonella* spp., at least the tests specified 8.5.3.2 to 8.5.3.4 shall be performed. The test specified in 8.5.3.5 and 8.5.3.6 can also be performed when the results of the other confirmation tests do not give a clear identification. The summary of biochemical reactions interpretation is presented in table 1.

8.5.3.2. TSI agar (B.8)

Streak the agar slant surface and stab the butt. Incubate at 37°C for 24h ±3h.
Interpret the changes in the medium as follows:

a. Butt

- Yellow: glucose positive (glucose fermentation)
- Red or unchanged: glucose negative (no fermentation of glucose)
- Black: formation of hydrogen sulphide;
- Bubbles or cracks: gas formation from glucose

b. Slant surface

- Yellow: lactose and/or sucrose positive (lactose and/or sucrose fermentation)
- Red or unchanged: lactose and sucrose negative (no fermentation of lactose or sucrose)
- The majority of the typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and (in about 90% of the cases) formation of hydrogen sulfide (blackening of the agar)
- When lactose-positive *Salmonella* is isolated, the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar test only

8.5.3.3. Urea agar

Streak the agar slant surface. Incubate at 37°C for 24h ±3h

- If the reaction is positive, urea is hydrolysed, liberating ammonia. This changes the colour of phenol red to rose pink and later to deep cerise. The reaction is often apparent after 2h to 4h.
- Typical *Salmonella* cultures do not hydrolyse urea so that the colour of the urea agar will remain unchanged

8.5.3.4. L-lysine decarboxylation medium (LDC)

- Inoculate just below the surface of the liquid medium.
- Incubate at 37°C for 24h ±3h
- Turbidity and a purple colour after incubation indicate a positive reaction.
- A yellow colour indicates a negative reaction.
- The majority of the typical *Salmonella* cultures show a positive reaction in LDC.

8.5.3.5. Detection of β-galactosidase (Optional)

The β-galactosidase test can be used to distinguish *Salmonella* enterica subspecies arizonae and diarizonae and other members of the Enterobacteriace (all give a positive reaction) from other subspecies of *Salmonella* enterica (generally gives negative reaction)

- Suspend a loopful of suspected colony in a tube containing 0.25ml of saline solution

- Add one drop of toluene and shake the tube. Place the tube in a water bath set at 37°C and leave for 5 minutes. Add 0.25ml of the reagent for detection of β-galactosidase and mix
- Replace the tube in the water bath at 37°C and leave for 24h.
- A yellow colour indicates a positive reaction. The reaction often apparent after 20 min.
- If prepared paper discs are used for detection of β-galactosidase, follow the manufacturer's instructions.

8.5.3.6. Medium for indole reaction (Optional)

The indole test can be used when there is a need to differentiate *Salmonella* (indole negative) from E. coli and Citrobacter (both indole positive) as these oganisms can give typical reactions on some of the *Salmonella* isolation media.

- Inoculate a tube containing 5ml of the tryptone/tryptophan medium with the suspected colony
- Incubate at 37°C for 24h ±3h. After incubation, add 1ml of the Kovacs reagent
- The formation of a red ring (surface layer) indicates a positive reaction. A yellow-brown ring (surface layer) indicates negative reaction

Table: Interpretation of biochemical tests

Test (8.5.3.2 – 8.5.3.6)	***Salmonella* strains**													
	S. Typhi		S. Paratyphi A		S. Paratyphi B		S. Paratyphi C		S. Gallinarum biovar gallinarum		S. Gallinarum biovar pullorum		Other strains	
	Reaction	%[+c]	Reaction	%[+c]	Reaction	%[+d]	Reaction	%[+d]	Reaction	%[+c]	Reaction	%[+c]	Reaction	%[+c]
TSI acid from glucose	+	100	+	100	+	100	+	100	+	100	+		+	100
TSI gas from glucose	-[e]	0	+	96.1	+	96.1	+	96.1	-	0	+		+	92
TSI acid from lactose	-	2	-	0	-	0	-	0	-	0	-		-	1b
TSI acid from sucrose	-	0	-	0.6	-	0.6	-	0.6	-	0.6	-	0.6	-	1
TSI H_2S produced	+	97	-	10	+	100	+	100	Vf		Vf		+	92
Urea hydrolysis	-	0	-	0	-	0	-	0	-	0	-	0	-	1
Lysine decarboxylation	+	98	-	0	+	95	+	100	+	95	+	95	+	95
B-galactosidase reaction	-	0	-	0	-		-		-	< 10	-	< 10	-	2b
Production of indole	-	0	-	1.2	-	1.2	-	1.2	-	1.2	-	1.2	-	1

8.5.4. Serological testing

8.5.4.1. General

The pure colonies showing typical biochemical reactions for *Salmonella* are tested for the presence of *Salmonella* O- and H-antigens (and in areas where *Salmonella* Typhi is expected, also for Vi-antigen) by slide agglutination using polyvalent antisera. The pure colonies are cultured on non-selective agar medium and tested for auto-agglutination. Use the antisera according to the manufacturer's instructions

The following tests (8.5.4.2 to 8.5.4.5) are the minimum required for serological testing of *Salmonella* spp.

8.5.4.2. Elimination of auto-agglutinable strains

- Place a drop of saline solution on a clean glass slide. Using loop, disperse part of the colony to be tested in the saline to obtain a homogenous and turbid suspension
- Rock the slide gently for 5 to 60 seconds, observe the suspension against dark background. If the the bacteria have formed granules, it indicates auto-agglutination and serological confirmation will become complicated.

8.5.4.3. Examination for O-antigens

- Using one non-agglutinating pure colony, proceed according to 8.5.4.2 using one drop of polyvalent anti-O sera in place of saline solution.
- If agglutination occurs, this is considered a positive reaction.

8.5.4.4. Examination for Vi-antigens (Optional)

- Using one non-auto-agglutnating pure colony, proceed according to 8.5.4.2 using one drop of anti-Vi sera in place of saline solution.

8.5.4.5. Examination for H-antigens

- Using one non-auto-agglutinating pure colony, proceed according to 8.5.4.2 using one drop of polyvalent anti-H sera in place of saline solution
- If agglutination occurs, this is considered a positive reaction.

8.5.5. Inerpretation of biochemical and serological reactions

The interpretation of the confirmatory tests carried out on the colonies used is given in table below:

Table: Interpretation of confirmatory tests

Biochemical reactions	***Auto-agglutination***	***Serological reactions***	***Interpretation***
Typical	No	O-and H-antigens positive (and Vi positive if tested)	Strains considered to be *Salmonella*

Typical	No	O-and/or H-antigens negative	Presumptive *Salmonella*
Typical	Yes	Not tested because of auto-agglutination	
No typical reactions	-	-	Not considered to be *Salmonella*

Strains that are confirmed as *Salmonella* spp. can be further typed to serovar level. If required, confirmed strains can be sent to recognized *Salmonella* reference centre for definitive typing (serotyping, phage typing, molecular typing).

9. Isolate Storage

Inoculate the *Salmonella* isolates in 15%- 20% LBB and store at -80°C for future references

10. Waste disposal

As per the SOP for laboratory waste management

11. References

1. ISO 6579-1:2017, Microbiology of food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp.

Composition and preparation of culture media and reagents

1. Buffered peptone water

Composition

Enzymatic digest of casein	10g
Sodium chloride	5g
Disodium hydrogen phosphate dodecahydrate (Na2HPO4·12H2O) 9	9g
Potassium dihydrogen phosphate (KH2PO4)	1.5g
Water	1000ml

Preparation

- Dissolve the components in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization pH is 7.0 ± 0,2 at 25 °C.
- Dispense the medium into flasks of suitable capacity to obtain the portions necessary for the test.
- Sterilize for 15 min in the autoclave set at 121 °C.

2. Rappaport-Vassiliadis medium with soya (RVS broth)

Solution A: Composition

Enzymatic digest of soya	5g
Sodium chloride	8g
Potassium dihydrogen phosphate (KH2PO4)	1.4g
Dipotassium hydrogen phosphate (K2HPO4)	0.2g
Water	1000ml

Solution A: Preparation

- Dissolve the components in the water by heating to about 70 °C if necessary.

- The solution shall be prepared on the day of preparation of the complete RVS medium.

Solution B: Composition

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	400g
Water	1000ml

Solution B: Preparation

- Dissolve the magnesium chloride in the water.
- As this salt is very hygroscopic, it is advisable to dissolve the entire contents of $MgCl_2 \cdot 6H_2O$ from a newly opened container, according to the formula. For instance, 250 g of $MgCl_2 \cdot 6H_2O$ is added to 625 ml of water, giving a solution of total volume of 788 ml and a mass concentration of about 31.7 g per 100 ml of $MgCl_2 \cdot 6H_2O$.
- The solution may be kept in a dark glass bottle with tight stopper at room temperature for at least 2 years.

Solution C: Composition

Malachite green oxalate	0.4g
Water	100ml

Solution C: Preparation

- Dissolve the malachite green oxalate in the water.
- The solution may be kept in a brown glass bottle at room temperature for at least 8 months.

Complete medium: Composition

Solution A	1000ml
Solution B	100ml
Solution C	10ml

Complete medium: Preparation

- Add to 1 000 ml of solution A, 100 ml of solution B and 10 ml of solution C.
- Adjust the pH, if necessary, so that after sterilization pH is 5.2 ± 0.2.
- Before use, dispense into test tubes in 10 ml quantities.
- Sterilize for 15 min in the autoclave set at 115 °C.
- Store the prepared medium at 3°C ± 2°C. Use the medium the day of its preparation.

NOTE: The final medium composition is: enzymatic digest of soya, 4,5 g/l; sodium chloride, 7,2 g/l; potassium dihydrogen phosphate ($KH_2PO_4 + K_2HPO_4$), 1,44 g/l; anhydrous magnesium chloride ($MgCl_2$), 13,4 g/l or magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 28,6 g/l; malachite green oxalate, 0,036 g/l.

3. Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn)

Base medium: Composition

Meat extract	4.3g
Enzymatic digest of casein	8.6g
Sodium chloride (NaCl)	2.6g
Calcium carbonate ($CaCO_3$)	38.7
Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$) 47	47.8g
Ox bile for bacteriological use	4.78g
Brilliant green	9.6g

Water	1000ml

Preparation

- Dissolve the dehydrated basic components or the dehydrated complete medium in the water by boiling for 5 min.
- Adjust the pH, if necessary, so that pH is 8.2 ± 0.2 at 25 °C.
- Thoroughly mix the medium.
- The base medium may be stored for 4 weeks at 3 °C ± 2 °C.

Iodine-iodide solution: Composition

Iodine	20g
Potassium iodide (KI)	25g
Water	100ml

Preparation

- Completely dissolve the potassium iodide in 10 ml of water, then add the iodine and dilute to 100 ml with sterile water. Do not heat.
- Store the prepared solution in the dark at ambient temperature in a tightly closed container.

Novobiocin solution: Composition

Novobiocin sodium salt	0.04g
Water	5ml

Preparation

- Dissolve the novobiocin sodium salt in the water and sterilize by filtration.
- Store for up to 4 weeks at 3 °C ± 2 °C.

Complete medium: Composition

Base medium	1000ml
Iodine-iodide solution	20ml
Novobiocin solution	5ml

Preparation

- Aseptically add 5 ml of the novobiocin solution to 1 000 ml of base medium
- Mix, then add 20 ml of the iodine-iodide solution. Mix well.
- Dispense the medium aseptically into sterile flasks of suitable capacity to obtain the portions necessary for the test.
- The complete medium shall be used the day of its preparation.

4. Xylose lysine deoxycholate agar (XLD agar)

Base medium: Composition

Yeast extract powder	3g
Sodium chloride (NaCl)	5g
Xylose	3.75g
Lactose	7.5g
Sucrose	7.5g
L-Lysine hydrochloride	5g
Sodium thiosulfate	6.8g
Iron (III) ammonium citrate	0.8g
Phenol red	0.08g
Sodium deoxycholate	1g
Agar	9g to 18g[a]
Water	1000ml

[a]: Depending on the gel strength of the agar

Preparation

- Dissolve the dehydrated base components or the dehydrated complete base in the water by heating, with frequent
- agitation, until the medium starts to boil. Avoid overheating.
- Adjust the pH, if necessary, so that after sterilization pH is 7.4 ± 0.2 at 25 °C.
- Pour the base to tubes or flasks of appropriate capacity.
- Heat with frequent agitation until the medium boils and the agar dissolves. Do not overheat.

Preparation of the agar plates

- Transfer immediately to a water bath at 44 °C to 47 °C, agitate and pour into plates. Allow to solidify.
- Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven set between 37 °C and 55 °C until the surface of the agar is dry.
- Store the poured plates for up to 5 days at 3 °C ± 2 °C.

5. Nutrient agar

Composition

Meat extract	**3g**
Peptone	5g
Agar	9g to 18g
Water	1000ml

Preparation

- Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization pH is 7.0 ± 0.2 at 25 °C.
- Transfer the culture medium into tubes or bottles of appropriate capacity.
- Sterilize for 15 min in the autoclave set at 121 °C.

Preparation of nutrient agar plates

- Transfer about 15 ml at 44 °C to 47 °C, agitate and allow to solidify.
- Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven set between 37 °C and 55 °C until the surface of the agar is dry.
- Store the poured plates for up to 5 days at 3 °C ± 2 °C.

6. Triple sugar iron agar (TSI agar)

Composition

Meat extract	**3g**
Yeast extract	3g
Peptone	20g
Sodium chloride (NaCl)	5g
Lactose	10g
Sucrose	10g
Glucose	1g
Iron (III) citrate	0.3g

Sodium thiosulfate	0.3g
Phenol red	0.024g
Agar	9g to 19g
Water	1000ml

Preparation

- Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization pH is 7.4 ± 0.2 at 25 °C.
- Dispense the medium into test tubes or dishes in quantities of 10 ml.
- Sterilize for 15 min in the autoclave set at 121 °C.
- Allow to set in a sloping position to give a butt of depth 2.5 cm to about 5 cm.

7. Urea agar (Christensen)

Base medium: Composition

Peptone	**1g**
Glucose	1g
Sodium chloride (NaCl)	5g
Potassium dihydrogen phosphate (KH2PO4)	2g
Phenol red	0.012g
Agar	9g to 18g
Water	1000ml

Preparation

- Dissolve the components or the dehydrated complete base in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization pH is 6.8 ± 0.2 at 25 °C.
- Sterilize for 15 min in the autoclave set at 121 °C.

Urea solution: Composition

Urea	**400g**
Water	1000ml

Preparation

- Dissolve the urea in the water.
- Sterilize by filtration and check the sterility.

Complete medium: Composition

Base	950ml
Urea solution	50ml

Preparation

- Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to 44 °C to 47 °C.
- Dispense the complete medium into sterile tubes in quantities of 10 ml.
- Allow to set in a sloping position.

8. L-Lysine decarboxylation medium

Composition

L-Lysine monohydrochloride	**5g**
Yeast extract	3g
Glucose	1g
Bromocresol purple	0.015g
Water	1000ml

Preparation

- Dissolve the components in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization pH is 6.8 ± 0.2 at 25 °C.
- Transfer the medium in quantities of 2 ml to 5 ml to narrow culture tubes with screw caps.
- Sterilize for 15 min in the autoclave set at 121 °C.

9. β-Galactosidase reagent

Buffer solution: Composition

Sodium dihydrogen phosphate (NaH2PO4)	**6.9g**
Sodium hydroxide, 10 mol/l solution	3ml
Water	50ml

Preparation

- Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a volumetric flask.
- Adjust the pH to 7.0 ± 0.2 at 25 °C with the sodium hydroxide solution
- Add water to a final volume of 50 ml.

ONPG solution: Composition

o-Nitrophenyl β-D-galactopyranoside (ONPG)	**0.08g**
Water	15ml

Preparation

- Dissolve the ONPG in the water at approximately 50 °C.
- Cool the solution.

Complete reagent: Composition

Buffer solution	**5ml**
ONPG solution	15ml

Preparation

- Add the buffer solution to the ONPG solution.

10. Reagents for Voges-Proskauer (VP) reaction

VP medium: Composition

Peptone	**7g**
Glucose	5g
Dipotassium hydrogen phosphate (K2HPO4)	5g
Water	1000ml

Preparation

- Dissolve the components in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization pH is 6.9 ± 0.2 at 25 °C.

- Transfer the medium to tubes in quantities of 3 ml.
- Sterilize for 15 min in the autoclave set at 121 °C.

Creatine solution (*N*-amidinosarcosine): Composition

Creatine monohydrate	**0.5g**
Water	100ml

Preparation

- Dissolve the creatine monohydrate in the water.

1-Naphthol, ethanolic solution: Composition

1-Naphthol	**6g**
Ethanol, 96% purity	100ml

Preparation

- Dissolve the 1-naphthol in the ethanol.

Potassium hydroxide solution: Composition

Potassium hydroxide	**40g**
Water	100ml

Preparation

- Dissolve the potassium hydroxide in the water.

11. Reagents for indole reaction

Tryptone/tryptophan medium: Composition

Tryptone	**10g**
Sodium chloride (NaCl)	5g
DL-Tryptophan	1g
Water	1000ml

Preparation

- Dissolve the components in the boiling water.
- Adjust the pH, if necessary, so that after sterilization it is 7,5 ± 0,2 at 25 °C.
- Dispense 5 ml of the medium into each of several tubes.
- Sterilize for 15 min in the autoclave set at 121 °C.

12. Kovacs reagent

Composition

4-Dimethylaminobenzaldehyde	**5g**
Hydrochloric acid, ρ = 1,18 g/ml to 1,19 g/ml	25ml
2-Methylbutan-2-ol	75ml

Preparation

- Mix the components.

13. Semi-solid nutrient agar

Composition

Meat extract	**3g**
Peptone	5g
Agar	4g to 9g
Water	1000ml

Preparation

- Dissolve the components in the water, by heating if necessary.
- Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.
- Transfer the medium to flasks of appropriate capacity.
- Sterilize for 15 min in the autoclave at 121 °C.

Preparation of agar plates

- Pour into small sterile Petri dishes, about 15 ml of the freshly prepared medium.
- Do not allow the agar plates to dry.

14. Physiological saline solution

Composition

Sodium chloride (NaCl)	8.5g
Water	1000ml

Preparation

- Dissolve the sodium chloride in the water.
- Adjust the pH, if necessary, so that after sterilization pH is 7.0 ± 0.2 at 25 °C.
- Dispense quantities of the solution into flasks or tubes so that they will contain 90 ml to 100 ml after sterilization.
- Sterilize for 15 min in the autoclave set at 121°C.

Annexure 09: SOP for Kirby-Bauer Disk Diffusion Susceptibility Test

1. Introduction

The discovery of antimicrobials has transformed the practice of medicine in human and animal health. The availability and use of antibiotics also contributed to progress in public health, animal health, food safety and security. However, these immeasurable benefits are under enormous threat from the emerging and increasing antimicrobial resistance (AMR) which has spread worldwide. Multidrug resistance in organisms most commonly result from the overuse and misuse of antimicrobial agents. Due to these, there is an urgent global call to prevent, control and manage AMR.

Surveillance of antimicrobial resistance (AMR) tracks changes in microbial populations, permits the early detection of resistant strains of public health animal health and food safety importance, and supports the prompt notification and investigation of outbreaks. Surveillance findings are needed to inform clinical therapy decisions, to guide policy recommendations, and to assess the impact of resistance containment interventions.

2. Scope

This SOP describes the procedures for conducting Kirby-Bauer disc diffusion test for Antimicrobial Sensitivity Testing for *Salmonella spp, Escherichia coli, Enterococci spp*. and *Campylobacter spp*. in food animals and their products.

3. Objective

To determine the prevalence and trends of antimicrobial resistance bacteria in food animals and their products.

4. Principles

A standardized inoculum of the organism is swabbed onto the surface of a Mueller-Hinton agar plate. Filter paper discs impregnated with standardized concentration of an antimicrobial agents are placed on the agar. After overnight incubation, the diameter of the zone of inhibition around each disk is measured. The size of the zone is inversely proportional to the MIC of the organisms. Using CLSI guideline, a qualitative report of susceptible, intermediate, or resistant can be obtained.

5. Equipment and Consumables

a. Antibiotic disc dispenser or Forceps
b. Ruler to measure inhibition zones
c. Vortex mixer
d. 34˚ to 35˚ C ambient air incubator
e. Turbidity meter for McFarland's turbidity determination.
f. Sterile petri plates
g. Sterile cotton swab
h. Antimicrobial discs

6. Culture media and consumables

a. Mueller-Hinton Agar
b. Normal saline

7. Antibiotic Susceptibility Testing

7.1 Preparation of Inoculum

- Using sterile loop or swab, transfer four or five isolated colonies of similar colony morphology grown overnight on nonselective medium to 5ml sterile normal saline aliquot.
- 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.

7.2 Inoculating Plates

- Bring agar plates to room temperature before use, but avoid prolonged exposure to elevated temperatures.
- 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.
- 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.
- Allow inoculated plate to stand for at least 3 minutes but no longer than 15 minutes before applying disks.

7.3 Application of Antimicrobial discs to agar plate

- After removing unopened containers of disks from freezer or refrigerator, allow them to equilibrate to room temperature (requires at least 1 hr.) prior to opening to minimize condensation. Do not use disks beyond expiration date
- Apply disks to agar surface by using antibiotic disc dispenser or sterile forceps.
- Apply gentle pressure with sterile forceps to ensure complete contact of disk with agar.
- Do not place disks closer to each other than 24mm from center to center.
- Place no more than 9 disks on a 150-mm plate.
- Place no more than 5 disks on a 90-mm plate.
- Do not relocate a disk once it has made contact with agar surface, because antimicrobial diffusion begins instantly.

7.4 Incubation

- Incubate plates within 15 minutes of disk application.
- Invert plates and stack them no more than 5 high.
- Incubate for 16 to 18 hours at 35±2˚C in an ambient air incubator.
- In case of Campylobacter spp. incubate in an anaerobic condition.

7.5 Reading plates

- Each plate should be examined after overnight incubation (16-18 hours), for confluent growth and circular zones of inhibition.

– The measuring device should be held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background.

The diameters of the zones of complete inhibition, including the diameter of the disc, should be measured to the nearest whole millimeter with Vernier callipers or a ruler.

7.6. Reporting Results

7.6.1 Reference Range:

– Use WHONET /CLSI standard (Latest version) guidelines for interpretation of inhibition zone ranges.

7.6.2 Reporting Format:

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

8. Quality Control

8.1 QC Strains

8.1.2Reference strains used are recommended in CLSI Standard (Latest version).

a. *Escherichia coli* ATCC 25922

8.1.3 Maintenance of QC strains

a. Maintain permanent stock cultures at –20˚C or –70˚C in tryptic soy broth with 15-20% glycerol.
b. Maintain working stock cultures for up to 1 week. Subculture each week for no more than 3 successive weeks.

8.2 Frequency of QC Testing

– Perform QC daily until acceptable results from 20 (or 30) consecutive days of testing have been obtained.
– Proficiency in performing QC tests is confirmed if for each drug, no more than 1 out of 20 or 3 of 30 results are outside the accuracy limits.
– After validation of proficiency, frequency of QC testing can be reduced from daily to weekly.
– Results are reviewed for acceptability and recorded on the Quality Control log sheets before reporting results.

9. Antibiotic Panels

Table: list of antimicrobials for specific organisms

Antimicrobial class	***E.coli***	***Salmonella* spp.**	***Campylobacter* spp.**	***Enterococcus* spp.**
Amioglycoside	Gentamicin	Gentamicin	Gentamicin Streptomycin	Gentamicin Streptomycin
Amphenicol	Choramphenicol	Choramphenicol		Chloramphenicol
Carbapenem	Meropenem and Ertapenem	Meropenem and Ertapenem		

3rd generation cephalosporin's	Ceftriaxone/ cefotaxime	Ceftriaxone/ cefotacime		
4th Generation cephalosporin's	Cefepime			
Quinolones	Ciprofloxacin Nalidixic acid	Ciprofloxacin Perfloxacin/	Ciprofloxacin Nalidixic acid	
Macrolides			Erythromycin	Erythromycin
Glycopeptides				Vancomycin
Glycylcylines				Tigecycline
Oxazolidinones				Linezolid
Penicillins	Ampicillin	Ampicillin	Ampicillin	Ampicillin
Beta-lactam/beta-lactamase inhinitor combination	amoxicillin-clavulanic acid	amoxicillin-clavulanic acid	amoxicillin-clavulanic acid	amoxicillin-clavulanic acid
Polymixins	Colistin**	Colistin**		
Streptogramins				Quinupristin-dalfopristin*
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	Tetracycline
Sulpjonamides/Trimethropim	Co-trimoxazole	Co-trimoxazole		

10. References

1. Harmonized Test Protocol for Isolation, Identification and ABST profiling of Salmonella in Human, Animal and Food products in Bhutan through One Health approach-AGISAR, WHO, Bhutan.
2. CLSI Guidelines-M100S-Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition.
3. CLSI Guidelines-VET08-Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 4th Edition.
4. Standard Operating Procedures, Bacteriology, Antimicrobial Resistance Surveillance and Research Network, Indian Council of Medical Research, 2nd Edition, 2019.

Disk diffusion QC ranges for non-fastidious organism from CLSIM100S .

Antimicrobial Agent	Disk Content	*Escherichia coli* ATCC®[a] 25922	*Staphylococcus aureus* ATCC® 25923	*Pseudomonas aeruginosa* ATCC® 27853	*Escherichia coli* ATCC® 35218[b,c]	*Klebsiella pneumoniae* ATCC® 700603
Amikacin	30 µg	19–26	20–26	18–26	–	–
Amoxicillin-clavulanate	20/10 µg	18–24	28–36	–	17–22	–
Ampicillin	10 µg	15–22	27–35	–	6	–
Ampicillin-sulbactam	10/10 µg	19–24	29–37	–	13–19	–
Azithromycin	15 µg	–	21–26	–	–	–
Azlocillin	75 µg	–	–	24–30	–	–
Aztreonam	30 µg	28–36	–	23–29	**31–38**	**10–16**
Aztreonam-avibactam	**30/20 µg**	**32–38**	**–**	**24–30**	**31–38**	**26–32[d]**
Carbenicillin	100 µg	23–29	–	18–24	–	–
Cefaclor	30 µg	23–27	27–31	–	–	–
Cefamandole	30 µg	26–32	26–34	–	–	–
Cefazolin	30 µg	21–27	29–35	–	–	–
Cefdinir	5 µg	24–28	25–32	–	–	–
Cefditoren	5 µg	22–28	20–28	–	–	–
Cefepime	30 µg	31–37	23–29	24–30	–	–
Cefetamet	10 µg	24–29	–	–	–	–
Cefixime	5 µg	23–27	–	–	–	–
Cefmetazole	30 µg	26–32	25–34	–	–	–
Cefonicid	30 µg	25–29	22–28	–	–	–
Cefoperazone	75 µg	28–34	24–33	23–29	–	–
Cefotaxime	30 µg	29–35	25–31	18–22	–	**17–25**
Cefotetan	30 µg	28–34	17–23	–	–	–
Cefoxitin	30 µg	23–29	23–29	–	–	–
Cefpodoxime	10 µg	23–28	19–25	–	–	**9–16**
Cefprozil	30 µg	21–27	27–33	–	–	–
Ceftaroline	30 µg	26–34	26–35	–	–	–
Ceftaroline-avibactam	30/15 µg	27–34	25–34	17–26	27–35	21–27[d]
Ceftazidime	30 µg	25–32	16–20	22–29	–	**10–18**
Ceftazidime-avibactam	30/20 µg	27–35	16–22	25–31	28–35	21–27[d]
Ceftibuten	30 µg	27–35	–	–	–	–
Ceftizoxime	30 µg	30–36	27–35	12–17	–	–
Ceftobiprole	30 µg	30–36	26–34	24–30	–	–
Ceftolozane-tazobactam	30/10 µg	24–32	10–18	25–31	25–31	17–25
Ceftriaxone	30 µg	29–35	22–28	17–23	–	**16–24**
Cefuroxime	30 µg	20–26	27–35	–	–	–
Cephalothin	30 µg	15–21	29–37	–	–	–
Chloramphenicol	30 µg	21–27	19–26	–	–	–
Cinoxacin	100 µg	26–32	–	–	–	–
Ciprofloxacin	5 µg	30–40	22–30	25–33	–	–
Clarithromycin	15 µg	–	26–32	–	–	–
Clinafloxacin	5 µg	31–40	28–37	27–35	–	–
Clindamycin[e]	2 µg	–	24–30	–	–	–
Colistin	10 µg	11–17	–	11–17	–	–
Delafloxacin	**5 µg**	**28–35[h]**	**32–40[h]**	**23–29[h]**	**–**	–
Dirithromycin	15 µg	–	18–26	–	–	–
Doripenem	10 µg	27–35	33–42	28–35	–	–
Doxycycline	30 µg	18–24	23–29	–	–	–
Enoxacin	10 µg	28–36	22–28	22–28	–	–
Eravacycline	20 µg	16–23	19–26	–	–	–
Ertapenem	10 µg	29–36	24–31	13–21	–	–
Erythromycin[e]	15 µg	–	22–30	–	–	–
Faropenem	5 µg	20–26	27–34	–	–	–
Fleroxacin	5 µg	28–34	21–27	12–20	–	–
Fosfomycin[f]	200 µg	22–30	25–33	–	–	–
Fusidic acid	10 µg	–	24–32	–	–	–
Garenoxacin	5 µg	28–35	30–36	19–25	–	–
Gatifloxacin	5 µg	30–37	27–33	20–28	–	–
Gemifloxacin	5 µg	29–36	27–33	19–25	–	–
Gentamicin[g]	10 µg	19–26	19–27	17–23	–	–
Gepotidacin	**10 µg**	**18–26**	**23–29**	**–**	**–**	**–**
Grepafloxacin	5 µg	28–36	26–31	20–27	–	–
Iclaprim	5 µg	14–22	25–33	–	–	–
Imipenem	10 µg	26–32	–	20–28	–	–

Kanamycin	30 µg	17–25	19–26	–	–	–
Lefamulin	**20 µg**	**–**	**26–32**	**–**	**–**	–
Levofloxacin	5 µg	29–37	25–30	19–26	–	–
Levonadifloxacin	**10 µg**	**27–33[h]**	**32–39[h]**	**17–23[h]**	**–**	–
Linezolid	30 µg	–	25–32	–	–	–
Linopristin-flopristin	10 µg	–	25–31	–	–	–
Lomefloxacin	10 µg	27–33	23–29	22–28	–	–
Loracarbef	30 µg	23–29	23–31	–	–	–
Mecillinam	10 µg	24–30	–	–	–	–
Meropenem	10 µg	28–34	29–37	27–33	–	–
Methicillin	5 µg	–	17–22	–	–	–
Mezlocillin	75 µg	23–29	–	19–25	–	–
Minocycline	30 µg	19–25	25–30	–	–	–
Moxalactam	30 µg	28–35	18–24	17–25	–	–
Moxifloxacin	5 µg	28–35	28–35	17–25	–	–
Nafcillin	1 µg	–	16–22	–	–	–
Nalidixic acid	30 µg	22–28	–	–	–	–
Netilmicin	30 µg	22–30	22–31	17–23	–	–
Nitrofurantoin	300 µg	20–25	18–22	–	–	–
Norfloxacin	10 µg	28–35	17–28	22–29	–	–
Ofloxacin	5 µg	29–33	24–28	17–21	–	–
Omadacycline	30 µg	22–28	22–30	–	–	–
Oxacillin	1 µg	–	18–24	–	–	–
Pefloxacin	5 µg	25–33	–	–	–	–
Penicillin	10 units	–	26–37	–	–	–
Piperacillin	100 µg	24–30	–	25–33	12–18	–
Piperacillin-tazobactam	100/10 µg	24–30	27–36	25–33	24–30	–
Plazomicin	30 µg	21–27	19–25	15–21	–	–
Polymyxin B	300 units	13–19	–	14–18	–	–
Quinupristin-dalfopristin	15 µg	–	21–28	–	–	–
Razupenem	10 µg	21–26	–[k]	–	–	–
Rifampin	5 µg	8–10	26–34	–	–	–
Solithromycin	15 µg	–	22–30	–	–	–
Sparfloxacin	5 µg	30–38	27–33	21–29	–	–
Streptomycin[g]	10 µg	12–20	14–22	–	–	–
Sulfisoxazole[j]	250 µg or 300 µg	15–23	24–34	–	–	–
Tedizolid	20 µg	–	22–29	–	–	–
Teicoplanin	30 µg	–	15–21	–	–	–
Telavancin	30 µg	–	16–20	–	–	–
Telithromycin	15 µg	–	24–30	–	–	–
Tetracycline	30 µg	18–25	24–30	–	–	–
Ticarcillin	75 µg	24–30	–	21–27	6	–
Ticarcillin-clavulanate	75/10 µg	24–30	29–37	20–28	21–25	–
Tigecycline	15 µg	20–27	20–25	9–13	–	–
Tobramycin	10 µg	18–26	19–29	20–26	–	–
Trimethoprim[j]	5 µg	21–28	19–26	–	–	–
Trimethoprim-sulfamethoxazole[j]	1.25/23.75 µg	23–29	24–32	–	–	–
Trospectomycin	30 µg	10–16	15–20	–	–	–
Trovafloxacin	10 µg	29–36	29–35	21–27	–	–
Ulifloxacin (prulifloxacin)[l]	5 µg	32–38	20–26	27–33	–	–
Vancomycin	30 µg	–	17–21	–	–	–

Abbreviation: ATCC®, American Type Culture Collection.

Annexure 10: SOP for Detection of ESBL-producing Enterobacteriaceae (Disc method)

1. Introduction

Extended-spectrum β-lactamases (ESBLs) are enzymes hydrolyzing most penicillin's and cephalosporins, including oxyimino-β-lactam compounds but not cephamycin's and carbapenems. Most ESBLs belong to the Amber class A of β-lactamases and are inhibited by β-lactamases inhibitors: clavulanic acid, sulbactam and tazobactam. ESBL production has been observed mostly in Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pnuemoniae*, but all other clinically-relevant Enterobacteriaceae species are also common ESBL-producers. In many areas, ESBL detection and characterization is recommended or mandatory for infection control purpose. ESBL detection involves two important steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harboring ESBLs. The second step is a confirmation test which evaluates the synergy between an oxyimino cephalosporin and clavulanic acid, distinguishing isolates with ESBLs from those that are resistant for other reasons.

2. Scope

This SOP describes the procedures for conducting Disc tests for detection of ESBL-producing Enterobacteriaceae in food animals and their products.

3. Objective

To detect the ESBL-producing Enterobacteriaceae in food animals and their products.

4. Principles

4.1 ESBL Disc Screening

Disk-diffusion method for ESBL screening can be performed using cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone according to EUCAST and/or CLSI guidelines. Since the affinity of ESBLs for different substrates is variable, the use of more than one of these agents for screening improves the sensitivity of detection.

However, it is adequate to use the couple cefotaxime (or ceftriaxone) and ceftazidime. If only one drug can be used, cefpodoxime is the most sensitive indicator cephalosporin for detection of ESBL production may be used for screening. However, it is less specific (high number of false positive) than the combination of cefotaxime (or ceftriaxone) and ceftazidime and only the latter compounds are used in the confirmation testing.

4.2 ESBL Disc Confirmation

Enterobacteriaceae suspected to be producers of ESBLs enzymes may be submitted to the follow confirmation tests: Combination Disc Test (CDT) and/or Double-Disc Synergy Test (DDST). These tests permit to evaluate the inhibition of ESBL activity by Clavulanic acid.

4.3 Combination Disc Test (CDT)

For each test discs containing cephalosporin alone (cefotaxime, ceftazidime, cefpodoxime) and in combination with clavulanic acid are applied. The inhibition zone around the cephalosporin disc combined with clavulanic acid is compared with the zone around the disc with the cephalosporin alone. The test is positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without.

4.4 Double-Disc Synergy Test (DDST)

Discs containing cephalosporin (cefotaxime or ceftriaxone, ceftazidime, cefepime) are applied next to a disc with clavulanic acid, amoxicillin + clavulanic acid or ticarcillin + clavulanic acid. Positive result is indicated when the inhibition zones around any of the cephalosporin discs are augmented in the direction of the disc containing clavulanic acid. The distance between the discs is critical and 20 mm center-to-centre has been found to be optimal for cephalosporin 30µg discs; however, it may be reduced (15mm) or expanded (30 mm) for strains with very high or low resistance level, respectively.

5. Equipment and Consumables

a. Antibiotic disc dispenser or Forceps
b. Ruler to measure inhibition zones
c. Vortex mixer
d. 34˚ to 35˚ C ambient air incubator
e. Turbidity meter for McFarland's turbidity determination.
f. Sterile petri plates
g. Sterile cotton swab
h. Antimicrobial discs

6. Culture media and consumables

a. Mueller-Hinton Agar
b. Normal saline

7. Procedure

7.1 Test procedure

- Using a fresh pure culture, prepare a suspension of the test organism equal to 0.5 McFarland Standard.
- Using a sterile cotton swab spread the adjusted suspension over the entire area of a Mueller Hinton agar plate.
- Apply **the** discs onto the inoculated plate, ensuring sufficient space between individual discs to allow for proper measurement of inhibition zones.
- Incubate at 35±2°C for 18-24 hours.

7.2 Interpretation

At the end of the incubation period, measure the inhibition zone and interpret as indicated in the following.

Method	Antibiotics disc	Confirmation is positive if
Combination disc test (CDT)	Cefotaxime alone and Cefotaxime with Clavulanic acid * CTX 30 µg	≥ 5 mm increase in inhibition zone of cephalosporin with

	CTL 30+10 µg	Clavulanic acid deemed positive
	Ceftazidime alone and Ceftazidime with Clavulanic acid * CAZ 30 µg CAL 30+10 µg	
	Cefpodoxime alone and Cefpodoxime with Clavulanic acid ** FEP 30 µg FEL 30+10 µg	

7.3 Reporting Results

7.3.1 Reference Range:

Use WHONET / CLSI standard (Latest version) guidelines for interpretation of inhibition zone ranges.

7.3.2 Reporting Format:

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

8. Quality Control

10.1. QC Strains

2. Reference strains used are recommended in CLSI Standard (Latest version).
    a. *Escherichia coli* ATCC 25922
3. Maintenance of QC strains
    a. Maintain permanent stock cultures at –20˚C or –70˚C in tryptic soy broth with 15-20% glycerol.
    b. Maintain working stock cultures for up to 1 week. Subculture each week for no more than 3 successive weeks.

10.2. Frequency of QC Testing

1. Perform QC daily until acceptable results from 20 (or 30) consecutive days of testing have been obtained.
2. Proficiency in performing QC tests is confirmed if for each drug, no more than 1 out of 20 or 3 of 30 results are outside the accuracy limits.
3. After validation of proficiency, frequency of QC testing can be reduced from daily to weekly.
4. Results are reviewed for acceptability and recorded on the Quality Control log sheets before reporting results.

9. References

i. Harmonized Test Protocol for Isolation, Identification and ABST profiling of Salmonella in Human, Animal and Food products in Bhutan through One Health approach-AGISAR, WHO, Bhutan.
ii. CLSI Guidelines-M100S-Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition.
iii. CLSI Guidelines-VET08-Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, 4th Edition.

iv. Standard Operating Procedures, Bacteriology, Antimicrobial Resistance Surveillance and Research Network, Indian Council of Medical Research, 2nd Edition, 2019.

Annexure 11: SOP for Bacterial Isolate Transportation

1. Introduction

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

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

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

2. Purpose:

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

3. Equipment/materials:

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

4. Chemicals/reagents

4.1 Nutrient agar

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

5. Procedure:

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

5.1.1 Inoculation of slant

- Inoculate the slant by transferring pure isolates (Refer preparation of slants in annex) with an inoculating loop from a single-colony microorganism on a plate to the slant's surface.
- Move the loop across the surface of the slant and recap the tubes.

- Incubate the slant until there is evidence of growth, then put the tube in a refrigerator.

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

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

5.1.2 Packaging of samples

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

The recommended procedure for packing samples is as follows:

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

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

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

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

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

5.1.3 Transport of isolates

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

- Infectious substances, which can include diagnostic specimens, are not permitted to be shipped as checked luggage or as carry on luggage and must be shipped as cargo.

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

5.2.1 Freeze Drying (Lyophilisation)

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

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

5.2.2 Lyophilisation Process

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

5.2.3 Packaging & Transport of culture isolates

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

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

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

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

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

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

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

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

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

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

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

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

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

6. Safety

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

7. References

- Basic Practical Microbiology, A Manual, Society for Microbiology 2006, ISBN 0 95368 383 4
- https://microbeonline.com accessed on 2020
- OIE terrestrial manual 2008, Chapter 1.1.1 collection and shipment of diagnostic samples.
- OIE terrestrial manual 2008, Chapter 1.1.3 Transport of Biological Materials.
- IremOmurtag, Fuat Aydin, Peter Paulsen, Friederike Hilbert & Frans J.M. Smulders(2011).Simple media and conditions for inter-laboratory transport of *Campylobacter jejuni*isolates,Veterinary Quarterly,31:2,73-75,DOI: 10.1080/01652176.2011.585797.

Annexure: Composition

8.1 Nutrient agar:

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

Preparation of Nutrient agar slant

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

1. Dissolve the dehydrated medium in the appropriate volume of distilled water i.e., 23 gm dehydrated nutrient agar (*see the manufacturer instruction*) in 1000 ml distilled water.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder

3. Sterilized the medium by autoclaving (121°C for 15 min)
4. Dispense the medium into tubes *(i.e. 3 ml to make nutrient agar slopes)*. Leave the agar medium to solidify. Making sure the medium inside the tubes is at a slanted position relative to the test tubes.
5. Date the medium and give it a batch number.
6. Store in a cool dark place.

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

8.2 Mueller Hinton agar

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